

28.071.2(04)

К-852

КРЫМСКИЙ СИМПОЗИУМ

Труды
Том 1

CRIMEAN MEETING

Proceedings

Volume 1



Joint Institute for Nuclear Research

CRIMEAN MEETING

**MODERN PROBLEMS OF GENETICS, RADIOBIOLOGY,
RADIOECOLOGY AND EVOLUTION**

*Third International Conference, Dedicated to
N. W. Timofeeff-Ressovsky*

Third Readings after V. I. Korogodin & V. A. Shevchenko

**RADIOBIOLOGICAL ISSUES
PERTAINING TO ENVIRONMENTAL SECURITY AND
ECOTERRORISM**

NATO Advanced Research Workshop

Alushta, Ukraine, 9–14 October 2010

Proceedings

Volume 1

Dubna • 2012

Объединенный институт ядерных исследований

28.07.2(04)
К-852



КРЫМСКИЙ СИМПОЗИУМ

151034
СОВРЕМЕННЫЕ ПРОБЛЕМЫ ГЕНЕТИКИ,
РАДИОБИОЛОГИИ, РАДИОЭКОЛОГИИ И ЭВОЛЮЦИИ

*Третья международная конференция,
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*Третьи Чтения памяти
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УСПЕХИ РАДИОБИОЛОГИИ: БЕЗОПАСНОСТЬ
ОКРУЖАЮЩЕЙ СРЕДЫ И ЭКОТЕРРОРИЗМ

Конференция по научной программе НАТО

Алушта, Украина, 9–14 октября 2010 г.

Труды симпозиума

Том 1

Дубна • 2012
Объединенный институт
ядерных исследований
БИБЛИОТЕКА

УДК 577.391(042+091)
ББК 28.071.2я434+28.081.28я434
К85

Под общей редакцией *В. Л. Корогодиной, К. Мотерсил, К. Сеймура*
Составитель *В. Л. Корогодина*

Обложка и макет *В. Л. Корогодиной*

Фотографии любезно предоставлены участниками конференции.

Editors: *V. L. Korogodina, C. Mothersill, C. Seymour*

Composed by *V. L. Korogodina*

Title page design: *V. L. Korogodina*

Photos are the courtesy of participants of the conference.

Крымский симпозиум: «Современные проблемы генетики, радиобиологии, радиоэкологии и эволюции», Третья междунар. конф., посвященная Н. В. Тимофееву-Ресовскому; Третьи Чтения памяти В. И. Корогодина и В. А. Шевченко; «Успехи радиобиологии: безопасность окружающей среды и экотерроризм», конф. по научной программе НАТО (Алушта, Украина, 9–14 окт. 2010 г.): Труды симпозиума / Под общ. ред. В. Л. Корогодиной, К. Мотерсил, К. Сеймура; Сост.: В. Л. Корогодина. — Дубна: ОИЯИ, 2012. — Т. 1. — 377 с., [10] с. фото.

ISBN 978-5-9530-0347-6

Сборник включает статьи и доклады, представленные на Крымском симпозиуме (Алушта, 9–14 октября 2010 г.). В первый том вошли оригинальные статьи по генетике и радиобиологии. Статьи опубликованы в авторской редакции.

Издание представляет интерес для широкого круга читателей.

Crimean Meeting: «Modern Problems of Genetics, Radiobiology, Radioecology and Evolution», Third Intern. Conf., dedicated to N. W. Timofeeff-Ressovsky; Third Readings after V. I. Korogodin & V. A. Shevchenko; «Radiobiological Issues Pertaining to Environmental Security and Ecoterrorism», NATO Advanced Research Workshop (Alushta, Ukraine, 9–14 Oct. 2010): Proc. / Eds.: V. L. Korogodina, C. Mothersill and C. Seymour; Comp. V. L. Korogodina. — Dubna: JINR, 2012. — V. 1. — 377 p., [10] p. photos.

ISBN 978-5-9530-0347-6

The collection contains papers presented at the Crimean Meeting (Alushta, 9–14 October 2010). Volume 1 includes original articles on modern genetics and radiobiology. The papers are published in the authors' wording.

УДК 577.391(042+091)
ББК 28.071.2я434+28.081.28я434

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ядерных исследований, 2012

ISBN 978-5-9530-0347-6

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Финансовая поддержка

Программа НАТО «Наука в целях мира и безопасности»
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ГЕНЕТИКА. ПРОБЛЕМЫ МЕДИЦИНСКОЙ ГЕНЕТИКИ
GENETICS. PROBLEMS OF MEDICAL GENETICS

A TEST OF KIMURA'S MUTATION-RATE CONJECTURE*

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Abstract- Mutation is the first of the four great engines of evolution and the prime source of heritable variation in all living and semi-living organisms and their molecular parasites. As a result, the processes and products of mutation have been deeply explored at both the phenomenological and the mechanistic levels for more than a century. There has been notable progress at both levels over the last half century, with the result that the phylogeny of mutation has become fairly well codified, especially the rates and kinds of mutation in as diverse organisms as could be probed. These explorations began to coalesce in the 1990s and soon comprised a small number of broad generalizations. These include very high genomic mutation rates in riboviruses and some higher eukaryotes, and strikingly similar genomic mutation rates in most DNA microbes. However, highly informative exceptions have recently been observed.

Keywords: mutation, mutation rate, ribovirus, thermophile

1. Introduction

My scientific hobby for the last forty years has been the exploration of the phylogeny of mutation, specifically the rates of mutation in as many organisms as could be probed and the kinds of mutations that result. These explorations began to coalesce in the 1990s and soon comprised some broad generalizations. More recently, I and several colleagues sought exceptions to these generalizations in order to test particular hypotheses, with sometimes surprising results.

At the beginning, it is important to stress that mutation has long been accepted to be a highly evolved process shaped by natural selection (Fishier, 1930; Sturtevant, 1937; Kimura, 1967). For instance, most mutations are deleterious and thus reduce the average fitness of progeny, providing a basis for adaptive evolution based on mutation down-modifiers. As such, the mutational target of an organism is its whole genome, or at least that fraction which is subject to purifying or adaptive selection. Thus, the most interesting parameter is usually the *genomic* mutation rate μ_g , although it is sometimes

* Radiobiology and Environmental Security / Eds. C.Mothersill, V.Korogodina, C.Seymour. Springer, 2012. P.13-19

subdivided in various ways. However, the only part of the genomic mutation rate that is subject to selection is that fraction that can generate deleterious or adaptive mutations, so that spacer DNA that shows no traces of purifying selection is of a very limited significance in this respect; accordingly, I will sometimes refer to the fraction of the genome in which new mutations are subject to selection as the *effective* genome and the corresponding genomic mutations rate as μ_{eg} . This is close to the total genome in most microbes but may be a small fraction of the genome in higher eukaryotes.

Mutation rates are not often measured starting with a specific mutation-reporter locus. The mutation frequency f is determined by counting mutants among total organisms screened. The frequency is then converted into a rate μ using equations that reflect the mode of chromosome replication, for instance, iterative copying (the stamping-machine model) or exponential replication. Although complex equations are sometimes required, there exist very simple equations that suffice for many situations. Thus, for riboviruses, which replicate by a stamping-machine mechanism, $\mu = f/2c$ where c is the number of sequential infection cycles that generated the test population (Drake & Holland, 1999). For geometrically replicating organisms (such as numerous microbes), provided that the population is initiated with a number of organisms small enough to contain no mutants and then grows until it has experienced many (more than 30) mutations, $\mu = f/\ln(N\mu)$ where N is the final population size (Drake, 1991; Rosche & Foster, 2000); the median rate from five or more cultures is the best measure.

The rate per locus is then converted into a rate per average base pair, which is finally converted into a genomic rate. It is important that the mutation-reporter locus be free of selective forces and certain other behaviors that bias mutation frequencies, and that it be large enough to mirror the genome as a whole. The main difficulty with mutation-reporters, which are usually protein-encoding sequences, is that they report base-pair substitutions (BPSs) inefficiently because most BPSs do not produce a mutant phenotype. Because of the efficiency and low cost of DNA sequencing, this constraint can now sometimes be overcome by calculating BPS rates using only BPSs that create chain-terminating mutations, which are expected to be scored efficiently.

Other modes of measuring mutation are sometimes used. One involves massive genomic sequencing. This has the advantage of scoring mutations without regard to their phenotypes but may have the disadvantages of young technologies.

2. Mutation Rates Codified

Table 1 presents an overview of rates of spontaneous mutation across major groups of organisms. They fall into three categories.

Riboviruses and retroelements (including retroviruses and retrotransposons) have the highest rates per genome replication, and when these are presented per "generation" (two replications for riboviruses, three for retrotransposons, the rates are close to the maximum that can be tolerated without extinguishing the population (variously called

“error catastrophe” or “mutational meltdown”). Most of the published values are based on very short reporter sequences and thus provide uncertain values when extrapolated to the whole genome, which may account for the wide range of values.

Table 1. Rates of mutation in diverse categories of organisms

Group	μ_{eg} per genome replication	μ_{eg} per “generation”
Riboviruses	0.1–1	0.2–2
Retroelements	0.03–0.43	0.08–1.3
Most DNA microbes	0.003–0.005	(same)
Some animals	0.004–0.014	0.04–1.6

Ribovirus rates are from Drake & Holland (1999) and Malpica *et al.* (2002). Retrovirus rates are from Drake *et al.* (1998). DNA-microbe rates are from Drake (2009). Animal rates are from Drake *et al.* (1998).

Most DNA-based microbes have rates far lower than do the RNA-based microbes, in the neighborhood of 0.004 per genome replication. For the DNA-based microbes, a genome replication usually occurs once per cell division.

Several animals (*Caenorhabditis elegans*, *Drosophila melanogaster*, *Mus musculus*, *Homo sapiens*) also have rates (per effective haploid genome) in the neighborhood of 0.004 per genome replication in the germline, but animals often experience large numbers of germline replications per sexual generation (mostly in the male) and therefore have very high rates per effective genome per sexual generation. As a result, therefore, animals to some extent resemble the RNA world. The highest rate, 1.6 mutations per haploid genome per generation, belongs to humans, and suggests that any long-extended increase in this rate might extinguish the species.

All but one of the mutation rates considered thus far were determined using strains well adapted to the laboratory growing under generally benign conditions and should be viewed as *basal* mutation rates. For most of these organisms, the basal rates are so large that even small increases could be disastrous. However, because of their much lower basal mutation rates, the DNA microbes can tolerate sharply increased rates, at least for a while. However, under diverse stresses, bacterial mutation rates often increase transiently, or at least for as long as the stress persists [e.g., Petrosino *et al.* (2009), Gibson *et al.* (2010)]. More generally, a considerable fraction of mutation spectra from all categories of life contain one or more mutants bearing two or more mutations, where the “multiples” are usually far more frequent than anticipated from a random distribution of mutations and are likely to arise from bouts of transient hypermutability such as might result from errors of transcription, translation, or folding in proteins involved in diverse DNA transactions (Drake *et al.*, 2005). In addition, closely spaced complex mutations are sometimes produced by DNA primer extension using ectopic templates, but these can usually be recognized by finding the ectopic donor sequences

(Schultz and Drake, 2008). All of these kinds of supra-basal mutation rates may well contribute to evolution, but to an unknown extent.

3. Informative Exceptions

The single most remarkable aspect of comparative mutation is the very narrow range of genomic rates among the DNA microbes, and perhaps also among animals if the estimates in Table 1 are correct. Because genome sizes vary by about 6000-fold among the microbes that make up that entry in the table, average mutation rates per base pair necessarily vary inversely by about the same factor, an observation that is about two decades old (Drake, 1991) but remains unexplained: while much is known about fidelity mechanisms in many of these model organisms, it is not known why “0.004” is such a strongly preferred value.

One of the most frequently evoked hypotheses has its origins in the work of Motoo Kimura, who pointed out that down-modifiers of mutation rates should be adaptive because most mutations are deleterious, at least within the effective genome (the latter concept not being anticipated at that time because of the lack of DNA sequences to reveal the large fractions of many genomes that consists of DNA not subject to strong purifying selection). As he appreciated, however, there will always be a cost associated with further reducing the error frequency (Kimura, 1967). (He also noted that up-modifiers of mutation rates would be inefficiently selected through the adaptive mutations they might produce because the favorable alleles would soon be separated from the mutator allele by recombination, an efficient process in frequently sexual organisms and not necessarily sufficiently absent from bacteria to prevent such selection, although the issue is complex.) In any case, the Kimura conjecture implies an equilibrium mutation rate, but does not imply that the equilibrium would be the same in such a diverse set of organism with so sharply differing life histories and molecular mechanisms for maintaining replication fidelity, especially when it encompasses a 6000-fold variation in average mutation rate per base pair.

An experimental test of the Kimura conjecture might consist of artificially increasing the mutation rate with a physical or chemical mutagen and then determining how the overall rate adjusts during subsequent evolution in the laboratory, but there are many pitfalls in this approach, one being uncertainty about the time required to reach equilibrium and another being about the limited set of environments that can be explored in the laboratory. Our first test of the conjecture involved characterizing mutation in the hyperthermophile *Sulfolobus acidocaldarius* because we wondered if growth at extreme temperatures might introduce so much DNA damage as to force the mutation rate upwards. However, the result was just the opposite: mutation rates were lower in *S. acidocaldarius* than in the mesophilic microbes examined to date (Grogan *et al.*, 2001). We then considered the impact of temperature on the average base pair in a protein-encoding gene, knowing that one of the easiest kind of conditional mutation to

isolate historically in many microbes was a temperature-sensitive allele. It seemed reasonable to surmise that many neutral missense mutations in mesophiles would become deleterious at higher temperatures, in which case purifying selection against nonsynonymous mutations would be expected to be more intense in thermophiles than in mesophiles. This turned out to be correct: the estimator dN/dS (where N refers to nonsynonymous mutations and S to synonymous mutations) averaged to about 0.14 in mesophilic DNA microbes but fell to about 0.09 in thermophiles (Friedman *et al.*, 2004). Also, because *S. acidocaldarius* was the first archaeon to be examined for its mutational propensities, we repeated the analysis in a bacterial thermophile, *Thermus thermophilus*, and observed again that the mutation rate was lower than seen in mesophiles (Mackwan *et al.*, 1008). The more reliable of the mesophile and thermophile rates are listed in Table 2 and were calculated using only chain-terminating mutations to estimate BPS rates.

Table 2. Rates of spontaneous mutation in DNA microbes.

Organism	Genome (nt)	μ_b	μ_g	$\mu_g(\text{BPS})$	$\mu_g(\text{indel})$
Phage M13	6.4×10^3	7.5×10^{-7}	0.0048	0.0038	0.0010
Phage λ	4.9×10^4	5.3×10^{-8}	0.0026	0.0022	0.0004
Herpes simplex virus	1.5×10^5	1.8×10^{-8}	0.0043	0.0035	0.0008
Phage T4	1.7×10^5	1.8×10^{-8}	0.0038	0.0030	0.0008
<i>Escherichia coli</i>	4.6×10^6	6.4×10^{-10}	0.0030	0.0025	0.0004
<i>Saccharomyces cerevisiae</i>	1.2×10^7	3.6×10^{-10}	0.0044	0.0041	0.0003
<i>Schizosaccharomyces pombe</i>	1.3×10^7	2.4×10^{-10}	0.0030	0.0026	0.0004
Mesophile mean			0.0038	0.0032	0.0006
<i>Thermus thermophilus</i>	2.1×10^6	3.2×10^{-10}	0.00067	0.00054	0.00013
<i>Sulfolobus acidocaldarius</i>	2.2×10^6	1.7×10^{-10}	0.00037	0.00011	0.00026
Thermophile mean			0.00052	0.00033	0.00019

Values are from Drake (2009). The value for phage λ is for its rates when growing lytically. Where a mutation reporter was impacted by an extreme indel hotspot, the posted values are for the rates without the hotspot; the overall profile is only modestly impacted by this procedure. The total genomic rate μ_g is fractionated into its base-pair substitution and indel components, $\mu_g(\text{BPS})$ and $\mu_g(\text{indel})$, respectively.

Not only are total genomic rates seen to be reduced in thermophiles (by roughly 7-fold), but the main cause of this decrease is a 10-fold decrease in BPS rates. These results provide a striking confirmation of the concept of a reciprocal relationship between the impact of mutations and the genomic mutation rate, as expected from the Kimura conjecture.

Acknowledgements

Research conducted in the author's laboratory was supported by funds allocated to project number ES065016 of the Intramural Research Program of the NIH, National Institute of Environmental Health Sciences.

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HYPERMUTABILITY ASSOCIATED WITH DOUBLE-STRAND BREAK REPAIR*

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Abstract- Double-strand breaks (DSBs) are the most toxic kind of DNA damage caused by ionizing radiation as well as by a number of other environmental factors and drugs. DSBs lead to gross chromosome rearrangements, genetic disease, and cancer or cell death. However cells can be programmed to generate DSBs in their own DNA. Programmed DSBs are a key element of many biological functions such as meiotic recombination and segregation, adaptive immunity, regulation switches and viral life cycles. Either damage-induced or programmed DSBs should be repaired in order to retain cell viability. Over the last years it has been established that DSB repair can be associated with up to 10,000-fold increase in frequency of base substitutions and small insertions/deletions (indels). This *localized* hypermutability represents additional genotoxic threat as well as a potential for generating rare multiple mutant alleles with high fitness without overloading the rest of the genome with mutations.

Keywords: DNA repair, mutagenesis, double-strand breaks, multiple mutations

1. A challenge of multiple mutations in a single ORF

Even a single mutation in DNA can alter biological functions either to a benefit or to a detriment of a cell or an organism. Thus important balance must be maintained between limiting mutation frequency to avoid harmful changes on one hand and on the other hand allowing the level of mutagenesis which can generate sufficient amount of rare adaptive changes fueling evolution. In various species the rates of spontaneous mutations on a genome scale are kept down by a firewall of replication fidelity and repair systems (Kunkel *et al.* 2000; Friedberg *et al.* 2006). Based on studies with mutation reporters mutation rate per genome in various species is low; no more than one out of a hundred new cells would carry a single new mutation in the entire genome (Drake 1999; Drake *et al.* 1999). Thus accumulation of multiple mutations in a genome over just few generations would be extremely unlikely. Even less likely would be the

* Radiobiology and Environmental Security / Eds. C.Mothersill, V.Korogodina, C.Seymour. Springer, 2012. P. 1-12

incidence of simultaneous changes in a single gene; however multiple mutations in a gene would carry the strongest biological effects, i.e. reduction in gene function, increase in gene function or even creation of a novel function. The stronger potential of multiple mutations is evident for the case of gene inactivation, since the majority of base pair substitutions (bps) and even some of small insertions and deletions (indels) would leave the gene function on a biologically sufficient level. Multiple mutations are also more likely to generate changes increasing fitness. Limited number of studies aimed to generate enzymes with enhanced or even new activity established that this can be achieved primarily by multiple mutations (Camps *et al.* 2007; Romero *et al.* 2009). Importantly, multiple mutations showed *sign epistasis*, i.e. a condition when individual changes within a multiple mutant are neutral or even deleterious (Weinreich *et al.* 2005; Kogenaru *et al.* 2009). On the evolution scale it translates into a requirement of multiple mutations to avoid *fitness valleys* (i.e. steps with reduced fitness within the succession chain of mutations resulting in alleles with high fitness) by following *fitness ridges* involving only the succession of mutation steps that do not lead to fitness reduction (Wright 1932; DePristo *et al.* 2005). As established by comparisons across wide range of taxons sign epistasis as well as fitness ridges and valleys are the present characteristics of protein evolution (Povolotskaya *et al.* 2010). However, fitness valleys are not a concern if multiple mutations of the advantageous alleles occur by simultaneous or closely timed events. Multiple mutations that appear to be simultaneous or coordinated in time (*chronocoordinate*) were also detected in normal mouse and human tissues (Colgin *et al.* 2002; Wang *et al.* 2007) and in tumors (Chen *et al.* 2008; Chen *et al.* 2008; Kashuba *et al.* 2009). While the fraction of such mutations is small they may play bigger role only in some categories of cancers especially in cancers associated with high density of DNA damage (see below).

Since beneficial mutations represent only a tiny subset of all possible changes, only very small group of multiple mutations can be expected to bring high fitness. In order to get a specific set of simultaneous specific mutations in a single ORF with a realistic probability, individual changes should have a very high chance. Based on the maximal estimate of spontaneous mutation rate for as 10^{-9} per nt (Drake 1999; Drake *et al.* 1999) even the simultaneous mutations of three specific bases become practically impossible (10^{-27}), because it would require unrealistic amounts of biological material (for example 10^{27} yeast cells would weigh 100 billion tons). Several orders of magnitude greater mutation rates are required to make simultaneous multiple mutations realistic. For example, the rate of 10^{-4} per nt would allow a simultaneous mutation of three specified nucleotides to be found among 10^{12} cells which corresponds to just 100 grams of yeast or around 1 kg of human cells. Such high mutation probabilities are impossible on a genome-wide scale even for a single cell generation. A minimal approximation for 40,000 one-kilobase ORFs (minimal estimate for a diploid human genome) gives around 4,000 mutations, which would create intolerable mutation load

by coincidence of allelic recessive lethals and by inactivation of haplo-insufficient genes.

2. Pathway generating multiple mutant alleles

The genome-wide mutation overload can be avoided if high mutation density would be generated only in a small section of a genome – a phenomenon we define as *localized hypermutability* (LHM). Some regions of a genome could be permanently more prone to mutations than others. The presence of at-risk motifs capable of forming DNA structures that are poor substrates for DNA repair and mutation avoidance systems (Gordenin *et al.* 1998; Zhao *et al.* 2010) and chromatin structure and modification (Hassa *et al.* 2005) are among possible causes. Studies of adaptive mutagenesis in *E. coli* by Rosenberg *et al.* (Harris *et al.* 1994; Rosenberg *et al.* 1994) indicated that double-strand break (DSB) repair could be mutagenic. Soon after that Jeff Strathern and colleagues have established in the model yeast system using a defined site-specific DSB repaired by homologous recombination that the repair in fact is associated with up to several hundred fold increase in mutation frequency in the area around a DSB (Strathern *et al.* 1995; Rattray *et al.* 2003). Thus there is a potential for LHM to emerge in any given place of the genome, if there is a DSB followed by DSB-repair. Later the hypermutability of DNA adjacent to a site-specific DSB has been shown also in *E. coli* thus establishing the generality of the phenomenon across microbial taxons (Ponder *et al.* 2005; Galhardo *et al.* 2007). Recently we confirmed one of the sources of LHM proposed by the Strathern group – hypermutability of long single-strand DNA (ssDNA) formed by strand-biased 5'→3' DNA degradation (resection) (Yang *et al.* 2008). The second proposed source – error-prone DNA synthesis creating two new strands in the course of filling a double-strand gap was demonstrated recently by the Haber lab (Hicks *et al.* 2010). In all systems the rates of mutation per nt in the absence of applied DNA damage ("spontaneous") was close to 10^{-6} per nt initially estimated by the Strathern group (Rattray *et al.* 2002) (see also Table 1). Not surprisingly this level of hypermutability produced only single mutations.

The rates of spontaneous LHM associated with DSB repair vary around the estimated value of *in vivo* error rates in yeast cells carrying double defect in DNA polymerase proofreading and in post-replicative mismatch repair (MMR) (1.5×10^{-6} per nt in the *URA3* gene (Morrison *et al.* 1993). Importantly, combination of proofreading and MMR defects did not produce multiple *URA3* mutations even through growing yeast cells for several generations. There are no indications so far that significantly higher *in vivo* rate of errors capable of producing simultaneous multiple mutations within a single ORF could be achieved during synthesis on long undamaged templates. However, very high mutation density can be achieved if LHM is associated with DNA damage. The striking case of *programmed* damage-induced increase of mutation

frequency by about million-fold as compared to genome-wide rate has been well established for a small region of the Ig-locus in genomes of immune B cells ((Odegard *et al.* 2006; Liu *et al.* 2009) and Table 1). The increase in somatic mutation (somatic hypermutability –SHM) is confined to a small region within the Ig locus. SHM is driven by a specialized enzyme – activation-induced deaminase (AID), which converts a part of cytosines into uracils in the SHM region. Since this region is involved in determination of the affinity to an antigen, SHM results in a very fast accumulation of multiple mutant alleles providing sufficient material for selecting cells producing antibodies with several orders of magnitude greater affinity to the antigen. Because of the specially organized cell division control the very same cells also have proliferation advantage over the cells producing antibodies with lower affinity. LHM can bring not only high fitness alleles with improved or new function. It also increases the frequency of gene inactivation. Albeit with much lower efficiency, AID expressed in immune cells is mutagenic for several other genomic regions, which makes them prone for undesired changes (Unniraman *et al.* 2007; Liu *et al.* 2008; Liu *et al.* 2009). However, since LHM is mostly confined to a small region within the Ig-locus, it can generate multiple mutant alleles with high fitness without overloading genome with additional mutations and thus avoiding accumulation of lethal or low fitness alleles in other genes.

Can damage-induced LHM operate in a *non-programmed* mode? The existence of multiple powerful repair systems enables living cells to repair vast number of damages through a single cell cycle. Even the number of endogenous damages in normal human cells is estimated in tens of thousands (Lindahl *et al.* 2000). The tolerable number of damages that can be caused by exogenous sources can be orders of magnitude greater reaching a density of one damage per several thousand nt (Resnick *et al.* 1972; Wheatcroft *et al.* 1975; Santos *et al.* 2006; Pachkowski *et al.* 2009). Unrepaired damages often lead to mutations, if copied by error-prone translesion synthesis (TLS) DNA polymerase (Plosky *et al.* 2004; Friedberg 2005). A cell with high density of DNA damage will inevitably die if it lacks DNA repair throughout the genome. However, the lack of repair in a small section of the genome could be tolerated. In this situation error-prone TLS during replication of the damaged section can produce a stretch of multiple mutations. Most of DNA repair systems operate in double-strand DNA (dsDNA). However, damage in rare stretches of single strand DNA (ssDNA) would be often left unrepaired and thus lead to mutation. Coincidence in formation of large stretches of ssDNA with DNA-damage could, in principle, be a source of LHM, if damaged ssDNA is capable of recovery to ds-state. However, if long ssDNA with multiple damages is lost because of degradation or cell death triggered by checkpoint reaction, the opportunity for multiple mutations will be lost.

We sought to determine if long stretches of ssDNA formed around DSB or at uncapped telomeres can recover with multiple mutations (Yang *et al.* 2008). For that purpose we developed special genetic systems in model eukaryote, yeast *S. cerevisiae* where stretches of long ssDNA can be formed around inducible site-specific double

strand break or uncapped telomeres formed by holding a telomere-capping *cdc13-1* mutant in non-permissive temperature (Figure 1, Table 1). In these model systems we observed the frequencies of damage-induced LHM comparable to those observed in programmed SHM within Ig-locus (Table 1). Importantly, we observed up to 6 simultaneous mutations in a single ORF, while mutagenesis in other genomic locations was barely detectable.

Table 1. Mutation frequencies associated with various kinds of localized hypermutability.

Cause of hypermutability (species)	Mutation frequency per megabase	References
DSB-repair in F'-episome (<i>E. coli</i>) ¹	19	Gonzalez <i>et al.</i> 2008
DSB-repair, gap filling (yeast) ²	0.7	Hicks <i>et al.</i> 2010
DNA adjacent to repaired DSB (yeast) ³	17	Rattray <i>et al.</i> 2002
ssDNA adjacent to DSB repair (yeast) ⁴	13	Yang <i>et al.</i> 2008
Damage-induced LHM (yeast) ⁵	300	Yang <i>et al.</i> 2008; Yang <i>et al.</i> 2010
SHM, adaptive immunity (humans) ⁶	1,000	Odegard <i>et al.</i> 2006; Liu <i>et al.</i> 2009

¹ *E.coli* mutations selected in F' codA,B genes with mutation target size 848 bp used for calculations as determined in the referenced work.

² Mutations in the *URA3* gene of *Sacharomyces cerevisiae* occurring during a double-stranded (ds) gap repair, (minimal estimate of mutation target of 125 bp as determined by (Lang *et al.* 2008)

³ Frequency of spontaneous mutations in the vicinity of a DSB repaired by homologous recombination. Site-specific DSB was induced next to chromosomal *CAN1* gene and repaired by homologous recombination with a truncated copy of *CAN1* in the same chromosome. This system did not allow distinguishing between hypermutability in transiently formed ss DNA (as in), or hypermutability during repair of a ds-gap (as in (Hicks *et al.* 2010))

⁴ In this system *CAN1* reporter gene was placed in the vicinity of a DSB that was repaired by a short oligonucleotide, so *CAN1* sequence did not participate in a recombination act therefore the most likely hypermutable intermediate was a stretch of transient ss DNA next to a DSB.

⁵ Average frequencies of mutations induced by UV-C (45 J/m²) and MMS (30 min in 11.8 mM (0.1%) MMS) in yeast ssDNA around DSB or next to uncapped telomere (see also Figure 1).

⁶ Approximate frequency of mutations associated with somatic hypermutation in the Ig-genes.

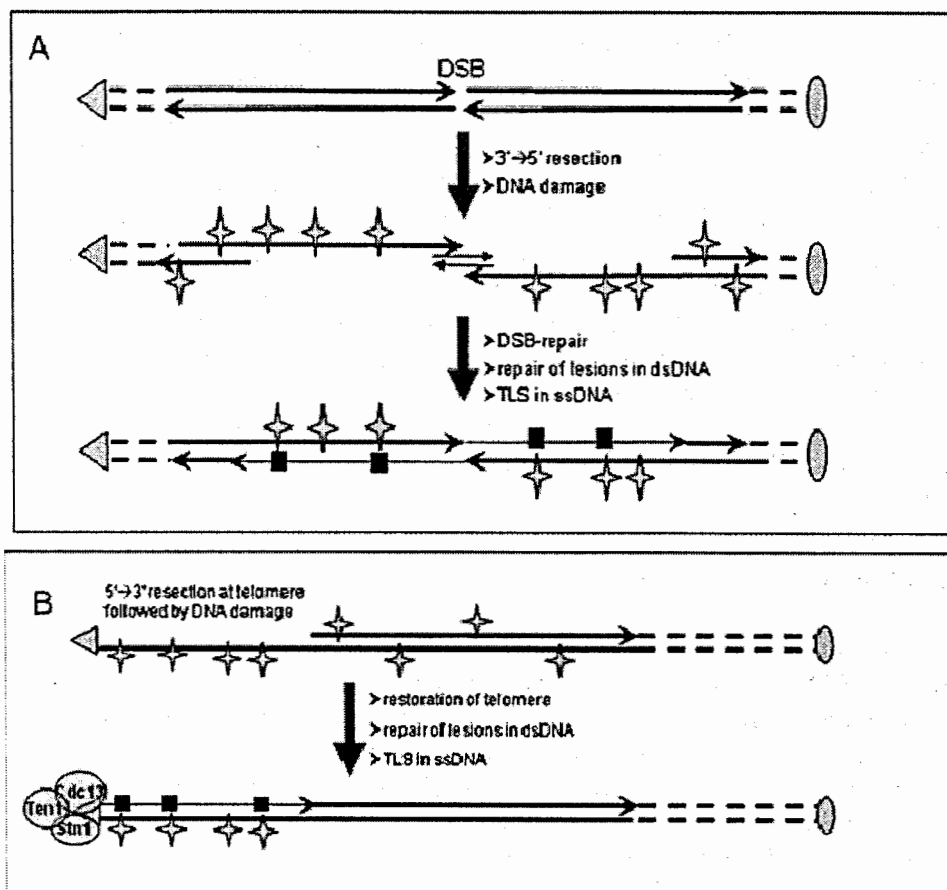


Figure 1. Damage-induced localized hypermutability associated with transient regions of single-strand DNA generated at double-strand breaks and uncapped telomeres.

Damage-induced LHM caused by two different kinds of damaging agents, ultraviolet light (UV) and methylmethane sulfonate (MMS) completely relied on TLS polymerase Pol η . Strand-biased mutation spectrum of UV-induced mutations (Yang *et al.* 2008) indicated that mutations are caused by TLS in damaged ssDNA. In a later work we found that in the case of MMS mutations were caused by ssDNA-specific damage (predominantly N-3-methyl cytosine), so the damage was inflicted after DNA became single-stranded (Yang *et al.* 2010). Importantly, we observed a large number of strand-biased multiple mutations (up to 6 widely spaced changes in a 4 kb ORF). Thus, yeast cell is set to generate simultaneous multiple mutations in a single ORF by mechanism of damage-induced LHM.

Experimental approaches and conclusions summarized in this figure were described in (Yang *et al.* 2008; Yang *et al.* 2010). **(A) Double-strand break (DSB).** Long ssDNA can be generated around a DSB by 5'→3' resection if DSB repair is delayed. While DNA damage in ds regions (grey stars) can be repaired by major repair pathways, such as BER, NER, PRR, damage in ss DNA often stays unrepaired. Repair of inducible site-specific DSB in was allowed by adding oligonucleotides complementary to the ends of the break. Trans-lesion DNA synthesis (TLS) is required to build a complementary strand on the damaged DNA template. Some TLS events will create wild type sequence, however error-prone TLS can generate mutations (blue boxes) at many sites of DNA damage. **(B) Uncapped telomere.** Long ssDNA can be generated by 5'→3' resection at telomeres that transiently lost capping protein complex. This was achieved by shifting *cdc13-1* mutant to non-permissive temperature (37°C). Restoration of the telomere cap and dsDNA was allowed by shifting to permissive temperature (23°C) after applying DNA damage. Multiple mutations were generated by error-prone TLS similar to (A).

3. Mechanisms of damage-induced LHM

In our experiments LHM was observed after restoration of damaged ssDNA formed at unprotected DNA ends such as DSBs and uncapped telomeres. Long ssDNA was well documented at unprotected DNA ends in pro- and eukaryotic microbes, however resection tracts may be less long in mammalian systems (Amundsen *et al.* 2003; Sartori *et al.* 2007; Shrivastav *et al.* 2008; Mimitou *et al.* 2009). It is worth note that unlike in microbial systems mammalian resection studies mostly rely on detection of ss DNA-interacting proteins rather than direct monitoring of ssDNA formation. The prevailing current view that the resection machinery is conserved through eukaryotes but in normal conditions end-resection capacity in mammalian cells is limited. Shorter resection tracts were also suggested based on high capacity of mammalian cells to non-homologous end-joining (NHEJ) or to microhomology mediated end-joining (MMEJ). These pathways efficiently operate with blunt or minimally degraded DNA ends and thus quickly eliminate the substrates of end-resection. An additional factor reducing the size and number of resection tracts could be the ssDNA binding proteins inhibiting resection. Even if normal resection tracts in mammalian cells are shorter than in yeast all conserved proteins participating in resection are there so infrequent long resection tracts are possible especially when inhibitors of resection would not act.

Another potential source of long ssDNA is uncoupling between unwinding and DNA synthesis in leading and/or lagging strands of replication fork that can be formed by infrequent spontaneous miscoordination and/or in response to DNA damage blocking DNA polymerase (Lopes *et al.* 2006; Hamdan *et al.* 2010). However, the frequency of uncoupling and sizes of ss DNA regions are not well documented so far.

There were also communications about very long stretches of ssDNA formed in cultured cancer cells (Bjursell *et al.* 1979). The origin of and mechanism producing this form of ssDNA are unknown. However, if it can restore to ds state this could be one more source of damage-induced LHM.

In principle damage-induced LHM should not be necessarily associated with ssDNA. It can originate from any cause that would inhibit DNA repair from the time of damage through the next DNA replication. While several factors such as chromatin type, nucleosome position or transcription status can affect efficiency of DNA repair and/or mutation frequency (see Introduction) there were no demonstration of really strong, multi-fold effects of these factors leading to clusters of simultaneously occurring multiple mutations. We expect that with accumulation of whole-genome mutagenesis data (see e.g., (Nishant *et al.* 2009; Nishant *et al.* 2010; Zanders *et al.* 2010)) will reveal important information about pathways and molecular mechanisms of damage-induced LHM.

4. Biological context and evolution implications for damage-induced LHM

Examples of wide spread clusters of multiple mutations (*mutation showers*) have been detected among mutation spectra in mice and humans (Colgin *et al.* 2002; Wang *et al.* 2007) however the mechanisms generating these clusters were not addressed. Hypermutability and mutation clusters in our experiments (Yang *et al.* 2008; Yang *et al.* 2010) were caused by damaging artificially formed ssDNA around inducible site specific DSB or in the vicinity of uncapped telomeres in G2-arrested *cdc13-1* mutant yeast. However, ssDNA can be formed by resection at unprotected ends of spontaneous or damage-induced DSBs. Importantly, the vast number of DNA damaging agents can induce both DSBs and mutagenic base or nucleotide damage (Friedberg *et al.* 2006). For example we demonstrated that base alkylation by methyl methanesulfonate (MMS) can result in DSBs via faulty repair of closely opposed damages (Ma *et al.* 2008; Ma *et al.* 2009) as well as in high frequency of base substitutions in artificially formed ssDNA around site-specific DSB (Yang *et al.* 2010). However, there were no or very small fraction of multiple mutations found within spectra of spontaneous or damage-induced forward mutations in common mutation reporters. Mutation reporters targeted to detect low frequencies of clustered multiple mutations are under development in our lab.

Mutations have generated all current variety of DNA sequences over the time of biological evolution. On the evolution timescale localized increases in the numbers of mutation accumulated during human evolution from a common ancestor with chimpanzees have been associated with meiotic DNA breaks (Lercher *et al.* 2002; Pollard *et al.* 2006; Dreszer *et al.* 2007; Duret *et al.* 2008; Berglund *et al.* 2009). These studies have identified a number of regions (*human accelerated regions* – HARs) in which over the past ten million years of primate evolution much more mutations have

accumulated than over preceding hundred million years of mammalian evolution. Interestingly, association was detected between hotspots of meiotic recombination in human males and HARs. Another distinct feature of HARs is a mutation bias of A-T or T-A pairs changing into G-C or C-G pairs. One explanation is that there is biased gene conversion (BGC) in which G-T and C-A mismatches are more frequently corrected towards G-C and C-G as compared with correction towards A-T and T-A. This would lead to increased fixation of G-C and C-G mutant base pairs. However HARs could also reflect the increased mutability around meiotic DSBs which can be further enhanced by endogenous damage to ssDNA formed around DSBs triggering male meiotic recombination. Recently, based on analysis of the vast amount of human sequencing data it was concluded that the increased rates of base substitutions on an evolution, a population and even on a single tumor or cell line timescales are associated with rearrangement breakpoints which could at least in part be caused by hypermutability of break-associated ssDNA ((De *et al.* 2010) and references therein). Another study associated increased mutation rates in the human evolution line with late replicating regions of genome which could be also associated with higher frequency of breakage during mitotic divisions in the germline or/and with increase in ssDNA formation (Stamatoyannopoulos *et al.* 2009). These observations together with >1,000-fold increase of mutation frequency in damaged ssDNA as compared with dsDNA regions demonstrated in our experiments makes us to speculate that significant fraction of mutations in nature may come from error-prone translesion synthesis in ssDNA. Future studies integrating model experiments with genotoxic factors and whole genome mutation analyses on various timescales will shed light on the role of damage induced LHM in evolution, biology of species as well as in human health.

Funding note

This work was supported by intramural research funds from NIEHS to Chromosome Stability Group under project Z-01-ES06507

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RELIABILITY SYSTEMS IN PLANTS AS A BASIS FOR EVOLUTION

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Abstract- Reliability of cell and multicellular organism is governed by the action of special systems among which are six types of DNR repair, renewal of membranes, self-assembling of molecular machines on the cell level as well as repopulation processes and cell selections on the level of tissue. These systems reveal and failures in molecular structures due to stochastic process and determine the future trends in the cell development. Reliability systems may be thought of as a basis for the trouble free operation in any living beings and evolution processes.

Keywords: Failures in biological system, DNA repair, signaling, correction of genome, evolution

1. Introduction

Whole world of living beings demonstrates very important inherent property of all cells and multicellular organism: very complex in structural and functional organization cells with tender and fragile supermolecular structures are characterized by invariant reliability of functioning. Determination «reliability» of organism or cell means that during interval of time sufficient to perform the main vital function cells and multicellular organism, as a rule, are exemplified by a faultlessness within the framework of the defined quantitative values of the tension of environment factors.

The faultlessness of functioning of the systems of organism in the proper terms guarantees safety of species. Naturally, the cytogenetic, metabolic and physiological processes in organism are followed by failures in functioning one or another systems. The clearing of these failures represent a reliability of organism. Naturally, reliability of the systems of organism is the result of a long evolution.

Reliability of organism can be described on the basis of a conception of area of reliability under which understand phase space, where as co-ordinates use characteristics of the tension of separate environment factors (temperature, humidity, intensity of light, sources of energy, some mineral elements etc) is taken to mean. The simplest phase space can be two-dimensional when as the basic factors of environment only temperature and humidity are taken into account.

Using a picture of area of reliability it is convenient to appeal to another very important conception of adaptation. Essence of adaptation consists of capacity of cells or multicellular organism to conform to new conditions of environment going out outside the area of reliability. Due to adaptation the area of reliability can broaden in one or another direction of its fields.

Functioning of any biological system, as a rule, is accompanied by appearance of spontaneous damages to molecular or more complex ultrastructures of cells. Among other things, cellular membranes can lose the functional ability by virtue of the fact that lipids are perpetually subjected to peroxyde oxidation and molecules of DNA undergo spontaneously deterioration of this structure.

Character of failures induced by stress factors depends on nature of the last. So, failures in reply to salt stress, naturally, must differ from failures, coming because of action of ultraviolet rays or surplus of heavy metals in environment. Therefore methods of removal of these failures should be different.

Ability of plants to form special systems designed to removal of failures or consequences of deterioration in structure and function of organism is an integral part of vitality. These systems are known as the System of Reliability [Grodzinsky, 1983].

2. The main failures in biological systems

Structural and functional failures in biological systems are recognized. To the first belong Deviations from the canonical forms of structures at the level of molecules, supermolecular structures, cells and multicellular organism on the whole relate to the first type of failures.

Violations of one or another physiological reaction, metabolism, growth, morphogenesis and reproduction fall to the second type of failures. It is difficult to reciprocate the structure and functional failures while the first are always responsible for functional anomalies at the level of molecules can result in failures functional, conditioned that the change of molecules, cells or tissue. Functional failures can be stipulated by distortions in the regulatory mechanisms. of the alarm systems. In this case, too, the structural failures give rise to functional failures.

Three general cell structures suffered failures in biological systems are recognized. These structures are DNA, membranes and supermolecular complexes due to processes of self-assembling.

2.1. DAMAGES OF DNA

Structural damages of unique compounds play a key role in the vital functions of organism on different levels of organization. Examples are found in damages of DNA. Distortions of the structure of this molecule centre around failures of many processes of fundamental importance. There are many types of damages in DNA, among which are depurinated DNA, molecules with single- or double-strand breaks, DNA duplex with cyclobutanic dimers of thymidine, chemical modifications of bases, covalent binding of DNA with nuclear proteins. The failures of more complicated nature can be added to these damages in DNA, namely, distortions in the structure of chromatides and chromosomes.

The structure of DNA within cells is constantly damaged under influence of endogenous and exogenous factors. Dehydration of cells, fluctuations of temperature, presence of substances with mutagenic properties, ionizing radiation, an increase of concentration of active forms of oxygen (free-radical forms, hydroxyl radical, superoxide, hydrogen peroxide, oxide of nitrogen) are responsible for failures formation as well as errors in replication of DNA [Tuteja & oth., 2001].

Absorption of ultraviolet rays by molecules of DNA is accompanied by formation of different type of damages. Greater part of these damages related to modification of nitrogen bases which results in formation of cyclobutanic pyrimidine dimers and pyrimidine-pyrimidone photoproducts. Exactly these damages meet most often. The generation of cyclobutanic dimers of pyrimidines owe to covalent binding of thymidine or cytidine residues adjoining to each other [Sutherland, 1977]. Thymine, cytosine and thymine-cytosine dimers can exist in different conformations. These stereoisomers occur with different frequency. Cis-isomers of thymine dimers are found most often. Such damages of DNA present a large danger for a cell, because they are accompanied with distortion of cell division, complications of replication and transcription as well as deterioration of some regulator functions [Britt, 1999].

Besides cyclobutanic dimers pyrimidine(6-4)pyrimidone photoproducts are appear under influence of ultraviolet rays. These failures arise up due to formation of linkage between 6th and 4th atoms of nearby pyrimidine residues. Their output is below than an yield of cyclobutanic dimers at the action of ultraviolet. Pyrimidine cyclobutanic dimers are characterized by high stability and they do not exposed to the acid hydrolysis.

Appearance of cyclobutanic dimers in DNA of cell is followed by distortion in tertiary structure of this macromolecule, by complication of functioning, that entails braking of replication and transcription, and also appearance of point mutations. As a result survivability of cells impose to severe ultraviolet irradiation, as a rule, goes down.

Amongst the damages of DNA there are the cross-link of nucleotides, various covalent binding of alkyl residues (methyl, ethyl, propyl, butyl residues) to the nitrogen bases, in particular thymine glycols, oxidation of guanine under influence of active

forms of oxygen, tacking of methyl residue to guanine, resulting in formation of O⁶-methyl-guanine. As a result of break of glycosidic linkage uniting nitrogen bases with a deoxyribose takes place and depurinated or, rarer, depyriminated areas in the molecule of DNA arise.

During replication of DNA errors of coupling of nucleotides occur and incomplementary pairs of nucleotides arise in DNA duplex in place of base-pairing A-T or G-C. These incomplementary bases are referred to as mismatches. Although polymerase complex, carrying out a replication of DNA, after every insertion of nucleotide does a step back and checks up the rightness of including of nucleotide though much errors take place, and mismatches as unintaglated wrong pairing of nucleotides. Replacement of false bases in daughter's filaments of DNA duplex take place also in the case of oxidation of guanine because arising up here 8-oxoguanine during replication couple not only with cytosine but also with adenine. As a result of this event bases replacements occur in daughter's filament entailing mutational changes.

There are such types of DNA damages under the action of ionizing radiations: single- and double-strand breaks. Sugar-phosphate bonds undergo breakage in a result of radiation-chemical reactions and single-strand breaks appear in a macromolecule. As a rule, breaks of DNA are the result of attack of free radicals, arising up at radiolyses of water.

The double-strand breaks of molecules of DNA arise up as a result of accumulation of single-strand breaks in the opposed filaments of the DNA duplex. Single - and double track double-strand breaks are distinguished: in the first case these breaks are formed along one track of ionized particles, in the second – at the near coincidence of two tracks. Ratio between single-strand and double-strand breaks at the irradiation of the isolated cells is close to the value 50.

The linkage DNA-protein are very dangerous damage of DNA structure. Covalent bonds, appearing between the proteins of nuclear matrix and nitrogen bases of DNA, collapse neither under influence of organic solvents nor at the processes of denaturizing.

Thus we can see that there are very dangerous failures in structure of DNA with which are related substantial malfunctions in cells and organism, foremost replication and, accordingly, stopping of cell division. Sometimes structural failures in DNA arise from cascade process of the structure realignment. The advent of these failures is accompanied with a severe sacrifice in cell divisions, loss in the inherited information and, in final analysis, to cell death or mutation.

2.2. MAIN DAMAGES OF CELL MEMBRANES

Oxidation of some lipid molecules constituent membrane cell system can initiate a chain reactions of oxidation of other lipid molecules. As this takes place, many intrinsic

failures occur, namely, permeability and bioelectric potential of plasmalemma vary within wide limits, ability to recognize the signals of cell-cell interactions and other systems related to membranes break down. To the damages of molecular components of membranes such functional failures are related, as an distortion in compartmentation of metabolic pools, and also abnormality in an ability of cells to perception of positional information.

It should be emphasized that the failures in the alarm systems of cells unbalance the control of cell-cell interactions, maintenance of different forms of homeostasis from ionic to metabolic and upset the equilibrium in the dynamics of cellular populations. Thus cells and organisms can lose a capacity for adequate reaction on biotic stresses that will be accompanied by development of bacterial and fungal infections.

Failures in membrane system can affect the time perception in organism. The mechanisms of counting out of time play a determining role in implementation of vital strategies. The mechanisms of the so-called biological clock to the present time are investigated mainly in a phenomenological plan. Three basic mechanism of memorizing of time is known: on the sum of effective temperatures, on a photoperiodic reaction and, presumably, on adding up of effects, caused by gravity waves. These mechanisms are related to the cell membranes.

The scale of damages of membranes can appear so considerable, that the functional failure of this structure acquires catastrophic sizes.

2.3. ABNORMALITY OF SELF-ASSEMBLING PROCESSES – FAILURES IN MOLECULAR MACHINES

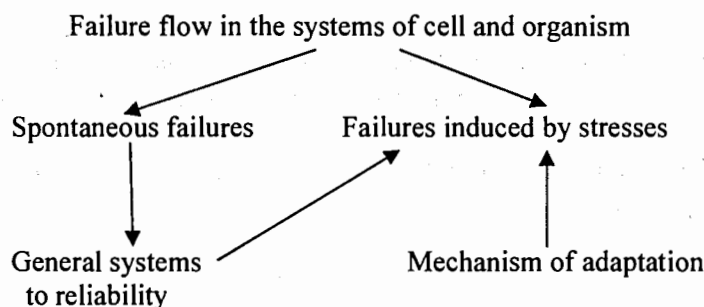
Self-assembling of some molecules of kind proteins, DNA, RNA, lipids take place in cell structures and in the issue supramolecular complexes are generated. These complexes play a role of the "molecular machines" which are responsible for many processes particularly DNA replication, transcription, biosynthesis of proteins, gene regulation, transportation of substances through membranes, formation of the electron transport chains in mitochondria and chloroplasts, enzymatic reactions. These molecular machines permit to put into effect negentropic processes within cells.

The foundation of self-assembling is the phenomenon of molecular recognition that is responsible for formation of complicated molecular complexes. The complementation of the spatial structure of molecules determines molecular interactions. Naturally, any chemical modifications in constituents of self-assemblages gave birth to its degradation. Degradation of the self-assemblages should be considered as failures of a great importance. The rates of some metabolic reactions reflect the frequencies of these failures.

2.4. FAILURE FLOW IN BIOLOGICAL SYSTEM

It should be emphasized that the basic systems of reliability cure the nascent failures in the molecular structure of cells, where as the mechanisms of ontogenetic adaptation provide a realignment of metabolism and morphogenesis for the purpose to shape stress tolerance in cells or multicellular organism.

Failures are characterized by frequency of their appearance. There is a good reason to think that there are flows of failures of certain types in cells. Counteraction to failures is provided with both the general systems of reliability of organism and adaptation mechanisms. These flows are shown in the following chart:



3. Function of the Reliability System in biological objects

The main aim of the Reliability Systems consists in removing of failures or consequences of malfunctions related to failures in biological objects.

3.1. PHOTOREACTIVATION OF DNA

Survivability of cells irradiated with ultraviolet radiation can substantially increase under influence of visible light. Effect of renewal of viability in cells exposed to ultraviolet rays after the action of visible light got the name of photoreactivation. First this phenomenon was discovered in bacteria by A.Kelner [Kelner,1959].

It was shown that special enzymes, photolyases take part in photoreactivation. Photolyases are enzymes the action of which depend on flavine adenine dinucleotide (FAD). These enzymes absorb dark blue or near ultraviolet light. Absorption of light is provided by a presence in the photolyase molecule of two chromophor groups. A decoupling between pyrimidine rings takes place due to energy of light absorbed by photolyase. The specific binding of photolyase to cyclobutanic pyrimidine dimers or to pyrimidine(6-4)pyrimidone photoproducts is accompanied with break of the proper linkages due to energy of quanta of light absorbed by photolyase. The role of enzyme

consists of transmission of energy of electron excitation to one of pyrimidine as a condition of dimer monomerization. As a result of this process there is a complete restructuring of DNA, its repair occurs. This unique reaction is based on the use of not chemical but energy of quanta of light.

CPD photolyases responsible for repair of dimers have been found out in many types of bacteria, fungi, plants, invertebrates and vertebrates. 6-4 PP- photolyases related to repair of pyrimidine(6-4)pyrimidone photoproducts are displayed in the less number of species among which are fruit fly, mulberry silkworm, rattlesnake.

Spectrum of action of photoreactivation is different in various plant species. There are the action spectrums with one or a few maximums of absorption. A maximum of absorption in the action spectrum of photoreactivation is found out in a wide band from 330 to 450 nm. It should be noted that the spectrum of action does not coincide with the spectrum of absorption of the isolated photolyase. Number of molecules of photolyase in cells is very insignificantly: only 8 – 10 molecules provide reliable repair of cell exposed to ultraviolet.

Photoreactivation has been demonstrated in many plant species related to different taxonomic units [Saito & oth, 1969].

3.2. DNA REPAIR

The continuous functioning of the systems of DNA repair is required for the normal vital functions of cells suffered spontaneously nascent errors in the structure of this macromolecule. Naturally, it must exist not alone system of its repair as there are many types of such errors.

3.2.1. *Methyltransferase pathway of DNA repair*

Failures in the form of O⁶-alkylated guanine and O⁴-alkylthymine undergo a repair due to special proteins – methyltransferases which intercept a methyl residue from O⁶-methylguanine and O⁴-methylthymine. As a result the correct structure of DNA is restored. Methyltransferase releasing nucleotides from a methyl can not in subsequent be delivered from a methyl residue because this alkyl is bound very firmly with protein molecule. In this plan methyltransferases can not be referred to genuine enzymes because a release from substrate after a reaction is characteristic for the last. The continuous synthesis of methyltransferase is needed for providing a repair of errors. It has been proved that about 100 molecules of methyltransferase are synthesized in the cell of E.coli for one minute [Soyfer, 1997].

3.2.2. *Repair depurinated sites in DNA*

Glycosidic bond opening between nitrogen bases and sugar lead to formation of depurinated sites in DNA (AP). These sites can be filled with the proper bases with

renewal of glycosidic linkage during replication of DNA in accordance with the rules of complementary nucleotide pairing. Enzymes providing this pathway of DNA repair are given the name DNA-insertases. Direct repair of DNA is carried out in this case.

3.2.3. *Excision repair of DNA*

There are repair pathways when damaged sites are cut from the DNA molecule and thereafter originated gaps are closed with undamaged structures.

Reparation of N-alkylated purines and other modified bases in DNA takes place with participation of specific N- glycosilase- enzymes splitting bonds between the modified bases and desoxyribose. N-glycosilases are also involved in corrections of errors in the form of spontaneous deaminization of cytosine or adenine when these bases are converted accordingly into uracil or hypoxanthine. Nascent gaps are filled with bases in place of the modified basin accordance with the rules of complementation under influence of N-glycosilases.

Uracil-N-glycosilase plays a very important role as an antimutagene factor responsible for elimination of failures related to the errors of replication when dUTP is included in place of dTTP in the structure of DNA molecule. This error is not recognized by DNA polymerases and it will be copied in daughter's filaments of DNA at further replication if this damage will not be removed by uracil-N-glycosilase.

Once the ends of breaks in the filament of DNA are not exposed to chemical modification and hydrogen bonds support the structure of DNA duplex, the DNA filament can be restored by DNA-polynucleotide ligases.

Number of mechanisms of DNA repair are handled by more complicated pathways which are based on synthetic processes. Excision DNA repair is at the heart of these mechanisms. Broadly speaking this pathway of reparation consists of cutting the damaged sites in polynucleotide sequence and filling the nascent gaps with new fragments of molecule to DNA which are synthesized on the proper area of opposite filament used as a matrix. Excision repair consists of the followings stages: recognition of damage, incision of polynucleotide chain, delete of part single-strand DNA and correction of error. A gap is filled up with the fragment of DNA synthesized on the polynucleotide sequence of opposite uncrippled thread of DNA duplex. DNA repair is completed with restoration of an initial undamaged structure. Significance of storage of the inherited information in form of the DNA duplex is distinctly clear: DNA repair is not possible without duple-strand structure.

Clearly excision reparation of DNA is provided by numerous gene products. As pointed out above, there are various chemical modifications of nitrogen bases of DNA and these damages are recognized with special enzymes. Thus uracil glycosilase recognizes uracil in DNA, PD-glycosilases inquire the cyclobutanic dimers of pyrimidines DNA-glycosilase, G-T-glycosilases identify mismatches, MUTY-DNA glycosilase exposes DNA containing G-and mismatch et cetera [Friedberg & oth.,1995].

Glycosilases join in with the damaged bases and tear the glycosidic linkage between base and deoxyribose. As a result depurinated or depyrimidinated sites arise in DNA duplex. The last are identified by AP-endonuclease which makes an incision in the thread of DNA, resulting in formation of single-strand break. Phosphodiesterase break off the bond in sugarphosphate in the depurinated site. Thus a gap arise in thread of DNA opposite uncrippled thread o DNA duplex. DNA a polymerase inserts in this gap complementary fragment of polynucleotide chain and polynucleotide ligase provides joining of the ends of gap.

Exactness of identification of the modified bases by glycosilases is very precise. Nuclease reaction resulting in formation of gaps carries out a multienzyme complex consisting of endonucleases encoded by the genes of *uvrA*, *uvrB* and *uvrC*. Formation of long gaps is carried out due to the synthesis of an appropriate fragment in the course of DNA polymerase reparative replication and polynucleotide ligase generate linkagwe between the ends of gap. As excision reparation proceeds successfully can take place so long as the local threads of DNA duplex undergo a stripping down, besides the enzymes mentioned before other enzymes, in particular, responsible for stabilization of local denaturated DNA as well as factors conditioning locations of sugarphosphate bond breakage in molecule of DNA assist in this process. The cuts are done on each side of the damaged nucleotide. A single uncrippled nucleotide is inserted in a gap due to participation of polymerases. In the described mechanism of reparation a gap can be minimal just in one nucleotide. There are more complicated situation in excision reparation when more extensive gaps are formed as a result of exonuclease activity. Sometimes gaps are long in thousands of nucleotides.

3.3. REPARATION OF MISMATCHES

A wrong pairing of nucleotides can arise during replication. The nascent error affects daughter's, but not matrix thread of DNA. This error of replication is named as mismatch. Repair of mismatches is determined by event of formation optimal strategy of genome correction. There are three main responses on failures in genome: apoptosis, proliferative death of cell or reparation of DNA. The stage of choice of process which will provide reliability of organism is very important. Prosperity of species but not individual cell is the top priority of vital strategy of organism. Therefore in many cases when there are errors genetic code of cell organism will prefer an apoptosis. This phenomenon is often observed in case micro- and macrosporogenese. The alarm systems participate in the choice of concrete mechanism of DNA repair. For example, cyclobutanic dimers are removed by direct photoreactivation as well as by excision repair.

Repair of mismatches is necessary to carry out in daughter's thread of DNA duplex as it will be used in next replication. Therefore there must be a mechanism which allows to distinguish daughter's thread and initiate reparation of mismatches exactly in this

polynucleotide sequences. Such mechanism was found out: GATTS methylase attaches a methyl residue to adenine in a matrix thread after completion of replication. Genes of MUTH, MutL, and MUTS *uvrD* (helicase V) take part in reparation of mismatches.

3.4. REPARATION OF DOUBLE-STRAND BREAKS

Excision mechanism of reparation of DNA can not be directly used in relation to the double-strand breaks. A complementary synthesis of uncrippled fragment of DNA can not truly be carried out as far as repair mechanisms in matrix area of opposite thread of macromolecule is damaged.

Two different mechanism of reparation of cuts is known: homologous recombination and unhomologous reunion of ends of double-strand breaks.

Reparation by an unhomologous reunion is carried out in all phases of cellular cycle. Distortions in complementation of nucleotides, when the thread ends in a double-strand breaks are sewn together with covalent linkage can take place at this mechanism. By other words, reparation of double-strand breaks can be inexact, erroneous. Direct linkage of ends of the threads of DNA at the place of double-strand breaks in such state, that their association is possible, occur with the participation of cohesine and hRad50 which contact with both ends of the breaks. Reparation by the unhomologous fastening of ends of double-strand breaks is usually accompanied by forming of deletion.

Reparation by homologous recombination is possible mainly in S- G2 – phases of cell cycle. At this mechanism of A reparation a gap nascent in every threads of DNA duplex is built up on the identical uncrippled polynucleotide sequence involved in the segment of DNA from the nearest surroundings of the damaged site of DNA. Such segment can be chosen, for example, in a sister chromatid and this kind of DNA repair is made possible only in postreplicative or synthetic phase of cellular cycle.

Difficult processes which provide realization of recombination reparation of double-strand breaks, for example, local denaturation of DNA, hydrolysis of proteins related to DNA in the site of reparation, displacement of nearby segment of polynucleotide sequence complementary to the damaged site are controlled by products of *recA* gene. It is urgent that the segment of DNA duplex which should take part in reparative replication as a matrix have passed a replication.

There are many repetitive nucleotide sequences in genomes of eucaryotes and homologous recombination here is always possible. However fraught such kind of DNA repair is fraught with chromosome translocations. There is a mechanism of suppression of this phenomenon which is usually negative for a cell.

The complex of DNA polymerases can in somewise slip damages in a matrix thread during the replicative synthesis if damages of DNA were saved, for example, cyclobutanic pyrimidine dimers have been saved in a matrix DNA to the moment of replication. Under this condition gaps generate in the daughter's thread. These gaps can

be build up by means of using an uncrippled thread that has arisen in the replication. This is also a homologous recombination consisted of using a conformable part of the thread from a tetraploid set of DNA in a reparative synthesis of DNA fragment filling a gap. The described process is enough complicate as a presence of sister duplex of DNA, local denaturation of this duplex as well as some protease reactions and undercutting of uncrippled thread of DNA are needed for the correct filling of gaps. As marked already the product of *recA* gene takes part in these processes. Apparently, this type of reparation is possible only after doubling of genetic material in a cell, when it passed a synthetic phase. Therefore this type of reparation is named as post-replicative DNA repair.

More than 40 years passed from the moment of opening of post-replicative DNA repair but its mechanisms are not until now exposed the end. However it is known that this kind of repair is the main reason for an induction of mutations.

3.5. SOS DNA REPAIR

The cells are doomed to proliferative death when they conserve the damages of DNA to the phase of replication. The replication in this case can not usually occur. In individual cases cells restore genetic structures so that its division became possible but in so doing an exactness of genetic information will be lost. Another mechanism of DNA repair is known. It lies in an ability of the polymerase complex to form daughter's threads in spite of the fact that DNA damages are contained in matrix threads. As a result a cell restores a capacity for a division though both matrix and daughter's threads of DNA contain errors. *RecA* protein and products of SOS-genes, among which *LexA*, *uvrA*, *uvrB*, *UvrD* take part in this post-replicative reparation of DNA feels like assumption of errors (error-prone repair system). In SOS-reparation takes part. Control above expression of these genes carries out by repressor *LexA*.

Mechanism of DNA repair of this kind got the name of SOS-reparation. This reparation is the very dangerous phenomenon for somatic and reproductive cells because the cells doomed to death will appear inevitably in cellular generations.

3.5.1. *Misrepair*

It was marked at the review of mechanisms of DNA repair that at some from them exact renewal of canonical structure of nucleotide sequence after the removal of errors does not be achieved and recovered failures is retained in the cell genome. The fact of conservation of errors in genome provides reason to talk about the presence of erroneous reparation (misrepair, error-prone reparation). It is hardly probably that conservation of errors in genome can be conserved of as a display of certain biochemical random events since there are kinds of DNA repair among the systems of reparation which can provide complete faultless reparation of DNA.

3.6. REPARATION IN MEMBRANES AND SUPRAMOLECULAR PRODUCTS OF SELF-ASSEMBLINGS

Recovery of membranes is accomplished by membrane flow function of which consist in transfer of new lipid components from the Golgi apparatus to membranes. New lipid materials are used in recovery of oxidated sites in membrane. The rate of renewal of lipids in membrane materials is representative of the failure frequency in membrane. This rate varies over a wide range.

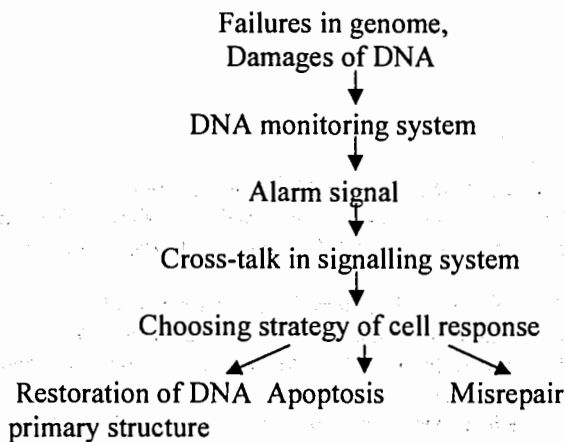
Synthesis of new proteins and the substances of other nature is necessary for spontaneous recovery of self-assemblings in cell. Degradation and recovery of these self-assembling “molecular machines” make up the principal component in the cell metabolism. The products of disintegration of molecular machines are subjected to hydrolysis. New components for self-assembling processes are synthesized. There is precise control over this synthetic processes.

3.7. SIGNAL SYSTEM IN THE MITIGATION OF THE FAILURE CONSEQUENCES

The failures in any biological systems may be regarded as signals activating the Systems of Reliability. There are many signal system in cells and these systems are involved in signalling processes which are implicated in the realization of responses of biological systems on the advent of failures [Szumiel, 2008].

DNA damages are perceived as signals for the system of DNA monitoring. As a result various strategies of cell responses on failures in DNA can be formed. All these strategies should be optimal in relation to a well-being of species as a whole. Misrepair should be mentioned among the strategies of the cell responses on failures in genome since this kind of DNA repair essential for increasing of variability. The foundation of evolution of species is the variability related to misrepair of DNA.

Signaling processes related to the DNA repair schematic are here shown.



4. Conclusion

There is a great depth of meaning in existence of repair mechanisms. The availability of several modes of DNA repair is worthwhile. On the one hand complete reparation of genome gives the most effective conservation of hereditary characters in species, and on the other modes of error-prone DNA repair, has also certain biological meaning as the process of variability necessary for the evolution of species is supported due to erroneous reparation. Thus the erroneous reparation of DNA may be thought of as a principal reason of the variability which is often referred to by name an indefinite variability. If reparation in the all cases of its display was faultless and complete, evolution would be impossible.

It is not inconceivable that the choice of mode of DNA repair as a result of the cross-talk process is controlled by the requirement of species in that the certain level of variability determines an optimal adaptive strategy of organism.

By reliability of the biological system is meant that organization of the processes in cells minimizes flow of failures and provides recovery of system. Reliability of organism is expressed through all forms of its stability. In this connection testing of reliability of cell and multicellular structure is carried out on the different forms of stability as well as on an assessment of an ability of biological system to forestall formation of molecular or supramolecular damages appearance of which entails physiological or cytogenetic failures in cells and organism or presence of mechanisms the action of which consist in remedying these damages (reparation of DNA, membranes, supramolecular structures).

A capacity for adaptation in regard to certain factors which can acquire a stress tension was formed during the evolution of species.

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FLAVONOID BIOSYNTHESIS GENES IN WHEAT AND WHEAT-ALIEN HYBRIDS: STUDIES INTO GENE REGULATION IN PLANTS WITH COMPLEX GENOMES*

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Abstract- Pigmentation by flavonoid pigments is the oldest trait employed for studies in genetics. In the current chapter, we describe application of the flavonoid biosynthesis (FB) genes as a model in the study of regulatory-target gene relationships in allopolyploid wheat genome (*Triticum aestivum*, AABBDD, $2n=6x=42$) and gene functioning in a foreign background in wheat-alien hybrids. Investigation of this multicomponent gene system showed that FB gene regulation cuts across genomes of allopolyploid wheat, the regulatory FB genes contribute more to the functional divergence between the diploid genomes of allopolyploid wheat than do the structural genes, and a good cooperation of the wheat and alien FB gene systems is observed in wheat-alien hybrids.

Keywords: allopolyploids, wheat, flavonoid biosynthesis, multicomponent gene system, genetic mapping, gene regulation

1. Introduction

Bread wheat (*Triticum aestivum* L., $2n = 6x = 42$) is derived from a complex hybridization process involving three diploid species carrying the three homoeologous genomes A, B and D (Kihara, 1944, 1954). The progenitors of the A, B and D genomes (*T. urartu*, *Aegilops speltoides* and *Ae. tauschii*, respectively) are closely related, and

* Radiobiology and Environmental Security / Eds. C.Mothersill, V.Korogodina, C.Seymour. Springer, 2012. P. 31-42

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the gene content in these three genomes is largely conserved. Thus, there are commonly three representatives of each single copy gene present in bread wheat. They are referred as homoeologous genes. Due to allopolyploid nature of wheat chromosome engineering technologies can be successfully applied to its improvement by introgression of alien genetic material from some other cereal species. It is of interest how genetic material derived from different species or genera manages to coexist in a common nucleus. The mechanisms underlying adaptation of different genomes in a common nucleus are known to be acting at the different levels of cell organization. Besides changes in genome size (Ozkan et al., 2003) and genome structure (Song et al. 1995; Pestsova et al., 1998; Ozkan et al., 2001; Kashkush et al., 2002; Salina et al., 2004; Ma and Gustafson, 2008), alteration of gene expression in allopolyploids in comparison with the diploid parents is observed. This chapter will focus mainly on the regulation of gene transcription in multicomponent flavonoid biosynthesis gene system in allopolyploid wheat and wheat-alien hybrids.

Flavonoid substances are divided into 12 classes. Eight of the 12 classes are known to be either plant pigments or precursors of the pigments. Different plant organs can be colored with flavonoid pigments. Mutations in the genes determining coloration are nonlethal and confer easily scorable phenotypes, therefore flavonoid biosynthesis (FB) gene system provides a colorful tool for studies in genetics and biotechnology. FB genes contributed to understanding and discovery of many genetic and epigenetic phenomena, such as basic underlying *principles of heredity* (Mendel, 1865) and gene polymery (Nilsson-Ehle, 1909, 1914), transposable elements and epigenetic gene regulation (McClintock, 1956), posttranscriptional gene silencing (Napoli et al., 1990) and paramutations (Brink, 1956). FB also has contributed to understanding of the organization of metabolic pathways in plants (Quattrocchio et al., 2008; Winkel, 2008). Recently, FB genes have been used as a model to investigate how genes function in a foreign background in wide hybrids and to study interaction between structural genes and their homoeologous regulatory genes in allopolyploid wheat genome (Khlestkina et al., 2008a, 2009a; Khlestkina, 2010).

2. Biological role of flavonoids

Coloration of different plant organs (stems, flowers, seeds etc.) with flavonoid pigments is not a luxury. It plays multiple biological functions in plants. Flavonoids are known to be important for plant growth, development and reproduction, as well as for biotic and abiotic stress tolerance.

In 1883, Charles Darwin noted in his book that pigmented canes, grapes and onion are more resistant to diseases than white varieties of these species, and that red wheat is hardier in northern climates than white wheat (Darwin, 1983). Later, many researchers reported relationships between pigmentation intensity (or content of certain flavonoid

substance and transcriptional level of FB structural genes) and level of resistance to different stressors (or extent of stress-induced damage) (Parker, 1962; Mehdy and Lamb, 1987; Bahler et al., 1991; Singh et al., 1995; Christensen et al., 1998; Chalker-Scott, 1999; Hoch et al., 2003; Giovanini et al., 2006). In particular, pigmentation of plant organs with anthocyanins is important for plants defense against UV-light, cold, draught, salinity stress, heavy metals, irradiation exposure, nutrient deficiency and fungal diseases (Izdebski, 1992; Chalker-Scott, 1999; Farrant, 2000; Bogdanova et al., 2002; Nozzolillo et al., 2002; Ryan et al., 2002; Lachman et al., 2005; Nagata et al., 2005; Suzuki et al., 2005; Plaza et al., 2009; Pozolotina et al., 2007; Tereshchenko et al., 2010).

Furthermore, flavonoids affect plant growth, development and reproduction, membrane permeability and seed germination, and prevent pre-harvest sprouting (Gfeller and Svejda, 1960; Miyamoto et al., 1961; Freed et al., 1976; Khan et al., 1996; Clegg and Durbin, 2000; Debeaujon et al., 2000; Himi et al., 2002; Sasaki and Takahashi, 2002; Peer and Murphy, 2008).

3. Genetic bases of flavonoid biosynthesis in wheat

Flavonoid biosynthesis genes are divided into the regulatory and the target genes. FB enzymes are encoded by the structural genes representing the target genes in the network. Regulatory genes control tissue-specific expression of the target genes. Genetic bases of flavonoid biosynthesis have been intensively studied in maize, rice, barley, *Arabidopsis*, petunia and morning glory (Mol et al., 1988; Chopra et al., 2008). In wheat, until recently, information on flavonoid biosynthesis gene system was very scant. Different wheat plant organs may be colored with flavonoid pigments such as anthocyanins (grain pericarp, coleoptile, culm, leaf blade, leaf sheath, auricle, anther and glume; McIntosh et al., 2008; Khlestkina et al., 2008b), proanthocyanidins (seed coat; Miyamoto and Everson, 1958) and phlobaphenes (glume; Khlestkina, 2010). In wheat genome, at least 70 loci were found, either determining pigmentation of different organs or encoding FB enzymes (McIntosh et al., 2008). Some of the genes determining organ pigmentation may encode transcriptional factors regulating expression of the FB structural genes (Himi and Noda, 2005; Khlestkina et al., 2010a; Khlestkina et al. unpublished).

3.1. GENES DETERMINING PIGMENTATION OF DIFFERENT ORGANS

Three genes determining red coleoptiles coloration were reported in hexaploid wheat, localized on homoeologous chromosomes 7A (Sears, 1954), 7B (Gale and Flavell, 1971) and 7D (Jha, 1964). Microsatellite mapping of these genes showed them to be homoeoloci which were designated *Rc-A1*, *Rc-B1* and *Rc-D1*, respectively

(Khlestkina et al., 2002). Two genes controlling purple culm were localized on chromosomes 7B (Kuspira and Unrau, 1958) and 7D (Maystrenko 1992). Later they and the third gene on chromosome 7A were mapped in homoeologous positions (loci designations: *Pc-A1*, *Pc-B1* and *Pc-D1*) in close linkage with the *Rc-1* loci (Khlestkina et al. 2009b, 2010a). Furthermore, novel genes determining purple leaf blade color (*Plb-A1*, *Plb-B1* and *Plb-D1*) and purple leaf sheath color (*Pls-A1*, *Pls-B1* and *Pls-D1*) were mapped in close linkage with the *Rc-1* and *Pc-1* genes (Khlestkina et al. 2009b, 2010a). Two genes determining purple anther (*Pan-A1* and *Pan-D1*) were mapped on chromosomes 7A (Blanco et al., 1998) and 7D (Khlestkina et al. 2009b) in a short distance from *Rc-A1* and *Rc-D1*, respectively. Comparative mapping in wheat and maize demonstrates that the wheat *Rc/Pc/Plb/Pls/Pan* gene cluster locates in syntenic region in comparison with the maize genome region carrying locus *c1* encoding Myb-like regulatory protein and determining anthocyanin pigmentation in maize (Devos et al., 1994; Khlestkina et al., 2008b). Furthermore, the *c1* gene was used as a probe in Southern hybridization-based mapping in wheat, and its homolog was mapped to the chromosomes 7A, 7B and 7D in position highly comparable with that of the wheat *Rc/Pc/Plb/Pls/Pan* gene cluster (Li et al., 1999).

Two complementary genes determining purple grain pericarp, *Pp1* and *Pp3*, were mapped to non-homoeologous chromosomes 7B and 2A, respectively in both hexaploid (Dobrovolskaya et al., 2006) and tetraploid wheats (Khlestkina et al., 2010b). Comparative mapping in wheat, rice and maize demonstrates that the *Pp3* gene locates in syntenic region of the rice and maize genomes carrying loci *Pb* (*Ra*; Hu et al., 1996; Wang and Shu, 2007) and *Lc* (*R*; Ludwig et al., 1989), respectively, encoding Myc-like regulatory protein determining anthocyanin pigmentation.

Three genes determining red auricles, *Ra1*, *Ra2*, *Ra3*, were assigned to non-homoeologous chromosomes 1D (Gulyaeva, 1984), 4B and 6B (Melz and Thiele, 1990), respectively.

The pigmentation traits described above are due to anthocyanin biosynthesis. In wheat, seed coat, glumes and awns may be colored with proanthocyanidins or phlobaphenes. Three genes determining red seed coat color, *R-A1*, *R-B1* and *R-D1*, were mapped to the homoeologous chromosomes 3A, 3B and 3D (Flintham and Gale, 1995). Three homoeologous loci determining red, smokey-grey or black glume color, *Rg-A1*, *Rg-B1* and *Rg-D1*, were mapped to chromosomes 1A, 1B and 1D (Khlestkina et al., 2006, 2009c). Homeoloci determining black (*Bla*) or red (*Raw*) awn color are closely linked to the *Rg-1* genes (Panin and Netzvetayev, 1986; Börner et al., 2002).

3.2. GENES ENCODING FLAVONOID BIOSYNTHESIS ENZYMES

The genes for phenylalanine ammonia-lyase (*Pal*) were cloned (Liao et al., 1996; Li and Liao, 2003) and mapped (Li et al., 1999) to chromosomes of homoeologous groups 3 and 6. The genes for chalcone synthase (*Chs*) were cloned (Yang et al., 2004) and

mapped (Li et al., 1999) to chromosomes of homoeologous groups 1 and 2. The genes encoding chalcone-flavanone isomerase (*Chi*) and anthocyanidin-3-glucoside rhamnosyltransferase (*3Rt*) were cloned and mapped to chromosomes of homoeologous group 5 (Li et al., 1999; Khlestkina et al. 2009a; Tereshchenko and Khlestkina, unpublished). The genes for flavanone 3-hydroxylase (*F3h*) and dihydroflavonol-4-reductase (*Dfr*) were cloned and mapped to chromosomes of homoeologous group 2 (Khlestkina et al., 2008a) and 3 (Himi and Noda, 2004; Munkvold et al., 2004), respectively. The anthocyanidin synthase (*Ans*) genes were cloned and mapped to chromosomes of homoeologous group 6 (Himi et al., 2006).

4. Regulation in flavonoid biosynthesis gene systems in wheat and wheat-alien hybrids

Mapping the genes participating in pigmentation traits formation in wheat allowed to choose proper genetic models for the further investigation of FB gene regulation in allopolyploid wheat and wheat-alien hybrids.

4.1. TRANSCRIPTION OF FLAVONOID BIOSYNTHESIS STRUCTURAL GENES IN WHEAT

4.1.1. Transcription of flavonoid biosynthesis genes in different organs

Analysis of wheat near-isogenic lines showed that wheat *R* gene determining red seed coat color is a tissue-specific Myb-like transcriptional activator of the FB structural genes (Himi and Noda, 2005).

Using wheat 'Chinese Spring' with green coleoptile and chromosome substitution line 'Chinese Spring'('Hope' 7A) with red coleoptile, Ahmed et al. (2006) showed that *Rc-A1* gene may be a transcriptional activator of the structural genes *Dfr*, *Ans* and *Ufgt* (UDPG flavonol 3-0-glucosyl transferase). The use of such models as substitution, recombinant and introgression lines demonstrated that *Rc-A1*, *Rc-B1* and *Rc-D1* activate transcription of the *F3h* gene in colored wheat coleoptiles (Khlestkina et al. 2008a, 2010a). Transcriptional activating function of the *Rc-1* genes is in agreement with the comparative mapping in wheat and maize, suggesting *Rc* to be an orthologue of the maize *c1* gene encoding Myb-like regulatory protein determining anthocyanin pigmentation (Devos et al., 1994; Khlestkina et al., 2008b). The gene *Pc-1* which is closely linked to *Rc-1* was shown to activate transcription of the *Chs*, *F3h* and *Ans* genes in purple culm (Tereshchenko and Khlestkina, unpublished). Probably, the wheat *Rc/Pc/Plb/Pls/Pan* gene cluster in wheat chromosome 7 originates from the single ancestor Myb-like gene-orthologue of the maize gene *c1*.

Using wheat near-isogenic lines we demonstrated that the *Rg-1* genes activate *Chi* transcription in red and black glumes, whereas the *Pp3* gene activate the *Chs*, *F3h* and

Ans genes in purple pericarp. Furthermore, analysis of expression of the candidate gene for *Pp3* in pericarp of the isogenic lines confirmed the suggestion resulted from comparative mapping in wheat, rice and maize that *Pp3* is a Myc-like transcriptional activator for the structural FB genes (Khlestkina, 2010; Tereshchenko and Khlestkina, unpublished).

4.1.2. Transcription of homoeologous genes in wheat

In 70 - 99 % homoeologous gene series of allopolyploids, all homoeologues are reportedly co-expressed (Comai et al., 2000; Kashkush et al., 2002; Bottley et al., 2006). Analysis of transcriptional level of individual gene copies showed that expression levels of co-expressed homoeologues can sometimes be equal (Morimoto et al., 2005; Shitsukawa et al., 2007; Khlestkina et al., 2008a) or vary (Nomura et al., 2005; Appleford et al., 2006; Shitsukawa et al., 2007). However until recently the question remained as to whether, in an allopolyploid, the interaction between regulatory and target is genome-specific, or whether regulation cuts across genomes. To clarify this, relationships between structural genes and their homoeologous regulatory genes were studied using the *Rc-F3h* gene pair as a regulatory-target model (Khlestkina et al., 2008a). It was shown that each dominant *Rc-1* allele affects the expression of the three *F3h-1* homoeologues equally, but the level of *F3h* expression was dependent on the identity of the dominant *Rc-1* allele present. The lack of any genome-specific relationship between *F3h-1* and *Rc-1* implies an integrative evolutionary process among the three diploid genomes, following the formation of hexaploid wheat. Another conclusion made from this study was that the regulatory genes contribute more to the functional divergence between the wheat genomes than do the structural genes themselves (Khlestkina et al., 2008a).

4.2. GENE REGULATION IN WHEAT-ALIEN HYBRIDS

Alien germplasm keeps stirring interest as a source of genes useful for crop plant species. Novel phenotypes can be obtained using *transgenic* technologies or chromosome engineering approaches. Finely coordinated work of the FB genes of different species (Lloyd et al., 1992; Bradley et al., 1998; Ahmed et al., 2003; Butelli et al., 2008) or *vice versa* their inability to cooperate (Quattrocchio et al., 1998; Bovy et al., 2002) were shown by *transgenic* technologies.

Alternatively to transgenic plants, a wide range of the wheat-alien hybrids provides an important tool to investigate relationships between foreign regulatory and target genes. Using such wheat-rye chromosome substitution and addition lines, it was shown that the rye regulatory *Rc* gene can activate the wheat target gene *F3h* and *vice versa* wheat *Rc* induces expression of rye *F3h*. However, lower level of expression of rye *F3h* in comparison with that of the two wheat orthologues in the wheat-rye chromosome

substitution line 2R(2D) was observed (Khlestkina et al., 2009a). It was suggested that transcriptional dominance observed in the substitution line was a result of post-hybridization changing methylation patterns of rye *F3h* or was due to divergence between rye and wheat *F3h* cis-regulatory elements, which although did not prevent activation of rye *F3h* governed by the wheat *Rc* gene, affected specifically expression level of the gene (Khlestkina, 2010). Analysis of wheat *F3h* transcription in wheat-*Aegilops* chromosome addition and substitution lines, wheat-rye chromosome addition line and wheat-barley chromosome substitution line demonstrated that each of the *Aegilops*, rye or barley *Rc* genes is able to activate wheat *F3h-1* genes. However, the observed compensatory effect of the alien chromosomes was partial, and the bigger genetic distance between wheat and a donor species was, the lower transcriptional level of wheat *F3h-1* genes was observed (Khlestkina, 2010).

5. Conclusion

Pigmentation traits formation in wheat is based on the functioning of the multicomponent gene system including the genes controlling biosynthesis of flavonoid pigments anthocyanins, proanthocyanidins and phlobaphenes in different plant organs and the genes encoding flavonoid biosynthesis enzymes (structural genes). The genes determining pigmentation of different organs are the tissue-specific regulatory genes activating transcription of the structural genes. During pigmentation traits formation in wheat, coordinated work of the regulatory and structural genes located in different diploid genomes composing allopolyploid wheat genome is observed. When some wheat components of this gene system are substituted by orthologous genes from *Aegilops*, rye or barley, the partial compensatory effect takes place.

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MOLECULAR CHANGES IN RADIATION INDUCED THYROID CARCINOMAS IN MICE*

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Abstract: Thyroid carcinomas arising from follicular epithelial cells are the most common endocrine malignancy in man. During studies performed on the population of the Marshall-Islands and after the accident at the Chernobyl nuclear power plant in 1986 a large increase in benign thyroid nodules and thyroid cancer, especially among children was shown. Thyroid follicular carcinomas are categorised into 3 histotypes: papillary, follicular, and undifferentiated (anaplastic). Papillary thyroid carcinomas are the predominant type of the thyroid cancer in patients exposed to external radiation, particularly in children. The classic oncogenic genetic alterations commonly seen in thyroid cancer include RET/PTC rearrangements, Ras point-mutations, PAX8-peroxisome proliferator-activated receptor γ (PPAR γ) fusion oncogene and BRAF mutation. For some families that share the FNMTC syndrome (familial follicular nonmedullary thyroid carcinoma) a predisposition to thyroid tumors has been described. The genes responsible for FNMTC have been identified through linkage analyses of the affected families, TCO (thyroid tumors with cell oxyphilia), PRN1, and NHTC1. This study investigates gene changes in radiation induced follicular thyroid carcinoma. Following low dose exposure of the thyroid by off-targeted irradiation of the alpha-emitter ^{227}Th Thorium, 5 cases of Follicular thyroid carcinomas (FTC) and 1 case of thyroid hyperplasia developed in a highly susceptible mouse strain. In such cases, Comparative Genomic Hybridization (CGH) for numerical changes was performed on the whole genome. Copy number loss affecting the entire chromosome 14 in three out of six cases was observed. A similar pattern of chromosome 14 deletions was already reported in thyroid tumours of other mouse strains following high-dose exposure or oncogene activation and therefore suggests that this deletion is not associated with genetic predisposition in different mouse strains to thyroid tumourigenesis

Keywords: mice, radiation induced follicular thyroid carcinoma

* Radiobiology and Environmental Security / Eds. C.Mothersill, V.Korogodina, C.Seymour. Springer, 2012. P. 307-316

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1. Introduction

1.1. EPIDEMIOLOGY

Malignant late effects in the thyroid gland following irradiation currently attract much attention and have been widely investigated for various aspects. The first investigations were performed on the Marshall Islands population contaminated from nuclear-weapon test explosions (Takahashi *et al.* 1997). In this study, a clear increase of benign thyroid nodules was seen in all age classes exposed to the nuclear fallout (Takahashi *et al.* 1999a; Takahashi *et al.* 1999b). However malignant thyroid carcinoma appeared mainly in patients that were exposed as children.

Thyroid gland late effect studies showed great importance after the Chernobyl nuclear power plant accident in 1986 when a significant increase in the incidence of thyroid cancer among children in Ukraine and Belarus was observed. The regions affected by the fallout from the Chernobyl NPP accident were heavily contaminated with different radioisotopes, with ¹³⁷Cs and ¹³¹I being the most pronounced. The incidence of thyroid carcinomas in children in affected regions increased from less than 1 per million to more than 80 per million. In this category of patients papillary thyroid carcinoma was the predominant type of thyroid cancer (Baverstock *et al.* 1992; Stsjazhko *et al.* 1995; Richter *et al.* 2004). The relative risk for thyroid cancer has been found to be significantly higher for patients who were exposed during childhood as compared to those exposed at an older age (Nikiforov *et al.* 1996). At present there still remains an increasing trend of thyroid malignancy among population in the aforementioned regions (Sumner 2007).

Another study involved investigation of an Israeli population, mainly childhood, treated for tinea capitis in 1950 showed increase in long term relative risk of thyroid cancer formation (Sadetzki *et al.* 2006). Now a new study investigates population of Serbia that was also treated for tinea capitis with ionising radiation in 50th. Late health consequences can be expected in treated people (Shvarts S 2010).

1.2. CLASSIFICATION OF THYROID TUMOURS

On a global scale thyroid carcinomas are the most common endocrine neoplasm with an incidence of roughly 1%. Approximately 95% of all thyroid tumours arise from thyroid follicular epithelial cells whereas 5% of thyroid tumours are medullar thyroid carcinomas of parafollicular C-cell origin. Thyroid follicular cells are responsible for iodide uptake and thyroid hormone synthesis. They may undergo neoplastic transformation into carcinoma of 3 histotypes: papillary, follicular, and undifferentiated (anaplastic).

Papillary thyroid carcinomas are the most common thyroid tumour in countries with sufficient iodine diet and comprise up to 80% of all thyroid malignancies. Follicular

thyroid carcinomas are more prevalent in regions with insufficient iodine intake and represent approximately 10-20% of all thyroid malignancies. Up to 7% of all thyroid tumours are represented by the familial form of nonmedullary thyroid carcinoma (FNMTC). FNMTC is defined by the presence of differentiated thyroid cancer of two or more first-degree relatives. Thus diagnosis of FNMTC may be reduced due to the presence of coexisting syndromes such as familial adenomatous polyposis (FAP) or Gardner's syndrome. In addition it is also difficult to specifically attribute malignancy to FNMTC when exposure to environmental factors such as ionising radiation is known to increase the risk of developing thyroid carcinomas (Fagin 1997; Pal *et al.* 2001; McKay *et al.* 2004; Sturgeon & Clark 2005).

Anaplastic thyroid carcinomas are refractory to iodine uptake and characterised by a poor prognosis. Medullary carcinomas or C-cell carcinomas arise from calcitonin-secreting cells and in 20-25% are associated with inherited syndromes (multiple endocrine neoplasia) and familial medullary thyroid carcinomas. In the remaining 75% cases, medullary carcinomas are sporadic.

1.3. GENE CHANGES IN THYROID CARCINOMAS

The aetiology of most thyroid cancers is not clear. Some might develop as a late effect following exposure to ionising radiation. However mutations of DNA repair genes could also lead to gene rearrangements and development of the diseases.

The classic oncogenic genetic alterations commonly seen in thyroid cancer include RET/PTC rearrangements (cytogenetically detectable as an intra-chromosome 10 translocation), Ras point-mutations, PAX8-peroxisome proliferator-activated receptor γ (PPAR γ) fusion oncogene and BRAF mutation.

External radiation is a well-known exogenous agent that can cause thyroid carcinomas, specifically papillary thyroid carcinomas. The predominant molecular alteration in these tumours are chromosomal rearrangements affecting the RET proto-oncogene. This results in the synthesis of chimeric proteins, composed of the catalytic Tyrosine-Kinase domain of the RET proto-oncogene and variable C-terminal domain fusion to different genes. There are three major forms of rearrangement with RET/PTC1, RET/PTC2 and RET/PTC3 being the most frequent variants. RET/PTC1 is most often found in patients who were exposed to external radiation. On the other hand, RET/PTC3 is most often found in papillary thyroid carcinomas that developed within the first decade after exposure to the Chernobyl accident fall-out. RET/PTC3 is often associated with a solid variant of papillary thyroid carcinoma, exhibiting a more aggressive clinical appearance while RET/PTC1 does not. RET rearrangements are detected in 15-25% of papillary thyroid carcinomas (Komminoth 1997; Nikiforov *et al.* 1997; Santoro *et al.* 2006; Lin *et al.* 2008).

Follicular carcinomas are characterised by somatic rearrangements in the gene encoding the nuclear receptor PPAR γ (the peroxisome proliferator-activated receptor

gamma). PPAR γ rearrangements identify in approximately 25-30% of follicular carcinomas. PAX8-PPAR γ is a thyroid-specific mutation and one of the members of a family of PPAR γ rearrangements in follicular carcinomas (Farid 2004).

Somatic point mutation in the BRAF gene has also been identified in thyroid cancer (Namba *et al.* 2003). BRAF gene produces the protein BRAF, which is involved in cell signalling and in cell growth. BRAF gene is located on chromosome 7 and a mutation that alerts valine 599 to glutamine acid (V559E) in the BRAF kinase domain has been identified in 35-45% of papillary thyroid (Xing 2005).

Activation of Ras mutations are found in benign and malignant follicular neoplasms, and rarely in papillary thyroid carcinomas. Mutations in the Ras genes (H-Ras, K-Ras and N-Ras) arise from single base substitutions at codons 12, 13 or 61. These mutations have been found in 10-15% of all human cancers and in up to 50% of follicular thyroid malignancies (Fagin 2002). In addition the p53 tumour suppressor gene also appears to play a role in the genesis of anaplastic thyroid cancers (Fagin *et al.* 1993).

A predisposition to thyroid tumors has been described for some families that share the FNMTC syndrome (familial follicular nonmedullary thyroid carcinoma). The genes responsible for FNMTC have been identified through linkage analyses of the affected families, TCO (thyroid tumors with cell oxyphilia), PRN1, and NHTC1 (Pal *et al.* 2001; McKay *et al.* 2004).

In addition to these point mutations and chromosomal translocations, little is known about the contribution of DNA copy number changes in thyroid cancerogenesis after radiation exposure.

1.4. AIM OF THE STUDY

To use an experimental animal model of radiation-induced thyroid carcinoma in a genome-wide screen of DNA alteration using array CGH. Comparison of gene alteration in the genome of mouse strains with different sensitivity to irradiation after induction of the thyroid tumours.

2. Material and Methods

2.1. TISSUES AND GENOMIC DNA EXTRACTION

Follicular thyroid carcinomas (FTC) for CGH studies developed in FVB/N mice following low-dose exposure to the thyroid by off-targeted radiation of bone-seeking alpha-emitter ²²⁷Th. Following necroscopy, they were fixed in 5% formalin, embedded in paraffin and histologically analysed in the Institute for Pathology (Figure 1). DNA from those tumours were extracted using the Qiagen DNAease kit (Qiagen Inc). Case, diagnosis and sex of mice are shown in Table 1.

Reference DNA from mice tails was obtained by digestion of a tail tip in a 750 μ l of lysis buffer overnight. The buffer was composed of 50mM Tris, pH 8 100mM ethylene diaminotetraacetic acid (EDTA), 100mM NaCl and 1% sodium dodecyl sulfate (SDS) with 0.5mg/ml proteinase K at 55°C. After adding to the mixture 250 μ l of 6M NaCl and centrifuging for 10 min at 10000rpm, supernatant was transferred to a fresh tube and DNA precipitated by adding one volume of isopropanol. DNA was pelleted, washed twice for 15 minutes with 75% ethanol, air-dried, and resuspended in 200 μ l of H₂O (Ampuwa).

Table 1. Case, diagnosis and sex of mice

Case number	Tissue	Diagnosis	Sex
032	Thyroid gland	FTC	male
457	Thyroid gland	FTC	male
909	Thyroid gland	FTC	male
910	Thyroid gland	FTC	female
1026	Thyroid gland	FTC	male
1375	Thyroid gland	FTC	male



Figure 1. FTC developed in mouse of FVB/N mouse strain exposed to Th-227

2.2. WHOLE GENOME AMPLIFICATION

Whole genome amplification of the thyroid tumor DNA was done using GenomPlex® Complex Whole Genome Amplification (WGA) kit (Sigma) following the protocol of the manufacturer. The quality of the WGA DNA was than qualitatively determined by loading 10 μ l of the amplified DNA onto 1.5% agarose gel.

2.3. COMPARATIVE GENOMIC HYBRIDIZATION (CGH) ON BAC ARRAYS

CGH is a technique that can identify and map DNA copy number changes in tumours relative to a normal tissue in a single hybridisation experiment (Ried *et al.* 1997). 1 Mb CGH arrays containing approximately 3000 BAC probes that cover the entire genome (Codelink DNA array slides). For each experiment, 450 ng of either female or male reference DNA and 450 ng of tumour DNA were labelled with Gy3-dCTP and Cy5-dCTP (Perkin Elmer Life Science), respectively, in a random-primed labelling reaction. Fluorescent labelled tumour and reference DNA of the opposite sex

were co-hybridised with a presence of mouse cot-1 DNA, (to block repetitive sequences) washed and dried in an automated TECAN HS400 hybridisation station. Co-hybridization was performed 'sex-mismatch', meaning that male tumour DNA was hybridized with female reference DNA and opposite and the expected difference in number of X-chromosomes could serve as an internal control for the experiment. The slides were scanned with a TECAN Laser Scanner and the red-to-green intensity ratio determined for each BAC clone with an array analysis software (GenePix Pro 6.0, Axon Laboratories) (Figure 2).

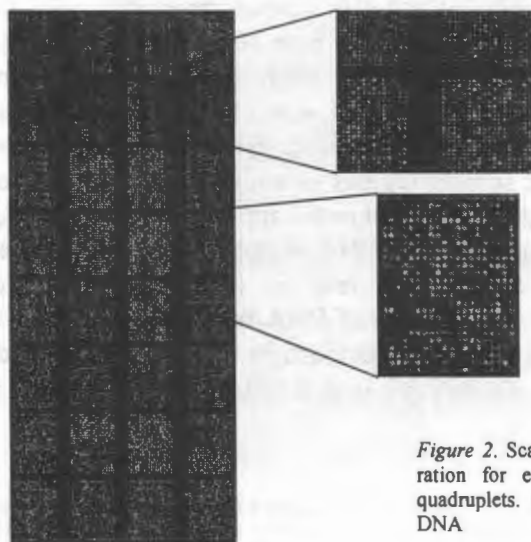


Figure 2. Scan of the 1Mb CGH array slide with measured intensity ration for each BAC clone. All BAC clones are repeated in quadruplets. Green channel: Tumour DNA; Red channel: reference DNA

For every single BAC clone on the array, a shift in the intensity ratios of the tumour DNA fluorescence signal to the reference DNA fluorescence signal is indicative of DNA copy number changes in the tumour sample. For all genomic regions where the tumour DNA carries the normal two copies, the related BACs on the array show an equal intensity of green (i.e. tumour DNA) and red (i.e. reference DNA) fluorescence intensity. In case of a region of chromosome or genes being lost or deleted, the tumour DNA hybridizes less intense and hence causing an overall colour-shift to red. In contrary, if the tumour cells acquired a DNA-copy number in a certain genomic region, this would result in an increased hybridisation signal of the tumour DNA onto the related BAC clone, equivalent to a colour-shift towards green.

Further analysis of the data was performed with CAPweb array CGH evaluation platform, which was accessible via Internet server.

3. Results

3.1. ARRAY CGH ANALYSES

In three out of six cases (032, 910 and 1026) a deletion of the entire chromosome 14 was observed. In addition, case number 910 also suggested a deletion of the whole chromosome 7. The remaining three cases (457, 909 and 1375) did not show significant chromosome copy number changes. The total number of BAC spots showing a useful hybridisation signal varied between 75% and 97%. The expected 'sex-mismatch'-signal could be observed only in case numbers 032, 457 and 910, whereas cases 909, 1026 and 1375 failed to pass this qualitative quality parameter. For case 1026 a 'sex-mismatch'-signal could be found in a repeated experiment after using whole-genome preamplification where deletion of the chromosome 14 was confirmed. This Control hybridisation produced 53% detectable BAC clones. All cases exhibit significant noise, which probably masked the detection of smaller regions of copy number changes on other chromosomes. One of the reasons that could lead to the appearance of huge noise and loss of a number of BAC clones could be that DNA samples consisted of small DNA fragments (partially degraded DNA). A PCR reaction with different sets of primers was performed to determine the length-range of DNA fragments presented in investigated samples. The results in Figure 3 clearly demonstrate that DNA samples do not contain fragments much longer than ~200bp. All array CGH profiles are shown in Figure 4.

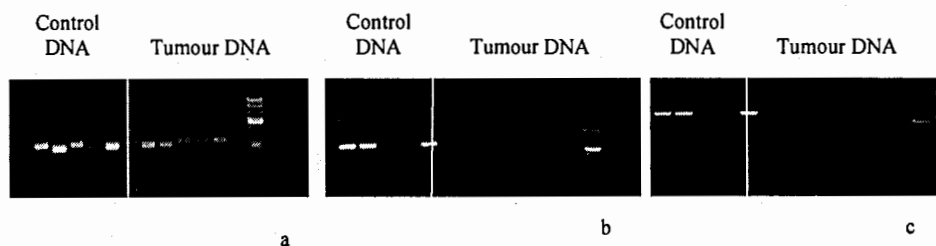


Figure 3. Gel-electrophoresis of PCR products: (a) show fragments of 180bp range; (b) show fragments of 530bp range; (c) shows fragments of 650bp range. Tumours DNA were loaded with next order of cases 457, 909, 910, 1026 and 032 respectively.

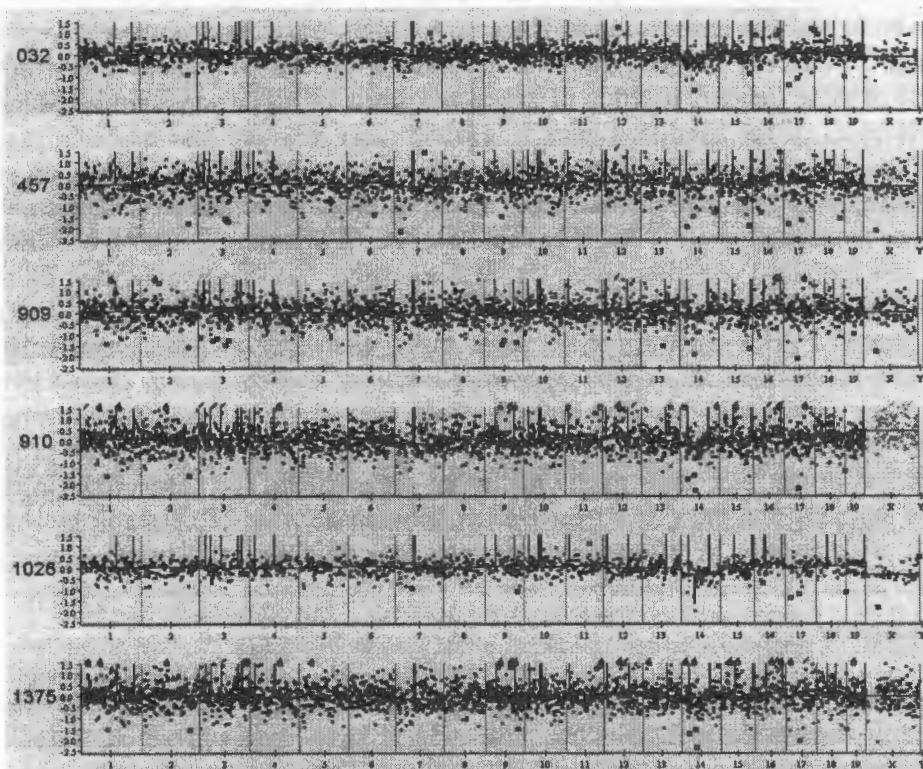


Figure 4. Result of array CGH analysis. Numbers along abscissa relate to chromosome, from left to right is the distance from p-arm telomere to q-arm telomere. Numbers on the ordinate axis give relative copy number changes in tumour DNA as a logarithm to base 2. From top to bottom case 032, 457, 909, 910, 1026 and 1375. Each single point represents hybridisation of tumour DNA relative to normal DNA on one distinct genomic BAC probe. Dots in black are classified as unchanged in tumours copy numbers, dots in red classified as reduced copy numbers and dots in green as increase in copy numbers. BAC clones taken into squares are outliers.

4. Discussion

This study aimed to investigate gene changes in radiation induced follicular thyroid carcinoma. CGH was performed for numerical changes within the whole genome in 5 cases of follicular thyroid carcinomas and 1 case of thyroid hyperplasia that developed following low dose exposure of the thyroid by off-targeted radiation of the alpha-emitter ^{227}Th in strain FVB/N. Using array CGH we could show deletions of the entire chromosome 14 in 3 out of 6 cases. Despite the fact that DNA obtained from formalin fixed paraffin embedded tissue was not of high quality and one case failed to show internal control signal, after performing whole genome amplification, deletion of chromosome 14 in that particular case was confirmed. These observations correspond to data from recent studies where follicular thyroid carcinomas were induced by ^{131}I in

backcrossed mice (G.Hözlzimmer, GSF Pathology) (GC 2007). Therein, 5% of mice (F1-hybrids and backcrosses of C3H/He and C57BL/6), treated with relatively high doses of ^{131}I (activity 111 kBq), developed follicular thyroid carcinomas after exposure. In 38% of these tumours deletion of the chromosome 14 was also observed. Moreover in 75% cases of the irradiated mice G. Hözlzimmer detected loss of heterozygosity (LOH) on chromosome 14. Because injection of the alpha-emitter ^{227}Th used in the present study targets predominately the mouse skeleton, the gamma-dose from ^{227}Th daughter products (mainly ^{233}Ra) that could expose the thyroid gland must be estimated to be low (personal communication Dr. M.Rosemann). The observation that follicular thyroid carcinoma in both high doses ^{131}I treated C3H x C57BL/6 mice and low dose ^{227}Th treated FVB/N mice share the same histological type and the same molecular alteration implies that the congenital predisposition of FVB/N mice is caused by very early steps of the carcinogenic process of thyrocytes. In contrast, both the gross-histomorphology of a tumour as well as large chromosomal alterations are probably determined in the later steps of the malignant progression. The observation in recent studies suggests that there is no correlation between deletion of chromosome 14 and genetic predisposition in different mouse strains to thyroid tumourigenesis.

5. Summary

Our observations suggest that congenital predisposition of FVB/N mice is caused by very early steps of the malignant transformation of thyrocytes, without any association to deletion of chromosome 14 in tumours itself.

6. Acknowledgments

I would like to thank Head of the Department of Radiation Cytogenetics Helmholtz Center Munich Prof. Dr. H. Zitzelsberger and to the Head of the Master course in Radiation Biology University College London Prof. Claus R. Trott.

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CYCLIN-DEPENDENT KINASE CDC28: INVOLVEMENT IN CELL CYCLE REGULATION, GENOME STABILIZATION, CHECKPOINTS, AND REPAIR

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Abstract- The CDK family of enzymes is required for the G₁-to-S-phase and G₂-to-M-phase transitions during the cell-division cycle of eukaryotes. In addition, such process as checkpoint-control and repair are also regulated by phosphorylation of CDK. In the budding yeast, *Saccharomyces cerevisiae*, the CDK/CDC28 kinase is the best studied. In this review the molecular mechanisms of CDC28 functioning will be considered.

Keywords: protein kinase CDC28; yeast *Saccharomyces cerevisiae*; cell cycle regulation; repair, checkpoint

Abbreviations: CDKs, cyclin-dependent kinases; CDC, cell division cycle; APC/C, anaphase promoting complex/cyclosome; IR, ionizing radiation, HU, hydroxyurea; HR, homology recombination; NHEJ, non-homology end joining

1. Introduction

Cell cycle dependent kinases (CDKs) play a central role in cell cycle regulation (Mendenhall and Hodge, 1998). There are 7 CDK and 8 cyclins in the human and one central CDK1/CDC28 and 7 cyclins in budding yeast. CDKs are highly homologous (40-75 % similarity among hCDK1 through hCDK7 and 62 % for hCDK2 and yCDC28). At present time it is possible a 3MD computer simulation of kinase complex which facilitates understanding of interactions between the kinase subunit, cyclin, substrate, and regulator proteins at kinase complexes in the wild type and mutant forms (Kholmurodov and Koltovaya, 2009). Yeast CDC28 is an excellent model for studying CDK regulation and their role in cell division and genetic tolerance. CDC28 is active and required during all phases of the cell cycle. Lesions of CDC28 functioning have pleiotropic manifestations: for example the *cdc28-srm* mutation decreases the mitotic

stability of the native chromosomes and recombinant circular mini-chromosomes, increases cell sensitivity to damaging agents, and fails the cell cycle arrest in G₀/S, G₁/S, S/G₂, G₂/M (Devin et al., 1990; Koltovaia et al., 1998; Kadyshevskaja and Koltovaia, 2009). It seems likely that in budding yeast CDC28 mediates cellular response induced by DNA damage including checkpoint control and repair. The well known checkpoint genes *RAD9*, *RAD17*, *RAD24*, and *RAD53* have been found to belong to one epistasis group called the *RAD9*-group as regards cell sensitivity to γ -radiation (Koltovaia et al., 2008a). An analysis of the radiosensitivity of double mutants has revealed that the mutation *cdc28-srm* is hypostatic to each of the checkpoint mutations *rad9 Δ* and *rad24 Δ* , and additive to *rad17 Δ* and *rad53* (Koltovaia et al., 2008b). Thus epistatic interactions have demonstrated *CDC28* belongs to this epistasis group and a branching *RAD9*-dependent pathway. *CDC28* can also participate in a minor mechanism involved in determining cell radiation sensitivity independently of the mentioned *RAD9*-dependent pathway.

In the human, CDK have proto-oncogenic properties. Failure to arrest the cell cycle in CDK mutant contributes to cancer development and may cause resistance to standard treatments. Major advances in the understanding of cell cycle regulation mechanisms provided a better knowledge of the molecular interactions involved in human cancer. This progress has led to the promotion of new therapeutic agents.

Moreover, the components of the cell cycle progression are probably involved in other non-cancerous diseases (for example, mitochondrial diseases) and their role must be defined. In yeast, *CDC28* influences the mitochondrial genome stability (Devin et al., 1990). In undisturbed yeast cells, *CDC28* was required for a high frequency of mitochondrial *rho*⁻ mutations. The effects of the *cdc28-srm* mutation on spontaneous and induced *rho*⁻ mutability seem to be opposite to its effect on the rate of the loss of natural chromosomes and recombinant plasmids. However, the raised mitotic stability (compared to the normal level) of the mitochondrial genome in *cdc28-srm* cells may in fact reflect a relative decrease in the stability of the mitochondrial *rho*⁻ plasmids which presumably form upon the recombination of the so-called excision sequences and replace the normal mitochondrial DNA in the course of *rho*⁻ mutagenesis. Molecular mechanisms of *CDC28* participation in cell tolerance were considered – in particular the hypothesis that links genetic stability in yeast to changes in the chromatin and mitochondrial genome organization dependent on the gene *CDC28*.

Although protein kinase activity of Cdc28p is under multiple, complex controls, the protein product is stable and naturally occurs in excess. Transcriptional and translational regulation of Cdc28p has not been considered important. There have been many excellent reviews of how *CDC28* activity is generated and regulated (Mendenhall and Hodge, 1998; Viallard et al., 2001). There is one important aspect in studying of yeast kinase *CDC28*. Kinase is essential, operates during all cell cycle and has numerous substrates (~600). The choice of *cdc28* mutants is very important, because mutations may disturb limited (not all) number of processes. Now we know mutants blocking

these or those processes. From this point of view the mutation obtained by us (*cdc28-srm*) is very interesting and perspective.

Because of a limited volume of article let us consider some of the mentioned problems.

2. Regulation of cell cycle by CDC28

The kinases catalyze the transfer of γ -phosphate in adenosine triphosphate (ATP) to a protein substrate. The eukaryotic cycle is coordinated by several related Ser/Thr protein kinases (specifically phosphorylate Ser or Thr in protein substrates), each consisting of a catalytic cyclin-dependent kinase (CDK) subunit and a regulatory cyclin subunit. During each phase of the cell cycle, Cdks form a complex with specific cyclins that activate Cdks and help target them to their substrates (Bloom and Cross, 2007). Waves of CDK activity drive events of the cell cycle through phosphorylation of key substrates. To accomplish these waves of activity, CDK associates with different regulators throughout the cell cycle. The expression of several of these regulators is periodic which serves to limit their window of action to the proper time in the cell cycle. The transient appearance of these cyclin-CDK complexes drives cell cycle events such as cell growth (CDK4/cyclin D and CDK6/cyclin D in G_1), DNA replication (CDK2/ cyclin E in G_1/S and CDK2/cyclin A in S) and cell division (CDK1/cyclin A and CDK1/cyclin B in G_2 and M) in human.

The budding yeast *Saccharomyces cerevisiae* is a suitable eukaryotic model and possesses five CDKs (CDC28, Pho85, Kin28, Ssn3, and Ctk1). The best studied CDC28 is the central coordinator of the major events of the yeast cell division cycle. The first wave of CDK activity occurs when Cdc28p associates with G_1 cyclins and small protein Cks1. The G_1 cyclins are Cln1p, Cln2p, and Cln3p. Cdc28p/Cln3p activity is required for setting the size threshold at which cells pass through START (commitment to duplication and division). Once committed Cdc28p/Cln3p inactivates a repressor of G_1 transcription, Whi5p, which in turn leads to active SBF (Swi4p-Swi6p) and MBF (Mbp1p-Swi6p), transcription factors that promote transcription of *CLN1*, *CLN2*, and genes required for S-phase. Cln1p and Cln2p are important for initiating polarized growth at the site of bud emergence, promoting spindle pole body (SPB) duplication and inhibiting Sic1p and Cdh1p, two CDK inhibitors. During G_1 , Sic1p binds and inhibits the growing pool of Cdc28p/B-type cyclin complexes. Towards the end of G_1 , Cdc28p/Cln1p and Cdc28p/Cln2p complexes phosphorylate Sic1p and target it for degradation. The absence of Sic1p allows a wave of CDK/B-cyclin activity that drives DNA replication and entry into mitosis.

The initiation of DNA replication is regulated by three protein kinase classes: cyclin-dependent kinases (CDK), Dbf4p-dependent kinase (DDK) and the DNA damage checkpoint kinases. CDK phosphorylation of two key initiation factors, Sld2p

and Sld3p, promotes essential interactions with Dpb11p, whereas DDK acts by phosphorylating subunits of the Mcm2-7p helicase (Zegerman and Diffley, 2010). CDK has an additional role in replication by preventing the re-loading of Mcm2-7p during the S, G₂ and M phases, thus preventing origin re-firing and re-replication. During the G₁ phase, both CDK and DDK are down regulated, which allow origin licensing and prevents premature replication initiation.

B-type cyclins CLB1, CLB2, CLB3, CLB4, CLB5, and CLB6 regulate Cdc28p during S, G₂, and M phases. Cdc28p association with CLB5 and CLB6 drives DNA replication. B-type CDKs target two origin recognition complex (ORC) subunits, Orc2p and Orc6p, to inhibit helicase loading (Chen and Bell, 2011). Helicase loading by ORC is inhibited by two distinct CDK-dependent mechanisms. Independent of phosphorylation, binding of CDK to an "RXL" cyclin-binding motif in Orc6p sterically reduces the initial recruitment of the Cdt1p/Mcm2-7p complex to ORC. CDK phosphorylation of Orc2p and Orc6p inhibits the same step in helicase loading. CDK phosphorylation specifically blocks one of the two Cdt1p-binding sites on Orc6p. Consistent with the inactivation of one Cdt1p-binding site preventing helicase loading, CDK phosphorylation of ORC causes a reduction of initial Cdt1p/Mcm2-7p recruitment but results in nearly complete inhibition of Mcm2-7p loading. In addition to being a target of both CDK inhibitory mechanisms, the Orc6p RXL/cyclin-binding motif plays a positive role in the initial recruitment of Cdt1p/Mcm2-7p to the origin, suggesting that this motif is critical for the switch between active and inhibited ORC function at the G₁-to-S-phase transition.

Association with Clb3p, Clb4p, and Clb5p promotes maturation and separation of spindle pole bodies, and proper spindle segregation while Cdc28p association with Clb2p (and to some extent Clb1p, Clb3p, and Clb4p) promotes entry into mitosis and triggers a switch in bud growth from polarized to isotropic. The metaphase to anaphase transition occurs when securin (Pds1p), an inhibitor of DNA segregation is destroyed by the proteasome. Mitotic CDK activity is required to target Pds1p for degradation by directly phosphorylating Pds1p and activating the anaphase promoting complex/cyclosome (APC/C^{Cdc20}) by phosphorylating the regulatory subunit Cdc20. Once DNA is segregated, exit from mitosis (spindle disassembly, cytokinesis and transition to the next G₁) requires that mitotic CDK activity be turned off. This is accomplished by degradation of mitotic cyclins and inhibition of remaining mitotic activity by Sic1p. In the absence of mitotic CDK activity, G₁-cyclins can once again accumulate.

3. Role of CDC28 in checkpoints

The cyclin-dependent kinase CDC28 is an attractive target for the G₁ and G₂ checkpoints, as it is involved in the regulation of the cell cycle progression and is necessary for DNA replication and mitosis (Mendenhall and Hodge, 1998). However,

the mechanism of CDC28 inactivation and involvement in the regulation of the checkpoints is not yet clear.

We demonstrated importance of CDC28 in G₁/S arrest (Kadyshevskaja and Koltovaia, 2009). In early G₁, the kinases CDC28/CLN1,2 activate the Swi6/Mbp1 factor of the transcription of the CLB5 and CLB6 cyclins involved in the assembly of a prereplicative complex (phosphorylating Cdc6p, Orc and Mcm proteins) and DNA replication genes.

DNA damages activate the kinase Rad53, which phosphorylates and inactivates the Swi6 coactivator of transcription of the G₁-cyclins (Sidorova et al., 2003). Hyperexpression of cyclins suppresses the checkpoint defect. Mutations of the catalytic subunit also abrogate the cell cycle arrest. We observed a decrease in the cell cycle delay in G₀ or G₁ upon UV light irradiation in the *cdc28-srm* mutant, but the activity of the kinase complexes in the *cdc28-srm* mutant was not studied (Kadyshevskaja and Koltovaia, 2009). However enlarge form of cells points out to a probably alteration of kinase activity associated with CLN3 which mediates cell size control. For mutation *cdc28-5M* was shown not only effect on the arrest caused by UV-induced damage but also a decrease of kinase activity, including that associated with CLB5 and CLB6 (Li and Cai, 1997). Failure of both checkpoint activation and homologous recombination (HR) in G₁-arrested *cdc28-as1* cells after induction of a homothallics switching HO endonuclease break was shown by Ira et al. (2004). It can be assumed that the G₁ checkpoint controls the activity of some of kinase complexes by regulating cyclin transcription. The inhibition of cyclin transcription or the mutations of the catalytic subunit decreasing affinity to G₁-cyclins inactivate the G₁ checkpoint. MD-simulation of *cdc-srm* kinase structure show shift of PSTAIRE loop which modulate cycline interaction (Koltovaya and Kholmurodov, 2010).

The involvement of CDC28 in the S and intra-S checkpoints was not revealed. The mutations *cdc28-srm* (Kadyshevskaja and Koltovaia, 2009), *cdc28-5M* (Li and Cai, 1997), and *cdc28-Y19F* (Sorger and Murray, 1992), or the inhibition of CDC28 kinase activity (Sorger and Murray, 1992) had no influence on the S checkpoint but some effects of *cdc28-srm*, *cdc28-5M* and *cdc28-as1* on HU-sensitivity observed by us and Enserink et al. (2009). HU inhibits DNA replication by depleting cells of dNTP precursors, presumably by inhibiting ribonucleotide reductase, and causes wild-type cells to checkpoint-dependent arrest cell division in the phase S (Allen et al., 1994). The *cdc28-srm* mutation also influences the checkpoint-dependent arrest of the replicative *cdc6-1* and *cdc9-1* mutants in the late S/G₂ and G₂ respectively (Kadyshevskaja and Koltovaia, 2009). Moreover, *cdc28-srm* increased the frequency of conversion, crossing-over and chromosome loss (Koltovaya et al., 1998; Devin et al., 1990). It is known that defects in the replicative S checkpoint and in the intra-S checkpoint, rather than in the G₁ or G₂ checkpoints, cause an increase in the level of spontaneous rearrangements in the genome (Muyng et al., 2001). It is possible that the influence of CDC28 on the checkpoint in the phase S is not yet determined.

Wild type cells had recovered after 40 min from arrest for 3 h with HU and completed bulk DNA synthesis as showed FACS analysis (Enserink et al., 2009). The *cdc28-5M* mutant also completed DNA synthesis after 40 min but exited from M phase slower than wild type cells. A portion of *cdc28-5M* mutants with sub-G₁ DNA content induced by HU and accumulated, indicating a certain degree of mitotic catastrophe, which is consistent with a study implicating Cdc28p in prevention of mitotic catastrophe (Kitazono and Kron, 2002). Therefore, although Cdc28p is important for cell survival it does not appear to have a major role in either the activation or down-regulation of DNA damage or S checkpoint.

Recovery from checkpoint is down-regulated by the phosphatases Ptc2,3 and Pph3 that dephosphorylate Rad53p (Leroy et al., 2003; O'Neil et al., 2007). In wild type cells Rad53p was largely dephosphorylated 1 h after release from HU arrest and almost completely dephosphorylated 2 h after release, indicating down-regulation of the checkpoint. The degree of Rad53 dephosphorylation in *cdc28-as1* mutants was identical to that of wild type cells, indicating that CDC28 is not required for turning off the checkpoint (Enserink et al., 2009).

More directly determination of kinase functions showed that Cdc28p was not effect on checkpoint activation in S phase. Synchronization *cdc28-as1* cells in S phase did not reveal more sensitivity to killing by HU or MMS and was not essential for the formation of Ddc2p foci (Enserink et al., 2009). Ddc2p functions in the initiation of DNA damage checkpoint activation by mediating the interaction between Mec1p and PCNA-like Rad17p-Ddc1p-Mec3p clamp (Majka et al., 2006). It agrees with findings that Cdc28p by itself is not essential for Rad53p activation (Barlow et al., 2008). But although the frequency of Ddc2p focus formation was not affected by Cdc28p activity, the intensity of Ddc2p foci was lower in 1-NM-PP1-treated *cdc28-as1* mutants. However MMS- (Enserink et al., 2009) and 4-nitroquinoline 1-oxide- (Ira et al., 2004) induced Rad53p activation is independent of Cdc28p, phleomycin-induced Rad53p phosphorylation was partially reduced in 1-NM-PP1-treated *cdc28-as1* mutants (Enserink et al., 2009), indicating that checkpoint activation by phleomycin-induced DNA damage may be partially dependent on Cdc28p.

Origin firing is inhibited during the S phase when DNA damage or replication fork stalling activates the checkpoint kinases. Analogous to the situation in the G₁ phase, the checkpoint kinase Rad53 inhibits both CDK- and DDK-dependent pathways, which acts redundantly to block further origin firing (Zegerman and Diffley, 2010). Rad53p acts on DDK directly by phosphorylating Dbf4p, whereas the CDK pathway is blocked by Rad53p-mediated phosphorylation of the downstream CDK substrate, Sld3p. This allows CDK to remain active during the S phase in the presence of DNA damage, which is crucial to prevent re-loading of Mcm2-7p onto origins that have already fired. These results explain how checkpoints regulate origin firing and demonstrate that the slowing of S phase by the 'intra-S checkpoint' is primarily due to the inhibition of origin firing.

The relative lack of importance of the intra S-phase checkpoint for survival of DNA damage (Kumar and Huberman, 2004) does not mean that the checkpoint is not important for the preservation of genome stability. Indeed, reduction of genome stability (in contrast to reduction of viability) may account for the correlation between loss of the intra S-phase checkpoint and cancer in human (Petrini, 2000).

CDC28 is involved in the arrest in the phase G₂. There is a cell wall integrity checkpoint (Suzuki et al., 2004). Cell cycle arrest in G₂ is reportedly due to inhibition of Clb2p induction and requires components of the dynactin complex. Bud emergence required a stable polarization of the actine cytoskeleton beginning at the end of G₁ and its control by cyclins CLN1,2. Phosphorylation of Y19-CDC28 by the kinase Swel was removed by the phosphatase Mhl1 causing an increase in kinase activity in G₂. In the case of bud emergence disruption, phosphorylation of Y19 is critical for the morphological checkpoint, which delays nuclear division in G₂ and prevents occurrence of binuclear cells. A delay of nuclear division is completely eliminated by *cdc28*-Y19F preventing tyrosine phosphorylation or by over expression of *MIH1* (Sorger and Murray, 1992). Thus, the direct targets of the morphological checkpoint are the kinase Swel and phosphatase Mhl1. However, this mechanism does not function in the case of the spindle body checkpoint (Lew and Burke, 2003) or DNA damage-inducible checkpoint (Chen and Sanchez, 2004), which also induces an arrest in G₂.

Mitosis in budding yeast is regulated at several points. The spindle assembly is controlled in G₁ soon after the START, and the mitotic segregation of chromosomes is controlled later in the metaphase/anaphase; in addition, the exit from mitosis is also controlled. Different stages of mitosis are orchestrated by two or more complexes of CDC28 with B-cyclin. Some of these forms (CDC28/CLB3 and CDC28/CLB4) are required for the initiation of mitosis and for the formation of the mitotic spindle, and other forms (CDC28/CLB1 and CDC28/CLB2) are necessary for the exit from mitosis and for the completion of the cell cycle.

Mitotic catastrophe of *cdc28*^{CST} mutants at non-permissive temperature allow to suggest requiring Cdc28 function in mitotic progression *per se* from an essential mitotic surveillance function. Cdc28 may participate actively in regulating the order and completion of mitotic events such as bipolar kinetochore attachment, separation of sister chromatids, and/or spindle elongation. An alternative mechanism is a substrate-specific defect. For example, deregulation of the anaphase-promoting complex by loss of subunit phosphorylation might affect the dependence and kinetics of anaphase (Rudner and Murray, 2000).

For transition metaphase/anaphase degradation of the inhibitor of the separation of sister chromatids, securine Pds1, are required. The mitotic form of the kinase CDC28 takes part in the activation of the proteasome APC/C^{Cdc20} by phosphorylating the regulatory subunit Cdc20 and following the ubiquitin-dependent degradation of the anaphase inhibitor Pds1 and the cyclins CLB5 and CLB3. The DNA damage checkpoint causes a delay in mitosis through the inhibition of Pds1p ubiquitination due to

phosphorylation of securine by CDC28 (Sanchez et al., 1999). Phosphorylated securine Pds1 mediates binding and nuclear localization of separase Esp1 (Agarwal and Cohen-Fix, 2002) which cleave one of the subunits of the cohesine complex (Med1p/Scc1p) responsible for the cohesion of chromatids. The second pathway includes the Rad53p-dependent phosphorylation of Cdc20p that inhibits the interaction between the Cdc20p and Pds1p (Agarwal et al., 2003) and also mediates the inducible phosphorylation of the subunit of the cohesine complex Scc1 (Sidorova and Breeden, 2003).

The exit from mitosis is associated with the inactivation of CDC28/CLB2. With the arrest of the cell cycle in the G₂/M, the kinase Rad53 maintains the high activity of CDC28 through the inhibition of the kinase Cdc5 involved in the degradation of cyclins and the exit from mitosis (Sanchez et al., 1999).

CDC28 is required for checkpoint activation and HR after induction of HO endonuclease break during the G₂ phase of the cell cycle (Ira et al., 2004). In our experiments with *cdc28-srm*, no defects in the DNA damage-induced G₂ checkpoint and replication block (HU) were detected, but disturbances in the G₂-arrest were observed in the replicative *cdc6* and *cdc9* mutants at nonpermissive temperature. However, using the *cdc28-as1* [F88G] mutation was shown that CDC28 is required for activation by a double-strand DNA break of Mec1p-dependent arrest in G₂ (Ira et al., 2004). Moreover, in the mutant *cdc28-M* abrogating cell cycle arrest induced by DNA-damage a decrease of the CLB2-associated kinase activity required for the exit from mitosis is observed (Li and Cai, 1997). Thus, the different mutant kinase subunit alleles evoke different consequences of the dysfunction of CDC28 and phenotypic properties. It is very interesting to study the specificity of kinase structural rearrangements and their functional role.

4. Participation of CDC28 in repair

Checkpoint genes participate not only in checkpoints but in repair too. It is known that mutations *rad9Δ*, *rad17Δ*, and *rad24Δ* decrease the efficacy of NHEJ (de la Torre-Ruiz and Lowndes, 2000), *rad53* – NHEJ (de la Torre-Ruiz and Lowndes, 2000) and HR (Glaser et al., 1990), *cdc28* – HR (Koltovaya et al., 1998), NHEJ (Ira et al., 2004), and BIR (Tamburini and Tyler, 2005). We have investigated the interactions between checkpoint genes in the determination of sensitivity to ionizing radiation of diploid strains (Koltovaya et al., 2008a). *CDC28* gene belongs to a single, though branched, *RAD9* epistasis group; for instance, kinases *RAD53* and *CDC28* are attributed to different branches of the *RAD9*-dependent pathway. Mutation *cdc28-srm* is epistatic to *rad24Δ*, the others analyzed interactions are additive. *CDC28* does not belong to the *RAD6* and *RAD52* epistasis groups mediating the repair of a major portion of DNA radiation lesions via post-replication repair and HR.

Our results confirmed by results of Enserink et al. (2009). Authors conclude that Cdc28p functions in a genetic network that supports cell viability during DNA damage. Analyzing haploid strains they find genetic interaction of mutation *cdc28-as1* with *rad52*, *rad53* and absence of interaction with *rad17Δ* and *rad24Δ*. *CDC28* also showed strong genetic interactions with components of the post-replication repair pathway, particularly *RAD6* (Enserink et al., 2009). *CDC28* genetically interacts with a wide range of pathways involved in genome stability (HR, sister chromatid cohesion, DNA replication and DNA damage checkpoint, spindle checkpoint, mitotic exit networks and chromatin remodeling), thus underscoring the importance of Cdc28p in this process but mechanisms are not completely understood.

DSBs are repaired by two participial mechanisms: NHEJ and HR. HR is the most accurate DSB repair mechanism but is generally restricted to the S and G₂ phases of the cell cycle, when DNA has been replicated and a sister chromatids are available as a repair template. By contrast, NHEJ operates throughout the cell cycle but assumes most importance in G₁. The choice between repair pathways is governed by CDKs (Aylon et al., 2004; Caspari et al., 2002; Ira et al., 2004; Esashi et al., 2005), with a major site of control being at the level of DSB resection, an event that is necessary for HR but not NHEJ, and which take place most effectively in S and G₂. To initiate HR one strand of the broken DNA duplex is resected in the 5'→3' direction generating ssDNA, that can anneal with a homologous DNA duplex. In *S. cerevisiae* effective resection and HR require sustained Cdc28/C1b (Cdk1/cyclin B) kinase activity (Aylon et al., 2004; Caspari et al., 2002; Ira et al., 2004). The cell cycle control of DSB resection results from the phosphorylation by CDK of an evolutionary conserved motif in a DNA endonuclease Sae2 (Huertas et al., 2008). Sae2p is targeted by the Mec1 and Tel1 kinases in response to DNA damage. Mre11p is recruited quickly to DSB sites, and then replaced by the HR protein Rad52 as ssDNA is formed in S and G₂ cells.

Cdc28/Cdk1-mediated Sae2p phosphorylation modulates the balance between NHEJ and HR during the cell cycle. The commitment to DSB resection is highly regulated to ensure that the cell engages the most appropriate DNA repair pathway, thereby optimizing genome stability. Endonuclease Sae2 with the MRE complex facilitates resection in S/G₂ by mediating an endonucleolytic cleavage close to the DNA brake, thus generating a clean end that can serve as an efficient substrate for nuclease such as MRX and Exo1p. Sae2p activity might be particularly important to initiate resection at DSBs that contain covalently bound proteins that would otherwise resist exonuclease action. Sae2p might also initiate resection at radiation induced DSBs that are resistant to exonuclease because they bear protein-DNA crosslinks or complex damage to bases at their termini. By contrast, at sites of clean DSBs, *SAE2* deletion would only slow down resection and ensuing HR, thus explaining why *sae2* mutants are not as sensitive to radiation as other HR mutants.

The study of the role of the *CDC28* in checkpoints and repair arouses great interest and should be continued.

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DE NOVO MUTATIONS IN Y-CHROMOSOME STR LOCI REVEALED IN PATERNAL LINEAGES OF SIBERIAN TUNDRA NENTSI POPULATION*

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Abstract. Y-chromosome short tandem repeats (STR) markers are widely used in human population genetic studies and forensic applications. Estimation of Y-STR mutation rate has a key role for dating the origin of Y chromosome lineages and for paternity tests. Previous studies demonstrated significant interlocus difference in mutation rate and the positive correlation to STR repeat length. The different ethnic groups and various Y-chromosome haplogroups defined by single-nucleotide polymorphisms were characterized by different Y-STR mutation rates. To date no Y-STR pedigree mutation studies were reported in native Siberian populations. Siberian Tundra Nentsi population possesses many unique genetic features and represents a good model for genealogical studies because of large family sizes, available ancestry information and relatively isolated life style in the extreme north environment. We thoroughly selected 50 paternal lineages with deep genealogical depth from 2 to 6 generations with the total number of 330 males. The number of descendants varied from 2 to 25 per paternal lineage, in average 7 males per lineage. We also included in our analysis six Komi, three Russian and one Khant families who lived in the same villages. Totally 34 STR loci from non-recombining part of Y-chromosome were studied: DYS19, DYS390, DYS391, DYS393, DYS385A, DYS385B, DYS426, DYS388, DYS392, DYS439, DYS389-1, DYS389-2, DYS458, DYS447, DYS449, DYS459, DYS454, DYS464, DYS455, DYS457, DYS448, H4, DYS607, CDY-1, CDY-2, DYS460, YCA-1, YCA-2, DYS576, DYS570, DYS438, DYS456, DYS442, and C4. Eleven males (4 %) had different

* Radiobiology and Environmental Security / Eds. C.Mothersill, V.Korogodina, C.Seymour. Springer, 2012. P. 371-380

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alleles in 6 or 7 STR loci compared to their paternal ancestors. We believe that those men were born out-of-wedlock or were adopted. Traditionally native Siberian people adopt and raise all children who lost their parents. Mutations were observed in 21 out of 34 Y-STR loci. We found relatively higher number of mutation gains (60%) versus losses. Our study has thus shown that the chosen Y-STR loci represent a powerful tool to estimate mutation rates for forensic and population genetic purposes.

Keywords: Y chromosome, short tandem repeats, de novo mutation, tundra nentsi

1. Introduction

The past two decades witnessed an explosion in data from the Y chromosomes in human populations. The inability of the Y chromosome to undergo recombination over most of its length means that the genotype of any individual is traceable with certainty to a single lineage (consisting of the father, the paternal grandfather, one paternal great-grandfather, etc.). Because Y chromosomes have unisexual transmission, migration and genetic drift will have quite different effects on the population genetic structure of Y chromosomes when compared to autosomes, particularly due to the difference in effective population size. Compared to the mitochondrial genome containing 16.5 thousand base pairs, Y chromosome numbers approximately 60 million base pairs, and provides the researchers with a potentially more powerful instrument (Underhill, Kivisild, 2007).

The low level of polymorphism on the on the NRY hindered research for many years. By the end of 1990s a novel mutation detection method and direct sequences have led to discovery of many new Y-specific single nucleotide polymorphisms (SNPs). More than 200 SNP's on the NRY were identified by 2001 (Underhill et al., 2000; Hammer et al., 2001). Almost 600 mutations were incorporated into the Y chromosome binary haplogroup tree (Karafet et al., 2008).

Y-STRs are a short tandem repeat (STR) on the Y chromosome (Fig.1). Due to their diversity, simple structure and the unique feature of being inherited by the paternal lineage, Y chromosome STRs (Y-STRs) have become a valuable tool in evolution studies, forensic medicine, medical genetics and genealogy analysis (Kayser et al, 2004; Kayser 2007). Distinct Y chromosomes identified by STRs are designated "haplotypes," as suggested by de Knijff (2000). Comparing haplotypes of two men with known mutation rates for Y-STRs, one can assess the number of generations to their closest ancestor by paternal lineage.

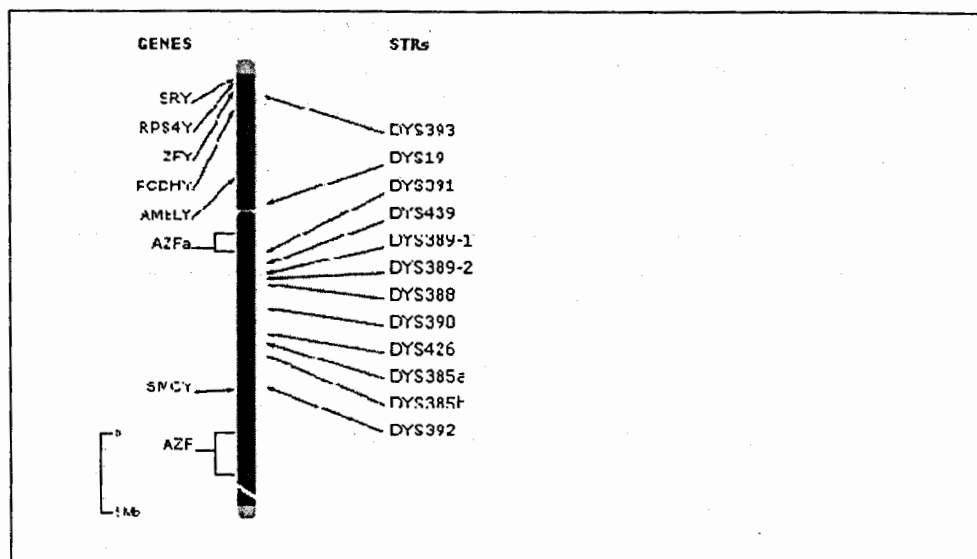


Figure 1. Schematic diagram of human Y chromosome

To date, about 500 polymorphic Y-STRs have been discovered. The most noted, well-described and widely used are DYS19, DYS385, DYS389I, DYS389II, DYS390, DYS391, DYS392, DYS393 и YCAII (Gusmao, Alves, 2005). (Table 1). Less used are DYS434, DYS435, DYS436, DYS437, DYS438, DYS439, DYS460, DYS461, GATA A10, GATA C4, GATA H4 (White et al, 1999; Ayub et al, 2000) Many Y-STRs have been found recently, and even more still are to be discovered (Ballantyne et al, 2010).

Microsatellites show high levels of polymorphism. For example, the quadruplet TAGA (DYS19) may be repeated from 10 to 19 times, the triplet ATA (DYS388) – 10-16 times, DYS385 contains from 7 to 28 repeated quadruplets GAAA etc. The most informative Y-STRs have been chosen as genealogical markers. At present, standard genealogic DNA tests are conducted using 12, 17, 25, 37 or 67 markers, although 6 markers is often already enough to attribute the given haplotype, depending on the combination of repeat numbers, to one of the Y-chromosome haplogroups, from A to R.

For the effective use of these microsatellite markers, it is necessary, besides further improving and reduction in price of typing methods, to create comprehensive databases that take into account regional and ethnic attributes of people, which will allow comparing STR variants and estimating its variation in different populations. It is also important to refine on a convenient and simple classification of these repeats, as for some Y-STR with a simple repeat structure, it is easy to find the consensus, and for others, characterized by a complex structure, it is often a problem. For instance, some obliged changes were made in the classification to include motives of the Y-STRs DYS19 and DYS390 (the first ones to be described), that turned out to be variable as new data had emerged. To prevent such changes, according to the general guidelines of

the International Society for Forensic Genetics (ISFG), “alleles should be named considering the variable and non-variable repeats” (Gill et al, 2001).

Table 1. Structure of some Y-STRs, corresponding GeneBank accession numbers and sizes (Gusmao, Alves, 2005).

Marker	GeneBank	Sequence	Size
DYS19 (DYS394)	X77751	(TAGA)3TAGG(TAGA) _n	143-179
DYS385	Z93950	(AAGG) ₆₋₇ (GAAA) _n	240-312, 324
DYS389 I	G09600	(TCTG) ₃ (TCTA) _n	235-267
DYS389 II	G09600	(TCTG) _n (TCTA) _n N ₂₈ (TCTG) ₃ (TCTA) _n	343-387
DYS390	G09611	(TCTG) _n (TCTA) _n (TCTG) ₀₋₁ (TCTA) ₀₋₄	188-232
DYS391	G09613	(TCTG) ₃ (TCTA) _n	128-160
DYS392	G09867	(TAT) _n	181-217
DYS393	G09601	(AGAT) _n	104-140
DYS437	AC002992	(TCTA) _m (TCTG) _n (TCTA) ₄	180-196
DYS438	AC002531	(TTTTC) ₁ (TTTTA) _{0,1} (TTTTC) _n	201-241
DYS439	AC002992	(GATA) _n	232-260
DYS460 (GATA A7.1)	G42675	(ATAG) _n	105-133
DYS461 (GATA A7.2)	G42671	(TAGA) _n (CAGA) ₁	144-172
GATA A10	G42674	(TCCA) ₂ (TATC) _n	150-178
GATA C4	G42673	(TCTA) ₂ [(TCTA) ₂ (TGTA) ₂] _{2,3} (TCTA) _n	238, 246-274
GATA H4	G42676	(AGAT) ₄ CTAT(AGAT) ₂ (AGGT) ₃ (AGAT) _n N ₂₄ (ATAG) ₄ (ATAC) ₁ (ATAG) ₂	268-292

2. Materials and methods

A Tundra Nentsi population of the size of about 2000 people from Pur district of Yamalo-Nenets Autonomous Okrug has been under study since 1992. A comprehensive gene pool survey including the reproductive and demographic indices, health evaluation, and the genetic analysis employing the state-of-the-art methods and molecular genetic markers has been performed. The population has been extensively studied including the classic blood group marker polymorphism rates as well as the mtDNA and Y-chromosome variabilities (Osipova et al., 1996; Duzhak et al, 2001; Karafet et al., 2002, Tambets et al., 2004).

We attempted to achieve 2 goals – first, to study Y-chromosome microsatellite haplotype diversity in the Siberian population of Tundra Nentsi, and, second, to evaluate the Y-chromosome microsatellite mutation level associated with the adaptation of the Nentsi population to the technogenic environment.

In the present study, we are primarily concerned with the second aspect, which appears important due to the prevalence of chemical and radiation mutagenic factors in the human habitats. A multidisciplinary radioecology and genetic study by the Siberian Branch of the Russian Academy of Sciences was carried out over the period of 1997-2007 (Osipova et al, 1999, 2002; Korovkina et al, 2004). It has been shown that the areas inhabited by the Nentsi are saturated with the longlived isotopes emerged from the nuclear tests at the Novaya Zemlya Test Site and deposited into a prolonged food chain “lichen-reindeer-human”. The current absolute contamination values (according to the Radiation safety standards of Russian Federation, 1999), however, are not hazardous to health. The present-day mean caesium-137 content in 237 samples of lichen was 153 Bq/kg (varying from 7 to 685 Bq/kg), while in 17 samples of reindeer meat the mean value was 118 Bq/kg (17 samples). The circulating caesium-137 values were measured in the urine of aboriginal people of Pur district (1-44 Bq/kg in 65 samples), as well as in the placentas of parturient women (1-27 Bq/kg in 64 samples). The aboriginal people appear to be affected by the low and ultra-low doses of internal ionizing radiation. The mechanism of action of those doses, especially, in the susceptible cohorts (including pregnant women) is significantly different than that of the short-term external irradiation (as it is shown by Burlakova E.B. with co-authors as well as by other researchers). During gestation, and, particularly, at critical stages of embryogenesis, teratogenic or embryotoxic exposure may occur (Osipova et al, 2002).

The results of our 5-year long cytogenetic study confirm the negative effects of exposure to hazardous technogenic factors on human genome. The study was performed by a routine classical method of differential staining. The group under study was 369 people from the exposed region, including 250 of aboriginal people and 119 locals of European descent. As follows from the results presented in the Table 2, the total chromosome aberration frequency in all groups is significantly higher than the control level. The frequencies of dicentric and ring chromosomes indicative of radiation were significantly higher than the control level in all groups except the aboriginal children.

In the group of adult aboriginal population alone (N=178), a significant increase in the total chromosome aberration rate (3.23 ± 0.21) as well as the frequencies of rings and dicentric chromosomes (0.44 ± 0.10) is observed.

The classical cytogenetic approach, thus, indicates at a stochastic influence of mutagenic factors on the cellular genetic mechanisms of the aboriginal population, possibly, causing an elevated level of disabilities in the local population.

FISH analysis with rRNA probe was performed unfortunately on a single local patient, year of birth 1957. (Grafodatsky, Rubtsov, personal communication). It demonstrated the presence of a marker chromosome carrying ribosomal genes in 50%

of as much as 300 metaphases analysed. The patient was clinically infertile and died at the age of 50.

Table 2. Results of cytogenetic analysis of aboriginal and non-aboriginal population of Yamalo-Nenets Autonomous Okrug (Osipova L.P. et al.).

		Number of people	Number of cells (total)	Total cells with aberrations (N) dispersion % mean% $\pm$ m	Dicentrics and ring chromosomes (N) dispersion % mean% $\pm$ m
Aboriginal population	total	250	26631	860 0 – 23,8 ***3,23 $\pm$ 0,21	92 0 – 5,5 ***0,35 $\pm$ 0,07
	adult	178	18642	691 0 – 23,8 ***3,71 $\pm$ 0,27	82 0 – 5,5 ***0,44 $\pm$ 0,10
	children	72	7989	169 0 – 18 ***2,12 $\pm$ 0,32	10 0 – 1,3 0,13 $\pm$ 0,08
	control	105	10472	162 0 – 5 1,55 $\pm$ 0,24	12 0,11 $\pm$ 0,06

Significance levels: significantly higher*** ($p < 0,001$); significantly higher ** ($p < 0,01$); significantly higher * ($p < 0,05$); and (without asterix) – non-significantly higher ($p > 0,05$) than the control level

Recently, an elevated level of different kinds of pathologies with underlying genetic causes, including the congenital heart and vessel disfunctions, eye pathologies, allergic diseases and neuropsychic abnormalities has been observed among the children in the population under study.

Over the period of 1992-2008, when the population monitoring of the Samburg group of Tundra Nentsi has been carried out, we collected blood samples from about 80% of the total population together with all individual information required. All survey participants gave informed consent. From the overall pool of participants we constructed 50 paternal genealogical lineages ("extended families") totaling 330 people including the family founders and their descendants. Those lineages differed in the temporal depth of information available (from 2 to 7 generations) and the number of descendants (from 2 to 25 descendants, with a mean value of about 7 descendants in a family). About 70% of male family founders in this sample were Tundra Nentsi. The others were represented by the Forest Nentsi ($n=6$), Komi ($n=6$), Russians ($n=3$), and Khanty (1), who resided in the same area and used to marry the Tundra Nentsi women. Since the peak exposure to the short-lived radionuclides has been in its highest values in the period of 1955-1963, we specifically focused on that time frame, tentatively

considering September, 1955 to be the starting date of the technogenic exposure in the form of ionizing radiation (IR).

In our work, we used the following 34 Y-chromosome STR loci as the markers: DYS19, DYS390, DYS391, DYS393, DYS385A, DYS385B, DYS426, DYS388, DYS392, DYS439, DYS389-1, DYS389-2, DYS458, DYS447, DYS449, DYS459, DYS454, DYS464, DYS455, DYS457, DYS448, H4, DYS607, CDY-1, CDY-2, DYS460, YCA-1, YCA-2, DYS576, DYS570, DYS438, DYS456, DYS442, and C4. We genotyped these loci in 5 two multiplex reactions according to Redd et al. (2002) with slight modifications.

Experimental study of Y-chromosome microsatellite polymorphisms was carried out in 2004-2005, in the period of work stay of L.P.Osipova at the University of Arizona, Tucson (in the laboratory of Prof. Michael Hammer).

3. Results and discussion

Analysis of our results, first, discovered 11 nonpaternity cases (4% of the participants under study). The minority of those were the children born out of wedlock, from the fathers, who arrived to the area to work at oil and gas fields. The rest were either unrelated adopted orphans or the adopted relatives, who changed their family name to the adoptive father's family name. The traditional ethical rules in the aboriginal people populations were aimed at the avoidance of orphan hood. Typically, the children, whose parents have died, were adopted either by the families of their close relatives or by the childless families. Although genealogical information has been collected with maximal scrutiny, the high resolution of STR markers did not allow to unambiguously attribute those 11 individuals to the putative parental lineages. It was important to distinguish nonpaternity from mutation. We defined nonpaternity as a mismatch between fathers and sons at ≥ 2 Y STR loci. In 11 cases of nonpaternity, a mismatch occurred in at least 6-7 STR markers.

It is relevant to present here two paternal genealogical trees, in which the de novo mutations were identified unambiguously.

In the first family tree presented in Fig. 2 (paternal line #5, family founder Ader Elsi, who was a Tundra Nenets), we identified a two de novo mutation in CDY locus. It is apparent that the mutation event occurred in the germ line of Michail, the member of Ader family born in 1940 and departed in 1970. It is from him that the "marker" branch of paternal lineage stemmed, within which the CDY 40-40 variant is replaced by CDY 39-39 variant, which is further inherited. That mutation may be considered as induced by technogenic exposure, since his elder son, Nikolai, was born in 1961 (the younger son, Vyacheslav was born in 1966). This genealogy includes 5 generations of descendants, for whom the reliable information about their relation was available.

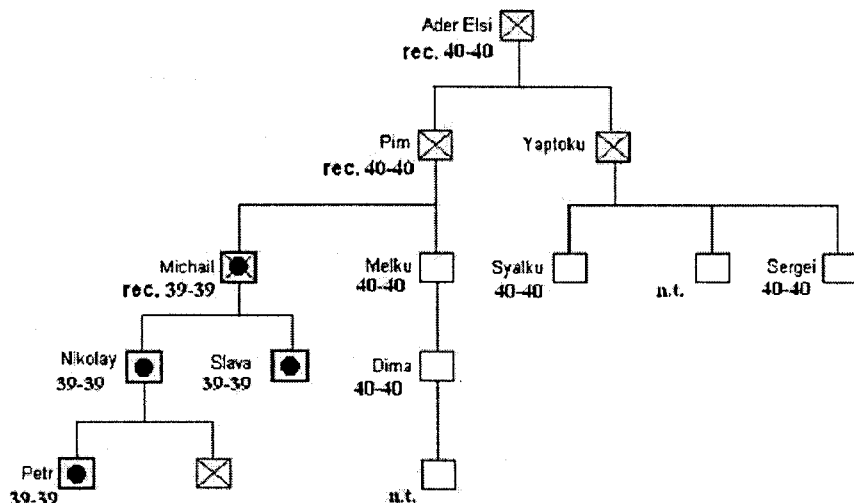


Figure 2. Genealogy of Tundra Nentsi, de novo mutation in CDY locus. Squares with black dots designate individuals with mutation. "Rec" stands for "reconstructed".

The founder of the second family was the Forest Nenets Aivasedo Lelyu, who settled among the Samburg Tundra Nentsi and married the Tundra Nentsi woman. The de novo mutation in CDY-2 locus was shown to occur in his grandson, Nakoti (Fig. 3).

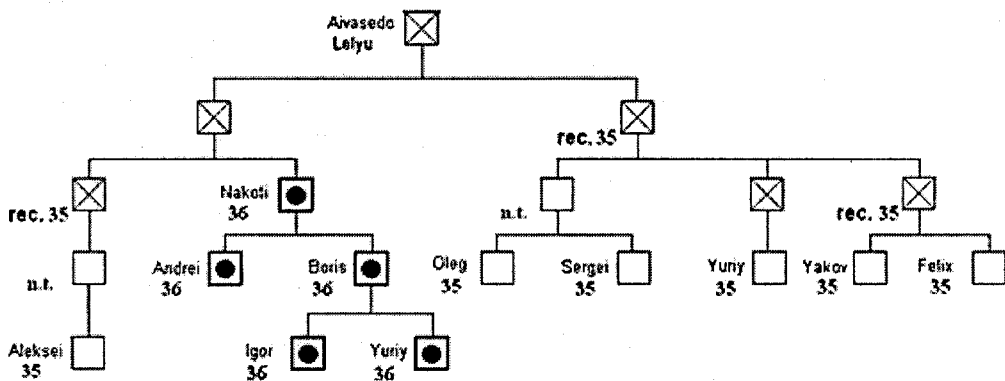


Figure 3. Genealogy of Forest Nentsi, de novo mutation in CDY-2 locus. Squares with black dots designate individuals with mutation. "Rec" stands for "reconstructed".

In our sample, we discovered 59 "de novo" mutations, which, typically, involved a single locus. The mutations directed to an increase of repeat number prevailed (60% vs. 40%).

The "de novo" Y-chromosome STR loci mutations were discovered in 28 pedigrees out of 50, i.e., in more than a half of cases. Out of 34 Y-STR loci employed, as much as 21 loci were involved in mutation process, which confirms the high adequacy of the

selected markers for the study of mutation component (among other microevolutionary factors) in effect in human populations. The highest mutability was identified in CDY locus, which was responsible for one third of all STR mutations. This locus provides increased male relative differentiation. Mutations in DYS449 and DYS464 loci occurred three times less frequent.

4. Conclusion

The selected markers are highly suitable for the study of mutation component (among other microevolutionary factors) in effect in human populations, for forensic identification purposes, and for evolutionary studies. For example, the men born out of wedlock often turn to the geneticists with a request to try to identify the ethnic origin of their biological father.

High frequency of the discovered "de novo" Y-STR mutations, in fact, put more questions, than they provide the answers. First, the selected populations resides at high latitudes with a severe climate. The possibility that the Y-microsatellite mutation rate in the High North populations is increased cannot be ruled out. In order to investigate the effect of high latitudes on the observed mutation rates of Y-STR loci, similar studies in the other populations within the same climatic zone of residence-should be carried out. Our male database includes such aboriginal populations of Siberia and Altai under demographic monitoring.

We are currently unable to make an unambiguous and mathematically accurate statement about a direct relation between the level of microsatellite mutability and the technogenic ionizing radiation. This stays to be a working hypothesis, though.

Currently, the obtained results with the consideration of the number of meiotic events are carefully analyzed with the use of different statistical methods, since the different mutation rates for the particular Y-STR loci has been published. We find important to obtain the correct estimates of those rates for a selected population, and we are planning to expand our male population sample and the number of paternal lineages to this end.

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ENDLESS PURSUIT OF DNA DOUBLE-STRAND BREAK ENDS*

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Abstract. In the pursuit of radiation-induced cellular damage that manifests in cell killing and genome instability, but is reversible by the phenomenon of liquid holding, DNA double-strand breaks (DSBs) have been shown to be the major culprit. It is now widely accepted that DSBs provide both beneficial and deleterious effects and that elaborate systems were evolved to address their detection, processing and repair. Understanding the mechanisms of DSB induction, processing and eventual biological consequences as well as just the detection of DSBs remain a challenge--especially for randomly induced breaks. Given the large number of extensive reviews available and the limited space here, I have chosen to focus on summarizing recent novel findings from our group using the budding yeast *Saccharomyces cerevisiae* that are relevant to many molecular and genetic aspects of DSB repair and impact on genome stability. Included are the systems we have developed that address i) induction of random primary and secondary DSBs; ii) processing of DSB ends; iii) genetic control of DSB induction and repair; iv) genome instabilities associated with DSBs including rearrangements and hypermutability associated with resected ends; and v) physical factors that determine the transition from DSB to chromosome break or recombination. Using a circular chromosome, we find that resection of random, dirty-end DSBs induced by ionizing radiation or derived from MMS single-strand damage is rapid and is primarily due to the MRX complex. Interestingly, the transition from DSB to chromosome break at a unique DSB in yeast is largely prevented by the nuclease function of exonuclease 1 as determined from separation of florescent markers that flank a DSB. Based on a tetraploid gene-dosage model, the role of the chromosome structural complex cohesin is not only to enhance DSB repair between sister chromatids, but it also directs recombinational repair events to sisters thereby preventing loss of heterozygosity. Notably, DSBs greatly sensitizes cells to localized mutability as well as gross rearrangements. Overall, these findings demonstrate the genomic vulnerability to DSBs and the genetic investment in their orderly processing.

Keywords: DNA repair, double-strand breaks, resection, MRX, RAD52, mutations, genome stability, ionizing radiation, UV, chromosome breaks, tetraploidy, recombination

* Radiobiology and Environmental Security / Eds. C.Mothersill, V.Korogodina, C.Seymour. Springer, 2012. P. 245-258

1. Overview

All organisms possess intricate networks for genome duplication and maintenance that assure stability in the face of internal and external environmental stresses or during normal growth and development. The stability is provided through gene expression networks and protein interactions that assure coordination of normal replicative processes and responses to potential genome threats. In addition, all organisms are capable of detecting double-strand breaks (DSBs) and correcting them in an efficient and timely manner. In fact, several years prior to the molecular characterization of DSBs, repair and genetic controls it was clear that yeast cells had a tremendous potential to withstand ionizing radiation insults either through liquid holding [1] or change in cell cycle [2].

It is important to have a full understanding of how DSBs and other types of lesions arise and contribute to genome instability. While there is an upside to DSBs including antibody diversity and chromosome segregation in meiosis, the downside is that DSBs can lead to a variety of genome destabilizing events including incorrect restitution of broken molecules, chromosome breaks, mutation, growth inhibition, disease and death. In the multistep process leading from normal to cancer cells, human chromosomal DNA frequently exhibits genetic instability resulting in mutations, rearrangements and/or aneuploidy. Chromosome breakage, which might arise from a DSB, has been hypothesized to be an initiating event in many of these processes. DSBs at various stages of the cells cycle checked and can generate checkpoint responses. Abrogation of checkpoint functions, for example loss of p53 in human cells, may contribute to genetic instability and eventual neoplastic progression.

Three pathways of DSB repair have been characterized in yeast and human cells: recombination, single-strand annealing and end-joining. Efficient repair of DSBs in yeast generally involves *RAD52*-dependent recombination provided that there is homologous or related (diverged) DNA available. This repair mechanism was originally proposed to require a resected, invasive 3' DNA end supplied by the DSB and a homologous chromosome or sister chromatid as template [3]. Several features of this model have proven correct, as for the case of synthesis dependent, single strand annealing, as well as resection after radiation-induced DSBs as described below. (For a discussion of models and implications see [4].) While it was envisioned as being largely error free, subsequent experiments have clearly demonstrated that the repair may be at-risk for errors. Deletions and rearrangements can also occur from the *misrepair* of DSBs. For example, deletions can occur between homologous, directly repeated sequences flanking a DSB via single-strand annealing in the presence or absence of *RAD52*. Misrepair of DSBs can also result from illegitimate recombination between related DNA sequences thereby reshaping the genome (as described below). Another pathway involving direct nonhomologous end-joining (NHEJ) was described first in

mammalian systems and subsequently characterized in yeast. This DSB repair pathway is mediated by the yeast homologues of human Ku70 and Ku80.

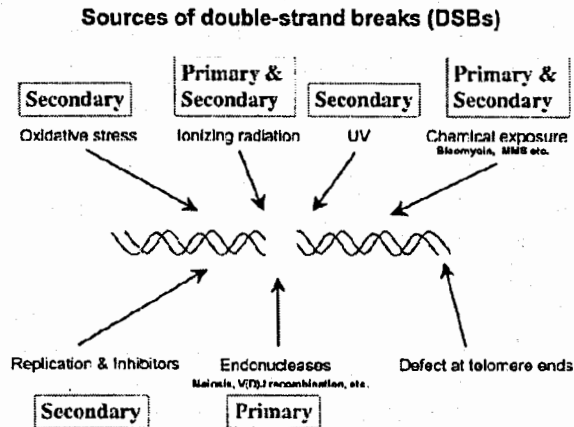


Figure 1. Sources of double strand breaks(DSBs)

The choices in DSB repair pathways may depend on the nature and number of breaks as we have shown for the case blunt-end [5] vs staggered-end DSBs [6] produced by endonucleases, opportunities for homologous interactions and chromosome organization. Figure 1 describes many of the sources of DSBs either as primary events, secondary during processing of lesions, or even during replication resulting from collapsed forks. Our Chromosome Stability Group has developed multi-pronged approaches to understanding the appearance of a variety of DSBs created by different agents and situations, including replication and ARMs (at-risk motifs) associated DSBs [7 -9] along with mechanisms of repair and genomic changes resulting from DSBs in budding yeast. There are several reviews over the past few years that address many of the issues related to DSB induction, repair and consequences. In light of this, I have chosen to briefly review our recent approaches with the budding yeast *Saccharomyces cerevisiae* which have led to novel systems and findings that address many molecular and genetic aspects of DSB repair and impact on genome stability. (This summary is based on a recent unpublished report to the Scientific Board of NIEHS that also addresses studies on the human p53 damage response network [10].)

2. Radiation-induced random DSBs and subsequent molecular events

HIGHLIGHTS

- *Repair of randomly induced, dirty-end breaks is highly efficient in G2 cells*
- *Development of a robust system for detecting resection and recombinants using circular chromosomes*
- *Resection at γ -induced DSB ends is rapid and predominantly controlled by RAD50 (MRX)*

Armed with new tools, many of which we developed, we revisited our early studies that provided the first evidence for DSB repair via recombination [3, 11] with a view towards understanding chromosomal variation in repair and the genomic consequences of random DSBs induced by ionizing radiation (IR) and other agents. Radiation, which is commonly used in cancer therapy, has proven a model agent for instantaneous induction of random DSBs (unlike *in vivo* enzymatic induction of DSBs). For most chemical and IR inducers of DSBs, there is the issue of repair at “dirty” ends and the formidable challenge of studying molecular events associated with random DSBs.

The 16 chromosomes of haploid yeast are between ~230 and 3000 kb. Since the individual chromosomes can be displayed with neutral pulse-field gel electrophoresis (PFGE), we have chosen to address repair in individual chromosomes (*i.e.*, detected as complete chromosome restitution) and genetic controls. DSB induction by IR and subsequent repair have been extensively examined in haploid and diploid, logarithmically growing cells arrested in G2, since they have the highest potential for recombinational repair (either sister chromatids or homologous chromosomes; also discussed below in terms of the role for cohesin). A unique addition to defining events at random DSBs is our use of circular chromosomes (Figure 2). A single DSB leads to a unique, unit-size linear molecule detectable by PFGE unlike the circular chromosomes that remains in the starting well. This innovation has provided accurate measurements of DSBs as well as closely-spaced single-strand breaks (SSBs) [12] and the opportunity to study resection.

We established that there are 0.07 DSBs/mb-krad [13]. Repair can be detected at about 30-60 minutes after IR, with about half the DSBs repaired in 1 hour at a dose inducing ~140 DSB in the haploid G2 genome. The time scale for complete restitution to full-size chromosomes is related to size, consistent with a time-dependent component in the random repair of DSBs. Thus, the smallest chromosomes are repaired the fastest, which may indicate differences in ability of radiation to induce chromosome changes. The ribosomal DNA region located on Chr XII contains nearly 200 large rDNA repeats is subject to different repair rules for IR induced DSBs than the rest of the genome, consistent with spontaneous alterations in rDNA. We investigated further the roles of *RAD52*, *RAD51* and both *RAD50* and *MRE11* (MRX) in repair and restitution.

We wanted to address events shortly after IR, a task that we anticipated would be particularly difficult given that DSBs are random. Surprisingly, the circular chromosomes that received a single break (*i.e.*, a unique linear band in PFGE) developed a unique property—slower mobility—within minutes after irradiation as diagrammed in Figure 2. We established that the slower mobility, referred to as PFGE-shift, was due to single strand tails generated by resection.

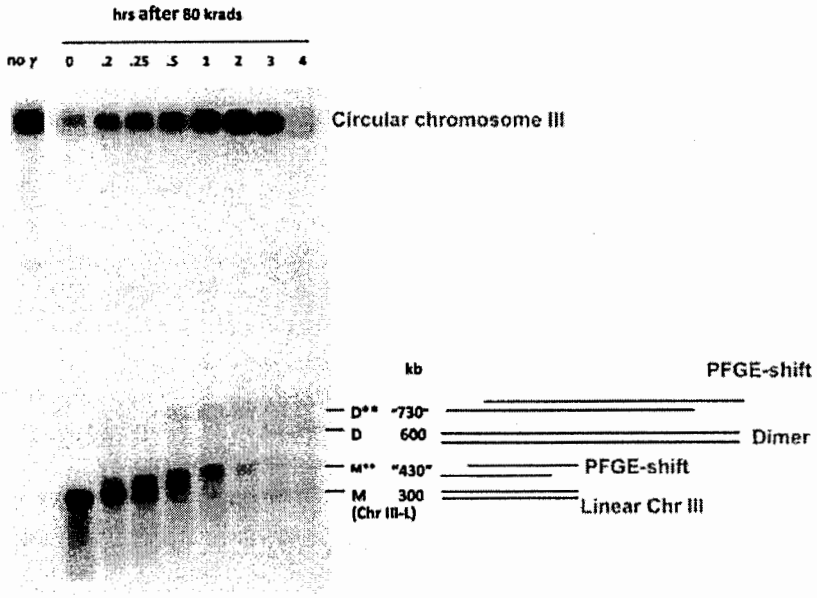


Figure 2 Single DSBs in circular chromosomes lead to linear molecules which exhibit PFGE-shift with time of incubation

This has provided a novel, robust assay and led to the first direct measurement of resection at random breaks, opening opportunities to address the efficiency, consequences and genetics of resection [13]. Within 10 min after IR of WT cells arrested at G2/M, there is a near synchronous PFGE-shift of the linearized circular molecules, corresponding to resection of a few hundred bases. Resection continues so that by the time of significant repair of DSBs at 1 hr there is about ~1-2 kb resection per DSB end. The PFGE-shift is comparable in WT and recombination-defective *rad52* and *rad51* strains. However, in *rad50* and *mre11* null mutants the initiation and generation of resected ends at radiation-induced DSB ends is greatly reduced. Thus, the Rad50/Mre11/Xrs2 complex is responsible for rapid processing of most damaged ends into substrates that subsequently undergo recombinational repair. Among the few molecules exhibiting shift in the *rad50* mutant, the residual resection is consistent with resection at only one of the DSB ends (also recent unpublished results).

In addition to these findings, we were able to directly assess sister chromatid recombination associated with the DSBs. Within 1 hr after irradiation, double-length linear molecules are detected in the WT and *rad50*, but not *rad52*, strains providing the first real time description of recombination induced by random DSBs. These recombinants were largely resection and *RAD50* independent and provide a unique opportunity to address DSB-induced recombination.

3. DSB-associated genome instability: radiation-induced random DSBs and genome plasticity

HIGHLIGHTS

- *Randomly induced DSBs frequently generate chromosome aberrations*
- *IR-induced aberrations are primarily associated with breakpoints in repeat (Ty) sequences*

Investigations into chromosomal changes in unselected survivors (20 to 40% survival) revealed remarkably high levels of chromosome alterations even though repair is expected to be maximal in 2n-4C cells [14]. Because we employ diploid cells and the only requirement is survival, our approach enables us to address a broad range of changes. Studies from other labs of spontaneous and damage-induced chromosome changes have generally used haploid cells in which there is selection for changes in small regions and where many chromosome aberrations are lethal.

Nearly half the radiation survivors had at least one apparent chromosomal change based on karyotypic analysis with pulse-field gels. This led us to investigate chromosomal changes by Comparative Genome Hybridization (CGH) microarray analysis through a collaborative effort with Tom Petes at Duke. We found that duplication or deletions were common (~100 among 37 colonies). Surprisingly, the incidence of chromosome aberrations was not related to chromosome size and, therefore, frequency of DSBs. Nearly 90% of the breakpoints occurred in the vicinity of multiply repeated (although diverged) sequences, with some repeats being much hotter than others and subsequent sequencing demonstrated that the breakpoints occurred within the repeats. Thus, while repair between sister chromatids is highly efficient, repeat regions—particularly TY elements—in the genome can compete in the repair process.

Based on the appearance of tripartite events and our measured frequency of DSBs, we concluded that the two ends of a radiation-induced DSB in a repeat sequence can independently interact with other repeats in the genome. This has obvious implications for damage in the human genome. Thus, randomly-induced DSBs can have profound genomic effects and lead to nonrandom genomic changes opening the diploid genome to dramatic changes.

4. DSB associated genome instability: DSBs, ssDNA and localized hypermutability

HIGHLIGHTS

- *Single-strand DNA formed at DSBs and uncapped telomeres is at risk for hypermutation and multiple mutations*
- *Strong potentiation of weak mutagens in single strand DNA*

A common mechanism of repair of DNA lesions involves excision and resynthesis utilizing the intact template strand. This implies that damaged single-strand DNA (ssDNA) might be at high risk for loss or genome instability. Based on the systems we had developed to study DSB repair by oligonucleotides [15, 16] including RNA-containing oligonucleotides [17], we were uniquely poised to address the vulnerability of ssDNA to spontaneous and damage-induced changes including mutagenesis. Unlike the lesions induced in dsDNA, most lesions in ssDNA are not repairable. In these studies which were initiated and developed by Dmitry Gordenin [see his report in this volume], we found that multiple lesions caused by ultraviolet light-C (UV) and MMS can be tolerated in ssDNA and that they lead to extremely high frequencies of single and multiple mutations.

Using our uniquely designed yeast systems [18], up to 20 kb of persistent ssDNA can be generated at the ends of a chromosomal DSB or at uncapped telomeres in *cdc13-1* temperature-sensitive mutants. Cells are then exposed to a DNA damaging agent. The mutagenic consequences of the lesions in the ssDNA can be assessed with mutation reporters that are included in the regions that experienced ssDNA.

Since nearly all the UV-induced mutations correlated with pyrimidines in the nonresected strand [18] and the majority of MMS-induced mutations could be related to damaged cytosines in the nonresected strand which provided a unique mutation signature [19], ssDNA was concluded to be highly vulnerable to mutagenesis. Consistent with this finding, there was a striking multiplicity of strand-biased mutations, with up to 6 mutations separated by hundreds of nucleotides after a nonlethal dose of damage. These results established that long stretches of transient ssDNA can be restored to the dsDNA state even when they contain multiple lesions. The ssDNA was greater than 1,000-fold more mutable than the dsDNA in the rest of the genome. The high levels of induced, as well as spontaneous, mutagenesis were largely attributable to bypass by the DNA polymerase zeta (controlled by *REV3* as well as *REV1*).

These findings are contrary to commonly held views of mutation independence across the genome. They reveal novel mechanisms of mutation and biological consequences. Environmental toxicants and drugs that can lead to and/or generate lesions in single strand DNA are of special health concern since single-strand regions in the genome may be highly prone to mutation and subsequent disease. This is particularly relevant since transient stretches of ssDNA are formed during 5'-end-

resection of DSBs, as discussed above, as well as during DNA replication. Abnormalities in DNA metabolism, such as replication fork uncoupling, delay in recombination repair of a DSB or in telomere recapping after replication could be sources of long ssDNA stretches (thousands of bases). Our findings establish long ssDNA as extremely risky to genome stability as well as a potential source of adaptive evolution and cancer [see report by Gordenin in this volume] without severe mutation load in the rest of the double-strand genome, where repair of lesions is efficient.

5. Base damage-associated DSBs and implications for SSB repair

HIGHLIGHTS

- *System developed to monitor induction and repair of DSBs caused by DNA base damage*
- *Chromosomal DSBs can result from interrupted or misrouted base-excision repair*
- *Coordinated polymerase δ and Rad27/Fen prevent transition of closely-opposed lesions to DSBs*

Endogenous metabolism or environmental factors such as oxidizing and alkylating agents produce a wide variety of lesions in cellular DNA. The genomes of mammalian cells experience from 10,000 to 200,000 modifications per day. Most lesions are repaired by a network of proteins that are part of an elaborate, multi-step base excision repair (BER) system that generate single-strand break (SSB) intermediates. Importantly, defects in BER can lead to malignancies and are associated with age-associated disease, including neurodegeneration. While BER enzymes have been characterized biochemically, BER functions within cells are much less understood, in part because replication bypass and DSB repair can impact resistance to base damage. To investigate *in vivo* BER, we examined repair of methyl methanesulfonate (MMS) induced DNA damage in haploid G1 cells in the absence of replication bypass and recombinational DSB repair [12].

Following methylation of DNA bases, abasic sites (AP sites) are generated by glycosylases. The AP sites are heat-labile and give rise to SSBs during sample preparation for PFGE at a high temperature (55°C) while few SSBs are formed at lower temperatures (30°C). If closely spaced on a chromosome, SSBs will result in a secondary chromosomal DSB that is detectable by PFGE. Assuming that pairs of single strand lesions separated by less than 10 nt are detectable as DSBs, the number of secondary DSBs appearing after MMS treatment corresponded to 2,000–10,000 base lesions per haploid genome. These DSB studies were facilitated by our approach of simultaneous probing of a circular and a linear chromosome using a common sequence; the system can detect 2 to 40 DSBs per yeast genome. (Note that in this system DSB measurements are independent of the amount of DNA entering the gel.)

The heat-labile sites associated with DSBs were efficiently repaired after incubating G1 stationary cells for 24 hr in buffer. Because the cells were also haploid, the repair could not occur through recombination. Consistent with a proposed role for BER in repair of heat-labile sites, the repair required the glycosylase *MAG1*. Simultaneous deletion of AP-endonuclease genes *APN1* and *APN2* led to a large number of DSBs even for samples processed at 30° C, indicating that heat-labile sites can be converted into SSBs within the cell by other BER enzymes (possibly Ntg1 or Ntg2 lyases) and are not efficiently repaired thereafter. Consistent with this, DSBs formed in *apn1 apn2*-deletion strains were not repaired and even increased in number during post-treatment incubation in buffer. These DSBs were suppressed by a *mag1* deletion suggesting the DSB resulted from downstream processing of AP-sites *in vivo*. Thus our approach provided direct physical evidence that Apn1 and Apn2 not only repair cellular base damage but also prevent break accumulation that can result from AP sites being channeled into other BER pathway(s).

Our findings provide the first direct evidence that interrupted or misrouted base-excision repair of clustered lesions can lead to the *in vivo* accumulation of chromosomal DSBs. In subsequent experiments [12], we described a pathway for the prevention of DSBs from clustered lesions through the coordinated repair of closely-opposed lesions. Using single and multiple mutants that impede interaction of DNA polymerase δ and 5'-flap endonuclease Rad27/Fen1 with the PCNA sliding clamp, we found that lack of coordination between these components during long-patch base excision repair of alkylation damage can result in the generation of many DSBs within nondividing haploid cells. This contrasts with efficient repair of nonclustered MMS-induced lesions, as measured by quantitative PCR and S1 nuclease cleavage of SSB sites.

We conclude that closely-opposed single-strand lesions are a unique threat to the genome and that repair of closely-opposed strand damage requires greater spatial and temporal coordination between the participating proteins than widely-spaced damage in order to prevent generation of DSBs. In our recent unpublished studies (Ma *et al.*, in preparation) we have gone on to describe how MMS can lead to DSBs in *apn1 apn2* cells and that the DSBs are subject to resection and recombinational repair, similar to findings with radiation-induced DSBs.

6. Chromosome structure and DSBs: single molecule analysis of DSB to chromosome aberration transitions

HIGHLIGHTS

- *Chromosomal continuity is not disrupted by a DSB*
- *The transition of nearly all DSBs to a chromosome break is controlled by MRX & EXO1*

Since DSBs are powerful sources of chromosome instability and rearrangements, we set out to understand the relationship between a DSB and a cytologically detectable chromosome break (CRB) and what factors might influence the transition from DSB to CRB. In collaboration with Kerry Bloom (University of North Carolina, Chapel Hill) we developed a yeast-based system that provides for chromosome analysis in real time following the induction of a single DSB by an inducible I-SceI endonuclease [20]. The system (Figure 3) utilizes the fluorescent labeled proteins tetR-CFP and LacI-GFP marking each side of a DSB and Spc29-RFP fusion to identify the spindle poles. The tetR-CFP and LacI-GFP proteins bind multiple repeats of their operator target sequences ~5 kb from the DSB site. This arrangement enabled us to investigate the development of a CRB following DNA breakage and the relation to spindle pole separation and sister chromatid separation in wild type and various end-joining or end-processing RMX mutants.

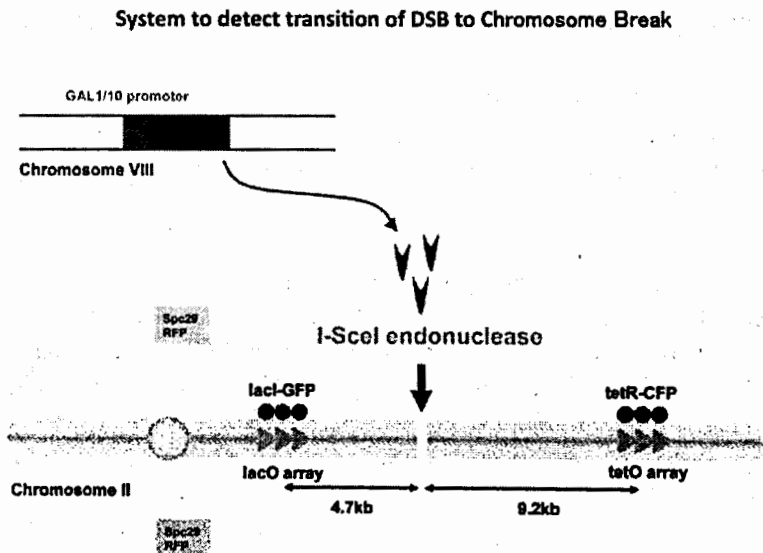


Figure 3 System to detect transitions of DSB to chromosome break

Previously, we showed that nearly all wild type cells were arrested at the G2/M phase in response to an unrepaired DSB signaling event [20]. The centromere-containing and acentric broken DNA fragments remained in close proximity, which led us to conclude that a break in DNA does not develop into a CRB. (Similar results were reported using an identical fluorescent markers nearly 50 kp from an induced DSB.) This physical association may be important in DSB repair. We went on to identify some factors that help prevent a DSB to CRB transition. There is a requirement for the complex, since 10 to 20% of chromosomes exhibited a CRB when any of these genes were mutated. However, it is not the end-joining or end-processing MRX function that prevents chromosome breakage. Instead, it is the tethering feature of the complex through the Rad50 component. We proposed that this function, in addition to repair, is likely to play an important role in preventing chromosome instability. These studies were recently extended to other mutants. Remarkably, deletion of the exonuclease 1 (*EXO1*) gene increases the frequency of cells with a CRB to ~40% [21]. Importantly, in the *exo1 rad50* double mutant nearly all DSBs transition to CRBs. Since the *exo1* nuclease function specifically affects the DSB to CRB transition, the findings have led us to conclude that some aspect of resection plays a major role in preventing a DSB to CRB transition.

7. Chromosome structure and DSBs: limiting cohesin leads to genome instability

HIGHLIGHTS

- *Development of genetic system to identify limiting components controlling genome stability*
- *Cohesin is limiting for DSB repair.*
- *Cohesin defines the sister chromatid as a donor for DSB repair, preventing recombination between homologous chromosomes and loss of heterozygosity (LOH)*

The repair of DSBs via homologous recombination (HR) is an evolutionarily conserved process that is relatively error free. While HR may have a deleterious effect on genome stability through nonallelic recombination or LOH, these risks might be reduced by restricting HR to sister chromatids. Although the sister chromatin cohesion complex (cohesin) facilitates DSB repair between chromatids, its role in HR and repair when homologous chromosomes are present has not been addressed. We examined the consequences of changes in cohesin level on HR and DSB repair as well as the role that cohesin might play in directing events between sister chromatids vs homologous chromosomes [22]. Using tetraploid yeast, gene dosage could be varied 4-fold for the essential sister chromatid cohesin sub-unit *MCD1* as well as other key players in HR. Survival and global DSB repair are compromised in *MCD1* simplex (single-copy)

strains that are γ -irradiated at G2/M when sister chromatids are present. Importantly, γ -induced HR between homologous chromosomes was actually increased ~ 10 times over that in WT strains (4 copies), while there was no difference in spontaneous HR rates. The survival and HR frequency were comparable to WT tetraploid yeast irradiated in G1, when only homologous chromosomes are present. The results for UV and IR were similar although to a lesser extent for the DNA replication inhibitor hydroxyurea. This system has provided the first direct evidence that the cohesin complex is both limiting in DSB repair and that it has a dual role in HR, namely, promotion of recombinational repair between sister chromatids and suppression of recombination between homologues (Figure 4). The suppression would prevent loss of heterozygosity (LOH) through recombination and segregation of sister chromatids.

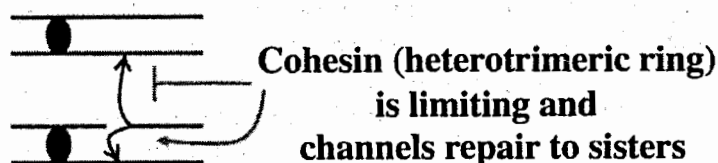


Figure 4 Cohesin (Heterotrimeric ring) is limiting and channels repair to sisters

Thus, reduction in cohesin may have important consequences to genome change and evolution. Cells with moderately reduced cohesin levels (*MCD1* simplex) have a reasonable fitness but exhibit increases in DNA damage-induced recombination between homologous chromosomes, even when survival is high. The promiscuous recombination which can cause elevation in LOH may also lead to more nonallelic recombination, reshaping the genome through structural variations [22]. While the present results indicate a general role of cohesin in control of HR, our approach can provide useful insights into genome dynamics as well as genetic processes associated with tetraploidy and consequences of cell-to-cell variation in limiting proteins. In addition, the experimental design can be used to search for subtle changes in factors that are limiting for genome stability, especially when combined with modest (*i.e.*, high survival) levels of genotoxic environmental stress. Our system utilizes normal, wild type proteins and native gene expression regulation, eliminating the uncertainty associated with mutations and controlled gene expression.

8. Summary

Overall, our approaches have established the efficiency of repair of random DSBs in yeast and the genomic vulnerability associated with these breaks. Based on results with a unique cut site, broken DNA ends are held in close contact which could facilitate

recombinational repair. In addition the physical interaction between sister chromatids appears to enhance opportunities for repair. Importantly, the ends have a strong potential for genome instability either by interacting with homologous chromosomes and/or sequences across the genome or through resection of ends, making them highly vulnerable to mutagenesis. These approaches have helped elucidate a portion of the considerable genetic investment that all cells have in the detection and orderly processing of DSBs.

Acknowledgements

I would like to thank all the past and present members of the Chromosome Stability Group who have contributed to the many studies and papers summarized here, many of whom are indicated in the references. Included among these exceptional researchers are Wenjian Ma, Yong Yang, Shay Covo, Jim Westmoreland, Stephaen Roberts and especially Dmitry Gordenin for development of several ideas and systems that have been employed in this work. Special thanks to Kerry Bloom at the University of North Carolina, Chapel Hill, and Tom Petes at Duke University as well Lucas Argueso. This work was supported by intramural research funds from NIEHS to M.A. Resnick under projects Z01-ES021016 and Z-01-ES065073

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REDUCED RB1 EXPRESSION CAUSES IMPAIRED GENOME STABILITY IN BONE-CELLS AND PREDISPOSE FOR RADIATION-INDUCED OSTEOSARCOMA*

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Abstract: The risk of cancer after exposure to ionizing radiation is currently defined only as a function of the received dose. Genetic factors that modify individual susceptibility to radiation-induced cancer are excluded from the risk assessment. We report the mapping of QTLs that confer increased susceptibility to radiation-induced osteosarcoma in the mouse. The strongest candidate locus, on chromosome 14, contains a functional polymorphism weakening the efficiency of the Rb1 promoter. The Rb1 allele associated with increased susceptibility is preferentially retained during allelic loss at the Rb1 gene in radiation-induced tumors. In combination with allelic losses of CDKN2a /P16, an upstream regulator of Rb1, 100 % of all analyzed tumors exhibit a defect affecting this pathway. Alpha-irradiation of knockout mice with a bone-specific expression reduction of Rb1 or P16 confirms that these genes can alter the susceptibility for osteosarcoma, either by increasing the tumor-risk or by shortening their latency. These results suggest that common germ-line polymorphisms causing impaired expression of known tumor-suppressor genes can modify individual susceptibility to radiation-induced cancer.

Keywords: radiation-induced cancer; osteosarcoma; mouse model; genetic modifier; inherited susceptibility; alpha-emitter; incorporated isotopes; instability; radiation-risk; individual predisposition

1. Introduction

Ionising radiation has been identified as a carcinogenic agent through epidemiological studies of cohorts exposed to relatively high doses, such as A-bomb

* Radiobiology and Environmental Security / Eds. C.Mothersill, V.Korogodina, C.Seymour. Springer, 2012. P. 353-364

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survivors and those receiving therapeutic or accidental irradiation. The dose response data from these groups is used in extrapolating the risk of radiation-induced cancer to low-exposure groups (UNSCEAR, 2000). Such population-based extrapolations carry with them a degree of uncertainty, and cannot accurately predict outcome in individual cases. This is especially true in those individuals carrying a germ-line mutation that predisposes them to the carcinogenic effects of ionising radiation e.g. P53, Rb1, and ATM (Hahn et al., 1998; Cox, 1994). However, common gene polymorphisms also may influence individual susceptibility to the carcinogenic effects of ionising radiation, as they do other malignancies (Ponder, 1990; Hemminki and Mutanen, 2001; Zhang et al., 2003).

Uncertainties of tumor causality preclude studies in man that are designed to identify genetic effects on radiation carcinogenesis. However, mouse models show both direct causality and strain-dependent differences in radiation-induced tumor incidences, suggesting that modifiers of susceptibility can be identified using the mouse (Groch et al., 1997; Santos et al., 1998).

Skeletal deposition of bone-seeking alpha particle-emitting radionuclides (e.g. Ra, Pu, Am), results in highly localized irradiation of the bone-forming cell compartment. The incidence of osteosarcoma in man and mouse is increased by a number of orders of magnitude following such exposures (Fry, 1986; Luz et al., 1991). Inbred BALB/c mice show a high incidence of osteosarcoma following injection of ^{227}Th (4 Gy skeletal dose), whilst at the same dose CBA strain animals are relatively resistant (Fig. 1). The polygenic basis of the strain difference was established in irradiated (BALB/c x CBA) F1 hybrid animals. These show an intermediate sensitivity as compared to their parental strains, indicating the action of multiple low-penetrance modifier genes.

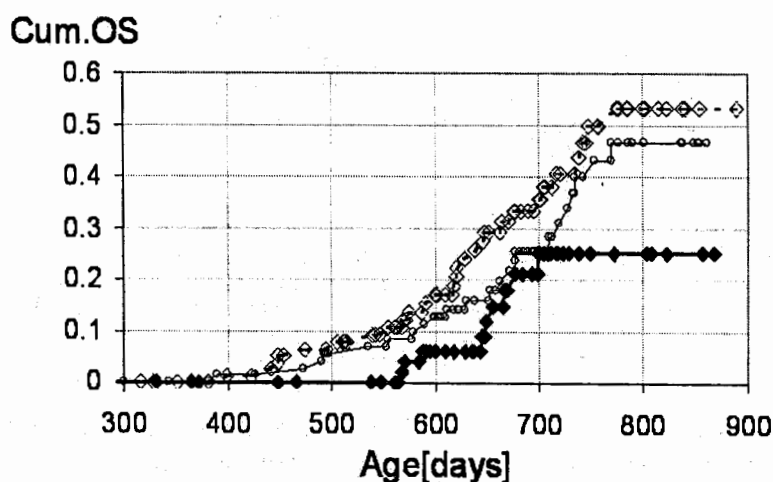


Figure 1. Cumulative incidences of osteosarcoma development following injection with 185 Bq/g ^{227}Th at the age of 100 days showing greater sensitivity of BALB/c strain (open diamonds) compared to CBA/CA strain (closed diamonds). The F1 hybrid animals (open circles) are of intermediate susceptibility.

The variable penetrance of osteosarcoma in certain familial cancer syndromes (Meisner et al., 1979) also suggests the existence of yet unknown susceptibility genes in man. We describe in this report mapping, identification and first functional studies of such susceptibility genes in radiation-induced osteosarcoma in mice.

2. Genome-wide mapping of murine susceptibility genes

We have previously employed genetic mapping techniques in backcrossed mice strains to identify loci in the genome that harbour potential susceptibility genes for alpha-radiation induced osteosarcoma (Rosemann et al., 2002; Rosemann et al., 2006). These genome-wide screens for quantitative trait loci (QTL) established a total of 6 distinct chromosomal sites that modify osteosarcoma predisposition in the mouse strains BALB/c, CBA/CA, C3H/H and 102/Nhg (Fig. 2).

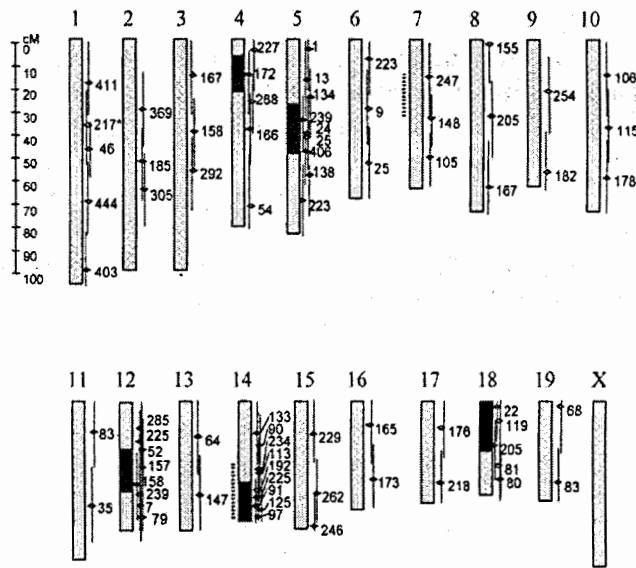


Figure 2. Approximate positions (derived from MGD database) of BALB/c / CBA polymorphic microsatellite markers are given as black diamonds. Numbers are related to the name of each marker in the MIT panel (* exception is 217, that was originally D3Mit217, but has now been allocated to chromosome 1). Red lines indicate the sweep-radius of each marker, i.e. the interval for which linkage to susceptibility genes could be detected. Chromosomal regions showing linkage of osteosarcoma susceptibility with BALB/c alleles (red) and CBA/CA alleles (blue) are shown together with two osteosarcoma susceptibility loci mapped in preceding study (hatched blue bars) (repr. from Rosemann et al., 2006).

Animals inheriting the high-risk genotypes at five of these loci showed a 100% osteosarcoma incidence, with an average latency of 430 days. In contrast, animals inheriting low-risk gene variants at these five loci exhibit a marked tumor resistance, with only 1 out of 11 animals developing osteosarcoma with a latency of 680 days (Fig. 3).

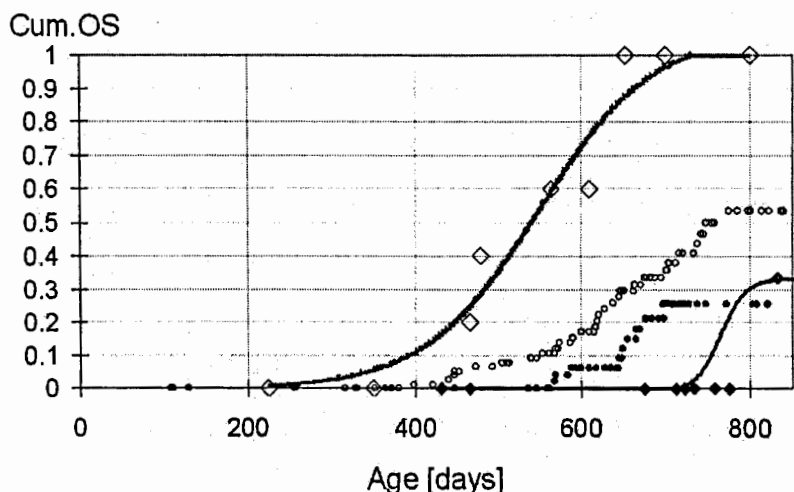


Figure 3. Additive effect of five susceptibility loci on tumor incidence in backcross F2 animals grouped according to their genotypes. Those inheriting high-risk genotypes at all 5 susceptibility loci (open diamonds) show higher sensitivity than parental BALB/c (open circles) or CBA/CA mice (closed circles). F2 animals inheriting low-risk genotypes at all 5 loci exhibiting lowest osteosarcoma frequency (1/11) with the longest latency time (closed diamonds).

Markers between D14Mit234 and D14Mit97 showed linkage with the BALB/c-allele being associated with an increased osteosarcoma resistance. Of 24 animals developing tumors all but 5 were heterozygous (CBA and BALB/c allelotypes) at these markers. This is in contrast to tumor-free animals, where only 10 of 36 animals were heterozygous ($p=0.0002$). QTL mapping using a normalized, censored latency time $t(L)C$ as a trait parameter indicated that the likelihood ratio statistic for a QTL in this interval exceeds 20, equivalent to $LOD = 4.39$ (Rosemann et al., 2006). Significantly, this region on chromosome 14 overlaps with the osteosarcoma susceptibility locus we previously mapped in a different mouse cross (Rosemann et al., 2002); giving a compound LOD score of 5.44. Haplotype analysis was carried out for all 169 mice using additional markers placed between D14Mit234 and D14Mit97 (Fig. 4). The lowest number of recombinants was found for markers D14Mit192 and D14Mit225, which span only a 3 Mbp interval (Ensembl 2010).

2.1. SOMATIC LOSSES-OF-HETEROZYGOSITY IN TUMORS

To further narrow down the genomic position of an tumor susceptibility gene we analysed a set of radiation-induced osteosarcoma in (BALB/c x CBA) F1 hybrid mice for the presence of somatic gene alterations (loss-of-heterozygosity, LOH). In the interval originally mapped by F2 linkage analysis, we now also found LOH in 13 out of 17 tumors (Fig. 5). Interestingly, there was a preferential loss of the BALB/c-allele, with 10 of the 17 tumors (58.8 %) showing loss of the BALB/c allele, compared to only 3 of 17 (17.6%) tumors showing loss of the CBA-allele (Gonzalez Vasconcellos et al.,

2010). This preferential loss of the BALB/c allele in the tumors suggests that the BALB/c gene variant may have a tumor suppressor function in these animals.

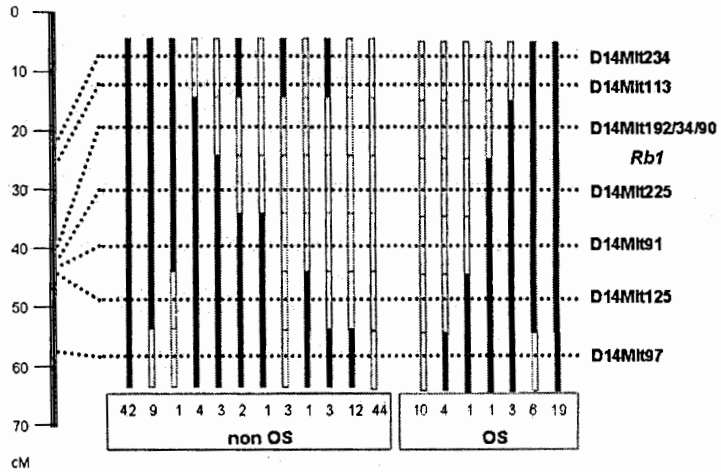


Figure 4. Haplotype analysis of radiation-osteosarcoma susceptibility with additional markers to narrow down the principal susceptibility locus on chromosome 14. Germline haplotypes in 44 mice with and 125 mice without tumor are shown here with tracks of BALB/CBA heterozygote genotypes (dark bars) and BALB/BALB homozygote genotypes (open bars).

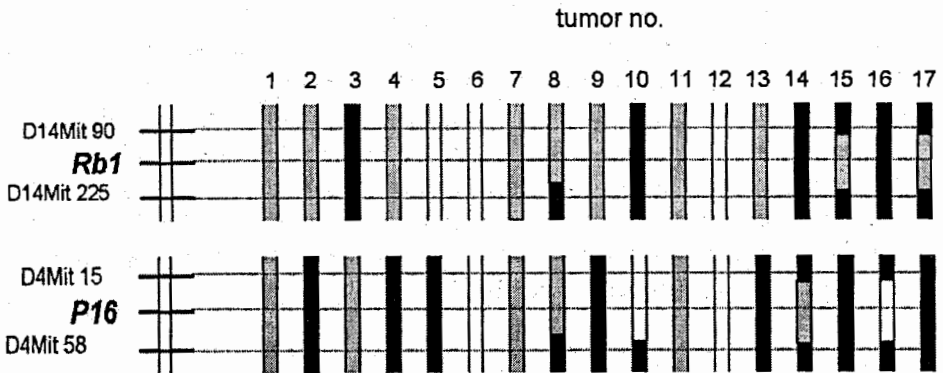


Figure 5. Pattern of allelic-loss in 17 radiation-induced osteosarcoma found in BALB/c x CBA/CA F1 hybrid mice. Two loci in the mouse genome, encompassing RB1 on chromosome 14 and encompassing P16 on chromosome 4 are shown. Allelotyping was done for intra-genic SNPs of the two genes and for microsatellite-markers flanking them proximal and distal (repr. from Gonzalez Vasconcellos et al 2010). Black: retention of heterozygosity; grey: loss of maternal BALB/c allele; white: loss of paternal CBA/CA allele.

The smallest common region affected by allelic losses in the analyzed tumors was defined by the proximal marker D14Mit192 (72.3 Mbp) and the distal marker D14Mit225 (75.3 Mbp). Within this 3 Mpb region on murine chromosome 14 maps the *Rb1* tumor suppressor gene (73.6 Mbp), which is a strong candidate for both an osteosarcoma- and a radiation sensitivity predisposing gene. Patients carrying germline *Rb1* mutations show an increased incidence of sporadic osteosarcoma (Hansen et al., 1985; Gilman et al., 1986; Draper et al., 1986), as well as a propensity to develop post-therapy osteosarcoma in the radiation field (Eng et al., 1993).

3. Inherited hypomorphic variations of the *Rb1*-gene

Sequencing of the entire *Rb1* gene transcript revealed no strain-specific differences in the *Rb1* coding region, implying that the protein function is not impaired in either of the two strains. Within the 1190bp upstream of exon 1, however we identified a TCGCCC hexanucleotide duplication that is present only in the BALB/c allele, positioned between nt 1053 and nt 1084 (Fig. 6, base numbering according Genebank M86180). This duplication lies 177 bp upstream from the first exon, and is 5' to the predicted binding sites for Sp1, ATF and E2. This alteration has therefore the potential to change the *Rb1*-transcription in the BALB/c strain relative to the CBA/CA strain.

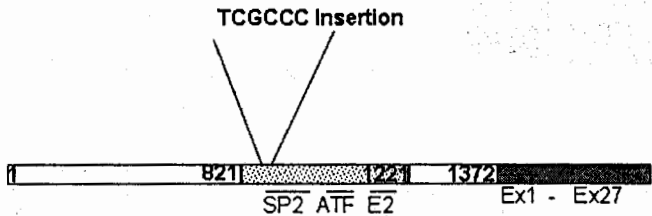


Figure 6. Sequence of the *Rb1* promoter region between nt1053 and nt1084 determined for strains BALB/c and CBA/CA, showing position of the BALB/c specific hexanucleotide insertion relative to the SP1, ATF and E2 core binding sites (base numbering according to Genebank accession no. M86180).

In whole embryo mRNA from both strains we could indeed find a 50% reduced *Rb1* expression in the CBA/CA strain (183 AU, CI 138 – 228) as compared to the BALB/c strain (232 AU, CI 165 – 299, $p=0.038$, t-test) (Fig. 7).

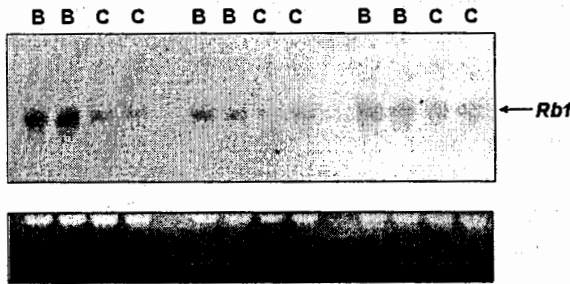


Figure 7. *Rb1* expression in BALB/c and CBA/CA embryo RNA by northern blot analysis. Lower row: 18S and 28S RNA as loading control.

When we used the genotype of this Rb1 promoter variant to stratify the entire BALB/c x CBA F2 backcross into either BALB/BALB homozygotes (B/B) or BALB/CBA heterozygotes (B/C) it was obvious, that mice inheriting the B/C genotype have a significantly shorter latency and higher overall osteosarcoma incidence than mice with the B/B genotype. At 500 days after tumor induction more than 40% of the B/C mice developed osteosarcoma as compared with just 12% tumors in mice of B/B genotype ($p=0.0007$, Log-Rank Test) (Fig. 8).

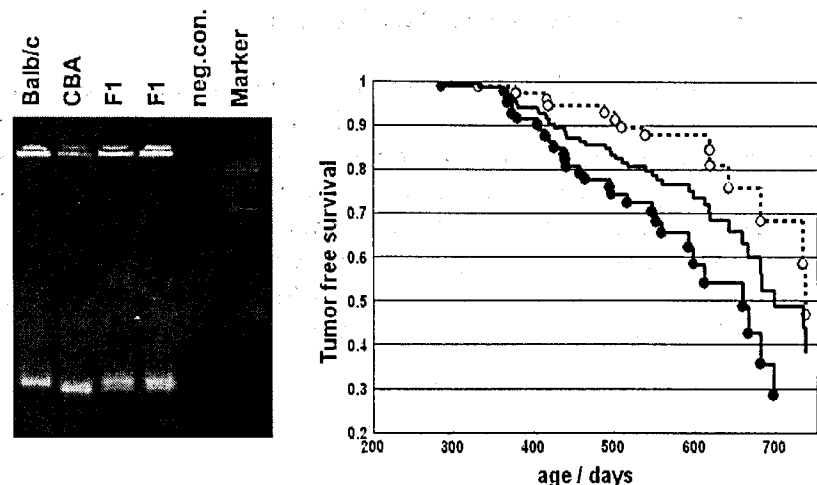


Figure 8. Kaplan-Meier plot of osteosarcoma development in BALB x CBA backcrossed mice grouped according to their genotypes at the Rb1 promoter. Difference between the B/B (dashed line, open circles) and the B/C genotypes (filled line, black circles) is highly significant ($p=0.0007$, Log Rank test). Intermediate line (without symbols) represent the pooled cohort of both genotypes.

4. The Rb1 pathway and other affected elements

The Rb1 protein acts as a growth regulator and tumor suppressor by controlling transition of the G1/S cell cycle checkpoint in conjunction with the p16 tumor suppressor (Zacksenhaus et al., 1993). Temporary arrest at this checkpoint is an essential event in the post-irradiation DNA damage repair process, allowing efficient repair before entry into S-phase (Brugarolas et al., 1999). The lower level of transcription of Rb1 from the CBA promoter may be expected to lead to an inefficient arrest, and hence the entry of cells with unrepaired damage to enter S phase. A similar mechanism has been proposed for the increased incidence of pristane-induced plasmacytoma is associated with inheritance of a p16 promoter variant in BALB/c mice (Zhang et al., 2003). It should be noted that the p16 locus did not show any association

with increased tumor susceptibility in our study nor is there any evidence of an association between human P16 germline mutations and osteosarcoma.

4.1. REDUCED RB1 AND P16 GENE EXPRESSION SHOW DIFFERENT EFFECTS ONTO OSTEOSARCOMA DEVELOPMENT

To test how Rb1 and P16 germline variants differ in their effects onto radiation osteosarcomagenesis, we used heterozygote P16 knock-out and Rb1 knock-out mice and induced tumors again by Th-227 incorporation (Gonzalez-Vasconcellos, 2010). Both mouse lines had a pre-existing defect in one copy of either the P16- or the Rb1 gene in all bone cells, and were compared in this experiment with their wildtype littermates (i.e. with a set of mice on the same genetic background, but with the normal state of both target genes).

Out of 42 animals derived from conditional Rb1 knockout mating, 13 developed osteosarcoma with a median latency time of 402 days. Of these 13 tumors, 11 were found in 24 mice that had already a single copy of the *Rb1* gene lost in every normal bone cell. Of the remaining 18 mice without a pre-existing *Rb1* defect in bone, only 2 were diagnosed with an osteosarcoma (median latency 392 days). As shown in Fig. 9, the time course of osteosarcoma induction is similar in heterozygote Rb1 knockout and in wildtype mice, but the tumor incidence is significantly different ($p=4 \cdot 10^{-5}$ Fisher-Yates exact test).

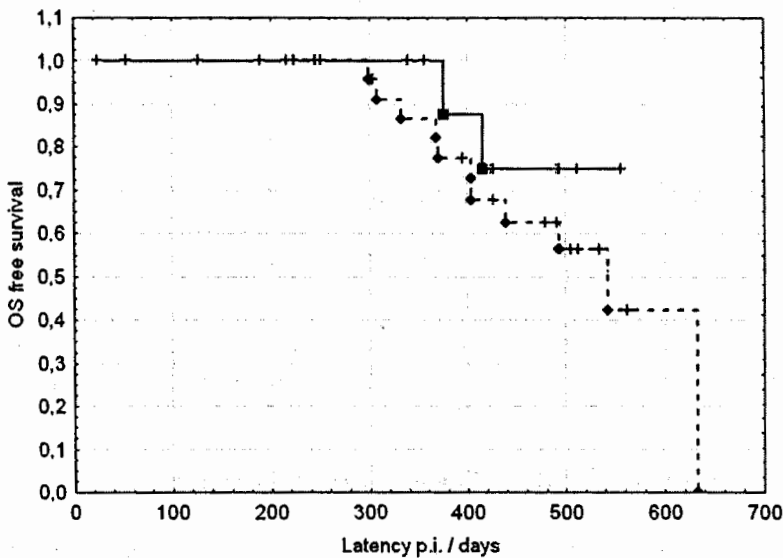


Figure 9. Kaplan-Meier curve for development of osteosarcoma in female FVB/N-RB1LoxP x CreCol mice after 227Thorium injection. All mice carried heterozygote or homozygote alleles of the conditional RB1-LoxP allele (repr. from Gonzalez Vasconcellos et al 2010). dashed line: Mice inheriting Cre-Recombinase --> RB1-deletion in normal bone; full line: Mice not inheriting Cre-Recombinase --> RB1 functionally normal.

Of a total of 70 animals from the P16 knockout breeding, 24 developed osteosarcoma (Fig. 10, median latency time 413 days). Of those 24 tumors, 14 were found among 36 mice with a preexisting defect in one copy of the P16 gene (median latency 355 days). Of the remaining 34 mice without a pre-existing P16 germline defect, 10 were diagnosed with an osteosarcoma (median latency 445 days). The relative numbers of tumors in the heterozygote P16 knockout and in the wildtype mice were statistically not different ($p=0.28$, Fisher's exact test), but they arise much earlier in P16 $^{+/-}$ mice as compared to their wildtype littermates ($p=0.018$, Mann-Whitney Test).

We can therefore conclude that a germline defect of the P16 gene, leading to a 50% reduced expression, changes the tumor growth kinetics, but not the tumor incidence rate. In contrast, a reduced Rb1 expression due to an congenital variant has the capacity to increase osteosarcoma risk, but not the latency time or tumor kinetics.

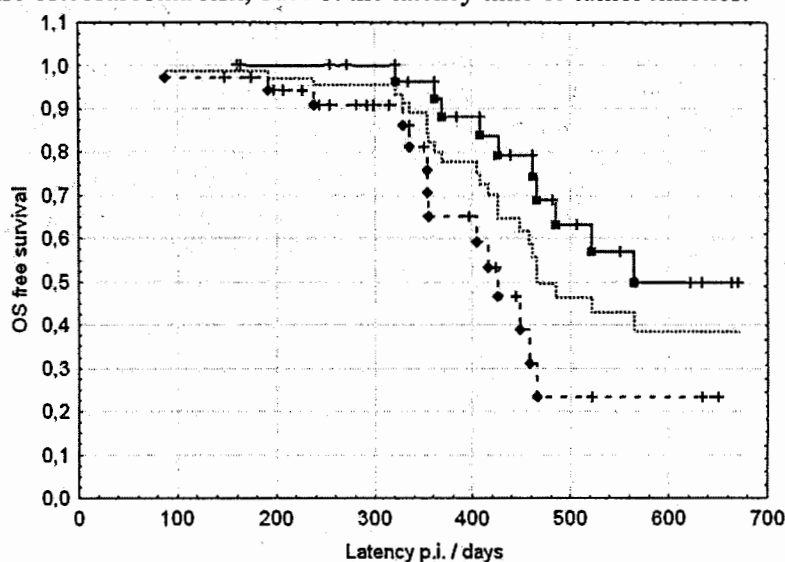


Figure 10. Kaplan-Meier curve for development of osteosarcoma in female C57/BL6-P16 k.o. mice after ^{227}Th injection. Littermate mice are either P16 $^{+/-}$ heterozygote or P16 wildtype (repr. from Gonzalez Vasconcellos et al 2010). dashed line: Mice inheriting P16 $^{+/-}$ germline defect; full line: Mice inheriting wildtype P16; dotted line: pooled cohort of both genotypes

5. Rb1 increases genome instability following radiation-exposure

Whereas Rb1 inactivating germline mutations are known to predispose for radiation-induced osteosarcoma in man (Eng et al., 1993), such an association is not yet known for hypomorphic Rb1 alleles (i.e. alleles that result only in a lower gene-expression). To understand why a 50% reduced Rb1 expression in normal osteoblast cells is sufficient

to confer an increased tumor risk, we investigated the acute effects of a gamma-irradiation in in-vitro cultures of osteoblasts from the above described heterozygote Rb1 knockout mice and their wildtype littermates. Phenotypically normal osteoblasts with a monoallelic Rb1 loss (i.e. with a 50% reduced gene expression) after 2Gy gamma-irradiation exhibit a significantly increased number of post-mitotic genome defects, such as anaphase bridges (Fig. 11a) and micronuclei (Fig. 11b). Both are indicative of an increased genomic instability in the heterozygote Rb1 knockout osteoblasts.

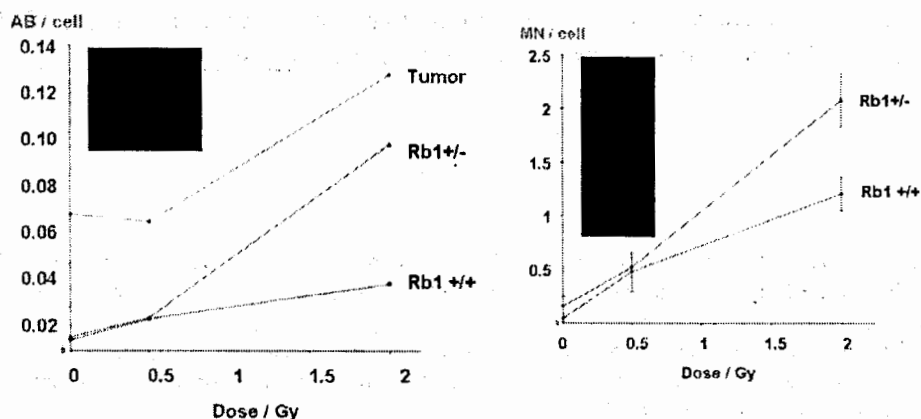


Figure 11. Anaphase bridges (left) and micronuclei (right) in Rb1^{+/-} and Rb1^{+/+} osteoblast cultures in-vitro after gamma irradiation with 0.5 and 2 Gy. For Anaphase bridges, data for an tumor cell line established from one of the radiation-induced osteosarcoma is shown as comparison.

6. Conclusion

We have shown here that a reduced expression of 50% of an important tumor susceptibility gene (Rb1) is sufficient to increase the risk for radiation induced osteosarcoma from 11% to 46%. This reduced Rb1 expression causes an increase of radiation-induced genomic instability in phenotypically normal osteoblasts. Both observations taken together suggest, that in normal wildtype osteoblasts the radiation damage can be tolerated up to a certain level whilst in cells with reduced Rb1-expression the induced genomic instability quickly exceeds a threshold and hence causes an overproportional rise in tumor incidence.

Genetic mapping of additional modifier loci in the outbred BALB/c x CBA strains could also show that, in addition to a principal susceptibility gene such as Rb1 other loci in the genome exist which, in an additive manner, can further contribute to an individual tumor risk. Whether susceptibility genes exist at these loci, or if they rather harbour regulatory elements remains elusive. Such regulatory elements could control expression of susceptibility gene *in-trans*, or could even modulate entire cellular

pathways. Albeit a deeper functional knowledge of such heritable susceptibility factors in man is still missing, such factors could in principle be used already today to predict an individual tumor risk following occupational, medical or accidental radiation exposure.

Acknowledgement

This work was supported by a grants from the European Commission (EURATOM) FIGH-CT99- 00001 and from the German ministry BMBF (KVSF 03NUK007).

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STUDY OF *ARABIDOPSIS THALIANA* PATTERNS OF EXPERIMENTAL MUTATIONAL VARIABILITY*

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Abstract. A summary of results of prolonged genetic and physiological research initiated by N.V.Timofeev-Ressovsky and carried out based on the unique genetic collection of the mutants of the classical model object – *Arabidopsis thaliana* – under the leadership and immediate participation of P.D.Usmanov in the Laboratory of Physiological Genetics of the Genetic Department of the Institute of Plant Physiology and Genetics of the Academy of Sciences of the Republic of Tajikistan. The article contains the following: the summary of development of the *Arabidopsis* genetic collection; the results of the study of the *Arabidopsis* mutation variability; creation of the wide spectrum of mutant forms differing by their phenotype, the study of their genetic nature and physiological and biochemical peculiarities; the analysis of the genotypic variability of the photosynthetic apparatus characteristics in order to ascertain the mechanism of the photosynthesis genetic control and interrelations between the cell organelle (karyon, chloroplasts, mitochondrion). Based on the complex genetic, physiological and biochemical study of the mutant lines from the mentioned *Arabidopsis* collection, there have been established experimental model systems for ecological and genetic research, and the systems for evaluation of the impact of extreme factors and anthropogenic influence on the gene pool of plant populations and analysis of genetic mechanisms of plants adaptation.

Keywords: *Arabidopsis*; mutants; phenotype; genotype; markers; experimental models; test-systems

* Radiobiology and Environmental Security / Eds. C.Mothersill, V.Korogodina, C.Seymour. Springer, 2012. P. 317-330

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1. INTRODUCTION

At the end of 50th of the previous century special attention has been paid to the creation of collections of such plant species which offered special advantage as objects of scientific research in different divisions of biology. *Arabidopsis thaliana* (L) Heynh – the “plant drosophila” – can be attributed to this category with confidence. Good fruit inception at self-pollination and controlled cross-pollination; relative simplicity of cultivation both in soil and on growth medium with agar in test tubes, Koch and Petri dishes with all possible regulation of root nutrition, light and temperature regimes. Due to the abovementioned characteristics *Arabidopsis* has started being used as a model object in genetic, physiological and biochemical research (Kvitko, Muller, 1961; Usmanov, Muller, 1970; Ivanov, 1974).

The Central Seed Bank with hundreds of samples of populations and genetically pure lines of *Arabidopsis* (Kranz, 1978) has been established in Frankfurt-am-Main (Germany). Publication of the special annual journal “*Arabidopsis* Information Service” has been initiated there. *Arabidopsis* researchers from many countries joined around it. A perfect model object – “plant drosophila” – *Arabidopsis* first emerged in Tajikistan at the beginning of 60th of the previous century following the example of N.V.Timofeev-Ressovsky. Being in Tajikistan, N.V.Timofeev-Ressovsky in his reports, lectures and speeches had repeatedly reiterated that a successful choice of the initial material for an experimental research had the decisive importance for the successful solving of the correctly formulated scientific task. In his article “Genetics and plant physiology” (Timofeev-Ressovsky, 1968) he proved the importance of a joint approach of genetics and physiology to the solution of the fundamental practical as well as the most important practical problems of the modern biology. N.V.Timofeev-Ressovsky was one of the first who emphatically urged to use *Arabidopsis* mutants to study the genetic fundamentals of the photosynthesis.

This period is supposed to be the beginning of the unique genetic collection of *Arabidopsis* for genetic and physiological research (including mutagenesis) under the leadership and the immediate participation of P.D.Usmanov, who was a worthy pupil and, later on, a worthy friend of the unforgettable N.V.Timofeev-Ressovsky.

It is known that the *Arabidopsis* genus includes 14 species, some of which are available in the Central Asia. Some species are available in the North Africa, the Mediterranean, Europe and Siberia. “The Flora of the USSR” (1939) includes 5 species. The mentioned number will grow up to 8 with the inclusion of *A.Wallichii* (Hook.f.et Thom) N.Busch, *A.bursifolia* (D.C.) Botsch, isolated recently from other genuses, and a new species *A.Korshinski* Botsch. Seven species of the abovementioned eight are available in the territory of the Central Asian republics and five in Tajikistan (Yunusov et al., 1969). N.I.Vavilov has rightly pointed out that the unique climatic, ecologic and geographic conditions of Tajikistan stipulate its rich morpho-physiological diversity. Tajikistan is an acting evolutionary arena, one of the loci of the intensive form-building

processes. Five species of the *Arabidopsis* genus growing in Tajikistan represent a natural polyploid set: *A. thaliana*, $2n = 10$; *A. Wallichii*, $2n = 16$; *A. mollissima*, $2n = 26$; *A. pumila*, $2n = 32$; *A. korshinski*, $2n = 48$ (Fig.1), as well as 8 ecotypes of *Arabidopsis*: Anzob, Vakhsh, Tajik, Kurama, Shugnan, Rengantau, Turkestan, Kharangon, and populations at different altitudes: Kondara, 1100 meters above the sea level; Khodja-Obi-Gharm, 1800 meters; Sorbo, 2200 meters; and Shokhdora, 3400 meters.

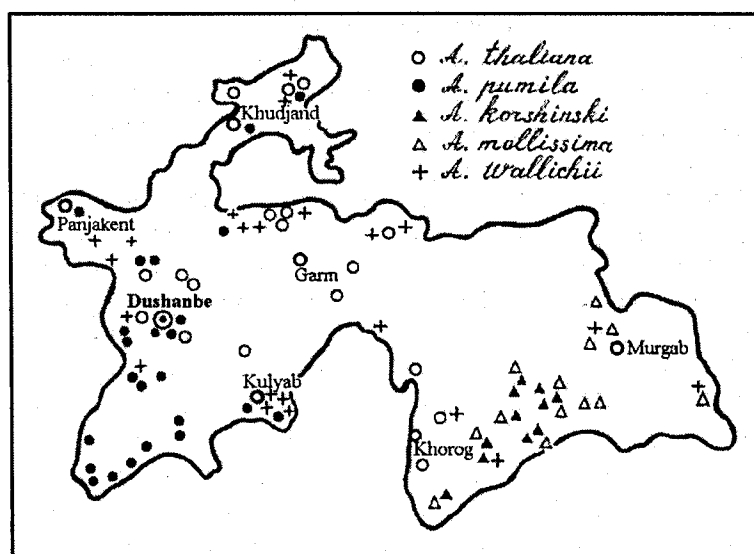


Figure 1. Map of Tajikistan with the marked locations of growth of the *Arabidopsis* species.

2. MATERIALS AND METHODS

Mainly, the work on the cultivation of the *Arabidopsis* mutant forms and their collection, study and experiments have been carried out at the Biological station "Siekukh" of the Institute of Plant Physiology and Genetics of the Academy of Sciences of the Republic of Tajikistan. It is located on the southern slope of the Ghissar range at the altitude of 2300 meters above the sea level, 73 km north of Dushanbe. Plants were grown on special experimental plots annually starting from June to September-October.

Arabidopsis was also grown in the greenhouse of the Genetics Department (Dushanbe, 800 meters above the sea level) as well as in laboratories on agar in test tubes and Koch dishes (aseptic culture) under the light installation in strictly control condition (Veleminsky and Gichner, 1964; Ivanov, 1974).

Methods of experimental mutagenesis, genetic analysis, cytological, biochemical and biophysical methods were used in this work. The experimental data obtained underwent statistical processing according to the commonly accepted methods.

Currently, the genetic collection of *Arabidopsis* of the Genetics Department of the Institute of Plant Physiology and Genetics contains more than 400 samples – different races, populations, ecotypes and mutant forms

3. RESULTS

3.1. THE MUTATION ANALYSIS OF THE PHOTOSYNTHETIC APPARATUS CHARACTERISTICS

During the study of patterns of induction of photosynthetic mutations in *Arabidopsis* by mutagens we followed the common radiobiological experimental methodology, where the main condition is the plotting of "the dose-effect" (DE) curves in the maximum range of doses, possible for the characteristics under study (Timofeev-Ressovsky et al., 1968).

Ionizing radiation – X-rays (XR) and gamma radiation (GR); protons and alpha rays (P and α); and supermutagens: nitrozomethylurea (MNU), nitrozomethyl biureth NMB), nitrozoethylurea (NEU), ethylene imine (EI) and ethyl methane sulphonat (EMS) have been used as mutagens.

It has been established for the most of the genes, determining the characteristics of the photosynthetic apparatus that they react in a different manner to the mutagen impact. To compare the genetic impact caused by different mutagens in *Arabidopsis*, the index of "the degree of sterility" was used as a standard curve (Muller, 1963). Comparison of the frequency of chlorophyll mutations caused by NMB, NMU and XR at the different degrees of sterility has shown that at a low level of sterility (up to 8%) mutation yield curves of the mentioned mutagens are practically congruent. This indicates the identical impact of the compared mutagens on a cell, i.e. that the lethal and mutagen effects have common mechanisms in this case. There is the most clear-cut distinction between mutagens at the interval of sterility from 10% to 30%. The maximum number of mutations is noted at the use of NMB, then NMU and XR, though the comparison of the genetic impact at higher levels of sterility leads to unclear results. The abovementioned allows making a very important methodical conclusion: evaluate the relative genetic efficiency of the mutagens under study, it is necessary to plot DE curves on many experimental points and compare them with each other. Otherwise, the conclusions will be erroneous.

The phenotypic spectra have been plotted (Fig.2) for the evaluation of the specificity of the mutagen impact in induction of different levels of mutations. In this figure, the mutants are arranged according to the increasing frequency of mutations of the *viridis*

class. Therefore, according to the efficiency of causing the mutation frequency of this class, the mutagens arrange as follows: NMU, NMB, P, XR and GR, alpha rays, EMS, NEU and EE. According to mutations of the *xantha* type this order looks as follows: EI, α -particles, EMS, P, NEU, XR and GR, NMB and NMU.

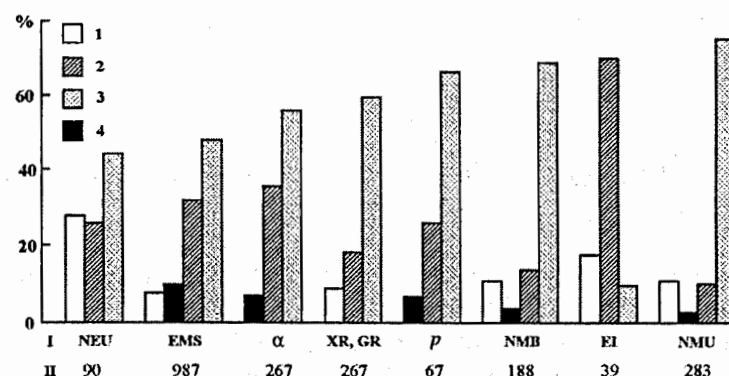


Figure 2. Phenotypic spectra of chlorophyll mutations induced by different mutagens. I – mutagens; II – number of the mutant families. 1 – albina; 2 – xantha; 3 – viridis; 4 – others.

The Figure 2 shows that the heavy charged particles in the spectrum do not have mutations of the *albina* type. Possibly, it is indicative of the specificity of the P and α -particles impact on the genetic apparatus (Usmanov, 1984).

It should be pointed out that the share of the chlorophyll mutations is significant among the experimentally caused mutations (Robbelen, 1963; Ivanov, 1974; Gostimsky, 1981; Sidorova, 1981; Usmanov, 1984; Usmanova, 1990). Therefore, the chlorophyll mutations are widely used in the mutation research as a handy test-system for the quantitative characteristic of the mutation variability of plants.

In order to obtain the point (chlorophyll and morphological) and the genome (polyploidy) mutations of *Arabidopsis* its seeds and pollen grains of the race *Dijon*, *Enkheim* and *Columbia* were exposed to X-rays and processed by EMS and colchisine. For the X-rays (Fig.3) the linear correlation of the yield of the chlorophyll mutations according to the dose of radiation is established at the maximum of 32 kg, while for EMS the exponential correlation of the yield of mutations at the maximum of 24 mM (Usmanov, Usmanova; 1980; Usmanova, 1982).

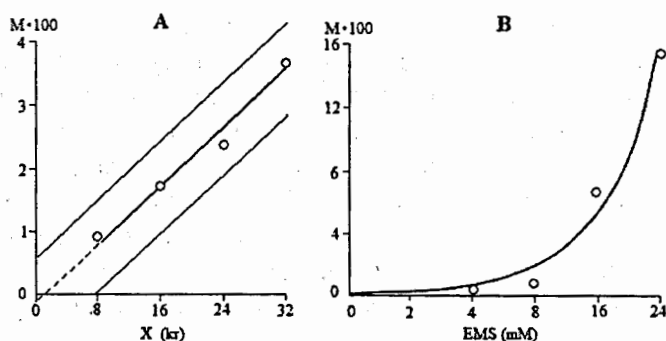


Figure 3. Dependency of the recessive chlorophyll mutations frequency in the *A.thaliana* (A – Dijon race; B – Columbia race) on doses of the X-ray irradiation of the pollen and the EMS concentration (processing of seeds). On the X-axis: dose is in kr; concentration is in mM. On the Y-axis: average number of mutations (M) per 100 diploid cells.

The impact of the UV ($\lambda=254$ nm) on the pollen, and the impact of the natural UV radiation on the pollen and plants of *Arabidopsis* has been studied. Trial experiments have shown that the UV radiation ($\lambda=254$ nm) in the interval of 60-120 minutes caused inactivation of the pollen grains. The results of the basic experiments have shown that the frequency of the dominant embryonic lethal mutations increased naturally according to the radiation dose.

UV dose, min	0	8	16	24	32
Embryos under study	2,026	924	1,132	1,054	1,202
Lethal among them, %	4.1	14.5	33.5	52.8	68.4
Average number of embryos in a seedpod	48	51	52	53	48

During the special experiments the lack of photo-reactivating impact of the light and the dark repair during or shortly after the UV irradiation of the pollen has been proved. The results obtained during the study of the mutation variability of *Arabidopsis* and the variability of the genotypic structure of the model populations of *Arabidopsis* and wheat allow to assert that the UV-B is the factor of selection impacting the direction of micro-evolutionary processes in the populations of the higher plants. Most probably, this is the role of the high mountain UV radiation in the process of the morphogenesis of the higher plants (Usmanov et al., 1980; 1987, 1994).

3.2. GENETIC ANALYSIS AND EVALUATION OF THE NUMBER OF GENES DETERMINING THE PHOTOSYNTHETIC APPARATUS CHARACTERISTICS

The mutational and hybridologic methods of analysis of the genotype structure allowed carrying out the inventory of karyogenes determining the photosynthetic

apparatus characters. To receive the objective evaluation of the number of genes determining the character "the chlorophyll insufficiency", two successive stages of the experimental work should be carried out. At the first stage the frequency of mutations is determined by any mutagen, and the phenotypic spectrum of mutations is plotted. At the second stage determine the number of genes for a definite phenotypic character (phene) by the cross-breeding on the allelism. The second stage of the experiments is very labour-intensive, as is known. And, it is advisable that one should use the method without that stage, or bring it to the minimum. This idea was assumed as a basis of our method. To evaluate the number of genes determining any character, one can limit him/herself by the first stage of the experiment, and instead of the second stage use the following formula:

$$n_i = \frac{P_{ij}}{P_{oj}},$$

where n_i is the number of genes determining A_i – the character of the plant ($i=0,1,\dots,N$); P_{ij} is the frequency of mutations P_{ij} character under the influence of B_j – mutagene ($j=1,2,\dots,M$). The values of n and P_{oj} are determined from the reference experiment over the A_o character (in our case A_o is the *fuska* character), and P_{ij} is ascertained during the experiment on determination of the phenotypic spectra of mutations. In order to calculate the number of genes we accepted $n=12$ (adopted from Muller's work (Muller, 1972) by the *fuska* character, and $P_{ij} : P_{oi} = 40$ (ascertained in our experiments independently of Muller). Then, it follows from the offered formula (as a result of calculation), that $n_i \sim 500$, i.e. the mentioned number of the genes of the *Arabidopsis* genotype determines the characters of the chlorophyll genesis and the lamella system of chloroplasts (Usmanov, 1984).

One of the main results of the genetic analysis was a significant expansion of the available collection by new genetically pure lines of *Arabidopsis*, whose chromosomes are marked with multiple signal genes. These and other genetically pure lines of *Arabidopsis* essentially alleviate the labour-intensive genetic analyses and are used by scientific research groups.

During some special experiments some photosynthetic mutants of *Arabidopsis* were cross-bred with the tester lines *vc'er*, *gl'an* and *lu'co* in order to determine the coupling groups. For the random sampling, including 34 genes, determining the photosynthetic apparatus characters, the following distribution of the mutant genes on the chromosomal coupling groups was obtained. In the first group: *atroc*, *atvi 1*, *atvicos 1 and 2*, *cif 1*, *cif 3*, *flavi 4*, *vimac 2*; in the second group: *sid*, *xa 1-9*, *viluts 1*; in the third group: *almac 2*, *bf*, *clavi 17*, *lu 1*, *tr 2*; in the fourth group: *as 3*, *ch 13*, *xa 1-5*, *xas 1-1*, *1-2*, *1-3*, *1-4*, *1-5*, *vimac 6*; in the fifth group: *cla*, *chlotti*, *flavi 1-1*, *1-2*, *1-3*, *1-4*, *1-5*, *flavici*, *tr 1*. It follows from the above, that the genes determining the photosynthetic apparatus characters are located in all five chromosomal coupling groups of *Arabidopsis* (Usmanov, 1984).

It is significant that in the overwhelming majority of cases the photosynthetic mutations induced by ionizing radiation and chemical mutagens in *Arabidopsis* are the monofactorial disjoining recessives, i.e. they have a nature of the point (gene) mutations. Along with this, some cases showed that the phenes of the chlorophyll insufficiency can be stipulated by both chromosome mutations located in the first and the third chromosomes, and by mutations of the plastid genes.

3.3. PHENE ANALYSIS AND PHENE GENESIS OF THE PHOTOSYNTHETIC APPARATUS

The research of patterns of the genotypic variability of plants has found that the genotypic differences (the genotypic environment) of plants stipulated by the point and chromosome mutations have a significant impact on the character of the interactions and interrelations of the cell organelle. The received mutant forms of *Arabidopsis* differed by the big phenotypic variability and this phenomenon allowed to study the phenotypic display and photosynthetic mutations for determination of the adaptive potentials of the mutant genotypes.

Variation of the composition of the agar nutritive medium had a significant impact on the *Arabidopsis* mutants. As a result, some lethal mutants of *Arabidopsis* reached the phase of fruitification (without nutritive additions lethal seedlings usually die at the stage of cotyledonary leaves within 2-18 days from the date of emergence of seedlings). The cultivated plants were typical dwarfs evenly coloured yellow (79 xa, 127 xa) and whitish (9al), i.e. the normalization did not take place by the character of the colour of plants (Usmanov et al., 1978). Analysis of the ultra-structural organization of the cell components has shown that the plastid completely lacked the lamella structures; however, the genetic blockage of the development of the plastids caused the growth of the number of mitochondrion:

Lines	The number of plastids	The number of mitochondrion
En	8±0.14	9±0.13
79 xa	3±0.13	15±0.20
127 xa	3±0.14	16±0.17

It is interesting to point out that the growth of the number of mitochondrion was accompanied with the good development compared to the norm of their membranous organization. This fact speaks well of the idea on the functional interchangeability of the energy transforming organelle of the plant cell (Gofmann, 1971).

The results show that the energy needed for the biosynthesis ensuring growth and development of the mutant plants is delivered exclusively by mitochondrion which works with the increased functional load in the lack of the normal chloroplasts.

During the study of the biochemical mutant *virido-albina* 40/3 the new evidence of the availability of competitiveness between chloroplasts for the product of metabolism, amino acid leusine, has been received. The leusine deficit caused by the impact of the mutant gene leads to creation of the undeveloped plastids and the normal chloroplasts inside the same cell (the so-called, pseudo-mixed cells, in contrast to the real, characteristic of the cytoplasmic chloroplast mutants). Addition of leusine into the nutritive medium (D,L – 200 mg/liter) led to the finalization of the chloroplast genesis and complete recovery of the chloroplasts ultra-structure, and extinction of the pseudo-mixed cells in the mutant. The concrete mechanism of the formation of hetero-plastids, i.e. the pseudo-mixed cells, in contrast to the real mixed cells, formed under the influence of the plastid mutations, has been found (Abdullaev et al., 1972).

Based on the experimental data (Usmanov et al., 1978) and the results of many other authors, the patterns of the phylogenetic, the ontogenetic, the paratypic and the mutational variability of the photosynthetic membranes systems of the representatives of different systematic plant groups have been analyzed. It allowed to establish the homology of the variability of the photosynthetic membranes stipulated by the mutation events both the genome and the plastome, and by the environmental conditions, in the cells of prokaryotes and eukaryotes.

The mutational variability of the chloroplasts of the higher plants depicts, practically, all types of structures represented in the scheme on the lower floors of the evolutionary spiral. This testifies to the fact that the chloroplasts of the higher plants preserve the historic chronicle of emergence and improvement of the photosynthetic membranes in their genotype.

It is necessary to draw attention to the point mutations of the genetic systems of the cell, which almost always lead to the emergence of the types of the membrane systems located on the lower floors of the evolutionary spiral.

To study the genetic control of the dimensions and the number of chloroplasts both the gene and the genome mutations were used. The dimensions and the number of the chloroplasts in the cells of the cancellous mesophyll of the first pair of the real leaves were studied on the five species of *Arabidopsis* genus growing in Tajikistan, and forming the polyploid row ($2n=10, 16, 24, 32$ and 48 ; (Fig.4).

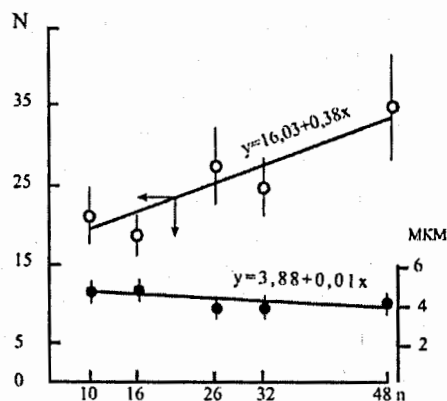


Figure 4. Interrelation between number of chromosomes (n), chloroplasts diameter (dark circles) and average number of chloroplasts (light circles - N) in cells of cancellous mesophyll in five species of the *Arabidopsis* genus.

The results of the regression analysis have shown that the increase of the number of the chromosomes correlates positively with the number of the chloroplasts. For the coefficient of the regression, which characterizes the angle of the curve slope according to the dimensions of the chloroplasts, a reliable difference from a zero was not found ($b=0.014 \pm 0.013$; $P=0.38$). This testifies to the lack of correlation between the alteration of the number of chromosomes, on the one hand, and the dimensions of the chloroplasts, on the other hand (Usmanov, Usmanova, 1973; Usmanov, 1977). Therefore, the control of the number of the chloroplasts in the cell is carried out irrespective of the control of their dimensions, i.e. the number of the chloroplasts in the cell is controlled by the chromosome apparatus, but the dimensions are determined by the plastid genes. The established patterns were confirmed during experiments on the artificial polyploid series of *Arabidopsis* of *Dijon* and *Columbia* races ($2n=10$; $3n=15$ and $4n=20$), and sugar beet ($2n=18$; $3n=27$ and $4n=36$).

3.4. EXPERIMENTAL MODEL SYSTEMS

For ecological and genetic research several test-systems have been elaborated. They include the genetically studied heterozygous *Arabidopsis* plants split by the recessive lethal chlorophyll mutations of the *al*, *xa* and *ch* types (e.g., 104 *xa*, 127 *xa*, P 10/53 *xa*, etc.). The mentioned lines have a specific character – their splitting by the genotype corresponds to the ratio 1:2:1. As a result of the lethal mutations impact the mutant seedlings die in the heterozygous status in the phase of formation of the cotyledonary leaves. Consequently, during the period of fruitification in the second generation only two genotypic classes are registered: one part of the homozygous plants of the wild type and two parts of the heterozygous on the lethal factor plants, which, phenotypically, do not differ from the norm, but which are accurately identified by the embryo-test

method (Muller, 1963; Usmanov, Muller, 1970). These lines are elementary by their structure. They represent "the micro-populations", which being reproduced give plants attributable to two genotypic classes at the ratio 1:2. Shifts in this ratio under the influence of the natural factors allow analysis of the structural variability patterns impacted by the agents under study (Usmanov, 1984; Usmanov et al., 1994).

The study of impact of the lethal mutations in heterozygous state on physiological processes revealed some mutant heterozygotes of *Arabidopsis* (*9 al*, *127 xa*, *P20/31 xa*, *19 ch*, *29 ch*), which statistically exceed the initial race *Enkheim* in the growth, the development and the seed productivity. This indicates the effect of the monohybrid heterosis. The phenotypic display of the monohybrid heterosis depends both on the impact of the mutant genes in the heterosis state, and on the environmental conditions, even if the plants have been grown under the same conditions, but in different seasons (Usmanov, Usmanova, 2007). This experimental model can be used as a sample of the scientific approach for elaboration of effective genetic and selection methods of increase of the crop-productive power of agricultural plants.

A highly sensitive complex test-system was designed based on the semi-dominant chlorophyll mutation of the chlorine type with the recessive lethal impact. It allows the evaluation of the frequency of different types of mutations caused by physical agents or anthropogenic environmental pollutants with the minimal labour and time costs (Usmanov, Usmanova; 1990; Usmanov et al., 1997).

The complex test-system allows, after a single processing of air-dry seeds of *Arabidopsis*, taking of proper account of the mutations frequency on the induction of:

- The somatic mutations registered on the surface of the plants' leaves (the phase of four true leaves 14 days after seeding) attributable to different genotypic classes (+/+, chl/+, chl/chl);
- The recessive, embryonic and chlorophyll lethal mutations being established by the embryonic (germinal) test in the seed generations of plants M1, the generation grown from the seeds exposed to the mutagenic impact;
- The lethal mutations emerging in the fourth chromosome coupling group, taken into consideration in M2 generation (Mednik, Usmanov, 1982a, 1982b).

Among the mutations impacting the structure and the function of the photosynthetic apparatus, allelic mutations are of the biggest interest. Their establishment through the genetic analysis serves as a telling argument in favour of the fact that the structure of the same gene is the unit of variation.

As a result of the cross-breeding of eight chlorophyll mutants (random sampling), attributable to *chlorina* class, three independent genes have been established (*ch3*, *flavi*

1-1 and *ch5*), which irrespective of one another determine yellow-green coloration of plants. Based on one gene *flavi 1* six allelomorphic conditions compiling a series of multiple alleles have been determined: *flavi 1-1*, *flavi 1-2*, *flavi 1-3*, *flavi 1-4*, *flavi 1-5*, *flavi 1-6*, located in the fifth chromosome coupling group. Mutations in different sub-units of the same gene (*flavi 1*) determining the chlorophyll insufficiency, impacting in a different manner all the parameters of the meso-structure and the work of the photosynthetic apparatus, lead, as a rule, to the visible decrease of the index of photosynthesis (Usmanova, Usmanov P.D., 1983; Usmanova, Usmanov, 1989; Usmanova et al., 1991). The key role in suppression of the function of the photosynthetic apparatus in allelic mutants plays the patterns of the ultra-structural organization of the system of the photosynthetic membranes of chloroplasts (Usmanova, 1990).

The obtained results allowed offer the experimental model based on the use of allelic mutations of *Arabidopsis* for the study of mechanisms of sustainability and adaptation of the photosynthetic apparatus to the adverse environmental factors.

Special attention deserve the unique experimental models – “the gene – stem fasciation”, based on the coupling of the gene *cla* (the morphologic mutant, fasciated seed pod) with the tester line *lu*⁻*co* (the gene *lu* stipulates the altering yellow-green coloration of plants in the ontogenesis, the gene *co* determines a very delayed development), and the allelic mutations of the *flavi* series determining yellow-green coloration of plants and delayed development. All the signal genes *cla*⁻, *lu*⁻, *co* and *flavi*, as was established, are located in the same fifth chromosomal group of coupling (Usmanov, Startsev, 1979). For the first time it is shown that the gene *cla* in combination with the different genes *lu*⁻*co* and the genes from a series of multiple alleles (*flavi 1-1* to *flavi 1-5*) on the chlorophyll insufficiency leads to the emergence of the new mutant forms with strongly fasciated stem (up to 25 mm). The experimental model based on the fasciation character can be used for the study of the genetic control of the photosynthetic productivity, in general, and the hereditary heterosis, in particular.

For the first time an experimental model system consisting of 32 differently marked signal genes of the genetically pure lines of *Arabidopsis* has been elaborated. They were received due to multiple cross breedings between themselves and the initial *Columbia* race with the marker lines *tr*⁻, *vc*⁻, *er*⁻, *gl*⁻ and *an*⁻, and allowed the fruitful study of the phylogenetics of the complex physiological processes of the impact of the mutant gene in different genotypical media on the growth, the development and the productivity of plants. It is shown that different genotypical media has a different impact on the display of the genes *tr*⁻, *vc*⁻, *er*⁻, *gl*⁻ and *an*⁻, if evaluate their actions according to the fertility character (weight of 1000 seeds, the number of seeds in a pod and on one plant). A regular decrease of values of these indices was found out depending on the number of the mutant genes, introduced into the genotype. Maximal values of the weight of seeds, the number of the seeds in a pod and on one plant were registered when solitary genes *tr*⁻, *vc*⁻, *er*⁻, *gl*⁻ and *an*⁻ were introduced into the *Arabidopsis* genotype, while minimal

values were identified following the introduction of four and five mutant genes in the genotype of *Arabidopsis*. It is established that the variability of the weight of seeds and the number of seeds, formed on a single plant, depends on different combinations of the signal genes. The weight of seeds from a single *Arabidopsis* plant is a constant value on condition of introduction of one to three genes. At the introduction of four and five genes this index diminishes drastically (Usmanov, et al. 2000; Usmanov T.P., Usmanova O.V., 2007; Usmanov, 2007) (Fig.5).

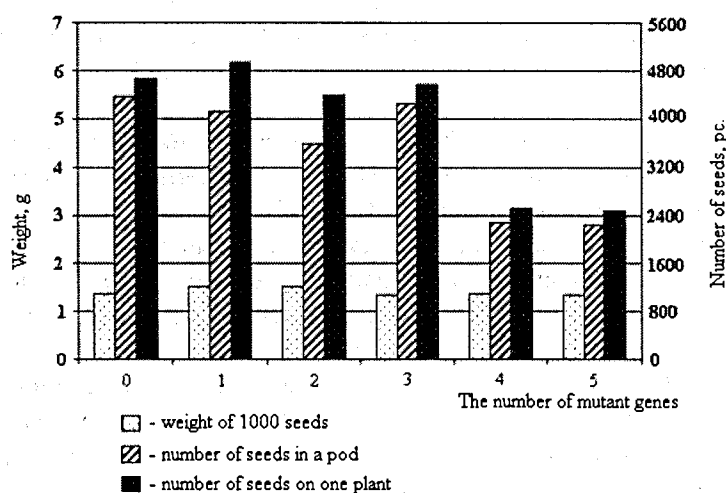


Figure 5. Variability of the weight of seeds from one plant depending on number of mutant genes introduced into the *Arabidopsis* genotype.

The "test-system" for determination of the impact of the factors of a space flight on the life activity of the higher plants including some *Arabidopsis* lines (*En, tr*, 310) was approved under the conditions of imponderability on orbital space stations "SALUT" (Author's Certificate № 919174. Published in the official bulletin of the State Committee of the USSR on inventions and findings, 1982, № 3, p.254; Usmanov et al., 1984).

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ВЛИЯНИЕ МОБИЛЬНЫХ ГЕНЕТИЧЕСКИХ ЭЛЕМЕНТОВ (МГЭ) НА НЕСТАБИЛЬНОСТЬ ГЕНОМА У *DROSOPHILA MELANOGASTER*

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Abstract-This study is dedicated to polymorphism for transposable elements in natural *Drosophila melanogaster* populations and the nature of spontaneous mutations in *D. melanogaster* laboratory stocks and strains sampled in the nature. Transposable element (TE) polymorphism in a natural population can manifest itself at different levels. Strains from one population may differ in TE quantity, quality, and distribution over chromosomes. The most common causes of high locus-specific instability are TE migration and cis-recombination of TE copies. Apparent spontaneous mutations for several genes recorded in highly mutable strain 3314 from Zvenigorodka (Ukraine) seem to be independent. Probably, the mutation in the lozenge gene is related to insertion of a P element, hybridizing with this locus, the more so that this allele demonstrates instability characteristic of insertion mutagenesis. The mutation in the yellow gene is likely to be caused by nucleotide substitutions, because no long DNA insertions have been found within this gene. The high TE polymorphism degree in various natural populations and the high rate of TE transposition may affect reproducibility in tests of the influence of environmental factors on genome instability in natural populations.

Ключевые слова: мобильные генетические элементы; *Drosophila melanogaster*; генетическая нестабильность; спонтанный мутагенез.

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1. Введение

Влияние малых доз радиации на живой организм и на последующие поколения – тема чрезвычайно дискуссионная, поскольку на данный момент существует несколько взаимоисключающих гипотез. Одни считают, что малые дозы радиации полезны (радиационный гормезис), поскольку активируют адаптивный ответ (Ruda and Kuzin, 1991; Turysheva et al., 2008). Есть данные, что длительность жизни некоторых видов животных увеличивается под влиянием малых доз радиации (Кузин, 1995; Moskalev et al., 2011). Экранирование животных от естественного фона радиации приводит к различным отклонениям от нормального развития (Кузинб 1995). Другие считают, что малые дозы радиации относительно более опасны, чем большие дозы, поскольку адаптивный ответ на внешнее воздействие включается только после некой пороговой дозы. Пока пороговая доза не достигнута, в геноме накапливаются повреждения (Бурлакова и др., 1999). Официально пока признана компромиссная линейно-беспороговая гипотеза, поскольку для жизни общества нужны нормативные документы, регламентирующие работу с радионуклидами (Маргулис, 1974). Согласно линейно-беспороговой гипотезе любая сколь угодно малая доза радиации оказывает повреждающее воздействие на геном. Потенциально опасной считается доза, удваивающая частоту спонтанного мутирования. Поскольку частота спонтанного мутирования может варьировать в природных популяциях в широких пределах, а системы репарации в клетках разнообразны, универсальны и активно функционируют, то и дозы, удваивающие частоту спонтанного мутирования, оказываются значительно выше естественного радиационного фона. Например, для человека острая доза, удваивающая частоту спонтанного мутирования в соматических тканях, считается равной 10 сГр. За всю жизнь человек получает примерно эту же дозу при естественном радиационном фоне в 15 мкР/час.

Причины спонтанного мутирования многочисленны и до конца не выяснены. Одним из источников генетической нестабильности является перемещение мобильных генетических элементов, которые найдены во всех изученных на данный момент организмах и занимают порой значительную часть генома. Особенно много МГЭ у растений, однако, эволюционное положение вида не зависит от количества мобильных элементов в геноме (DeBolt, 2010). Родственные виды могут значительно отличаться по содержанию МГЭ в геноме. Одни МГЭ относительно консервативны, другие видоспецифичны (Bartolomé et al., 2009). У *Drosophila melanogaster* описано около 100 семейств МГЭ, каждое из которых представлено одним – двумя десятками копий. Высокая гетерогенность по количеству, качеству МГЭ и полиморфизм по их распределению в геноме может влиять на генетическую нестабильность в природных популяциях.

Мы исследовали влияние МГЭ на нестабильность генома *Drosophila melanogaster* в лабораторных и природных линиях. Лocus-специфическая нестабильность может складываться из перемещения МГЭ и рекомбинации их друг с другом. Высокая частота спонтанного появления видимых мутаций в нестабильной линии 3314 может быть индуцирована как внедрением мобильных элементов в соответствующие гены, так, вероятно, и нуклеотидными заменами. Возможно, такого рода факты в числе прочих и обуславливают появление противоречивых гипотез при анализе влияния малых доз радиации на живой организм, особенно когда речь идет о природных популяциях, генетический фон которых неизвестен с точки зрения представленности МГЭ в геноме контрольных и опытных популяций при внешнем фенотипическом сходстве.

2. Материалы и методы

В работе использовали линии *Drosophila melanogaster*, выделенные из природы (Умань и Звенигородка) - коллекция лаборатории генетики популяций ИЦиГ СО РАН (Новосибирск), линию *y cn bw sp* с полностью секвенированным геномом (Bloomington).

Блот-гибридизацию с P-32 клонированным *hobo* делали по Саузерну после рестрикции по *XhoI* геномной ДНК исследуемых линий как описано ранее (Sambrook et al., 1989).

FISH - анализ проводили на политенных хромосомах слюнных желез личинок третьего возраста. ДНК зондов метили dUTP-bio (Медиген, Новосибирск). Детекцию сайтов гибридизации проводили с помощью авидин-FITC на флуоресцентном микроскопе "Axioskop" 2 Plus (ZEISS) с черно-белой CCD-камерой VC-44 (PCO:) в центре коллективного пользования (ИЦиГ СО РАН, Новосибирск). Использовали пакет программного обеспечения ISIS3, фирмы METSYSStems GmbH.

ПЦР проводили с различными парами праймеров, используя наборы реактивов производства «Медиген» (Новосибирск) по прилагаемым прописям.

3. Результаты и обсуждение

МГЭ могут обеспечивать внутривидовой полиморфизм как на межпопуляционном, так и на внутривидовом уровне. В одних популяциях (или особях) МГЭ может присутствовать, в других его может и не быть. Особи могут отличаться друг от друга по числу копий каждого конкретного МГЭ в геноме, по наличию полноразмерных копий или их отсутствию (Рис. 1). В изосамочьих линиях, полученных из природной популяции *Drosophila melanogaster* Умани (Украина), часть линий не содержит полноразмерного

варианта *hobo*-элемента (Рис. 1). Набор дефектных вариантов *hobo* уникален для каждой из этих линий, хотя все они имеют родственное происхождение и содержат встройку одного и того же варианта *hobo* в регуляторной части гена *yellow* (Захаренко и др. 2004; Zakharenko et al. 2000). Число вариантов дефектных копий также может быть значительным, и может приближаться к числу копий МГЭ на геном, хотя некоторые варианты дефектных копий могут доминировать (Zakharenko, Perepelkina, 2009; Рис 1 и 2). Например, в геноме линии *у cn bw sp* доминирует дефектная копия *hobo* длиной в 1,4 т.п.о. (Рис 2).

Невозможно найти два одинаковых генома по распределению МГЭ у дрозофилы, даже если это дрозофилы из одной и той же популяции. Даже в изогенной линии распределение мобильных элементов в хромосомах может меняться со временем (Захаренко и др., 2007, Рис 3). В геноме полностью секвенированной линии *у cn bw sp Drosophila melanogaster* локализация *mdg1* со временем не изменилась (сайты 93F и 98C), в то время как число *hobo*-элементов на представленном фрагменте хромосомы увеличилось в три раза (Рис. 3). Часть старых копий *hobo* за это время исчезла (Zakharenko et al., 2007), при этом исчезли в основном дефектные производные одного размера (1.4 т.п.о.).

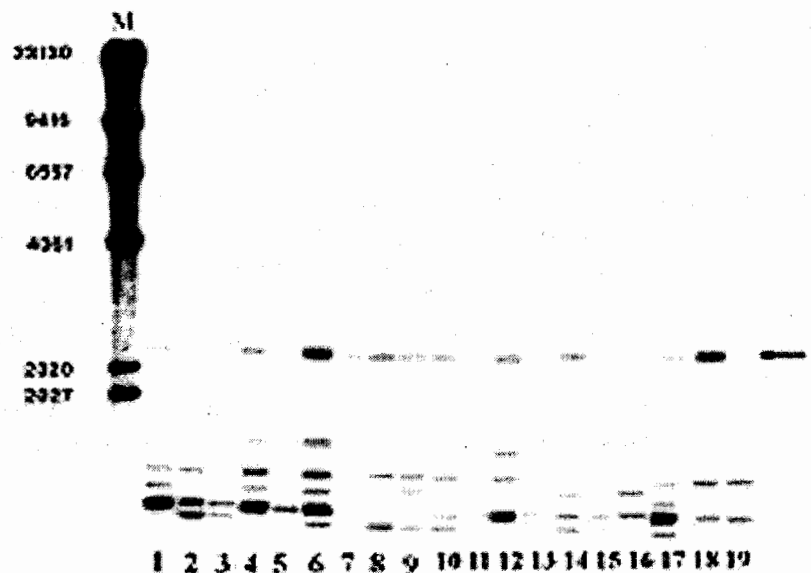


Рис 1. Саузерн-блот-гибридизация P^{32} – зонда (ДНК *hobo*-элемента) на геномную ДНК 19-ти изосамочьих линий из природной популяции *D. melanogaster* (г. Умань, Украина) после рестрикции *Xho*I. Стрелкой обозначена полноразмерная копия *hobo*. М – маркерная ДНК (λ -Hind III).

Большая часть копий МГЭ дрозофилы дефектна (Рис. 1, 2) и не способна продуцировать белковый продукт, хотя в некоторых случаях дефектные копии способны к неавтономному перемещению. Перемещаются МГЭ с помощью ферментов, которые сами же и продуцируют. Считается, что в некоторых случаях возможна кроссиндукция, когда перемещение одного МГЭ определяется активностью другого. Одни авторы считают, что активность МГЭ определяется внешними факторами (радиационное воздействие, тепловой шок) (Аникеева и др., 1994; Васильева и др., 1995; Забанов и др., 1995; Бубенщикова и др., 2002; Васильева, Ратнер, 2000), другие такой зависимости не находят (тепловой шок, перекись водорода, дихлорфос, γ -радиация) (Arnault, Biemont, 1989; Arnault et al., 1991; Arnault, Dufournel, 1994, Arnault et al., 1997; Захаренко и др.,).

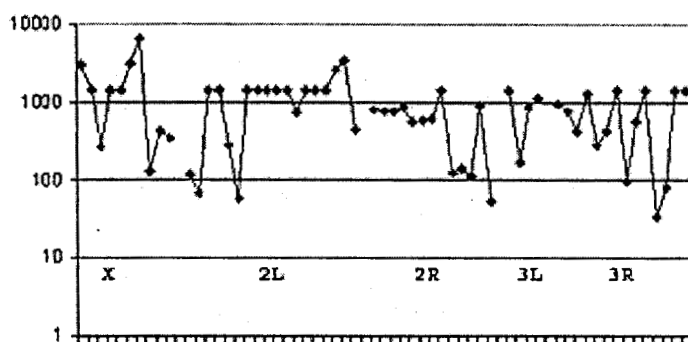


Рис 2. Представленность *hobo*-элемента и его дефектных производных в геноме линии *u sn bw sp Drosophila melanogaster*. По оси абсцисс – распределение *hobo* по хромосомам, по оси ординат – логарифм длины *hobo*-элемента. Доминирует дефектный вариант *hobo* длиной 1400 пар оснований.

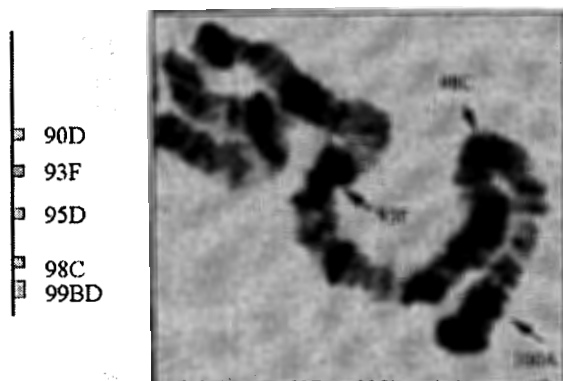


Рис. 3. Локализация ретротранспозона *mdg1* (сайты 93F и 98C) и *hobo*-транспозона на фрагменте третьей хромосомы линии *u sn bw sp Drosophila melanogaster* по данным *in silico* (1992 г., слева) и FISH (2005 г., справа).

Из природных популяций *D. melanogaster* можно выделить линии, обладающие высоким уровнем спонтанной генетической нестабильности. Из популяции *D. melanogaster* (Звенигородка, Украина) была выделена высоконестабильная линия 3314, в которой было найдено несколько спонтанно

возникших видимых мутаций. Спонтанные мутации в отводках линии 3314 были обнаружены в генах *yellow* (1A5), *white* (3B6) и *lozenge* (8D5-8D6) (Рис. 4). С активностью исследованных мобильных генетических элементов (транспозонов *P* и *hobo* и ретротранспозонов *mdg1* и *mdg2*) по нашим данным может быть связано лишь появление мутации *lz*, поскольку в сайте 8D в соответствующей линии обнаружен сайт гибридизации *P*-элемента (Рис. 5). Мутация *lz* проявляла свойственную инсерционному мутагенезу нестабильность, ревертируя к нормальному фенотипу. При возврате к нормальному фенотипу *P*-элемент из сайта 8D исчезает (Рис. 5).

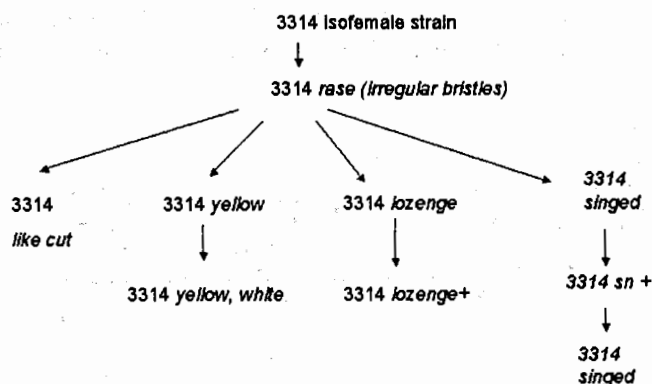


Рис 4. Схема возникновения видимых мутаций в линии 3314 *D. melanogaster* из Звенигородки.

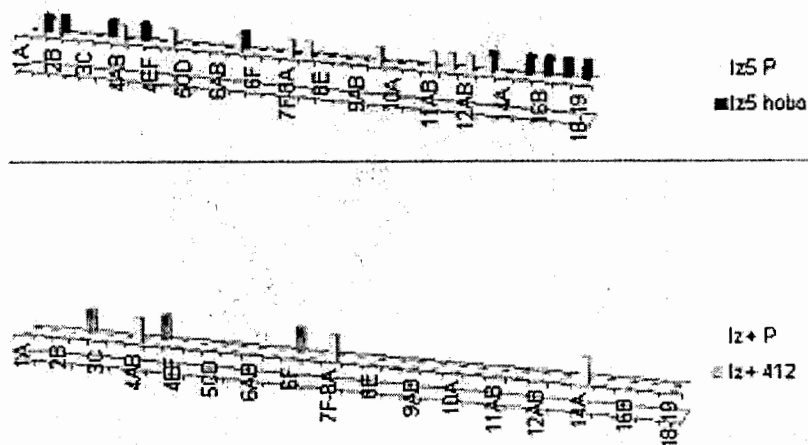


Рис 5. Распределение сайтов гибридизации *P*-элемента, *hobo*-элемента, *Dm412* на X-хромосоме производных по гену *lozenge* линии 3314 *Drosophila melanogaster*

В мутантном гене *yellow* (в производной линии 3314 *yellow*) встроено не обнаружено. Длина ПЦР-продуктов, полученных с разными наборами праймеров (Рис. 6) с ДНК мутантного гена, совпадала с длиной соответствующего фрагмента в контрольном образце на электрофореграммах в агарозном геле. Это означает, что больших инверсий или делеций в этом гене нет и что мобильные элементы скорее всего не причастны к появлению мутации в гене *yellow* этой линии. Таким образом, появление видимых мутаций в разных локусах этой линии имеет, видимо, различную природу.

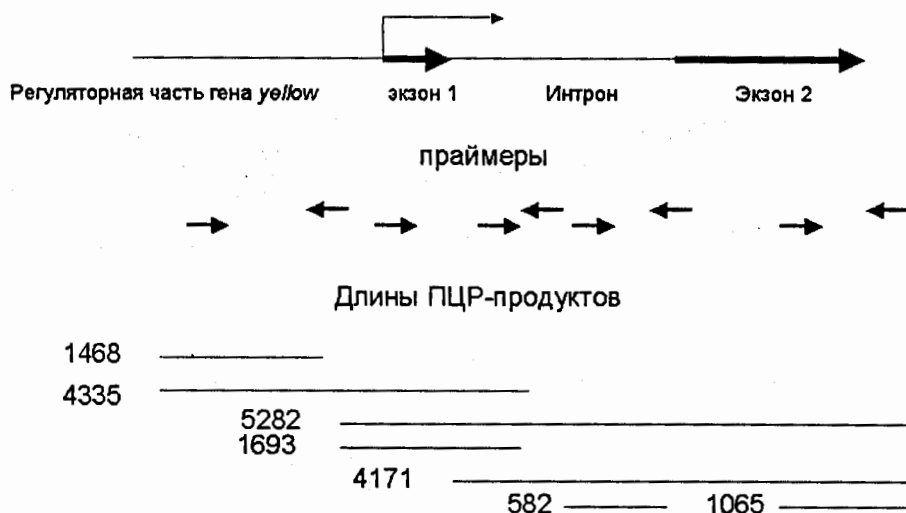


Рис. 6. Структура гена *yellow* *D. melanogaster* и схема ПЦР-анализа гена *yellow* из отводки 3314 *yellow*.

Распределение МГЭ на X-хромосомах исследуемых линий чрезвычайно вариабельно, что свидетельствует о высокой скорости перемещения МГЭ, взятых в анализ (Рис 5, 7). Обычно повышенная активность в конкретной линии характерна лишь для отдельных типов МГЭ. В нашей ситуации активны все исследованные МГЭ, что типично для вспышки мутабельности. Мутантные отводки сохраняли в лаборатории благодаря скрещиванию исключительных самцов с самками со сцепленными X-хромосомами. Возможно, нестабильность генома линии 3314 по мутациям *lz*, *y*, *sn* индуцируется или, по крайней мере, отчасти усиливается скрещиванием с балансерной линией в последующих поколениях, поскольку скрещивание географически и/или генетически удаленных родителей у дрозофилы может индуцировать гибридный дисгенез, проявляющийся в различных генетических нарушениях. Нестабильность в геноме линии 3314 может быть обусловлена также дисгенными скрещиваниями за счет генетической гетерогенности природной популяции или нарушениями в системе репарации (см, например, Коваленко и др., 2006; Захаренко и др., 2007; Zakharenko and Perepelkina, 2009).

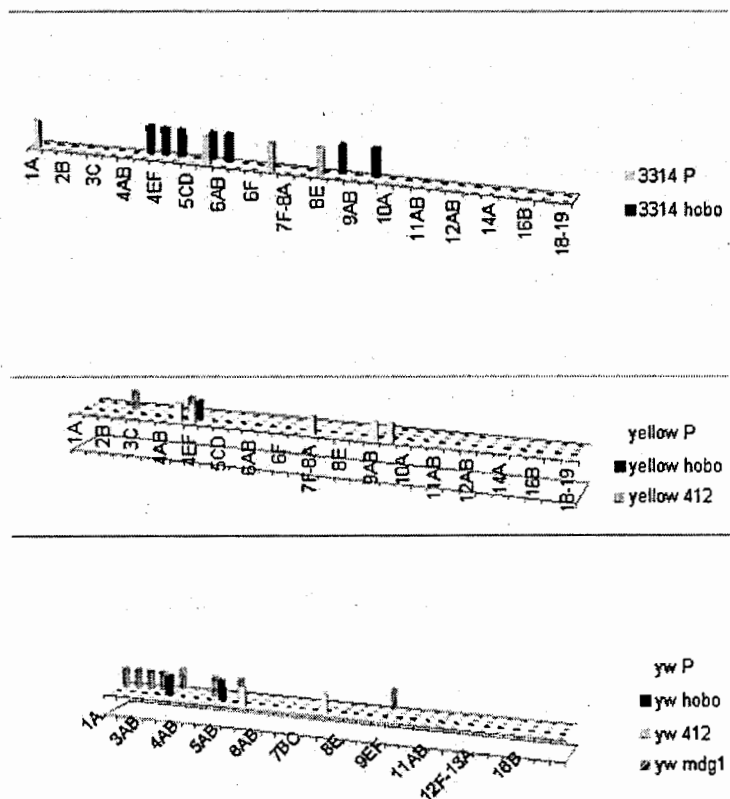


Рис. 7. Распределение сайтов гибридизации *P*-элемента, *hobo*-элемента, *Dm412* и *mdg1* на X-хромосоме линии 3314 strain *Drosophila melanogaster* и ее производных.

В ряде случаев у *Drosophila melanogaster* обнаруживали локуспецифическую нестабильность, обусловленную внедрением МГЭ в интрон или регуляторную зону гена (Zakharenko et al., 2000, Захаренко и др., 2004). Нестабильность в этом случае складывается из перемещения МГЭ и рекомбинации их друг с другом. Благодаря рекомбинациям между разнонаправленными МГЭ, возможна быстрая смена фенотипов от мутации к норме и обратно. Частота мутирования в таких локусах может достигать $10^{-1} - 10^{-2}$ на сайт на геном за поколение и может быть обусловлена инверсиями и реинверсиями между двумя разнонаправленными МГЭ (Eggleston et al., 1996). Инверсии, окаймленные двумя МГЭ, находят в природных популяциях (Sniegowski and Charlesworth 1994; Casals et al., 2003). Однако образование многочисленных внутривхромосомных инверсий, отличающих один вид дрозофил от другого, не связан с мобильными элементами. Благодаря почти

полному сиквенсу 12 геномов дрозофил показано, что более чем у половины из 29 проанализированных эволюционно закрепившихся инверсий на концах находятся дубликации уникальных последовательностей (Ranz et al., 2007).

Значимость МГЭ для эволюции до конца не понята, поскольку одни считают МГЭ паразитической компонентой генома, другие отводят ей значительную роль в эволюции. С одной стороны появился термин «экзонизация» мобильных элементов, когда фрагменты МГЭ становятся частью функционирующего гена и входят в состав экзонов (Lipatov et al., 2005). Из МГЭ, например, сформированы теломеры у *Drosophila melanogaster* (Pardue and DeBaryshe, 2008). С другой стороны, МГЭ накапливаются в неактивных районах хромосомы (интеркалярном гетерохроматине, хромоцентре, в межгенных спейсерах).

Высокая гетерогенность по МГЭ в природных популяциях и высокая скорость перемещения МГЭ, усложняет исследование влияния внешних факторов (в том числе влияние малых доз радиации) на генетическую нестабильность в природных популяциях. Возможно, этот факт в числе прочих и обуславливает такое разнообразие противоречивых гипотез при анализе влияния малых доз радиации на живой организм.

Благодарности

Работа была частично поддержана грантами РФФИ 09-04-00213а и 09-04-00872-а, грантом Б23.29 и Б23.30 Президиума РАН.

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Перед кафе «Дубна». Слева направо, первый ряд: Ю. Сошкина, Е. Степченкова (заслонена), Т. Дутова, Е. Аззам, А. Корсаков, К. Мотерсил, В. Корогодина, С. Розенберг, К. Виера, Н. Горбушин; второй ряд: Н. Мордкович, О. Пронина, С. Рушковский, В. Королев, Е. Даев, О. Афанасьев, К. Афанасьева, М. Роземан, Е. Хлесткина, О. Усманова, Г. Эрцгребер, Е. Исаева, А. Чинья; третий ряд: В. Суслов (позади), О. Ковальчук, С. Чанкова, Н. Зюзиков, И. Хохуткин, Н. Кузьмина, Е. Антонова, В. Ядровская, А. Моисеев, С. Инге-Вечтомов, Д. Горденин, Л. Намолован, В. Новикова

In front of coffee house «Dubna». From left to right, first row: Yu. Soshkina, E. Stepchenkova (hidden), T. Dutova, E. Azzam, A. Korsakov, C. Mothersill, V. Korogodina, S. Rosenberg, C. Viera, N. Gorbushin; second row: N. Mordkovich, O. Pronina, S. Rushkovsky, V. Korolev, E. Daev, O. Afanasiev, K. Afanasieva, M. Rosemann, E. Khlestkina, O. Usmanova, G. Erzgraeber, E. Isaeva, A. Cigna; third row: V. Suslov (behind), O. Koval'chuk, S. Chankova, N. Zyuzikov, I. Khokhutkin, N. Kuz'mina, E. Antonova, V. Yadrovskaya, A. Moiseev, S. Inge-Vechtormov, D. Gordenin, L. Namolovan, V. Novikova



К. Сеймур
и К. Мотерсил

C. Seymour
and C. Mothersill

Дж. Дрейк
J. W. Drake



С. Г. Инге-Вечтомов
S. G. Inge-Vechtomov



Р. М. Алексахин
R. M. Alexakhin





Добро пожаловать в пансионат «Дубна». М. Резник
Welcome to holiday house «Dubna». M. Resnick



Молодые ученые. Слева направо: стоят — И. Прокофьев, Л. Намолован, А. Жук, Ю. Сошкина, Н. Кузьмина, Е. Плюснина, Е. Степченкова, К. Афанасьева, Л. Ялковская, Н. Мордкович, Е. Александрова, Е. Антонова, Е. Хлесткина; сидят — О. Афанасьев, А. Буздин, С. Рушковский, А. Дикарев; лежит — В. Суслов

Young scientists. From left to right: stand — I. Prokopiev, L. Namolovan, A. Zhuk, Yu. Soshkina, N. Kuz'mina, E. Plusnina, E. Stepchenkova, K. Afanasieva, L. Yalkovskaya, N. Mordkovich, E. Alexandrova, E. Antonova, E. Khlestkina; squat — O. Afanasiev, A. Buzdin, S. Rushkovsky, A. Dikarev; lying — V. Suslov

Перерыв на кофе. Coffee break

Т. Хинтон и Д. Гудков
T. Hinton and D. Gudkov



А. Буздин и Л. Захаренко
A. Buzdin and L. Zakharenko

Прием. Welcome party

Три грации. С. Розенберг,
К. Вьера, О. Ковальчук
Three Graces. S. Rosenberg,
C. Viera, O. Kovalchuk



С. Инге-Вечтомов, В. Корогодина
S. Inge-Vechtomov, V. Korogodina



Церемония награждения
Awarding ceremony

Р. М. Алексахин,
С. Г. Инге-Вечтомов

R. M. Alexakhin,
S. G. Inge-Vechtomov



С. А. Гераськин,
Р. М. Алексахин

S. A. Geras'kin,
R. M. Alexakhin



П. Морозик
P. Morozik

Слева направо: М. Голубовский,
В. Корогодина, М. Резник

From left to right: M. Golubovsky,
V. Korogodina, M. Resnick



Слева направо: Л. Захаренко, Е. Александрова,
Т. Хинтон, Е. Аззам, Н. Рябokonъ

From left to right: L. Zakharenko, E. Alexandrova,
T. Hinton, E. Azzam, N. Ryabokon



Слева направо: М. Дуранте, А. Чинья,
М. Роземан

From left to right: M. Durante, A. Cigna,
M. Rosemann



Слева направо: Ю. Дуброва,
Л. Осипова, А. Рубанович

From left to right: Yu. Dubrova,
L. Osipova, A. Rubanovich

**Экскурсия
в заповедник Карадаг**

**Excursion
to Karadag reserve**





Покорители вершин. Peak conquerors



После обсуждения проблем экологии Крыма. Слева направо: Р. Горбунов, О. Парубец, В. Лапченко, Н. Мильчакова, В. Боков, В. Большаков, А. Морозова, В. Корогодина, В. Тарасенко, Н. Горбушин, Т. Бобра, А. Чинья

After discussion on the ecology problems of Crimea. From left to right: R. Gorbunov, O. Parubets, V. Lapchenko, N. Mil'chakova, V. Bokov, V. Bol'shakov, A. Morozova, V. Korogodina, V. Tarasenko, N. Gorbushin, T. Bobra, A. Cigna



М. Голубовский. M. Golubovsky

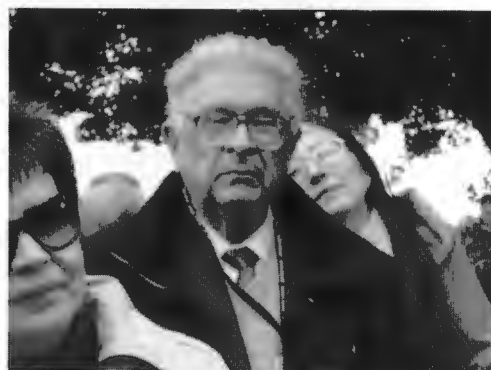


С. Гераськин. S. Geras'kin



Е. Исаева, А. Марков
E. Isaeva, A. Markov

Экскурсия в Воронцовский парк Excursion in Vorontsovsky Park



Р. М. Алексахин. R. M. Alexakhin



С. Чанкова, Б. Петров
S. Chankova, B. Petrov

**РАДИОБИОЛОГИЯ. ПРОБЛЕМЫ
РАДИАЦИОННО-ЗАГРЯЗНЕННЫХ ТЕРРИТОРИЙ**

**RADIOBIOLOGY. PROBLEMS
OF RADIATION-POLLUTED TERRITORIES**

MICRO - ALGAE AS A MODEL SYSTEM FOR STUDYING OF GENOTYPE RESISTANCE TO OXIDATIVE STRESS AND ADAPTIVE RESPONSE*

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Abstract. Here we discuss the possible contribution of DSB DNA repair and chaperone systems for the formation of genotype resistance to oxidative stress and the possible correlation between cells' genotype resistance and the magnitude of adaptive response (AR). Mutant strains of *Chlamydomonas reinhardtii* showing different levels of gamma- rays, paraquat- and zeocin-resistance as well as species *Chlorella* isolated from habitats with extreme environmental conditions are used as a model system. On the basis of results obtained it could be speculated that genotype resistance to oxidative stress may not always correlate with the initial level of DSB induced, but rather with higher DSB repair capacity and higher content of constitutive HSP70B. Our results concerning the relationship between genotype resistance to oxidative stress and adaptive response confirm our previous suggestion that up-regulated DSB DNA rejoining could be considered as one of the mechanisms involved in the formation of AR in this organism. New data are provided that strains with a relatively lower genotype resistance demonstrate a stronger AR. On the other hand the higher genotype resistance of strains did not abrogate their competence to adapt. A relationship between DNA repair capacity estimated on the basis of single dose treatment and the DNA repair capacity estimated on the basis of the AR is obtained.

Keywords: genotype resistance, adaptive response, DSB DNA induction, repair capacity, chloroplast chaperones, paraquat, zeocin, heat shock

* Radiobiology and Environmental Security / Eds. C.Mothersill, V.Korogodina, C.Seymour. Springer, 2012. P. 19-30

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1. Introduction

Organisms are continuously subjected to naturally occurring changes and additionally to disturbances originated by human activity. The impact of these changes on whole ecosystem depends on the response of single organism. During the evolution many defense strategies have been developed in organisms to minimize/overcome the potential harmful action of environmental stress and to increase cell tolerance or adaptation to oxidative stress.

The term adaptive response (AR) generally refers to the phenomenon that low-dose exposure to a genotoxic agent can protect cells from a subsequent damaging high-dose exposure (Joiner et al., 1996, 1999; Wolff, 1998; Patra et al., 2003; Asad et al., 2004; Girigoswami and Ghosh, 2005; Yan et al., 2006; Dimova et al., 2008). Accumulating data in the literature strongly suggest that some defense mechanisms probably underlying the AR could involve acceleration of DNA repair, *de novo* synthesized proteins; activation and/or partial contribution of the antioxidant system, chaperone accumulation, more efficient detoxification of free radicals etc. (Miura, 2004, 2010; Coleman et al., 2005; Lanza et al., 2005, Dimova et al., 2008). A well known hypothesis is that the AR may be triggered by some low level of primary induced DNA double-strand breaks (DSB) which could probably serve as a signal for activation of DSB repair (Boreham, and Mitchel, 1991; Wolff, 1998; Matsumoto et al., 2004, Chankova et al., 2009).

For example, it has been previously shown by us that model *C. reinhardtii* strains could develop strong radiation-, zeocin (Zeo)- or paraquat (PQ)-induced AR involving accelerated rejoining of DSB (Chankova and Bryant, 2002; Chankova et al., 2005a, 2007; Dimova et al., 2008, 2009). Intriguingly, heat pretreatment (37-42°C) was not sufficient to induce a statistically significant increasing of DSB in *C. reinhardtii* strain CW15 and did not induce AR to test dose gamma rays irradiation in the form of accelerated DSB rejoining (Chankova et al., 2009).

At present, however, there is limited evidence whether there could be a correlation between genotype resistance (i.e. single cell radio-/chemo-resistance) and the capability to develop AR. The published data are still insufficient and the relationships between cell resistance and the ability to develop an AR are difficult to predict. For example, it has been proposed that certain tumors which are difficult to cure by X-rays may exhibit a strong induced radio-resistance (Ward, 1988). Some radio-sensitive tumor lines and cells of ataxia telangiectasia patients do not show an AR in the form of induced radio-resistance (Lambin et al., 1994). On the other hand Zasukhina et al. (2000) reported that the level of radiation-induced AR was the same in blood cells of patients with Bloom syndrome (human autosomal recessive disorder with chromosomal instability and increased risk of malignancy at an early age) as that in cells from healthy donors. For better understanding of some mechanisms involved in the formation of genotype resistance and adaptive response (AR) in plants, mutant strains of *C. reinhardtii* with

different level of radio- and chemo-resistance as well as algae from habitats with extreme environmental conditions have been used as a model system.

Our hypothesis is that resistant to oxidative stress mutant strains and algal species isolated from habitats with extreme environmental conditions would have similar and/or more efficient cellular defense mechanisms.

We address two main questions: What is the possible contribution of DSB DNA repair and chaperone systems for the formation of genotype resistance to oxidative stress? Is there a relationship between genotype resistance to oxidative stress and the magnitude of adaptive response (AR) in micro- algae?

2. Why micro-algae have been chosen as a model system?

Unicellular green algae have been developed into a “good” test-system in modern ecotoxicology and for studying of genotype resistance and AR for several reasons: cell/organism, with a simple life cycle, relatively inexpensive experimental conditions and methods for cultivation, a growing array of tools and techniques for molecular genetic studies, quick methods with good resolution, sensitivity, potential for extrapolation of results obtained to higher plants, easily isolated mutants with different levels of radio- and chemo-resistance etc (Chankova et al., 2005a, 2005b, 2007; Dimitrova et al., 2007; Dimova et al, 2008, 2009).

On the other hand an understanding of the molecular mechanisms of genotype resistance to oxidative stress and adaptive response exhibited by mutant strains of *Chlamydomonas reinhardtii* may lead us to a deeper understanding of plant cells response to oxidative stress in general. This organism is particularly interesting from the point of view of its powerful adaptive response to radiation (Bryant, 1968, 1975, 1976; Boreham and Mitchel, 1991; Chankova and Bryant, 2002), and in addition it promises to provide indirectly further insight into the adaptive response of normal and malignant human cells at low radiation doses (Joiner et al., 1996, 1999). Up to now, most studies of repair on *C. reinhardtii* have focused on nuclear and extra nuclear DNA using mutant repair-deficient strains (Small, 1987; Vlcek et al., 1987, 1995, 1997; Podstavkova et al., 1992, Miadokova et al. 1994). However, radio resistant mutants are scarce (Chankova et al., 2000, 2001, 2005a). We have been interested in the question about whether radio resistant and chemo- resistant strains of this organism would, in addition to its resistance in single-dose experiments, show an adaptive response to oxidative stress in split-dose experiments. The main characteristics of *C. reinhardtii* strains and *Chlorella* species are presented in Table 1.

3. Genotype resistance to oxidative stress

Previously it was described by us that radio resistant genotype of *Chlorella vulgaris* and *Chlamydomonas reinhardtii* would be characterized with increased SSB DNA repair

efficiency (Chankova et al., 1994, 2000), higher levels of constitutive SOD, SH-group, pigments, especially carotenoids and chl" a" (Chankova et al., 1990, 2000), and stability of the ultrastructural cell components or/and of the presence of cell wall (Chankova et al., 2000). Furthermore we aimed to throw more light on the contribution of initial level of DSB DNA, repair efficiency and constitutive levels of HSPs for the formation of genotype resistance.

Table 1 Characterization of strains and species of micro-algae

C. reinhardtii strains	Characteristics
137C	Wild type (WT), (Smith, 1946); St Petersburg collection
AK-9-9	Obtained by chemical mutagenesis (Chankova, Frolova and Hromov-Borisov, 1990, St Petersburg University), Can res, radioresistant, cross-resistant to Zeo and PQ (Chankova et al, 2000; 2001; 2005; 2008; Dimova et al, 2008)
CW15	Culture Collection of Algae and Protozoa (CCAP 11/32CW15+), Ambleside, UK; cell-wall-less with WT radio resistance (Darling 1993; Chankova et al, 2001)
H-3	Hybrid strain, obtained by Chankova and Bryant (Chankova et al, 2000, 2005), radio resistant, cross - resistant to Zeo and PQ (Dimova et al, 2008, 2009).
Chlorella species	
<i>Chlorella kessleri</i>	Mesophilic, isolated from USA freshwater, axenic, mainly used for fundamental research, from Czech collection
<i>Chlorella sp.</i>	Antarctic – psychrophilic, isolated from soil on the island of Livingston, the South Shetland archipelago
<i>Chlorella vulgaris</i> strain 8/1	Thermophilic strain, isolated in 1968 from the thermal spring (t = 40-50° C) near by Petrich (Bulgaria); during 20 years laboratory cultivated at room temperature

In order to test our hypothesis that algal species isolated from habitats with extreme environmental conditions could be used as a model for the investigation of genotype resistance to oxidative stress, the responses of three *Chlorella* species to UV-B irradiation, Zeo and heat treatment are compared. A large range of doses of UV-B irradiation, $\lambda = 312\text{nm}$ (BLX, Life Technology, UV crosslinker), Zeo concentrations and temperatures are applied. As a first step cells' response of *Chlorella sp* (antarctic), *Chlorella vulgaris* 8/1 (thermophilic) and *Chlorella kessleri* (mesophilic) is examined on the basis of various endpoints - spot-test, micro-colonies assay and growth rate. Species resistance to oxidative stress is evaluated on the basis of LD₂₀, LD₅₀ and LD₉₀

(Chankova et al., 2005a, b). Our pilot results show that *Chlorella* species differ in their response. The most pronounced growth rate and cell survival are calculated for *Chlorella* sp. It is also found that *Chlorella* species are photo reactivation proficient and differ in their capacity to overcome harmful effect of UV-B. Species resistance to UV-B induced stress could be arranged in the following order depending on post irradiation conditions: *Ch.sp*>*Ch.vulgaris*8/1=*Ch.kessleri* (samples with photo-reactivation) and *Ch.sp*>*Ch.vulgaris*8/1>*Ch.kessleri* (samples without photo-reactivation).

3.1. ON THE ROLE OF INITIAL LEVEL OF DSB DNA FOR THE FORMATION OF GENOTYPE RESISTANCE

In our paper (Dimova et al., 2008) we describe that the level of DSB DNA induced by single PQ treatment is higher in strain CW15 comparing with that for strain 137C. No statistically significant increasing in the level of DSB is found for the radio-resistant strains H-3 and AK-9-9 even at high concentrations. For the same strains an increasing of DSB induced by single Zeo treatment in a concentration depended manner up to about 100 $\mu\text{g ml}^{-1}$ is shown (Dimova et al., 2009). Above this concentration the dose response curves levelled out to a plateau. As a whole, no statistically significant differences are observed between DSB values in strains 137C, AK-9-9 and H-3. One way ANOVA with Tukey multiple comparison test confirm that these three strains have a similar Zeo resistance in terms of DSB induction in the range of 10–300 $\mu\text{g ml}^{-1}$. The level of DSB is higher for strain CW15 (Chankova et al., 2007; Dimova et al., 2009).

3.2. ON THE ROLE OF DSB DNA REPAIR EFFICIENCY FOR THE FORMATION OF GENOTYPE RESISTANCE

To clarify the contribution of DNA repair factor in the formation of genotype resistance to oxidative stress, repair capacity of strains is analyzed. Our results indicate that differences in Zeo resistance not always correlate with the initial level of DSB induced, but rather with higher DSB repair capacity (Table 2).

Table 2 Repair capacity of *C. reinhardtii* strains after single dose treatment with Zeo and PQ. Mean data from at least 3 independent experiments.

Treatment	ZEO	PQ	ZEO	PQ	ZEO	PQ	ZEO	PQ
Recovery time	1h		2h		3h		4h	
137C	1,37	1,44	1,35	1,46	1,44	2,11	1,61	2,74
CW15	1,10	1,30	1,14	1,96	1,25	2,49	1,30	2,56
H-3	2,42		3,70		5,54		10,42	
AK-9-9	1,81		2,23		2,91		2,96	

These results are in agreement with the findings of other authors (Eguchi-Kasai et al., 1991; Ramotar and Wang, 2003). Moreover, DNA repair has been pointed out as probably the most crucial mechanism employed by cells to avert bleomycin-induced genotoxicity (Schaue and McBride, 2005). However, it is likely that, apart from DNA repair, other factors such as chromatin structure, mutations, cell cycle, altered DNA damage checkpoint response, defective apoptosis, or cell wall maintenance, etc. could also play a critical role toward determining cell resistance or sensitivity (Bao et al., 2006; Chalmers, 2007; Strasser et al., 2007).

3.3 ON THE ROLE OF CHLOROPLAST HSP70B/CHAPERONE FOR THE FORMATION OF GENOTYPE RESISTANCE

Heat stresses proteins (HSPs) as a key components contributing to cellular homeostasis take a special place among defense systems (Wang et al., 2004, Niu et al., 2006, Huang, Xu, 2008; Timperio et al., 2008). A special role in protecting cells from oxidative and heat stress belongs to HSP70 proteins, that together with co-chaperones proteins take part in the formation of chloroplast "foldosome" (Schroda et al., 2009). There are evidences of the involvement of chloroplast HSP70B/chaperons in refolding of denatured proteins (Drzymalla et al., 1996), in the biogenesis of thylakoid membranes, the synthesis or assembly of components of the new reaction centers and in the protection and repair of photosystem II during and after photoinhibition (Schroda et al., 1999; Schroda, 2004).

To throw more light on the possible role of HSP70B protein in the formation of cell resistance to temperature induced stress we addressed two questions: whether *Chlorella* species that differ in their response to oxidative stress would differ in their content of constitutive HSP70B and whether *Chlorella* species would differ in their response to heat shock (Fig 1 and Fig 2). Western blot analysis is used to measure HSP70B content 2 and 4 h after HS treatment (Yurina and Kloppstech, 2001; Chankova et al., 2009). Results presented in Fig. 1 show different stress response of algal species.

About 60% higher content of HSP70B is found for *Ch. kessleri* after the treatment with 39° C /30 min and 42° C/5 min and no statistically significant changes are obtained after the treatment at 45 ° C/5 min. The lack of induction of protein at 45 ° C corresponds well with our survival experiments (data not shown) where about 50% cell death is determined. Suppressed protein synthesis is possible also at this temperature. The only temperature that can induce heat stress in both species isolated from habitats with extreme environmental conditions *Ch. sp* (antarctic), and *Ch. vulgaris* 8/1 (thermophilic) is 45 ° C/5 min. The thermophilic strain 8/1 shows a slight HSP70B induction, but psychrophilic *Chlorella sp* demonstrates the increased HSP70B induction, indicating the occurrence of stress state in these cells. Small changes of HSP70B content in thermophilic *Ch. vulgaris* 8/1 may indicate participation of other defense factors. In Fig 2 (I) the relative contents of constitutive HSP70B are presented.

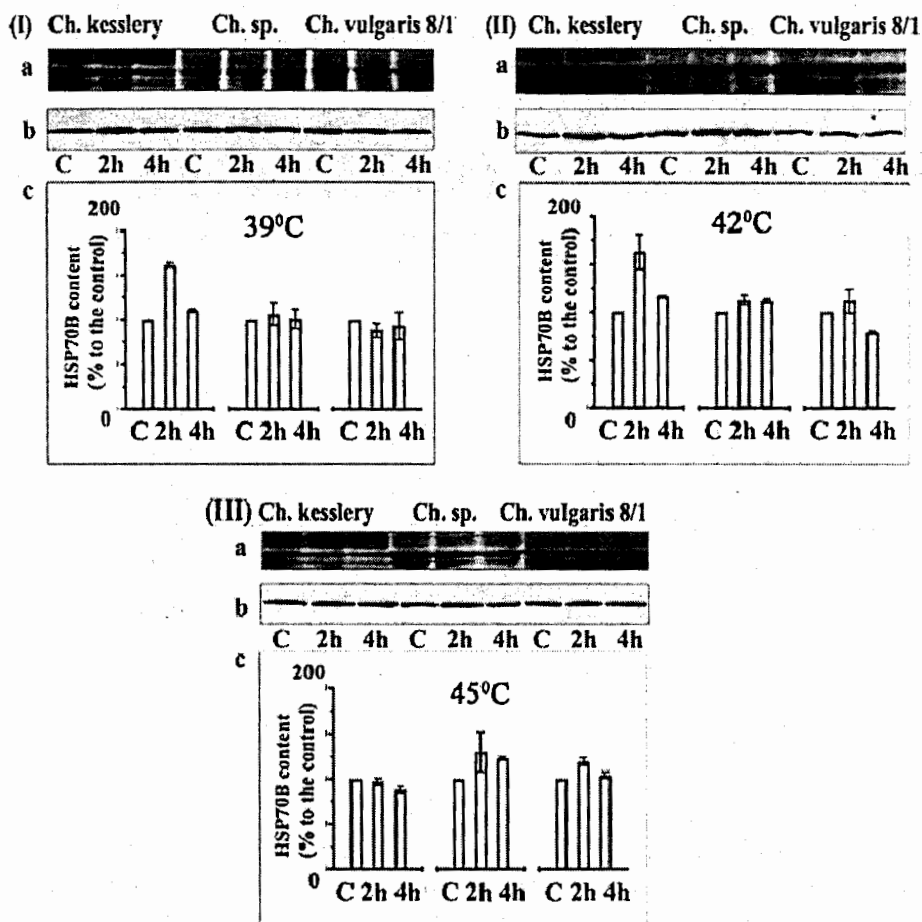


Fig.1 Comparison of HSP70B level in *Chlorella* species after heat stress: I – 39°C/30 min, II - 42°C/5 min and III - 45°C/5 min; a- Coomassie R-250 staining. The same amount of total protein at the gel was applied. Equal application of the protein on the gel was controlled by Coomassie R-250 staining; b - Western blot analysis of HSP70B level in *Chlorella* species incubated at heat stress; c – results of densitometry of HSP70B contents.

Markedly higher content of HSP70B is measured for *Ch. Sp* (Antarctic) comparing with other two species at different experimental conditions. The difference between HSP70B contents for *Ch. kessleri* and *Ch. vulgaris 8/1* is found to be insignificant. Incubation of *Ch. sp.* (antarctic) at low temperature did not lead to a decrease in HSP70B level (Fig. 2, II). This result speaks in favor of the assumption that increased HSP70B content in Antarctic algae is evolutionary fixed sign promoting to the survival

of cells under extreme conditions. It is interesting that *Ch. sp.* (antarctic) is also characterized by increased activity of superoxidedismutase and catalase at high temperatures, and increased efficiency of antioxidant system than other strains (Malanga et al, 2008; Nedeva and Puneva, 2009).

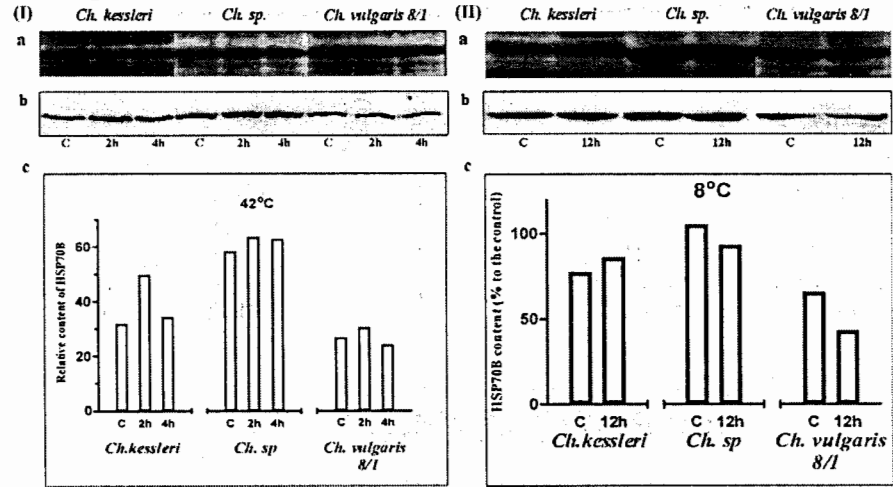


Fig 2. Effect of higher (I - 42°C/5 min) and lower (II - 8°C/12 h) temperature treatment on HSP70B level in *Chlorella* species.

a - Coomassie R-250 staining; b - Western blot analysis of HSP70B level in *Chlorella* species incubated with heat stress; c - results of densitometry of HSP70B contents.

On the basis of results obtained it could be possible to speculate that genotype resistance to oxidative stress may not always correlate with the initial level of DSB induced, but rather with higher DSB repair capacity and higher content of constitutive HSP70B. To show whether algae from habitats with extreme environmental conditions have evolutionary developed more effective cellular defense mechanisms further experiments must be done.

4. Is there a relationship between genotype resistance to oxidative stress and the magnitude of adaptive response?

Little is currently known about the correlation between cells' genotype resistance and their response to radiation (Silva et al., 2007). The magnitudes of Zeo induced AR in *C.reinhardtii* strains presented in Fig 3 show that all strains have the capacity to develop AR.

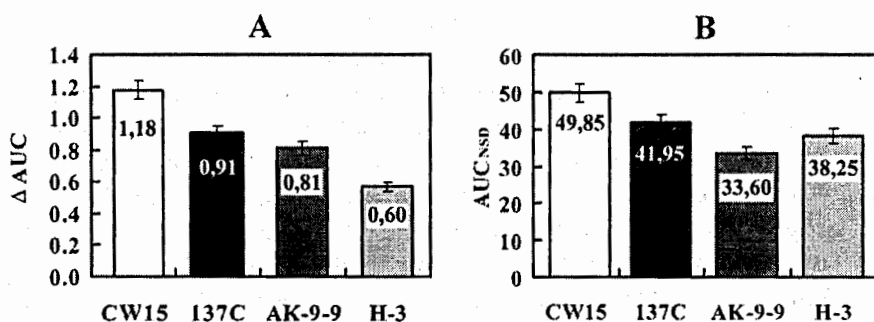


Fig. 3 A) Magnitude of AR measured as an area between curves representing the fraction of DSB remaining following single test Zeo treatment and following split Zeo treatment (ΔAUC); B) Magnitude of AR measured as an area under the curves representing the normalized split dose micro-colony survival (AUC_{NSD}) (Serafin et al, 2003)

A stronger AR is demonstrated in strains with a relatively lower genotype resistance. Interestingly, it was obtained that the higher genotype resistance of strains H-3 and AK-9-9 did not abrogate their competence to adapt. Our observation that the magnitude of induced AR in *C. reinhardtii* depends on the cell genotype is in agreement with the suggestion that the development of AR in human lymphocytes probably depends on genetic factors and not only on physiological ones (Mortazavi et al., 2003a; Tapio and Jacob, 2007).

Quite different is the picture presented in Fig 4.

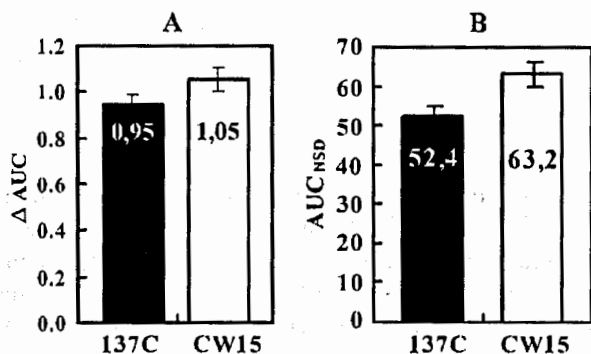


Fig.4 A) Magnitude of AR measured as an area between curves representing the fraction of DSB remaining following single test PQ treatment and following split PQ treatment (ΔAUC); B) Magnitude of AR measured as an area under the curves representing the normalized split dose micro-colony survival (AUC_{NSD}). (Serafin et al, 2003)

Strong PQ induced AR is manifested in strains with WT resistance - 137C and CW15 and no/or very slight PQ-induced AR in resistant strain AK-9-9 and H-3 (about

3%). No AR has been also induced when heat pretreatment has been used several hours before test treatment with gamma-rays or heat shock (Chankova et al, 2009).

Looking for the reason we have analyzed the level of DSB initially induced by pretreatment with low conditioning doses of PQ and heat. The absence of statistically significant higher level of DSB DNA induced by priming (conditioning) treatment is obtained for both cases. Our data add evidence to the hypothesis that a certain primary level of DNA damage could serve as a triggering event signalling the activation of DNA repair systems (Boreham and Mitchel, 1991; Wolff, 1998; Matsumoto et al., 2004; Leonard, 2007; Dimova et al., 2008; Chankova et al., 2009). The analysis of our results could suggest that the level of DSB DNA that can trigger an AR in *C. reinhardtii* strains should be at least 1.5-fold higher than those in control non-treated cells. Comparing repair capacity of strains, a relationship between DNA repair capacity estimated on the basis of single dose treatment and the DNA repair capacity estimated on the basis of the AR is obtained.

5. Conclusion

Our results concerning the relationship between genotype resistance to oxidative stress and adaptive response, confirm our previous suggestion that up-regulated DSB DNA rejoining could be considered as one of mechanisms involved in the formation of AR in this organism. New data are provided that strains with a relatively lower genotype resistance demonstrate a stronger AR. On the other hand the higher genotype resistance of strains did not abrogate their competence to adapt. A relationship between DNA repair capacity estimated on the basis of single dose treatment and the DNA repair capacity estimated on the basis of the AR is obtained.

Acknowledgements

This work was funded under the projects: D-002-317 "Antarctic algae – model system for oxidative stress resistance", Bulgarian Ministry of Education, Young and Science; "Molecular mechanisms of induced resistance in plants to oxidative stress", agreement between RAS and BAS; "WETLANET", FP7 CSA – SUPPORT ACTION, contract No. 229802, 2008; Russian Foundation for Basic Research (project № 06-04-48923a), Program MCB RAS.

We thank Prof. Michael Schroda (Albert-Ludwigs-Universität Freiburg, Institut für BiologieII, Germany) for the antiserum against HSP70B.

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PHEROMONES AND ADAPTIVE BYSTANDER-MUTAGENESIS IN MICE*

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Abstract. The genomic instability has been studied by cytogenetic analysis of chromosome aberrations in somatic and germ cells of CBA mouse strain. It was shown that volatile substances (VS) excreted by unisex groups of animals into the environment induce genomic instability in conspecifics of same sex depending on the state of VS-donors. Different pretreatments of donor animals modified the cytogenetic effect of their VS in recipients. The meaning of such pheromonal "bystander" effects for animal fitness is discussed.

Keywords: mouse, pheromones, mitotic disturbances, meiotic disturbances, bystander effect.

1. Introduction

Many social animals share some mechanisms in the formation of their response to stressors with man depending on degree of their genetic homology. Lots of external factors could become stressors if acted too long or too strong, etc. Direct action of some physical or chemical stress-inducing agents on recipient organism of an animal (including human beings) activates its hypothalamic-pituitary-adrenal axis. That is a sign of stress reaction accordingly H. Selye (1950). In spite of the great importance of studying different negative consequences of the stress state for man the significance of

* Radiobiology and Environmental Security / Eds. C.Mothersill, V.Korogodina, C.Seymour. Springer, 2012. P.153-162

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genetic changes during stress is still underestimated. Meanwhile, it is possible to suggest that inside different organs and tissues there are many types of sensitive cells which genome serves as target for different stressors. By means of special intraorganismic and intracellular messengers (neuropeptides, hormones, transcriptional factors etc.) these external factors can substantially disturb target cell genomes. That influences, in turn, functional activity of agitated cells, then tissues, organs and finally whole organism viability. Nevertheless we know almost nothing about the precise genetic mechanisms of such stressor action. But what we know that in human beings and in some other animals there are so-called psycho-social stressors and their action can spread rapidly among big groups even without direct contact with these factors. And again neither fine mechanisms nor possible genetic consequences of stress widening are well known till now.

In some animals the social way of their life has led to forming of specific mechanisms of spreading of a stress state among groups and populations. Therefore, impact of genetic disturbances can be amplified drastically also. For studying genetic consequences of stress mouse model of at least one "bystander" mechanisms by means of chemocommunication looks as the ultimate model.

Data obtained show that different chemosignals and especially pheromones serve as stress inducing factors in the house mouse. Some pheromonal treatments in mice activate hypothalamic – hypophyseal axis, induce adrenal hypertrophy and inhibit development of accessory glands, reproductive and immune function. Behavioral changes after these pheromonal exposures also correspond to a stress state in the recipient animals.

It looks like mouse female pheromone 2,5-dimethylpyrazine (DMP) is a good enough example of such stress inducing signal. This puberty-delaying mouse volatile chemosignal, the emission of which is under the control of adrenal glands (Drickamer, McIntosh, 1980) excreted to the environment by "together caged" (overcrowded) females. The maintenance of laboratory mice under overcrowded condition could be accepted, with the caution, of course, as a modeling of dense natural populations which is considered as a natural stress situation (Christian, 1961; Lecyk, 1967).

It is shown in mice that an exposure with female pheromones changes *c-fos* activity in accessory olfactory bulbs of males (Guo et al., 1997; Muroi et al., 2006) and in testes (Daev, Dukelskaya, 2005). It decreases drastically noradrenalin level in the mucous membrane of the nasal cavity and in the testes tunic of recipient male mice (Daev et al., 2000). DMP delays puberty of immature conspecifics (Jemiolo, Novotny, 1994), decreases significantly the reproductive fitness of male (Daev, 2003; Daev, Dukelskaya, 2004) and female mice (Jemiolo, Novotny, 1993), suppresses immune system (Daev et al., 2007a). DMP is also aversive chemosignal (Daev et al., 2007b). There are some other well known pheromonal stress effects in rodents such as activation *c-fos* in hypothalamus and limbic area (Fiber, Swan, 1995; Jin et al., 1996, Wysocky et al., 1995), changes in the level of gonadotropin-releasing factors (Dlusen et al., 1981;

Dudley et al., 1996) and pituitary hormones (Maruniak et al., 1978; Singer et al., 1988; Xu et al., 2000), adrenals hypertrophy (Archer, 1969; Wuensch, 1979), inhibition of sexual maturation (Lawton, Whitsett, 1979) and suppression of immune system (Surinov et al., 2004).

Up until now, little has been known or speculated about pheromonal influences on the genetic machinery and genetic structural integrity of the recipient cells which lies in the very beginning of at least primer-effects of pheromones. The similar is true for a meaning of this "bystander" effect of stress spreading for groups and populations of recipient animals.

Here we wish to report on the effect of chemosignals originating from intact or stressed (by overcrowded caging) mouse donors on genome stability in bone marrow and germ cells of recipient mouse males. Using conventional microscopic techniques, we have observed mitotic disturbances in dividing cells of the bone marrow (anaphase-telophase) and meiotic disturbances in germ cells of the testes of the mouse males.

2. Materials and Methods

2.1. MATERIAL

The inbred CBA/LacSto or CBA/LacStoRap mice used in all experiments were initially purchased from "Stolbovaya" or "Rappolovo" animal centers (Moscow region and Saint-Petersburg region, Russia, correspondingly). Isolated animals (donors of chemosignals) or unisex groups of animals (5 per group of tester animals and 7-8 per group of chemosignal donors) were maintained under standard conditions (cage size 22×30×10 cm), food and water provided ad libitum. Bedding was changed twice a week. After two weeks of adaptation, the animals were used for the experiments.

2.2. TREATMENT PROCEDURE

2.2.1. Treatment of chemosignal donor animals

We collected pheromonal cues emitted from mouse females or males to the environment. We used mainly the protein fraction of dialyzed urine pooled from 10 isolated or crowded females (VS_{if} or VS_{cf}), correspondingly. Single animals were caged separately at least two weeks before urine sample collection. Fresh urine individual samples were immediately pooled and centrifuged (MPW-310, 5000 rpm, 15 min). Then 2 ml of supernatant were dialyzed against 0.9% NaCl ("Servapore" membrane #44145, $t=1-4^{\circ}\text{C}$, 48 hrs, 2 l × 3 changes of salt solution). The volume of samples with

main urinary proteins was maintained constant and they were prepared directly before the experiment.

To receive different chemosignals we used diverse pretreatments of donor animals. For some experiments (to escape additional stress of handling of donor animals) we used just soiled bedding from 10 isolated donor females (SB_{if}). Animals were placed separately onto sawdust bedding for 5 days. Then soiled beddings from all donor cages were pooled and mixed. In parallel, we used as stressor soiled bedding from overcrowded females (SB_{cf}). One more stressor was water solution (0,01%) of the mouse female pheromone 2,5-DMP (Aldrich, 98%).

We used also soiled bedding from whole body irradiated CBA males (4Gy, 0.7cGy/sec, ⁶⁰Co, "Gammacell 220"). Soiled bedding had been collected for 24 hrs after irradiation of donor male groups and placed under the wire mesh bottom of standard cage with recipient animals. Direct contact with soiled bedding was prevented. One more donor group was stressed by swimming (Daev et al., 2007b). Similarly, control animals were exposed to soiled bedding of intact males.

2.2.2. *Treatment of chemosignal recipients*

For an exposure of animals to the pheromonal volatile stimuli, cotton balls were soaked in 1.5 ml of a respective VS or DMP solution and placed inside perforated plastic capsules. Each capsule was attached to the external side of the cage lid, at a distance of 2-3 mm from the lid's wiremesh, to prevent a direct contact of the animals with sample. An exposure to distilled water served as a control.

During experiment with soiled bedding half of the recipients own bedding was replaced by the same volume of corresponding sample. The treatment of the recipient animals was continued for 24 hrs. Clean bedding was used as a control.

All exposures lasted 24 hrs, after which the animals were sacrificed by cervical dislocation. Their bone marrow was rinsed from femoral bones and fixed in a methanol/glacial acetic acid (3:1) mixture. Testes were fixed the same way after tunic removal. The material was stored at -4°C until its microscopic analysis.

2.3. CYTOGENETIC ANALYSIS

Small pieces of fixed bone marrow tissue (0.2-0.5 mm) were placed in a drop of 2% acetoorcein for 30-45 min. They were subsequently rinsed in 45% acetic acid to remove an extra stain and gently squashed between a cover slip and a microscopic slide. The dividing cells at the anaphase-telophase stages were analyzed microscopically. The bone marrow cells of mice are dividing with a relatively high rate to yield a number of cells sufficient for further statistics. A careful analysis of the anaphase-telophase stage can thus provide an accurate estimation of the factors associated with mutagenic

activity, in fixed materials, without any additional pretreatment. In our experiments, the use of the metaphase method was avoided because it demands colchicine or colcemid and hypotonic treatments which all could affect the frequency of chromosomal aberrations.

Anaphase-telophase plates without overlapping or significant shifts were analyzed (more than 200 per each animal). The frequencies of abnormal divisions with a single bridge, a single fragment and a delayed chromosome as well as cells with two and more aberrations were registered. Total frequency of mitotic disturbances (MD) was counted.

Germ cells were removed from seminiferous tubules by shaking and suspended in fixative solution. Then drops of suspension were placed on slides and dried. Slides were stained as it was described earlier.

2.4. STATISTICS

Internal homogeneity among each recipient group was checked through the Chi-square contingency test for all types of abnormalities. Differences between groups were estimated by Chi-square criterion (Motulsky, 2003). In order to simplify the data for visual clarity, the frequencies (%) and their errors ($m\%$) were subsequently calculated.

3. Results and Discussion

Our present results show that chemosignals from crowded females increase frequency of mitotic disturbances in bone marrow cells of recipient mouse males. The results of one experiment are presented in Table 1.

DMP induces stronger change in MD frequency than chemosignals of crowded females in their soiled bedding (SB_{cf}). We obtained similar results for the exposure with VS_{cf} (Fig. 1, B). At the same time pooled chemosignals from isolated females (SB_{if}) act in opposite direction. They decrease the MD level in bone marrow cells of recipient males (Table 1, Fig. 1, B).

Our data provide evidence that the quality of chemosignals excreted by females depend on their state. Stressed by overcrowding donor females emit to the environment chemosignals which affect recipient's organism and destabilize genome of its dividing bone marrow cells. Since genome instability is a sign of stress state (Daev, 1994; 2007) we propose that chemosignals from overcrowded females spread the information about unfavorable environmental condition and thus induce stress in their neighbor conspecifics. DMP (which production in mouse females depends on group density and adrenals activity (Jemiolo, Novotny, 1993; 1994), as we suggest, is one of the possible messengers. But except DMP females definitely excrete to the environment many other sex specific substances which "positively" affect recipient males. It could explain more weak influence of SB_{cf} in comparison with pure DMP action. The same explanation is

applicable to “positive” effect of SB_{if} (Table 1, Fig. 1, B). Sex-specific “positive” chemosignals stabilize chromosomes structure and mitotic divisions when DMP does not produced by isolated females.

We investigated also action of different female chemosignals on dividing bone marrow cells in mouse female recipients. Our results show that chemosignals in soiled bedding and purified volatiles from urine of crowded females induce mitotic disturbances in bone marrow cells of recipient females. DMP acts in a same manner. The chromosome aberration patterns as well as degree of elevation were not different either in case of soiled bedding or purified volatiles. It was shown earlier that sensitivity of females to DMP depends on their stage of estrous cycle (Daev et al., 2007c).

As we have mentioned previously mouse male chemosignals can serve as stress-factors. Therefore we compare the DMP effect with the influence of other chemosignals originated from stressed or intact males in mitotic and meiotic cells of recipient males. It is shown by ana-telophase analysis that DMP increases total frequency of meiotic disturbances at anaphase-telophase II stage (Fig.2).

Chemosignals of stressed by physical stressor males (after swimming) act in similar manner. Finally, volatiles from irradiated males elevate frequency of meiotic disturbances approximately twice.

Table 1. Frequency of mitotic disturbances in bone marrow cells of CBA mouse males after their exposure with different chemosignals originated from mouse females (%).

Treatment of recipients	N	n	Number of cells with MD	Total frequency of MD (% ± m%)	Significant differences
Bclean	9	2994	102	3,4 ± 0,33	
SBcf	10	4320	225	5,2 ± 0,32	
DMP	10	3506	306	8,0 ± 0,46	
SBif	10	4982	87	1,7 ± 0,18	

N – number of animals; n – number of cells; B_{clean} – control (clean bedding); SB – soiled bedding (cf, if – after crowded or isolated females, correspondingly); DMP – 2,5-dimethylpyrazine; ends of vertical lines show on significantly different values (χ^2 -criterion, $P < 0,01$).

Our results are in a good agreement with data that volatile chemosignals from mouse males stressed by swimming or after whole body irradiation induce the same mitotic disturbances in intact recipient males (Daev et al., 2007a; 2010). It was shown

also similar genome destabilization in germ cells of stressed mouse males (Filkina, 2009).

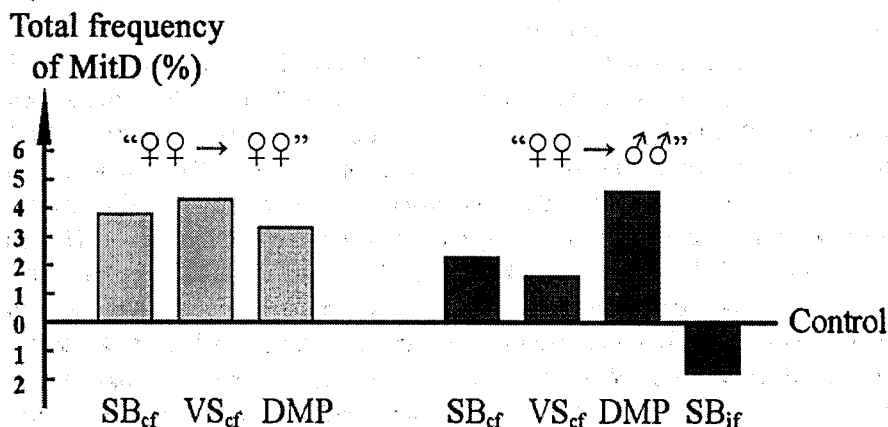


Fig.1. Effect of different chemosignals originating from mouse females in dividing bone marrow cells of mouse females (A) and males (B) of CBA strain. Differences in total frequencies of mitotic disturbances (MitD) between corresponding control and experimental groups are shown. SB_{cf} – soiled bedding of crowded females; VS_{cf} – volatile substances from urine of crowded females; DMP – 2,5-dimethylpyrazine solution; SB_{if} – pooled soiled bedding of isolated females. All the differences from control are significant; * - difference between effect of SB_{if} and all other effects (Chi-square test, $P < 0,01$).

To our mind cytogenetic disturbances in target cells are a general sign of stress reaction of an organism at an intracellular level. Such influence could be a mechanism by which volatile chemosignals effect development and fitness of recipient animals described earlier. With the aid of that mechanism animals could regulate their population density and structure via “chemosignals – genome integrity of target cells” interactions (Daev, 1994; 2007).

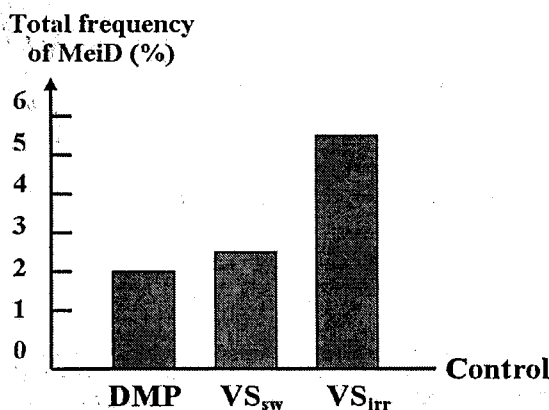


Fig.2. Effect of 2,5-dimethylpyrazine (DMP) and volatiles from CBA mouse males stressed by swimming (VS_{sw}) or from irradiated males (VS_{irr}). MeID – meiotic disturbances. All differences between experimental groups and corresponding controls are significant (Chi-square test, $P < 0,01$).

4. Conclusion

Our data supports the hypothesis that the integrity of target cell genome is decreased under stress conditions. Cytogenetic disturbances can serve as a universal sign of organismic stress. Pheromonal "bystander-effects" demonstrate that the olfactory mechanism in mice is working effectively to spread a stress state and genetic instability among conspecifics. Definitely there are other different specific and unspecific pathways of indirect widening of environmental influences among communicating animals. But the importance of searching of such pathways is still underestimated as well as mechanisms themselves that are not easily analyzed. Especially it concerns to the genetic consequences of such "bystander-effects". Therefore, we should look for appropriate models to study them at all levels. And research of mouse chemocommunicative mechanisms looks as good possibility to investigate the problem.

The research is supported by RFBR grant N 09-04-00693.

Acknowledgements

We are grateful to Prof. B.P.Surinov for his help in conducting our experiments with irradiation and to Alex Pryor from University of Cambridge, UK, for correcting the manuscript.

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GENOMIC INSTABILITY IN THE OFFSPRING OF IRRADIATED PARENTS*

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Abstract. The review describes the phenomenon of epigenetic transgenerational effects detected in the offspring of irradiated parents. The results of some recent studies showing elevated mutation rates in the non-exposed offspring of irradiated parents are presented and discussed. The possible mechanisms and implications of transgenerational instability are also discussed.

Keywords: instability, radiation, germline

1. Introduction

The target theory, independently developed in 1947 by Timofeeff-Ressovsky and Zimmer, and Lea (Timofeeff-Ressovsky and Zimmer, 1947; Lea, 1947), still represents the cornerstone of radiation biology and radiation protection. According to the modern interpretation of it, the effects of ionising radiation on the exposed cell are attributed to the initial DNA damage at the sites (targets) either directly hit by photons or affected by free radicals arising as a result of the localised ionisation of water (Lehnert, 2007). The main prediction of the target theory is that mutation induction almost exclusively occurs in the directly exposed cells at the non-repaired and mis-repaired damaged sites. In other words, everything, including the recognition of radiation-induced damage, its repair and mutation induction, happens in the exposed cells. There is another important prediction of the target theory postulating that the magnitude of mutation induction is directly related to the amount of radiation-induced DNA damage and the ability of exposed cell to repair it, meaning that the yield of radiation-induced mutations strongly depends on the dose- and dose-rate of exposure (UNSCEAR, 2001). The target theory also lays the foundation of a linear-no-threshold risk model, whereby the risk of human exposure to ionising radiation is linearly related to the dose of exposure without a threshold (BIER, 2006). However, a number of recent studies have clearly demonstrated the existence of non-targeted effects of ionising radiation. They include

* Radiobiology and Environmental Security / Eds. C.Mothersill, V.Korogodina, C.Seymour. Springer, 2012. P.127-140

mutation induction occurring at tandem repeat DNA loci, which are not directly targeted by ionising radiation (Sadamoto et al. 1994; Dubrova et al. 1996; 1998a), radiation induced genomic instability, detected among the non-exposed progeny of irradiated cells/organisms (Morgan, 2003a; 2003b; Dubrova, 2003), and bystander effect, whereby an elevated mutation rate is detected in the non-exposed cells, which receive signals from the irradiated neighbours (Morgan, 2003a; 2003b; Lorimore et al. 2003). Taking together, these observations imply that the non-targeted effects of ionising radiation can manifest over a period of time much longer than previously thought and may therefore be regarded as a component of the genetic risk of ionising radiation. Although a recent UNSCEAR report has concluded that the existing experimental data on non-targeted effects 'are insufficient to justify modifications of current risk estimates' (UNSCEAR, 2008), further analysis of this phenomenon will be important in improving their accuracy.

In this review the hereditary non-targeted effects of ionizing radiation will be presented and discussed. According to the results of some recent publications, these effects can manifest in the germline of directly exposed parents as well as in their non-exposed offspring. As the phenomenon of non-targeted mutation induction in the germline of irradiated mammals has previously been discussed in detail (see Dubrova et al. 1998b), this review therefore describes the transgenerational non-targeted effects of parental exposure to mutagens, including ionizing radiation. The studies presented here were designed to test the hypothesis that non-targeted effects induced in the germline of directly exposed parents could manifest in the offspring, affecting their mutation rates, cancer predisposition and other characteristics. This paper also presents some recent findings which were not discussed in our previous reviews (Dubrova, 2003; Barber and Dubrova, 2006).

2. Experimental data

The ability of ionising radiation to induce mutations in the germline of exposed parents has been known for almost a century. The development of the specific locus test in the late '50s by William Russell gave rise to a number of comprehensive studies aimed to establish the germline effects of ionising radiation and some chemical mutagens in mice (reviewed by Searle, 1974). Although these studies for the first time provided sound estimates of the genetic risk of high-dose exposure to ionising radiation in humans (UNSCEAR, 2001), they were designed to evaluate mutation induction in the germline of exposed parents. The detection of transgenerational effects requires a thorough analysis of the offspring of exposed parents. The first evidence for unusually elevated mutation rate in the first-generation (F₁) offspring of exposed parents was obtained in the early '60th (reviewed by Aurebach and Kilbey, 1971). For example, a substantially elevated incidence of mosaicisms was detected in the F₁ offspring male

Drosophila exposed to number of alkylating agents (Mathew, 1964; Lee et al. 1970). Another strong evidence for transgenerational effects was obtained by Luning and coworkers, who studied the frequency of dominant lethal mutations in the germline of non-exposed offspring of irradiated male mice (Luning et al. 1976). Although the results of these studies provided the first indication for the transgenerational destabilisation of the F_1 genome, for the long time they had remained inconclusive. One of the main reason for this was the lack of a sensitive technique for mutation detection in the germline and somatic tissues.

In the 1990th, we and others proposed that expanded simple tandem repeat (ESTR) loci can provide a sensitive approach for monitoring radiation-induced mutation in the mouse germline (Dubrova et al. 1993; Sadamoto et al. 1994). ESTRs consist of relatively short repeats, 5-10 bp long, and belong to the most unstable loci in the mouse genome with mutation rate as high as 0.05 per cell division (Bois et al. 1998; Hardwick et al. 2009). In the early studies, the analysis of ESTR mutation induction was restricted to the germline (Dubrova et al. 1993, 1998a; 2000a; Barber et al. 2000; Vilarino-Guell et al. 2003), but with the development of single-molecule PCR it was extended to the somatic tissues (Yauk et al. 2002). Given that according to our results the dose-response of ESTR mutation induction in male mice exposed to ionising radiation and chemical mutagens is remarkably similar to that for protein-coding genes (Dubrova et al. 1998a; 2000a; Barber et al. 2000; Vilarino-Guell et al. 2003; Dubrova, 2005; Glen et al. 2008), we therefore used them for the analysis of transgenerational instability.

Our first published results show that ESTR mutation rates are substantially elevated in the germline of non-exposed first- and second-generation offspring of irradiated males (Dubrova et al. 2000b; Barber et al. 2002). This remarkable stimulation of mutation in the F_1/F_2 germline following paternal irradiation (transgenerational instability) is reminiscent of the above mentioned phenomenon of delayed radiation-induced genome instability in somatic cells. Most importantly, our data demonstrate that the phenomenon of radiation-induced genomic instability is not restricted to a certain inbred strain of mice, as roughly equally elevated ESTR mutation rate was detected in the germline of three different inbred strains – CBA/Ca, C57BL/6J and BALB/c (Barber et al. 2002).

Using single-molecule PCR, we also analysed ESTR mutation frequencies in DNA samples extracted from the germline (sperm) and somatic tissues taken from the F_1 offspring of male mice, exposed to 1 or 2 Gy of acute X-rays (Barber et al. 2006, 2009; Hatch et al., 2007). Figure 1a summarises the results of these studies, clearly showing that ESTR mutation rates are equally elevated in the germline and somatic tissues of F_1 offspring of irradiated male mice. Transgenerational changes in somatic mutation rates were also observed by studying the frequency of chromosome aberrations (Vorobtsova, 2000; Slovinska et al. 2004), micronuclei (Fomenko et al. 2001) and *lacI* mutations (Luke et al. 1997) in the F_1 offspring of irradiated male mice and rats. It should be noted that the most compelling data addressing somatic instability in the F_1 offspring of

irradiated males were obtained from the analysis of somatic reversions of the pink-eyed unstable mutation (Shiraishi et al. 2002) and mutations at the mouse *hprt* locus (Barber et al. 2006).

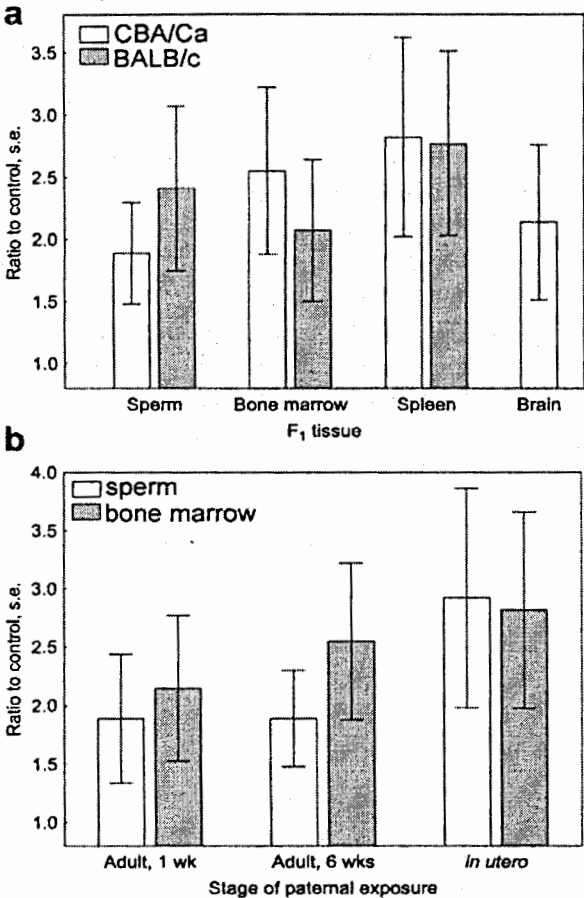


Figure 1. ESTR mutation frequency in the F₁ offspring of irradiated male mice. a, Frequency of ESTR mutation in the germline and somatic tissues in the F₁ offspring irradiated males from two different inbred strains of mice (data from: Barber et al. 2006; 2009). b, Frequency of ESTR mutation in the F₁ offspring BALB/c mice irradiated in utero or during adulthood (data from Barber et al. 2009).

The abovementioned results clearly imply that exposure to ionising radiation results in the induction of a transgenerational signal in the germline of exposed parents which can substantially destabilise the genomes of their offspring. As far as potential implications of these data for radiation protection are concerned, the important question is – for how long the exposed males can ‘remember’ the history of irradiation. In a number of our publication, we have addressed this issue. In these studies, male mice were mated over a considerable period of time following exposure to acute X-rays during adulthood (Barber et al. 2002; 2006; Hatch et al. 2007). In our recent study we also analysed the F₁ offspring male mice irradiated *in utero* during the early stage of development (Barber et al. 2009). Figure 1b summarises the results of these studies, showing that the magnitude of the increases in ESTR mutation frequency in the F₁

offspring of male mice irradiated during adulthood or prenatally does not significantly differ.

These data suggest that some as yet unknown marks of paternal irradiation can survive even massive epigenetic reprogramming during the early stages of development and, being passed to the offspring, destabilise their genomes. Our results therefore imply that the transgenerational effects may be regarded as an important component of the long-term genetic risk of ionising radiation. The analysis of mutation frequencies in the germline and somatic tissues of F₁ offspring of irradiated males has provided important clues on the possible mechanisms of transgenerational instability.

As in many studies the effects of paternal irradiation were studied in the offspring conceived by the exposed males and non-irradiated females, the very design of these studies allowed to establish the F₁ mutation rates at the alleles derived from the both parents (Shiraishi et al. 2002; Barber et al. 2002; 2006). Figure 2 presents the results of the two studies analysing the frequency of somatic mutations in the offspring of irradiated male mice. Thus, Shiraishi and co-workers reported equally elevated frequencies of somatic reversions at the mouse pink-eyed unstable locus in both alleles derived from the irradiated fathers and the unexposed mothers (Figure 2a).

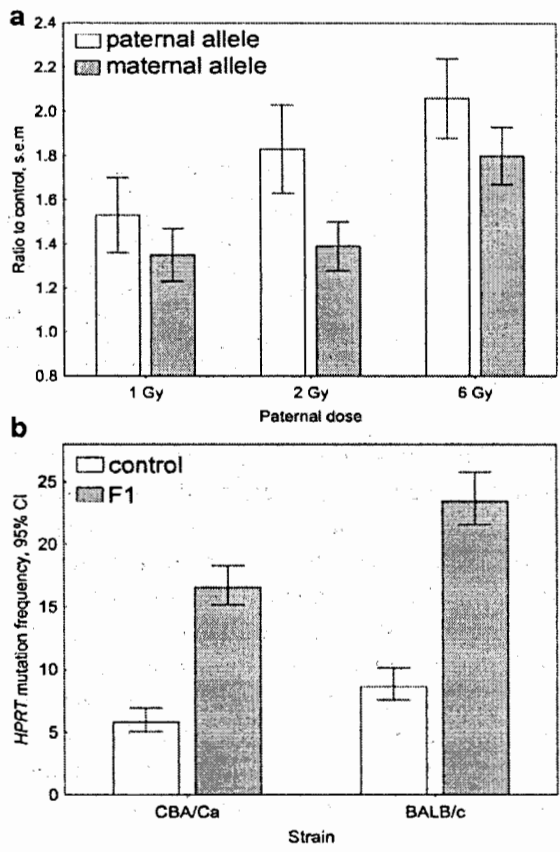


Figure 2. Transgenerational increases in the frequency of somatic reversions of the pink-eyed unstable mutation (a) and *hprt* mutation (b) in the F₁ offspring of irradiated male mice (data from Shiraishi et al. 2002; Barber et al 2006).

In our study, the increased frequency of mutation at the mouse *hprt* locus was found in the male F₁ offspring of male mice (Figure 2b). Given that the *hprt* locus is X-linked, the results of this study are therefore in line with the abovementioned pink-eyed data, as an elevated mutation rate was found at the F₁ locus derived from the non-exposed mothers. These results clearly imply that transgenerational instability is attributed to a genome-wide destabilisation.

3. Mechanisms

The data presented in the previous chapter clearly show that mutation rates in the germline and somatic tissues of offspring of irradiated males are substantially elevated. It should be stressed that the abovementioned results are at odds with the main predictions of target theory, as the effects of parental irradiation manifest in the non-exposed offspring, i.e. mutation induction in these animals are clearly off target and therefore the phenomenon of transgenerational instability belongs to the newly discovered non-targeted effects of ionising radiation. The key question is – what are the mechanisms underlying transgenerational instability? In a number of publications it has been suggested that the phenomenon of radiation-induced genomic instability observed either in the clonal progeny of irradiated cells or in the offspring of irradiated parents cannot be ascribed to conventional mechanisms such as a mutator phenotype and is most likely a result of epigenetic events (Morgan, 2003a; 2003b; Lorimore et al. 2003; Dubrova, 2003). This conclusion is based on the two sets of experimental data, showing that: (i) *in vitro* and *in vivo* radiation-induced genomic instability persists over a long period of time after the initial exposure; (ii) the number of cells/organisms manifesting radiation-induced genomic instability is too high to be explained by the direct targeting of any group of genes. As far as the transgenerational effects of paternal exposure are concerned, there are two main issues which should be addressed. The first step to elucidate the yet unknown mechanisms is to investigate the initial cellular events triggering an instability signal in the exposed germline. The analysis of the transmission of such a signal to the offspring and its manifestation represents another essential step in the clarification of this issue.

Given that exposure to ionising radiation produces a wide spectrum of DNA lesions (Frankenberg-Schwager, 1990), the analysis of its transgenerational effects may not provide enough evidence for a specific type of DNA damage triggering an instability signal in the directly exposed cells. However, it has been suggested that radiation-induced complex double-strand DNA breaks (DSBs) may constitute one of the signals that initiate the onset of genomic instability (Limoli et al. 1997). To verify whether this particular type of DNA damage can trigger an instability signal in the mouse germline, we have studied the effects of paternal exposure to the alkylating agent ethylnitrosourea (ENU) on the manifestation of genomic instability in the offspring of treated male mice.

In contrast to irradiation, exposure to ENU mainly causes alkylation of DNA at the N- and O- positions, resulting predominantly in base substitution mutations and seldom leads to any measurable increases in the yield of DSBs (Shibuya and Morimoto, 1993). This work revealed a number of striking similarities between the transgenerational effects of paternal exposure to this mutagens and ionising radiation (Figure 3).

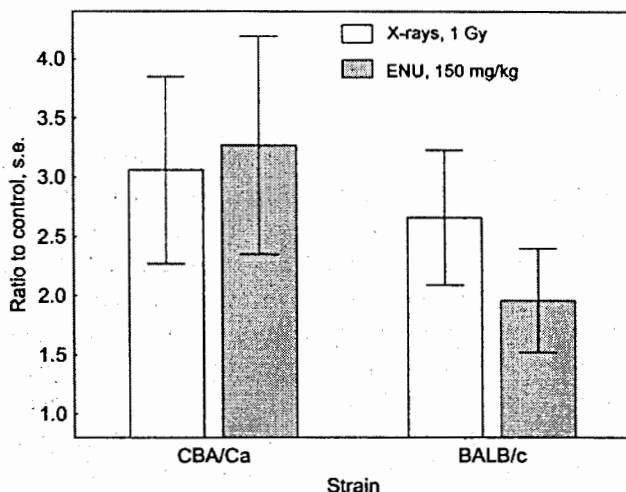


Figure 3. ESTR mutation rates in the F₁ germline of ENU-exposed and irradiated male mice (data from Barber et al. 2002; Dubrova et al. 2008).

Given the profound differences in the spectrum of ENU- and radiation-induced DNA damage, our data suggest that transgenerational instability is not attributed to a specific sub-set of DNA lesions, such as DSBs, but is most probably triggered by a stress-like response to a generalised DNA damage. Our data are in line with the results of previous studies showing that exposure to some chemical carcinogens and mutagens can result in a delayed increase in mutation rate in somatic cells (Limoli et al. 1997; Bardelli et al. 2001; Li et al. 2001; Gowans et al. 2006) or affect the fitness of the offspring of exposed male rats (Hales et al. 1992).

Given that the transgenerational changes can affect mutation rates at tandem repeat DNA loci, protein coding genes and chromosome aberrations, it therefore appears that the F₁ genomes could contain a variety of types of DNA damage. To compare the amount of DNA damage in the F₁ offspring and controls, we evaluated the amount of endogenous single- and double-strand DNA breaks, measured by the alkaline Comet and γ -H2AX assays (Barber et al. 2006). This analysis revealed an abnormally high level of DNA damage in the F₁ bone marrow and spleen cells (Figure 4a). Given that in tissues with a high mitotic index, such as bone marrow and spleen, the life-span of cells containing deleterious lesions such as single- and double-strand breaks is restricted since these types of DNA damage are not compatible with replication, these data clearly

demonstrate that transgenerational instability is an ongoing process occurring in multiple adult tissues.

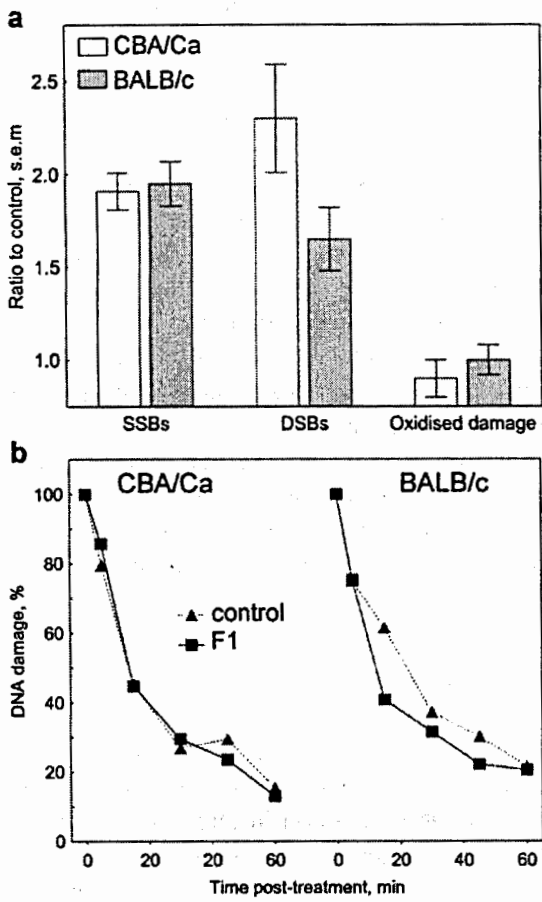


Figure 4. DNA damage and the efficiency of DNA repair in control males and the F₁ offspring of irradiated male mice. **a**, Endogenous DNA damage (single- and double-strand breaks) and the amount of oxidative damage in the F₁ offspring of irradiated males. **b**, Efficiency of DNA repair in the *ex vivo* irradiated bone marrow cells (data from Barber et al. 2006).

The presence of a persistent subset of DNA lesions in the offspring of irradiated males may either be attributed to an oxidative stress or reflect their compromised ability to repair of a certain type of endogenous damage. The involvement of oxidative stress/inflammatory-type response in the delayed increases in mutation rates in the progeny of irradiated cells has long been suspected (Morgan, 2003a; Lorimore et al. 2003). Reactive oxygen species are the major source of endogenous DNA damage, including single- and double-strand breaks, abasic sites, and a variety of nucleotide modifications (Jackson and Loeb, 2001). However, according to our data obtained by the FPG Comet assay the F₁ offspring of irradiated males did not show any alteration in the level of oxidatively damaged nucleotides (Figure 4a). Using the alkaline Comet, we

also measured the efficiency of repair of single-strand DNA breaks and other alkali labile sites in the F₁ *ex-vivo* irradiated bone marrow samples (Figure 4b). According to our data, the efficiency of DNA repair in the offspring of irradiated parents and controls is similar.

These data are in line with the results of some early studies, comparing the spectra of directly induced and delayed mutations at the *hprt* locus, detected in the progeny of irradiated cells (Little et al. 1996). According to the results of this study, the spectrum of delayed mutations, resulted from the ongoing instability substantially differed from that for directly induced and, in the same time, was very close for the spectrum of spontaneous mutations.

These data imply that radiation-induced genomic instability may result from the enhancement of the process of spontaneous mutation and therefore cannot be attributed to compromised DNA repair, which would affect the structure of delayed mutations. Given this, a genome-wide destabilisation of the F₁ genome could be attributed to replication stress. For example, some recent data suggest that in human precancerous cells the ATR/ATM-regulated checkpoints are activated through deregulated DNA replication, which leads to the multiplicity of DNA alterations (Bartkova et al. 2005; Gorgoulis et al. 2005). A detailed analysis of the expression profiles in the F₁ tissues should elucidate the still unknown mechanisms underlying the phenomenon of radiation-induced genomic instability.

4. Transgenerational instability and genetic risk

The abovementioned results showing that the offspring of exposed parents are genetically unstable may represent a serious challenge to the existing paradigm in radiation protection, according to which mutation induction in directly exposed cells is regarded as the main component of the genetic risk of ionising radiation for humans (UNSCEAR, 2001). Given that in the offspring of irradiated parents an elevated frequency of mutations is detected at a number of fitness-related endpoints, including chromosome aberrations and gene mutations, it would therefore appear that the transgenerational effects could impair the development. As, according to our data (Barber et al. 2006), the majority of the F₁ offspring of irradiated males manifest transgenerational instability, the number of affected offspring may substantially exceed that predicted by the target theory, which is based on the assumption their elevated mortality and morbidity can only be attributed to mutations induced in the germline of exposed parents. Indeed, the results of some animal studies show that paternal exposure to ionising radiation and some chemical mutagens can affect a number of fitness-related phenotypic traits in the offspring. For example, it has been reported that the rate of dominant lethal mutations is significantly elevated in the germline of F₁ offspring of male mice and rats, exposed either to ionising radiation (Luning et al. 1976) or

cyclophosphamide (Hales et al. 1992). The results of other studies showing decreased proliferation of early embryonic cells and increased frequency of malformations in the F₂ offspring of irradiated parents are also consistent with these observations (Wiley et al. 1997; Pils et al. 1999).

Given that tumour progression is attributed to accumulation of oncogenic mutations, the long-term destabilisation of the F₁ genome could also predispose them to cancer. In a number of studies, the incidence of cancer in the offspring of irradiated male mice exposed to recognised carcinogens was analysed (Nomura, 1983; Vorobtsova et al. 1993; Lord et al. 1998; Hoyes et al. 2001). Their results show an elevated incidence of cancer among the carcinogen-challenged offspring of irradiated males. The data on significantly elevated incidence of leukaemia in the children of male radiation workers from the Sellafield nuclear reprocessing plant (Gardner et al. 1990) are in line with these results.

It should be stressed that further analysis of the clinical impact of transgenerational instability is currently limited because of lack of the reliable experimental data in humans. So far, just a handful of experimental studies addressing this important issue have been conducted and their results are far from being consistent. For example, a recent publication on the transgenerational effects of post-Chernobyl paternal irradiation showed an elevated frequency of chromosome aberrations among the children of exposed fathers (Aghanjanyan and Suskov, 2009), whereas Tawn and coworkers failed to detect significant changes among the children of childhood cancer survivors (Tawn et al. 2005). On the other hand, the same group of authors reported an elevated G₂ chromosomal radiosensitivity in the children of survivors of childhood and adolescent cancer (Curwen et al. 2005). Given that the design of these studies, as well as the cohorts of irradiated families analyzed by the authors, dramatically differ, the comparison of their results remains highly problematic. The important issue of the transgenerational effects of parental exposure in humans should therefore be addressed in the carefully planned and executed studies, the results of which will provide experimentally based estimates of the delayed effects of radiation in humans.

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BIOPHYSICS OF HEAVY IONS*

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Abstract- Densely ionizing radiation, such as heavy ions, produce biological damage which is different from that normally produced by sparsely ionizing radiation, such as X- or γ -rays which are a large component of the natural radiation background. In fact, as a result of the different spatial distribution of the energy deposited, along the core and penumbra of the track, DNA lesions are exquisitely complex, and difficult to repair. RBE factors are normally used to scale from X-ray to heavy ion damage, but it should be kept in mind that RBE depends on several factors (dose, dose rate, endpoint, particle energy and charge, etc.) and sometimes heavy ions produce special damages that just cannot be scaled from X-ray damage. The special characteristics of heavy ions can be used to treat tumors efficiently, as it is currently done in Japan and Germany.

Keywords: Heavy ions, radiobiology, high LET, densely ionizing radiation, particle therapy

1. Introduction

The biological effectiveness of ionizing radiation strongly depends on the linear energy transfer, or LET, and it is well known that it is, for many endpoints, higher than sparsely ionizing radiation for LET values between 50 and 200 keV/ μ m in water. This different biological effectiveness is normally attributed to the different spatial distribution of lesion density in the DNA. While physicists know very well, from nuclear emulsions, how different a track of a heavy ion is compared to photons, more recently this could be visualized directly in mammalian cells, exploiting markers of DNA lesions such as phosphorylated histone γ H2AX (Fig. 1) [1] or the accumulation of GFP-tagged repair

* Radiobiology and Environmental Security / Eds. C.Mothersill, V.Korogodina, C.Seymour. Springer, 2012. P.365-370

proteins, such as 53BP1 (Fig. 2) [2]. Clearly, heavy ions produce “streaks” of DNA lesions in the cell nucleus, and the density of lesions increase with LET (Fig. 1), and they can be hardly repaired or moved following exposure (Fig. 2). This observation begs the question of whether the damage induced by heavy ions is different from that produced by X-rays. The answer is unfortunately not simple: even if the DNA damage is more difficult to repair, this may lead to an increased cell killing, but not necessarily to increased late risk: a dead cell cannot represent a risk, although the bystander effect may play a dominant role in explaining the effectiveness of high-LET radiation for late effects.

Fig. 1

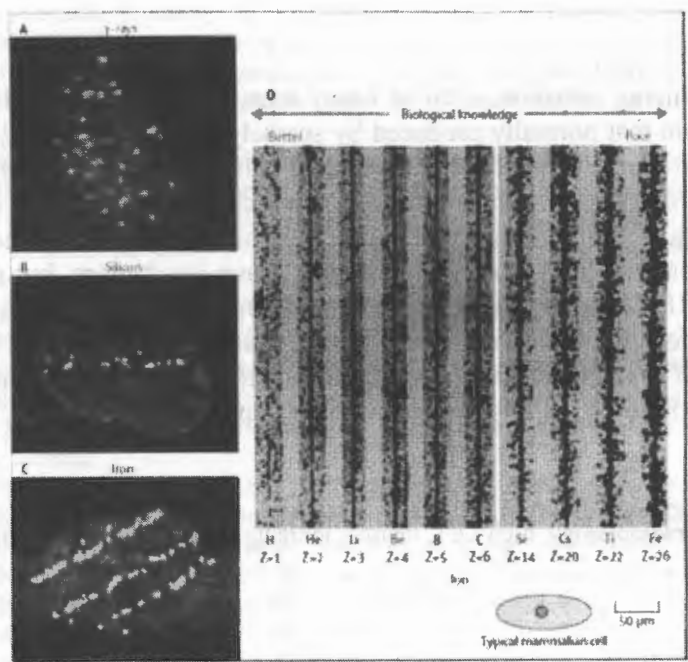


Figure 1 Three nuclei of human fi broblasts exposed to (A) γ -rays, (B) silicon ions, or (C) iron ions; and immunostained for detection of γ -H2AX. Every green focus corresponds to a DNA double-strand break. In the cell exposed to sparsely ionising γ -rays (A), H2AX foci are uniformly distributed in the nucleus. Cells exposed to heavy ions show DNA damage along tracks—one silicon (B) and three iron (C) particles, respectively. Spacing between DNA double strand breaks is reduced at very high-LET. (D) Tracks of different ions, from protons to iron, in nuclear emulsions, show increasing LET as charge, Z, increases. From ref. [1].

Fig. 2

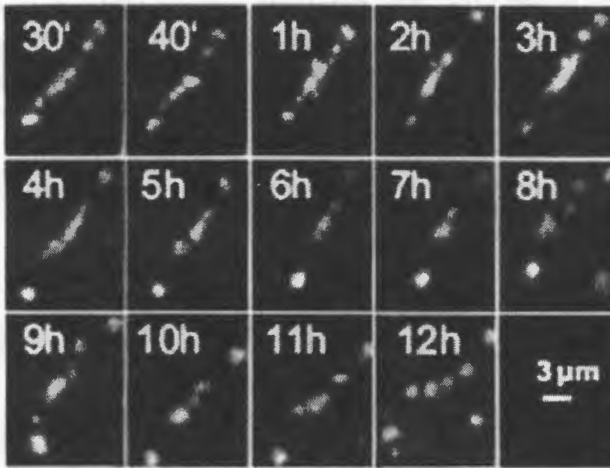


Figure 2 Quantitative analysis of the motion of DNA double-strand breaks (DSBs) after high LET irradiation. Time-dependent changes of a single Ni-ion-induced 53BP1-GFP streak in a human tumor cell showing the typical motional behavior of individual proteins along the trajectory over the time course of 12 h after irradiation. Compared to Fig. 1, these pictures show the evolution of the damage in living cells, exploiting GFP-tagged proteins expressed in the cell, instead of fixing and staining the samples. From ref. [2].

2. Heavy ion radiobiology

Because heavy ions are not present on Earth, their study is not relevant for radiation protection, and neither it has been for radiation therapy for many years. However, heavy ions are now often used in therapy [3] and they represent a major risk for human space exploration [4] (Fig. 3).

2.1. PARTICLE THERAPY

The rationale of oncological particle therapy is simply based on the different energy deposition of charged particles (the Bragg curve) and photons (exponential attenuation). Fig. 4 immediately suggests that charged particles have a better energy deposition pattern than X-rays for therapy, as recognized by Wilson already in 1946. Protontherapy is today widely spread in the world, and is considered a cutting-edge technology, with clinical results at least comparable to X-ray IMRT. However, apart from the favorable dose distribution, protons don't really add biological advantages, as their RBE is close

to 1. On the other hand, heavy ions combine an increased biological effectiveness to a high RBE, and reduced oxygen enhancement ratio (OER), in the Bragg peak. Carbon ions are for instance low-LET (about $10 \text{ keV}/\mu\text{m}$) in the entrance channel, but high-LET (up to $80 \text{ keV}/\mu\text{m}$) in the Bragg peak, thus providing sparing of the normal tissue and high effectiveness in the tumor. The clinical results, so far based on a fairly limited number of cancer patients (about 5000) are indeed very good, and after the clinical trials in NIRS (Japan) and GSI (Germany), several new centers are under constructions in Europe and Asia.

Fig. 3

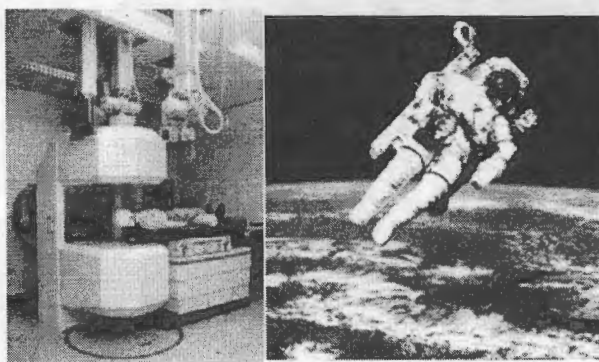


Figure 3 Interest for radiobiology of heavy ions is linked to two main applications: cancer therapy (left) and protection of astronauts in long term space missions (right). In one case, we are interested to exploit the ability of heavy ions to kill cells; in the second, to protect the crews from long-term late effects. Although the exposure conditions are very different (high dose, fractionated, localized irradiation in therapy; low dose, chronic, whole body in space), the two topics share several research topics, such as studies on stochastic risk of heavy ions, or on radioprotectors (Table 1).

2.2. RADIATION PROTECTION IN SPACE

Although protons are by far the most common particle in space radiation, heavy ions play a major role because energy deposition increase with z^2 , and the RBE increases with LET. Therefore, heavy ions are nowadays acknowledged by space agencies as a major barrier to human space exploration. Cancer risk is of course the main concern, because it is well documented that radiation can induce cancer, but the RBE of heavy

ions is not known, due to the lack of epidemiological studies and the only limited animal studies, performed at particle accelerators [4]. In addition to cancer, several others late effects cause concern, including damage to the central nervous system, cataracts, risk of cardiovascular diseases, and hereditary effects. Both NASA and ESA support large experimental campaigns to study these effects, considering that space agencies are now shifting their programs to exploration.

Fig. 4

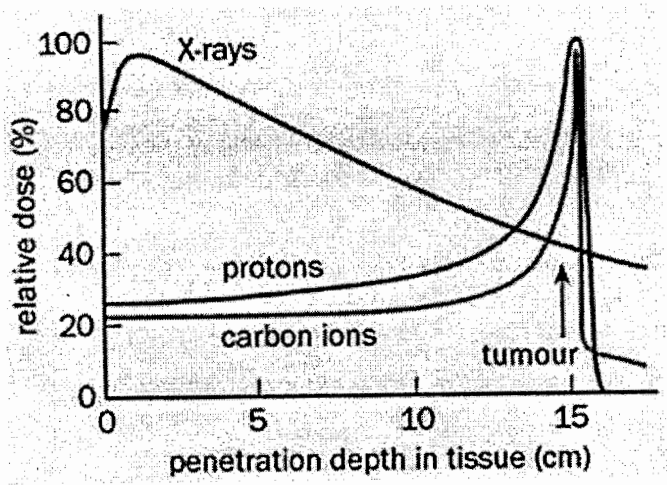


Figure 4 Rationale of using charged particles for cancer therapy. While X-rays deposit more energy on the surface than in the tumor, the opposite holds for charged particles, such as protons and carbon ions.

The NASA program is based at the Brookhaven National Laboratory (Upton, NY), whereas the European program is based at GSI (Darmstadt, Germany).

Notwithstanding the large differences in exposure conditions (high dose, fractionated acute partial-body exposure in therapy; low dose, chronic whole-body exposure in space), the two topics share several research topics as summarized in Table 1.

Table 1 – Some research topics relevant for both hadrontherapy and space radiation protection.

	Hadrontherapy	Space Radiation Protection
Particles	H and C.	All ions from H to Ni.
Maximum energy	~ 400 MeV/n	~ 10 GeV/n
Dose	60-80 Gy-eq. in the target volume. Dose to the normal tissue depend on the treatment plan.	50-150 mSv on the Space Station, up to 1 Sv for the Mars mission
Exposure conditions	Partial-body, fractionation (2 Gy-eq./day in the target volume)	Total-body, low dose-rate (1-2 mSv/day)
Individual radiosensitivity	Patient selection, personalized treatment planning	Personalized medical surveillance of the crewmembers
Mixed radiation fields	Effects of primary particles and fragments for tumor cell killing and side effects	Cosmic radiation is a mixed field. Effects of shielding.
Late stochastic effects of heavy ions	Risk of secondary cancers in patients	Risk of cancer in astronauts
Normal tissue deterministic effects	Early and late morbidity	Cataracts, CNS damage, other late degenerative effects
Radioprotectors	Protection of the normal tissue, but not of the tumor. Drugs.	Protection from heavy ions at low doses and protons at high doses (solar particle event). Dietary supplements
Biomarkers	Predicting risk of secondary cancers or late morbidity	Reducing uncertainties in risk estimates
Bystander effect	Role in tumor cell killing	Role in stochastic risk at very low fluence

3. Conclusion

Biological effects of densely ionizing radiation are becoming a key topic in radiobiology, because of the interest for heavy ions coming from space radiation protection and particle therapy. Large experimental campaigns are currently under way at accelerators, and it is likely that they will lead to a reduction of the uncertainty on the late risk of heavy ions.

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REPRODUCTIVE EFFECTS FROM CHRONIC, MULTIGENERATIONAL, LOW DOSE RATE EXPOSURES TO RADIATION*

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Abstract. Relatively few experiments have been conducted on the effects to organisms following long-term exposures to low levels of radiation. Even fewer studies have examined the effects of radiological exposures to multiple generations of organisms. Speculations that damage will accumulate and be greater with each passing generation are plausible. Alternative, opposing views that adaptive response and repair mechanisms will counter the effects, such that damage does not increase with each generation, are equally plausible. Few data exist to support one hypothesis over the other, particularly for chronic, low-level exposures to vertebrate organisms. Our research explored exposures of low-dose irradiation to multiple generations of a model vertebrate organism, Japanese medaka (*Oryzias latipes*), one of the most widely used fish in comparative mutagenesis and carcinogenesis studies. A unique outdoor irradiation facility allowed us to examine effects to five generations of medaka that were continuously irradiated to different dose rates. The dose rates bracketed the IAEA guideline for acceptable chronic exposures to aquatic wildlife (10 mGy d⁻¹), and thus

* Radiobiology and Environmental Security / Eds. C.Mothersill, V.Korogodina, C.Seymour. Springer, 2012. P.219-232

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were a test to see if the guidelines were applicable for multigenerational exposures. The effects on reproductive endpoints that might influence population dynamics were examined. Our intention was to test the hypothesis that multigenerational exposures to low dose rate irradiation are no more damaging, as measured by reproductive characteristics that could potentially impact a population, than damage incurred from exposure to a single generation. The data suggest that total accumulated dose may be more meaningful than dose rate when trying to predict effects to populations of chronically exposed organisms. Such knowledge is needed because chronic exposure to low levels of radiation is a more likely scenario for nuclear workers, and to wildlife exposed to routine releases from nuclear facilities.

Keywords: medaka; radioecology; dose-effect relationship; chronic exposure; low dose-rate; reproduction

1. Introduction

Exposure regimes influence the biological effects caused from ionizing radiation. A dose received over a few seconds will have a different biological effect compared to the same dose received over an organism's life time. Current knowledge of how responses vary from different radiological exposure regimes is inadequate to allow confident predictions about the effects that might result from low-level, chronic exposures. Most effects data have been derived from acute, high dose rate experiments. Relatively few experiments have been conducted on the effects to vertebrates following long-term exposures to low levels of radiation. Even fewer studies have examined the effects of radiological exposures to multiple generations of organisms. Speculations that damage will accumulate and be greater with each passing generation are plausible. Alternative, opposing views that adaptive responses, repair and compensating mechanisms will counter the effects, such that damage does not increase with each generation, are equally plausible. Few data sets exist to support one hypothesis over the other, particularly for chronic, low level exposures to vertebrate organisms. Such knowledge is needed because chronic exposure to low levels of radiation is a more likely scenario for nuclear workers, and to wildlife exposed to routine releases from nuclear facilities. Regulation of the latter is becoming a necessity with recent changes in regulations (Euratom, 2010) and as international committees place an emphasis on developing protection criteria specific to non-human biota (ICRP, 2009).

Our research explored multigenerational exposures of low dose rate irradiation to a model vertebrate organism. A unique outdoor irradiation facility allowed us to examine effects to five generations of vertebrate animals that were continuously irradiated to different dose rates. The dose rates bracketed the IAEA guideline for acceptable chronic

exposures to aquatic wildlife, and thus were a test to see if the guidelines were applicable for multigenerational exposures. The effects on reproductive endpoints that might influence population dynamics were examined. Our intention was to test the hypothesis that multigenerational exposures to low dose rate irradiation are no more damaging, as measured by reproductive characteristics that could potentially impact a population, than damage incurred from exposure to a single generation. Additionally, the vertebrate model that we chose, Japanese medaka (*Oryzias latipes*), are one of the most widely used fish in comparative mutagenesis and carcinogenesis studies. Shimada and Shima (1998) showed that the mutational response of the medaka male germ cell was close to that of the mouse, and therefore medaka are a viable non-mammalian model system for risk assessments from environmental mutagens.

2. Methods

2.1. LOW DOSE RATE IRRADIATION FACILITY (LoDIF)

LoDIF is an outdoor, gamma-irradiation array consisting of 40 fiberglass, open-air tanks designed to house aquatic organisms such that all stages of their life cycle (from eggs to breeding adults) can be continuously irradiated (Hinton et al. 2004). This aspect of the LoDIF, as well as the ability to replicate the experimental design and irradiate large numbers of organisms, makes it a unique facility. Each tank, or mesocosm, is parabolic in shape, 2.4 m in diameter and holds approximately 965 L of water with a maximum depth of 41 cm (**Figure 1**). The mesocosms are of a flow-through design and receive water from a nearby lake at rates that were adjusted to about 1 L min⁻¹.

The mesocosms allow manipulative experiments to be conducted on whole organisms in conditions more natural than laboratory settings, similar to what has been successfully used in ecology and population biology (Wilbur, 1987; Rowe and Dunson, 1994). Mesocosms allow the researcher to apply specific treatments in a more controlled environment than large-scale field tests. The LoDIF facility is arranged into eight 6.5 m x 10.5 m blocks, with each block containing five mesocosms, suitable for randomized block experimental designs (**Figure 2**).



Figure 1. Photo of a single mesocosm, showing the dimensions of the tank and a ^{137}Cs irradiator with collimated shielding positioned above it. The LoDIF has 40 such mesocosms installed over a 0.4 ha area. A single container is shown directly underneath the irradiator. Such containers house 10 to 20 fish. Several containers can be placed in a circle around the central beam of the irradiator such that each is exposed to the same dose rate. Precise geometry measurements and mapping of the irradiation field with TLDs allowed accurate estimates of the dose received by the fish.



Figure 2. Photo of a portion of the LoDIF facility, showing a pad with five mesocosms, three of which have sealed Cs-137 sources above them (cylinders mounted on frames that cross the mesocosm). Eight replicate pads exist within the LoDIF, resulting in 40 mesocosms.

Irradiation treatments can be applied *via* specially designed irradiators mounted on steel frames and placed over three of the five individual mesocosms within each of the eight blocks (Figure 2). The two unirradiated mesocosms within each block were intended to serve as controls. (The controls tanks received a dose rate of 0.1 mGy d^{-1} due to scatter of radiation from the nearby irradiated mesocosms; discussed below). Each irradiator contains a 0.74, 7.4 or 74.0 MBq sealed ^{137}Cs source within a lead container, collimated to deliver an exposure to animals residing in the tank below. The 7.4 MBq source strength and associated geometry was designed to deliver a mean dose rate of approximately 10 mGy d^{-1} . The other two source strengths give mean dose rates that are factors of 10 less than, and greater than 10 mGy d^{-1} . Thus, the facility was

designed specifically to determine if populations of aquatic organisms are truly protected by what is currently thought to be appropriate dose rates by the International Atomic Energy Agency (10 mGy d^{-1}).

Our experiments used a fish species as a vertebrate model. All life stages of the fish, from egg to reproductive adult, were continuously irradiated within the LoDIF under controlled conditions. Living conditions within the LoDIF were favorable for the species to grow and reproduce. Fish were maintained in mixed gender groups of ~ 20 individuals per container ($\times 8$ blocks ~ 160 adult fish per treatment), and placed within the gamma field to obtain specific dose rates.

2.2. MODEL ORGANISM AND LIFE CYCLE EXPOSURES

We selected a species of small fish, the Japanese medaka (*Oryzias latipes*), as our model vertebrate organism. The genetics, developmental biology, embryology, and specific developmental stages of medaka have been extensively characterized (Yamamoto 1975). Medaka is the leading fish species used to identify and predict human health effects following toxicant exposure. Medaka are recognized as important comparative animal models for addressing questions related to a variety of processes and diseases, including cancer, DNA repair, mutations, infectious diseases, aging, developmental biology, endocrine disruption, and genetics. The use of medaka in biomedical and environmental research, especially as a carcinogenesis model related to identifying and predicting human health effects from toxicant exposure, has received considerable attention (Wittbrodt et al. 2002; Winn et al. 2000; Shima and Shimada 2001).

The species has numerous advantages for experimental laboratory and field studies including: small size (about 2.5 cm in length); a short maturation time of 8 to 10 weeks; and a prolific capacity to reproduce (6 to 30 eggs d^{-1}). The life history characteristics of medaka are such that several generations of fish can be exposed within a relatively short time period. We used a Kyoto-Cab Medaka wild-type strain, courtesy of Hiroshi Mitani (Tokyo University), with a low background of embryonic abnormalities (Furutani-Seiki, et al. 2004).

Female medaka spawn daily and retain their egg clutch externally on their abdomen for several hours. Males fertilize the eggs externally while the clutch adheres to the female. Fertilization occurs at dawn. To obtain the embryos, clusters of eggs were removed from females that were chronically exposed to the various dose rate treatments within the LoDIF. The eggs were immediately brought into the laboratory for cleaning and evaluation. Egg clutches were gently rubbed to remove attached chorion filaments and to isolate individual eggs. Eggs were examined under a stereoscopic microscope to determine if fertilized. The number of viable eggs, and number of dead eggs per gravid female were recorded. Cleaned, viable eggs were placed in petri dishes with 1 ppm methylene blue water, returned to the LoDIF within hours after the formation of the

clutch, and exposed to the same irradiation dose rate treatment as the parents (**Figure 3**). The health status of eggs was monitored daily until hatching. Newly hatched fry remained in the LoDIF, also under the same radiation treatment as their parents. Hatching success per radiation treatment was recorded. Fry were contained in small buckets with fine screen mesh to allow in- and out-flow of water (**Figure 4**). Subadults were moved to larger buckets. Densities of fish were kept similar among the irradiation treatments to normalize potential confounding variables of crowding, food resources, lighting, competition, etc. Subadults matured, reached breeding conditions, and were monitored accordingly, while continuously being irradiated.



Figure 3. Special containers housed fish eggs and were exposed under known geometries with doses measured by TLDs. The photo depicts one of several egg exposure arrangements that were tested.

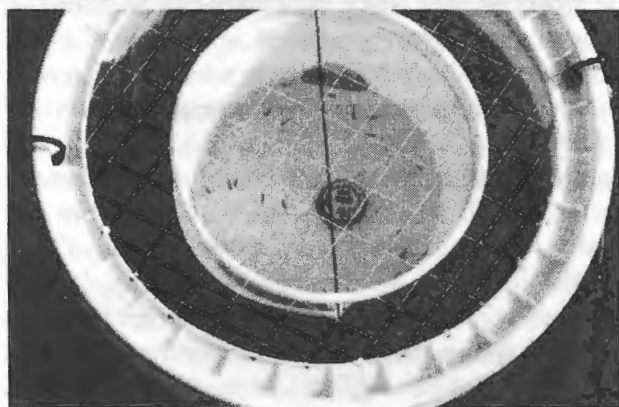


Figure 4. Several medaka fish are observable within a hatchling container used for irradiating small fry. The grey circle at the top of the inner bucket is a screened port for water to flow. Similar, rectangular ports are seen as dark patches on the right and left of the larger container. The circular object at the bottom of the inner container is a water temperature recording device.

The process described above was repeated such that five generations of medaka were exposed to the radiation treatments; from eggs through breeding adults. Several containers of fish were placed within each mesocosm, each under a known and specific exposure geometry in which the dose rate was known from measured TLD readings. The numbers of fish within each treatment were sufficiently large that cohorts of fish could be removed for specialized analyses, while leaving sufficient stock for producing the next generation of fish. Samples of fish were routinely removed for genetic and

molecular analyses (results to be reported elsewhere). The irradiators were shut down for a cumulative time of about two days per month for maintenance or special care of the fish. Feeding was done remotely and did not require turning off irradiators.

3. Experimental Design

These experiments started with the introduction of the founding generation, as subadult medaka, into the LoDIF on 17 February 2006. These fish were termed G0; subsequent generations were designated as G1 through G5. Fish were continuously exposed to four dose rate treatments (0.1; 2.4; 21; and 221 mGy/d). Treatments were replicated 8 times within the 8 pads (**Figure 2**), and ~20 fish of mixed gender were housed within each holding container (**Figure 1**) for each replicate. Thus a total of ~640 fish were used for the G0 group (160 fish per dose rate treatment, with 8 blocks of 20 fish each, x 4 treatments). G0 fish were exposed for 94 days at which time their eggs were collected and the G1 generation exposure began. The experiment was completed on 17 September 2008, with the collection of G5 eggs from the fifth generation of chronically exposed adults (G4).

3.1. DOSIMETRY

Thermoluminescent dosimeters (TLDs) were placed within the exposure field to obtain mean dose rate estimates. The precision with which dose rates can be estimated within the facility reduces the uncertainties often associated with dose-response relationships. Dose rates varied according to the life history stage in which the fish were exposed (egg, fry, adult) due to the type of enclosures required to house the fish and their geometries relative to the ^{137}Cs sources.

The total dose received by each generation of fish was dependent on the dose rate of the treatment and the length of exposure period. The latter varied, particularly for fish that over-wintered within the LoDIF. Their exposure period was longer. Over-wintering was necessary to maintain the fish lines for the following spring. Natural temperature and light conditions were not conducive for the medaka to breed in the winter, thus we were required to wait until spring for the next generation of fish to be produced. The accumulated doses for each generation [G(n)] at the time eggs were collected for start of the next generation [G(n+1)] are given in **TABLE 1**.

3.2. REPRODUCTIVE ENDPOINTS

Reproduction is regarded by the IAEA to be a more sensitive endpoint than mortality following exposure to radiation (IAEA, 1992). Changes in reproductive output are also thought to impact populations of biota. With the exception of endangered species, or

humans, populations are the level of biological organization managed for health from contaminant exposures. Thus, we chose to examine several reproductive-related endpoints in the medaka exposed to chronic irradiation within the LoDIF.

TABLE 1. Mean dose received by each parental group [G(n)] at the time the next generation of eggs [G(n+1)] were produced as a function of the four dose rate treatments (0.1; 2.4; 21 and 221 mGy/d). Total dose from one generation to the next were not the same due to different exposure durations (required overwintering of fish before the next generation could be produced).

				Dose rate treatments (mean mGy / d)			
G(n) producing G(n+1)	Date G(n) placed in LoDIF	Date G(n+1) collected from LoDIF	Exposure days of G(n) in LoDIF	Control 0.1	Low 2.4	Medium 21	High 221
				Resulting Cumulative Dose (mGy)			
Go - G1	17-Feb-06	25-May-06	94	9	220	1,970	20,800
G1 - G2	25-May-06	8-Sep-06	102	10	240	2,150	None *
G2 - G3	8-Sep-06	29-May-07	254	25	590	5,340	None *
G3 - G4	29-May-07	30-Aug-07	90	9	210	1,890	None *
G4 - G5	30-Aug-07	17-Sep-08	371	37	850	7,800	None *

* Effects at the highest dose rates were sufficient that subsequent generations could not be maintained.

A suite of data was collected in the LoDIF facility at the time eggs were harvested for propagation of the next generation. Procedures for processing the eggs were described under Section 2.2. Data collected included: 1) number of gravid females; 2) total number of eggs produced; 3) total number of live eggs produced; 4) total number of eggs that hatched; and 5) total number of hatchlings that survived for 15 days. Each of these reproductive parameters is related to the successful recruitment of young into the next generation of animals. The sensitivity of each parameter varies in response to contaminants and other stressors. Compensating mechanisms and adaptation can result in non-linear responses, and in what may seem like opposing responses to stress. A good example is the work of Blaylock (1969), where fish exposed to radiation suffered a reduction in the number of fertile eggs; but an increase in the number of eggs produced, such that the net effect in total fertile eggs produced did not differ from controls. Ultimately, it is the integration of all these reproductive parameters that determines the size of the next population cohort.

Thus, herein, we elected to combine the above parameters into an *Index of Reproductive Success* (IRS), by multiplying the number of eggs produced per female, times the percent fertility of the eggs, times the percent of fertile eggs that were still alive as fry 15-days post hatching. Fifteen days is a critical threshold for medaka. Fry that survey that period have a reasonable probability of reaching adulthood. We used the following parameter to compare treatments and to test our hypothesis:

$$\text{IRS} = \text{Eggs} \times \text{Fertility} \times \text{Survival}$$

Where: IRS = Index of Reproductive Success; Eggs = mean # of eggs per female; Fertility = mean percent fertility; and Survival = mean percent survival of fry (15 days post-hatching). Uncertainties, expressed as standard deviations (Stdv), were propagated as:

$$\text{Stdv IRS} = \sqrt{\left(\frac{\text{Stdv Eggs}}{\text{Eggs}}\right)^2 + \left(\frac{\text{Stdv Fertility}}{\text{Fertility}}\right)^2 + \left(\frac{\text{Stdv Survival}}{\text{Survival}}\right)^2} \times \text{mean IRS}$$

Effects to reproductive success, as measured by the reproductive index described above, were compared among the various LoDIF dose rate treatments (TABLE 1); and also compared among the various generations of exposed fish.

4. Results and Discussion

After 94 days of exposure to the various dose rate treatments, the reproductive abilities of the founding G0 fish were examined. The mean numbers of eggs produced were not affected by the irradiation treatments. However, significant reductions occurred in the number of live eggs and in the number of hatchlings produced from fish exposed to the highest dose rate. These reductions were so strong that we were unable to populate the high dose rate group for an additional generation. Their mean Index of Reproductive Success was only 0.05 ± 0.1 . Thus, the remaining multigenerational experiment within the LoDIF involved fish from the three lower dose rate treatments (0.1; 2.4 and 21 mGy d⁻¹).

4.1. LOW DOSE-RATE FIELD DATA

Figure 5 shows the mean IRS for five generations of fish continuously exposed to three dose rate treatments within the LoDIF. No differences appear to exist in IRS among the three dose rate treatments for generations G0, G1 or G3. A reduced IRS appears to have occurred at the highest dose rate of 21 mGy/d for generations G2 and G4.

The overall exposure times in days are also shown for each generation in Figure 5. Generations G0, G1 and G3 were exposed for similar lengths of times (~ 90 to 100 days); whereas G2 and G4 were exposed considerable longer (G2 = 254 days and G4 = 371 days). The latter two are the generations in which the IRS decreased for the fish exposed to the highest dose rates. The longer exposure periods resulted in fish in G2 and G4 acquiring greater total doses (TABLE 1 presents total dose per generation). The fact that the 21 mGy d⁻¹ treatment appears to have been impacted during the generation (G2) where total doses were twice as great; "recovered" the following generation (G3) when total doses were halved; and then suffered damage again in G4 when total dose

were three time greater suggests that reproductive effects, as measured by the IRS, are likely driven by total dose, and less by a multigenerational mechanism or dose rate.

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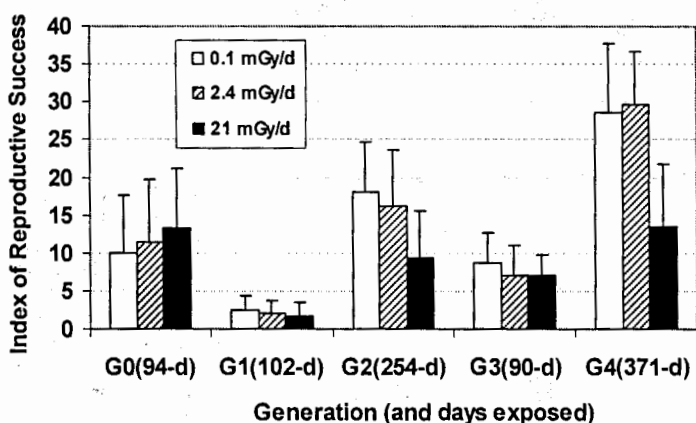


Figure 5. LoDIF Field Data. Five generations of medaka, continuously exposed to three dose rate treatments. Data show an Index of reproductive success (IRS) for each generation. The exposure days for each generation are also shown.

Note that even at the lowest dose rate of 0.1 mGy d⁻¹ there is considerable differences in IRS between generations (**Figure 6**). Differences in temperatures, food resources, and seasons are factors known to influence egg production (Koger et al., 1999). Such factors definitely occurred within the outdoor conditions of the LoDIF, and contributed, to some unknown extent, in fish from the same dose rate treatments having differences in IRS from one generation to the next.

The lowest dose rate of 0.1 mGy d⁻¹ occurred in the “controls” due to scatter radiation from the nearby irradiated mesocosms. 0.1 mGy d⁻¹ is two orders of magnitude lower than the IAEA guideline considered to be safe for populations of aquatic organisms. It is also less than the 0.2 mGy d⁻¹ benchmark value proposed as protective of the environment, based on species sensitivity distributions (Andersson, et al., 2009). If we consider the 0.1 mGy d⁻¹ to represent control conditions, then the data can be normalized to the same IRS across generations for the 0.1 mGy d⁻¹ treatments, and we can thus attempt to remove the variation caused from temperature, light regimes, and

food resources. The direction and amount of normalization required for each generation within the 0.1 mGy d^{-1} treatment was then applied to the other two dose rate treatments, resulting in the normalized data graphed in Figure 7.

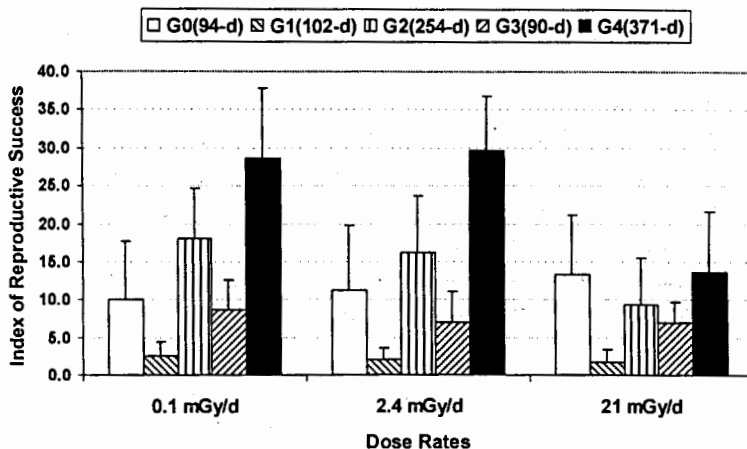


Figure 6. LoDIF Field Data. Index of reproductive success for five generations of medaka exposed to three dose rate treatments.

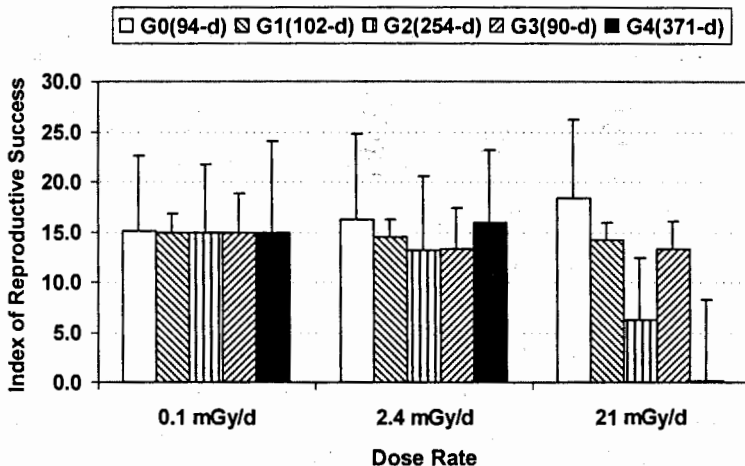


Figure 7. LoDIF Field Data normalized to remove seasonal outdoor temperature, lighting and food resource variation. All data were normalized such that the IRS for the lowest dose rate treatment for each generation were equal. The normalized mean IRS value for G4 at 21 mGy d^{-1} was 0.0 ± 8.1 .

Analysis of the normalized data suggests that the IRS was only reduced in the 21 mGy d^{-1} treatments, and then only for G2 and G4 populations (the two generations with the longest exposure times and thus the highest total accumulated doses). Thus normalization supports the theory that the reproductive effects observed in the IRS are more likely due to the accumulation of a total dose, rather than due to the confounding factors of dose rate and multigenerational phenomena. Actually, it is more likely that dose, dose-rate and generation are interacting in complicated, non-linear ways. More advanced statistical procedures are currently being used on this data set to see if their respective contributions can be better illuminated.

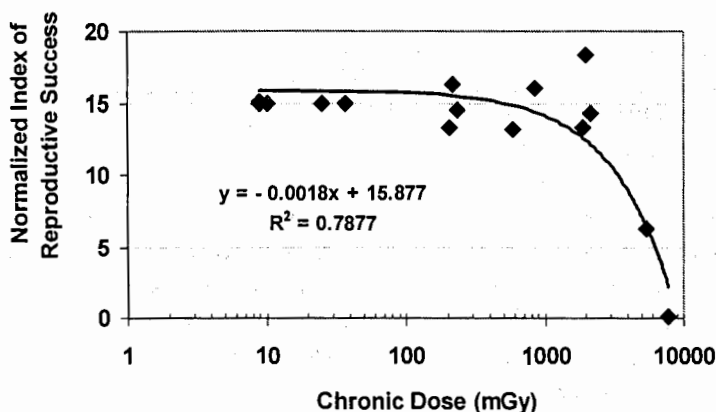


Figure 8. Dose response curve for the normalized LoDIF data when all treatments and generations are combined.

If total accumulated dose turns out to be the dominant factor contributing to a decrease in the IRS endpoint, then a dose response curve can be drawn from the entire normalized data set (Figure 8). The curve, on log-linear axes, suggests that a threshold dose of approximately 3 Gy is needed before a significant reduction in the IRS occurs. At the IAEA suggested dose rate guidance of 10 mGy d⁻¹, a total accumulated dose of 3 Gy could be obtained in 300 days. This period of time is easily achievable within the 2 to 5 year life time typical of medaka living outdoors (Shima and Mitani, 2004).

Overall, these data represent a valuable contribution to the science of radiation-effects studies. They suggest that total accumulated dose may be more meaningful than dose rate when trying to predict effects to populations of chronically exposed organisms. The data are rare in that few other studies have examined multiple generations of a vertebrate animal under different chronic dose rate treatments (Table 2). The data from several other LoDIF experiments (including some acute exposures to medaka at the same doses) are being analyzed. We are actively conducting more rigorous statistical analyses of the data presented herein.

Acknowledgments

Funding was provided by the Environmental Remediation Sciences Division of the Office of Biological and Environmental Research, U.S. Department of Energy, through Financial Assistance Award DE-FC09-96SR18546 to the University of Georgia Research Foundation. All experimental protocols were approved by the University of Georgia's Animal Use and Ethics Committee (# A2005-10169). The animal husbandry assistance of Laura Marsh was most appreciated.

TABLE 2. Other reproductive effects documented in fish following chronic exposures to radiation.

Fish Species	mGy/d	Reproductive Effects	Reference
<i>Gambusia affinis</i>	4	increased frequency of dead embryos, offset by larger brood sizes	Blaylock, 1969
<i>Rutilus rutilus</i>	> 5	reduced fecundity	Peshkov et al., 1978
<i>Hypophthalmichthys molitrix</i>	10	6 % sterility when living in Chernobyl Cooling Reservoir	Belova et al. 1993; as cited by Sazykina and Kryshev, 2003
<i>Tilapia mossambica</i>	30 - 40	total sterility from chronic ⁹⁰ Sr exposure	Voronina 1973; as cited by Sazykina and Kryshev, 2003
<i>Poecilia reticulata</i>	40	57 % decrease in fecundity, chronically exposed	Woodhead, 1977
<i>Oryzias latipes</i>	65	sterility and unfertilized eggs increased	Hyodo-Taguchi, 1980
<i>Ameca splendens</i>	185	complete sterility when exposed for 190 d (~35 Gy)	Rackham and Woodhead, 1984

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ROLE OF EPIGENETIC CHANGES IN RADIATION-INDUCED GENOME INSTABILITY*

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Abstract. Ionizing radiation (IR) is an important diagnostic and treatment modality, yet it is also a potent genotoxic agent that causes genome instability and carcinogenesis. While modern cancer radiation therapy has led to increased patient survival rates, the risk of radiation treatment-related complications is becoming a growing problem as radiation poses a threat to the exposed individuals and their progeny. Radiation-induced genome instability, which manifests as an elevated mutation rate (both delayed and non-targeted), chromosomal aberrations and changes in gene expression, has been well-documented in directly exposed cells and organisms. However, it has also been observed in distant, naïve, out-of-field, 'bystander' cells and their progeny. Enigmatically, this increased instability is even observed in the pre-conceptually exposed progeny of animals, including humans. The mechanisms by which these distal effects arise remain obscure and, recently, have been proposed to be epigenetic in nature.

Epigenetic alterations which comprise mitotically and meiotically heritable changes in gene expression that are not caused by changes in the primary DNA sequence, are increasingly being recognized for their roles in health and disease. Three major areas of epigenetics—DNA methylation, histone modifications and small RNA-mediated silencing, are known to have profound effects on controlling gene expression. Yet, the exact nature of the epigenetic changes and their precise roles in IR responses and IR-induced genome instability still need to be delineated. Here we will focus on the nature of epigenetic changes in directly exposed and bystander tissues. We will also discuss the emerging evidence that support the role of epigenetic deregulation in transgenerational effects.

* Radiobiology and Environmental Security / Eds. C.Mothersill, V.Korogodina, C.Seymour. Springer, 2012. P.53-66

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Keywords: radiation, bystander effects, genome instability, transgenerational effects, epigenetics, DNA methylation, histone modifications, small RNAs.

. Epigenetics – a Brief Historical Overview of a Science Reborn

The term ‘epigenetics’ (outside of genetics) was introduced by developmental biologist Conrad Hall Waddington well before the discovery the molecular structure of DNA itself. Waddington’s model proposed that epigenetics describes how genes within a multicellular organism interact with other genes and their environment to yield a certain phenotype (Van Speybroeck 2002). In later works by Robin Holliday, epigenetics was characterized as the cellular “mechanisms of temporal and spatial control of gene activity during development” (Holliday 1990).

After the discovery of DNA as the genetic material, the field of epigenetics was overshadowed by studies focused on the role of changes in DNA sequence between various normal and pathological conditions. It was not until the near completion of the human, and several other genome projects, that an interest in epigenetics was ‘reborn.’ By this time, many researchers had come to understand that genome sequences failed to fully explain the complexity of cellular processes, regulation or, most importantly, the fine-tuning of cell-organism-environment interactions.

One can not discount the importance of the genetic code: it is indispensable for life. Besides containing the coding information for RNAs and proteins, the DNA sequence also harbours a variety of gene expression guidelines within regulatory sequences such as promoters, enhancers and other elements. Nevertheless, it has become clear that regulatory elements may function differently under certain conditions or cell states without requiring any DNA sequence alterations. However, it has been noted that these changes are usually accompanied by chromatin modifications. Thus, it has become necessary to focus on the non-sequential characteristics of the genome: those aspects not detailed in the genetic code but, rather outside of it, the ‘epi-genetics’ so to speak. And indeed, in practice the term “epigenetics” has been adopted to refer to the information contained in chromatin rather than in the actual DNA sequence (Jaenisch and Bird 2003; Bird 2007).

. What is Epigenetics?

Modern science defines epigenetics as meiotically and mitotically stable alterations in gene expression that are not based on DNA sequence changes and involve processes that impact chromatin structure such as DNA methylation, histone modifications and genomic imprinting (Bird 2007; Weber and Schubeler 2007; Weidman, Dolinoy et al. 2007). More recently, RNA-mediated silencing was proposed as another epigenetic phenomenon (Bernstein and Allis 2005).

2.1. DNA METHYLATION

Cytosine DNA methylation was the first epigenetic alteration identified and has become the most widely studied (Feinberg and Vogelstein 1983; Feinberg 2004). It is known to be associated with inactive chromatin state and in most cases with the repressed gene expression activity (Jaenisch and Bird 2003; Klose and Bird 2006; Weber and Schubeler 2007) and, thus, is crucially important for the regulation of gene expression, silencing of parasitic sequences, X-chromosome inactivation and overall, for the normal development, cell proliferation and proper maintenance of genome stability of a given organism (Robertson 2002; Jaenisch and Bird 2003). In mammals, DNA methylation occurs predominantly in the context of CG dinucleotides which are methylated 60–80 % of the time (Weber and Schubeler 2007). This methylation occurs specifically at cytosine residues and requires the activity of DNA methyltransferase (DNMT) enzymes to form 5-methylcytosine. In mammals, three DNA methyltransferases (DNMT1, DNMT3a and DNMT3b) are primarily responsible for establishing and maintaining DNA methylation patterns at CpG sites (Robertson 2001; Rountree, Bachman et al. 2001; Goll and Bestor 2005). DNMT1 is the major enzyme involved in maintaining the existing pattern of DNA methylation following DNA replication (Liang, Chan et al. 2002). It localizes to the replication fork, where it can directly modify nascent DNA immediately after replication (Goll and Bestor 2005; Jirtle and Skinner 2007; Weber and Schubeler 2007). In contrast, Dnmt3a and Dnmt3b are *de novo* methyltransferases that target unmethylated and hemi-methylated sites in response to intra- and inter- cellular signals that are not yet fully elucidated (Okano, Bell et al. 1999; Goll and Bestor 2005; Weber and Schubeler 2007). Deregulation of these proteins is frequently recognized in diseased states and likely is involved in the altered methylation patterns that are a hallmark of numerous diseases (Goll and Bestor 2005; Jirtle and Skinner 2007; Weber and Schubeler 2007).

Altered global DNA methylation patterns are a well-known characteristic of cancer cells (Weidman, Dolinoy et al. 2007). Frequently, cancer cells are characterized by global genome hypomethylation with concurrent hypermethylation of selected CpG islands within gene promoters (Jaenisch and Bird 2003; Baylin and Chen 2005; Baylin and Ohm 2006; Weidman, Dolinoy et al. 2007). The global loss of DNA methylation at CpG dinucleotides was the first epigenetic abnormality identified in cancer cells, and it has been linked to the activation of transposable elements, elevated chromosome breakage, aneuploidy, increased mutation rates and, thus, to the phenomenon of a global genomic instability (Robertson and Wolffe 2000; Robertson 2001; Weber and Schubeler 2007; Weidman, Dolinoy et al. 2007).

2.2. HISTONE MODIFICATIONS

Changes in DNA methylation do not appear to be isolated, independent events.

Such changes accompany global chromatin deregulation and, sometimes, histone modifications (Jenuwein and Allis 2001; Jaenisch and Bird 2003). The term 'histone code' is used to describe the plethora of potential covalent modifications that may occur on several of the core histones. These modifications, many of which are still not fully understood, allow for an enormous amount of flexibility and complexity of the regulatory responsibilities of chromatin and include (not exclusively) acetylation, methylation, ribosylation, ubiquitination, sumolation and phosphorylation. Although the full scope of their effects remains mostly unexplored, many of these modifications are known to alter the spatial arrangement of the DNA strand, thus, changing the accessibility for numerous DNA associating proteins. Frequently, these spatial rearrangements impact transcriptional regulation. Examples of such modifications include histone acetylation, methylation and phosphorylation (Jenuwein and Allis 2001; Weidman, Dolinoy et al. 2007).

Acetylated histone tails lose their positive charge reducing their affinity for the negatively charged DNA and, thus, lead to a more relaxed chromatin packaging. The increasingly relaxed acetylated loci experience increased transcriptional activation, while histone deacetylation has an opposite effect—transcriptional repression (Jenuwein and Allis 2001). Histone methylation is not so straight forward. Methylation can result in different transcriptional consequences depending upon the residue affected (Cheung and Lau 2005; Saha, Wittmeyer et al. 2006). For example, methylation of lysine 9 of histone H3 is associated with chromatin compaction and gene silencing, while methylation of lysines 4 or 27 of histone H3 results in chromatin relaxation and transcriptional activation. Additionally, histone residues can be either mono-, di- or tri-methylated, and again, lead to different transcriptional profiles (both between the degree of methylation and the recipient residues) (Cheung and Lau 2005; Saha, Wittmeyer et al. 2006; Weidman, Dolinoy et al. 2007; He, Dunn et al. 2008). In a practical example, it has been shown that tumours undergo a massive loss of tri-methylation at lysine 20 of histone H4 (Fraga, Ballestar et al. 2005; Tryndyak, Kovalchuk et al. 2006). This loss occurs along with DNA hypomethylation and is linked to chromatin relaxation, aberrant expression and has been suggested to be a universal marker for malignant transformation (Sanders, Portoso et al. 2004; Fraga, Ballestar et al. 2005).

Although influencing transcription and expression is considered the paramount purpose of the epigenome, it also has been implicated in identifying DNA lesions, recruiting repair complexes and facilitating in the repair process. Phosphorylation, another important facet of the histone code, (He, Dunn et al. 2008) is best studied in the modification of histone H2AX (a member of the H2A family). The phosphorylation of H2AX is crucially important for the repair of DNA strand breaks and the maintenance of genome stability (Celeste, Difilippantonio et al. 2003). As H2AX becomes phosphorylated at serine 139 (γ H2AX) it accumulates in foci at the break sites, possibly as one of the earliest cellular responses to double strand breaks (DSBs) (Rogakou, Pilch et al. 1998; Pilch, Sedelnikova et al. 2003; Sedelnikova, Pilch et al. 2003). A direct

correlation exists between H2AX phosphorylation and the number of radiation induced DSBs (Celeste, Fernandez-Capetillo et al. 2003). Again, it appears that this mechanism does not act exclusively as γ H2AX recruits histone acetyl transferase (HATs) to relax the neighbouring chromatin.

2.3. SMALL RNAS – NEW REGULATORS OF GENE EXPRESSION

Another mechanism of epigenetic control is mediated through small regulatory RNAs (Bernstein and Allis 2005) with a particular interest in microRNAs (miRNAs). miRNAs are abundant, small, single-stranded, non-coding RNAs that regulate gene expression and are conserved across species (Hwang and Mendell 2006; Sevignani, Calin et al. 2006). To control the translation of the target mRNAs, miRNAs associate with the RNA-induced silencing complex (RISC) proteins and bind to the 3'UTR of mRNAs, thus, serving as translational suppressors that regulate protein synthesis (Hutvagner and Zamore 2002). Regulatory miRNAs impact cellular differentiation, proliferation, apoptosis and, possibly, even predisposition to cancer (Hwang and Mendell 2006; Fabbri, Ivan et al. 2007). Aberrant levels of miRNAs have been reported in a variety of human cancers (Volinia, Calin et al. 2006; Wiemer 2007). Furthermore, it has been suggested that small RNAs may be involved in the regulation of chromatin packaging (Grewal and Moazed 2003; Bernstein and Allis 2005).

Another class of short, non-coding RNAs are 29-30 nucleotides long and form complexes with Piwi proteins (piRNAs) (Aravin, Sachidanandam et al. 2007; Carmell, Girard et al. 2007; Aravin, Sachidanandam et al. 2008; Das, Bagijn et al. 2008; Farazi, Juranek et al. 2008). This novel class of small RNA molecules, discovered in 2007, are expressed in the mammalian germline and have been linked to silencing of retrotransposons and other germline genetic elements (Carmell, Girard et al. 2007; Aravin, Sachidanandam et al. 2008; Das, Bagijn et al. 2008).

2.4. EPIGENETICS AND ENVIRONEMENT

Epigenetic regulation appears to be vitally important for all organisms—especially eukaryotes, and during this decade it has become increasingly apparent that epigenetic changes modulate the molecular, cellular and organism responses to a changing environment (Minamoto, Mai et al. 1999; Jaenisch and Bird 2003; Wade and Archer 2006; Jirtle and Skinner 2007; Weidman, Dolinoy et al. 2007). Through interaction with their surrounding environment, living organisms are constantly exposed to a variety of stressors including physical, chemical, biological and social. Although these interactions may be positive and negative, all of them may potentially re-shape not only the genome, but also the epigenome. The body of work supporting this hypothesis continues to grow as more effort is applied to investigating the molecular players involved in communicating the signals and effecting the epigenetic changes. The full

potential of epigenetics and the role it plays in maintaining a balanced physiology cannot be fully appreciated until all the mechanisms are fully researched and understood: a daunting, albeit, critically important task.

3. Direct Effects of Radiation Exposure

Besides diagnostic and therapeutic medical radiation exposures, which usually represent finite doses, most exposure comes from more ubiquitous, unobvious sources that tend to supply chronic levels of radiation. These include background radiation, cosmic rays, radioactive waste, radon decay, nuclear tests and accidents (e.g. Chernobyl and other nuclear power plants). It is important to note that all living organisms are exposed to ionizing radiation (IR) on a day-to-day basis (both purposeful and unavoidable) yet, the underlying mechanisms of such exposure is not fully understood. On one hand, IR is a well-known cancer-inducing agent. The carcinogenic potential of IR was recognized soon after its discovery, when the first radiation-induced tumor was reported in 1902 (Chauveinc, Giraud et al. 1998; Little 2000; Little 2003). Over the past decade, intense research efforts have been made to elucidate the cellular and molecular mechanisms of radiation-induced carcinogenesis in mammalian cells. On the other hand, radiation is one of the primary clinical methods used for detecting and fighting human malignancies: medical practices that are responsible for prolonging countless lives. It can be difficult to justify the use of a known mutagen and carcinogen in such clinical methods without fully understanding the proximal, distal, immediate and persistent effects that are caused by radiation exposure, which makes investigations into the direct and indirect effects of IR much more imperative.

IR can disrupt a variety of processes in exposed cells including gene expression, mitochondrial processes, cell cycle arrest and apoptotic cell death (Amundson and Fornace 2003; Amundson, Lee et al. 2003; Criswell, Leskov et al. 2003; Fei and El-Deiry 2003; Iliakis, Wang et al. 2003; Powell and Kachnic 2003; Andreev, Eidelman et al. 2006; Jeggo and Lobrich 2006; Rodemann and Blaese 2007; Valerie, Yacoub et al. 2007). Perhaps its most important characteristic is that IR is a potent DNA damaging agent capable of producing lesions such as cross linking, nucleotide base damage and single and double strand breaks (Little 2000; Huang 2003). The accumulation of DNA damage caused by IR in conjunction with the disrupted cellular regulation processes can lead to carcinogenesis (Little 2000).

4. Epigenetic Changes in the Directly Exposed Tissue

Direct radiation exposure strongly influences epigenetic effectors. DNA damaging agents including ionizing radiation have been reported to affect DNA methylation patterns (Kalinich, Catravas et al. 1989; Tawa, Kimura et al. 1998; Kovalchuk, Burke et

al. 2004). Acute exposures to low LET x-rays or γ -rays were noted to result in global hypomethylation (Kalinich, Catravas et al. 1989; Tawa, Kimura et al. 1998). Surprisingly, this effect was not a generic response as it was later shown that the IR exposure leads to a profound dose-dependent and sex- and tissue- specific global DNA hypomethylation (Pogribny, Raiche et al. 2004; Raiche, Rodriguez-Juarez et al. 2004; Koturbash, Pogribny et al. 2005; Pogribny, Koturbash et al. 2005; Loree, Koturbash et al. 2006). Furthermore, the changes in methylation status did not appear to occur indiscriminately throughout the genome, but rather may be focused to particular loci as it was noted that IR exposure also affects methylation of the promoter of the p16 tumour suppressor in a sex- and tissue-specific manner (Kovalchuk, Burke et al. 2004). These changes in methylation patterns correlated with the radiation-induced alterations in the expression of DNA methyltransferases, especially *de novo* methyltransferases DNMT3a and DNMT3b suggesting that the changes are part of purposeful radiation-induced mechanism and not just another immediate, direct radiation-induced structural change (Raiche, Rodriguez-Juarez et al. 2004; Pogribny, Koturbash et al. 2005). Most importantly, the radiation-induced global genome DNA hypomethylation appeared to be linked to genome instability in the exposed tissue (Pogribny, Raiche et al. 2004; Raiche, Rodriguez-Juarez et al. 2004; Pogribny, Koturbash et al. 2005; Loree, Koturbash et al. 2006).

DNA methylation is closely connected with other components of chromatin structure and although much attention has been given to the radiation-induced changes in DNA methylation, histones have been largely overlooked. Among the histone modifications that change upon radiation exposure, phosphorylation of histone H2AX has been studied most intensively. Recent studies have also indicated that radiation-induced global loss of DNA methylation may correlate with the changes in histone methylation, specifically with the loss of histone H4 lysine tri-methylation (Pogribny, Koturbash et al. 2005).

5. Epigenetic Determinants of the Indirect Radiation Effects: Bystander Effect

Despite a significant body of evidence that points towards the epigenetic nature of radiation-induced bystander and transgenerational effects, until recently few studies addressed the exact nature of epigenetic changes related to the indirect radiation response. The pioneering work of Kaup and colleagues has shown that DNA methylation is important for the maintenance of radiation-induced bystander effects in cultured cells. Using cultured human keratinocytes, they demonstrated that the dysregulation of DNA methylation profiles in naïve cells exposed to media from irradiated cells persists for 20 passages. Over a similar period of culture under similar conditions, these cells also exhibited an increased and persistent level of chromosome

and chromatid aberrations, reproductive cell death, apoptosis and other signs of genome instability (Kaup, Grandjean et al. 2006).

Epigenetic changes were also shown to be important in whole-tissue- and whole-organism-based bystander effect models. The reconstituted 3D human tissue model offers an excellent alternative to cell cultures. The recent study by Sedelnikova and colleagues examined bystander effects in two reconstructed human 3D tissue models—bronchial epithelial and full-thickness skin. Following microbeam irradiation of cells located in a thin plane through the tissue, a variety of biological endpoints were analyzed in distal bystander cells (up to 2.5 mm away from the irradiated cell plane) as a function of post-exposure time (0 hours – 7 days). In bystander cells, they detected a significant increase in the levels of phosphorylated H2AX; apoptosis (persistent); micronuclei formation; loss of nuclear DNA methylation; growth arrest (persistent) and an increasing number of senescent cells. Of a special interest is the observed loss of DNA methylation in bystander cells as it may be indicative of an epigenetic nature of bystander effect in 3D human tissue models (Sedelnikova, Nakamura et al. 2007).

Further insight into the role of epigenetic changes in the bystander effect comes from animal-based studies, where irradiation was shown to induce DNA damage and modulate the epigenetic effectors in distant bystander tissues. The Kovalchuk and Engelward laboratories pioneered *in vivo* studies on the role of epigenetic changes in radiation-induced bystander effects. To analyze *in vivo* bystander effects, they developed a mouse model whereby half of an animal body was exposed to radiation, while the other half was protected by a medical grade shield (Koturbash, Rugo et al. 2006). This model was used to monitor the induction and repair of DNA strand breaks in the unexposed cutaneous tissue. In addition to this well-established endpoint, the authors also explored the possibility of epigenetic mechanisms (*i.e.* DNA methylation and alterations in DNA methyltransferases and methyl-binding proteins) in the generation and/or maintenance of a radiation-induced bystander effect in the unexposed cutaneous tissue. They have shown that radiation exposure to one half of the body leads to elevated levels of DNA strand breaks, and altered levels of key proteins that modulate methylation patterns and silencing in the bystander half of the body at least 0.7 cm from the irradiated tissue. These are some of the first data to clearly demonstrate that the epigenetically regulated bystander effects occur *in vivo* in distant tissues. Importantly, these epigenetic changes in bystander tissues are not due to the insufficient shielding or radiation scattering (Koturbash, Rugo et al. 2006).

To be relevant for carcinogenesis, the epigenetic manifestations of bystander effects should accumulate and/or persist over a long period of time. To investigate the possibility that the localized X-ray irradiation induces persistent epigenetically modulated bystander effects in distant tissues, Koturbash and colleagues monitored the occurrence of epigenetic changes (*i.e.* DNA methylation, histone methylation and miRNA expression) in spleen tissue 7 months after the localized cranial irradiation. This analysis has revealed that the localized cranial radiation exposure leads to the

decreased levels of global DNA methylation. It also alters the levels of key proteins that modulate methylation patterns and silencing (i.e. *de novo* methyltransferase DNMT3a and methyl-binding protein MeCP2) and contributes to the reactivation of the LINE1 retrotransposon in the bystander spleen, located at least 16 cm from the irradiation site. Importantly, it is the first evidence that down regulation of DNMT3a and MeCP2 is probably triggered and maintained by higher activity of a small regulatory RNA, microRNA *miR-194*. These experiments have demonstrated that *miR-194* is up-regulated in the bystander rat spleen. These data have also clearly demonstrated that the bystander effect occurs *in vivo* in distant tissue, persists over a long period of time, and are epigenetically regulated (Koturbash, Boyko et al. 2007).

The observed altered expression of *miR-194* in the bystander rat spleen was quite intriguing and promoted further studies of microRNAome changes in bystander tissues. Using the microRNA microarray platform, microRNAome patterns have been profiled in skin and spleen tissues of mice subjected to sham treatment, whole-body or head exposure. The radiation exposure led to significant alterations in the microRNA expression profiles in bystander skin and spleen (Koturbash, Zemp et al. 2008). The pronounced microRNAome alterations can also be seen in the bystander tissues using the 3D model (Kovalchuk, Zemp et al. 2010). These data suggest that the microRNA expression changes really occur in bystander tissues. Their exact function in the bystander effect still has to be delineated. Furthermore, due to their small size and high stability, microRNAs may be plausible candidates for the bystander signal.

6. Transgenerational Effects

Targeted effects of radiation are caused by direct interaction of ionizing particles with genetic material. Direct germline mutations are transmitted to subsequent generations in accordance to the laws of classical genetics and may lead to a whole spectrum of deleterious effects including, but not limited to, dominant lethality and congenital malformations (Mole 1979; Goldman 1982).

A growing body of data shows that heritable effects of radiation exposure are not limited to those caused by targeted mutations. A number of studies have shown *de novo* mutations in the non-exposed progeny of an irradiated parent that resemble radiation induced genomic instability (Luning, Frolen et al. 1976; Luke, Riches et al. 1997; Carls and Schiestl 1999; Shiraishi, Shimura et al. 2002; Slovinska, Elbertova et al. 2004; Streffer 2006). Early work that focused on the study of radiation-induced, dominant, lethal mutations in mice, unexpectedly, showed an excess of intrauterine deaths of fetuses fathered by pre-conceptually irradiated (at spermatogonial stage) mice. This effect continued to the F2 generation (Luning, Frolen et al. 1976). Later, the discovery of hypermutable mini satellite loci (short tandem repeats in human and mouse genome) provided an invaluable biomarker for the study of radiation-induced germ line

mutations (Dubrova, Jeffreys et al. 1993). Pioneering works by Dubrova *et al* showed increase of germ line mutation rates in the progeny of fathers exposed to radiation as a result of the Chernobyl accident (Dubrova, Nesterov et al. 1996; Dubrova, Nesterov et al. 1997). The same methods were used to detect the increased frequency of germ line mutations in multi-generation families living close to the Semipalatinsk nuclear test site and among the Techa River population (Dubrova, Bersimbaev et al. 2002; Dubrova, Ploshchanskaya et al. 2006; Akleev, Dubrova Iu et al. 2007).

Animal studies conducted by the same group showed that germ line mutations at mini satellite loci were induced at a frequency that was an order of magnitude higher than is expected in the case of direct mutations (Dubrova, Plumb et al. 1998; Dubrova, Plumb et al. 2000). Importantly, the increased rate of mini satellite mutations was still observed in F2 progeny of irradiated male mice and this trait was exhibited by most of the offspring in violation of Mendelian laws (Barber, Plumb et al. 2002). Induction of transgenerational genomic instability was influenced by the dose, type of irradiation and stage of germ cell development (Fan, Wang et al. 1995; Niwa, Fan et al. 1996; Dubrova, Plumb et al. 1998).

Transgenerational genomic instability may be linked to transgenerational radiation induced carcinogenesis that manifests itself as an increase of cancer risk in the unexposed progeny of irradiated parents. Increased predisposition to tumour formation in the progeny of irradiated mice was first demonstrated by Nomura (1982; 1983; 1989; 2004). This effect, although slightly carcinogenic, was transmitted to subsequent generations in a non-mendelian manner and was greatly enhanced upon application of secondary carcinogens (Vorobtsova and Kitaev 1988; Vorobtsova, Aliyakparova et al. 1993). A number of experiments were designed to confirm and expand initial findings by Nomura. Daher *et al* (1998) reported marginally significant increase of leukemia incidence in the progeny of X-ray irradiated N5 mice, as well as earlier onset in leukemia development in the progeny of mice injected with tritium. Similarly, modulation in leukemia/lymphoma development was observed after in BDF1 and CBA mice after pre-conceptual treatment with plutonium 239, followed by secondary exposure to methylnitrosourea (Lord, Woolford et al. 1998). A lifetime study that included 4279 mice showed that pre-conceptual treatment to a high, acute dose of X-Rays leads to increased hematopoietic malignancy in the female and bronchioloalveolar adenocarcinomas in male progeny. On the other hand the same study failed to show increased tumour induction by secondary exposure to urethane (Mohr, Dasenbrock et al. 1999). The exact manifestations of radiation induced transgenerational carcinogenesis were shown to depend on the dose, type of irradiation, stage of germ cell development and genetic background of the model organism (Draper 1989; Lord 1999; Nomura 2006).

To date, the exact molecular mechanism of radiation induced transgenerational effects remains unclear: the high frequency of induction and the fact that these effects are present in most, if not all, of the offspring are incompatible with the involvement of

genetic mutations. Radiation induced free radicals could be considered, however, their short lived nature and negligible cytoplasmic component in sperm make them an unlikely candidate. Manifestations and the mode of transmission of this effect suggests that it has to be fixed across multiple loci in most if not all of the germ cells produced by affected gonad. It is tempting to hypothesize that these changes are memorialized in the germ cell through epigenetic mechanism. DNA methylation marks at imprinted loci and certain repeat elements were shown to be resistant to epigenetic reprogramming events in developing embryos (Hajkova, Erhardt et al. 2002; Lane, Dean et al. 2003). A series of recent studies described the ability of sperm-bound small RNA to trigger developmental changes in the embryo that could be transmitted to subsequent generations in non-mendelian manner (Rassoulzadegan, Grandjean et al. 2006; Rassoulzadegan, Grandjean et al. 2007; Grandjean, Gounon et al. 2009). No data is available on the ability of histone marks to trigger similar responses, however, histones were shown to be present in sperm covering genomic areas responsible for embryonic development (Brykczynska, Hisano et al. 2010).

A preliminary study conducted by Kovalchuk's group found significant loss of cytosine DNA methylation in the thymus of the progeny after paternal exposure to X-rays. The loss of DNA methylation was paralleled by a significant decrease in the levels of maintenance (DNMT1) and *de novo* methyltransferases DNMT3a, DNMT3b and methyl-CpG-binding protein (MECP2). They also noted significant accumulation of DNA strand breaks in the thymus (Koturbash et al, IJROPBP, 2006). Localized cranial irradiation of male rats leads to accumulation of DNA lesions and loss of global DNA methylation in mature sperm (Tamminga, Koturbash et al. 2008). Loss of DNA methylation along with dysregulation of DNA methylation enzymes was also found in the progeny of cranially exposed male rats (Tamminga, Koturbash et al. 2008). A microarray expression profiling revealed altered miRNA levels in the thymus of adult male mice pre-conceptually exposed to 2.5 Gy of X-Ray (Filkowski, Ilnytsky et al. 2010). Changes in miRNAome were paralleled by dysregulation in miRNA processing machinery and decreased expression of LSH (Filkowski, Ilnytsky et al. 2010)—a chromatin remodelling factor implicated in the regulation of DNA methylation at repeatable elements (Muegge 2005). DNA methylation profiling of repeats in the same samples revealed loss of CpG methylation at LINE1 and SINEB2 retrotransposable elements (Filkowski, Ilnytsky et al. 2010).

Cumulative evidence points to the epigenetic origins of radiation-induced transgenerational genomic instability and cancer predisposition, however, their exact mechanism remains unclear. Future research in this area has to rely on the use of microarray technology, next generation sequencing and bioinformatic approaches in order to extract functionally relevant, causal changes influencing epigenetic reprogramming and genomic stability across generations. Such research has both practical and fundamental value, as it may offer an understating of how genotoxic factors contribute to complex disease by altering our epigenome across generations.

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RADIATION-INDUCED ADAPTATION: CHROMOSOMAL INSTABILITY AND SELECTION

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Abstract- Our investigations were performed to study the general regularities of the adaptation and instability processes induced by low-dose-rate radiation by means of the statistical modeling. The modeling is based on investigations in lab and of consequences of nuclear station and nuclear tests fallouts for natural populations and inhabitants, and shows the adaptation processes which are accompanied by instabilities coupled with selection. We consider the connections between the intercellular and intracellular instability processes. Synergism of radiation, high temperatures, and aging is discussed. Approaches to risk assessment of chromosomal instability are presented in plant cells and human blood lymphocytes. Risks have been analyzed as functions of radiation intensity in ecology and in descendents of persons exposed to radioactive fallout from nuclear tests. These ideas can be used in fields as diverse as radiotherapy and social sciences.

Keywords: adaptation, chromosomal instability, statistical modeling, seeds of plant, blood lymphocyte

1. Introduction

Chromosomal instability was observed long years ago [1, 2]. In 1970 V.I. Korogodin and his colleagues showed that yeast cells instability is coupled together with accumulation of chromosomal abnormalities [3] induced by low radiation effects or non-optimal environment conditions [4]. They noted a connection of the phenomenon of instability with evolution [3], which was mentioned earlier by R.K. Herman and N.B. Dworkin [5].

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In mathematical biology, R.A. Fisher [6], J.H. Gillespie [7], and H.A. Orr [8] investigated fitness to the environment conditions by modeling. Orr showed a universal character of distributions with tails and connection of this phenomenon with selection in evolution models [8]. Statistical modeling was used to analyze the appearance of multiple mutations in blood lymphocytes of persons who worked in a radiochemical enterprise [9] and patients with chromosome instability syndrome [10]. These authors suggested a hypothesis based on queues theory that distributions' tails were related with DNA repair [11].

Our statistical model of adaptation is based on time-dependent accumulation of abnormalities and selection [12, 13]. To investigate features of adaptation processes, we used the following data: own studies of lab-irradiated selected pea seeds, pure line [14] and plantain seeds collected in natural populations growing near the radiation sources [15, 16]; investigations on blood lymphocytes of persons living in the Tyumen and Irkutsk regions [17, 18]; data published by N.P. Bochkov et al. on blood lymphocytes of radiochemical enterprise' workers [9]. The statistical modeling of the appearance of abnormal cells, abnormal chromosomes in cells and proliferation activity of cells, were performed for these objects [19-24]. Our adaptation hypothesis was formulated on the basis of the statistical modeling.

Our aims were to analyze regularities of adaptation, its processing across the generation and to approach the risk assessments of chromosomal instability. To demonstrate these regularities, we used data on the pea seeds (pure line) irradiated in lab; on plantain seeds collected near the Balakovo (middle Volga) Nuclear Power Plant (NPP) and on blood lymphocytes of samples of persons living in the Tyumen (Far North) and Irkutsk (Siberia) regions.

2. STATISTICAL MODELING

We have used the maximum-likelihood method for approximations. The following statistical criteria were chosen to estimate the regression: (i) R_{adj} is the determination coefficient corrected for degrees of freedom [25] (equivalent to the T-criterion known in radiobiology [26]); (ii) the AIC criterion (the Akaike criterion imposes limitation on the minimal information distance between the model and experimental distributions) [27]; (iii) the BIC criterion (selection of the most probable models from the ensemble under the condition of a priori equal probability of any of them) [28]. The most sensitive test for our tasks is T-criterion [26], which encourages good efficiency of approximation and fines for using large numbers of parameters.

The best hypothesis with respect to the majority of criteria was preferred. For close values of criteria, the simpler hypothesis was chosen. For a given number of experimental points, the number of model parameters is, as a rule, larger than the optimal one [29]; therefore, the stability of distributions was verified. The verification

consisted in variation of the length of the partitioning interval upon construction of histograms. The interval length $(D_{\max} - D_{\min})/M$ was taken as the basis. Here, $(D_{\min}, D_{\max})$ is the interval of data variation, $M = [\log_2(N)] + 1$ is the number of partition intervals, and N is the number of data points. The verification showed the stability of the distributions.

The program FUMILI [30] was used. The search for optimal values of the model parameters was carried out in two stages. First, the initial approximation for the model parameters was found by the random search method [31] or the simulated annealing method [32]. Then the parameter values were refined using regular procedures BFGS [33] or Newton [31]. Three parameters were optimized.

We have examined the hypotheses of single-component normal, binomial, lognormal, Poisson, and geometric distributions and their composition [12, 13, 21].

3. NON-LINEARITY INDUCED BY LOW-DOSE RATE IRRADIATION. LAB EXPERIMENTS ON PEA SEEDS

3.1. MATERIALS AND METHODS

Seeds: Pea seeds (*Pisum arvense*), selected line Nemchinovsky-817 (received from Moscow, Nemchinovka, Agriculture Institute) were used in the laboratory experiment. For this kind of seeds, the reported quasi-threshold radiation dose, which corresponds to the inflection of the survival curve from a shoulder to mid-lethal doses, is 10–20 Gy [34]. The pea seeds were collected at the end of July of 1996 and 1997 in the field of the Agriculture Institute (Moscow region). The seeds collected in 1996 were stored in the refrigerator until April of 1997 ($T = 3-4^\circ\text{C}$ and relative humidity = 13–14%). Then the first fraction of seeds was tested (stored in the refrigerator ≈ 8 months, “young”); the second was kept in the refrigerator until April of 1998 (≈ 20 months, “old”); and the third was stored in the refrigerator for 8 months and then held outdoors until June. These seeds were stored without sunlight and precipitation, but the temperature ranges during the day and the night reached $30-32^\circ\text{C}$ and $16-18^\circ\text{C}$, respectively (these are higher for the Moscow region); the relative humidity of $\approx 80\%$ was usual for this region. The duration of heat stress was 2 months (“young seeds with heat stress”). The effects of humidity, and temperature profiles and extremes on the seeds were not considered in this study. In 1997, seeds were collected again. They were stored in the refrigerator ($T = 3-4^\circ\text{C}$ and relative humidity = 13–14%) until April of 1998 and then all seeds were tested (“young”). Before the tests, the water content of the seeds was stabilized at 16% humidity in a dessicator with 18% sulfuric acid, as described in [14].

Seed sprouting and fixing: Seeds of all groups were germinated on wet filter paper in petri dishes at 25°C until seedling roots reached 30 ± 4 mm, the length which

corresponds to the first mitoses. Before it seedling growth is due only to swelling without cell division. Seedlings were fixed in ethyl alcohol and acetic acid (3:1) and stained with acetoorcein. Seedlings less than 26 mm after 13 days were scored as non-surviving because too few cells reached the first mitosis (the mitotic index in the shoot zone is already known to increase in parallel with the size of the seedling [14]). The first fixation was started when approximately 1/3 of the population had germinated. After 13 days we ended the fixation. Prolonging the germination period (up to 6 weeks) increased the survival (S) by only 2-3% and these rootlets were small and brown. The methods of seed sprouting and fixing have been described in [14].

Determination of cells with chromosomal abnormalities: Ana-telophases were scored for chromosomal abnormalities CAs containing chromosome bridges and acentric fragments.

Determination of mitotic index: The mitotic index MI of seedling meristem cells was scored as the percent of cells in mitosis.

Irradiation of seeds: Pea seeds were gamma-irradiated at room temperature with 7 cGy ^{60}Co at 0.3, 1.2, or 19.1 cGy/h. A set of three thermoluminescence detectors TLD-700 was used to examine each dose. TLD were randomly placed with seeds into glasses, which were located at 14, 56, and 111 cm from the source. The overall uncertainty of dose rates and doses was 10-13% and ~3-5% at 14 cm (dose rate of 19.1 cGy/h) and 56-111 cm (dose rates of 0.3-1.2 cGy/h) from the source, respectively. The elapsed time of irradiation was 24 h (0.3 cGy/h), 6 h (1.2 cGy/h), and 23 min (19.1 cGy/h). The calibrated dose rate from the source was 0.362 cGy/h at 1 m and the ambient levels were 0.12-0.15 $\mu\text{Sv/h}$. The beta-irradiation shielding of seeds was glass beakers (wall thickness ~ 1.2 mm).

Annual radiation exposure is ~0.10-0.12 $\mu\text{Sv/h}$ and ^{137}Cs soil concentrations are ~5-10 Bq/kg in the fields of the Agriculture Institute. The applied analytical methods were standard and are described elsewhere [35, 36]. These values do not exceed the average radiation values over the Moscow region [36]. The accumulated external and internal doses were calculated and summed using the Brian-Amiro model [37] and estimated at the level of ~0.3 cGy for pea seeds by using the published transfer factors [36].

Statistics: In various components of the experiment with young and old peas, we took 125-174 seeds to determine S and usually analyzed ~2000 ana-telophases for each component. In the tests with "young+heat" peas we took 40 seeds and analyzed ~1500 ana-telophases for each component. More than 1000 cells in each seedling were studied to determine the MI value. The data were processed with standard statistical methods [38, 39] and statistical criteria [25-28].

3.2. DOSE RATE NON-LINEARITY OF AVERAGED BIOLOGICAL VALUES

Tabl. 1 presents survival of seeds, and the numbers of proliferated and abnormal cells. The current observations over all seeds groups have shown that dose rates of 0.3,

1.2, and 19.1 cGy/h induced both cell elimination and cells with CAs (CCAs), which can be modified by aging and additional heat stress. As a rule, cell elimination dominates at the dose rate of 0.3 cGy/h, and CCAs - at 19.1 cGy/h. Dynamics of the MI as well as frequency of cells with CAs at the first mitoses were considered in [19]. This analysis has revealed that irradiation with a dose rate of 0.3 cGy/h stimulates the MI increasing and earlier seed germination; its period can be shorter (young and heat-stressed seeds) than in the control while seed survival decreases; irradiation with 19.1 cGy/h delays seed germination and does not decrease the CCA frequency. The seed survival diminishes significantly in aging and heat-stressed seeds.

Cell cycles can be perturbed by irradiation [40 - 42] that includes the cell cycle delay [41, 42] as well as stimulation to proliferation [42, 43]. The cell cycle delay was shown at the dose rate of 19.1 cGy/h in all groups of seeds, and the stimulation to divide in resting cells was found at 0.3 - 1.2 cGy/h in the groups of young and old seeds. This effect of mitotic stimulation was virtually eliminated by the addition of heat stress [19]. Perhaps stimulation compensates for cell elimination in the seed groups exposed only to radiation at these dose rates, while aging and exposure to heat appear to be more significant forces for decreasing viability.

Table 1. Effects of ionizing irradiation on pea seeds populations at different dose rates and storage conditions

Dose rate, cGy/h	N	1-S, %	CA %	MI
Young seeds				
0	149	8.0	10.7±1.1	5.8 ± 0.4
0.3	150	16.0*	7.8±1.0*	7.6±0.5*
1.2	150	7.3	11.4±1.4	6.5±0.6
19.1	146	5.5	9.1±1.2	6.0±0.5
Old seeds				
0	125	21.6	13.1±1.0	5.1±0.5
0.3	128	28.1*	12.5±0.9	8.6±0.6*
1.2	174	20.7	15.8±1.4*	5.4±0.4
19.1	149	28.2*	13.1±1.7	4.6±0.5
Young seeds with heat stress				
0	40	7.5	11.2±1.1	5.9±0.3
0.3	40	32.5**	8.2±0.7*	5.5±0.5***
1.2	42	62.5**	6.1±1.0*	6.0±1.2***
19.1	40	50.0**	15.4±2.2*	4.5±0.3*

The standard error is shown. The difference with non - irradiated control: * - $p < 0.05$; ** - $p < 0.001$; *** - $p > 0.05$.

Fig. 1 presents some of the experimental data as a function of dose rates. The $(1 - S)$ value is connected with the number of proliferated meristem cells, which depends on the stimulated and eliminated cells. The CCA frequencies and MI values depend on the dose rate non-linearly (χ^2 -criterion, $p < 0.05$) (Tabl. 1, Fig. 1 A, B). It is the reason that $(1 - S)$ depends on the dose rate in a non-linear manner (χ^2 -criterion, $p < 0.05$) (Fig. 1 C).

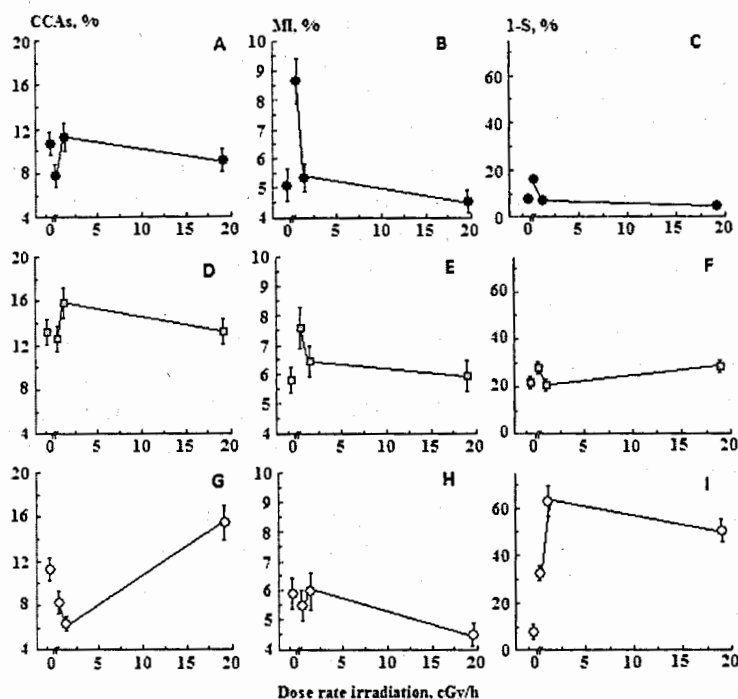


Figure 1. Frequency of cells with chromosomal abnormalities (CCAs) (A, D, G), mitotic index (MI) (B, E, H), and non-survival ($1-S$) (C, F, I) versus dose rate irradiation. A-C, young seeds (black circles); D-F, old seeds (grey squares); G-I, young seeds with heat stress (heat stress duration 2 months) (open circles). Values of the intact control seeds are separated from the irradiated ones by breaks.

Some authors interpret the non-linearity of the CCA frequency at low doses by different repair systems acting at different dose rates [44, 45]. It has been shown that stress leads to accumulation of mutations [46] as well as chromosome aberrations [3]. On the other hand, the bystander effect is documented at low doses in various investigations [47]. We have examined the hypothesis of bystander effect by statistical modeling.

The statistical analysis has revealed that frequency of seeds on the number of cells with CAs does not distribute normally, but displays tails. It assumes a model of abnormal cell appearance, which postulates a division of the seeds into two subpopulations. In the first subpopulation, cells with abnormalities appear independently and are Poisson-distributed among seeds [12]. In the second

subpopulation there is an enhancing factor that describes the distributions' tails. This enhancement might be due to bystander effect under the influence of oxidizing stress [13].

3.3. MODELING OF APPEARANCE OF CELLS WITH CAS IN SEEDLINGS' MERISTEM

The enhancing factor can be described by either geometric [11, 48] or lognormal [49] distributions. The fitting analysis of the observed data verified that the seed populations consisted of two Poisson (^sP) and geometric (^sG) subpopulations (χ^2 criteria, AIC [27], BIC [28], and T [26]) [12]. The geometric distributions are characterized by correlations between the events [50]. The modeling parameters ^smP and ^smG (the sample means) as well as ^sN_P and ^sN_G (the relative values) are shown in Tabl. 2. The values of the Poisson (^sN_P) and geometric (^sN_G) distributions were calculated as the ratios of a Poisson or geometric component to the entire tested seed population. In the dose-rate interval 0.3–1.2 cGy/h the ^smG values tended to increase ($p < 0.05$), whereas the ^smP ones did not change significantly ($p > 0.05$) in comparison with the control. In the groups of young and heat-stressed seeds, the ^sN_G values decreased in the dose-rate interval 0.3–1.2 cGy/h (in old seeds group, aging decreased ^sN_G too strongly and the statistical analysis is invalid). The ^sN_G quota decreased more than the ^sN_P quota ($p < 0.05$). As a rule, the parameters of the ^sP and ^sG subpopulations showed different behavior in the interval of 0.3 – 19.1 cGy/h for all groups of seeds (heterogeneity criterion [39], $p < 0.05$).

In young seeds, the value of $(1 - S)$ correlated strongly with the MI ($|R|_{1-S,MI|CA} = 0.83$), and not with CCA frequency (Tabl. 3). This indicates that in young seed populations, the stimulation of proliferation is the general mechanism to regulate the survival of the seeds irradiated with 0.3 – 19.1 cGy/h.

In old seeds, there is a correlation between $(1 - S)$ and CCA frequency ($|R| = 0.66$), which is due to the connection of seeds survival with the N_G subpopulation ($|R|_{1-S,P|G} = 0.03$ and $|R|_{1-S,G|P} = 0.65$). The CCA frequency also correlates significantly with ^sN_G ($p < 0.05$) ($|R|_{CCA, P|MI} = 0.69$; $|R|_{CCA, G|MI} = 0.99$). In addition, these values do not correlate with the radiation dose rate (Tabl. 3). The ^sG subpopulation is considered to play an important role in aging seeds, because the non-surviving fraction increases primarily as a result of diminution of ^sG seeds, and the CCA frequency also increases with the sample mean of the geometric distribution.

Table 2. Results of mathematical modeling of appearance of cells with abnormalities

Dose rate cGy/h	Number of seeds	Number of ana- telofases	Sample means		A value of a subpopulation	
			^5mP	^5mG	$^5\text{N}_\text{P}$	$^5\text{N}_\text{G}$
Young seeds						
0	60	2202	1.6±0.3	2.8±0.4	0.34±0.07	0.33±0.07
0.3	60	2294	1.1±0.2	11.5±4.3	0.60±0.01	0.01±0.10
1.2	60	2774	1.5±0.2	2.6±0.6	0.50±0.04	0.09±0.09
19.1	60	2144	0.8±0.2	6.7±1.2	0.58±0.04	0.08±0.09
Old seeds						
0	60	918	1.9±0.3	0.0±1.0	0.45±0.00	0.00±0.08
0.3	60	2419	1.7±0.2	0.0±1.0	0.64±0.00	0.00±0.10
1.2	60	2021	2.5±0.3	10.1±1.6	0.55±0.03	0.06±0.09
19.1	60	593	1.9±0.3	0.6±2.4	0.40±0.00	0.00±0.08
Young seeds with heat stress						
0	40	3367	1.1±0.2	13.3±1.0	0.59±0.08	0.31±0.12
0.3	40	2560	1.1±0.2	∞	0.53±0.05	0.11±0.11
1.2	40	1235	1.1±0.3	0.0±0.2	0.31±0.03	0.05±0.08
19.1	40	1088	1.1±0.3	7.3±0.8	0.22±0.08	0.28±0.07

The combined effect of irradiation and heat stresses disturbed the correlations of $(1 - S)$ with both CA frequency ($|R| = 0.001$) and MI value ($|R| = 0.13$). A strong correlation is observed between $(1 - S)$ and $^5\text{N}_\text{P}$ ($|R|_{1-S, \text{PIG}} = 0.98$) as well as $^5\text{N}_\text{G}$ ($|R|_{1-S, \text{GIP}} = 0.99$). Irradiation plays an important part in the case of heat-stressed seeds, because CCA frequency is related with dose rate ($|R|_{\text{CCA, DIMI}} = 0.81$).

Table 3. Partial correlations between the biological parameters, values P (N_P is designated by P) and G (N_G is designated by G) distributions, and dose rate irradiation

	Young	Old	Heat stress
$ R _{1-S, \text{CCA/MI}}$	0.28	0.66	0.001
$ R _{1-S, \text{MI/CCA}}$	0.83	0.21	0.13
$ R _{\text{G, P/MI}}$	0.95	0.18	0.16
$ R _{1-S, \text{PIG}}$	0.21	0.03	0.99
$ R _{1-S, \text{GIP}}$	0.31	0.65	0.98
$ R _{\text{CCA, P/MI}}$	0.37	0.69	0.42
$ R _{\text{CCA, G/MI}}$	0.19	0.99	0.11
$ R _{\text{CCA, DIMI}}$	0.06	0.01	0.81
$ R _{\text{MI, DICCA}}$	0.34	0.46	0.26
$ R _{1-S, \text{DIMI}}$	0.49	0.45	0.47
$ R _{1-S, \text{DICCA}}$	0.53	0.61	0.39

Irradiation with 0.3 – 19.1 cGy/h induces two regulatory mechanisms aimed at seed survival, stimulation of proliferation and the bystander effect. Stimulation of proliferation is sufficient to regulate viability in young seeds. In aging seeds, high CCA frequency is independent of the dose rate and is caused by sensitivity of cells in the ^5G subpopulation. Combination of irradiation and heat stresses induces another response of seeds. CCA frequency depends on dose rate, and seed survival correlates with both P- and G- mechanisms of CCA appearance. It should be noted that

bystander effects lead to both enhanced CCA frequencies and cell elimination. In the

bystander effects lead to both enhanced CCA frequencies and cell elimination. In the current studies the cell elimination was observed mainly at 0.3 cGy/h and enhanced CCA frequencies mainly occurred at 19.1 cGy/h. This summary agrees with the conclusion of C. Mothersill *et al.* [51] that the phenomenon of instability consists of the complete processes of mutation and lethal events, which do not correlate.

The effect of low dose rates was apparent not only as CCAs but also as increased values of $(1 - S)$ and MI, which require additional regulatory mechanisms. These are bystander effect and stimulation of proliferation (Tabl. 3). We conclude that the CCA frequency on the average can't be a sufficient criterion to define stress conditions because other mechanisms regulate the seed viability and variability.

3.4. MECHANISMS THAT REGULATE THE SEEDS' VIABILITY AND VARIABILITY

The non-surviving fraction of seeds $(1 - S)$ was increased by even a single oxidizing factor but especially (up to 62%) under the combined effect of multiple oxidizing factors. In all groups of seeds, $(1 - S)$ was non-linear at the interval of 0–19.1 cGy/h irradiation and increased significantly at a dose rate of 0.3 cGy/h (Tabl. 1). For young seeds, the values of $(1 - S)$ correlated strongly with MI ($|R|_{1-S, MI/CCA} = 0.83$), while the CCA frequency did not ($|R|_{1-S, CCA/MI} = 0.28$) (Tabl. 3). These results suggest that in young seed populations, stimulation of proliferation is the general mechanism to regulate the survival of the seeds irradiated with 0.3 – 19.1 cGy/h.

For old seeds, the effects of irradiation were less pronounced on cell survival (30% increase at 0.3 cGy/h compared with 100% at the same dose rate for young seeds), but the impact of aging was dramatic, with 2.5–5-fold increases in $(1 - S)$ (Fig. 1). In aging seeds, CCA frequency and non-survival were correlated ($|R|_{1-S, CCA/MI} = 0.66$), both values were independent of dose rate (Tabl. 3). In this case, high CCA frequency was specified by sensitivity of G-cells, and the correlation between MI and $(1 - S)$ was low ($|R|_{1-S, MI/CCA} = 0.21$) (Tabl. 3).

The greatest mortality was observed in young+heat stressed seeds. When compared with the young seed group, the significantly increased $(1 - S)$ values indicate that heat stress produces a more significant impact of seeds than aging does (Tabl. 1). The combined effect of irradiation and heat stresses disturbed the correlations of $(1 - S)$ with both CCA frequency ($|R| = 0.001$) and MI value ($|R| = 0.13$). In the heat-stressed group, the CCA frequency depended on dose rate ($|R| \sim 0.98 \div 0.99$) and seed survival was correlated with both P- and G- mechanisms of CCA appearance (Tabl. 3).

MI increased significantly at 0.3 cGy/h for young and old seeds but not for young+heat stressed seeds. One can view the combined CCA and MI data in Tabl. 1 as indicating that young and old seeds exposed to the lowest dose experienced a mitotic stimulus. Perhaps stimulation compensates for cell elimination in the groups exposed only to irradiation with tested dose rates (non-surviving fraction of young seeds increased up to 16% only), while aging and exposure to heat appear to be more

significant forces for decreasing viability (non-surviving fractions of old and heat-stressed seeds increased up to 28-62%).

In all groups, the CCA frequency decreased significantly at the lowest dose rate (0.3 cGy/h) ($p < 0.05$) that showed cell elimination. The CCA numbers were not normally distributed, but displayed tails. This result suggests that some CCAs were enhanced and correlated. The CCA frequency correlated with $(1 - S)$ in old seeds ($|R|_1 - S_{CCA|MI} = 0.66$) (Tabl. 3), but not in both young and heat-stressed seeds. Therefore, the CCA frequency cannot be a sufficient criterion for the viability of plant population under radiation stress.

The simulation of CAs justifies the hypothesis that there are two subpopulations of seeds. In the first subpopulation, the CAs appear independently and are Poisson-distributed. In the second, the appearances of CAs are correlated. The simulation supported both Poisson and geometric mechanisms in meristems, which contribute to seed survival (Tabl. 3). In the dose-rate interval of 0.3–1.2 cGy/h, the mG values tended to increase ($p < 0.05$), whereas mP ones did not change significantly ($p > 0.05$) in comparison with the control. The $(1 - S)$ value increased due to the elimination of cells and the failure of seeds to germinate in the geometric (particularly the Poisson) subpopulation (Tabl. 2). We can conclude that two mechanisms induced by stress conditions regulate the number of proliferating and abnormal cells. These are the bystander effect accompanied by selection, and stimulation of resting cells to divide. We would like to speculate that stress is an instrument to adapt populations in their ecological niches. Adaptation includes increasing variability (quickly elevating the sample mean mG) and dramatic decreasing the number of germinated seeds; a new genotype of the surviving seeds is supported by repair mechanisms and stimulation to division of resting cells.

3.5. ADAPTATION PROCESSES IN MERISTEM AND CELLS. CHROMOSOMAL INSTABILITY

Distribution of cells in root meristem of unirradiated seeds on the number of chromosomal abnormalities: The geometric distribution of cells ($^{\circ}G$) on the number of CAs in the control group is shown in Tabl. 4. It appears that correlations between the appearance of CAs originated up to "success" [48], which could be considered in this case as adapting of the genome to conditions. This suggests an adaptive process in intact cells: a DNA-damaging process coupled with selection, which checks fitness of cells. We can imagine that an additional factor influences a sensitive subpopulation of cells in the first place, and its distribution on the number of CAs could be geometric ($^{\circ}G$) or Poisson ($^{\circ}P$). Thus, experimental data could be compounded of $^{\circ}G$ and $^{\circ}G$ (or $^{\circ}P$) that corresponds to distributions of resistant ($^{\circ}G1$) and sensitive ($^{\circ}G2$ or $^{\circ}P$) fractions of cells. In the cases of aging and heat-stressed seeds, a sum of $^{\circ}G1$ and $^{\circ}P$ distributions

is observed (Tabl. 4). This is expected because high temperature and aging are strong mutagenic factors [52, 53].

Table 4. Statistical modeling of the CAs in root meristem cells of unirradiated pea seeds

Year	CA frequency	Number of ana-telophases	Number of cells with CA number					T criterion / χ^2 criterion (p < 0.05)			
			0	1	2	3	≥4	°G	°G1+°G2	°G1+°P	°P
Young											
1997	0.17	3443	2948	426	55	11	2	25/+	269/+	181/+	794/-
1997	0.12	2199	1931	238	25	5	-	22/+	451/-	1434/-	113/-
1996	0.08	1097	1022	67	5	2	1	3/+	22/+	2345/+	10/+
Young seeds with heat stress											
1996	0.11	4020	3663	274	68	11	4	1365/-	320/+	113/+	1614/-
Old											
1996	0.10	906	823	77	4	1	1	0.20/+	0.20/+	0.01/+	0.09/+

The standard error of CA frequency is ≤ 0.01 .

Distribution of cells in root meristem of irradiated seeds on the number of chromosomal abnormalities: In young seeds irradiated at 0.3 cGy/h the modeling has shown two cG distributions (Tabl. 5). We can assume that the second distribution cG2 reflects additional damaging processes in the sensitive subpopulation. At 1.2 and

Table 5. Statistical modeling on pea seeds irradiated with 7 cGy at low-dose intensity or without irradiation

Dose rate, cGy/h	N ana- telophases	CA frequency	Number of cells with CA number					T criterion / χ^2 criterion (p < 0.05)			
			0	1	2	3	≥ 4	$^{\circ}G$	$^{\circ}G1+^{\circ}G2$	$^{\circ}G1+^{\circ}P$	$^{\circ}P$
Young											
0	2199	0.12	1931	238	25	5		22/+	451/-	1434/-	113/-
0.3	2139	0.08	1971	145	19	4	1	188/+	21/+	333/+	246/-
1.2	1843	0.07	1637	191	13	2		249/+	1492/-	49/+	119/+
19.1	1965	0.08	1788	162	10	5		53/+	565/+	98/+	35/+
Heat											
0	4020	0.11	3663	274	68	11	4	1365/-	320/+	113/+	1614/-
0.3	2723	0.09	2517	172	26	8		314/-	44/+	378/+	370/-
1.2	1380	0.08	1296	68	9	6	1	44/-	19/+	283/+	48/-
19.1	1274	0.18	1092	143	29	6	4	139/+	10/+	42/+	231/-
Old											
0	906	0.10	823	77	4	1	1	0.20/+	0.20/+	0.01/+	0.09/+
0.3	2378	0.13	2109	236	27	5	1	0.05/+	0.01/+	0.01/+	0.50/+
1.2	1977	0.15	1729	207	38	3		0.18/+	0.12/+	0.06/+	0.90/+
19.1	586	0.11	527	53	5	1		0.04/+	0.02/+	0.01/+	0.30/+

The standard error of CA frequency is ≤ 0.01 .

19.1 cGy/h, the cP distribution gradually displaces cG2 distributions. Analyses of the combined effect of radiation and high temperature reveal domination of the G regularities of the CAs appearance (heat stress) and addition of a P component (aging) (Tabl. 5).

Tabl. 6 presents general characteristics and modeling values of the appearance of both cells with CAs in meristem and the CAs in cells. In the young seeds, a negligible value of seeds' G distribution (${}^sN_G$) on the number of cells with abnormalities, is coupled with a negligible value of cells' G2 (${}^cN_{G2}$) distribution at 0.3 cG/h that is accompanied by decreasing frequencies of R1, R2, and survival (Tabl. 6). It is basic to think that intensive radiation-induced bystander effects in meristem lead to intensive DNA damage in its cells, which together decrease the survival of cells and seeds. Poisson distributions of seedlings and cells are observed at 1:2 and 19.1 cGy/h. At 19.1 cGy/h, the parameters ${}^s mP$ (0.85 ± 0.20) and ${}^c mP$ (0.09 ± 0.02) have low values (Tabl. 6).

Table 6. Seed non-viabilities (1 - S) of both cells with CAs (R1) in seedling meristems and CAs in meristem cells (R2), parameters of the distributions of seeds on the number of cells with abnormalities as well as distributions of cells on the number of CAs.

Dose rate	1-S, %	R1	Distributions of seeds on the number of CCAs				R2***	Distributions of cells on the number of CAs					
			^s N _p	^s mP	^s N _G	^s mG		^c N _{G1}	^c mG1	^c N _{G2}	^c mG2	^c N _p	^c mP
Young													
0	8.0	10.7	0.33	1.60	0.34	2.8	0.12	1.00	0.88				
0.3	16.0*	7.8*	0.60	1.14	0.01	11.5	0.08	0.93	0.94	0.07	0.67		
1.2	7.3	11.4	0.50	1.52	0.09	2.6	0.07	0.26	0.87			0.74	0.06
19.1	5.5	9.1	0.58	0.85	0.08	6.7	0.08					1.00	0.09
Heat													
0	7.5	11.2	0.59	1.1	0.31	13.3	0.11	0.87	0.03			0.13	0.66
0.3	32.5**	8.2*	0.53	1.1	0.11	20.0	0.09	0.70	0.02	0.30	0.25		
1.2	62.5**	6.1*	0.31	1.1	0.05	0.0	0.08	0.94	0.04	0.06	0.64		
19.1	50.0**	15.4*	0.22	1.1	0.28	7.3	0.18	0.85	0.11	0.15	0.60		
Old													
0	21.6	13.1	0.45	1.9	0.00	0.0	0.10					0.99	0.10
0.3	28.1*	12.5	0.64	1.7	0.00	0.0	0.13	0.98	0.12	0.01	0.69		
1.2	20.7	15.8*	0.55	2.5	0.06	10.1	0.15	0.74	0.19	0.26	0.02		
19.1	28.2*	13.1	0.40	1.9	0.00	0.6	0.11	0.12	0.32			0.88	0.08

The difference from the unirradiated control: *p < 0.05; **p < 0.001; ***standard error ~8–12%; ****standard error < 0.03. Standard errors of the parameters of distributions do not exceed 20–30% (of the sample means) and 10–15% (of the relative values).

In aged seeds, the negligible values of the ${}^5N_G$ and ${}^6N_{G2}$ distributions are observed at 0.3 cGy/h, which corresponds to the increased seed survival ($p < 0.05$). This increase could be due to the stimulation of cell proliferation in the old seeds irradiated at 0.3 cGy/h ($p < 0.05$) [19]. The ${}^5G$ component is also revealed in the seeds irradiated at 1.2 cGy/h. The ${}^6P$ distribution is observed in the control and 19.1 cGy/h groups, its sample mean being low ($0.08-0.10 \pm 0.03$) (Tabl. 6) [12].

In the heat-stressed seeds, the lowest values of the ${}^5N_G$ and ${}^6N_{G2}$ distributions are observed at 1.2 cGy/h. The ${}^6G2$ distributions are revealed at 0.3 and 19.1 cGy/h; seed survival is decreased at all these dose rates, and especially at 1.2 cGy/h dose rate. In the control group, the ${}^6P$ distribution of cells on the number of DNA damages is characterized by an increased sample mean (0.66 ± 0.12). All the mentioned above could mean the increased late processes in all the groups of the seeds induced by heat stress. The comparison of the "old" and "heat" groups has shown that heat stress induces late processes accompanied by selection stronger than the processes produced by aging.

To analyze quantitatively the combined effects induced by irradiation and heat, we used a synergism coefficient (Fig. 2) offered by Petin *et al.* [54]. These authors characterize the appearance of synergic lethal damages as $N_x = N_1 + N_2 + \min\{p_1N_1 + p_2N_2\}$, where N_1 , N_2 , and N_x are the numbers of the lethal damages induced by the first, second, and combined treatments; p_1 and p_2 are sublethal damages produced simultaneously. Then, the coefficient of synergism can be calculated as $K_{syn} = (N_1 + N_2 + \min\{p_1N_1 + p_2N_2\}) / (N_1 + N_2)$. We calculated the synergism values using Tabl. 5, 6, and data on the numbers of cells with CAs reported earlier [12]. In the "heat" groups,

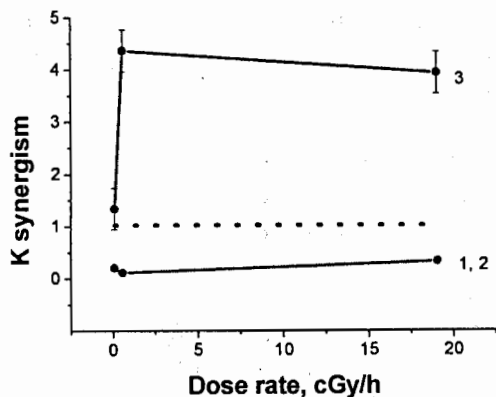


Figure 2. Synergism coefficients of the appearance of cells with CAs (1); CAs inside cells (2); as well as of seeds' non-survival (3) for group of irradiated heat-stressed seeds. Bottom of synergism is marked with a dotted line. Std. errors are shown.

both numbers of combined DNA damages and combined damaged cells decrease ($K_{syn} < 1$) (Fig. 2, pl. 1, 2) that can be the result of elimination of "G2"- cells and "G"- seeds (Tabl. 6). Synergic characteristic of seeds' death (1 - S) increases strongly ($K_{syn} = 4$) (Fig. 2, pl. 3). We can conclude that adaptation to the combined effect of high temperature and low-dose irradiation is based on instability processes which lead to a dramatic synergistic death of cells and seeds.

3.6. SCHEME OF THE ADAPTATION MODEL

The adaptive process has three components [12, 13]: primary radiation injury, which depends on the intensity of radiation (I_{rad}); late injury, which depends on the intensity of intercellular "bystander" (I_{byst}) and intracellular regulatory (I_{reg}) mechanisms; and selection, which depends on repair systems and environment conditions (Fig. 3).

These components can be presented by different combinations of Poisson and

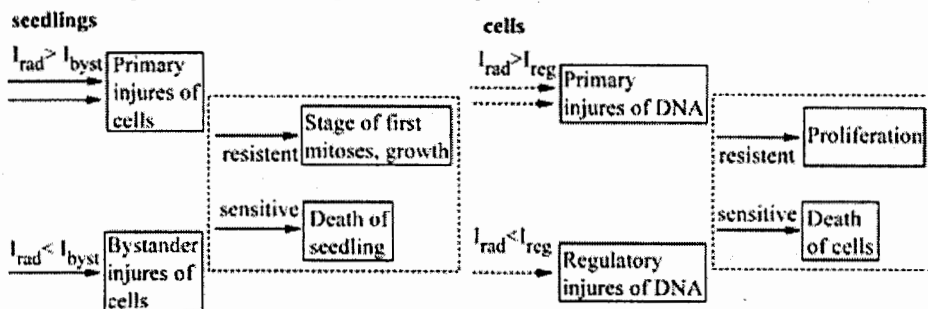


Figure 3. Scheme of the process of seedlings' and cells' adaptation [22]. Primary injury (I_{rad}) induces the intercellular bystander (I_{byst}) and intracellular regulatory (I_{reg}) processes. Primary injuries or late damages can be accumulated. As a result of selection, a stage of first mitoses is reached in resistant seedlings, the sensitive ones die. Intracellular processes (primary or regulatory damaging of DNA) lead to proliferation or death of cells.

geometric laws where P statistics describes the statistics of independent events [12, 13, 19] and G statistics characterizes statistics after selection [8, 10 - 13, 19]. The primary injury and late damage follow the Poisson or binomial law [48]. In both cases, the independently occurring damages can be approximated as increasing linearly with time, that is, by the Poisson law with the increased sample mean [12].

In practice, the observed P distribution can be a sum of P distributions on the numbers of primary and late damages. The first distribution dominates if radiation intensity exceeds the mechanisms of the late process [13]; its sample mean is therefore low [12]. For example, a dose rate of 19.1 cGy/h (time between two hits per cell = 5 sec) induces hits in cells in the first 2 min with the averaged number of hits per cell ≈ 33 . Tabl. 6 shows that this case corresponds to the lowest sample mean of the ^smP distribution of seedlings on the number of damaged cells (0.85 ± 0.20). Irradiation at dose rates of 0.3 and 1.2 cGy/h (time between two hits = 5 and 1.3 min, respectively) induces bystander effects which should prevail in these cases. The complete analysis of the pea data (Tabl. 6) reveals the correspondence of low ^smP values to low ^cmP values, which couple with high damage factor. The increased values of ^smP , ^cmP indicate late processes (for example, the control heat-stressed group, Tabl. 6).

Thus, in experiments on pea seeds, the distribution of seedlings on the number of cells with CAs can be approximated by $^s\text{P} + ^s\text{G}$, where the ^sP distribution corresponds to the subpopulation of seedlings in which the bystander effect is not accompanied by the

death of plants, and ^3G describes the bystander damage accompanied by seedling selection in the more sensitive fraction [12, 19]¹. A distribution of cells on the number of CAs can be presented as $^cG1$, $^cG2$, and ^cP distributions and their combination (Tabl. 5), [22]. The ^cP distributions mean primary DNA damage which, together with $^cG2$ distributions, describes the appearance of DNA damage in the sensitive fraction of cells. The appearance of the adaptive process in the resistant fraction of cells is described by the $^cG1$ distribution. When primary exposure intensity exceeds intracellular regulatory effects the Poisson law displaces the G-one [22].

4. ADAPTATION AND THE RISK OF GENOME INSTABILITY IN ECOLOGY. INVESTIGATIONS OF THE INFLUENCE OF NUCLEAR STATION FALLOUTS ON PLANT POPULATIONS

4.1. OBJECTS AND METHODS

Seeds: The plantain seeds (*Plantago major*) were used in the natural experiment. For this kind of seeds, the reported quasi-threshold radiation dose, which corresponds to the inflection of the survival curve from a shoulder to mid-lethal doses, is 10–20 Gy [34]. The plantain populations were located at sites within 80 km of the Balakovo Nuclear Power Plant (NPP) and in Chernobyl trace area (Saratov region), and in the Joint Institute for Nuclear Research (JINR) territory (Moscow region). In 1999 the temperatures during daylight hours reached 30–32° C in the Moscow region and 38–40° C in the Saratov one (it is the extreme for these provinces), and the seeds experienced elevated temperatures during the maturation period in nature [55]. The plantain populations were chosen in similar biotopes, which are located along reservoir banks.

Seeds were collected at the end of August in 1998 and 1999 from 20–30 plants. The seeds were refrigerated until the following April at $T = 3-4^{\circ} \text{C}$ and relative humidity = 13–14%.

Seed sprouting and fixing: Seeds of all populations were germinated on wet filter paper in petri dishes at 23°C until seedling roots reached 3.5 ± 2 mm, the length which corresponds to the first mitoses. Before it the seedling growth is due only to swelling without cell division. Seedlings were fixed in ethyl alcohol and acetic acid (3:1) and stained with acetoorcein. After 13 days the seedlings less than 1.5 mm were scored as non-surviving (1-S) because too few cells reached the first mitosis (the MI in the shoot zone is already known to increase in parallel with the size of the seedling [14, 56]). The first fixation was started when approximately 1/3 part of the whole seeds' population was germinated. After 13 days we stopped the fixation. Prolonging the germination

¹ The cells stimulated to proliferate are involved in the distributions.

period (up to 6 weeks) increases S by only 2-3%; some rootlets occurred to be too small and brown. The methods of seed sprouting and fixing have been described in [14, 15].

Cytogenetic analysis: Ana-telophases were scored for CAs containing chromosome bridges and acentric fragments. **Determination of mitotic activity:** The mitotic activity (MA) of seedling meristem cells was scored as the number of ana-telophases in apical meristem. It is known that this value reflects the MI in meristem [56].

Estimation of antioxidant status: Peeled seeds (1 g) were placed in 200 ml water at 60° C, allowed to cool to room temperature for 1 hour, and filtered. Antioxidant activities were studied with a photochemiluminescence method [57]. This method simulates the oxidation of tryptophan in proteins and the influence of antioxidants on this oxidation. The reporter compound is the dipeptide glycyltryptophan (Gly-Trp). Gly-Trp oxidation is induced using riboflavin as a photosensitizer. In the presence of riboflavin, oxidation occurs either via a radical (peroxide radicals $RO_2^{\cdot}$, or superoxide $O_2^{\cdot-}$) or via singlet oxygen. Riboflavin was at $2 \cdot 10^{-6}$ M, Gly-Trp at $2 \cdot 10^{-4}$ M in Na phosphate buffer (pH 7.4). The Gly-Trp/riboflavin solutions containing various amounts of seed or plant infusion were irradiated for 60 sec with a high-pressure mercury lamp (DRK-120). Light of $\lambda = 436$ nm was selected with a standard set of glass filters and the intensity of the filtered light was $15 \cdot 10^{15}$ quanta/s. A chemiluminescence signal was recorded 15 sec after irradiation was stopped. It was shown that the reciprocal relative

intensity of chemiluminescence I_0/I (where I_0 is a control intensity and I is the intensity in the presence of infusion) could be satisfactorily described as a linear function of the added infusion [58]. This dependence allowed us to find an amount of infusion, which inhibited chemiluminescence by 50% ($C_{1/2}$). The reciprocal value of $C_{1/2}$, linearly proportional to the inhibiting effect, was adopted as a measure of antioxidant status (AOS).

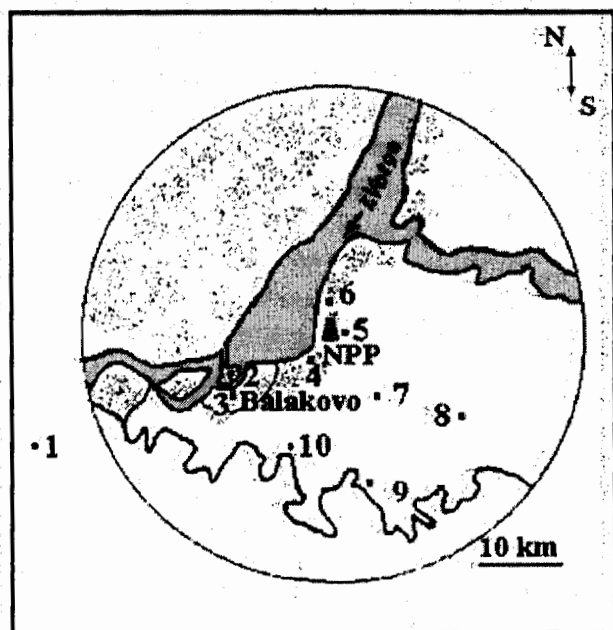


Figure 4. Locations of the selected plantain populations in the vicinity of the Balakovo NPP: 2 (P2); 3 (P3); 4 (P4); 5 (P5); 6 (P6); 7 (P7); 8 (P8); 9 (P9); 10 (P10). Populations P1, P11 are located 80 km and 100 km from NPP; P12 one is placed in the Moscow region.

Characteristics of sites and weather conditions: To investigate the possibility of radiation stress effects, the sites near the NPP (Fig. 4) and in

JINR territory were selected. The suitable site in Chernobyl trace territory was studied to compare the effects of low-dose rate irradiation provided by the atmospheric impact and the chronicle soil pollutions.

In 30-km zone of the NPP two sources of radioactivity are placed, which can influence the plantain populations: the atomic station (P2 – P6 sites) and the phosphogypsum dump (P8 – P10 sites). The sites were chosen in the view of the wind rose. For the most part, the NPP atmospheric fallouts [59] influence on populations P2 – P6 resulting from the direction of the winds in summer. Perhaps the populations P7 – P10 experienced the effects of phosphogypsum dump in this area [55]. The site P1 was on the left bank of the Volga (~80 km from NPP). The population P11 was chosen on the right bank of the Volga (100 km from the NPP) on a Chernobyl radioactivity-deposition track with well-characterized ^{137}Cs soil contaminations (average concentration ~30 Bq/kg, [60]). The Moscow region site P12 was selected within the JINR territory to know the both radiation exposures from the Institute facilities and soil pollution.

For the Saratov region, the annual rainfall near the Volga is 1.5 times higher than in steppe, therefore the microclimate of P1 – P6 sites is damper than that of P7 – P10 [55]. In 1999, the summertime high temperatures in the European part of Russia averaged 3°C above the normal one [55].

Determination of radioactivity and accumulative doses: For sites within a 100 km radius of the NPP, the annual γ -radiation dose rates (DR) and ^{137}Cs soil concentrations (C_{Cs}) varied little from the ranges ~0.10-0.15 $\mu\text{Sv/h}$ and ~5-10 Bq/kg reported in independent radiological surveys [55, 59]. In site P11 DR is ~0.10-0.15 $\mu\text{Sv/h}$ [55] and C_{Cs} is 30 Bq/kg [60]. DR is ~0.10-0.12 $\mu\text{Sv/h}$ and C_{Cs} is ~5-10 Bq/kg in site P12 (Dubna) [35]. These values (excluding the concentrations in site P11) do not exceed the average radiation values over the Saratov and Moscow regions [35, 36, 55].

Table 7. Soil contamination of radionuclides ^{137}C and ^{40}K (Bq/kg)

Radionuclide	Year	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12
^{137}C	1998	9	32	-	5	-	-	10	8	8	6	39	9
	1999	5	4	3	5	5	5	-	5	-	-	15	10
^{40}K	1998	360	330	-	550	-	-	470	450	460	700	450	580
	1999	440	340	400	410	230	500	-	410	-	-	320	600

We examined the upper 10–12 cm soil in the tested sites. Measurements were carried out using the low-background γ -spectrometers with a NaI(Tl) crystal as well as a Ge one, which were described in [35, 61]. The errors of detection efficiency of γ -quanta did not exceed 7%. Total errors of radioactivity determination for different isotopes were 20–40%. The artificial isotope ^{137}Cs soil contamination did not differ significantly in 1998 and 1999 (Tabl. 7), although usually the fluctuations can be observed in the

same site [35]. The data on C_{Cs} agree with published values [35-36, 55, 59-60] and do not correlate with NPP fallout.

The accumulated doses were calculated by using the Brian-Amiro model [37] and estimated of $\sim 1\text{-}3$ cGy for plantain seeds P1 – P6 using the published transfer factors [35]. We accounted the secondary wind rising in P7-P10 populations by means of [62]. The results did not differ significantly from 1998 to 1999 (for accumulated radiation doses and soil concentrations) in the tested sites.

Calculation of the NPP fallout and JINR accelerators' irradiation of seeds:

Plantain seeds were not irradiated in lab, they experienced the NPP fallout irradiation in nature (annual fallouts on isotopes: Kr ~ 2.5 TBq; Xe ~ 2.5 TBq, and I ~ 4.4 TBq [63], the dose rates are controlled by NPP administration).

Distributions of the particulate emissions and gases were estimated according to the Smith-Hosker model [64] based on NPP characteristics [63] and winds in summer near the ground in the NPP region [55]. The isotopes fallouts result in γ -irradiation mainly (mean energy ~ 1.1 MeV/ γ -quanta [65]). The relative DR values were calculated in the ratio to the dose in site P1 (Tabl. 8). The relative DR value in P7 site is higher than in P2, P3, P8 – P10 sites due to short half life of I isotopes, which do not reach the populations P2, P3, P8 – P10. This fallout irradiation is not chronicle and depends on location of populations. Intensity of irradiation is shown in Tabl. 8. We used the γ -quanta' LEP-dependence on their energy [65] and the NPP characteristics [63] to calculate a mean γ -quanta energy deposition per plant cell nucleus, which is 1.4 KeV.

The expected irradiation dose was calculated after the JINR facilities operation in the P12 site. In 1998 the calculated neutron dose level was 1 mSv (for two months), and the neutron dose rate level was $0.8 \mu\text{Sv/h}$ (the neutron background dose rate ~ 9.3 nSv/h [66]); the radiation level over all the particles exceeded the background $\sim$ twofold for

Table 8. Relative daily Balakovo NPP fallout (radioisotopes Kr, Xe, I) dose rates DR, experienced by populations, which were calculated in the ratio to the dose in the site P1

Populations	Relative dose rate DR	Intensity of γ -quanta per cell nucleus per min $\times 10^{-7}$	Intensity of γ -quanta per cell nucleus per 3 months
P1	1	$1.9 \cdot 10^{-4}$	$2.5 \cdot 10^{-6}$
P2	80	$1.7 \cdot 10^{-2}$	$2.2 \cdot 10^{-4}$
P3	80	$1.7 \cdot 10^{-2}$	$2.2 \cdot 10^{-4}$
P4	560	0.11	$1.4 \cdot 10^{-3}$
P5	5700	65	0.85
P6	1350	0.26	$3.4 \cdot 10^{-3}$
P7	340	$6.7 \cdot 10^{-2}$	$8.6 \cdot 10^{-4}$

the dose and $\sim$ eightfold for the dose rate. In 1999, the neutron irradiation did not increase. The averaged neutron energy was 5.5 MeV.

Chemical pollutions:

Chemical pollutions of the atmosphere and soil were published in [15, 55]. The analysis showed no correlations of the concentrations of the chemical pollutions with the NPP fallouts

or biological values [23]. We believe that chemical pollutions cannot be a reason of the biological regularities or imitate them.

Statistics: In the experiment with plantain seeds we used about 200 (1998) and 500 (1999) seeds and analyzed about 400-1000 (1998) and 900-3800 (1999) ana-telophases for each population. The data were processed using standard statistical methods [38] and statistical criteria [26 -28, 39].

4.2. ANALYSIS OF AVERAGED BIOLOGICAL VALUES

Tabl. 9 presents non-survival of seeds and values that characterize processes in seedlings meristems and their cells. For the populations located near the NPP (P2 – P6), the non-survival of seeds grew high (up to 80%) in 1999 in comparison with 1998. The mitotic activity is higher than the same value over all studied populations in 1998 ($p < 0.05$, ~ threefold), and higher than in P11, P12 plants ($p < 0.05$, ~ twofold) in 1999 [20].

Table 9. Antioxidant status and non-survival of seeds, frequencies of both cells with abnormalities in meristem and chromosomes with abnormalities in cells

Site	Number of seeds	Number of ana-telophases	AOS ($C_{1/2}$)	Non-survival 1-S, %	CCAs frequency	CAs frequency	Mitotic activity
1998							
P1	167	726	0.22	10.8	2.5±0.5	0.05	9.7±0.9
P2	152	942	0.16	34.2	3.1±0.8	0.03	6.0±0.6
P4	156	518	0.20	19.9	2.5±0.6	0.04	6.3±0.7
P7	149	763	0.50	13.4	1.3±0.3	0.02	10.9±1.0
P8	148	1047	0.80	31.8	3.2±0.8	0.03	7.8±0.8
P9	167	528	0.76	65.3	4.6±1.4	0.07	6.1±1.0
P10	153	231	0.66	29.4	3.2±0.7	0.05	9.7±3.0
P11	153	342	0.83	55.6	4.4±0.9	0.08	7.3±1.0
P12	148	1805	0.28	12.3	1.2±0.3	0.01	14.9±0.9
1999							
P2	500	2228	0.16	72.6***	3.2±0.4**	0.04	17.8±1.2***
P3	500	3827	0.33	32.6	5.4±0.6	0.06	21.9±1.3
P4	500	1035	0.22	83.6***	6.8±0.9***	0.09	17.5±1.4***
P5	500	2209	0.25	67.8	6.3±0.6	0.07	15.0±0.9
P6	500	2385	0.25	71.6	5.1±0.4	0.07	17.9±1.1
P11	500	2220	0.50	43.6*	5.5±0.5*	0.07	9.8±0.5**
P12	200	832	0.31	37.5***	5.6±0.8***	0.01	8.0±0.6***

Standard error of the CAs frequency does not exceed 0.01.

Comparing 1998 and 1999 data: * $p > 0.1$; ** $p > 0.5$; *** $p < 0.001$.

The correlations of the both frequencies of cells with abnormalities and chromosomes with abnormalities with non-survival of seeds were examined. The correlation between these values could mean the predominance of one mechanism influencing the non-survival of seeds. If such correlation is absent, we can assume that some mechanisms have approximately equal rights. In 1998, a strong correlation of the (1-S) value with both frequencies of cells with chromosomal abnormalities ($|r_{1-S, CCA}| = 0.92$, $df=7$, $p < 0.001$) [20] and the CAs ($|r_{1-S, CA}| = 0.76$, $df=7$, $p < 0.02$) was observed. In 1999, the correlation between the (1-S) value and CCA frequency disappeared ($|r| = 0.02$), and the correlation with CA frequency was non-confident statistically ($|r_{1-S, CA}| = 0.51$, $df=5$). These data indicated that some mechanisms acted in the seeds collected near the NPP in 1999. We can think that observed effects were induced by radiation and heat factors because the radiation factors were the same for the both years and the temperatures were extremely high in all sites in 1999.

The hypothesis of the combined effects of radiation and heat stresses was verified by our laboratory in the experiments on pea seeds [19].

4.3. STATISTICAL MODELING OF ADAPTATION PROCESSES IN SEEDLINGS MERISTEM AND THEIR CELLS

The statistical modeling was used to investigate mechanisms of adaptation and their dependence on irradiation. Tabl. 10 presents the parameters of distributions of seeds on the number of cells with abnormalities and cells on the number of chromosomes with abnormalities in 1998 and 1999. All distributions of seeds include Poisson and geometric components, and the Poisson one predominated in 1998. In 1999, the P-value decreased and the sample mean increased. It was expected because the heat stress increased (1999) the appearance of the reactive oxidative species (ROS) [67], which induced the DNA damages [68] and apoptosis [69]. The ROS increase the instability processes [70] that also results in the decreasing of the P-subpopulations.

In 1998 some populations of cells were geometric-distributed on the number of CAs. Two distributions of cells included the first and the second geometric components (their plantain populations located in high chemical-polluted sites [55]). One cell population was P-distributed (these plants were growing near the accelerator). In 1999, all distributions consisted of two-geometric or geometric plus Poisson components. The Poisson component was observed in the populations growing at the border of the sanitary zone, in Chernobyl trace territory and near the accelerator.

It is interesting that cells' distributions on the number of CAs can be geometric whereas seeds' distributions on the number of CCAs could have a Poisson component. We explain this fact by more intensive irradiation of meristem as the whole than of each separate cell. It suggests that irradiation effects are more pronounced in meristems than in cells.

Table 10. Parameters of distributions of plantain seeds on cells with abnormalities and distributions of meristem cells on chromosomes with abnormalities

Seeds	Distribution of seeds on the number of cells with abnormalities				Distribution of cells on the number of chromosomes with abnormalities					
	^smG	^sG	$^s\text{mP}^*$	P^*	$^c\text{mG1}^*$	$^c\text{G1}^*$	$^c\text{mG2}^*$	$^c\text{G2}^*$	$^c\text{mP}^*$	$^c\text{P}^*$
1998										
P1	1.10±0.05	0.17±0.03	0.22	0.41	0.01	0.42	0.30	0.07		
P2	0.05±0.05	0.10±0.03	0.17	0.45	0.03	0.26				
P4	0.07±0.04	0.06±0.02	0.17	0.64	0.04	0.38				
P7	0.14±0.06	0.27±0.04	0.19	0.38	0.02	0.52				
P8	0.10±0.06	0.07±0.02	0.26	0.39	0.03	0.34				
P9	2.40±0.09	0.01±0.01	0.36	0.19	0.07	0.39	0.28	0.04		
P10	2.30±0.06	0.04±0.02	0.32	0.46	0.03	0.35				
P11	0.70±0.10	0.09±0.02	0.45	0.21	non-confidence statistically data					
P12	0.06±0.04	0.12±0.03	0.21	0.69					0.01	0.9
av.	0.77±0.32	0.10±0.03	0.26	0.42						
1999										
P2	1.05±0.17	0.13±0.01	0.36	0.11	0.02	0.43	0.14	0.12		
P3	1.85±0.13	0.30±0.02	1.20	0.37	0.02	0.40	0.11	0.55		
P4	2.01±0.46	0.02±0.01	1.26	0.12	0.03	0.25			0.53	0.03
P5	0.08±0.31	0.04±0.01	1.20	0.26	0.06	0.50	0.23	0.05		
P6	2.45±0.42	0.04±0.01	0.96	0.21	0.02	0.36			0.22	0.12
P11	0.32±0.13	0.10±0.01	0.84	0.33	0.02	0.36			0.20	0.17
P12	1.81±0.34	0.12±0.02	0.36	0.39	0.02	0.19			0.35	0.03
av.	1.36±0.34	0.11±0.02	0.89	0.26						

* - Standard errors of the parameters do not exceed 20 - 30% (the sample means) and 10-15% (the relative values)

In addition to instability processes in meristems and their cells we present studies of stimulation of proliferation of resting cells, which contributes to adaptation significantly. Analysis of the distribution of seedlings on the number of proliferated cells (PC) has shown a sum of three lognormal distributions $\text{LN1}+\text{LN2}+\text{LN3}$ [12]. Stimulation of proliferation of resting cells is not a compensatory mechanism [43] and the number of PC is an independent value. We assume that the number of cells in a stationary phase can be described as a stationary random branch process. From the above mentioned it follows that cells' mitotic activity can be described by lognormal distribution of cells on the number of PC [12]¹ in a first mitoses phase. This conclusion

¹ The proof has been obtained together with V.B. Priezzhev

is in agreement with lognormal law described by A.N. Kolmogorov [49]. In the case of intensive selection it can be transformed into a geometric distribution [12].

4.4. REGULARITIES OF THE ADAPTATION PROCESSES INDUCED BY NUCLEAR STATION FALLOUTS IN PLANTAIN POPULATIONS

Here, we present data (1999) which demonstrate a strongly synergic adaptive process which leads to a high risk of instabilities. The data calculated for 1998 will be used as a control. The parameters of seed distributions (Fig. 5) and cells' distributions (Fig. 6) are shown as dependent of the seeds' antioxidant status (Fig. 5 A, B; Fig. 6 A, B) [22, 23] and the calculated fallout dose near the NPP (Fig. 5 C, D; Fig. 6 C, D).

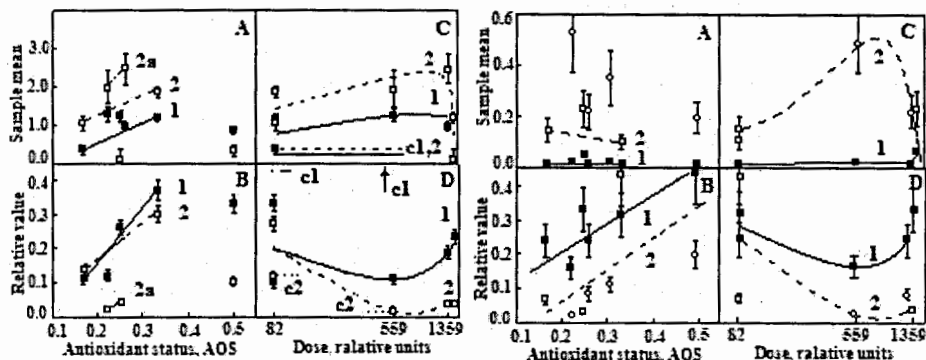


Figure 5. The parameters of ^6P and ^6G distributions of plantain seeds on the number of cells with CAs in root meristem of seedlings [20] versus antioxidant status (A, B) and the calculated relative dose (C, D). The sample mean (A, C) and value (B, D) of the ^6P and ^6G distributions are shown (pl. 1 and 2, respectively). Points with the same dose rate irradiation are connected (A, B). Control (1998) is designed by dotted lines (C 1, 2). The regressions, A: $y = -0.48 + 5.04 \cdot x$ ($p < 0.05$) (1), $y = 0.25 + 4.80 \cdot x$ ($p < 0.05$) (2); $y = -0.92 + 12.96 \cdot x$ ($p < 0.05$) (2a); B: $y = -0.15 + 1.43 \cdot x$ ($p < 0.05$) (1), $y = -0.04 + 1.02 \cdot x$ ($p < 0.05$) (2); $y = -0.10 + 0.53 \cdot x$ ($p < 0.05$) (2a); C: polynomial fits (1, 2); D: polynomial fit (1); $y = 100/x^{1.4} + 0.027$ (2).

Figure 6. The parameters of $^6\text{G1}$ and $^6\text{G2}$, ^6P distributions of plantain meristem cells on the number of CAs [13] for the seeds collected around the NPP versus antioxidant status (A, B) and the calculated relative dose (C, D). The sample mean (A, C) and value (B, D) of the $^6\text{G1}$ and $^6\text{G2}$, ^6P distributions are shown (pl. 1 and 2, respectively). Points with the same dose rate irradiation are connected (A, 1, 2). The regressions, B: $y = 0.73 \cdot x + 0.02$ ($p < 0.05$) (1), $y = 0.77 \cdot x - 0.12$ ($p < 0.05$) (2); C: polynomial fits (1, 2); D: polynomial fit (1); $y = 100/x^{1.4} + 0.027$ (2).

We combined the parameters of the $^6\text{G2}$ and ^6P distributions (Tabl. 10) because they characterize the damage to cells of the sensitive subpopulation. These parameters are correlated with the sensitive ^6G subpopulation ($p < 0.001$, $df = 5$, $n = 7$), which consists of surviving and more resistant seeds of the sensitive subpopulation [22]. Thus, the ^6G subpopulation can be considered as a group at risk of instability which leads to death of seeds.

Values of surviving sensitive subpopulations of seedlings (Fig. 5 D; pl. 2) and cells (Fig. 6 D; pl. 2) decrease with fallout dose rate ($p < 0.1$, $df = 3$, $n = 5$). At the border (558 and 1350) or inside (1500 r.u.³) the 10-km zone, approximately 70–80% of seeds died, and the value of surviving ^sN_G seedlings was about 2–3%. Meristem cells of these survived adapted seedlings consisted of the increased number of late or primary DNA damages ($p < 0.05$) [22] (Fig. 5 C, D; Fig. 6 C, D; pl. 2).

For the seeds collected in different populations of neighboring ecosystems (relative NPP fallout dose rate = 82 r.u.), the parameters of the ^sN_P and ^sN_G distributions (Fig. 5 B, pl. 1, 2) and the $^sN_{G2}$ distribution (Fig. 6 B, pl. 2) differed ($p < 0.05$) depending on the seeds' antioxidant status [22]. The picture shows increasing of the sample mean of ^sG -subpopulation with antioxidant status ($p < 0.05$) (Fig. 5 A, pl. 2).

Dependence of appearance of proliferated cells on dose rate irradiation: The proliferated cells (PC) can appear in the P- and G- subpopulations due to heterogeneity of proliferative pool [42], and the third subpopulation corresponds to the activated resting cells [12]. The analysis of the distribution of seedlings on the number of PC has shown a sum of three lognormal distributions LN1+LN2+LN3 [12].

Parameters of all distributions depend on antioxidant status (Fig. 7 A, B), but the second subpopulation is the most sensitive (Fig. 7 B). Dependence on the relative dose rate is presented in Fig. 7 C, D.

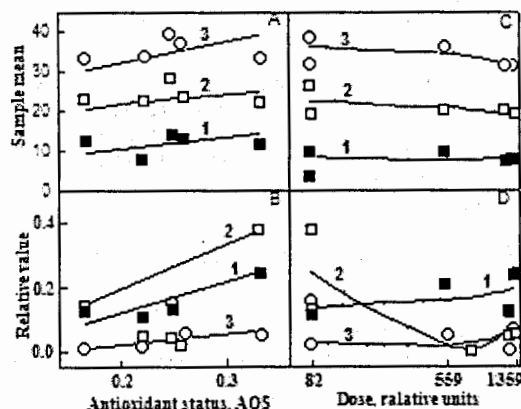


Figure 7. Dependence of sample mean (A, C) and relative value (B, D) of seeds' distributions on the numbers of proliferated cells in meristem of rootlet on antioxidant status (A, B) and relative dose (C, D). Plots of parameters of three lognormal distributions are marked with 1, 2, and 3.

The values of LN1 and P, as well as LN2 and G are correlated: $|r| = 0.83$ ($df = 5$, $p < 0.01$) and $|r| = 0.95$ ($df = 4$, $p < 0.01$), respectively [12]. It is supposed that distributions LN1 and LN2 describe resistant and sensitive subpopulations of the PC. The MI is related with G- and LN3-distributions (homogeneity criterion, $\chi^2 = 2.9$; $df=4$; $p < 0.001$). The value of the LN3-distribution increases significantly ($p < 0.05$) in the sanitary zone of the nuclear station (Fig. 7 D), thereby it corresponds to the

³ Evaluation of this relative dose rate can be less reliable because this population was located ≈ 100 m from the NPP in the shade of the smokestack.

increase of PC and CA numbers (because most of the resting cells are mutant [71]).

4.5. RISK OF CHROMOSOMAL INSTABILITY

At the border of the sanitary zone, the number of abnormal cells was negligible in 1998 (Fig. 5 C, pl. C 1, 2). The higher temperature (1999) increased it up to approximately one cell with abnormalities per meristem in fifty percent of seedlings for the P subpopulation and more than two cells for G-one (Fig. 5 C). It is enough to decrease seeds survival from eighty to twenty percent in P subpopulation (Fig. 5 D). In the resistant fraction, the risk of instability and accumulation of abnormalities can be calculated with P parameter, it is 20%. In the sensitive fraction, the risk of instability is coupled with selection and can be calculated with G-parameters; it is 1-2%. The risk of seeds' death is approximately 80%. Risks of the bystander and chromosomal instability processes increase with the decline of antioxidant status (Fig. 5 C), and increase with the fallouts dose (Fig. 5 D).

5. INSTABILITY PROCESS ACROSS GENERATION. CONSEQUENCES OF NUCLEAR TEST FALLOUTS FOR INHABITANTS

5.1. OBJECTS AND METHODS

Samples of individuals: At the beginning of the 1950s, nuclear tests were conducted in the Novaja Zemlja and Semipalatinsk sites, resulting in radioactive fallouts in the Far North and Siberia, including the Tyumen and Irkutsk regions. Random samples of persons living in settlements Samburg and Maloe Goloustnoe were studied. As a control, a group of individuals from the city of Novosibirsk, whose blood sample (1 ml) contained not less than 100 activated lymphocytes, was investigated. The samples of persons were divided into four groups corresponding to the individuals who were irradiated by fallouts, and their children, grandchildren, and great-grandchildren.

Cytogenetic analysis: Frequency of lymphocytes cells with abnormalities was determined as the ratio of the abnormal lymphocytes number to the proliferated ones. The results of the analyses of chromosomal breakages in blood lymphocytes of the persons living in these settlements are published in [18].

Radiation situation in tested sites: At present, ^{137}Cs contaminations are 153 and 118 Bq/kg (lichen and venison, respectively) in settlements Samburg of Tyumen region [17], and 55 Bq/m² (soil) Maloe Goloustnoe of Irkutsk region [72].

Methods of statistical modeling: In this case distributions of individuals on the occurrence frequency of abnormal lymphocyte cells in the proliferated ones are presented. This problem was investigated especially because both numbers of abnormal

and proliferated cells are independent values. It has been shown that values of frequency distributions can be also used to analyze instability processes [21].

Statistics: Blood samples of 163 (Samburg), 66 (Maloe Goloustnoe) and 40 (control) individuals were investigated.

5.2. STATISTICAL MODELING FOR PERSONS LIVING IN RADIATION - POLLUTED AREAS

To investigate the consequences of irradiation across generations, we analyzed distributions of individuals on the occurrence frequency of lymphocytes with CAs in blood samples of persons living in these settlements and of the control group. Distributions of individuals on the frequency of CAs in blood lymphocytes and approximations of these distributions are presented in Fig. 8.

The types of distributions for persons living in the Tyumen and Irkutsk regions are P, G, and P+G. Analysis of distributions for the parental generation on the frequency of lymphocytes with CAs has revealed a geometric component, whereas those for grandchildren (Irkutsk region) and great-grandchildren (Irkutsk and Tyumen regions) also follow a Poisson distribution. A tendency has been observed towards the decreased sample means of the P distribution across generations, but it remains higher ($3.1 \div 4.2$, $p < 0.05$) than the control value (1.4) (Tabl. 11). This tendency may reflect the death of individuals with small numbers of lymphocytes (these persons are "G distributed" [22]) and more resistant young individuals than the old ones.

5.3. CONSEQUENCES OF NUCLEAR TESTS FOR INHABITANTS FOUND BY STATISTICAL MODELING

We see that the sample mean of P-distribution varies a little, but exceeds significantly the control value. It means that the instability process continues across three generations of irradiated persons (Fig. 8). There are 3 abnormal cells of lymphocytes per blood sample for fifty percent of younger's living in the tested sites. In the control, this result is observed for five percent of individuals.

Distributions for older persons are characterized by G component. This indicates that resistance in persons plays the main role in selection.

The comparison of genome instabilities of the persons living in the Tyumen and Irkutsk regions has shown that the sample means of P distributions for both regions are approximately equal. Distributions of parents' and children's samples from settl. M. Goloustnoe and grandchildren's sample from settl. Samburg, have revealed a G component. Taking into account the absence of lymphocyte activation in many of the Samburg individuals (Tabl. 11), we can assume that the continued radiation effects are caused by the food chain of the North nations' "lichen-reindeer-man".

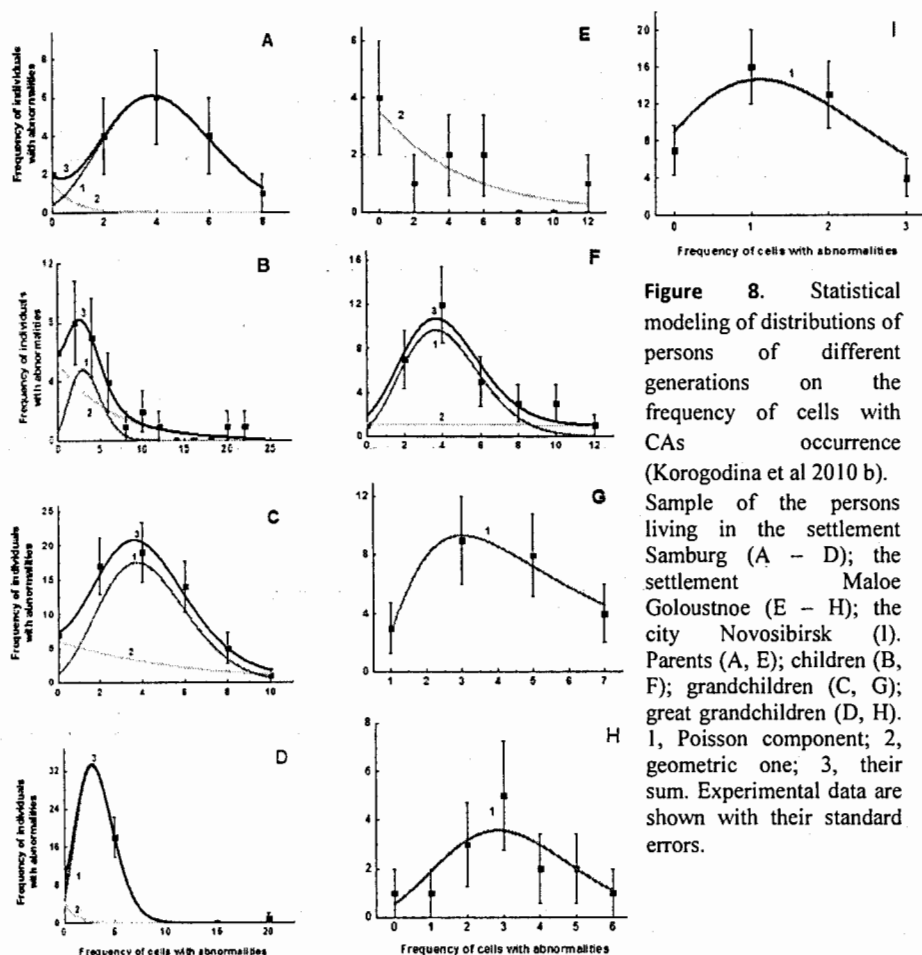


Figure 8. Statistical modeling of distributions of persons of different generations on the frequency of cells with CAs occurrence (Korogodina et al 2010 b). Sample of the persons living in the settlement Samburg (A – D); the settlement Maloe Goloustnoe (E – H); the city Novosibirsk (I). Parents (A, E); children (B, F); grandchildren (C, G); great grandchildren (D, H). 1, Poisson component; 2, geometric one; 3, their sum. Experimental data are shown with their standard errors.

5.4. RISK OF CHROMOSOMAL INSTABILITY FOR PERSONS

Bystander processes in resistant groups of persons result in increasing frequency of lymphocytes with CAs in blood samples, and the risk of these effects can be estimated with P parameters. The frequency of lymphocytes with CAs is 3.2 ± 0.3 for 50% of persons younger than 18 years old, whereas it is 5% in the control group (Tabl. 11). Selection (death of sensitive persons) is related with lymphocyte depletion: persons with lymphocyte deficiencies are those with blood diseases, who have been accounted in the presented statistics. They form a “group of risk” which can be determined by G value. The P and G values have demonstrated that the processes of genome instability and selection prevail in the oldest generation.

Table 11. Frequency of CAs in blood lymphocytes of persons living in Tyumen and Irkutsk regions, and Novosibirsk city, parameters of the most effective model of approximation.

Settl. Samburg, Tyumen region														
Age	N0	N1	Frequency										G, P	
			0	2	4	6	8	10	12	14	16	>18	mG/G	mP/P
50-80	24	7	2	4	6	4	1	0	0	0	0	0	0.2/1.7	4.2/15.4
39-49	36	4	6	8	7	4	1	2	1	0	0	2	5.0/19.3	3.5/11.1
18-38	71	7	7	17	19	14	5	1	0	0	0	0	3.2/18.5	4.2/44.7
Age	N0	N1	Frequency										G, P	
			0	5	10	15	20	0	0	0	0	0	mG/G	mP/P
< 18	29	-	10	18	0	0	1	0	0	0	0	0	-	3.3/28.1
Settl. Maloe Goloustnoe, Irkutsk region														
Age	N0	N2	Frequency										G, P	
			0	mG/G	mG/G	6	8	10	12	14	16	>18	mG/G	mP/P
60-80	10	3	4	1	2	2	0	0	1	0	0	0	3.0/9.9	-
40-60	32	4	1	7	12	5	3	3	1	0	0	0	5.8/7.3	4.2/24.6
Age	N0	N2	Frequency										G, P	
			0	1	2	3	4	5	6	7	8	9	mG/G	mP/P
18-40	23	1	1	1	3	6	4	4	3	1	0	0	-	4.0/23.7
<18	15	-	1	1	3	5	2	2	1	0	0	0	-	3.1/15.3
Novosibirsk														
Age	N0	N3	Frequency										G, P	
			0	1	2	3	4	5	6	7	8	9	mG/G	mP/P
-	40	40	7	16	13	4	0	0	0	0	0	0	-	1.4

The standard error of sample means $d(mP) = 10-15\%$, $d(mG) = 30-40\%$. The control group consisted of persons from Novosibirsk, the number of analyzed lymphocytes exceeding 100 in each blood sample. N0, total number of examined persons; N1, number of persons in whose blood samples the lymphocytes were not activated; N2, number of persons in whose blood samples the number of activated lymphocytes did not exceed 30; N3, the number of persons in whose blood samples the number of analyzed lymphocytes exceeded 100.

6. DISCUSSION

Non-linearity induced by low -dose- rate irradiation: The dead fraction of seeds was increased by even a single oxidizing factor but especially under the combined effect of multiple oxidizing factors (Fig. 1). In all groups of seeds, value $(1 - S)$ was non-linear at the interval 0-19.1 cGy/h irradiation and increased significantly at a dose rate of 0.3 cGy/h (Tabl. 1). The non-linear dependence of cells with CAs number was also observed at this interval (Fig. 1). It is clear that stress induces "system replay", which is necessary to fit the organisms in their ecological niche. The adaptation strategy of

survival differs from the strategy induced by the middle-lethal impact which is based on the post-radiation recovery: irradiation – number of CAs – non-survival [73].

The statistical modeling of cells with CAs appearance clarifies the stress-induced non-linearity. The simulation of CAs justifies the hypothesis that there are two subpopulations of seeds. In the first subpopulation, the CAs appear independently and are Poisson-distributed. In the second, the appearances of CAs are correlated. The simulation supported both Poisson and geometric mechanisms in meristems, which contribute to the seed survival (Tabl. 2). In the dose-rate interval of 0.3–1.2 cGy/h, the ^5mG values tended to increase ($p < 0.05$), whereas ^5mP ones did not change significantly ($p > 0.05$) in comparison with the control. The $(1 - S)$ value increased due to the elimination of cells and the failure of seeds to germinate in the geometric (particularly the Poisson) subpopulation (Tabl. 2). We can conclude that mechanisms induced by stress conditions regulate the number of proliferating and abnormal cells. We would like to speculate that a stress factor induces the bystander mechanism coupled with selection (filtration) to adapt a population in its ecological niche. Adaptation includes increasing variability (quickly elevating the sample mean ^5mG) and dramatic decreasing the number of germinated seeds. N.W. Timofeeff-Ressovsky (1939) emphasized two requirements of evolution: sharp increasing of the evolution material coupled with partial death of a population because a new genotype would get an advantage only in this case [74].

Statistical model of adaptation: Genetic instability has long been considered as material for evolution [3, 5, 75]. Appearance of multiple mutations in blood lymphocytes of workers in radiochemical enterprises was analyzed by N.P. Bochkov *et al.* [9] and A.N. Chebotarev [11], who first used statistical modeling. This statistical approach was developed by R. Arutyunian *et al.* who showed appearance of multiple CAs in blood lymphocytes of patients with a syndrome of chromosomal instability, which can be presented as G- and P-distributions [10]. These three authors expressed an idea based on the queues theory and related these distribution tails with DNA repair [9]. The investigations and modeling of fitness distributions were provided by R.A. Fisher [6], J.H. Gillespie [7], and H.A. Orr [8]. Orr showed the universal character of distributions with tails in evolutionary models and the complementation of this phenomenon with selection [8]. In Russia, V.I. Korogodin and his colleagues provided a series of investigations in the 1970s which showed the connection of instability with summarized chromosomal aberrations [3] induced by low dose irradiation or non-optimal conditions [4]. The above presented model of adaptation is founded on the time-dependent accumulation of abnormalities and time-dependent selection based on repair filtering [12, 13]. Its parameters indicate selection (relative value of distributions) and intensity of damaging processes (sample mean of distribution).

Influence of irradiation dose rate on instability and selection: Investigations on pea seeds have shown that the parameters of the ^5G and $^{\circ}\text{G}_2$ distributions change at 0.3–1.2 cGy/h in comparison with the control group: the sample mean increases and the

relative value decreases. The P component is displayed at the higher dose rate (19.1 cGy/h) whose sample mean was low (Tabl. 6). We can observe the increased late processes with the low-dose-rate irradiation, which are displaced then by intensive primary damage to cells and DNA. Investigations on plantain seeds have shown that the parameters ^smG , both $^c\text{mG2}$, ^cmP increase with radiation intensity and then fall back (Fig. 5 C; Fig. 6 C, pl. 2). This fall of the sample mean is coupled with dramatic diminution of the relative values of $^s\text{N}_G$, both $^c\text{N}_{G2}$, and $^c\text{N}_P$ at high-dose-rate irradiation (Fig. 5 D; Fig. 6 D, pl. 2). Thus, the intensities of both late processes and selection depend on the irradiation dose rate. Intensive primary damage becomes dominant under the late process in the $^c\text{G2}$ subpopulation when $I_{\text{rad}} > I_{\text{reg}}$.

Similar conclusion results from the statistical modeling of CAs in blood lymphocytes of inhabitants and workers of radiochemical enterprises based on the data published in [9] (Tabl. 12). For the "inhabitants" and "workers irradiated with 0.18 ± 0.37 Sv" the risks of chromosomal instability in blood lymphocytes are 4% and 7%, respectively; the averaged number of CAs per cell is about 1. We can conclude that radiation effects are equal for these two groups of persons because the characteristics of the resistant as well as of the sensitive groups are almost identical. For the third group of persons, 97% of cells have primary damages with an averaged number per cell of about 0.04.

Table 12. Statistical modeling on blood lymphocytes of people who lived in t. Seversk and workers in its radiochemical industry

Group	Modeling	G1	mG1	G2	mG2	P	mP
Inhabitants	G1+G2	0.96	0.03	0.04	1.17	-	-
Workers, 0.18±0.37 Sv	G1+G2	0.92	0.03	0.07	0.82	-	-
Workers, 0.93±1.57 Sv	G1+P	0.03	1.49	-	-	0.97	0.04

Standard errors of parameters did not exceed 10-15%. Data on chromosomal aberrations are published in [9]. The hypotheses "P" and "G" do not satisfy the criterion χ^2 ($p > 0.05$).

Dependence of relative values of P and G distributions on antioxidant status: In plantain investigations the dependence of relative values of P- and G- distributions on antioxidant status has been revealed (Fig. 5 B, pl. 1, 2). This supposes that antioxidants influence selection is related with radioprotection in plants; this conclusion agrees with the results of investigations published in [76].

Dependence of sample mean of distributions on antioxidant status: The sample mean of ^sG -subpopulation depends on antioxidant status ($p < 0.05$) (Fig. 5 A, pl. 2). This result agrees with investigations which showed the involvement of energy metabolism in the production of bystander effects by radiation [77]. No influence of

antioxidant status on the sample means $^{\circ}\text{mG1}$, $^{\circ}\text{mG2}$ and $^{\circ}\text{mP}$ was revealed in these plantain studies (Fig. 6 A), perhaps, because the sample sizes were not sufficient. The hypothesis has been made that the phenomenon of hypersensitivity can be a result of oxidative stress [78]. The authors of this paper have investigated effects of low-dose radiation on human blood lymphocytes within the range from 1 to 50-70 cGy, that is higher than around the NPP. In our calculations, each seedling's meristem experiences the influence of even one g-quantum per 3 months in the 20-km NPP zone [13]. We suppose that multiple DNA damages could appear due to the affected mass cellular structures. This conclusion agrees with the opinion of C. Mothersill and C. Seymour concerning the role of epigenetic factors in low-dose effects [79].

Involvement of stimulation of proliferation in adaptation process: The mathematical analysis has shown that proliferated cells' pool consists of three subpopulations: two of them represent heterogeneity of cells, - resistant plus sensitive fractions, and the third one -the resting cells stimulated to proliferation. The first and second fractions are identical with $^{\circ}\text{P}$ - and $^{\circ}\text{G}$ -subpopulations of cells. The resting cells are often abnormal; therefore the third subpopulation can be G-distributed in the first place [12]. So, stimulation of proliferation has a double mission: increasing of cells' pool and involvement of new genetic material for selection.

Continued adaptation across generations: The induction of genome instability by low-dose irradiation is well documented [80]. The studies of genetic instability across generations of low-dose-rate irradiated mice which indicated the genetic instability in the F1, F2, and F3 generations from the irradiated males were published in [81]. For persons living in settlements Samburg and Maloe Goloustnoe the mathematical analysis has shown chromosomal instability which continues across three generations of irradiated individuals, because the mP values of distributions of youngsters' samples have increased (Tabl. 11; Fig. 8). Model characteristics indicate the significance of resistance in individuals (youngsters' samples) which plays the main role in selection in blood lymphocytes: samples of older individuals are characterized by the geometric distribution on the number of lymphocytes with CAs (Fig. 8) and on lymphocyte depletion (Tabl. 11). Finally, it has been found that the intensity of bystander processes and selection are higher in settlement Samburg, and this can be related with longer irradiation due to the food chain of "lichen-reindeer-man". All the mentioned above should be considered as components of the adaptive process.

What γ -quanta can induce bystander effects? In our laboratory studies, irradiation with 0.3 cGy/h (intensity ~ 1 γ -quanta/cell nucleus/min) is most effective to induce bystander effects [19]. On the other hand, the number of primary Poisson damages (mP) should be minimal at this dose rate. A probability of double hits/nucleus/min would increase with the dose rate, whereas bystander effect decreases significantly [19]. It means that one γ -quanta can induce the bystander effect.

In natural experiments, γ -rays are mostly a part of irradiation near the NPP; their intensity is ~ 1 γ -quanta/nucleus/3 months in immediate proximity to NPP (P5). It means

that each nucleus could be damaged in the vegetation period. In P4, P6 populations (the border of the sanitary zone), the number of NPP fallout γ -quanta is 10^{-3} times lower. In all these populations, the bystander effect was observed significantly. It seems not too incredible because the modern investigations have shown that irradiation comparable with the background induces changes in cellular membranes [82]. Many researchers relate the bystander effect with the appearance of pores in cellular membranes [82].

Calculation of risks of instability in ecology and epidemiology: The risk of bystander effects coupled or not by selection processes, can be calculated by means of the parameters of G and P distributions. The risks of chromosomal instability can be determined with values of the G distribution of cells on the CAs number. A P distribution with a low mP value indicates primary abnormalities, but the increasing mP value indicates reduction of late processes [12].

7. CONCLUSION

Effects of low-dose-rate irradiation can be described by means of the model of adaptation. A statistical model of adaptation has been developed on the concepts of time-dependent accumulation of abnormalities and time-dependent selection based on repair filtering. Its parameters indicate intensity of damaging processes (sample mean of distribution) and selection (relative value of distributions).

Low-dose-rate irradiation increases sample means and decreases relative values of G distributions, signs of late damage processes and selection. Relative values of P and G distributions increased with antioxidant status, indicative of an inverse relation between selection and antioxidant status. Investigations on plantain seeds have shown the influence of antioxidant status on sample means of P and G distributions of seedlings on the number of cells with abnormalities, assuming involvement of oxidants in bystander mechanisms.

Synergism of irradiation and heat stresses is a result of late damage processes which leads to decreasing numbers of CAs and cells with CAs ($K_{syn} < 1$) and then - to the death of seedlings ($K_{syn} = 4$).

In old seeds, the statistical modeling has revealed P distributions on the number of CAs characterized by low sample means in control seeds and the seeds irradiated at 19.1 cGy/h. This result means that these P distributions are formed by cells with primary damages involving strong mutagenic effects of aging and irradiation at 19.1 cGy/h.

Statistical modeling on the basis of investigations of blood lymphocytes of samples of four generations of people living in settl. Samburg (Tyumen region) and settl. Maloe Goloustnoe (Irkutsk region) has shown increasing sample means of P distributions of youngsters' samples of settl. Samburg and M. Goloustnoe over the control level. This

result has confirmed bystander processes in blood lymphocytes. Age, region and national traditions are also important factors in radiation-induced adaptive processes.

The model parameters can be used to calculate risks of adaptive and instability processes. The risk of the bystander effect in tissues coupled or not with selection processes, can be calculated with the parameters of G and P distributions. Risks of chromosomal instability can be estimated with values of the G distribution of cells on CAs numbers. A P distribution with a low mP value indicates primary abnormalities, but the increasing mP value means reduction of late processes.

Acknowledgements

The authors would like to express their gratitude to V.B. Priezzhev and G.A. Ososkov for their consultations on the mathematics and statistics. We thank Yu.A. Kutlakhmedov, R.M. Aroutyunian, and V.G. Petin for helpful discussions and comments on the manuscript. The authors are grateful to J.W. Drake for his friendly support and consideration of our investigations of mutation processes and evolution.

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EFFECTS OF BYSTANDER FACTORS INDUCED IN VIVO IN BLOOD OF PEOPLE AFFECTED BY CHERNOBYL ACCIDENT*

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Abstract. The purpose of this work was the analysis of the effects of bystander factors from blood sera of people affected by the Chernobyl accident on human keratinocyte cell culture (HPV-G cells). A new method was developed for evaluation of bystander factor presence *in vivo* in blood of people irradiated by the Chernobyl accident. Affected population groups included liquidators of the Chernobyl accident and people living and working in areas of Gomel region contaminated by radionuclides. The analysis has shown that bystander factors persist in Chernobyl liquidator blood samples for more than 20 years since irradiation. The data suggest that blood sera contain bystander factors, which are able to induce micronuclei and decrease metabolic activity of HPV-G cells.

Keywords: Bystander effect, Chernobyl, liquidators, keratinocytes, micronuclei, alamar blue, melanin, melatonin

* * Radiobiology and Environmental Security / Eds. C. Mothersill, V. Korogodina, C. Seymour. Springer, 2012. P. 177-186

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1. Introduction

For a long time it was considered that genetic effects of irradiation are related only to direct DNA damage. But a lot of evidence suggests the existence of a phenomenon of information transfer from irradiated cells to non-irradiated (so-called radiation induced bystander effect, RIBE) (Nagasawa and Little, 1992; Mothersill and Seymour, 1997). Such bystander factors could be induced not only by ionizing radiation, but also observed under different pathological conditions (Emerit, 1994). The specific nature of these factors and mechanism of RIBE are still unknown (Baverstock and Belyakov, 2010).

In recent years some evidence of RIBE induced *in vitro* was obtained, while the effects *in vivo* are not enough studied.

Effects of bystander factors induced *in vivo* in blood of people affected by the Chernobyl accident are of particular interest.

For the first time such studies were performed by Dr. I. Emerit (Emerit, 1990), when it was shown that blood sera from liquidators of the Chernobyl accident or residents of areas contaminated by radionuclides contained an increased level of bystander factors. These factors increased the frequency of chromosome aberrations and micronuclei in own lymphocytes of serum donors even decades since irradiation.

In Emerit studies own lymphocytes of serum donors were used as a test-system. But these cells were already exposed to ionizing radiation, as a result, the level of chromosome aberrations and micronuclei was increased there. At the same time, human peripheral blood lymphocytes under *in vitro* conditions could be subcultured only for 2-4 days, therefore it is impossible to evaluate serum effects for a longer period.

In the present study a new method was developed which allows evaluation of the effects of bystander factors from sera of different groups of populations on immortalized culture of human keratinocytes. This method has serious advantages as compared to that which was used earlier: cells could be cultivated for a longer period of time (re-cultivation once a week), they are more sensitive as compared to human peripheral blood lymphocytes, experiments could be easily repeated, allowing simple comparison and interpretation of the obtained results.

Using this method, we evaluated the level of damaging bystander factors in blood of areas irradiated by the Chernobyl accident.

The study of the nature and possibility of modification of bystander factors, circulating in blood serum of populations, affected by the Chernobyl accident, will facilitate better understanding of radiation damage mechanisms. Knowledge of these mechanisms is increasingly important for cancer radiation therapy –for using special correcting coefficients taking into consideration effects of bystander factors. The anti-tumor therapy could be more effective and safe. Revealing the nature of the damaging bystander factors will promote more exact dose evaluation and radiation risk for people, exposed to ionizing radiation, as the risk of radiation exposure at low doses, calculated

using direct and bystander effects, may be higher as compared to that calculated only from direct effects. The ability to neutralize and modify these factors may help to decrease significantly the effects of whole body radiation exposure.

2. Materials and Methods

2.1. CELL CULTURE

In the present study HPV-G cells (human keratinocytes, immortalized by human papilloma virus transfection), deficient in p53 were used as a test system. Cells were cultured in Dulbecco's MEM: F12 (1:1) medium supplemented with 10% Foetal bovine serum, 1% penicillin-streptomycin (1g per 100 ml), 1% L-glutamine and 1 µg/ml hydrocortisone. The cells were maintained in an incubator at 37 degrees centigrade, with 95% humidity and 5% carbon dioxide and routinely subcultured every 8-10 days.

2.2. AFFECTED POPULATIONS

The objects of investigation were blood sera from people affected by the Chernobyl accident: Chernobyl liquidators of 1986-1987 (22 persons), Polesky State Radiation Environmental Reserve workers⁵ (PSRER, 21 persons) and people, living in areas of Gomel region (GR) contaminated by radionuclides (15 persons). The analysed groups also included 4 persons with acute virus infection (flu) living in areas contaminated by radionuclides. The control group included clinically healthy people from non-contaminated areas, corresponding to the main group in age and sex (36 persons).

2.3. SERUM EXTRACTION

The blood samples were taken and placed in Vacutainers for serum extraction (Becton Dickinson, USA), centrifuged at 2000g for 10 minutes, and the serum was frozen and stored at -20°C before use. Before freezing, the sera were filtered through 0,22 µm filters (Nalgene, USA) in order to remove all residual cell components of the blood.

⁵ Polesky State Radiation and Environment Reserve (founded in 1988) is a territory of Gomel region, where humans cannot live because of the very high levels of radiation contamination. The territory of this reservation is 215.5 hectares.

2.4. RADIOPROTECTIVE SUBSTANCES

Antioxidant substances melanin and melatonin were used as radioprotectors. Melanin (Belarusian pharmaceutical association, Minsk) was used at 10 mg/l concentration, melatonin (Sigma, Germany) – at 10 mg/ml.

2.5. MICRONUCLEUS ASSAY

Micronuclei assay. After plating, cells were left at 37°C in the CO₂ incubator to be attached for 12 hours. The blood serum from affected populations was placed into 25 cm² flasks (NUNC, USA) (6000 cells per flask) 1-2 days after plating, and cells were placed again in the incubator for 1-2 h. Then cytochalasin B was added (7 µg/ml concentration) and the cells were incubated for 24 hours. The cell culture medium was removed, the cells were washed with PBS and fixed with chilled Karnua solution (1 part of glacial acetic acid and 3 parts of methanol, 10-15 ml 3 times for 10-20 min). Later flasks were dried and stained with 10% Giemsa solution. The micronuclei count was carried out under inverted microscope. The data are presented as the micronuclei (cells with micronuclei) frequency recorded per 1000 binucleated cells ± standard error.

2.6. ALAMAR BLUE ASSAY

Alamar blue (AB) assay. HPV-G cells were plated on 96-well microplates (NUNC, USA) at the concentration of 2×10^4 cells/well. After plating, cells were incubated for 24 hours to allow attachment to the bottom of the well. Then the medium was removed, cells were rinsed with phosphate buffered saline (PBS) and the blood serum from the Chernobyl accident populations was added to the cells together with melanin and melatonin as appropriate. Microplates were placed into the incubators. Twenty-four hours later, serum was removed, cells were rinsed with PBS and 100 µl of a 5% solution of Alamar Blue prepared in phenol red free DMEM media were added. Microplates were placed again into the incubators. Three hours later, fluorescence was quantified using a microplate reader (TECAN GENios, Grödig, Austria) at the respective excitation and emission wavelength of 540 and 595 nm, respectively. Wells containing medium and Alamar Blue without cells were used as blanks. The mean fluorescent units for the 3 replicate cultures for each exposure treatment were calculated and the mean blank value was subtracted from these results.

2.7. STATISTICAL ANALYSIS

All experiments were repeated at least three times, and within each experiment cultures were set up in triplicate. Results are expressed as the means ± standard errors.

2.7.1. *t*-test

When the distribution was normal, significance was determined using the *t* test. The level of significance was chosen as 95%; at $t \geq 2.67$, the difference is highly significant at $p < 0.01$, at $t \geq 1.96$, the difference is significant at $p < 0.05$. At $p > 0.05$, the difference is not significant.

2.7.2. Mann-Whitney U-test

The Mann-Whitney U test is a nonparametric alternative to the *t*-test for independent samples and was calculated using STATISTICA 8.0 (Statsoft, USA). The U statistics is accompanied by a *z* value (normal distribution variate value), and the respective *p*-value.

3. Results

3.1. STUDY OF EFFECTS OF BLOOD SERA FROM PEOPLE, AFFECTED BY THE CHERNOBYL ACCIDENT ON MICRONUCLEI FREQUENCY IN HPV-G CELLS

Table 1 presents data on the effects of blood serum samples from different population groups on the total micronuclei frequency in HPV-G cells (average data presented).

The micronuclei frequency in the controls indicates the level of spontaneous mutagenesis (it is comparatively low). The data from Table 1 show that the number of the cells with 2 and especially 3 micronuclei is very low as compared with the number of cells with one micronucleus.

As seen from Table 1, people exposed to chronic radiation (PSRER workers) have an increased level of bystander factors, expressed as a considerable increase in micronuclei frequency (almost 3 times as high as the control level – $248.03\% \pm 20.77$ as compared with $80.30\% \pm 13.14$, $p < 0.01$).

At the same time an increase in the number of the cells with more than one micronucleus was observed. Thus, the data clearly indicate that intensive chronic irradiation of PSRER workers significantly promoted accumulation of bystander factors in blood.

Similar results were observed after comparative analysis of the micronuclei frequency between the control group and the liquidators group (people exposed to acute radiation). The total micronuclei frequency of $273.7\% \pm 22.4$ and the frequency of cells with micronuclei of $235.6\% \pm 14.0$ in cells treated with serum from liquidators were significantly higher than those in the control group (in both cases $p > 0.01$). Also, an increase in the number of cells with more than one micronucleus was observed.

The level of micronucleus frequency induced by serum samples from the residents of contaminated areas of Gomel region is statistically significantly different from the control ($156.47\% \pm 11.22$ vs. $80.30\% \pm 13.14$ $p < 0.01$), but much lower than in people,

exposed to additional radiation influence (as compared to liquidators and PSRER workers in all cases $p<0.01$).

TABLE 1. The effects of blood serum samples from different groups of population on HPV-G micronuclei (MN) frequency (average data are presented)

	Frequency of cells with MN, ‰			Total number of cells with MN, ‰	Total MN frequency, ‰
	1 MN	2 MN	3 MN		
control	69,5±11,1	5,0±1,1	0,3±0,1	74,8±12,4	80,3±13,1
liquidators	200,6±12,2	30,6±2,6	4,6±1,1	235,6±14,0*†	273,7±22,4*†
PSRER workers	196,5±10,9	18,8±3,8	4,4±1,1	219,7±18,3*†	248,0±20,8*†
residents of GR	130,5±7,7	11,8±1,8	0,8±0,3	143,1±9,3*	156,5±11,2*
residents of GR with acute virus infection	190,2±6,6	87,7±4,8	23,3±2,3	301,2±7,8*†	435,6±8,4*†

* $p<0,01$ (compared to controls); † $p<0,01$ (compared to GR residents)

At the same time, the level of the micronucleus frequency induced by serum samples from the residents with acute virus infection at the active stage is higher than in all previous cases – the micronucleus frequency induced by the serum from these patients is $435.6‰\pm 8.4$, and the number of cells with micronuclei is $301.2‰\pm 7.8$. These figures are much higher than those for liquidators and PSRER workers (in both cases $p<0.01$).

3.2. STUDY OF THE EFFECTS OF BLOOD SERA FROM POPULATION GROUPS, AFFECTED BY THE CHERNOBYL ACCIDENT ON METABOLIC ACTIVITY OF HPV-G CELLS

The study was run to understand if there were any bystander factors observed in serum samples of these population groups which could affect cell metabolic activity. The blood serum samples for Alamar Blue analysis were taken from Chernobyl liquidators and residents of contaminated areas of Gomel region.

Figure 1 presents the average data for all these groups (metabolic activity of intact cells is taken as 100%). The viability of the cells treated with serum samples from non-irradiated individuals is very close to intact levels ($t=0.33$, $p<0.01$ – the difference is not significant). It means that the metabolic activity of these cells is not damaged by serum samples from healthy people, not increasing or decreasing significantly the viability of HPV-G cells.

Treatment of the cells with serum samples from Chernobyl liquidators clearly reduces the viability of HPV-G cells more than 1.5 times – from $24.89\pm 0.25 \times 10^3$ FU (intact cells) and $24.67\pm 0.62 \times 10^3$ FU (non-irradiated individuals) to $15.65\pm 0.82 \times 10^3$

FU (liquidators); $p < 0.01$ in both cases ($t = 10.79$ and 8.77 , respectively). Treatment of HPV-G cells with serum samples from residents of contaminated areas of Gomel region also reduces the viability of cells ($19.16 \pm 0.71 \times 10^3$ FU, $t = 7.62$ compared to intact cells, $p < 0.01$), but not as significantly as sera from liquidators.

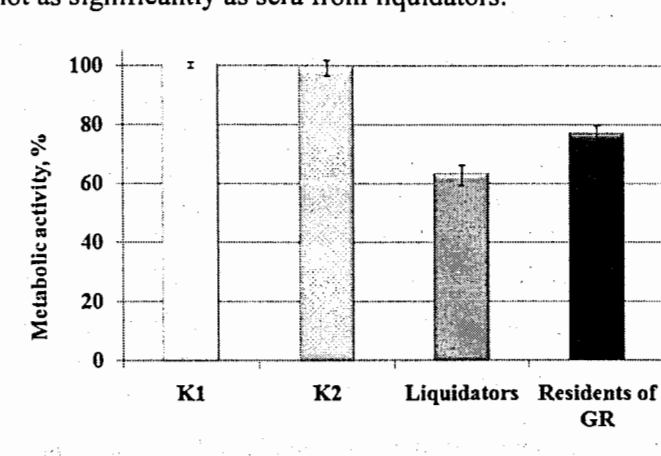


Figure 1. Cytotoxic effects of serum samples from the control groups, Chernobyl liquidators and residents of GR on metabolic activity of HPV-G cells (as a percentage of intact cells, average data for all groups of populations are presented).

Table 2 presents the results of the comparison between all groups using the Mann-Whitney U-test.

The analysis of differences between all groups showed that there was statistically significant difference (with at least $p < 0.005$) between all groups excluding intact cells vs. non-irradiated population group ($p > 0.05$). The highest difference was observed between liquidators vs. intact cells.

TABLE 2. Comparative analysis of 4 independent groups (intact cells K₁, non-irradiated individuals K₂, liquidators and residents of GR) using Mann-Whitney U-test.

Groups of comparison	Z	p
K1 vs. K2	0,39	>0,6
K1 vs. Liquidators	3,92*	<0,00001
K1 vs. GR residents	3,40*	<0,001
K2 vs. Liquidators	3,54*	<0,0005
K2 vs. GR residents	3,07*	<0,005
Liquidators vs. GR residents	3,03*	<0,005

* statistically significant at $p < 0,01$

3.3. STUDY OF THE EFFECTS OF ANTIOXIDANT SUBSTANCES ON BYSTANDER FACTORS

In previous studies (Mosse *et al.*, 2006) we have shown that bystander effect when induced *in vitro* by transfer of culture medium from irradiated cells to non-irradiated could be decreased using radioprotectors with antioxidant activity (melanin, melatonin). In the present investigation we have studied the possibility of these substances to neutralize bystander factors, induced *in vivo*.

Figure 2 presents the results of an attempt to modify the effects of blood serum samples from Chernobyl liquidators on metabolic activity of HPV-G cells using melanin and melatonin (average data).

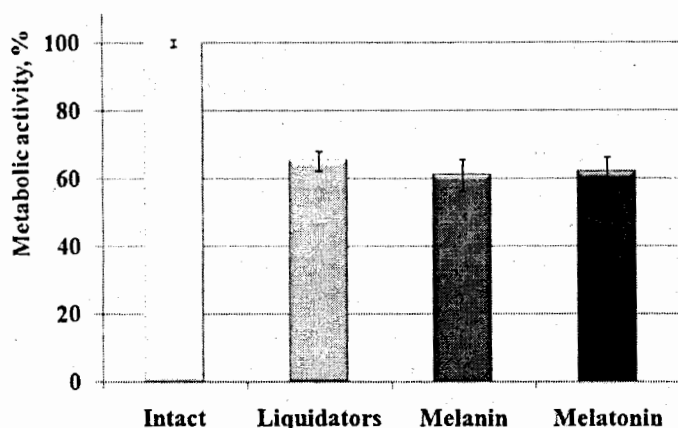


Figure 2. Effects of melanin and melatonin on HPV-G cells treated with blood serum samples from Chernobyl liquidators (control is taken as 100%).

As it can be seen from Figure 2, addition of melanin and melatonin to the medium together with serum samples from Chernobyl liquidators doesn't have any protective effect. The viability of cells treated with only serum samples ($16.24 \pm 0.73 \times 10^3$ FU) is almost the same as viability of cells treated with serum samples and melanin ($15.21 \pm 1.13 \times 10^3$ FU) or melatonin ($15.45 \pm 1.04 \times 10^3$ FU) – the difference is not significant ($t=0.77$ and $t=0.62$, respectively; $p>0.05$ in both cases).

4. Discussion

In similar studies on liquidators from Armenia (Emerit *et al.*, 1994; 1995) it was shown that only 42% of liquidators had an increased level of clastogenic factors (chromosome aberrations) as compared to the level of spontaneous mutations. In our experiments 100% of liquidators, 95% of PSRER workers and 82% of residents from contaminated

areas had an increased level of factors as compared to the control levels. A possible explanation of such difference could be the different protocols used in analysis. As mentioned above, HPV-G cells are much more sensitive to mutagenic factors as compared to peripheral blood lymphocytes. Another explanation could be that the cohort from Dr. Emerit studies (Emerit *et al.*, 1994) included liquidators of 1986-1988. After 1987 the dose limits for people engaged in clean-up works significantly decreased. Therefore liquidators from 1986 expressed higher levels of damaging factors as compared to liquidators of 1987-1988. In the present study liquidators of the consequences of the Chernobyl accident in 1986-1987 were analyzed, which were the most affected group and received the highest doses of radiation (higher than 250 mGy).

According to the results of the present study, bystander factors persist in the blood of Chernobyl liquidators for more than 20 years after the accident. In previous studies it was shown that irradiation of blood *in vitro* at a radiation dose of 500 mGy resulted in significant clastogenic activity and irradiated cells incubated in fresh culture medium continued to produce factors in culture as it was shown in studies (Emerit *et al.*, 1994).

Thereby, performed studies have shown that blood sera from population groups affected by the Chernobyl accident contain bystander factors, which are able to induce micronuclei and decrease metabolic activity of recipient cells. Pathological processes, such as acute virus infection, could significantly influence the level of bystander factors, increasing their damaging effect. The developed new method of evaluating of effects of damaging factors from blood sera has obvious advantages as compared to the assays which were used in previous studies (Emerit *et al.*, 1994) and may be applied in long-term monitoring of biological consequences of irradiation.

The results of the study of the mechanisms and nature of bystander factors circulating in blood stream of irradiated individuals make a major contribution in understanding non-direct effects of radiation *in vivo* and allows obtaining of new data on intercellular communications.

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SOME PROBLEMS OF RADIATION GENETIC EFFECT EVALUATION

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Abstract. There are a lot of questions about genetic effects of ionizing radiation. The main one is does ionizing radiation induce mutations in human? There is no direct evidence that exposure of parents to radiation leads to excess heritable disease in offspring. What is the difference between human and other species in which radiation induced mutations are easily registered? During evolution germ cell selection *ex vivo* has been changed to a selection *in vivo* and we cannot observe such selection of radiation damaged cells in human. Low radiation doses – are they harmful or beneficial? The “hormesis” phenomenon as well as radioadaptive response proves positive effects of low radiation dose. Can analysis of chromosomal aberration rate in lymphocytes be used for dosimetry? Many uncontrolled factors may be responsible for significant mistakes of this method. Some other questions need answers too.

Keywords: ionizing radiation, mutations in human, germ cell selection, low doses, hormesis, radioadaptive response, biodosimetry

1. Does ionizing radiation induce mutations in human?

Since we know that radiation can damage genes and chromosomes, we might expect that parental exposure to radiation would produce an increased incidence of inherited disease in the children of the exposed individuals.

It has been known that chromosome aberrations occur more frequently in somatic cells of people who have been exposed to radiation either in the course of their work, or because they live in an area in which the level of background radiation is abnormally

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high. Chromosome aberrations can be conveniently studied in lymphocytes. Aberrations are observed at dose equivalents of as low as a few tens of millisieverts. The biological and medical significance of such changes is not clear; there is an association between some types of chromosome aberration and some types of cancer and leukemia, but as yet no conclusive evidence that the chromosome damage actually causes the cancer.

The analysis of mortality and morbidity among the children of irradiated parents has provided neither positive nor negative results on germline mutation induction by ionising radiation. Nowadays there are no proves of radiation induced mutation existence in human.

Data on radiation hazards are regularly reviewed by two bodies - the United Nations Scientific Committee on the Effects of Atomic Radiation (UNSCEAR) and the Committee on the Biological Effects of Ionizing Radiations (BEIR). In turn, the reports of UNSCEAR and BEIR are reviewed by the International Commission for Radiological Protection (ICRP) who makes recommendations for radiation protection. The most recent major recommendations of ICRP were published in 2007. There is such statement in this issue:

“There continues to be no direct evidence that exposure of parents to radiation leads to excess heritable disease in offspring”. The 2007 Recommendations of the International Commission on Radiological Protection. (ICRP, 2007)

Data reviewed by the UNSCEAR and BEIR committees fall into three categories:

The survivors of the Hiroshima and Nagasaki bombings

Patients receiving radiotherapy

People who were exposed to radiation in the course of their work — occupational exposure.

1.1. THE SURVIVORS OF HIROSHIMA AND NAGASAKI

The survivors of Hiroshima and Nagasaki are by far the largest of these three categories. A total of about 100,000 people has now been followed up for more than forty years. The radiation doses reach up to about 5 Gy (500 rad), with an average dose of 0.27 Gy (27 rad). It should be noted that the doses are given in grays, because they were acute doses of low LET radiation. The radiation dose received by the survivors of Hiroshima and Nagasaki is thought to be due mostly to external gamma rays.

The largest group of people exposed to radiation is the survivors of Hiroshima and Nagasaki. The incidence of congenital abnormalities, stillbirths and child deaths has been examined in about 70,000 pregnancies in the survivors, and compared with pregnancies in a control group of people who were not exposed to radiation (Schull et al., 1981). There has been no difference detected in the incidence of congenital abnormalities or stillbirths; there has been a small increase in child deaths when both

parents were exposed to radiation, but it is not statistically significant, that is to say, it is a fluctuation which could have occurred by chance.

The white blood cells of 4230 children born to survivors have been studied to see if there were more chromosome aberrations than in the control group of 2202 children whose parents were not exposed to radiation. The number of children with chromosome aberrations was slightly higher among the exposed than among the controls, but again the difference was not statistically significant. However, because the average age of people in chromosome analysis was 24 years, genetic abnormalities that had resulted in death would obviously not be included, and this may have decreased the difference between the two groups.

No increase with parental dose and no effects of exposure on mortality and morbidity was registered.

There is as yet no convincing evidence of increased genetic damage in the survivors of Hiroshima and Nagasaki. It could be that the doses were too high to show an effect, and that the dose-response curve "turns over" at high doses - in other words, genetic effects may appear only if the radiation doses are low enough for the cells to retain their normal reproductive capacity. Some genetic injuries may have been eliminated through miscarriage; more seriously, recessive mutations may not have appeared yet.

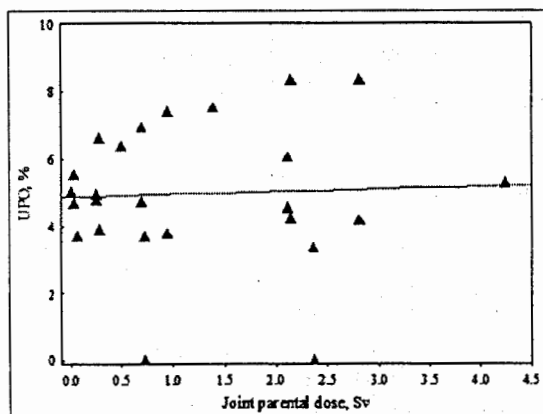


Figure 1. Congenital malformations, stillbirth & early mortality among the children of atomic bomb survivors of Hiroshima & Nagasaki (Otake *et al.*, 1990).

1.2. PATIENTS TREATED WITH RADIOTHERAPY FOR VARIOUS NON-MALIGNANT CONDITION

A small number of these patients subsequently developed cancer and it is possible to use these data to estimate radiation risks. The largest and most important group in this category is a group of 14,000 patients who were treated for ankylosing spondylitis, a disease causing stiffening of the spine. X-ray treatment of the spine was an effective

way of slowing this process, but was discontinued when it was realized that exposure of the bone marrow to radiation produced an increased risk of cancer. The average radiation dose to the bone marrow in a single course of treatment was 3.8 Gy (Lewis et al., 1988). Patients who received a single course of therapy have been followed up for an average of 24 years (Darby et al., 1987). The number of deaths from cancers other than leukemia was higher by 28% than in the general population of England and Wales, and leukemia deaths were three times as large as expected.

Many other smaller groups medically exposed to radiation have been studied, including: infants who received X-ray therapy for enlarged thymus glands; young women receiving multiple fluoroscopies during treatment for tuberculosis; women given radiation treatment for mastitis (inflammation of the breasts); and patients who received Thorotrast (containing naturally radioactive thorium dioxide) which was used as a contrast agent (like barium) in diagnostic radiography.

1.3. PEOPLE WHO WERE EXPOSED TO RADIATION IN THE COURSE OF THEIR WORK

The most important groups within this category are miners, particularly uranium miners. Groups in Canada, the USA, Czechoslovakia and Sweden have been studied. Uranium miners breathe radon (a decay product of uranium) into their lungs. Radon and some of its decay products emit alpha particles which can cause lung cancer. A more detailed discussion of radon hazards is included in the Appendix.

Another group in this third category consists of women who worked with luminous paint containing radium. These women have an increased incidence of bone or breast cancer (Baverstock et al., 1981; Rowland et al., 1983).

There is the fourth group of irradiated people:

1.4. LIQUIDATORS OF THE CHERNOBYL ACCIDENT

This group consists of about 320 000 people, received 170 mSv in 1986 and 130 mSv in 1987. Livshits et al., 2001, Kiuru et al., (2003) observed no minisatellit mutation increase in liquidator children. Sleboset et al. (2004) analyzed lymphocyte DNA and did not found any statistically proved increase of mutation level in children of irradiated fathers.

1.5. SOME DIFFICULTIES

It is important to note that most of the data referred to concerns exposure to low LET radiation; there is little information on high LET radiation effects in humans, apart from the uranium miners (where the radiation dose is largely due to inhaled radionuclides emitting alpha particles). It was originally thought that the Hiroshima survivors had

been exposed to a mixture of neutrons and gamma rays; however, a recent reassessment of the doses indicates that the neutron contribution to both Hiroshima and Nagasaki irradiation was quite low.

Even though there seem to be plenty of data on low LET radiation, there are two major difficulties with many of these data. The first is the uncertainty associated with many of the radiation doses-in some cases we do not know precisely how much radiation was actually received.

The second difficulty is that, with a few exceptions, nearly all the data concern exposures to doses of 200 mGy (0.2 Gy) or more - about ten times the radiation doses that would be classified as 'low level'. It is generally assumed that the line of excess cancer incidence versus dose (the dose- response curve) goes down to the origin of the graph, i.e. no matter how small the radiation dose, there is always some risk. But how do we extrapolate the graph down to the origin? Is it a straight line or a curve? - and if a curve, what sort of a curve?

Indeed there are considerable statistical problems involved in estimating the radiation risks in human. The confidence intervals are very large - up to many times greater than the ICRP risk factor at the upper limit, and consistent with a negative factor at the lower limit.

1.6. EVOLUTION DIRECTION

So, no mutation incidence increase in offspring of irradiated people was registered. What are the causes of this? Methods of induced mutation analysis in human are not satisfactory and don't allow detection induced recessive mutations, but why wasn't dominant mutation growth registered? What is the difference between human and animals which easily demonstrate radiation induced dominant mutations?

During evolution from plants to human the number of egg cells is decreasing as well as the number of progeny. Plants produce a lot of seeds, insects can lay hundred eggs per day, fish and frogs produce thousands spawns but only two of them will be adult organisms if the population is in a equilibrium state. So, a very intensive selection eliminates damaged germ cells. If some cells are changed due to irradiation we can observe these damaged cells before elimination because selection occurs *ex vivo* at the different ontogenesis stages.

Small mammals have less egg cells and less progeny and human has a one-child pregnancy as a rule. Damaged human germ cells and zygotes are eliminated by a selection *in vivo*. Thus, the selection *ex vivo* has been changed to a selection *in vivo*.

We can study easily dominant lethal mutations in insects at the egg-larva stages, we can analyze these type mutations in mice, but for this we have to kill them and we cannot observe such selection of radiation damaged cells in human *in vivo*.

Maybe this is the main cause why mutation incidence increase in offspring of irradiated people was not registered. Besides nobody registered radiation induced

mutations in monkey, elephant and other mammals with one-child pregnancy – obviously also due to the germ cell selection in vivo.

This is only my attempt to explain why radiation induces mutations in human – it was not proved and this question still has no strict answer.

2. Low radiation doses – are they harmful or beneficial?

There is no direct evidence of negative influence of low radiation doses on heredity. All health human investigations in populations from regions with high radiation background have revealed no genetic effects and no harmful consequences for health and lifespan.

There are a number of areas in the world where the radiation doses from background are substantially higher than the UK average. In some of these areas, studies have been carried out to see if the incidence of cancer or other diseases is higher than in a similar area with a lower background level of radiation. Here are some of the more interesting ones:

2.1. During 1972-1975 in the Dong-anling and Tongyou areas of Guangdong province in China, a population of about 73,000 living in a high background area was compared with a control group of 77,000 people living in a nearby area where the background is lower. The control group received an average annual dose from external radiation of about 0.7 mSv, and the study group about 2 mSv. Although the latter was the 'high background' group, the average dose was comparable with the average background level in the UK. There were no significant differences between the two groups in the incidence of cancer, hereditary diseases, congenital deformities or spontaneous abortions (High Background Research Group, 1980).

2.2 There is an area of Kerala State, on the southwest coast of India, where there are deposits of the mineral monazite which contains thorium-232, uranium-238 and their decay products. As a result the population is exposed to an average external radiation dose of 4-5 mSv per year, that is, about two or three times the average total dose from background radiation in the UK. A group of about 70,000 individuals have been studied. There is some evidence of an increased incidence of chromosome aberration, and also some evidence of an increase in Down's Syndrome, although this is controversial (Kochupillai et al., 1976; Sundaram, 1977). The incidence of many other effects has been examined, including other cancers, congenital abnormalities, infant mortality and longevity, but no significant effects have been found; however, a group of the population which received more than 20 times the normal background radiation exposure had the lowest value of fertility index and the highest value of infant mortality (Hanson and Komarov, 1983).

2.3. The third area of interest is in parts of the states of Iowa and Illinois in the USA, where there is a significant concentration of radium-226 in the drinking water (average activity 0.17 Bq per liter). These areas do show an increased incidence of bone and lung

cancer when compared with control populations from similar communities with a radium-226 concentration in water of less than 0.04 Bq per liter. However, the interpretation of these findings is not straightforward; for example, the death rate from bone cancer in Chicago, which has drinking water with a very low radium content, is higher than in the exposed population of Illinois and Iowa (Hanson and Komarov, 1983).

2.4. Knox et al. (1988) investigated the cancer death rates in childhood over Great Britain and related them to outdoor gamma radiation exposure levels. There was no simple relation between cancer rates and gamma radiation; for example, areas with the lowest gamma ray dose rates had the highest cancer rates. Several studies, including those of the populations in Kerala and China, show an increased incidence of chromosome aberrations when the background radiation level is higher, but the medical and biological significance of these changes is not clear (Pohl-Ruling and Fischer, 1983; Zufan and Luxin, 1986).

With the possible exception of the Iowa and Illinois data, most of the studies on high background areas show to date no clear effect of radiation exposure on health. The common opinion, that the main cause for this is probably the relatively small sample sizes that have been studied and the many factors that affect the incidence of cancer.

2.5. In the same time the "hormesis" phenomenon as well as radioadaptive response proves positive effects of low radiation dose.

There are a lot of data about stimulating effects of low radiation doses (hormesis)

Life is already accustomed to a low dose or dose rate of radiation, within the range of naturally occurring radiation. Radiation hormesis is explained, pointing out that beneficial effects are expected following a low dose or dose rate because protective responses against stresses are stimulated.

Jerry M. Cuttler and Myron Pollycove (2009) suggest that it is necessary to examine the scientific evidence of health effects of nuclear radiation, because negative images and implications of health risks derived by unscientific extrapolations of harmful effects of high doses must be dispelled.

Radon is a product of uranium radioactivity in the natural environment. A scientific test of the LNT model, as normally used, clearly disproved the LNT hypothesis.. Lung cancer mortality is *lower* in US counties where the radon concentration in homes is *higher* (Cohen, 1995 - figure 2).

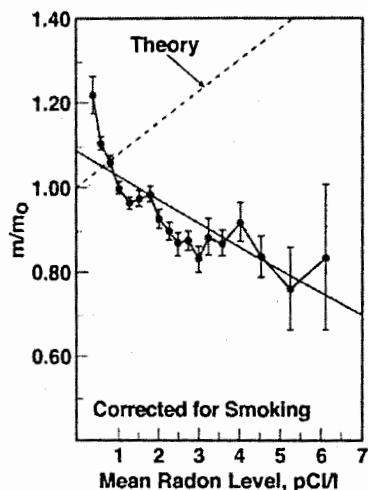


Figure 2. Lung cancer mortality rates compared with mean home radon levels by U.S. county and comparison with linear model by BEIR IV (Cohen 1995)

“Hormesis is an evolutionary conserved process characterized by nonlinear biphasic dose-response in which low doses of stressful activity stimulate adaptive responses that increase function and resistance of the cellular organism to stress, in contrast to inhibitory responses to high doses that decrease resistance and function” (Calabrese, 2008a).

Radiation hormesis involves non-linear, biphasic dose responses of prevention and repair to another stressful challenge: alteration of DNA and other molecules by ionizing radiation and by endogenous metabolic leakage of free oxygen radicals as reactive oxygen species.

Epidemiologic studies of human populations in high background residential radiation or chronic intermittent occupational or medical radiation exposure demonstrate a positive hormetic response of decreased mortality and cancer mortality rates (Luckey 1980, 1991, Pollycove and Feinendegen, 2001).

“Four decades of genomic, cellular, animal, and human data have shown that low-dose ionizing radiation stimulates positive genomic and cellular responses associated with effective cancer prevention and therapy and increased life span of mammals and humans. Nevertheless, this data is questioned because it seems to contradict the well demonstrated linear relation between ionizing radiation dose and damage to DNA without providing a clear mechanistic explanation of how low-dose radiation could produce such beneficial effects. This apparent contradiction is dispelled by current radiobiology that now includes DNA damage both from ionizing radiation and from endogenous metabolic free radicals, and coupled with the biological response to low-dose radiation” (Pollycove and Feinendegen, 2008).

The above mentioned positive human response to chronic, increased DNA damage by low-dose radiation is achieved by increased stimulation of: cellular antioxidant prevention of DNA damage by free radicals, enzymatic repair of DNA damage,

immunologic destruction of DNA damaged cells by “killer” T lymphocytes (Liu, 2007), and self destruction (apoptosis) of DNA damaged cells. These studies and similar ones in mice, rats and dogs have led to successful clinical trials in patients (Pollycove and Feinendegen 2008). Acceptance of current radiobiology would facilitate additional, urgently needed clinical trials of low-dose radiation (LDR) cancer therapy.

American scientist Luckey (1991) also claimed that low radiation doses can be beneficial for health and recommended to use them in medicine

The main proof of stimulating effects of low radiation doses is radioadaptive response in which a low priming dose protects cells or organism against a high second dose. This phenomenon is well-known and nobody has doubts in its existence. Adaptive response decreases effects of ionizing radiation approximately twice. Adaptive reaction can be induced by low radiation dose as well as by weak chemical mutagens and many other factors (Wolff, S. *et al.*, 1988). This is strong evidence of positive action of low radiation doses.

3. Biodosimetry or bioindication?

The frequency of chromosome aberrations is used widely as a method of estimating past exposure to radiation. Indeed if we irradiate human lymphocytes *in vitro* (cultured cells) with different radiation doses we shall receive a good correlation between a chromosome aberration level and a radiation dose (Fig. 3).

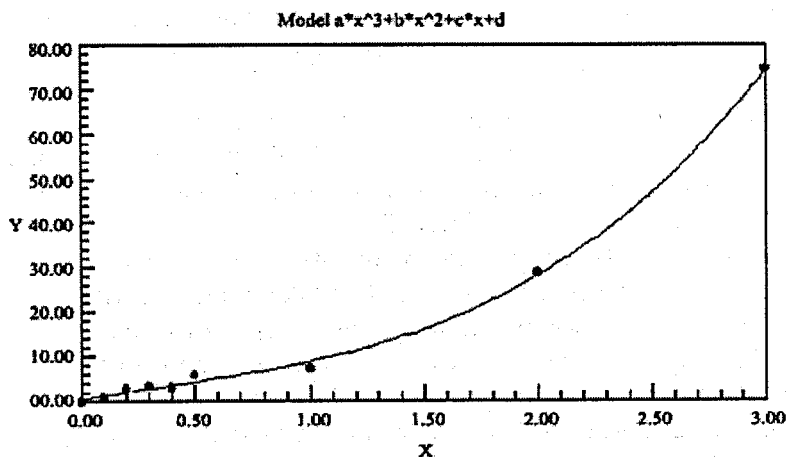


Figure 3. Dose-effect relationship for stable chromosome aberrations in human lymphocytes *in vitro*: x- radiation dose, y- aberration rate. (Melnov, S.B., 2002)

However there are many discrepancies when we analyze such a relationship in vivo. For example, if we compare doses, which were received by liquidators of Chernobyl accident in terms of physical dosimeter, and doses estimated with biodosimetry method, we reveal many contradictions. There are a lot of causes which change strongly the chromosome aberration level induced by irradiation in an individual organism.

First of all humans have different radiosensitivity, which is a quantitative character and its distribution in a population corresponds to binomial curve./1/. According to this 50% of the population have the mean values of radiosensitivity ($x \pm 0.67\sigma$), 95% of individuals have the radiosensitivity values equal to $x \pm 1.96\sigma$ and only 5% of the population are in the range of $x \pm 1.96\sigma$ to $x \pm 3\sigma$, with division into the supersensitive fraction (2.5%) and the superresistant one (also 2.5%). So radiosensitivity of a person can differ from the medium value in 2-3 sigma value (Ayala, 1987).

It means that reaction of people on the same radiation dose is different because radiosensitivity as well as other quantitative characters are caused by interaction of many polymeric genes determining a number of physiological and biochemical organism features (Ayala, 1987).

Genetic factors which influence radioresistance are as follows:

- Repair system activity
- Endogen radioprotectors and antimutagens
- Protein synthesis rate
- Amplification of Genes responsible for radioresistance
- Genetic mobile element action
- Adaptive response
- Bystander effect
- Hereditary diseases
- and so on

All these facts led to such conclusion: "Clearly, genetic predisposition is crucial and may even be more important than dose." (C.Mothersill, C Seymour, 2001).

Besides, radiosensitivity depends to a great degree upon environmental factors. That's why many factors which are not under control can change significantly biological effects of radiation and thereby can be responsible for serious mistakes of biodosimetry.

Environmental factors which influence radioresistance are as follows:

- Diet
- Physical activity

- Nervous-psychological state
- Hormonal state
- Drugs
- Virus diseases
- And so on

3.1. INFLUENCE OF SOME FERTILIZERS AND HERBICIDES ON RADIOSENSITIVITY.

Some of substances are present in our food — residual amounts of fertilizers or herbicides can be mutagenic or can influence mutagenic action of radiation. In the latter synergetic or antagonistic effects can be observed.

We studied cytogenetic effects of such fertilizers as sodium nitrite and nitrate and herbicide zenkor in mice and human cells. Reciprocal translocations in germ cells of mice were cytologically analyzed in metaphase of spermatocytes by the method, which is a modified Ivens' method. Human cells were cultured according to a standard method, the cytological test included dicentric-ring-fragment analysis.

Sodium nitrite and nitrate in concentrations of 10 and 100 mg/l respectively were added to drinking water for mice within 2,5; 5,0 and 7,5 months. It was found that these substances didn't possess mutagenic activity under such conditions. Acute or prolonged irradiation of treated mice with gamma-rays or neutrons 0,25; 0,5 and 5,0 Gy showed that NaNO_2 and NaNO_3 sensibilized significantly (2-4 times) cytogenetic action of ionizing radiation (fig.4). We have found the same effect in drosophila earlier.

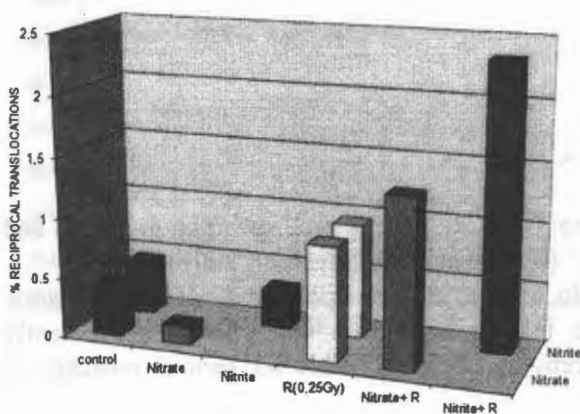


Figure 4. Influence of sodium nitrite and sodium nitrate on mutagenic action of radiation in mice germ cells.

Cytogenetic effects of zenkor were found to be completely different. This herbicide being injected intraperitoneally or perorally to mice increases the translocation rate in

germ cells and being added to culture media for human cells shows a mutagenic effect too. Irradiation of treated mice and human cells results in decreasing aberration levels in contrast to nitrite and nitrate. Chronic influence of zenkor and gamma-rays leads to more strict "antagonistic" effect than acute one (Mosse et al., 1997, 1998). Such effect can be explained by increased death of cells treated with both mutagenic factors — zenkor and irradiation. This explanation had been proved by our experiments with drosophila populations, in which combined action of these factors resulted in decreasing flies number. Thus different agricultural chemicals have different cytogenetic effects used in combination with irradiation.

So, NaNO_2 and NaNO_3 have no mutagenic effect and increase significantly the radiation induced mutation level. Herbicide zenkor was shown to be a weak mutagenic substance and prevent adaptive response. Genetic interaction of zenkor and gamma rays is antagonistic - mutation rate after the combined influence of both factors is lower than the expected sum of mutation rates induced by each taken separately (Mosse et al., 1997, 1998).

3.2. INFLUENCE OF SOME RADIOPROTECTORS AND RADIOADAPTIVE RESPONSE

In the same time many food stuffs contain radioprotectors or antimutagens. So, tea, coffee, cocoa, chocolate, mushrooms and others have melanin, which is very effective radioprotector not only against acute irradiation, but even against chronic one (Mosse et al., 1999, 2000, 2006).

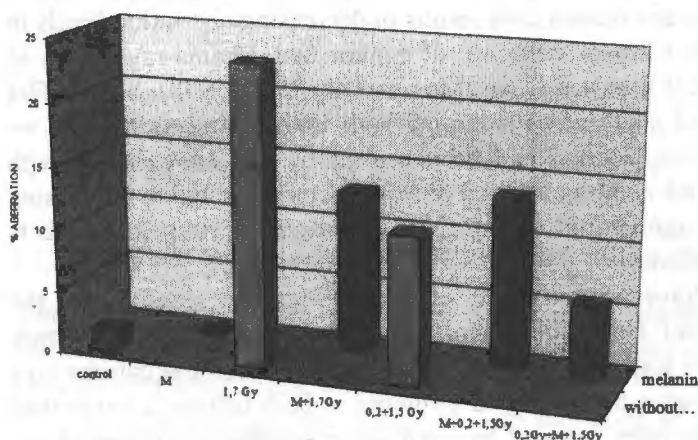
Thus, some chemicals can strongly influence results of biodosimetry. Some drugs, stress, virus diseases and so on can change biological effects of radiation too.

Radioadaptive response is one of the most significant factors which can be responsible for incorrect radiation dose evaluation.

We demonstrate an adaptive response in mice germ and bone marrow cells – rate of chromosome aberrations after 0,2 + 1,5 Gy was about half as much as after 1,7 Gy irradiation. Melanin influence on adaptive response has been studied. Melanin injection 2 hours before the first conditioning dose 0,2 Gy resulted in the same mutation level as before 1,7 Gy – adaptive response was not found. If melanin was applied between the first and the second doses both adaptive phenomenon and melanin protection led to 4-fold decrease in aberration rate (fig.5). So, effective radioprotectors are able to prevent adaptive response by suppressing conditioning dose effect or sometimes to decrease genetic effect of radiation.

All these uncontrolled factors may be responsible for significant mistakes of single radiation dose evaluation and the estimation of chronic radiation dose has much more problems. It is necessary to understand that biological methods show only a level of biological consequences of irradiation, but they do not allow estimation of exposed or absorbed radiation doses. That's why biological methods can be used for bioindication but not for biodosimetry.

Figure 5. Melanin influence on radioadaptive response in mice bone marrow cells



It is necessary to take into account that these factors are averaged over a population. That's why biological methods can be used for population but not individual radiation dose estimation.

4. Why did evolution preserve bystander effect?

There are a lot of questions concerning radiation induced bystander effect – is this effect positive or negative? Why did evolution preserve this phenomenon if it is negative? What happens after irradiation - adaptive response or bystander effect or both these phenomena simultaneously? May be they arise sequentially - at first bystander effect increases a number of damaged cells in order to switch on protect reactions in organism and induce adaptive response which appears about four hours later? All these questions have no answers yet.

5. Conclusion

Research into radiation effects is a particularly difficult area because we have no enough data to confirm or disprove a theory. Limited evidence, which can be interpreted in a different way, always induces a controversy. There are number of uncertainties in estimating radiation risks. Clearly we need to keep in mind a lot of limitations and qualifications because there are so many data and interpretation of many studies is contradictory. What we have discussed in this paper demonstrates that in this subject there are no simple answers.

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IMPLICATIONS FOR HUMAN AND ENVIRONMENTAL HEALTH OF LOW DOSES OF RADIATION*

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Abstract. Recent advances in our understanding of the mechanisms underlying the biological effects of low dose effects of ionizing radiation have revealed that similar mechanisms can be induced by chemicals in the environment. Therefore interactions between radiation and chemicals are likely and that the outcomes following mixed exposures to radiation and chemicals may not be predictable for human health, by consideration of single agent effects. Our understanding of the biological effects of low dose exposure has undergone a major paradigm shift. We now possess technologies which can detect very subtle changes in cells due to small exposures to radiation or other pollutants. We also understand much more now about cell communication, systems biology and the need to consider effects of low dose exposure at different hierarchical levels of organization from molecules up to and including ecosystems. We also understand at least in part, some of the mechanisms which drive low dose effects and which perpetuate these not only in the exposed organism but also in its progeny and even its kin. This means that previously held views about safe doses or lack of harmful effects cannot be sustained. ICRP and all national radiation and environmental protection organisations have always accepted a theoretical risk and have applied the precautionary principle and the LNT (linear-non-threshold) model which basically says that there is no safe dose of radiation. Therefore even in the absence of visible effects, exposure of people to radiation is strictly limited. This review will consider the history of the new discoveries and will focus on evidence for emergent effects after mixed exposures to combined stressors which include ionizing radiation. The implications for regulation of low dose exposures to protect human health and environmental security will be discussed.

* Radiobiology and Environmental Security / Eds. C.Mothersill, V.Korogodina, C.Seymour. Springer, 2012. P.43-52

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1. Biology of "non-targeted" effects

Within conventional radiobiology as accepted in the 1950's continuing through to the 1990's there was little consideration of epigenetic effects, because the traditional concept of radiobiology was based on target theory.^{1,2} For an effect to occur, radiation had to hit a defined target within the cell, assumed to be DNA. Assumptions about the number of targets hit could then be made from measurements of dose and dose rate.^{3,4} The evolution of non-targeted effect (NTE) radiobiology meant that at low doses the previous assumptions needed to be reconsidered in the light of the existence of non-DNA mechanisms.⁵⁻⁷ The mechanisms underlying radiation effects are not constant with respect to dose and it would now be generally accepted that low dose effects are mechanistically different to high doses effects. This is not to say the mechanisms are necessarily mutually exclusive but it does mean that NTE's will contribute more to the overall outcome at low doses where targeted effects are small. Targeted effects will predominate at high doses and in situations where NTE's have been inhibited or otherwise prevented. In terms of the progression of radiobiological thinking in this field, disease caused by radiation no longer had to be exclusively genetically based, but radiation could promote or exacerbate systemic disease. This disease could have been caused for example by a chemical mutagen.⁸⁻¹⁰ Equally, the radiation could facilitate a non-mutation based inflammatory type disease.¹¹⁻¹⁴ These concepts, although largely accepted theoretically by the radiobiology community, have been difficult to prove epidemiologically because of what are generally called "confounding variables" such as smoking, drinking, age, gender, or concurrent past or future exposures to the same or a different pollutant.^{15,16} These factors actually reflect the futility of trying to assign causation, as defined in epidemiology, to one agent when the doses are low! Others argue that radiation and many chemical "pollutants" might actually boost the immune system and be good.¹⁷⁻¹⁹ The hormetic argument has many interesting applications but is unproven with regard to multiple pollutants. This adds to the confusion and controversy surrounding low dose exposures. The essential point is that there will be huge individual variation due to involvement of epigenetic and non-targeted factors in the response.²⁰⁻²² At any one time we are as unique epigenetically as we are genetically. Epigenetic differences are linked to gender and lifestyle. In theory therefore a low dose of radiation could cause any number of effects ranging from beneficial to death-inducing disease depending on the context of the exposure and the interplay of factors such as cell communication, microenvironment, tissue infrastructure and a whole host of systemic variables which influence outcome from a cellular track of ionizing radiation.²³⁻²⁴

2. Is radiation "just another stressor" or is it unique?

Key developments leading to the current widespread acceptance of ionizing radiation as

“just another stressor” include:

1. The development of sensitive techniques such as m-FISH, for detecting chromosomal abnormalities.²⁵⁻²⁸
2. Studies showing that delayed or persistent sub-optimal survival (reproductive death) could be seen in surviving progeny of irradiated cells.²⁹⁻³²
3. The emergence of genomic instability as a mechanism by which low doses of radiation could cause delayed or persistent damage to chromosomes.³³⁻³⁷
4. The accumulation of knowledge of “bystander effects” whereby chromosome damage, death, DNA damage and various other consequences occur in cells receiving signals from cells irradiated with low doses of radiation.³⁸⁻⁴³
5. Criticism of the epidemiological research undertaken after the Hiroshima and Nagasaki bombs as ignoring the damage from residual radiation and fall out.⁴⁴⁻⁴⁵

The NTE paradigm emerged initially as a result of re examination of firmly held beliefs and some odd results in the laboratory which did not fit the DNA paradigm. Proof of the new hypotheses required the techniques such as molecular imaging, M-FISH, and SKY as well as the development of tissue culture techniques for human *normal* tissues which permitted functional studies to be performed.⁴⁶ Older studies tended to use high doses on a limited number of cell lines or highly inbred animal strains. These tended to thrive in the laboratory but were often unrepresentative of tissues in the outbred human or non-human.⁴⁷⁻⁴⁹

2.1. IMPORTANT CONSEQUENCE FOR RADIATION PROTECTION AND RISK ASSESSMENT OF NTE'S

2.1.1. *The concept of hierarchical levels*

Hierarchical levels stretch from the individual to smaller units (organs – tissues – cells – organelles – genes) and to bigger units - populations (multiple individuals/ single species) and ecosystems (multiple individuals and multiple species). Confusion in the low dose exposure field (both radiation and chemical) arise from lack of consideration of this concept. Most of the arguments about whether radiation is good or bad for you fail due to lack of consideration of the hierarchical level at which the effects occur and because most of the arguments are anthropocentric. For example cell death is seen as a bad effect but if it removes a potentially carcinogenic cell from the population of cells in a tissue it could prevent cancer starting and could be seen as good. Survival of cells is seen sometimes as desirable but if they survive with unrepaired or mis-repaired damage, they could facilitate development of a cancer. Similarly in non-human populations – death of radiosensitive individuals which cannot adapt to the changed (now radioactive and or chemically polluted) environment, could be good at the population level in evolutionary terms depending on the life stage and reproductive

status when the effects manifest, although death will be bad for the individual. It is only by considering responses in context, that any conclusions can be drawn about risk or harm.

2.1.2. *Spatio-temporal concepts*

There are two aspects to this – one is simply the age of the irradiated unit and the spatial deposition pattern of the ionizing energy. This concept is relevant across all hierarchical levels. Obvious considerations are the age or maturity of the cell, organ, life-form or ecosystem) receiving the track, the density of the energy deposition, the lifetime of the unit and its importance in the context of functionality of the higher hierarchical levels. Young units tend to be less stable and thus more vulnerable (or more adaptive?) than old or mature units because of their faster metabolic rate, higher rate of growth/cell division and at the ecosystem level, because of their less strongly developed interdependencies. There is also (usually), more redundancy in young units, for example there are more available individuals, better reproductive rates and better viability from young progenitors, whether cells or individuals. The other aspect is that the delayed effects of radiation and bystander effects mean that radiation effects are not fixed in time or space to the energy deposition along ionizing track. The effects can persist and manifest at distant points in time and space. These concepts are also discussed elsewhere.^{8,9}

2.1.3. *The importance of mixed exposure analysis*

Pollutants including radiation seldom occur in isolation. In fact most environmental radioactivity comes from radioisotopes which are chemical entities. This means that there is always a mixed exposure and that both the chemical and radioactive aspects need to be considered. Additive damage used to be an acceptable way to deal with mixed exposures (if any way were used!). The new field of non-targeted effects with the consequent realization that emergent properties can exist, which were not predictable from the individual agent dose response data, makes this no longer acceptable. The complexities of mixed pollutant scenarios call for a re-think of fundamental approaches to both epidemiological causation after low dose exposures to anything. They also question the need regulators have to regulate to a number (dose unit/exposure unit). Some of the issues concerning the latter position include the following:

- How to ensure compliance if there is no “safe” or legal limit?
- How to deal with multiple stressors especially if the interactions are not known
- How to correct for dose rate/ time of exposure

- How to deal with mixed chronic and acute exposures
- How to factor in possible adaptive, hormetic or antagonistic effects
- How to regulate in pristine versus dirty environments

The issues of legal causation are highly relevant to the former point but outside the scope of this review. Discussion of these issues can be found elsewhere.⁵⁰⁻⁵² Ultimately, in order to resolve these issues, more data are needed for mixed exposure scenarios using relevant species. Systems biology approaches involving close interaction between experimental biologists and modelers are also required

3. Data concerning low dose effects of mixed exposures

There are very little data where low dose exposures to multiple stressors/mixed contaminants involving radiation and a chemical are investigated. The field was reviewed by Mothersill et al⁵³ in 2006. Recent interest in non-targeted effects probably means more attention will be paid to this area in future. Gowans et al⁵⁴ have data showing chemical induction of genomic instability. Data from the authors' own and other laboratories shows that heavy metals singly or in combination can cause genomic instability.⁵⁵⁻⁶⁷ Delayed death and chromosome aberrations in human cells following nickel, titanium or cadmium exposure have been reported.⁶⁴⁻⁶⁷ Similar effects have been reported in fish cell lines,⁵⁸⁻⁶³ and more recently in live fish exposed to very low doses of gamma radiation 4-75mGy over 48hrs in the presence of heavy metals at levels just above background.^{68,69}

Organic pesticides and detergents such as prochloras, nonoxylphenol, nonoxynol and dichloroaniline have also been found to cause delayed lethal mutations in fish cells.^{57,58,61}

Chromium and vanadium used in implants and dentures lead to a variety of genetic and reproductive delayed effects in vivo and to multiple endpoints associated with non-targeted effects in vitro.⁶⁴⁻⁶⁷

4. Implications for environmental protection and human health

While many of the studies cited above are concerned with fish or in vitro cells rather than humans, the data show that non-targeted effects can be induced by low dose exposures to a number of environmental chemicals as well as ionizing radiation. This means that combined exposures to low doses of these agents cannot be regulated in isolation and that studies of potential mechanistic interactions are important. Radiation protection of humans could find use from the approaches which are being taken by the task groups within ICRP, IAEA and the US-DoE (see for example 70, 71) who have to

formulate policy to regulate exposure of non-human biota. Many of the issues involved such as dealing with non-cancer endpoints, mixed contaminants or chronic low dose exposure are real issues in human radiation protection.

5. Summary thoughts and recommendations

The challenge in the low dose exposure field is to tease out the “noise”. Noise is the euphemistic term we use when the level of the disease which is unattributable to our favoured causative agent, is too high to prove causation formally in any strict scientific or legal sense. Perhaps we should accept that we cannot assign causation and instead view ionizing radiation as one among many agents which *together* contribute to cause disease. Before we can do this it is vital to understand the key mechanisms and in particular to find areas of mechanistic commonality suggesting common causation. Biomarkers may be useful to identify possible common mechanisms and to validate their relevance across different hierarchical levels. If this is achieved it should be possible to model links between *effects* at one level e.g. cellular or individual leading to *harm and risk* at higher levels – in this example the individual or the population. Biomarker studies do need to be interpreted cautiously however because they are often used as surrogates for risk when in fact they may merely be pointing to change in the system. Without the back-up modeling and multi-level analysis of their relevance they may lead to false conclusions and confusion about the true risk of an inducing agent.

The problem of establishing causation following mixed exposures still exists as does the issue of what constitutes “harm”. In the non-human biota field, there is great concern about doing more harm than good, if action levels are enforced which might require “remediation” of a habitat – i.e. removal of contaminated vegetation and soil. This could cause much more harm to the ecosystem than the original stressor. In the realm of human protection against low dose stressors, issues might include the ethics of genetic screening to identify sensitive sub-populations. If a sensitivity marker were available, who should be tested and when? Should diagnostic screening be forbidden to these individuals because of their possible sensitivity to low doses of radiation? There are also issues regarding lifestyle choices and risk benefit analysis at the biological level. Evolutionary adaptation leads to a fitter population (of cells, individuals) by eliminating the weak units but how is that population changed? In dealing with concepts of adaptation to environmental stressors where “nature” sorts things out in the optimal way, is “nature’s way” to Nietzian for Man?

It would be good to conclude this reflection with a “way forward” but as we are still in the very early stages of accepting that radiation doses effects at low doses are non-linear, that multiple stressors impact the final outcome, and that what appears to be bad (or good) may be good (or bad)– it is perhaps best to recommend caution and consideration of these points rather than a great new regulatory framework!

Acknowledgements

I acknowledge continued support from the Canada Research Chairs program, NSERC, and the EU NOTE integrated project.

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EFFECTS OF CHRONIC GAMMA IRRADIATION ON REPRODUCTION IN THE EARTHWORM *EISENIA FETIDA*^{*}

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Abstract. In recent years ecological impacts of ionizing radiation have emerged as an important research field. But there are still considerable knowledge-gaps regarding biological effects of chronic irradiation in wildlife, particularly for endpoints related to reproduction. Reproduction is considered to be one of the most sensitive radiation-associated endpoints, and determines not only the fate of the single organism, but may also influence population dynamics and the balance of higher ecological units. Based on their radioecological properties and their important role in the soil ecosystem, earthworms have been identified by ICRP as one of the reference animals and plants (RAPs) to be used in environmental radiation protection. This paper will present results of a series of studies carried out on the effects of ionising radiation on earthworms, covering long term-effects on reproduction, as well as studies on biological processes such as recovery, acclimatization and adaptive response, using a number of molecular biomarkers. The aim is to show how the studies can improve predictions of the way individuals and populations respond to chronic exposures of ionising radiation.

Keywords: Environmental effects, ionising radiation, earthworm; reproduction, chronic exposure

1. Introduction

There are still considerable knowledge-gaps regarding biological effects of chronic irradiation in many wildlife species, particularly for endpoints related to reproduction

^{**} *Radiobiology and Environmental Security / Eds. C.Mothersill, V.Korogodina, C.Seymour. Springer, 2012. P.381-388*

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(Garnier-Laplace et al., 2008). In ecological risk assessment, impacts on populations of species are considered the most relevant for protection of ecosystems as a whole (Andersson et al., 2008; 2009). Since the reproduction capacity of adults and the sexual maturation rate of offspring are of great importance for population dynamics, it follows that information on the effects of chronic exposures on these endpoints is required (Stark et al., 2004; Alonzo et al., 2008). Based on their radioecological properties and their important role in the soil ecosystem, earthworms have been identified as one of the reference animals and plants (RAPs) by ICRP (2008), and they are also included as reference organisms in the framework for environmental risk assessment developed during the EU ERICA project (Beresford et al. 2007; Laarson , 2008). Invertebrates are generally known to be relatively radioresistant but as for most species, reproduction is known to be far more sensitive than mortality (UNSCEAR, 1996). After nuclear accidents the local radioactivity levels can be very high, but will show a rapid decline due to the disintegration of short-lived radionuclides, i.e., after the Chernobyl accident, approximately 80% of the accumulated doses were absorbed within three months of the accident (UNSCEAR, 1996). The long term effect of such high exposures on the population dynamics depends partly on whether the radiation induced damage is irreversible or if the organisms have the ability to recover after the exposure levels have declined. Mechanisms leading to acclimatization (physiological adjustments during the exposure history of individuals) or adaptation (inheritable traits acquired during the exposure history of the population), might also influence the long term effects of exposure. This paper summarises results from two studies. The main purpose of the first experiment (Hertel-Aas et al., 2007) was to establish dose (rate) response relationships for reproduction endpoints in the earthworm *Eisenia fetida* in two subsequently exposed generations (F0 and F1). In the second experiment worms were exposed at a high dose rate and the ability to recover after a complete radiation induced inhibition of the reproductive capacity were studied.

2. Exposure details

In both experiments the experimental set up was based on standardised OECD chemical ecotoxicology tests for earthworm reproduction (OECD, 2003). Briefly, ten worms were placed in each replicate container applied with 677 g moist artificial OECD soil (OECD, 2007), and moist horse manure was spread on the soil surface (1.5 g wet w/worm/week). The endpoints were assessed after defined exposure periods, after which the cocoons were transferred to small boxes, the soil replaced and hatchlings removed after registration. More details on the experimental set up and dosimetry can be found in Hertel-Aas et al. (2007).

2.1. DOSE-RESPONSE RELATIONSHIPS FOR CHRONIC EXPOSURE

In the first study (Hertel-Aas et al., 2007), *E. fetida* was exposed to external ^{60}Co gamma irradiation during 2 generations (F0 and F1). Adult F0 reproduction capacity (i.e., number of cocoons produced, cocoon hatchability and number of F1 hatchlings) in controls and at five absorbed dose rates (0.18, 1.7, 4, 11 and 43 mGy/h) was measured over a 13 week exposure period. Survival, growth and sexual maturation of F1 hatchlings were examined for 11 weeks. F1 adults were exposed for a further 13 weeks for registration of reproduction capacity.

2.2. RECOVERY

In the second experiment, adult F0 worms were exposed at 17 mGy/h for 10 weeks. The dose rate and exposure time were chosen to achieve an accumulated dose, causing a complete inhibition of hatchability, based on results from the first experiment. Thereafter the worms were transferred to a climate chamber at $21 \pm 0.5^\circ\text{C}$ for 16 weeks.

3. Results and Discussion

3.1. DOSE-RESPONSE RELATIONSHIPS

Accumulated doses are shown in Table 1 and impacts on reproduction endpoints in Fig. 1.

There was no radiation-induced effect on the viability, growth, sexual maturation or cocoon production rates in F0 or F1. For F0, hatchability of cocoons produced during the first four weeks was reduced to 60% at 43 mGy/h (98% in controls), and none of the cocoons produced at 5 - 13 weeks hatched. At 11 mGy/h a pronounced effect on cocoon hatchability was not observed until 9 - 13 weeks, when hatchability was reduced to 25%. Also, the number of hatchlings per hatched cocoon was reduced at the two highest dose rates, and the total number of F1 hatchlings per adult F0 produced during the 13 weeks exposure period was reduced to 17% and 57% compared to the controls at 43 mGy/h and 11 mGy/h, respectively. The total number of F1 hatchlings was reduced also at 4 mGy/h, but the effect was of borderline significance. At the end of adult F0 exposure, dissection and microscopic examination revealed atrophic male reproductive organs in worms exposed at 43 mGy/h. For adult F1, the hatchability of cocoons produced at 11 mGy/h was reduced to 45 - 69% during the 13-week exposure period, and the total number of F2 hatchlings produced per adult F1 was reduced to 37% compared to the controls. However, and in contrast to the results observed for F0, hatchability increased with time, suggesting a possible acclimatization or adaptation of the F1 individuals. This finding might be confounded by the increase in temperature during some of the F1 exposure periods (Fig.2).

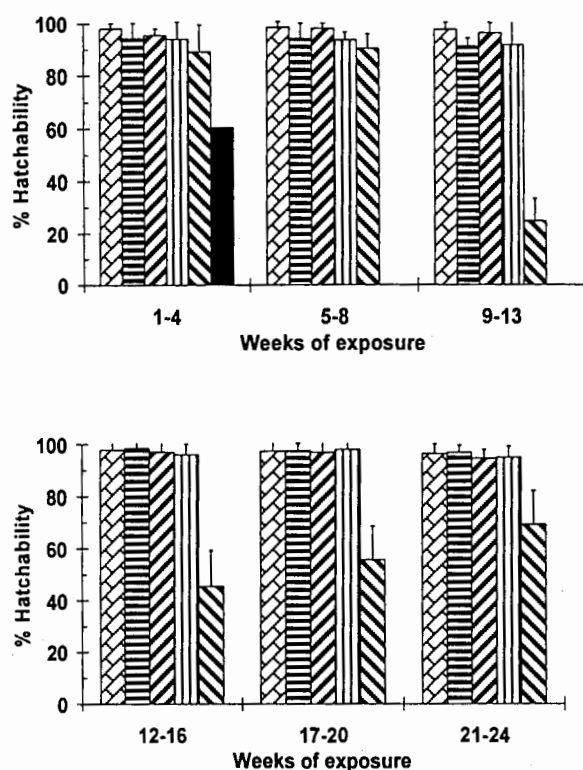


Figure 1. Hatchability of cocoons produced during different exposure periods, shown as accumulated percentages 8 weeks after first counting. a) F0 exposure, b) F1 exposure. Means $\pm$ SD of replicates.

Table 1 Dose rates and accumulated doses in F0 and F1 worms, are given as ranges due to the decay of ^{60}Co . Values are means $\pm$ SD (including 15% uncertainty).

Mean dose rate (mGy/h)	Accumulated dose (Gy)						
	F0			F1			
	Week 4	Week 8	Week 11	Week 13	Week 16	Week 20	Week 24
0.18 - 0.19	0.11 $\pm$ 0.02	0.23 $\pm$ 0.03	0.37 $\pm$ 0.05	0.39 $\pm$ 0.06	0.53 $\pm$ 0.08	0.63 $\pm$ 0.09	0.73 $\pm$ 0.11
1.7 - 1.8	1.1 $\pm$ 0.2	2.2 $\pm$ 0.3	3.6 $\pm$ 0.5	3.7 $\pm$ 0.6	5.0 $\pm$ 0.8	6.1 $\pm$ 0.9	7.0 $\pm$ 1.1
4.2 - 4.3	2.7 $\pm$ 0.4	5.4 $\pm$ 0.8	8.6 $\pm$ 1.3	9.0 $\pm$ 1	12 $\pm$ 2	15 $\pm$ 2	17 $\pm$ 3
11.2 - 11.5	7.1 $\pm$ 0.9	14 $\pm$ 2	23 $\pm$ 3	24 $\pm$ 4	33 $\pm$ 5	39 $\pm$ 6	45 $\pm$ 7
42.4 - 42.7	26 $\pm$ 4	53 $\pm$ 8	85 $\pm$ 13	-	-	-	-

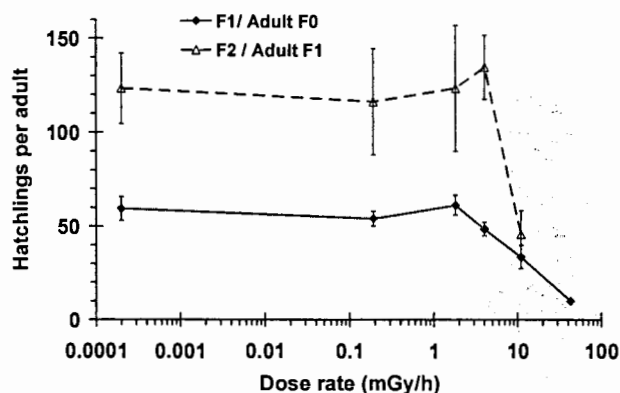


Figure 2. The total number of F1 hatchlings per adult F0, and F2 hatchlings per adult F1, produced during the 13 weeks reproduction periods. External dose to controls was 0.2 μ Gy/h. Values are means $\pm$ SD for replicate boxes. Control: $n = 12$; 0.19–11 mGy/h, $n = 4$; 43 mGy/h, $n = 1$.

3.2. RECOVERY

At 17 mGy/h, the hatchability of cocoons produced during week 7 and 8 was reduced to 6%, at which time the dose absorbed by the adult worms had reached 20 ± 3 Gy. None of the cocoons produced during week 9 to 10 hatched (absorbed dose 25 ± 4 Gy) (Fig. 3a). This was in good agreement with the results from the first experiment where there seemed to be a critical absorbed dose in F0 of around 20 Gy. Also the number of hatchlings per hatched cocoon and the numbers of F1 hatchlings produced per adult per week was significantly reduced during week 7-8 (Fig. 3b). At the end of the 10 week exposure period, the total number of F1 hatchlings produced per adult F0 was reduced to 51% compared to the controls. After transfer to the control area, none of the cocoons produced during the first 2 weeks by worms pre-exposed at 17 mGy/h area hatched, whereas after 3 to 4 weeks the hatchability increased slightly. A further increase was observed for cocoons produced during week 5 to 8, and during the next period the recovery seemed complete as the hatchability and the number of F1 hatchlings produced per adult F0 per week reached control levels. Furthermore, the number of F1 produced per adult F0 worm derived from the cocoons produced during week 13-16 was higher than for the controls.

4. Conclusion

In conclusion, the most sensitive endpoints examined in the current studies were the hatchability of cocoons and the number of hatchlings per cocoon. But relatively extensive exposure periods were needed for these effects to be expressed at lower dose rates. This illustrates the problems in extrapolating from acute to chronic studies, and the need for more ecologically relevant studies for understanding the potential impact of ionising radiation on non-human species. Short exposure times can lead to an

underestimation of the potential effects on such endpoints, and studies on reproduction in any species must be designed to avoid such errors.

a)

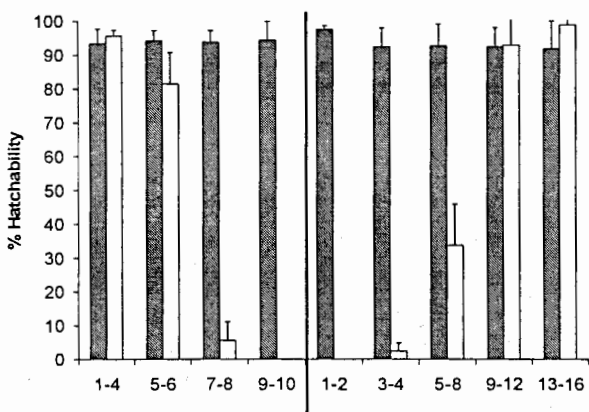
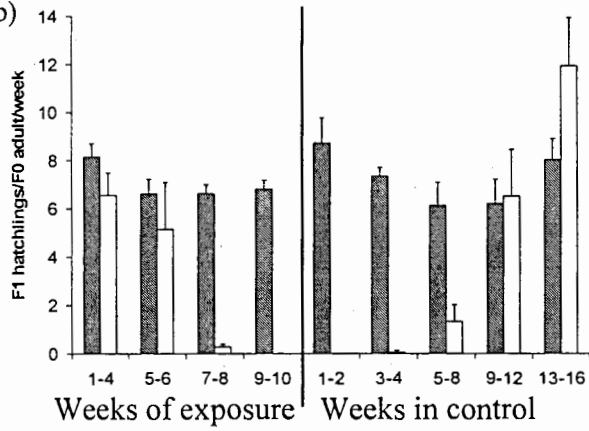


Figure 3. a) Hatchability of cocoons produced during different periods, shown as accumulated percentages 8 weeks after first counting. b) Number of F1 hatchlings produced per adult F0 per week derived from cocoons produced during the same periods. Means $\pm$ SD for replicate boxes. Controls, n = 3; 17 mGy/h n=3.

b)



The likely mechanism behind the reduction in hatchability is that damage is induced in male germ cells (i.e spermatogenic cells) directly, and/or that damage accumulates in the testes or seminal vesicles resulting in reduced sperm production or infertile sperm. In the F0 generation it appeared that inhibition of reproduction was related to a critical total accumulated dose of approximately 20 Gy. Although the lowest observed effect dose rates (4 mGy/h in F0 and 11 mGy/h in F1) were relatively high, they are comparable to those that may occur after major nuclear accidents. While earthworms showed the ability to regain their reproductive capacity after a complete radiation

induced inhibition of cocoon hatchability, more studies are required to better understand the underlying mechanisms.

Acknowledgment

This work was supported by the EU ERICA project (Contract: FI6R-CT-2004-508847).

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ASSOCIATION STUDY OF THE FREQUENCIES OF SPONTANEOUS AND INDUCED CHROMOSOME ABERRATIONS IN HUMAN LYMPHOCYTES*

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Abstract. The data on the frequencies of chromosome aberrations in lymphocytes of peripheral blood of 97 volunteers depending on genotypes by genes of xenobiotics detoxication before and after γ -irradiation with the dose of 1 Gy in vitro are presented. The frequencies of aberrations were estimated by analyzing not less than 500–1000 metaphases per person. The data of the cytogenetic analysis were compared with the results of PCR genotyping by the genes of xenobiotics detoxication (CYP1A1, CYP2D6, GSTM1, GSTT1, GSTP1, COMT, NAT2), genes of DNA repair (XRCC1, XPD, ERCC1, APEX1, RAD23B, OGG1, ATM) as well as by the genes of oxidative response and cell cycle regulation (SOD2, CAT, GCLC, MTHFR, Tp53). The frequency of spontaneous aberrations of chromosome type was reduced for homozygotes by the deletion of GSTM1 locus, especially for double homozygotes by the deletions of GSTM-GSTT1 genes. The mentioned frequency increased additively with the number of copies of the minor allele variants XPD*2251G and XPD*862A of gene of excision repair XPD ($p = 0.025$). The frequency of γ -induced chromosome aberrations proved to be elevated for the carriers of a minor allele OGG1*977G ($p = 0.011$). The significantly elevated number of γ -induced chromosome aberrations was also observed for the carriers of major alleles XRCC1*G1996 and XRCC1*C589 ($p = 0.002$). The frequency of γ -induced chromosome aberrations proved to be reduced for G/G homozygotes by a minor allele of poorly studied site CYP1A1T606G: 0.094 ± 0.006 against 0.112 ± 0.002 for the carriers of allele T ($p = 0.004$). The results of meta-analysis of the data of 14 works on the effect of genotype by GSTM1 locus on the frequency of cytogenetic anomalies in the control and exposed populations are discussed.

* Radiobiology and Environmental Security / Eds. C.Mothersill, V.Korogodina, C.Seymour. Springer, 2012. P.187-198

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Keywords: Genetic polymorphism, DNA repair genes, associative studies, chromosome radiosensitivity, gamma radiation in vitro, chromosome aberrations

1. Introduction

In this work, the results of our investigations (Sal'nikova, et al., 2009, 2010) aimed at the systematic search for genotypic associations of the radiosensitivity of human chromosomes are reviewed. These works are carried out using the bank of DNA of young healthy volunteers on the base of the previous thorough cytogenetic analysis of spontaneous and γ -induced (1 Gy *in vitro*) chromosome aberrations of blood lymphocytes. The existence of the DNA bank allows the recurrent control of new groups of polymorphic candidate genes for the presence of the association with the frequency and spectrum of chromosome aberrations.

It is well known that the revealed dependence of the radiosensitivity on the polymorphism of candidate genes often is not confirmed in other works. In our opinion, this is to a great extent related to a low statistical stipulation of the corresponding association studies (the analysis of no more than 100 metaphases per person for sampling of 20–60 donors). A distinctive feature of this work is the estimation of the frequencies of aberrations of chromosome type via scanning of many metaphase cells as well as a significant (for such studies) number of donors.

By now we have performed the search of genetic markers of the radiation risk among the genes of xenobiotics detoxication (*CYP1A1*, *CYP2D6*, *GSTM1*, *GSTT1*, *GSTP1*, *COMT*, *NAT2*), genes of DNA repair (*XRCC1*, *XPB*, *ERCC1*, *APEX1*, *RAD23B*, *OGG1*, *ATM*) as well as genes of oxidative response and cell cycle regulation (*SOD2*, *CAT*, *GCLC*, *MTHFR*, *Tp53*). All loci studied were characteristic of the functional polymorphism associated with the change in activity and/or in the amount of the corresponding enzyme as well as of associations with different biological effects and diseases.

2. Materials and Methods

The correlations of the frequencies of spontaneous and induced chromosome aberrations were studied with the samples of peripheral blood taken from 115 young (20–25 years old) healthy males (students of the Military Technical University, Balashikha). The investigation was sanctioned by the Ethic Commissions of the Institute of General Genetics, RAS and the Military Technical University.

The cytogenetic analysis was successfully carried out for 97 donors. Each sample of pure blood for the cytogenetic analysis was poured into two tubes: the first one was

exposed to γ -irradiation (Co^{60} , 1 Gy, dose rate of 1.37 Gy/min), and the second tube was used for analyzing the spontaneous chromosome aberrations.

The preparations with metaphase cells for estimation of chromosome aberrations were obtained by the standard method (Sal'nikova, et al., 2009). 500 (for γ -induced aberrations) or 1000 (for spontaneous aberrations) of metaphases of the first mitosis per person were analyzed. The differential assessment of aberrations of the chromosome type (dicentric and ring chromosomes, acentrics, atypical monocentrics) and of chromatid type (single and isochromatid fragments and exchanges) was carried out.

The genotyping was accomplished using the allele-specific tetraprimer PCR. The method allows the amplification of DNA fragments corresponding to the alternative alleles in one tube. The amplification products are separated via electrophoresis on an agarose gel without fluorescence labels. The studied polymorphic sites are listed in Table 1. In the case of deletion-insertion polymorphism (*GSTM1*, *GSTT1*) two genotypes were revealed: a "zero" one – homozygote by deletion (D/D) and a "positive" one carrying a functional allele in the homo- or heterozygote state (I/*). From here on * is related to an arbitrary allele.

The statistical analysis was carried out by the standard methods with a WinSTAT 2003.1 package integrated into Excel. All assessments of the group frequencies of aberrations were obtained as a result of the averaging of the individual frequencies for persons with the specific genotype. The corresponding errors reflected the intragroup variability of the frequencies of aberrations. For intergroup comparison, a nonparametric Mann–Whitney test was used.

3. Results

The frequencies of spontaneous and γ -induced chromosome aberrations for the carriers of different genotypes are given in Table 1. The distributions of allele frequencies for all loci studied were conform to the Hardy–Weinberg equilibrium and did not differ from the data for the groups of the residents of the Central region of Russia examined previously.

3.1. ASSOCIATION STUDIES OF SPONTANEOUS CHROMOSOME ABERRATIONS

Genotype by *GSTM1* locus had the most significant effect on the frequency of spontaneous chromosome aberrations. The frequencies of aberrations of the chromosome type for "positive" (I/*) and "zero" (D/D) genotype had very different distributions (Fig. 1c). In particular, in 57% persons, which are homozygote by *GSTM1* deletion, the cells with the aberrations of chromosome type were not found. For "positive" genotypes I/*, the corresponding index was equal to 27% (OR = 3.1; $p = 0.0095$ by the precise Fischer criterion). However, the differences between the average

Table 1. Average frequencies of spontaneous and induced aberrations per 100 cells ($\pm$ SE) for carriers of different genotypes. The cases of significant genotype differences are distinguished.

Loci and genotypes		Spontaneous aberrations			γ - induced aberrations (1 Gy in vitro)		
		#	Chromosome type aberrations	All aberrations	#	Chromosome type aberrations	All aberrations
CYP1A1	T/T	40	0.26 $\pm$ 0.04	0.94 $\pm$ 0.09	42	11.0 $\pm$ 0.3	12.1 $\pm$ 0.4
T606G	T/G	41	0.25 $\pm$ 0.05	0.87 $\pm$ 0.12	43	11.4 $\pm$ 0.3	12.9 $\pm$ 0.4
rs2606345	G/G	12	0.15 $\pm$ 0.06	0.68 $\pm$ 0.10	12	9.4 $\pm$ 0.6	10.4 $\pm$ 0.8
CYP1A1	T/T	81	0.24 $\pm$ 0.03	0.90 $\pm$ 0.08	84	11.1 $\pm$ 0.2	12.4 $\pm$ 0.3
T3801C	T/C	11	0.23 $\pm$ 0.07	0.74 $\pm$ 0.15	11	10.7 $\pm$ 0.5	12.0 $\pm$ 0.6
rs4646903	C/C	1	0.35	0.47	2	8.9 $\pm$ 2.9	9.8 $\pm$ 3.4
CYP1A1	A/A	88	0.24 $\pm$ 0.03	0.89 $\pm$ 0.07	91	11.0 $\pm$ 0.2	12.3 $\pm$ 0.3
A4889G							
rs1048943	A/G	5	0.29 $\pm$ 0.10	0.62 $\pm$ 0.10	6	10.3 $\pm$ 0.9	11.7 $\pm$ 1.1
CYP2D6 A1934G	G/A	28	0.27 $\pm$ 0.05	0.98 $\pm$ 0.11	28	11.3 $\pm$ 0.4	12.4 $\pm$ 0.5
rs3892097	G/G	65	0.23 $\pm$ 0.04	0.83 $\pm$ 0.08	65	10.8 $\pm$ 0.3	12.4 $\pm$ 0.3
GSTM1	D/D	35	0.20 $\pm$ 0.05	0.80 $\pm$ 0.09	39	11.2 $\pm$ 0.4	12.3 $\pm$ 0.4
	I/*	58	0.27 $\pm$ 0.03	0.92 $\pm$ 0.10	58	10.9 $\pm$ 0.3	12.3 $\pm$ 0.4
GSTT1	D/D	30	0.21 $\pm$ 0.05	0.97 $\pm$ 0.16	30	10.9 $\pm$ 0.4	12.2 $\pm$ 0.5
	I/*	62	0.26 $\pm$ 0.04	0.84 $\pm$ 0.07	67	11.0 $\pm$ 0.3	12.3 $\pm$ 0.3
GSTP1 A313G rs1695	A/A	47	0.24 $\pm$ 0.04	0.83 $\pm$ 0.08	48	10.8 $\pm$ 0.4	12.0 $\pm$ 0.4
	A/G	37	0.26 $\pm$ 0.05	0.87 $\pm$ 0.08	40	11.4 $\pm$ 0.3	12.7 $\pm$ 0.4
	G/G	9	0.17 $\pm$ 0.11	1.13 $\pm$ 0.49	9	10.5 $\pm$ 0.5	12.3 $\pm$ 1.2
COMT G1947A rs4680	A/A	23	0.28 $\pm$ 0.07	0.95 $\pm$ 0.12	26	11.5 $\pm$ 0.5	12.9 $\pm$ 0.5
	G/A	49	0.24 $\pm$ 0.04	0.80 $\pm$ 0.07	50	10.9 $\pm$ 0.3	12.1 $\pm$ 0.3
	G/G	21	0.21 $\pm$ 0.06	0.97 $\pm$ 0.22	21	10.6 $\pm$ 0.5	12.1 $\pm$ 0.7
NAT2	A/A	5	0.37 $\pm$ 0.16	0.92 $\pm$ 0.22	5	11.6 $\pm$ 1.1	12.6 $\pm$ 1.2
G590A	G/A	36	0.26 $\pm$ 0.05	0.94 $\pm$ 0.13	37	11.4 $\pm$ 0.4	12.9 $\pm$ 0.5
rs1799930	G/G	52	0.22 $\pm$ 0.04	0.83 $\pm$ 0.08	55	10.7 $\pm$ 0.3	11.8 $\pm$ 0.3
SOD2	C/C	27	0.22 $\pm$ 0.05	0.82 $\pm$ 0.10	28	10.7 $\pm$ 0.4	11.7 $\pm$ 0.4
C47T	C/T	43	0.24 $\pm$ 0.05	0.93 $\pm$ 0.12	45	11.3 $\pm$ 0.4	12.7 $\pm$ 0.4
rs4880	T/T	23	0.26 $\pm$ 0.05	0.84 $\pm$ 0.10	24	10.9 $\pm$ 0.5	12.1 $\pm$ 0.5
CAT	A/A	12	0.15 $\pm$ 0.08	0.88 $\pm$ 0.14	12	10.9 $\pm$ 0.5	12.2 $\pm$ 0.6
T21A	T/A	44	0.26 $\pm$ 0.09	0.84 $\pm$ 0.09	46	10.6 $\pm$ 0.3	11.8 $\pm$ 0.4
rs7943316	T/T	37	0.25 $\pm$ 0.05	0.92 $\pm$ 0.13	39	11.5 $\pm$ 0.4	12.9 $\pm$ 0.5
GCLC	C/C	78	0.25 $\pm$ 0.03	0.90 $\pm$ 0.08	81	11.1 $\pm$ 0.2	12.5 $\pm$ 0.3
C129T							
rs17883901	C/T	15	0.21 $\pm$ 0.06	0.74 $\pm$ 0.10	16	10.3 $\pm$ 0.6	11.4 $\pm$ 0.7

(continued)

(continued)

(continued)

Loci and genotypes		Spontaneous aberrations			γ - induced aberrations (1 Gy in vitro)		
		#	Chromosome type aberrations	All aberrations	#	Chromosome type aberrations	All aberrations
MTHFR C677T rs1801133	C/C	43	0.22±0.04	0.80±0.06	46	10.8±0.3	11.9±0.4
	C/T	41	0.29±0.05	1.02±0.13	42	11.4±0.3	12.8±0.4
	T/T	9	0.15±0.07	0.60±0.25	9	10.4±0.9	11.8±1.0
XRCC1 C589T rs1799782	C/C	84	0.21±0.03	0.82±0.07	87	11.10±0.23	12.41±0.28
	C/T	11	0.25±0.09	0.62±0.11	11	10.51±0.79	11.38±0.88
	T/T	1	0.00	0.68	1	7.80	9.00
XRCC1	G/G	44	0.23±0.04	0.88±0.12	45	11.53±0.31	13.02±0.41
G1996A	G/A	44	0.20±0.04	0.72±0.06	46	10.64±0.34	11.73±0.38
rs 25487	A/A	8	0.14±0.04	0.75±0.17	8	10.07±0.56	11.05±0.71
XPD	T/T	35	0.14±0.03	0.72±0.08	38	10.99±0.33	12.29±0.38
T2251G	T/G	44	0.23±0.04	0.79±0.11	44	11.04±0.34	12.28±0.44
rs13181	G/G	17	0.29±0.07	0.96±0.13	17	10.92±0.61	12.17±0.73
XPD	G/G	37	0.15±0.03	0.86±0.13	39	11.02±0.35	12.35±0.46
G862A	G/A	43	0.22±0.04	0.72±0.07	44	11.04±0.33	12.27±0.38
rs1799793	A/A	16	0.31±0.07	0.85±0.11	16	10.86±0.62	12.02±0.70
ERCC1	G/G	77	0.23±0.03	0.83±0.07	80	11.12±0.25	12.39±0.31
G262T	G/T	17	0.14±0.03	0.67±0.10	17	10.50±0.53	11.67±0.60
rs2298881	T/T	2	0.20±0.20	0.54±0.06	2	10.50±1.30	12.10±1.30
ERCC1	T/T	46	0.22±0.04	0.85±0.10	48	10.74±0.35	12.05±0.44
T354C	T/C	30	0.20±0.04	0.79±0.10	31	11.60±0.29	12.80±0.38
rs11615	C/C	20	0.20±0.05	0.67±0.09	20	10.70±0.51	11.94±0.59
APEX1 T444G rs1130409	T/T	35	0.24±0.05	0.77±0.06	35	10.90±0.40	12.09±0.45
	T/G	38	0.17±0.03	0.72±0.08	41	11.07±0.30	12.33±0.34
	G/G	23	0.23±0.05	0.94±0.20	23	11.03±0.53	12.42±0.73
RAD23B	C/C	75	0.20±0.03	0.82±0.07	78	11.09±0.25	12.40±0.30
C 746T	C/T	21	0.23±0.05	0.69±0.11	21	10.68±0.50	11.76±0.59
rs1805329							
OGG1 C977G rs1052133							
C/C	63	0.19±0.03	0.77±0.09	65	10.75±0.25	12.03±0.33	
C/G	27	0.24±0.05	0.81±0.08	28	11.16±0.48	12.33±0.53	
G/G	6	0.23±0.10	0.95±0.12	6	13.01±0.58	14.43±0.74	
G/G	49	0.22±0.04	0.78±0.05	51	11.00±0.31	12.11±0.34	
G/A	46	0.20±0.04	0.79±0.11	47	11.02±0.33	12.43±0.43	
G5557A rs1801516	A/A	1	0.10	1.60	1	10.40	1.80
Tp53	G/G	57	0.21±0.04	0.85±0.09	59	11.17±0.29	12.55±0.35
G215C	G/C	33	0.22±0.04	0.73±0.07	34	10.66±0.38	11.75±0.45
rs1042522	C/C	6	0.15±0.07	0.64±0.17	6	11.25±0.97	12.29±1.19

The cases of significant genotype differences are distinguished

frequencies of the aberrations of chromosome type proved to be at the limit of significance: 0.0020 ± 0.0005 for homozygotes D/D against 0.0027 ± 0.0003 for I/* ($p=0.049$ by the Mann–Whitney test).

An essentially reduced frequency of spontaneous aberrations of the chromosome type was observed in donors, which are double homozygotes by the deletions of *GSTM1-GSTT1* loci (11 persons). In this group, only 3 donors showed one aberration and 8 donors had no aberrations of the chromosome type. In double homozygotes by deletions, the differences in the average values comprise 0.0006 ± 0.0003 against 0.0027 ± 0.0003 for the rest of genotypes ($p = 0.018$ by the Mann–Whitney test).

The additive trend to an elevated frequency of aberrations of the chromosome type was observed in the carriers of minor alleles of gene *XPB* (excision repair of nucleotides – NER) in sites that were in linkage disequilibrium T2251G (Lys751Gln) and G862A (Asp312Asn). In this connection the regression analysis of the dependence of the frequency of chromosome aberrations on the total number of copies of minor allele variants *XPB**2251G and *XPB**862A was performed. Figure 2 shows the results of this analysis: the correlation coefficient for 96 observations $r = 0.228$ at $p = 0.025$. For group 4 (both sites are homozygote by minor alleles), the frequency of induced chromosome aberrations is significantly higher compared to group 0 (both sites are homozygote by major alleles): 0.0031 ± 0.0017 against 0.0014 ± 0.0003 at $p=0.026$ by the Mann–Whitney Test. The combinations with both major and minor homozygotes (i.e., 2251G/G-862G/G or 2251T/T-862A/A) were not found that results from a strong linkage disequilibrium of sites ($D' = 0.772$; $p = 2 \cdot 10^{-16}$).

Thus, the regression analysis allowed one to reveal the significant additive effects of minor alleles in two sites of *XPB* gene as to the frequency of spontaneous aberrations of the chromosome type, with the maximum effect being achieved in the carriers of double minor homozygotes. The residual loci showed no significant associations with the frequency of chromosome aberrations at the given level of sampling.

3.2. ASSOCIATION STUDIES OF INDUCED CHROMOSOME ABERRATIONS

The frequency of aberrations induced by γ -irradiation dose of 1 Gy *in vitro* was independent of genotype by *GSTM1* locus (Fig. 1d), but proved to be reduced for homozygotes G/G by a minor allele of gene *CYP1A1* T606G: 0.094 ± 0.006 against 0.112 ± 0.005 for the carriers of a major allele T ($p = 0.004$ by the Mann–Whitney test). The differences in the distributions of the frequencies of chromosome aberrations for these two genotypes are impressive (Fig. 1f). For the frequencies of spontaneous chromosome aberrations, the analogous tendency proved to be insignificant (Fig. 1e).

The significantly elevated level of induced aberrations of the chromosome type was revealed for homozygotes T/T by major allele of locus *CAT* T21A: 0.115 ± 0.004 against 0.106 ± 0.003 for the carriers of a minor allele A ($p = 0.0269$ by the Mann–Whitney test).

The increase in the frequency of induced aberrations of the chromosome type was associated with major variants of two sites of excision repair gene of bases (BER)

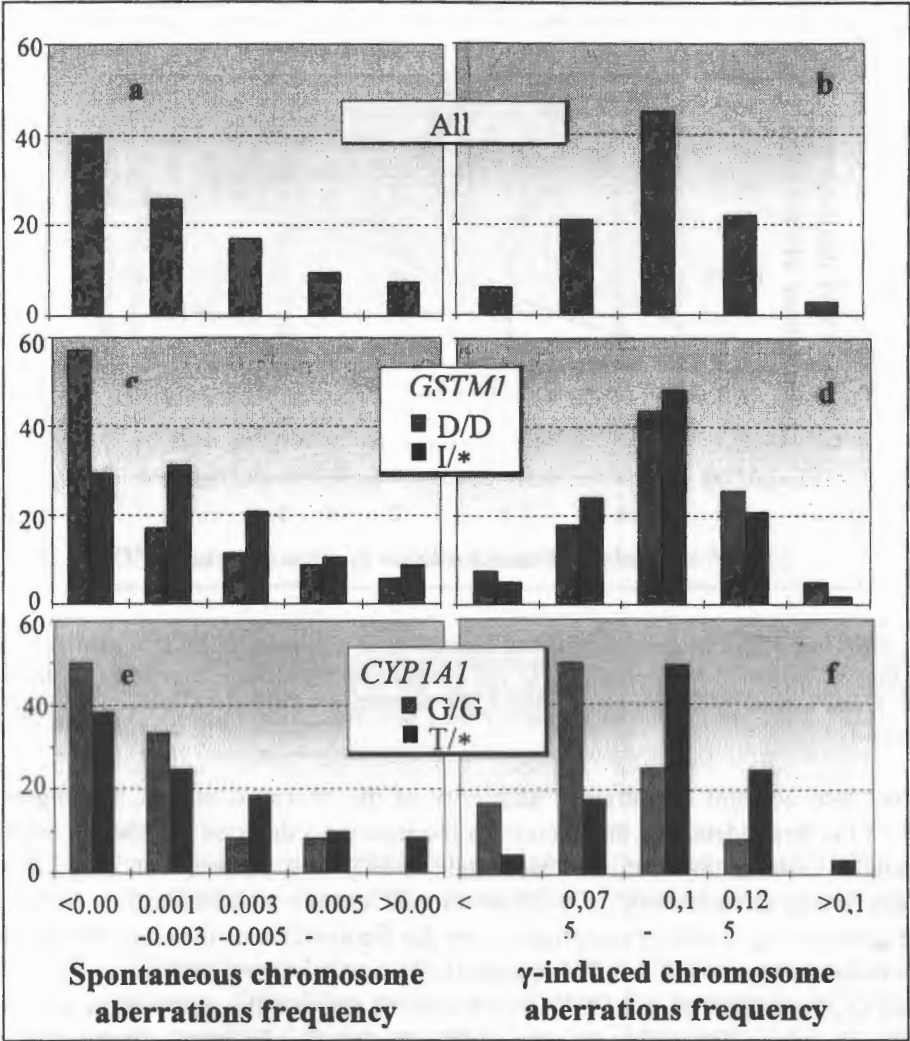


Figure 1. Distributions of frequencies of spontaneous and γ -induced aberrations of the chromosome type for all sampling (a, b) and for different genotypes by loci *GSTM1* (c, d) and *CYP1A1* T606G (e, f). In histograms (a, c, e), the extreme left group is composed uniquely of donors, in which spontaneous aberrations of the chromosome type were not found.

XRCC1: G1996A (Arg399Gln) and C589T (Arg194Trp). In spite of the strong linkage disequilibrium ($D' = 0.998$; $p = 0.006$), the statistically significant results were obtained for site G1996A only. In this case a major variant of site *XRCC1**G1996 was associated with the elevated frequencies of chromosome aberrations: 0.115 ± 0.003 against $0.106 \pm$

0.003 for the carriers of minor allele *XRCC1**1996A ($p = 0.007$). The analogous results were obtained for all types of aberrations: 0.0130 ± 0.004 against 0.116 ± 0.003 ($p = 0.006$). For other site of the same gene C589T (Arg194Trp), the effects were of the same type, but the differences were not significant.

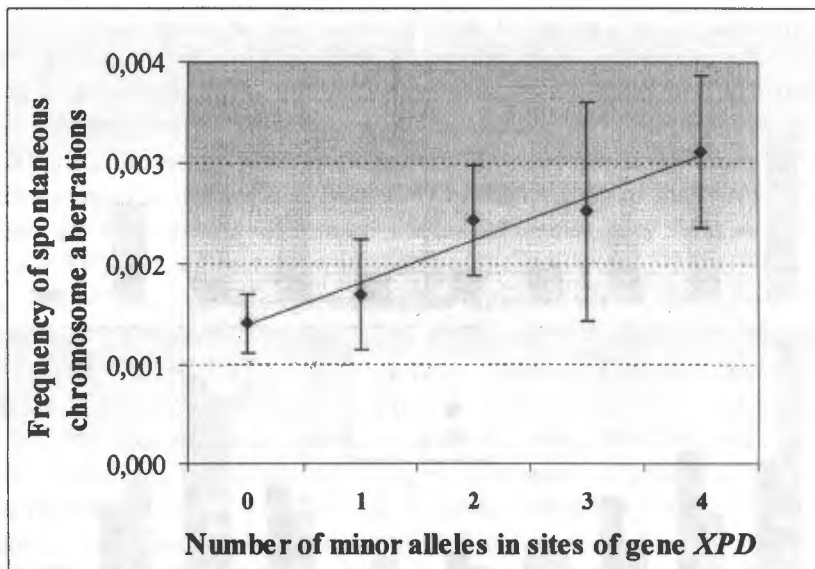


Figure 2. Frequency of spontaneous chromosome aberrations as a function of the total number of minor alleles in sites T2251G and G862A of gene *XPD*. The average aberrations frequencies ($\pm$ SE) for the carriers of 0, 1, 2, 3, and 4 minor alleles are given. Class 4, for example, is composed of double homozygotes by minor alleles.

Taking into account the obvious additivity of the observed effects, the regression analysis of the dependence of the aberration frequency on the total number of copies of minor allele variants in sites G1996A and C589T was carried out (Fig. 2). The regression line is characteristic of correlation coefficient $r = 0.303$ at $p = 0.002$. The extreme groups (Fig. 4) differ significantly by the frequencies of induced aberrations of the chromosome type: 0.117 ± 0.003 against 0.095 ± 0.005 ($p = 0.0008$).

Locus *OGG1* (BER) in site C977G (Ser326Cys) showed the association of a minor allele with increasing frequency of induced aberrations. The frequency of aberrations of the chromosome type for homozygotes by minor allele *OGG1**977G comprised 0.130 ± 0.006 against 0.109 ± 0.002 for the carriers of a major allele in homo- or heterozygote state ($p = 0.011$). For all aberrations, the corresponding differences were equal to 0.144 ± 0.007 against 0.121 ± 0.003 ($p = 0.024$). Most significant differences were observed for radiation-specific aberrations (dicentric and centric rings): 0.074 ± 0.005 for homozygotes by minor allele *OGG1**977G against 0.058 ± 0.001 for the rest of

genotypes ($p = 0.009$ by the Mann–Whitney test). For the frequencies of spontaneous aberrations, the analogous tendencies were not significant.

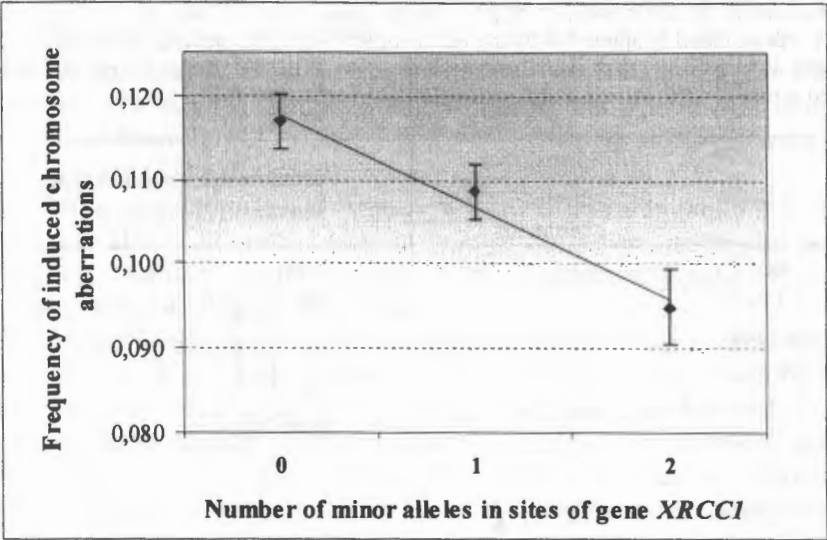


Figure 3. Frequency of induced chromosome aberrations as a function of the total number of minor alleles in sites G1996A and C589T of gene XRCC1. The average aberrations frequencies ($\pm$ SE) for the carriers of 0, 1, and 2 minor alleles are given. No carriers of 3 and 4 minor alleles were found.

4. Discussion

By now 24 polymorphic sites functionally associated with the chromosome damage are used in our study of radiosensitivity. The found effects are listed schematically in Table 2.

While on the subject of general tendencies, the following observations should be noted.

- 1) The association of the DNA polymorphism with the frequencies of cytogenetic damages is manifested to the greatest extent for aberrations of the chromosome type. In this case the significant associations of chromatide aberrations with the genetic polymorphism can be absent.
- 2) The frequencies of induced *in vitro* and spontaneous chromosome aberrations are associated with different groups of polymorphic genes.
- 3) The relative differences in the average levels of aberrations for the carriers of different genotypes are rather small: 10–20% for γ -induced aberrations ($p=0.002\pm0.01$) and 30–50% for spontaneous aberrations of the chromosome

type ($p = 0.02 \div 0.04$). The shifts and changes in the type of distributions of the aberrations frequency are seen to a much greater extent as the different genotypes are compared (Fig.1).

Table 2. Associations of allele variants of polymorphic genes with the frequency of aberrations of the chromosome type in blood lymphocytes by the results of this work and previous publications. The upward arrow indicates an elevated level of aberrations for the carriers of the specific genotype. The braces indicate the combined action of allele related to different sites

Locus	Spontaneous chromosome type aberrations	γ - induced chromosome type aberrations (1 Gy <i>in vitro</i>)
<i>XRCC1</i>		G/G $\uparrow$
G1996A	-	G/*
rs 25487		} $\uparrow$
<i>XRCC1</i>		C/*
C589T	-	($p=0.002$)
rs1799782		
<i>XPB</i>		
T2251G	*G $\uparrow$	-
rs13181	} $\uparrow$	
<i>XPB</i>		
G862A	*/A $\uparrow$	-
rs1799793	($p=0.025$)	
<i>OGG1</i>		
C977G	-	G/G $\uparrow$
rs1052133		($p=0.011$)
<i>CYP1A1</i>		
T606G	-	G/G $\downarrow$
rs2606345		($p=0.005$)
<i>GSTM1</i>	I/* $\uparrow$	
Ins-Del	($p=0.044$)	-
<i>CAT</i>		
T21A	-	T/T $\uparrow$
rs7943316		($p=0.017$)

- 4) The association of the DNA polymorphism with the frequencies of cytogenetic damages is manifested to the greatest extent for aberrations of the chromosome

type. In this case the significant associations of chromatide aberrations with the genetic polymorphism can be absent.

- 5) The frequencies of induced *in vitro* and spontaneous chromosome aberrations are associated with different groups of polymorphic genes.
- 6) The relative differences in the average levels of aberrations for the carriers of different genotypes are rather small: 10–20% for γ -induced aberrations ($p=0.002\div0.01$) and 30–50% for spontaneous aberrations of the chromosome type ($p = 0.02\div0.04$). The shifts and changes in the type of distributions of the aberrations frequency are seen to a much greater extent as the different genotypes are compared (Fig.1).
- 7) The differences between genotypes are often manifested while considering the combined effects of several sites in linkage disequilibrium related to the same gene, for example, as correlations of the aberrations number with the total number of minor alleles in these sites.
- 8) The elevated level of spontaneous chromosome aberrations is associated with the presence of minor allele variants (*XPD*, *GSTM1*). In this case the elevated radiosensitivity under irradiation *in vitro* is often associated with the presence of more common (major) allele variants (*XRCC1*, *CAT*, *CYP1A1*). *OGG1*, site C977G (Ser326Cys) was the unique gene, which showed the association of a minor rather than a major allele with the increasing frequency of induced aberrations.
- 9) Many polymorphic sites, for which an essential association with the radiosensitivity was suggested, did not show significant tendencies in the association with the frequency of aberrations (for example, *APEX*, *ATM*, *RAD23B*, *ERCC1*, *Tp53*).

In conclusion, the results of meta-analysis of the dependence of chromosome aberrations on genotype by locus *GSTM1* will be presented. The repeated attempts to find the association between the radiosensitivity and DNA polymorphism are related to this xenobiotics detoxication gene. It was suggested that the homozygotes by deletion (D/D – “zero” genotypes) are more radiosensitive than the carriers of a functional allele (I/* – “positive” genotypes). However, even in the first works on this subject (Karahalil et al., 2002; Marcon et al., 2003), the contradictory results were obtained.

For meta-analysis, the results of 11 studies of the control and/or exposed populations as well as the data of 3 works on the irradiation *in vitro* were chosen. Figure 4 shows the forest plot for the frequency ratio ($FR = p_1/p_0$), where p_1 and p_0 are the frequencies of cytogenetic disorders (aberrations of chromosomes or micronuclei) in carriers of “positive” and “zero” genotypes, respectively. The above data demonstrate no significant heterogeneity. The proportion of variation attributable to the heterogeneity (Higgins–Thompson I^2): $I^2 = 10.6\%$ (95%CI = 0.0–45.1%). According to the fixed-effect model the average value $FR (\pm SE)$ by overall data is equal to $1.03 \pm$

0.02 (95%CI = 0.98±1.07). Thus, by overall data, significant differences of the average *FR* from unity are not observed (two-tailed $p = 0.11$ for Z test).

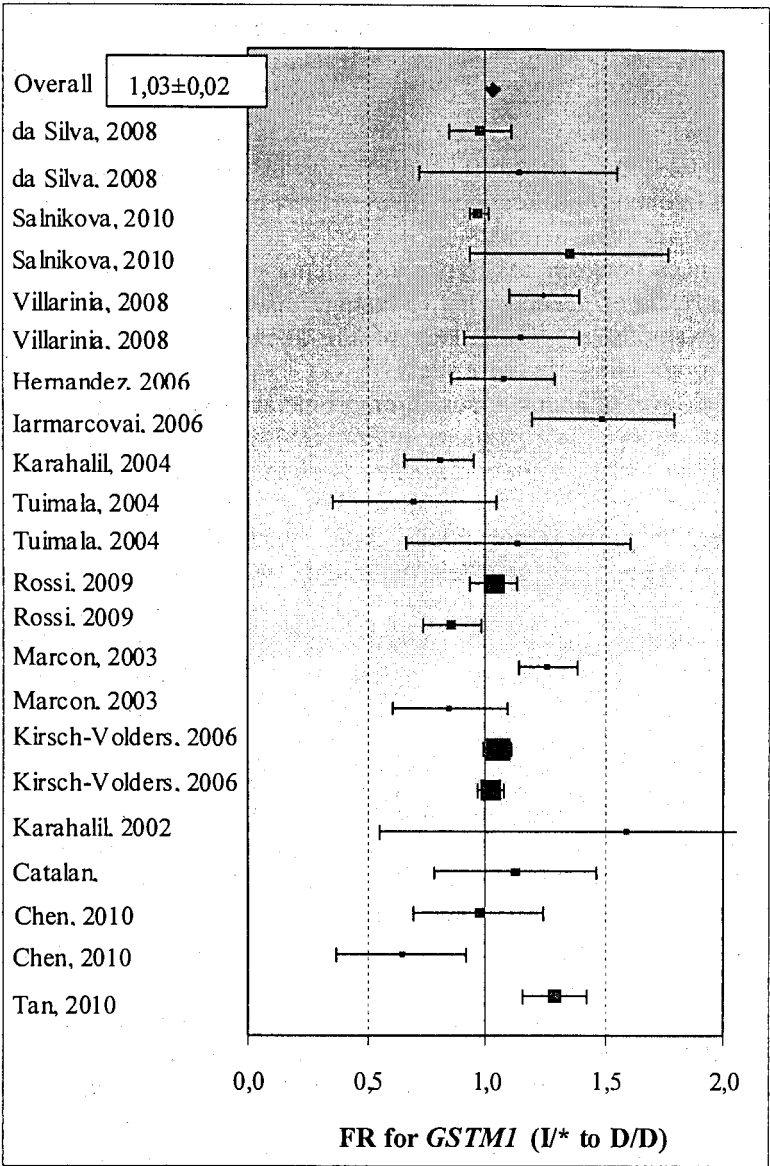


Figure 4. Meta-analysis of the data of 14 works (22 samplings) on the relationship of cytogenetic disorders in the carriers of different genotypes by gene *GSTM1*. The average values ($\pm$ SE) of the ratio of *FR* of cytogenetic effects for genotype I/* to the analogous value for D/D are given. The point size is proportional to the sampling volume.

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RADIATION AND TERROR*

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The best description of terrorism I have encountered is in this poem by Yeats, an Irish writer

The second coming

Turning and turning in the widening gyre
The falcon cannot hear the falconer;
Things fall apart; the centre cannot hold;
Mere anarchy is loosed upon the world,
The blood – dimmed tide is loosed, and everywhere
The ceremony of innocence is drowned;
The best lack all conviction, while the worst
Are full of passionate intensity.

-William Butler Yeats

This paper is presented to examine certain radiological aspects of terrorism, and to demonstrate that the perception of any event, and the consequent reaction to that perception, is more important than the reality of the event. Radiation, as a terrorist weapon, has been considered for a number of years. A report from 1969 (classified for 15 years) examined radiation sickness or death caused by surreptitious administration of ionizing radiation to an individual. It was initiated because of a suggestion that Alexander Dubcek, Czechoslovak Communist Party Leader, had radiation sickness due to radiation exposure or consumption of radioisotopes during his August 1968 captivity in Moscow.

This was not confirmed or denied by the report, which concluded that surreptitious administration of a lethal or sublethal dose to an individual was technically possible. It would however be technically difficult to achieve an immediately lethal dose, or for the subsequent illness to be undiagnosable, and at sub lethal doses the outcome would be uncertain. The final conclusion was "In view of the existence of other tried and true methods of political assassination, the use of ionizing radiation for this purpose would

* Radiobiology and Environmental Security / Eds. C.Mothersill, V.Korogodina, C.Seymour. Springer, 2012. P.67-70

seem unnecessarily cumbersome unless the enemy were to expect some compelling advantage peculiar to the administration of radiation". A striking example of this was the recent poisoning of the Russian security agent Litvinenko with Polonium 210 which served as a graphic and very public demonstration of the misfortune that could befall dissenters.

The object of terrorism is to strike terror into a general population, but the motives for terrorism may vary considerably. Simplistic terrorism, in historical terms, was primarily intended to cause regime change. Often the terrorism was an expression of alienation from the state apparatus of power, with disenfranchised intellectuals and middle classes giving some tacit support.

The success of terrorism in its primary objective, that of inducing a state of terror, means that it is useful to both revolutionaries, who wish to change the established order, and those who wish to consolidate power and enable more draconian powers for the existing government. There is also the possibility of a "third player" who may wish to manipulate events for financial or other reasons that may not be immediately obvious.

In terms of striking terror into a general population, a "dirty bomb" that ensures widespread radiological contamination is obviously the weapon of choice, and is aided by the fear of radiation that pervades most of the population. The bomb would have minor health effects, but would cause major panic, disruption and chaos. The panic caused would depend on the objectivity or sensationalism of the reporting, and the level of trust in the population of the government. The style of reporting would also to some extent depend on the trust of the media of the government, and so the argument could be made that the effect of terrorism in this context could depend entirely on the trust of the population in their government and institutions. There is no doubt that trust in government has eroded considerably over recent years. Consider the following quote from Lord Denning, a senior English judge.

Before his Lordship's court were the six men who had been convicted of the Birmingham pub bombings. They alleged that the West Midlands police had beaten them up, and were suing for damages. Denning realized that accepting the police had beaten them up meant accepting that the police had beaten false confessions out of them, which meant accepting that the police had framed innocent men for one of the worst IRA atrocities on the British mainland. 'This,' said Denning as he dismissed the case, 'is such an appalling vista that every sensible person in the land would say, "It cannot be right these actions should go any further."'

This amount of trust in the police or security forces now seems quaint and anachronistic. In modern times it is almost accepted that security forces will disregard the law, and abuse it when it suits their purposes.

It is also apparent that the extent of control over the media is important, and it is notable that most governments try to control at least certain aspects of communication, often under the cloak of "security." Control over the opinions of the media is important both prior to and after the terrorist event, as it allows a pre-determined response. An

example of this would be, "If a terrorist event occurs we will blame and retaliate against A even if we know A did not instigate or was involved in the terrorist event." The government can use the terrorist event to further its own policy objectives. Terrorism without objectives is anarchy. That is not to argue that the average person classified as a terrorist need necessarily be aware of the objectives, any more than a conventional soldier need know the plan of his generals. It does mean that in order to form a reasoned response the objectives have to be identified. This may change the question from "Who did this?" to "Who benefits from this?"

Another issue that arises in the context of a radiological dispersion device is that it is clearly designed to panic a population. If the population is panicked it becomes more vulnerable to secondary attacks, and dealing with the secondary attacks become more problematic. It also becomes more vulnerable to an authoritarian style government. It would be a challenge for any government not to feel itself pushed towards a more authoritarian response, which may actually be a response that was anticipated and hoped for. Perhaps the only logical response to a terrorist incidence is to stabilize the situation (calm, prevent further casualties) and then assess the situation in depth.

In terms of public policy it might be prudent to legislate in terms of hormetic models rather than linear (no threshold) models. The absolute certainty that any dose of X is harmful is not only scientifically wrong, it is unnecessarily alarmist. Everybody is aware that food is good, unless too much is eaten in which case it becomes bad. By not encouraging the same views for most substances, fear is encouraged because "any dose of radiation is harmful." As we become more regulated as societies, fear becomes a more pervasive weapon for governments to exercise control over the population. It could be argued that over-regulation itself leads to a fearful society, willing to take only sanctioned risks. If the society is fearful, any attacks designed to create fear will be more effective. It is ironic that in trying to control the population more closely the government both emulates and makes terrorism more effective. This quote from Neil Addison, Director of the Thomas More Legal Centre, eloquently supports this conclusion;

"Whether it is fox hunting, smoking, adoption agencies or microchips in rubbish bins, we are a society that is increasingly intolerant, repressive, regulated and untrusting and, in consequence, we have officials who are dictatorial, interfering and untrustworthy."

In this climate terrorism becomes more effective as a weapon.

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**ЛЕТАЛЬНОЕ ДЕЙСТВИЕ ИОНИЗИРУЮЩИХ ИЗЛУЧЕНИЙ,
РАЗЛИЧАЮЩИХСЯ ПО ВЕЛИЧИНЕ ЛИНЕЙНОЙ ПЕРЕДАЧИ ЭНЕРГИИ
(ЛПЭ), НА КЛЕТКИ БАКТЕРИЙ *ESCHERICHIA COLI* K12 РАЗНОГО
РЕПАРАЦИОННОГО ГЕНОТИПА**

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Abstract. The results of investigation of the biological efficiency of accelerated electrons with the energy of 7,5 MeV on their lethal action on the cells of *Escherichia coli* K-12 bacteria with the different reparation genotype are presented. The received results are discussed from the position of existing representations regarding the role of the balance of work of reparation enzymes in definition of distinctions in radiosensitivity of studied cells strains of bacteria to the action of an ionizing radiation of different quality.

Ключевые слова: электроны, бактерии, выживаемость, репарационный генотип.

Введение

Проблема относительной биологической эффективности излучений разного качества и защиты организма от их повреждающего действия всегда была

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ключевой задачей радиобиологии. С началом космической эры эта задача стала еще более актуальной. В космическом пространстве были обнаружены высокие уровни доз различных типов ионизирующих излучений, характеризующиеся широким зарядовым составом от электронов и протонов до ядер урана, энергетическим диапазоном от десятков эВ до 10^{18} – 10^{19} эВ и спектром линейной передачи энергии (ЛПЭ), определяющим характер локального воздействия на клетки и ткани биообъектов, от десятых долей кэВ/мкм до $2 \cdot 10^3$ кэВ/мкм [1].

В секторе биологических исследований ОИЯИ (г. Дубна) были проведены многоплановые эксперименты по моделированию радиационно-генетических эффектов космических излучений на пучках ускоренных тяжелых ионов, и выяснению механизмов, определяющих различия в биологической эффективности излучений, различающихся по ЛПЭ, на клетки разного генотипа. Сформулированы представления о том, что биологическая эффективность излучений разного качества определяется как физическими особенностями микрораспределения энергии разных типов излучений в генетических структурах клетки, в результате чего индуцируется широкий спектр первичных повреждений ДНК, так и биологическим фактором, направленным на репарацию этих повреждений. Снижение репарационной способности клеток с увеличением ЛПЭ, а соответственно и возрастание биологической эффективности, в значительной степени связаны с индукцией нерепарируемых прямых двойных разрывов ДНК, играющих ведущую роль в летальном действии плотноионизирующих излучений [2-3].

Эти исследования в ОИЯИ были проведены как на клетках низших и высших эукариот, так и прокариот. Мы принимали участие в экспериментах ОИЯИ, выполненных на бактериальных клетках [4]. Как логическое продолжение вышеуказанных работ нами было проведено исследование биологической эффективности ускоренных электронов по их летальному действию на клетки бактерий *Escherichia coli* K-12 разного репарационного генотипа.

В работе использованы следующие штаммы бактерий *E. coli* K-12, любезно предоставленные нам В.Н. Вербенко из коллекции ПИЯФ РАН: дикий тип АВ 1157 (thr-1 leu-6 pro A2 his-4 arg E3 lac Y1 gal K2 ara-14 xyl-5 mtl-1 tsx-33 str A31 sup E37); γ -резистентный мутант BL 1114 (Gam^r 444); радиочувствительный мутант АВ 2463 (rec A13).

Выращивание бактериальных культур проводили на полноценной питательной среде УЕР (дрожжевой экстракт – 10 г/л, пептон – 10 г/л, натрий хлористый – 10 г/л, агар-агар – 20 г/л) или МПА (мясная вода, пептон – 10 г/л, натрий хлористый – 5 г/л, агар-агар – 20 г/л) до стационарной фазы роста. Разведения клеточной суспензии для контрольных и облучаемых проб готовили в 0,85%-ом физиологическом растворе с таким расчетом, чтобы в каждой чашке выросло от 100 до 300 колоний. Облучение клеток электронами в монослое на поверхности “голодного агара” проводили на микротроне МК-7,5 Ереванского Физического

Института. Энергия электронов – 7,5 МэВ. Значение ЛПЭ – 0,3 кэВ/мкм. Мощность дозы 25 Гр/мин. Радиационные характеристики электронного пучка определяли на основе “An International Codes of Practice” IAEA TRS 277 [5], с помощью электрометра UNIDOS (PTW, Freiburg, Germany) и ионизационной камеры фермеровского типа 30001 с объемом камеры 0,6 см³, откалиброванных в SSDL IAEA. Мощность дозы определяли в воздухе с помощью камеры фермеровского типа снабженной колпачком из полиметилметакрилата.

Рентгеновскими лучами клетки облучали на установке РУП-200-20-5 (нефильтрованное излучение, напряжение на трубке – 200 кВ, сила тока – 14 мА, мощность дозы – 30 Гр/мин). Специально поставленные эксперименты показали, что способ облучения клеток (в монослое на поверхности “голодного агара” или в суспензии) не влиял на величину радиочувствительности клеток.

В качестве источника α -частиц использовали плоский α -источник ²³⁹Pu. Мощность дозы 22 Гр/мин. Облучение проводили в монослое на поверхности “голодного агара”.

Облучение клеток всеми типами излучений проводили при комнатной температуре. Выживаемость клеток определяли подсчетом макроколоний, вырастающих на среде УЕР через 24-48 часов при температуре 37⁰С. Опыты повторяли 3-5 раз. Стандартная ошибка определения средних значений выживаемости клеток, как правило, не превышала 5-10%.

Результаты и обсуждение

На рисунке 1 представлены результаты экспериментов по облучению клеток дикого типа, суперрезистентного и чувствительного мутантов *E. coli* электронами с энергией 7,5 МэВ.

Как можно видеть, у данных штаммов бактерий радиочувствительность клеток, значительно различается. В варианте опыта, когда клетки дикого типа до облучения выдерживались в физиологическом растворе в течение двух часов при комнатной температуре, радиочувствительность их несколько увеличивается, но различия значений чувствительности клеток дикого типа и чувствительного мутанта сохраняются.

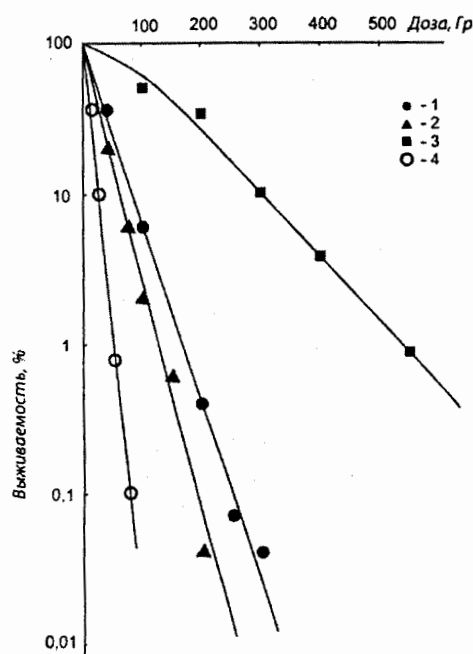


Рис. 1. Кривые выживания клеток бактерий *E. coli* K-12, облученных электронами: АВ 1157 – обычные условия облучения (1), АВ 1157 – 2-х часовое выдерживание в физиологическом растворе (2), BL 1114 (3), АВ 2463 (4).

По оси абсцисс – доза облучения, Гр; по оси ординат – выживаемость, %.

Ранее нами было показано, что уже в первые часы выдерживания клеток бактерий *E. coli* в так называемых «непитательных» средах, в зависимости от генотипа и исходной концентрации клеток, может наблюдаться как увеличение, так и уменьшение числа жизнеспособных клеток [6]. Исходя из вышесказанного, для уточнения данных по модификации радиочувствительности клеток дикого типа в условиях предрадиационного выдерживания их в «непитательной» среде, мы провели наблюдения по регистрации числа жизнеспособных клеток данного штамма, внесенных в физраствор в разных концентрациях (табл.1).

ТАБЛИЦА 1. Число жизнеспособных клеток *E. coli* K-12 дикого типа при выдерживании их в физиологическом растворе в разных концентрациях при комнатной температуре

Время инкубации, часы	Число жизнеспособных клеток, кл/мл				
0	$2,0 \cdot 10^3 \pm 0,21$	$3,0 \cdot 10^4 \pm 0,22$	$1,8 \cdot 10^5 \pm 0,12$	$4,2 \cdot 10^6 \pm 0,31$	$5,8 \cdot 10^7 \pm 0,23$
2	$2,1 \cdot 10^3 \pm 0,11$	$3,2 \cdot 10^4 \pm 0,20$	$1,7 \cdot 10^5 \pm 0,21$	$4,3 \cdot 10^6 \pm 0,28$	$6,0 \cdot 10^7 \pm 0,30$

Как следует из таблицы, в данных условиях опыта число клонообразующих клеток дикого типа не меняется.

Кривые выживания вышеуказанных клеток бактерий *E. coli* K-12 с разным репарационным генотипом при облучении их рентгеновскими лучами и α -частицами приведены на рис 2 и 3.

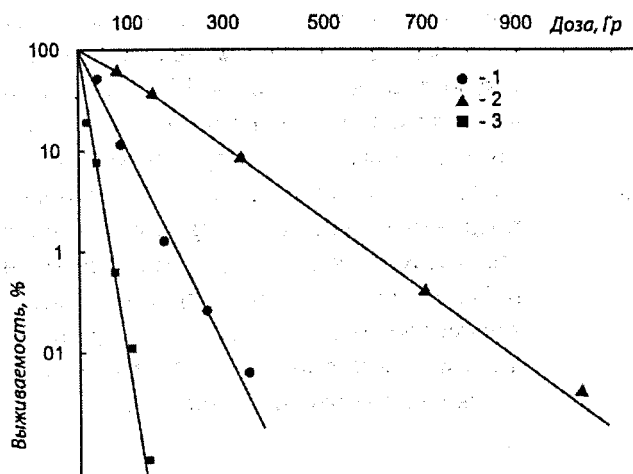


Рис. 2. Кривые выживания клеток бактерий *E. coli* K-12 штаммов АВ 1157 (1), BL 1114 (2), АВ 2463 (3), облученных рентгеновскими лучами.

По оси абсцисс — доза облучения, Гр; по оси ординат — выживаемость, %.

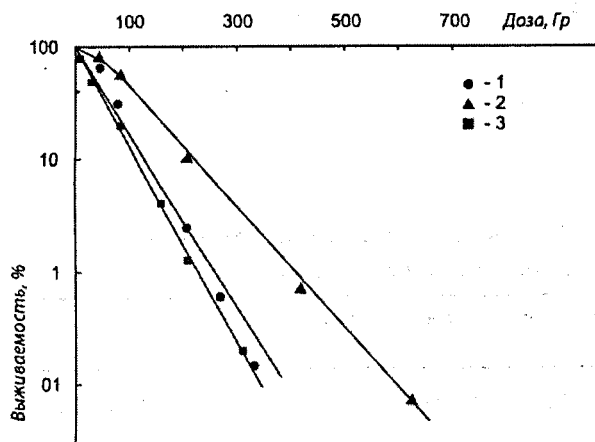


Рис. 3. Кривые выживания клеток бактерий *E. coli* K-12 штаммов АВ 1157 (1), BL 1114 (2), АВ 2463 (3), облученных α -частицами.

По оси абсцисс — доза облучения, Гр; по оси ординат — выживаемость, %.

В таблице 2 даны значения D_0 кривых доза-эффект клеток дикого типа и репарационных мутантов, представленных на рисунках 1-3.

ТАБЛИЦА 2. Значения D_0 кривых выживания клеток бактерий *E. coli* K-12, облученных ионизирующими излучениями разного типа.

Штамм	Вид ионизирующего излучения	Мощность дозы, Гр/мин	ЛПЭ, кэВ/мкм	D_0 , Гр
AB 1157	Электроны	25	0,3	$40 \pm 2,1$
	Рентгеновское излуч.	30	2,5	$44 \pm 1,5$
	Альфа-частицы	22	110	$52,5 \pm 0,9$
BL 1114	Электроны	25	0,3	$100 \pm 6,2$
	Рентгеновское излуч.	30	2,5	$115 \pm 5,6$
	Альфа-частицы	22	110	$77,7 \pm 6,3$
AB 2463	Электроны	25	0,3	$12 \pm 1,0$
	Рентгеновское излуч.	30	2,5	$15,8 \pm 1,0$
	Альфа-частицы	22	110	$50,4 \pm 2,5$

Из таблицы следует, что наибольшие различия в радиочувствительности клеток разного генотипа наблюдаются при облучении их рентгеновскими лучами и электронами. С увеличением ЛПЭ излучений, в данном случае при облучении α -частицами, имеет место нивелирование чувствительности всех использованных в эксперименте штаммов *E. coli* K-12.

Известно, что решающую роль в летальном действии излучений на клетки играют одностранные (ОР) и двунитевые разрывы (ДР) ДНК. Различают два типа ДР ДНК: прямые двунитевые разрывы (ПДР) и энзиматические двойные разрывы ДНК (ЭДР), возникающие из перекрывающихся брешей при расщеплении повреждений ДНК в результате действия репарационных ферментов. Реализация как одного ЭДР, так и ПДР ДНК в геноме клеток *E. coli* K-12 дикого типа и суперрезистентного мутанта приводит к летальному исходу. Выход ЭДР ДНК генетически детерминирован и определяется балансом нуклеазной и полимеразной активности репарационных ферментов. Большая устойчивость клеток суперрезистентного мутанта к излучениям с низкими значениями ЛПЭ объясняется лучшей координацией работы репарационных ферментов, приводящей к уменьшению выхода ЭДР ДНК. Низкая радиоустойчивость чувствительного мутанта связана с тем, что для клеток данного штамма губительна реализация не только ДР, но, преимущественно, и ОР ДНК. С возрастанием ЛПЭ излучений выход ЭДР падает, а ПДР резко увеличивается и становится преобладающим в суммарном выходе ЭДР и ПДР: в диапазоне значений величины ЛПЭ $\sim 150-200$ кэВ/мкм практически все ДР являются ПДР ДНК.

Таким образом, поскольку выход ЭДР ДНК с возрастанием ЛПЭ снижается пропорционально уменьшению выхода ОР, то уменьшаются и различия радиочувствительности изогенных мутантов *E. coli* K-12, обусловленные генетическим дефектом в системе репарации клеток одного мутанта (AB 2463), либо, наоборот, эффективной работой восстановительных систем клеток другого мутанта (BL 1114).

Авторы выражают глубокую благодарность М.Л. Петросяну и Л.А. Габриэляну за предоставление возможности, обеспечения условий и техническое содействие в проведении экспериментов на Микротроне МК-7,5, а также В.Н. Вербенко за предоставление штаммов *E. Coli*.

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DIRECT AND REVERSE BYSTANDER EFFECT BETWEEN IRRADIATED AND UNIRRADIATED ORGANISMS: THE MODULATING ROLE OF CHEMOSIGNALLING IN ECOLOGY*

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Abstract. It was shown that mice or rats exposed to sublethal dose of ionizing radiation are able to decrease the immune reactivity of intact animals when they kept together in the same cage. Even one individual can cause such a disturbances of immunity in a group of intact animals. The data indicate that at early stage after exposure to sublethal doses of ionizing radiation mice secrete volatile components (VC) in urine which decreased thymus dependent humoral immune response in intact animals (to 60-70% relative control). The stable effect observed as a result of one-day exposure allowed us to study the time course of secretion of immunosuppressive VC by irradiated animals. These VC possess attractive properties for the intact individuals. The irradiated mice showed an increased attractiveness to intact individuals. The biological significance of a combination of the immunosuppressive and attractive effects of VC is unclear. It is supposed, that mammals possess of the distant immunomodulating chemosignal system, directed for immunoreactivity of individuals with immunodeficiency state.

It was established that VC of intact mice restored the humoral immune response and other parameters of immunity in irradiated with a dose of 1 Gy animals. In this case, the irradiated recipients demonstrated an increase of humoral immune response to 140-170%. These VC of intact mice activated at irradiation mice phagocytic activity peritoneal macrophages. Exposure of rats for the third day after irradiation (1 Gy) to the VC of intact animals significantly increased the number of red blood cells, lymphocytes and granulocytes in the bloodstream. Thus, direct and reverse bystander chemosignaling between the irradiated and intact, irradiated and irradiated animals mediate the modification of immunity and behavioral reactions of recipients. Apparently these untargeted effects of radiation spreading from one individual to another can have a significant impact on the viability of the entire population of animals.

Keywords: ionizing radiation; bystander effect; chemosignaling; immunity

* Radiobiology and Environmental Security / Eds. C.Mothersill, V.Korogodina, C.Seymour. Springer, 2012. P.99-110

1. Introduction

As established previously, exposure to ionizing radiation, stress, immunodepressants induced in laboratory animals excretion with urine absent in norm volatile components (VC). Exposure of these VC to intact conspecifics reduced their immunoreactivity and the number of hemocytes (Surinov and Dukhova, 2004; Surinov, Isaeva and Dukhova, 2005). It was found later that such excretions have the properties of chemosignals attracting intact animals.

Combination of attractive and immunosuppressive properties inherent to VC was not explained in terms of biological feasibility a long time. In the published data has been reported only about the role of signaling in natural physiological conditions (Beauchamp and Yamazaki, 2003; Beauchamp and Yamazaki, 2003; Hurst and Beynon, 2004), in particularly (Novikov, 1988), in dissartotive mating of animals relevant for the viability of offspring or for limitation contact of healthy and infected individuals (Moshkin et al., 2002; Penn and Potts, 1998; Kavaliers and Colwell, 1995).

In the literature we are not found any information about chemosignalling induced by ionizing irradiation in animals with the exception of works of authors of this paper (Surinov and Dukhova, 2004; Surinov, Isaeva and Dukhova, 2005; Surinov, 2007; .

Meanwhile the advantage of use of such damage as ionizing radiation allowed much deeper to explore the issue of chemocommunication in pathological state and detect a number of previously unknown facts. For example, it was found that the secretion of attractive or aversive chemosignals depended on the dose of ionizing radiation and period after radiation injury (Surinov, 2007). As result is the assumption that the attractive chemosignals induced by sublethal doses of radiation provide with a communication aimed at improving the viability of the animals, whereas the aversive signals appeared in terminal period after exposure to lethal doses of radiation, probably, like an infection, may play a role in limitation of propagation of pathology in the group of animals (Surinov, Isaeva and Karpova, 2008).

This paper presents the results of further study the stimulating effect of urinary volatiles of irradiated and intact animals on the antibody forming capacity of individuals with reduced immune reactivity induced with sublethal dose (1 Gy) of ionizing radiation, or on these parameters in animals immunized with thymus dependent antigen.

2. Materials and Methods

2.1. ANIMALS AND PROCEDURES

Laboratory male mice of the inbred CBA strain and hybrids F1 (CBA x C57Bl/6), 2-3 months old (25-30g) where kept in standard plastic boxes by groups of 5-6 per box with

ad libidum food at room temperature (20-22°C) under a 14 light: 10h dark cycle. (In certain experiments are used the Wistar rats.

The whole-body irradiation of the animals was carried out with doses of 1 or 4 Gy using a ^{60}Co source with a dose-rate of 3,2 mGy/sec on "Gamma-Cell-220" equipment (Atomic Energy Canada Limited, Canada).

Donors urine volatile components (VC) were intact and irradiated with a dose 4 Gy individuals.

To obtain urine samples at the bottom of the box was placed sheet of filter paper (bedding). Access to this bedding was limited by a performed screen elevated above the bottom at 0.5 cm. Paper bedding containing of absorbed 72hs urine of irradiated or intact mice were transfer for one day in box with radiation at a dose of 1 Gy recipients under the mesh screen.

2.2. IMMUNOLOGICAL INVESTIGATIONS

To study the influence VC in the development of the immune response papers with urine samples were placed in boxes to the immunized animals. In 24hs after exposure with bedding animals were injected intraperitoneally with 0,2 ml of suspension of sheep red blood cells (SRBC) in dose 1×10^8 cell/mouse in medium 199. In 4 days mice were decapitated of under ester narcosis and determined the mass and cellularity of the spleen. The number of antibody forming cells (AFCs) in the spleen was determined by Cunningham method. In each of the studied groups was 5-7 individuals.

2.3. PHAGOCYTOSIS

Effect of VC on the phagocytic activity of peritoneal macrophages of mice-recipients was assessed by their ability to absorb latex particles of diameter 1.5 microns (firm DIA*M). Mice were decapitated under ether anesthesia. The suspension of macrophages obtained by washing the abdominal cavity 199 medium supplemented with 10% fetal calf serum. The reaction of phagocytosis set by mixing 1 ml of suspension of macrophages in a concentration 10^6 cells/ml with 1 ml of latex suspension at a concentration of 10 ul/ml. The mixture was incubated for 30 min at 37°C. Smears were fixed 30 min in 96% ethyl alcohol and dyed with Romanovsky-Giemsa. Counted the number of absorbed latex particles per phagocytic macrophage (phagocytic index).

Experiments were reproduced at least three times.

Statistic analysis of results we performed with use of Student's t-test.

3. Results and Discussion

3.1. IMMUNOLOGICAL INVESTIGATIONS

The ionizing radiation with 1 Gy causes suppression of immune reactivity in mice. Thus, three days after irradiation of animals, significantly reduced spleen weight, cellularity and the number of AFC induced by challenge with SRBC (Table 1). As a result of exposure of these mice during the third day after irradiation to VC of intact syngeneic animals led to the fact that the spleen cellularity and the number of AFC significantly exceeded these parameters in the irradiated animals not exposed to VC. Almost the same effects were observed after exposure to VC of individuals irradiated with a dose of 4 Gy.

A similar effect of these VC in the recipient mice on day 7 after irradiation (1 Gy) was also accompanied by a significant increase in the ability to the immune response to antigen (Table 1). In this case, unlike the effect observed on day 3 after irradiation, change in spleen mass and cellularity not detected.

We evaluated the immune response in mice irradiated with a dose of 1 Gy, at different times after their exposure to VC of intact or irradiated with Gy of individuals (Table 2).

As a result of exposure of these mice to VC of intact animals during the third day after exposure to ionizing radiation the spleen cellularity and the number of AFC was significantly higher than these parameters in the group of irradiated mice which is not exposed VC.

TABLE 1. Immunological parameters ($M \pm m$) in irradiated 1Gy male mice F1 (CBAx57Bl6) after exposition to volatile components (VC) of urine of intact mice in different time post irradiation periods

Groups of animals	Time after irradiation, days	Spleen		
		Mass, mg	Numer of cells, 1×10^6	AFC, 1×10^3
Control	-	130 ± 6.5 (100 ± 5.0)	189 ± 12.3 (100 ± 6.5)	121 ± 9.0 (100 ± 7.4)
Irradiated 1Gy	3	$94.7 \pm 3.8^*$ (72.8 ± 2.9)	$137 \pm 8.4^*$ (72.5 ± 4.4)	$52 \pm 3.1^*$ (42.9 ± 2.6)
Irradiated 1Gy + VC intact mice		$82.8 \pm 2.5^{**}$ (63.7 ± 1.9)	$134 \pm 14^*$ (70.9 ± 7.3)	$61.7 \pm 2.6^{**}$ (50.9 ± 2.2)
Irradiated 1Gy	7	110 ± 3.8 (84.6 ± 2.9)	164 ± 14 (86.8 ± 7.4)	50 ± 2.2 (41.2 ± 1.8)
Irradiated 1Gy + VC intact mice		105 ± 5.2 (80.8 ± 4.0)	145 ± 13 (77.2 ± 6.3)	$66.2 \pm 2.4^{**}$ (54.6 ± 2.0)

Note: in brackets - % to control

* - significant different ($p < 0.05$) from control;

** - significant differences ($p < 0.05$) from 1Gy.

TABLE 2. Dynamics immunological parameters ($M \pm m$) in irradiated 1Gy male mice CBA after one-day exposition to volatile components (VC) of urine of intact or irradiated (4Gy) syngenic mice in during the third day post irradiation period

Groups of animals	Time after exposition, days	Mass of spleen, mg	Number of cells at spleen, 1×10^6	AFC in spleen, 1×10^3
Irradiated 1Gy		101 $\pm$ 2.3 (100 $\pm$ 2.3)	80.0 $\pm$ 7.1 (100 $\pm$ 8.9)	104 $\pm$ 2.9 (100 $\pm$ 2.8)
Irradiated 1Gy + VC intact mice	1	83.6 $\pm$ 4.3* (82.8 $\pm$ 4.3)	83.3 $\pm$ 3.4 (104 $\pm$ 4.3)	126 $\pm$ 6.0* (121 $\pm$ 5.8)
Irradiated 1Gy+ VC irradiation 4Gy mice		86.0 $\pm$ 3.3* (85.1 $\pm$ 3.3)	103 $\pm$ 3.4* (129 $\pm$ 4.3)	125 $\pm$ 15.0 (120 $\pm$ 14.4)
Irradiated 1Gy		90.4 $\pm$ 1.8 (100 $\pm$ 2.0)	74.0 $\pm$ 5.1 (100 $\pm$ 6.9)	67.4 $\pm$ 9.0 (100 $\pm$ 13.4)
Irradiated 1Gy + VC intact mice	3	83.4 $\pm$ 5.2 (92.3 $\pm$ 5.8)	84.0 $\pm$ 9.8 (114 $\pm$ 13.2)	96.7 $\pm$ 6.0* (143 $\pm$ 8.9)
Irradiated 1Gy+ VC irradiation 4Gy mice		99.0 $\pm$ 4.0 (110 $\pm$ 4.4)	82.0 $\pm$ 12.4 (111 $\pm$ 16.8)	113 $\pm$ 6.8* (168 $\pm$ 10.0)
Irradiated 1Gy		83.4 $\pm$ 2.7 (100 $\pm$ 3.3)	90.0 $\pm$ 3.2 (100 $\pm$ 3.5)	85.0 $\pm$ 6.7 (100 $\pm$ 7.9)
Irradiated 1Gy + VC intact mice	7	102 $\pm$ 2.8* (122 $\pm$ 3.4)	116 $\pm$ 17.5 (129 $\pm$ 19.4)	183 $\pm$ 4.5* (215 $\pm$ 5.3)
Irradiated 1Gy+ VC irradiation 4Gy mice		115 $\pm$ 3.8* (138 $\pm$ 4.6)	104 $\pm$ 5.1* (115 $\pm$ 5.6)	195 $\pm$ 10.4* (229 $\pm$ 12.2)

Note: in brackets - % to irradiated mice; * - significant differences ($p < 0.05$) from irradiated 1Gy.

Within 3 days after exposure to VC of irradiated with 1Gy mice, showed a significant, approximately 1.5-fold, increasing of the number of AFC in the spleen.

In more remote periods, within 7 days after exposure, effects of VC on irradiated mice increased – the spleen cellularity and the number of AFC in was more than 2 times higher than that in animals not exposed to VC.

During this observation period is also increased as the mass and cellularity of the spleen, especially after exposure to VC produced by mice irradiated with 4 Gy.

Therefore, the single exposure of the irradiated with 1Gy mice to VC of of intact or irradiated (4 Gy) was accompanied by stimulation of the immunity. The effect not only on long-preserved, but strengthened over time.

The above data rather differ from previously described ones with respect to the impact of a single exposure with the same VC, but at different times after irradiation of the mice with a dose of 1 Gy.

In this case, stimulation of humoral immunity in the irradiated with the same dose of mice was less pronounced.

This confirms the above data relating to the development of immunopotentiating effect in the course of time after exposure of irradiated animals to VC.

These data are in fact the opposite to results obtained in the process of studying of the immune reactivity of intact mice exposed to VC produced by mice after exposure to 4 Gy. In the latter case, there was inhibition of humoral immunity, which lasted more than three days.

In one series of experiments were simultaneously evaluated the impact of VC produced by male mice-CBA after exposure to radiation with 4 Gy (3 days), on intact animals and irradiated with 1 Gy.

It was found that as a result of exposure of VC with intact or subjected to radiation with 1 Gy mice, there was a decrease or increase the number of AFC in the spleen up to $62.0 \pm 5.6\%$ ($p < 0.05$) and up to $95.6 \pm 17.5\%$, respectively.

In irradiated at a dose of 1 Gy mice not exposed to VC the number of AFC in the spleen was $58.3 \pm 1.9\%$ ($p < 0.05$) compared with the intact animals ($100 \pm 2.6\%$).

Therefore, postradiation VC have different directions in the action depending on the state of recipients. It should be emphasized that the described here immunostimulatory effect was revealed at relatively weakly expressed immunodeficiency, as in this case at deficiency caused by irradiation with a dose of 1 Gy.

Similar experiments, but with mice irradiated with a dose of 4 Gy, resulted in irregular results and in case of exposure to animals with 6 Gy immunostimulatory effect was not found.

Ability of chemosignals produced intact mice to increase the immune reactivity of irradiated animals also demonstrated on a model which has previously been used to confirm the immunosuppressive effect of irradiated mice to intact when the irradiated and intact animals in equal proportions contained in one box.

Assessment of the capacity to antibody genesis of the irradiated with a dose of 2 Gy male mice (10 animals in each group) contained during the two weeks together with intact animals showed that the number of AFC in the spleen in 1.4 times ($P < 0.05$) higher than that of in only irradiated mice kept separately (table 4).

Immunostimulatory effect observed under the combined housing in one box intact mice with individuals irradiated at a higher dose (4 Gy) was unstable and not reproduced in all series of experiments.

The presence in urine of chemosignals recovering under certain conditions relatively not too deep post-radiation damage was confirmed by experiments with rats in which investigated the number of formed elements in peripheral blood after exposure to the VC (Table 5). Exposure of irradiated (1 Gy) of rats during the third day of post-radiation period to VC of intact or irradiated animals caused quantitative changes in the cellular composition of blood.

Thus, three days after irradiation (1Gy) the number of red blood cells in rats was $7.75 \pm 0.24 \times 10^{12}$ /liter. Exposure of such animals with the components of irradiated or intact rats increased in recipients compared to 1.2 times.

Thus, three days after irradiation (1Gy) the number of red blood cells in rats was $7,75 \pm 0,24 \times 10^{12}$ /liter. Exposure of such animals with the components of irradiated or intact rats increased in recipients compared to 1.2 times.

Influence VC of the intact or irradiated (4Gy) animals on the intact or the irradiated (1Gy) rats also resulted in increase of the absolute number of blood lymphocytes and polymorphonuclear leukocytes, respectively, 1,7 and 1,9 times (Table 3).

TABLE 3. Cell structure of peripheral blood ($M \pm m$) irradiated in the dose 1 Gy (3 day) rat Wistar after exposition to VC intact or irradiated (4Gy) animals

Groups of animals	Erythrocytes, $1 \times 10^{12}/l$	Leucocytes, $1 \times 10^9/l$	Lymphocytes, $1 \times 10^9/l$	Neutrophils, $1 \times 10^9/l$
Irradiated	7.75 ± 0.24 (100 $\pm$ 3.1)	10.7 ± 1.4 (100 $\pm$ 13.0)	3.2 ± 0.27 (100 $\pm$ 8.4)	2.4 ± 0.45 (100 $\pm$ 18.8)
Irradiated + VC intact rat	$9.65 \pm 0.24^*$ (123 $\pm$ 3.1)	14.1 ± 1.5 (132 $\pm$ 14.0)	$5.5 \pm 0.87^*$ (172 $\pm$ 27.0)	$4.54 \pm 0.61^*$ (189 $\pm$ 25.4)
Irradiated + VC irradiated rat	$9.4 \pm 0.48^*$ (121 $\pm$ 6.2)	9.8 ± 0.7 (91.6 $\pm$ 6.5)	3.4 ± 0.3 (106 $\pm$ 9.4)	2.8 ± 0.18 (117 $\pm$ 7.5)

Note: * - significant differences ($p < 0,05$) from groups irradiated rat not exposition with VC.

TABLE 4. Immunological parameters ($M \pm m$, in brackets - % to control) in irradiated (1Gy) male mice CBA after co- contents during the third day with intact male

Groups of animals	Spleen		
	Mass, mg	Numer of cells, 1×10^6	AFC, 1×10^3
Intact	113 ± 5.6 (100 $\pm$ 5.0)	106 ± 6.8 (100 $\pm$ 6.4)	182 ± 8.3 (100 $\pm$ 4.6)
1Gy	$76.6 \pm 3.8^*$ (67.8 $\pm$ 3.4)	$72.0 \pm 2.0^*$ (67.9 $\pm$ 1.9)	141 ± 19.3 (77.5 $\pm$ 10.6)
1Gy + intact male	$86.4 \pm 3.8^*$ (76.5 $\pm$ 3.4)	$108 \pm 8.6^{**}$ (102 $\pm$ 8.1)	$228 \pm 32.4^{**}$ (125 $\pm$ 17.8)

Note: in brackets - % to intact control; * - significant differences ($p < 0,05$) as compared with intact animals;

** - significant differences ($p < 0,05$) as compared with irradiating animals.

TABLE 5. Immunological parameters ($M \pm m$, in brackets - % to control) in intact male mice CBA after co- contents during the third day with irradiated male

Version experiment	Mass of spleen, mg	Numer of cells at spleen, 1×10^6	AFC in spleen, 1×10^3
Control - content with intact individual	$104 \pm 1,7$ (100 $\pm$ 2,1)	$132 \pm 9,0$ (100 $\pm$ 7,1)	$90,1 \pm 4,9$ (100 $\pm$ 11,2)
Experiment - content with irradiated individual	$95,3 \pm 2,1$ (92,1 $\pm$ 2,0)	$107 \pm 6,4$ (81,1 $\pm$ 5,0)	$74,1 \pm 4,6$ (82,8 $\pm$ 6,0)*

Note: in brackets - % to irradiated mice; * - significant differences ($p < 0,05$) from control.

Among the findings of particular interest is the fact that the immunostimulatory effect on irradiated mice is intrinsic not only VC that are contained in the urine of mice, but also the intact individuals themselves. It was found that the presence even one intact male-mouse in the group of irradiated mice (1 Gy within three days) increased their immune reactivity (Table 4), and presence in group of intact animals only one irradiated male depressed their immune reactivity (Table 5). Hence, it is probably of VC produced by one animal is sufficient to demonstrate their chemosignal properties aimed at mobilizing the immune system.

In one series of experiments studied the effect of post-radiation VC on animal induced by non-radiation factors. As one of these models has been elected immune response to foreign antigen.

This antigens were sheep red blood cells (SRBC). To some extent this is model of response to infection. Mice recipients were exposed to VC gave off irradiated (4 Gy) individuals up to 1 day, immediately, 3 and 7 days after injection of SRBC.

The number of AFC in the spleen was evaluated 4 days after injection of antigen. As a result of exposure to post-radiation VC before the antigenic stimulus the number of AFC in the spleen decreased to $30.4 \pm 6.5\%$ relative to controls.

Such reaction is fully consistent with previously obtained data showing the immunosuppressive properties of post-radiation VC excreted in the urine of irradiated animals [2]. After exposure, VC to the recipient mice immediately after the administration of antigen an immune response is not changed. In that case, when the recipients were exposed to VC through 1, 3 or 7 days after immunization, the number of AFC increased compared with the control to 193 ± 11.0 , 122 ± 27.7 and $263 \pm 60.7\%$, respectively.

The presented here results of effects VC of irradiated mice on individuals before and after immunization showed the dual nature of the studied chemosignals – the direction of their influence on syngeneic animals depends on the state of the immune system. If before the immunization was observed suppression then after immunization - stimulation of the humoral immune response.

3.2. PHAGOCYTIC ACTIVITY

Study of the effect of VC on the phagocytic activity of peritoneal macrophages of irradiated mice showed (Table 7) that phagocytic index (number of particles absorbed by one of phagocytic macrophages) in mice-recipients after exposure to VC of intact animals increased in 1,4 times in comparison with irradiated unexposed animals.

This information is also, as in the case of antibody genesis, opposite the previously obtained data showing the inhibitory effect of post-radiation VC on phagocytosis.

induced by various factors, and individuals whose reaction to the signals depends on their physiological state.

Our data allow to revise the view at some biological traits of the bystander effect at the interorganismal level (Mothersill, and Seymour, 2001; Mothersill et al. 2007; Surinov, 2007).

TABLE 6. Immunological parameters ($M \pm m$) at male mice F1 (CBAxCS7Bl6) after exposition to volatile components (VC) irradiated (4Gy) individuals in different time relatively immunization

Groups of animals	Time exposition, day	Spleen		
		Mass, mg	Numer of cells, 1×10^6	AFC, 1×10^3
Control	1 day before immunization	95.4 $\pm$ 3.3	122 $\pm$ 9.8	23.0 $\pm$ 0.5
Exposition to VC irradiated		(100 $\pm$ 3.5)	(100 $\pm$ 8.0)	(100 $\pm$ 2.2)
		102 $\pm$ 3.9	141 $\pm$ 8.0	7.0 $\pm$ 1.5 *
		(107 $\pm$ 4.0)	(116 $\pm$ 7.0)	(30.4 $\pm$ 6.5)
Control	Immediately after immunization	119 $\pm$ 3.7	143 $\pm$ 5.0	27.5 $\pm$ 3.1
Exposition to VC irradiated		(100 $\pm$ 3.1)	(100 $\pm$ 3.5)	(100 $\pm$ 11.3)
		104 $\pm$ 6.4	135 $\pm$ 14.0	30.8 $\pm$ 2.9
		(87.4 $\pm$ 5.0)	(94.4 $\pm$ 9.8)	(112 $\pm$ 10.5)
Control	1 day after immunization	103 $\pm$ 5.0	142 $\pm$ 13.0	46.6 $\pm$ 2.9
Exposition to VC irradiated		(100 $\pm$ 4.9)	(100 $\pm$ 9.2)	(100 $\pm$ 6.2)
		102 $\pm$ 4.8	147 $\pm$ 10.2	89.9 $\pm$ 5.0 *
		(105 $\pm$ 5.0)	(93.4 $\pm$ 6.5)	(193 $\pm$ 11.0)
Control	3 day after immunization	77.4 $\pm$ 2.6	106 $\pm$ 6.0	18.0 $\pm$ 3.8
Exposition to VC irradiated		(100 $\pm$ 3.4)	(100 $\pm$ 5.7)	(100 $\pm$ 21.0)
		74.6 $\pm$ 1.9	83.3 $\pm$ 6.8 *	22.0 $\pm$ 5.0
		(96.4 $\pm$ 2.5)	(78.6 $\pm$ 6.4)	(122 $\pm$ 27.7)
Control	7 day after immunization	77.2 $\pm$ 1.9	112 $\pm$ 4.9	9.5 $\pm$ 1.5
Exposition to VC irradiated		(100 $\pm$ 2.4)	(100 $\pm$ 4.4)	(100 $\pm$ 16.0)
		76 $\pm$ 2.1	88.0 $\pm$ 6.7 *	25.0 $\pm$ 6.0 *
		(98.4 $\pm$ 2.7)	(78.6 $\pm$ 6.0)	(263 $\pm$ 60.7)

Note: in brackets - % to control; * - significant different ($p < 0.05$) from control

TABLE 7. The phagocytic activity ($M \pm m$) of peritoneal macrophages of irradiated (1 Gy; 3 dais) mice after exposure to the volatile components of urine of intact or irradiated (4 Gy) of individuals

Animals group	Phagocytic index
Intact	9.0 ± 0.4
Irradiated	$13.2 \pm 1.0^*$
Irradiated + VC of intact individuals	$18.5 \pm 0.6^{* **}$
Irradiated + VC of irradiated individuals	$12.8 \pm 1.2^*$

Note: * - significant different ($p < 0,05$) from control;

** - significant different ($p < 0,05$) from irradiated.

If previously considered that such an effect caused by unknown of post-radiation VC were directed only at the suppression of immunity, the above data demonstrate also the opposite effect - the impact VC of intact animals on irradiated individuals resulted in the restoration of immunity. The such phenomenon (immunostimulation) was observed also and after exposure VC to animals after antigenic administration.

Hence, in our experiments we found that the orientation of the immunomodulatory effect of chemosignals depended on time of exposure of VC relatively of the effects of ionizing radiation or antigenic stimulus, i.e. the physiological condition of the recipient. This manifests itself most clearly in respect of post-radiation effects chemosignals induced by exposure to radiation with a dose of 4 Gy, which causes immunosuppression in intact animals, but stimulate the immune response in individuals immunized with an antigen, or irradiated with low doses (1 Gy) of radiation.

Under certain conditions, the immunosuppressive chemosignals for intact animals have regenerating and stimulating activity in relation to immunocompromised by the radiative and nonradiative effects individuals.

Therefore, bystander effect in groups of animals can have both suppressive and repaired consequences. Dependence of these effects upon the state of the donor chemosignals, and individuals-recipients can evaluate them as manifestation of biologically advisable of mechanisms that allow animals with chemosignaling to recognize individuals with disturbance of immunity and provide them by stimulating and repairing effects.

4. Conclusion

The presented research findings demonstrate the ability of animals to produce chemosignals with previously unknown properties. Under certain conditions, they show the regenerating or stimulating activity in relation to other individuals who have a disturbance of immunity resulting from radiation and nonradiation effects. Therefore, bystander effect in groups of animals can have both suppress and repaired consequences. The direction of immunomodulatory effect is depends upon the status of

immune system of donors and recipients of chemosignals. It is probably argue for existence of biologically advisable mechanisms that allow animals using the chemosignaling to recognize individuals with immunity disorders and provide them a stimulating and repaired effect.

This work was supported by the Russian Foundation for Basic Research and the Government of Kaluga Region project No 09-04-97505.

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INTER-PLANT COMMUNICATION OF GENOME INSTABILITY IN RADIATION EXPOSED ARABIDOPSIS*

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Abstract. Bystander effect is the phenomenon of the response of naïve bystanding cells or organisms to the stress experienced by neighboring cells or organisms. It is well documented for animal cells grown in vitro, and there are some reports of similar response on the level of the whole organism and even between stressed and non-stressed organisms. Here we present the evidence of the existence of communication between stressed and non-stressed plants. We show that naïve plants neighboring plants exposed to X-ray or UVC exhibit similar increase in the frequency of homologous recombination as exposed plants. We present the evidence that communication signal is primarily airborne.

1. Introduction

‘Bystander effects’ is a term originally used in cancer therapeutics, and refers to the attempt at affecting a single type of cell within a heterogeneous population resulting in several types of cells being affected by the treatment (Freeman, et al., 1993; Morgan 2003a). Now the term bystander effect has been applied to a number of different phenomena whereby unexposed ‘units’ exhibit the molecular symptoms of stress exposure when adjacent or nearby ‘units’ are a subject to a stress. Indeed, a unit seems a peculiar subject; however, the unit here can be referring to neighboring cells, systemic cells, or even entire organisms.

Modern bystander effects are non-targeted effects of ionizing radiation exposure. They refer to naïve cells that were either in direct contact with irradiated cells or received an irradiation ‘distress’ signals from irradiated cells (Zhou, et al., 2000; Morgan, et al, 2002; Morgan 2003a; Morgan 2003b; Mothersill and Seymour, 2003; Mothersill and Seymour, 2004; Mothersill and Seymour, 2006; Morgan and Sowa, 2007). In these instances, bystander effects can include a wide variety of genetic alterations such as gross genome rearrangements, chromosome aberrations, sister chromatid exchanges, deletions, duplications, gene mutations, and amplifications (Zhou, et al., 2000; Huo, et al., 2001; Zhou, et al., 2002a; Zhou, et al., 2002b; Suzuki, et

* Radiobiology and Environmental Security / Eds. C.Mothersill, V.Korogodina, C.Seymour. Springer, 2012. P.87-98

al., 2003; Klovov, et al., 2004; Smilenov, et al., 2006; Hamada, et al., 2007). With further influences such as changes in gene expression, cellular proliferation, senescence, and cell death (Sawant, et al., 2001; Sawant, et al., 2002; Liu, et al., 2006; Lyng, et al., 2006; Sedelnikova, et al., 2007).

Other bystander-like effects are plasma- or blood-borne signals produced upon irradiation that direct chromosomal breakage and abnormalities in unirradiated tissues. These factors have subsequently been termed clastogenic factors (Mothersill and Seymour, 2001; Mothersill and Seymour, 2004; Morgan and Sowa, 2007). Similar to clastogenic factors are abscopal effects, in which radiation-induced changes occur outside the targeted area of irradiation, often in different organs (Mothersill and Seymour, 2001; Morgan, 2003a, Morgan and Sowa, 2007). A classic example is the bilaterally symmetric radiation-response, where only the left or right organ is exposed, but similar responses occur in both organs (Mothersill and Seymour, 2001; Morgan, 2003a; Koturbash, et al., 2006).

Bystander-like effects have also been shown in separate organisms. This can include the transgenerational transmission of radiation effects to offspring, especially in terms of 'bequeathing' genomic instability to cellular (Mothersill and Seymour, 2001; Morgan, 2003b; Morgan and Sowa, 2007) or organismal (Barber and Dubrova, 2006) progeny. Radiation-induced bystander phenomena have been shown to occur between cohabiting animals as well (Mothersill, et al., 2006; Mothersill, et al., 2007).

As such, bystander and bystander-like effects are known to be involved in a number of different processes in different organisms. The commonality lies in non-effected 'units' receiving signals from effected 'units' to produce a response that modifies homeostasis in the non-effected 'units.'

The field of radiation-induced bystander effects in plants, in the sense of cell-cell media transfer experiments, has yet to be explored. However, abscopal bystander-like effects are well known to occur in plants under a variety of biotic or abiotic stresses. The local application of biotic or abiotic stresses to plant tissue can lead to systemic changes in pathogen resistance (Grant and Lamb, 2006), methylation pattern (Boyko, et al., 2007), recombination rate (Filkowski et al., 2004), hormone levels (Schilmiller and Howe, 2006) and gene expression (Truman, et al., 2006). UV-triggered intra-plant bystander effect was shown to be in part dependent on the free radicals as application of radical scavenger prior to UVC irradiation resulted in bystander signal of substantially lower intensity (Filkowski et al., 2004). The first published work referring to ionizing radiation-induced abscopal effects in plants occurred in 2007 (Yang, et al., 2007). This laboratory demonstrated that direct and specific radiation to the shoot apical meristem of embryonic *Arabidopsis* with 1000 α -particles resulted in post-embryonic developmental defects in root formation.

Plants have long been known to communicate to one another via diffusible signals, or volatile organic compounds. This phenomenon was first discovered several decades ago when it was observed that non-herbivore attacked (bystander) plants residing next

to herbivore-attacked plants were emitting the same defensive signals and activating the same defensive machinery as the attacked plants (Baldwin, et al., 2006). The reason for this signalling, or eavesdropping as some would put it, has a number of purposes including the priming of defences before the arrival of the herbivore, the readying of pathogen defences to disease, or to attracting predators or parasitoids of the organisms causing stress (Jiménez-Martínez, et al., 2004; Baldwin, et al., 2006). Interestingly, some of the phytohormones implicated in these plant-plant signals are similar to the hormones involved in both the systemic acquired resistance and systemic wound signalling. Again, this plant-plant communication of damage or impending attack could be considered bystander-like phenomenon.

One of the signatures of the influence of bystander signal is the genome instability, including the increase in the homologous recombination frequency (HRF). Homologous recombination in plants plays dual roles as both a double-strand break repair mechanism as well as the mechanism responsible for increased genetic diversity during meiosis (Boyko, et al., 2007). In our laboratory and others, it has been shown that plants treated with ionizing radiation have increased levels of homologous recombination (Ries, et al., 2000; Filkowski, et al. 2004; Molinier, et al., 2005; Boyko, et al., 2006a; Molinier, et al., 2006). Further experiments have even shown that the local leaf treatment with UV-C leads to increased recombination rates in both the local and systemic leaves (Filkowski, et al., 2004). This systemic recombination signal (SRS) was also found in pathogen- (Kovalchuk, et al., 2003) and reactive oxygen stress-treated plants (Filkowski, et al., 2004).

It is possible that the SRS, as well as some of the other systemic signals, are transmitted not through the plant vasculature, which is the common perception, but through the immediate gaseous environment of the plant. If this was indeed the case, plants exposed to ionizing radiation with increased recombination rates may transmit the signal to neighbouring plants in a closed environment.

In this work, we show that plants neighboring X-ray- and UVC-irradiated plants exhibit similar changes in recombination frequency. We show that the bystander effect has two components: liquid, released through roots and airborne, released by leaves

2. Methods

2.1. PLANT LINES AND GROWTH CONDITIONS

For the experiments on liquid media, sterilized *Arabidopsis thaliana* (cv. 24) line 11 seeds were spread on sterile Whatman No. 1 filter paper in a divided (Fisher) or undivided (Fisher) Petri plates. A total of 4 mL of liquid MS media was added to the plates (2 x 2 mL in divided plates). In the solid media experiments, the same sterilized seeds were spread on divided or undivided Petri plates containing 25mL of solid MS

media (liquid media recipe plus 8 g/L agar (Sigma). The plates were stratified for 48 hours at 4°C, and then grown in Enconair (Winnipeg, Canada) growth chambers at 16/8 hours light/dark, at 23°C and 18°C, respectively.

Arabidopsis line 11 is a transgenic line that contains a homologous recombination (HR) reporter gene. This reporter line has two, non-functional, overlapping, truncated copies of the β -glucuronidase gene (GUS; Boyko, *et al.*, 2006b; Swoboda, *et al.*, 1994). If a strand break occurs anywhere in the region of homology in one of the two truncated GUS genes, the HR double-strand break repair pathway could repair it using the other strand as a template, potentially restoring the function of the GUS transgene (Figure 1A; Boyko, *et al.*, 2006b). In this case, sectors of blue will result after histochemical staining.

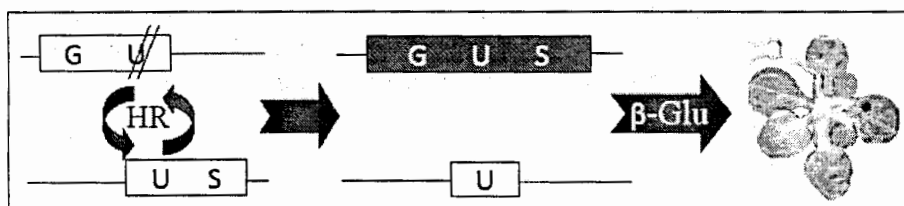


Figure 1. Representation of homologous recombination reporter line #11 in Arabidopsis. HR - homologous recombination; DSB - double strand break; β -Glu - GUS staining solution.

2.2. EXPERIMENTAL SET-UP

Ten-day old Arabidopsis line 11 plants were a subject to either X-Ray (XR) or UV-C (UV) irradiation while growing on either solid or liquid MS media in either undivided or divided Petri dishes. Half of the plates were covered with a shielding that protected the plants underneath, and the plants were irradiated. Seven days post-irradiation the plants were harvested separately as either irradiated (IR; not covered) or bystander (BS; covered) plants. An additional treatment was added, half-bystander ($\frac{1}{2}$ BS), in which the plates were covered as above, but only media was irradiated. 75-150 plants were counted per treatment, and the averages taken to represent the recombination rate for that experiment. The experiment was independently repeated three times, and statistics were performed on those three averages in MS Excel 2003.

2.3. RADIATION TREATMENTS

Plants were prepared for irradiation at 10 days post-germination. In the instance of UV-C irradiation, the Petri lids were removed and half of the plate was completely covered in tinfoil. The entire plate was then irradiated with 7000 ergs (~60 ergs/second) of UV-C. A set of control plates were completely covered with tinfoil and irradiated

under the same conditions to ensure there was sufficient UV protection. X-ray irradiation was done in a similar manner. Half of the Petri dish was covered with a 2.5 mm thick medical grade lead shield, and whole plates received 20 gray of X-rays (90 kV, 5 mA, ~18 mGy/sec). Again, a set of control plates were irradiated while completely covered with the lead shielding to ensure sufficient protection.

2.4. HISTOCHEMICAL STAINING

Whole plants were harvested seven days post-irradiation. These plants were then immediately placed in GUS staining solution (100 mg of 5-bromo-4-chloro-3-indolyl glucuronide substrate (GBT) in 300 mL of 100 mM phosphate buffer (pH 7.0), 0.05% NaN₃, and 1 mL dimethylformamide) and vacuum infiltrated for 10 min. Plants were incubated in the staining solution for 37°C for 48 hours, and then destained with 70% ethanol for 24 hours. The number of events per plant was then counted under a dissecting microscope, and the number of spots/plant (homologous recombination frequency) was calculated.

3. Results

The analysis of HRF in irradiated and non-irradiated bystander plants (Figure 2A) showed the increase in the case of X-ray and UVC exposures, although the effect of X-ray was more pronounced (Figure 2B,C). We hypothesized that the bystander effect on recombination frequency was triggered by the signal released by roots and transmitted through the liquid. To test whether the effect of the signal will be different depending whether plants were grown on liquid or solid media, we performed the experiments using both media (see Materials and methods for details). We found that in general HRF was higher in plants grown on solid media and that the bystander increase in HRF was more pronounced when plants were grown in the liquid media (Figure 2B,C).

To test for the presence of airborne bystander signal, we performed above mentioned experiments using a divider that did not allow liquid exchange but allowed the air exchange (Figure 3A). The analysis showed that there was the increase in HRF in both, irradiated and non-irradiated plants, regardless whether there was a divider present or not (Figure 3D,E). The results were similar for both, X-ray and UVC exposures.

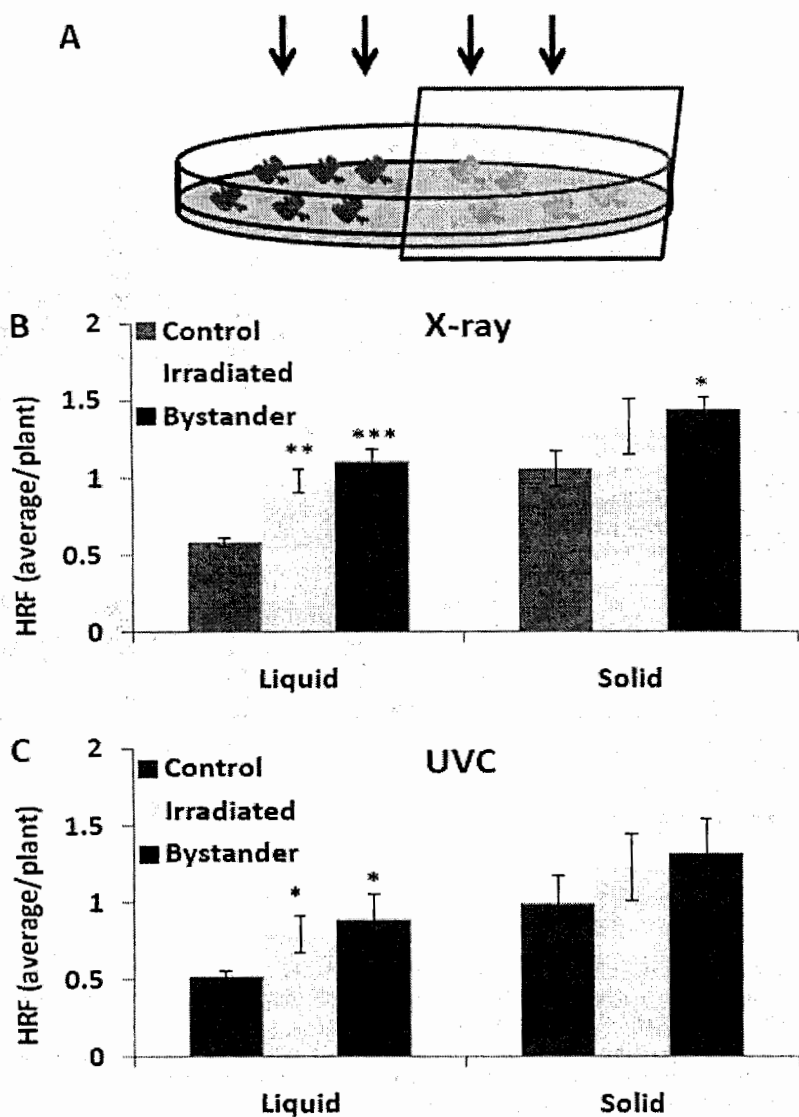


Figure 2. X-ray and UVC induce the HRF increase in both exposed and bystanding plants. Ten-day-old *Arabidopsis* line 11 plants were subject to either 20 Gy of X-Ray (XR) or 7000 ergs of UV-C (UV) irradiation while growing on either solid or liquid MS media in either undivided or divided Petri dishes.

A. Schematic presentation of the experiment. Half of the plates were covered with a shielding that protected the plants beneath from the radiation (Bystander), while the other half was uncovered (Irradiated).

B. X-ray exposure experiment. Y axis shows the average recombination frequency in exposed and "bystander" plants grown on liquid or solid medium. Asterisks show significant difference from control (one asterisk - $P < 0.05$; two - $P < 0.01$; three - $P < 0.001$).

C. UVC exposure experiment. Y axis shows the average recombination frequency in exposed and "bystander" plants grown on liquid or solid medium. Asterisks show significant difference from control ($P < 0.05$).

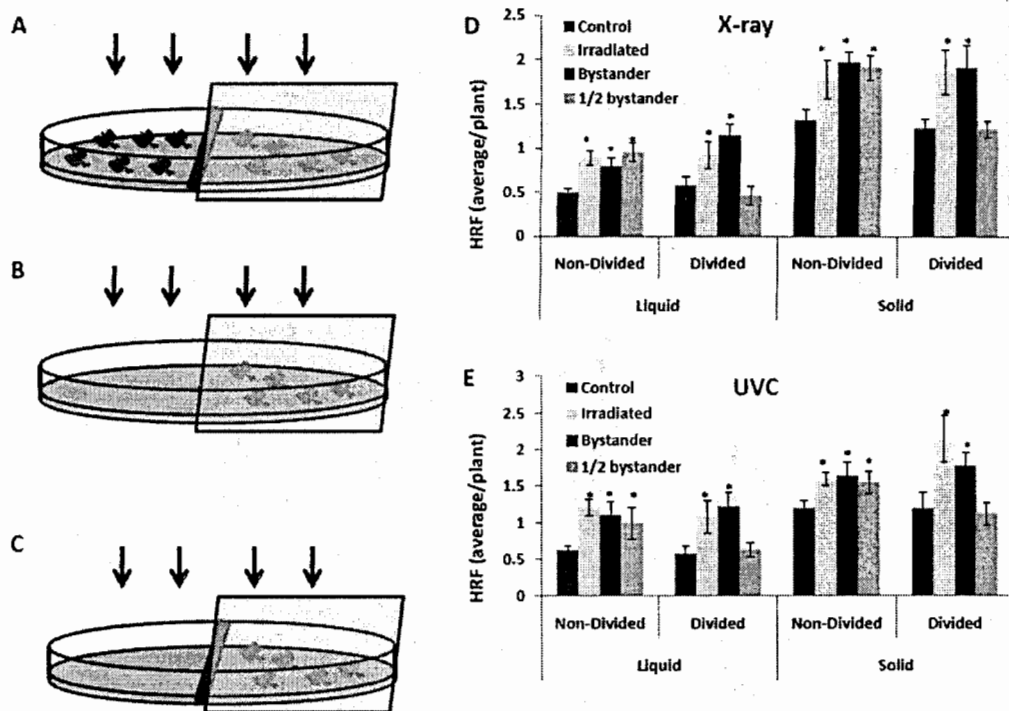


Figure 3. The signal triggering bystander effect is transmitted through liquid and air. Ten-day-old Arabidopsis line 11 plants were a subject to either 20 Gy of X-Ray (XR) or 7000 ergs of UV-C (UV) irradiation while growing on either solid or liquid MS media in either undivided or divided Petri dishes.

A. Schematic presentation of the experiment. Half of the plates were covered with a shielding that protected the plants beneath from the radiation (Bystander), while the other half was uncovered (Irradiated). The Petri dishes used contain the dividers separating the medium in which irradiated and non-irradiated plants are grown.

B. Schematic presentation of the experiment. Plants were only planted under the shielding and not in the non-protected area.

C. Schematic presentation of the experiment. Plants were only planted under the shielding and not in the non-protected area. The Petri dishes used contain the dividers separating the irradiated medium and non-irradiated plants.

D. X-ray exposure experiment. Y axis shows the average recombination frequency in exposed and "bystander" plants grown on liquid or solid medium. Asterisks show significant difference from control ($P < 0.05$).

E. UVC exposure experiment. Y axis shows the average recombination frequency in exposed and "bystander" plants grown on liquid or solid medium. Asterisks show significant difference from control ($P < 0.05$).

Next, we asked ourselves whether the bystander signal could have originated from liquid media itself as the result of water radiolysis or similar radical producing events. To test this, we placed plants only in one half of the plate that was protected by the shield; no plants was placed in non-shielded area. We used plates that did and did not have a divider (Figure 3B,C). The results showed that in the case of liquid media, there was an increase in HRF in neighboring plants when the divider was not used (Figure 3D,E; data point labelled as "1/2 bystander"). At the same time, no increase in HRF was observed when the divider was used. Similar trend was observed for the solid media, although the data were not significantly different.

4. Discussion

Homologous recombination frequency increases can be the result of up-regulation of the homologous recombination double-strand break repair pathway, and has been shown numerous times to respond to direct ionizing radiation (Ries, *et al.*, 2000; Filkowski, *et al.* 2004; Molinier, *et al.*, 2005; Boyko, *et al.*, 2006a; Molinier, *et al.*, 2006). This increase in HFR is a measure of genome instability, which generally refers to the susceptibility of the genome to mutations, rearrangements, and activation of mobile elements (Boyko, *et al.*, 2007).

Elevated levels of HRF are generally accountable through two mechanisms, the increase in the amount of double strand breaks, and/or the increase in the activity of homologous recombination repair machinery (Boyko, *et al.*, 2006b). In the case of ionizing radiation, it is not surprising that increased HRF occurs upon exposure, as ionizing radiation is well known to induce double-strand breaks in eukaryotic cells, most commonly through the production of genotoxic intercellular reactive oxygen species (Chatgililoglu and O'Neill, 2001; Filkowski, *et al.*, 2004).

However, here our data suggests that direct UV-C and X-ray exposure cause increases in HRF that are more pronounced in liquid than solid media. This is an interesting finding, as it would suggest that plants in liquid media are more susceptible to total plant irradiation. However, if one considers the framework of the experiment, the plants as well as the media are being irradiated. It may be possible that the irradiation of the media is creating free radicals that are affecting the plants global HRF. It is well documented that the radiolysis (X-ray) and photolysis (UV) of water produces substantial quantities of hydroxyl radicals ($\text{OH}^\cdot$) and hydrogen peroxide (H_2O_2) (LaVerne, 2000; Azarague, *et al.*, 2005). It is possible that irradiation of the media is producing these reactive oxygen species (ROS), which are then being taken in by the plant. Further, as the solid media is a semi-solid gel, it is possible that the ROS produced in solid media are less mobile, resulting in less uptake by the plant and causing less damage. These less-mobile ROS also explains the less dramatic increase in HRF in bystander plants in solid media.

These ROS produced by the irradiation of media could explain the bystander-like effect in the non-divided plates. It is likely that the ROS created by the radio- or hydrolysis of the media on the irradiated side of the plates can diffuse to the bystander side, causing similar effects in these plants. This is supported by the "1/2 bystander" treatments where only media is irradiated resulting in similar increases in HRF in undivided bystander plants.

Further, this increase is completely abolished in the "1/2 bystander" divided plates, suggesting the physical barrier separating the media is capable of halting the signal. This evidence supports the media diffusible signal hypothesis and removes any speculation of media produced gaseous signals.

Interestingly, however, is that bystander plants on divided plates that do contain irradiated plants do produce an elevated HRF. This would suggest that in addition to the media produced ROS causing elevated HRFs in bystander plants, the directly irradiated plants are producing a gaseous signal causing increased genome instability in neighbouring, unexposed plants. This is a truly phenomenal and novel finding. It is curious, however, why there is not an additive result in the increase in HRF in the bystander undivided plates. In this instance, the bystander plants are receiving both the putative aqueous ROS as well as the radiation-derived volatile signal. It may be possible that there is a certain level of saturation in this system, and HRF rates produced in this manner are regulated to a maximal level. Alternatively, as the irradiated plants covered much of the surface of the media, it could be the scenario that the plants are absorbing most of the dose and the media is receiving little radiation, resulting in the generation of only one prominent signal.

The ability of plants to communicate through the release of volatile organic compounds is not novel. In past it was observed that plants not attacked by insects (bystander), residing next to those that were attacked, were able to produce the same volatile signalling molecules and were activating the same defensive pathways as the attacked plants (Baldwin, *et al.*, 2006). A number of volatile organics have been studied over the years in an attempt to characterize these plant-plant interactions. Small highly volatile compounds, such as ethylene, methanol, isoprene, acrolein and some monoterpenes, can diffuse in the surrounding environment rapidly, limiting signalling to systemic leaves or very close neighbours (Baldwin, *et al.*, 2006). Heavier volatile compounds, such as methyl jasmonate (MeJA), methyl salicylate (MeSA), have been suggested to function over longer distances, as their slow dispersal will allow for the establishment of turbulence-resistant of plumes (Baldwin, *et al.*, 2006). In our case, since we are working in a limited air volume of a sealed Petri dish, the signalling of either type of volatile could be possible.

MeSA and MeJA has previously been implicated in intraplant stress responses and systemic signalling, with examples in systemic acquire resistance (SAR; Grant and Lamb, 2006; Park, *et al.*, 2007) and systemic response to wounding (SRW; Schilmiller

and Howe, 2005). Interestingly, both the plant-plant signalling of wounding (Baldwin, *et al.*, 2006) and pathogen infection have been shown before (Shulaev, *et al.*, 1997).

Similar to these systemic effects is the systemic recombination signal (SRS) discovered in our laboratory several years ago (Kovalchuk, *et al.*, 2003; Filkowski, *et al.*, 2004; Boyko *et al.*, 2007). In this model, the local application of virus, UV-C or rose Bengal (a ROS producing compound) resulted in the local and systemic induction of increased recombination rates. Indeed, this systemic induction of increased HRF may be similar to the plant-plant induction of HRF we show here.

The biological relevance of such a signal between plants is indeed quite perplexing. It has been postulated that the SRS generated in systemic leaves in plants helps drive increasing genetic diversity in plants, as their sessile nature forces them to adapt to survive (Boyko and Kovalchuk, 2008; Boyko *et al.*, 2010). However, overwhelming rates of recombination are not 'geno-healthy' either, as homologous recombination can cause the loss of heterozygosity at heterozygous loci. It is possible that this putative plant-plant signal evolved as a systemic signal as opposed to an interplant signal, and it is only in the context of this experiment where close proximately plants in a small gaseous environment are exhibiting this plant-plant-like phenomenon.

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BYSTANDER EFFECTS AND ADAPTIVE RESPONSES MODULATE IN VITRO AND IN VIVO BIOLOGICAL RESPONSES TO LOW DOSE IONIZING RADIATION*

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Abstract. We have utilized cellular and molecular approaches to characterize biological effects that are induced in normal mammalian cells and tissues exposed to low doses/low fluences of ionizing radiations that differ in their quality (i.e. linear energy transfer; LET). In human cells exposed to particulate radiations with high, but not low, LET character, the induced stressful effects were not only confined to the cells that have been directly targeted by the radiation, but involved a number of non-targeted and delayed effects. Chromosomal damage and oxidative changes in proteins and lipids were detected in cells exposed to alpha and high charge and high energy (HZE) particles and in their neighboring bystanders. Signaling events mediated via inflammatory cytokines and/or intercellular channels that comprise gap junctions were critical for the expression of the induced non-targeted effects. With relevance to health risks, the stressful changes in bystander cells were propagated to their progeny. In contrast, induced DNA repair and antioxidant defense mechanisms often attenuated the basal level of DNA damage and oxidative stress to below the spontaneous rate in tissues of animals and in cultured rodent and human cells exposed to low dose/low dose-rate γ rays, a low LET radiation. Together, our data suggest that low dose radiation-induced signaling events act to alter the linearity of the dose-response relation that is predicted by biophysical arguments. They show that the nature of the altered responses strongly depend on radiation quality.

Keywords: Low dose ionizing radiation, bystander effect, adaptive response, health risks, linear energy transfer

* Radiobiology and Environmental Security / Eds. C.Mothersill, V.Korogodina, C.Seymour. Springer, 2012. P.71-88

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1. Introduction

Although recent advances in biochemical, molecular, and epidemiological techniques have increased our understanding of the biological effects and health risks of low dose ionizing radiation (<100 mSv), great ambiguities in knowledge still remain. The quality (i.e. linear energy transfer; LET), dose and dose-rate of the radiation, the types of irradiated cells, their microenvironment and their metabolic state, as well as the variations in inherent radiation sensitivity are among the factors that can modulate the responses to low dose radiation. These characteristics and others are under intense investigation in many laboratories, including ours; the results are yielding a wealth of novel insights into the mechanisms that underlay cell and tissue responses to low dose/low fluence ionizing radiation¹. It is hoped that the elucidation of the mechanisms involved may alleviate the uncertainties in estimating low dose radiation effects on human health². Typically, human epidemiological studies would be ideal to assess such effects; however, they currently have limited statistical power due to the small size of the cohorts under study³.

Understanding the biological effects of low dose radiation is of immense, public, scientific and regulatory interest, as the frequency of human exposure to low dose radiation has been on the increase. In addition to exposures from natural sources (e.g. inhaled radon gas), the human population may be subjected to ionizing radiation during activities related to nuclear technology, mining, air travel and space exploration. Perhaps of greatest significance is the explosive growth in diagnostic radiology use where an increasing number of individuals, including children, are being *repeatedly* exposed to low dose radiation⁴.

Currently, for the purposes of radiation protection, the deleterious effects of ionizing radiation are assumed to have a linear dose response with no threshold². Two radiation-induced phenomena that were particularly recognized in the past three decades, namely adaptive and bystander effects, are thought to cause a challenge to these assumptions⁵. The propagation of damaging effects from irradiated to non-irradiated bystander cells would, presumably, result in supra-linear dose-response relationships. In contrast, the expression of adaptive responses that mitigate the initial damaging effects induced by radiation would suggest an infra-linear dose-response relationship or the existence of a threshold dose, below which there would be no risk. Although widely observed, the data confirming the expression of these two phenomena are not universal⁶⁻⁹. Moreover, the exact molecular steps by which adaptive and bystander effects are elicited remain unclear. Elucidation of these steps would clearly increase our understanding of the role of cellular processes that impact the health risks of low dose radiation exposures.

We have been intensely involved in examining the mechanisms underlying radiation-induced bystander and adaptive responses by using model cells and rodent tissues. Here, we summarize some of our findings in the context of a brief review of the latter phenomena. These findings provide strong support for the expression of both bystander and adaptive responses and reveal a critical role of the quality of the radiation in triggering either protective or stressful effects at low doses. Whereas, low doses of low LET radiations (highly energetic X rays, γ rays or protons) triggered processes that mitigated not only stressful effects induced by subsequent challenge doses of radiation, but also stressful effects due to endogenous oxidative metabolism¹⁰⁻¹³, high LET radiations (α particles and high charge, high energy (HZE) particles) resulted in the propagation of stressful effects from irradiated to non-irradiated bystander cells¹⁴⁻¹⁶. The data strongly support a role for intercellular communication and oxidative metabolism in the mediation of these responses. Induction of genomic instability and low dose hypersensitivity are other phenomena that are also thought to impact the health risks of exposure to low dose radiation. These phenomena are being investigated in different laboratories¹⁷⁻²⁰.

2. Interactions of Ionizing Radiation with Biological Matter

Ionizing radiation is energetic and penetrating. Many of its chemical effects in biological matter are due to the geometry of the initial physical energy deposition events, referred to as the track structure. The transfer of radiation energy to living tissues causes ionization of atoms and molecules and breaks chemical bonds, which initiates a series of biochemical and molecular signaling events that culminate in transient or permanent physiological changes²¹.

Ionizing radiation exists in either particulate or electromagnetic types. The ionizations and excitations that it produces tend to be localized, along the tracks of individual charged particles, in a pattern that depends on the type of radiation involved. Whereas the ionization events produced by fast electrons ejected from molecules traversed by high energy X rays or γ rays are well separated in space, those produced by certain charged particles, such as α and HZE particles, occur in dense columns along the particle path²². Such differences in ionization patterns mainly arise from differences in charge-to-mass ratio of the impacting particles.

Effects due to the track structure define the quality of the radiation and are commonly called linear energy transfer (LET) effects. In irradiated mammalian cells, which consist mainly of water, single energy deposition events cause bursts of reactive oxygen species (ROS) in and around the radiation track as well as in the intercellular matrix. Depending on the physiological state of the cell, these bursts of reactive species may alter the cellular redox environment, modify signaling cascades and normal biochemical reactions, and generate damage to cellular molecules and organelles²³. In

addition to the damages caused by water radiolysis products (i.e. the indirect effect), cellular damage may also involve reactive nitrogen species (RNS) and other species²⁴, and can occur also as a result of ionization of atoms on constitutive key molecules (e.g. DNA). The latter is known as the direct effect²¹. The ultimate result, of direct and indirect effects, is the development of biological and physiological alterations that may manifest themselves seconds or decades later. Genetic and epigenetic changes may be involved in the evolution of these alterations^{25, 26}. Intercellular communication among the irradiated cells²⁷, and between irradiated and non-irradiated cells^{14, 28}, as well as oxidative metabolism and DNA repair mechanisms are major mediators of the *system* responses to ionizing radiation exposure²⁹.

Because high LET radiation deposits greater amounts of energy per unit length of matter traversed, the possibility of multiple lesions in close proximity and short time frame is high³⁰. Consequently, for the same total dose absorbed, high LET radiation is more damaging to cells than low LET radiation²¹. The effects of LET, dose, and dose-rate in the cellular responses to low dose/low fluence ionizing radiation exposures continue to be intensely investigated. Here, we highlight the relevance of the latter characteristics of radiation in the expression of adaptive responses and in the nature of the biological effect propagated from irradiated to bystander cells in the exposed cell populations.

3. Ionizing Radiation-Induced Bystander Effects

The ionizing radiation-induced bystander effect is broadly defined as the occurrence of biological effects in unirradiated cells as a result of exposure of other cells in the population to radiation. Bystander effects have been mainly observed in high density cell cultures exposed to low fluences of α particles wherein only a small fraction of cells is irradiated³¹. Emerging data also indicate that bystander effects exist in cell cultures exposed to low doses of HZE particles³². They have also been noted in co-cultures of irradiated and unirradiated cells^{33, 34}. Stressful effects including up-regulation of stress-responsive proteins, genetic changes, induction of cell cycle checkpoints and cell death occur in both irradiated and non-irradiated cells of human and rodent origin at different stages of growth (reviewed in^{1, 18, 35-37}). More recently, strong evidence for bystander responses *in vivo* has been presented^{38, 39}. A few studies have also indicated that radiation-induced protective responses are mediated in a bystander manner in cell cultures exposed to low doses of low LET radiations⁴⁰ (and our unpublished data).

By using several biological endpoints to investigate non-targeted effects, including induction of DNA damage and various parameters of oxidative stress, our studies strongly support a role for LET, dose-rate and total absorbed dose in determining the nature and magnitude of the radiation-induced bystander effect and its persistence in progeny cells^{13, 41, 42}. Together with results generated by others, the data clearly show

that a given cell need not be directly irradiated to experience an ionizing radiation-induced biological response. Depending on cell type and radiation characteristics, distinct molecular interactions lead to propagation of either damaging or protective effects from irradiated to unirradiated cells and between irradiated cells. Gap-junction selectivity, secreted diffusible factors and oxidative metabolism are mediators of these effects³⁵.

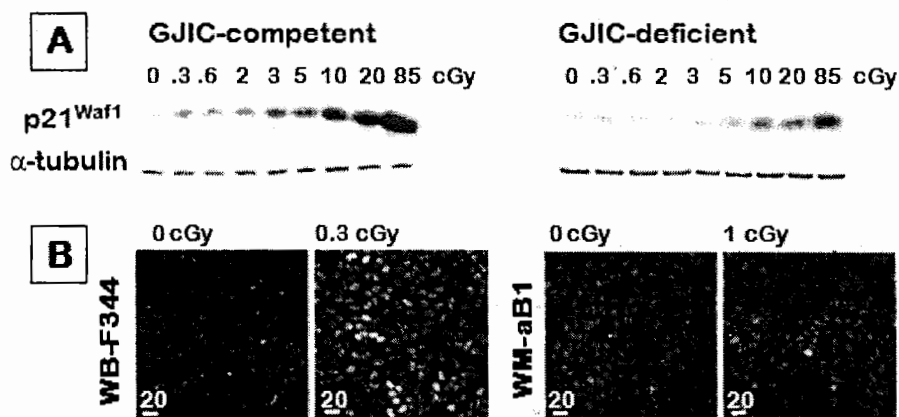


Figure 1 (A) Expression of p21^{Waf1} in protein lysates from gap junction communication-competent WB-F344 or gap junction communication-deficient WM-aB1 confluent cultures following exposure to α particles. Cells were harvested 4h after the exposure and proteins were examined by western blot analyses (B) *In situ* immunofluorescence detection of p21^{Waf1} expression in control non-irradiated WB-F344 cultures and in cultures exposed to 0.3 cGy of α particles. Expression of p21^{Waf1} in control non-irradiated and in 1 cGy-exposed cultures of GJIC-deficient WM-aB1 cells¹⁵

A direct evidence for the role of gap-junction intercellular communication (GJIC) in these processes was shown by our group and by others^{14, 15, 28}. The modulation of proteins involved in the p53/p21^{Waf1} stress-induced signaling pathway and induction of DNA damage in bystander cells were observed only in GJIC-proficient cell cultures. The data in Figure (1) describe p21^{Waf1} expression in sham-exposed and α particle-irradiated cultures from two related rat epithelial cell lines that differ in their ability to communicate via gap junctions. The WB-F344 cells are GJIC-competent, a function that is sensitive to inhibition by lindane and other chemicals that block junctional communication^{43, 44}. The WM-aB1 cells were derived from WB-F344 cells, but are deficient in GJIC^{15, 45}. Similar to WB-F344 cells, WM-aB1 cells express connexin43, a structural protein of gap junctions, however they are deficient in the ability to phosphorylate it, which renders them deficient in functional GJIC^{15, 45}. The western blot analyses data in Fig. (1A) show an increase in p21^{Waf1} levels in confluent WB-F344 cultures exposed to mean doses as low as 0.3 cGy. In WM-aB1 cell cultures, an increase in p21^{Waf1} levels is

significant only at mean doses of 5 cGy or higher. Therefore, the magnitude of the response in the GJIC-competent cells and the lack of p21^{Waf1} up-regulation in WM-aB1 GJIC-deficient cultures exposed to low mean doses strongly support the involvement of GJIC in the bystander gene expression response. This is further confirmed by the *in situ* immunofluorescence data in Fig. (1B) showing induction of p21^{Waf1} in confluent cultures exposed to a mean dose of 0.3 cGy. While small clusters of responding cells were observed in WB-F344 cells, only single isolated and presumably irradiated WM-aB1 cells invariably exhibited up-regulation of p21^{Waf1} after exposure to doses in the range of 0.3 to 1.0 cGy (Fig. 1B). At these mean doses to the monolayer, 1% or less of the WB-F344 or WM-aB1 cells would have their nuclei traversed by an α particle.

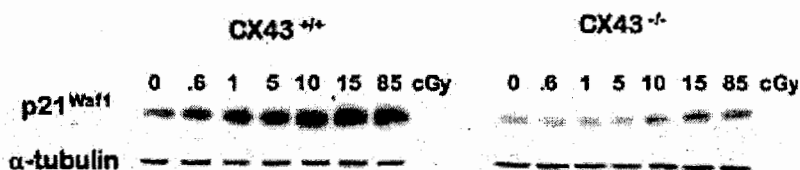


Figure 2 Western analyses of p21^{Waf1} level in lysates from isogenic wt or connexin43^{-/-} MEF cultures exposed to α particles. Cells were harvested 4h after the exposure¹⁵.

The WM-aB1 cells were transformed by mutagenesis of the WB-F344 parental cell line⁴⁶. To exclude effects due to mutagenesis other than loss of GJIC, we tested the induction of the p53/p21^{Waf1} signaling pathway after low fluence α particle irradiation of low passage mouse embryo fibroblasts (MEFs) from wt and isogenic knockout embryos for connexin43. Similar to WB-F344 and WM-aB1 cells (Fig. 1), the data in Fig. (2) indicate a lack of detectable increase in p21^{Waf1} level in connexin43^{-/-} cell cultures exposed to mean doses less than 10 cGy. In contrast, p21^{Waf1} was induced in wt cell cultures exposed to mean doses as low as 0.6 cGy. Collectively, these data support strongly the involvement of GJIC in the bystander gene expression response observed in confluent, density-inhibited cell cultures exposed to low fluences of α particles. They are relevant to estimation of the health risks of exposure to environmental radon. Radon accounts for 55% of the average annual radiation dose to the public in the USA and is considered to be the single largest naturally occurring environmental hazard. In fact, ~10-14% of lung cancer fatalities in the USA may be linked to radon and its α particle-emitting decay⁴⁷.

In addition to α particles, and with relevance to space exploration, we have also observed prominent stressful bystander effects in cell cultures exposed to low fluences of HZE particles⁴⁸. Our published and unpublished data indicate prominent induction of DNA damage, protein oxidation, lipid peroxidation and perturbations in mitochondrial functions, including mitochondrial protein transport and inactivation of the metabolic enzyme aconitase in bystander cells and in their progeny. Gap-junction communication was a major mediator of the propagation of these effects in the tissue culture systems used in our studies.

3.1. GAP-JUNCTION CHANNELS AND THE CELLULAR RESPONSE TO IONIZING RADIATION

Gap junctions are dynamic structures that are critical for diverse physiological functions⁴⁹. The intercellular channels that comprise gap junctions are formed by *connexin* protein. Each of the ~20 isoforms of connexin forms channels with distinct permeability properties. Though the properties of channels formed by each isoform differ, connexin pores, which vary in diameter, usually allow permeation of molecules up to ~1000Da, well above the size of most second messengers. Connexin channels have been shown to be highly selective among molecular permeants⁴⁹.

Evidence for the involvement of GJIC in propagation of bystander effects has been derived from studies with α particle, β particle, γ , and HZE radiations. These studies highlight the relevance of bystander responses to radiotherapy, diagnostic radiology, and risk of environmental and occupational exposures⁵⁰. Manipulation ($\downarrow\uparrow$) of connexin expression/gap-junction gating by pharmacological agents, forced expression by transfection, and connexin gene knockout studies have provided evidence for the participation of GJIC in radiation-induced bystander effects³⁵. This is particularly supported by the stabilization and up-regulation of connexin mRNA and protein by ionizing radiation Fig. (3)⁵¹. Examination by Northern and Western analyses of AG1522 normal human fibroblast cultures exposed to low fluences of α particles indicated that the *CONNEXIN43* gene is indeed activated. Relative to sham-irradiated controls, *CONNEXIN43* mRNA (Fig. 3A) and protein levels (Fig. 3B) were increased after exposure to mean doses ranging from 1 to 24 cGy. The similar increases at all doses suggest that low fluences of α particles induce molecular pathways that lead to maximal up-regulation of *CONNEXIN43*. The data in Fig. (3B) indicate increased expression in three protein bands detected by the antibody used. These bands were previously described to represent the native, phosphorylated and hyperphosphorylated isoforms of connexin43⁵². Low fluences of α particles up-regulate both the native and the post-translationally modified isoforms in confluent normal human fibroblasts⁵¹.

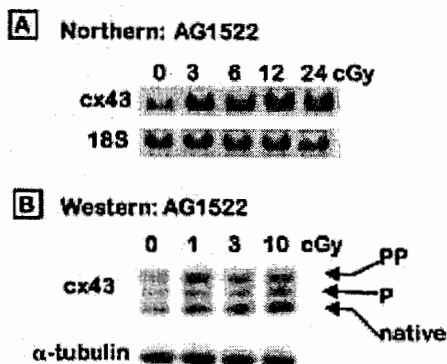


Figure 3. Upregulation of *CONNEXIN43* in α -particle-irradiated cell cultures. (A) Northern analyses of *connexin43* expression in AG1522 fibroblast cultures exposed to α -particles doses ranging from 0 to 24 cGy and held at 37°C for 6 h. (B) Western analyses of *connexin43* in AG1522 confluent cultures at 3 h after exposure to α particle doses ranging from 0 to 10 cGy under normal growth conditions⁵¹.

Participation of GJIC in stress-induced bystander effects is not unique to ionizing radiation; it has also been described in high density cell populations exposed to chemotherapeutic agents. Toxicity of these compounds was enhanced by functional gap-junction communication in target cells⁵³. Thus, many systems show that GJIC enhances the effects of toxic agents on targeted and untargeted cells. Direct intercellular communication may also lead to induction of protective effects that attenuate damage in targeted cells⁵⁴. The determinants and mechanism(s) of these effects, however, remain largely undefined. Our emerging data indicate that permeability properties of gap-junction channels have affect the nature of the induced bystander response. Different connexins form channels with *different* selectivities for various molecules including ions and highly similar second messengers⁵⁵.

Direct intercellular communication is not unique in propagating radiation-induced non-targeted effects. A wealth of data has also shown the critical importance of secreted diffusible factors in the expression of radiation-induced non-targeted effects³⁶. TGF- β , interleukin-8, serotonin and others have been implicated in propagation of bystander effects^{56, 57}.

3.2. OXIDATIVE METABOLISM AND BYSTANDER EFFECTS

Normal oxidative metabolism is a key endogenous generator of reactive oxygen and nitrogen species⁵⁸, and homeostatic control of normal cellular growth pathways is tightly dependent on oxidants⁵⁹. A disruption of the balance between oxidant production

and antioxidant defense alters the homeostatic cellular redox environment, resulting in a state of oxidative stress that promotes several pathological conditions including degenerative diseases and cancer⁶⁰. The endogenous targets of oxidants are diverse and include nucleic acids, proteins and lipids.

There is a strong connection between the generation of ROS and RNS and the damage that follows radiation exposure. Whereas ~60 ROS per nanogram of tissue were estimated to be generated from a hit caused by ¹³⁷Cs γ rays^{67, 68}, we can estimate that over 2000 ROS are generated from an α particle traversal, corresponding to a ROS concentration of ~19 nM in the nucleus of a normal human AG1522 cells²⁷. Such a ROS concentration can obviously cause extensive oxidative damage and may constitute a signaling event that triggers the spread of stressful effects from irradiated to neighboring bystander cells.

The involvement of ROS in the ionizing radiation induced bystander response was postulated by Nagasawa and Little³¹ in their initial report describing the induction of sister-chromatid exchanges (SCE) in bystander Chinese hamster ovary cells present in cultures exposed to fluences of α particles by which less than 1% of the nuclei were directly targeted. Evidence for such involvement was subsequently generated in studies involving various biological endpoints and irradiation modalities³⁵. Induction of stress responsive proteins, lethality and genetic changes (SCEs, mutations, chromosomal aberrations) in bystander cells was inhibited by superoxide dismutase (SOD) and other antioxidants³⁵.

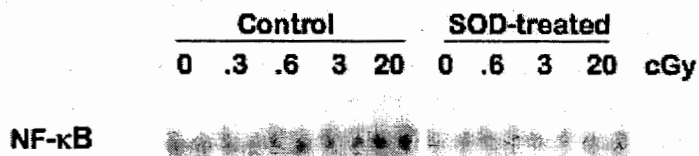


Figure 4. Electrophoretic mobility shift assay in α particle-irradiated AG1522 fibroblast cultures indicates activation of NF κ B DNA-binding by low mean doses at 30 min after exposure¹⁶.

ROS act as second messengers that regulate gene expression by signaling processes that involve activation of redox sensitive transcription factors⁶¹⁻⁶⁷. Consistent with a role for radiation-induced alterations in redox sensitive transcription factor activation in bystander cells in cultures exposed to low fluences of α particles, increases in the DNA-binding activity of NF κ B were observed in AG1522 normal human cell cultures exposed to doses as low as 0.3 cGy (Fig. 4). A similar level of increased DNA-binding activity was observed at 0.6 and 3 cGy although a 5-fold greater fraction of cell nuclei are traversed at 3 cGy than at 0.6 cGy. Importantly, when SOD (100 μ g/ml, 300 U/ml) was added to the culture 30 min prior to irradiation, the increase in NF κ B DNA-binding activity was inhibited (Fig. 4), further supporting the role of O₂⁻ in the bystander response of AG1522 cells.

Extensive data now indicate that the intracellular production of superoxide anions and hydrogen peroxide in both irradiated and bystander cells involves both the plasma bound NADPH-oxidase and mitochondria¹⁶. Of particular significance, our data strongly indicate that increased ROS levels following cellular exposure to α or HZE particles persist in progeny cells for many generations. This is manifested by increased oxidation of cellular proteins and disruption of mitochondrial physiology. In particular, decreased aconitase activity, which is involved in electron transport and regulation of gene expression, was observed, in bystander cells, 20 population doublings after exposure⁴⁸. Ectopic over-expression at the time of irradiation of the antioxidant enzymes superoxide dismutase or glutathione peroxidase, in bystander or irradiated cells, attenuated DNA damage and induction of stress-responsive proteins in the bystander cells (our data, unpublished). These results show that oxidative metabolism modulates non-targeted effects at the level of the irradiated and bystander cells.

Through *in vivo* experiments consisting of partial body irradiation of male Sprague-dawley rats with low fluences of HZE particles (energetic titanium or oxygen ions), stressful effects involving mitochondrial dysfunction were observed in non-targeted tissues 20 months after the exposure³⁸. Decreases in mitochondrial protein import as well as increases in antioxidant defense in non-targeted tissues were associated with perturbations in immune responses and inflammatory cytokines levels (e.g. interleukin-6) (unpublished). These data are consistent with the findings of others who showed that inflammatory-type responses involving oxidative stress occur after exposure to ionizing radiation^{68, 69}. In these *in vivo* experiments, activation of macrophages and neutrophil infiltration were not a direct effects of irradiation, but were a consequence of the recognition and clearance of radiation-induced apoptotic cells. The occurrence of such a response has been suggested to provide a mechanism for the interactions between irradiated and non-irradiated haemopoietic cells^{68, 69}. Such interaction was also observed in out of field experiments examining the genetic effects of partial organ irradiation. Antioxidants and nitric oxide synthase inhibitors attenuated these effects⁷⁰ strongly supporting the role of ROS and RNS in mediating bystander effects^{71, 72}.

Overall, several studies challenge the traditional paradigm that the important biological effects of ionizing radiation are due to DNA damage induced as a result of direct interaction of the radiation track with the cell nucleus. They indicate that irradiated and non-irradiated cells interact, and oxidative metabolism and intercellular communication have an essential role in signaling events leading to radiation-induced bystander effects. However, clear evidence explaining how these events occur is still lacking. Regardless, the occurrence of bystander effects implies that the modeling of dose response relationships based on the number of irradiated cells may not be a valid approach¹⁷.

4. Low LET Radiation-Induced Adaptive Responses

The “adaptive response” is a phenomenon generally induced by low dose/low LET radiation that protects cells and whole organisms against endogenous damage or damage due to a subsequent dose of radiation⁷³. Data generated over the last three decades suggest that exposure of mammalian cells, including human cells, to low doses of low LET radiation (e.g. X rays, γ rays, β particles) induces molecular processes that are different from those induced by high dose radiation⁷⁴. Such processes were found to be protective against stress measured by several biological endpoints⁵. Radiation-induced adaptive responses were dependent on the adapting dose, dose rate, expression time, culture conditions and stage of the cell cycle⁷⁵. Adaptive responses seem to be evolutionarily conserved as effects that protect against DNA damage in mammalian cells⁷⁶ mirror the evidence of radiation-induced protective mechanisms in prokaryotes and lower eukaryotes⁷⁷. Adaptive responses to ionizing radiation have also been detected *in vivo*^{78, 79}.

Of particular relevance to risk assessment, it was observed that low-dose/low LET radiation (0.1 – 10 cGy) decreases the frequency of neoplastic transformation to a level below the spontaneous rate in C3H 10T $\frac{1}{2}$ mouse embryo fibroblasts (MEFs) and in HeLa human hybrid cells^{11, 80}. It is noteworthy that these protective effects were seen only in irradiated cells that were allowed to incubate at 37°C before release from contact inhibition (Fig. 5), which suggests that time is required for expression of the protective effects.

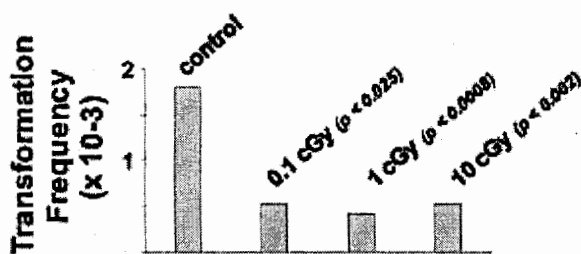


Figure 5. Low dose/low dose rate γ rays decrease the frequency of neoplastic transformation to a level below the spontaneous rate in C3H 10T $\frac{1}{2}$ mouse embryo fibroblasts¹¹.

Chronic exposure of C3H 10T $\frac{1}{2}$ MEFs to ^{60}Co γ -radiation at doses as low as 10 cGy protected the cells not only against damage from endogenous metabolic processes, but also against neoplastic transformation by a subsequent large acute radiation exposure¹². The induced resistance to neoplastic transformation correlated with increased ability to repair radiation-induced chromosome breaks¹⁰. Significantly, our recent proteomic

studies have identified novel proteins that were distinctly induced by low and not high dose γ rays. We have uncovered a role in DNA repair for the translationally controlled tumor protein, which was induced by almost 10-folds in cell cultures exposed to doses as low as 1 cGy (Zhang *et al.*, manuscript in preparation).

In addition to up-regulation of DNA repair mechanisms⁸¹, other processes may also modulate cellular responses to low dose/low dose-rate irradiation. Irradiation, under such conditions may affect the overall redox-state of the cell and its anti-oxidation potential, and may alter chromatin conformation, hence affecting the accessibility of DNA lesions to components of the DNA repair machinery. It may also induce mechanisms (e.g. apoptosis) that eliminate heavily damaged cells from the irradiated cell population⁸². Our data indicate that direct intercellular communication by gap-junctions¹³ is an important modulator of these effects. In addition, the induction of cell cycle checkpoints presumably provides more time for repair of radiation damage. Such effects may involve epigenetic events that could be transmitted to the progeny of low dose irradiated cells (Chaudhry *et al.*, manuscript in preparation).

Similar to its role in modulating bystander effects, oxidative metabolism is also a significant mediator of low dose, low LET radiation effects. Exposure of normal human fibroblasts maintained in 3-dimensional architecture to 10 cGy from γ rays delivered over 48 h reduced the frequency of micronucleus formation (a form of DNA damage) to levels similar or lower than background¹³. The effects correlated with up-regulation of cellular content of the antioxidant glutathione¹³. Extensive data have also shown that whole-body exposure of mice to low dose/low dose-rate γ rays up-regulates superoxide dismutase and alters mitochondrial functions in a manner that attenuates the generation of ROS (Li *et al.*, manuscript in preparation). We predict that such alterations provide a defense mechanism that allows the organism to cope with the radiation-induced oxidative stress. Together, the data suggest that mitochondria, which are active participants in oxidative metabolism, play a crucial role in low dose-induced adaptive responses.

5. Conclusion

Some of the mechanisms (e.g. junctional communication, oxidative metabolism) that underlie the bystander effect have been also implicated in the adaptive response to ionizing radiation. However, classical adaptive response protocols involving low LET radiation are clearly distinct from those of bystander studies conducted mainly with high LET radiation. In the adaptive response, cells are exposed to a small dose of low LET radiation. In contrast, cells traversed by an α or a HZE particle receive a substantial dose (10-70 cGy) and undergo a complex type of DNA damage. While similar mediators may modulate the same endpoint in both phenomena, the occurrence of opposite effects, such as pro-survival rather than cytotoxic effect, may reflect

changes in concentration of the inducing factor(s). For example, ROS have been shown to be a double-edged sword capable of inducing both proliferative or cell death effects depending on their concentration. Moreover, recent studies emphasized the effect of LET on the yield of water radiolysis products⁸³. Prevalence of different radiolysis species at the time of irradiation may induce dissimilar effects. However, the bystander effect and adaptive response could also be mediated by distinct mechanisms/mediating factors.

Due to limitations in the statistical power of current human epidemiological studies in assessing the health risks of low dose radiation exposures, mechanistic studies may be essential to understanding biological effects, and to help evaluating risks at low doses. Coupled with epidemiology, the knowledge of cellular and molecular processes that underlay low dose radiation-induced biological effects should further refine our estimates of radiation risks at low doses. The expression of stressful bystander effects in cell populations exposed to low fluences of high LET particles may contribute to the understanding of lung cancer incidence from environmental radon and degenerative diseases that may occur following deep space travel⁸⁴. Bystander effect studies may also enhance our understanding of biological effects that result from non-uniform distribution of incorporated radioactivity such as α particles emitted from radionuclides used in therapeutic nuclear medicine or released during nuclear accidents or terrorist activities⁵⁰. In particular, they offer avenues to characterize the nature of communicated signaling molecules and formulate strategies to protect normal tissue surrounding irradiated tumor targets. In contrast, the expression of adaptive responses in low dose/low LET exposed cell populations and the propagation of protective effects from irradiated to non-irradiated cells present in these populations may explain reported hormetic effects. They indicate that for some individuals, the risk from very small doses of radiation delivered at low dose-rate may be inexistent.

In conclusion, it is apparent that extensive *in vitro* and *in vivo* experimental evidence suggests that biological responses together with biophysical considerations likely determine the outcome of cellular exposure to ionizing radiation. Collectively, these studies should further contribute to the setting of radiation protection standards that would be effective in different exposure scenarios, applicable to men and women of all ages, and that must protect radiosensitive persons.

Acknowledgements

This research was supported by Grants DE-FG02-07ER64344 from the US Department of Energy (Low Dose Radiation Research Program), CA049062 from the NIH and NNJ06HD91G from NASA.

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LOW DOSE RESPONSES OF BONE MARROW TO X-RAYS IN VIVO*

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Abstract. Radiation risk at low doses is determined by linear extrapolation from high dose epidemiological data. In the last decade many non-targeted effects have been reported which may be relevant to low dose risk determination. To investigate cell responses at such low doses we used bone marrow cells of mice. We have not observed non-targeted effects long- or short-term post irradiation. Exposure below 50-100 mGy provides no evidence of a dose response for apoptotic signaling, bystander effects and low responses for p53 and p21 induction with significant individual variability. There is also no evidence for long-term chromosomal instability in the bone marrow at doses below 1 Gy. The data also demonstrate unexpected thresholds above which dose-dependent damage signaling is observed and the chromosomal instability phenotype is induced. The data are consistent with low dose X-irradiation being less damaging than would be expected from the LNT paradigm.

Keywords: DNA damage, in vivo, bone marrow, individual radiosensitivity, low doses

1. Introduction

Exposure to high doses of ionizing radiation unequivocally produces adverse health effects including malignancy. At lower doses epidemiology is not powerful enough for the purposes of radiation protection and the carcinogenic risk is estimated by

* Radiobiology and Environmental Security / Eds. C.Mothersill, V.Korogodina, C.Seymour. Springer, 2012. P.141-152

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extrapolation linearly proportional to radiation dose down to background levels (LNT model) relying heavily on radiobiological investigations and theoretical biophysical considerations¹. Importantly, dose is used as a surrogate for risk and averaged across a tissue rather than considered as dose to individual cells. In recent years, many observations of, so called, non-targeted effects indicate that radiation-induced lesions are not necessarily restricted to cells in which energy has been deposited. These observations include radiation induced bystander effects (responses exhibited by non-irradiated cells that have communicated with irradiated cells) and radiation-induced genomic instability (nonclonal effects arising in the descendants of irradiated cells)²⁻⁵. The phenomena have been regarded by some as being particularly important at low doses, potentially questioning the validity of a simple linear extrapolation of radiation risks from high to low doses⁵⁻¹⁰. To study *in vivo* responses of relevance to low dose effects of low-LET radiation and their potential risk, we investigated short-term (p53 pathway signalling and apoptotic responses) and longer-term (cytogenetic) effects in mouse bone marrow cells after exposure to very low doses of X-rays where, at the lowest doses studied, not all cells are irradiated and after 1.7 mGy approximately 40% of cells would not have received a single electron track^{11,12}.

1. Material and methods

1.1. ANIMALS AND IRRADIATION

C57BL/6 and CBA/Ca mice, susceptible or resistant, respectively, to both radiation-induced myeloid leukaemia¹³ and radiation-induced chromosomal instability in the bone marrow¹⁴ and exhibiting differences in the generation of bystander-type signals after exposure to high doses of low-LET radiation^{15,16} were sham- or X-irradiated as described previously¹⁷. Mice were bred and housed under conventional conditions and experiments conducted using adult mice of 8–12 weeks of age. Mice were sham-irradiated for controls or exposed to different doses of X-rays at a dose rate of 1 mGy/sec for low doses (up to 100 mGy) and 7.5 mGy/sec for high doses (0.5 Gy, 1 Gy and 3 Gy). Experiments were approved by local ethical review and carried out in accordance with Home Office Project License.

1.2. IMMUNOHISTOCHEMISTRY

At 3 hours post irradiation, femurs were removed, fixed and processed for immunohistochemistry using standard procedures¹⁸. Femurs were fixed by immersion in 4% buffered formalin for 48 hours and later were decalcified with EDTA, pH 7.0 for 10–12 days. All tissues were processed to paraffin wax using standard histological

processing schedules. Tissue sections 4 μm thick were stained using a peroxidase avidin-biotin-complex technique (Vector Elite ABC System, Vector Laboratories, UK) detected with an intensified AB/imidazole reaction. Antigen retrieval was performed by boiling in 10 mM citrate buffer, pH 6.0 for 15 min in a microwave oven. The CM5 polyclonal antibody to rodent p53 was provided by D.Lane and used at 1/80 000 (University of Dundee, UK). Purified rabbit anti p21/waf1 (Ab5, Oncogene Research Products, Nottingham, UK) was applied at 0.5 $\mu\text{g}/\text{ml}$. Caspase 3 antibody was purchased from Cell Signaling Technology (Cat N. 9664L). Sections were counterstained with haematoxylin. The numbers of positive cells and the total number of cells were counted by light microscopy with the aid of an eye-piece graticule. At least four independent areas were assessed per dose/time point for each individual animal and the decoded data pooled, tested for normality using the Shapiro-Wilk Test and differences between control and single experimental groups analyzed using the Student's t test with multiple comparisons of dose response data analyzed by Scheffé analysis of variance. Differences with $p < 0.05$ are stated as significant in the text.

1.3. TUNEL ASSAY

The same tissue sections and cytopspined cells as for immunochemistry were treated by Proteinase K (Sigma) at concentration 10 $\mu\text{g}/\text{ml}$ for 30 min at 37°C. Then Labeling the DNA ends were done using enzyme TdT (Invitrogen) and biotinylated-14-DCTP (Invitrogen) according the supplier instructions. The staining (using a peroxidase avidin-biotin-complex technique), scoring and statistical analysis are performed the same way as for immunochemistry.

1.4. IN VITRO ASSAY OF BYSTANDER STRESS RESPONSE

To assay for a radiation-induced p53 bystander effect in vitro, bone marrow cell suspensions obtained from C57BL/6 mice were divided into two aliquots; one was irradiated the other sham-irradiated. The irradiated aliquot was divided to provide two sources of signals for study of bystander effects. Two different assay systems were used: co-culture using transwells with 0.4 μm pores in which irradiated and sham-irradiated cells are separated by a polycarbonate membrane and media transfer in which sham-irradiated cells are suspended in medium obtained from irradiated cells. After incubation for 3h in 5% CO_2 in air at 37°C, cytocentrifuge preparations were prepared, immunostained, scored and analyzed as for tissue sections.

1.5. ASSAY FOR DELAYED CYTOGENETIC ABERRATIONS

At 30 and 100 days post-irradiation bone marrow cells were flushed from the femora of the irradiated mice. Metaphases were accumulated for 1 h by adding 0.02 $\mu\text{g}/\text{ml}$

colcemid to the marrow cells, suspended in α -MEM supplemented with 20% foetal calf serum at 37C. These cells were then suspended in 5ml hypotonic (0.55% w/v) potassium chloride (KCl) for 33 min incubation at 37C, then 2–3 ml KCl in sodium citrate was added (0.28 g KCl and 0.5 g sodium citrate in 100 ml distilled water) for 8 min. The cells were then fixed in suspension by adding 1–2 ml 3:1 methanol: acetic acid mixture to a final volume of 10 ml at room temperature. After 10–15 min, the cells were resuspended in at least two additional changes of the fixative mixture. Air-dried slides were aged for 10–14 days before Giemsa (15% in distilled water) staining. Chromosomal aberrations in coded metaphase preparations were recorded¹⁴, data obtained from 6–12 individual mice pooled after decoding and differences between the proportions of aberrant cells analyzed by the Fisher's exact test as described previously^{14,17}.

2. Results

2.1. LACK OF LOW DOSE-RESPONSE AND A THRESHOLD FOR DAMAGE RESPONSE SIGNALING AND APOPTOSIS

Hemopoietic cells express a p53-dependent cell cycle G1 phase arrest mediated by cyclin-dependent kinase inhibitor 1A (p21/Cip1/ CDKN1A) or p53-dependent apoptosis in response to a variety of stress stimuli. Using immunohistochemical techniques for p53 and p21 protein expression, preliminary time-response studies demonstrated that a maximal response could be detected at 3 hours after a range of doses in both C57BL/6 and CBA/Ca mouse strains and at 50 mGy irradiation for both endpoints in C57BL/6 mice. Samples for analysis in subsequent investigations were obtained 3h post-irradiation and there was no significant difference between C57BL/6 and CBA/Ca strains in the proportion of p53- or p21-positive cells in the femoral bone marrow of normal untreated 5 mice. After irradiation with 1.7, 5, 12 or 25 mGy X-rays, there was no significant increase in the proportion of p53-positive cells in C57BL/6 or CBA/Ca bone marrow and significant increases were not detected until exposure of 50 mGy for C57BL/6 bone marrow and 100 mGy for CBA/Ca bone marrow (Fig 1a). After 50 mGy (C57BL/6) and 100 mGy (CBA/Ca) exposure, there were correspondingly significant increases in the proportions of p21-positive cells (Fig 1b). Thus, for p53 and p21 expression there was no significant dose response over the range 0–25 mGy.

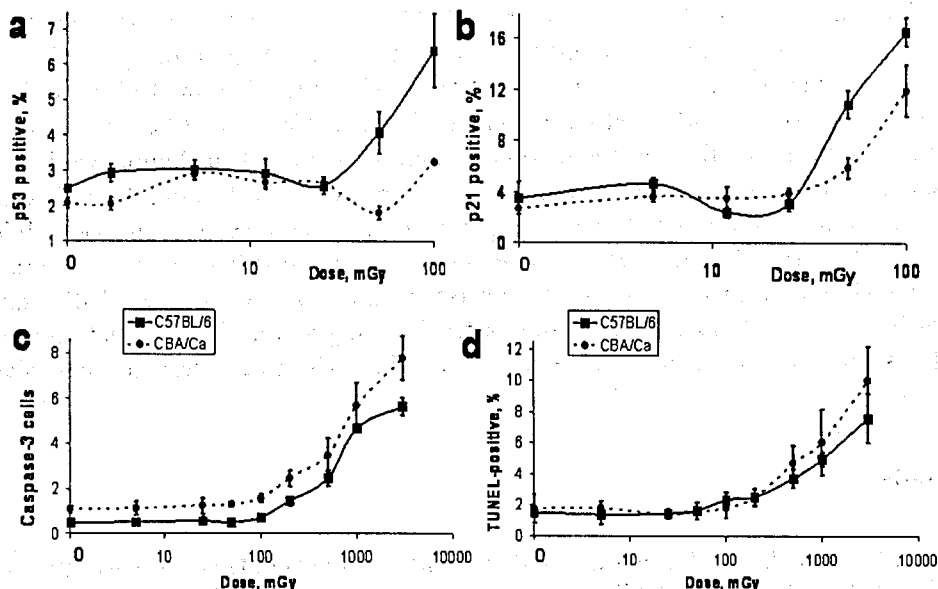


Figure 1. Dose responses in bone marrow after in vivo X-irradiation. C57BL/6 (■) and CBA/Ca (●) strains. Mean $\pm$ sem of % positive cells for p53 (a), p21 (b), Caspase-3 (c) or TUNEL (d).

To determine the proportion of cells responding to radiation-induced damage with an apoptotic response, the proportions of Caspase-3-positive cells and TUNEL-positive cells in the bone marrow of irradiated mice were determined. For both CBA/Ca and C57BL/6 mice, doses below 100 mGy produced no significant increase in Caspase-3-positivity (Fig 1c). After 200 mGy exposures the proportion of Caspase-3-positive cells is greater than controls for both CBA/Ca and C57BL/6 bone marrow and after 500 mGy exposures the proportion of TUNEL-positive cells is significantly increased (Fig 1d). For both strains and for both assays there are significant dose-dependent increases at higher doses. These data indicate that primary bone marrow cells do not show a short-term stress response to low levels of radiation-induced damage.

2.2. LACK OF AN IN VITRO LOW-DOSE BYSTANDER STRESS RESPONSE.

At the lowest X-irradiation doses studied, where not all cells in the bone marrow would have been irradiated, if bystander responses were operating they would be expected to enhance the consequences of irradiation producing a greater than expected response in one or more of the endpoints that we have studied. As there was no evidence of any enhancement at very low doses in vivo and most bystander experiments have used in vitro models an in vitro experiment was performed using a media transfer protocol and also a transwell insert culture dish system such that the non-irradiated cells shared the

culture medium but were not in direct contact with irradiated cells. Using the media transfer protocol (Table 1), the percentage of cells exposed to media obtained from 1.73 mGy irradiated cells expressing p53 (4.3%) was not greater than cells receiving media from sham-irradiated control cells (4.1%).

Table 1. Lack of bystander response in vitro.

Treatment	p53- Positive cells (%)			Caspase 3 positive cells (%)		
	Control	1.7 mGy	3 Gy	Control	1.7 mGy	3 Gy
Media transfer	4.1	4.3	3.6	3.8	4.2	4.2
Co-culture	3.0	2.9	2.9	3.1	3.4	3.3

Similarly, in a co-culture situation there was no evidence for a bystander-mediated increase in p53 expression with 2.9% cells expressing p53 not being greater than for cells in communication with sham-irradiated control cells (3.0%). Increasing the exposure dose to 3 Gy failed to produce a bystander-mediated increase in p53 positive cells. The same results are shown for Caspase-3 staining (Table 1). These data indicate that primary bone marrow cells do not show a short-term bystander response to low levels of radiation-induced damage and the question arises as to whether there is a delayed non-targeted effect.

2.3. A THRESHOLD FOR EXPRESSION OF RADIATION-INDUCED CHROMOSOMAL INSTABILITY

One of the longer-term radiation-induced non-targeted effects in bone marrow is the induction of a genotype-dependent chromosomal instability phenotype where ongoing chromosome breakage is reflected by significant increases in chromatid-type aberrations². Previously, 3 Gy X-irradiated CBA/Ca mice had been shown to demonstrate such instability^{14,19} and in the present study at 30 and 100 days after whole body irradiation with doses up to 500 mGy (Table 2) there was no evidence for the expression of cytogenetic aberrations (overall, 0.0041 aberrations per cell) being greater than the control levels (0.0044 aberrations per cell; $p = 0.5594$). However, consistent with previous data^{14,19}, 3 Gy (3000 mGy) X-irradiation resulted in a significant increase of chromatid-type aberrations ($p = 0.0020$) and the elevation after 1 Gy ($p = 0.0346$) was not significantly different from the elevated levels after 3 Gy ($p = 0.2887$).

Table 2. Dose-response for radiation-induced chromosomal instability

30-100 days post-irradiation.

Dose, mGy	Cells with aberrations		Aberrations / cell		Chromosome / Chromatid (30 days only)
	30 days	100 days	30 days	100 days	
0	4 / 908		0.004		3 : 1
1.7	0 / 132	0 / 175	0	0	-
5	1 / 146	0 / 193	0.007	0	0 : 1
12.1	3 / 346	1 / 174	0.009	0.006	0 : 3
25	0 / 205	-	0	-	-
50	1 / 446	-	0.002	-	1 : 0
100	3 / 611	-	0.006	-	2 : 1
500	1 / 384	-	0.003	-	1 : 0
1000	6 / 571	-	0.011	-	1 : 5
3000	12 / 730	6 / 275	0.016	0.022	2 : 10

3. Discussion

The findings of the present study provide no evidence for bystander enhancement of damage signaling and no dose-dependence of short-term (p53, p21 and apoptotic responses) in mouse bone marrow cells after exposure to very low doses of X-rays. Thus, there is no evidence of a linear response at low doses but instead an unexpected threshold where significant p53 pathway responses are initiated (approximately 50 mGy for C57BL/6 and 100 mGy for CBA/Ca bone marrow) which then increase linearly with dose above that threshold.

There maybe detection of irradiation at low doses by cells in some mice. Figures 1a, 1b show enhanced levels of p53 and p21 at doses up to the threshold. But this non-significant increase is due to much higher response of some mice (Fig. 2). At doses of 50 mGy (the threshold) and 100 mGy all responses are higher than the mean control level indicating that all mice demonstrate response to irradiation. At the lower doses most mice respond similarly to controls but in about 15% of mice the proportions of p53 positive cells are higher than in controls and those animals may represent the radiosensitive part of population. So, in the same population of inbred mice variability of responses to low dose radiation is observed.

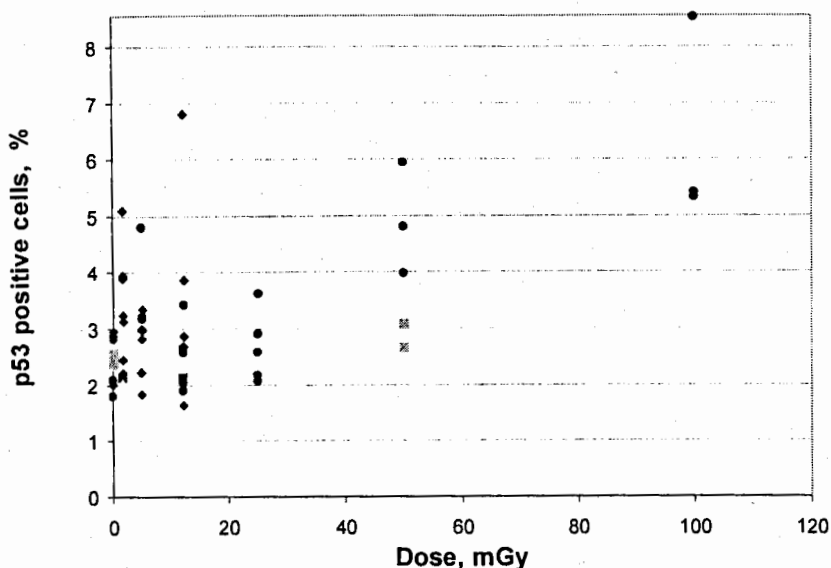


Figure 2. p53 responses in the bone marrow of C57BL/6 mice after exposure to low dose in vivo X-irradiation. Each dot is represented by a single mouse.

The chromosomal instability in bone marrow demonstrated in vivo after 3 Gy total body irradiation is consistent with previous studies of high doses of sparsely ionizing radiation^{14,17,19}. However, at the lowest doses studied in the present study, there was no evidence for such instability indicating that, similar to the damage response signalling, there is a threshold for the detection of this response. The threshold of 1000 mGy for expression of significant chromosomal instability is approximately an order of magnitude greater than the threshold for p53 pathway signalling. In contrast to these findings for sparsely ionizing low-LET radiation, our previous study of bone marrow cells irradiated with a low dose of densely ionizing high-LET alpha-particles (low fluence with a Poisson distribution and a mean of one particle per traversed cell) resulted in significant expression of chromosomal instability in vitro²³ and in vivo²⁴. Like our previous study of bone marrow cells irradiated with a low dose of alpha particles²⁴, many of the studies of non-targeted effects relate to cells exposed to low fluences of alpha-particles or microbeam-generated charged particles (densely ionizing high LET radiation). In these situations those cells that are traversed by a single particle receive a substantial dose (~ 0.3-0.5 Gy) and sustain damage that is much greater and more complex than is the case for cells irradiated with low doses of sparsely ionizing low-LET X- or γ -rays, where a single radiation track would deliver a dose of the order of 1 to a few mGy to the irradiated cell^{11,12}. It is important to be clear as to what is meant by a low and high dose to the individual cell or to population of cells in this context.

There are limited reports of radiation-induced non-targeted effects *in vivo*^{7,25,26} but evidence for such effects *in vivo* after low doses of low-LET radiation is generally lacking. Prior to the recent interest in bystander effects detected using *in vitro* systems there were many reports that clastogenic activity, capable of causing chromosome breaks in unirradiated cells, may be detected in blood plasma after high dose radiation exposures. Evidence for such clastogenic factors has been obtained from a number of sources including radiotherapy patients, atomic bomb survivors (31 years after exposure), Chernobyl salvage workers and a variety of individuals with chromosome instability syndromes and inflammatory disorders^{20-22,27}. Clastogenic activity is associated with lipid peroxidation products²⁸ and cytokines²⁹ and regarded as a biomarker of oxidative stress^{30,31}. Clastogenic factors may provide a mechanistic link to 'out of field' or abscopal effects where radiation treatment to one local area of the body results in an antitumor effect distant to the radiation site. This abscopal effect was originally described in 1953³² and clinical reports are generally descriptive³³ providing little insight as to mechanism. However, there are reports³⁴⁻³⁷ that implicate damage responses involving oxygen radicals produced as a result of the induction of inflammatory cytokines. It is also of interest the Japanese A-bomb survivors demonstrate evidence of persisting inflammation^{38,39}. Taken together, the various studies implicate inflammatory-type responses as contributing to potential health consequences of radiation exposure.

Considerable progress has been made in understanding the cellular and molecular events that are involved in the acute inflammatory response to infection but responses to tissue injury are much less well understood⁴⁰. The generation of clastogenic factors and induction of abscopal effects are associated with high dose exposures and a case can be made for tissue responses contributing secondary cell damage as a consequence of inflammatory responses to radiation-induced injury rather than any direct interaction between irradiated and non-irradiated cells. It is, therefore, far from clear how readily one may extrapolate simply from the *in vitro* investigations demonstrating direct interactions between irradiated and non-irradiated cells to *in vivo* scenarios. In the present study, using relevant exposures and a radiobiologically-relevant tissue, the findings provide no support for arguments that *in vivo* effects after very low doses of sparsely ionizing, low-LET radiation may be significantly greater than expected by extrapolation from high doses. On the contrary the data demonstrate the opposite as responses are less than expected and show that biological responses at low doses *in vivo* are not necessarily linearly related to dose and demonstrate, for the first time, a response threshold in primary bone marrow.

Acknowledgements

This work was supported by the NOTE IP 036465 (FI6R), Euratom specific programme for research and training on nuclear energy, 6th FP of the EC and by a specialist programme 07003 of Leukaemia and Lymphoma Research.

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Научное издание

КРЫМСКИЙ СИМПОЗИУМ

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РАДИОЭКОЛОГИИ И ЭВОЛЮЦИИ**

Третья международная конференция,
посвященная Н. В. Тимофееву-Ресовскому

Третьи Чтения памяти
В. И. Корогодина и В. А. Шевченко

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Конференция по научной программе НАТО

Труды симпозиума

Том 1

Д19-2012-144

Сборник отпечатан методом прямого репродуцирования
с оригиналов, предоставленных оргкомитетом.

Ответственная за подготовку сборника к печати *В. Л. Корогодина.*

Подписано в печать 02.12.2012.

Формат 70 × 100/16. Бумага офсетная. Печать офсетная.

Усл. печ. л. 23,68. Уч.-изд. л. 34,22. Тираж 135 экз. Заказ № 57852.

Издательский отдел Объединенного института ядерных исследований

141980, г. Дубна, Московская обл., ул. Жолио-Кюри, 6.

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