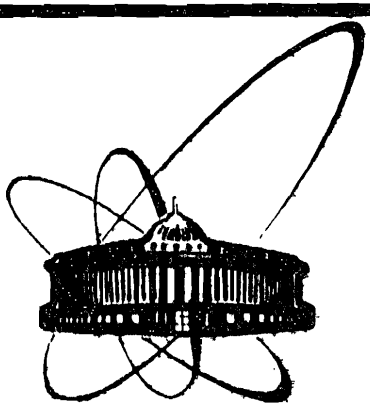

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THE INDUCTION OF REVERTANTS BY HEAVY PARTICLES AND γ-RAYS IN SALMONELLA TESTER STRAINS

Submitted to "Mutation Research"

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1987

INTRODUCTION

Salmonella mutagenicity test [1,2] is being extensively used for detection of mutagenic or potential cancerogenic agents. A set of histidine-requiring strains is used. Each tester strain contains a different type of mutation in the histidine operon. Therefore, according to the response of different tester strains the mutagens can roughly be divided into several classes:

a) frameshift mutagens - which stabilize the shifted pairing in TA 97 and TA 98 strains (2-nitrosofluorene, ICR 191).

b) base-pair substitution mutagens at G-C pairs - detected by TA 100 strain (sodium azid, methylating agents - MNNG)

c) base-pair substitution mutagens at A-T pairs - detected by TA 102 strain (X-rays, UV light, hydroxyperoxides).

Most of mutagens revert several tester strains causing several types of DNA injuries (e.g. MMS reverts both TA 100 and TA 102, 2-aminofluorene reverts both TA 98 and TA 100). Cross-linking agents such as psoralens and mitomycinC inhibit the growth of tester strains deficient in excision repair (TA 97, TA 98, TA 100). They are efficiently detected by the TA 102 strain which is uvrB proficient [3].

The mutagenesis in Salmonella tester strains is increased by pkM101 plasmid and in the case of the TA 102 strain by pAQ1 plasmid. The pkM101 plasmid contains an SOS-controlled muc gene increasing mutagenesis after SOS induction. The pAQ1 plasmid with his G428 mutation provides ≈ 30 copies per cell for back mutagenesis.

Ionizing radiation produces a wide spectrum of different types of DNA injuries [4]. γ-rays induce single strand breaks (SSB), double strand breaks (DSB), base damages - mainly t-products [5], sugar damages, apurine and apyrimidine sites,

cross-links and multiply-damaged sites with several nucleotides damaged. These DNA injuries can be further subdivided according to the structure of free strand ends. The character of DNA injuries is changed during DNA repair depending on the pool of repair enzymes and their induction in the course of repair process.

X-rays and γ-rays induce reversions in all Salmonella tester strains; its effectiveness, however, differs: mutation doubling doses of about 10^2 Gy are needed for plasmid free strains; mutation effectiveness is increased by one decadic order in plasmid containing strains [6]. The response of the Salmonella mutagenicity test to ionizing radiation can be further enhanced by the incubation of plated bacteria for several hours before irradiation [7]. The incubation increases the number of irradiated bacteria per plate approximately by one order.

The spectrum of DNA injuries varies with increasing LET of ionizing radiation [8]. More DSB and multiply-damaged sites, less SSB and base damages are induced. It can be, therefore, expected that the effectiveness of different ionizing radiations in the reversion of different Salmonella strains would differ.

Three strains have been used in our investigation: TA 98, TA 100, and TA 102. The procedure has been developed for their irradiation by heavy ions and α-particles. Simultaneous irradiations by some type of heavy ions and by γ-rays have been performed in order to establish more precisely relative biological effectiveness under the same conditions.

MATERIALS AND METHODS

Bacterial strains: the tester strains TA 98, TA 100, and TA 102 were provided by Dr.B.Ames, Department of Biochemistry, University of California, Berkeley.

Storage of the tester strains: overnight culture to which sterile glycerine had been added as a cryoprotective agent were distributed into sterile glass micro-tubes, closed, and

transferred to liquid nitrogen. Multiple copies of each strain were made and each experiment was started from a new micro-tube. No master plates were used. This technique provided better reproducibility of the experiments with plasmid containing strains.

Procedure for growing cultures: tester strain cultures have been grown using a shaker in Oxoid nutrient broth no. 2 to a density of about 2.10^9 cells per ml. To prevent loss of plasmids, 25 μg/ml ampicilin has been added. 2 μg/ml tetracycline has been added in the case of TA 102 strain.

Plates for the mutagenicity assay: 1,5% Bacto Difco agar with 0,5 mg/ml citric acid monohydrate has been used. The amount of agar per plate was 20 ml and this quantity was carefully controlled. Histidine and biotine were added in amounts of 0,1 ml per plate from the 120 μl/ml biotine + 100 μl/ml histidine solution prepared according to Maron and Ames [2]. The ingredients as well as 0,1 ml of washed cell suspension were either added to the top agar (0,6% Difco agar and 0,5% NaCl) or spread over the surface of petri plates. Owing to low penetration of accelerated ions we had to spread cell suspension on the surface of the bottom agar. The top agar was poured to the plates after irradiation. If ion irradiation was involved, the cell suspension was spread to a part of the petri plate only (≈10 cm^2). The agar with cells on the surface was cut to 10 pieces, irradiated, placed back to the petri plate and overlayered by 2,5 ml of the top agar (without ingredients) heated in the water bath.

Procedure of irradiation by heavy ions: agar pieces with cell suspension at the surface were put to sterile metal plates. Up to 11 metal plates can be inserted in a round magazine. The plates are then transported by remote control to the beam exit window. The exposure of various biological objects is monitored by an ionisation chamber set between the exit window and the biological sample. The device is described at [9].

Procedure of irradiation by α-particles: the cell suspension was spread on the surface of petri dishes. The geometry of α-particles dose fields was calculated and superposed for six different source-target positions. The superposition of these fields yielded nearly homogeneous irradiation of the whole petri plate surface. The source target distance was ≈ 1 cm. The initial average LET of α-particles was 115 keV/μm.

Procedure of irradiation by protons: the inside part of a petri plate about 5 cm in diameter could be irradiated. Owing to a great energy of protons which were used in our experiment, up to three plates could be irradiated at the same time on one another. Dose discrepancies due to energy losses do not exceed 2-3 per cent.

γ-irradiation was always performed simultaneously and in the same configuration of the experiment as irradiation by heavy particles. The sources of γ-rays were ^{137}Cs or ^{60}Co with dose rates 35 Gy/min and 3 Gy/min, respectively. All procedures of irradiation were done at room temperature.

Accelerators of heavy particles: $_4He$ ions with LET = 22 keV/μm and 72 keV/μm were obtained at the U-300 cyclotron of the Laboratory of Nuclear Reartions which accelerates helium ions up to ≈30 MeV. Protons with energy 100 MeV and LET = 0.73 keV/μm were obtained at the synchrocyclotron of the Laboratory of Nuclear Problems. The irradiation time ranged from several seconds (with helium ions) to several minutes (with protons and α-particles).

Counting of revertant colonies: his^- to his^+ revertant colonies were counted after 48 hours of incubation at 37°C under slight magnification. In order to reduce the influence of cell lethality on the results, the doses in our experiments did not exceed 20 Gy (the mean lethal dose amounted about 60 Gy).

RESULTS

The dose response curves for γ-rays are shown in Fig.1. In the region of doses up to 15-20 Gy all the curves were linear.

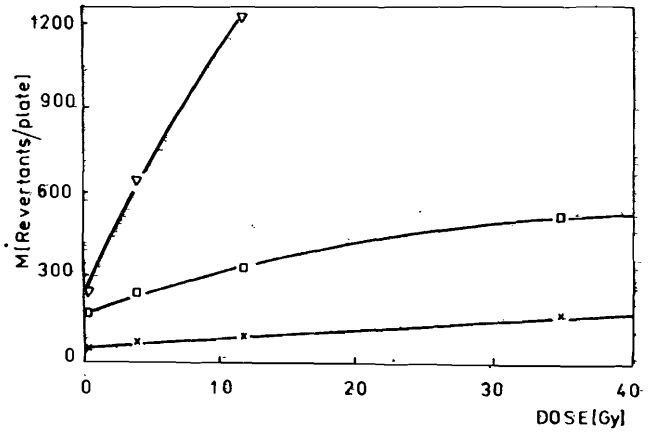

Fig.1. The number of induced revertants per plate as a function of the γ-radiation dose for three strains of Salmonella typhimurium: TA 98 (x), TA 100 (□), and TA 102 (▽). The dependences are linear for low doses.

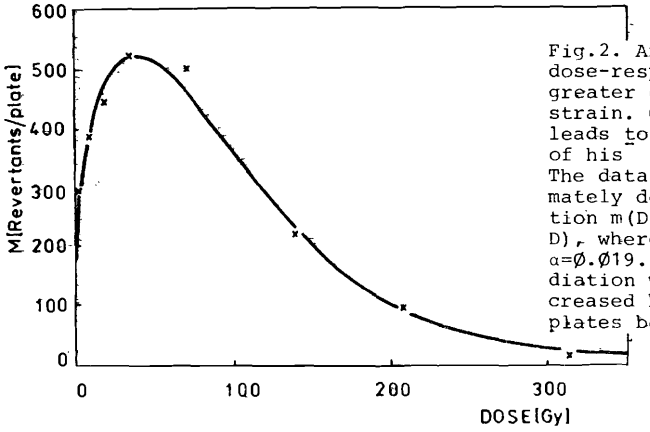

Fig.2. An example of the dose-response curve for greater doses (TA 100) strain. Cell lethality leads to decreasing number of his^- to his^+ revertants. The data could be approximately described by a function $m(D)=(m_o+q.D).\exp(-\alpha.D)$, where $m_o=182$, $q=24$, $\alpha=0.019$. The effect of radiation was somewhat increased by incubation of plates before irradiation.

Greater doses lead to some curvature of the dose-response dependence owing to cell killing. The exponential survival curves of Salmonella cells after γ-irradiation have the sensitivity $D_o=(60 +/- 5)$Gy for all three strains (data not shown). The difference between the total number of revertants per plate and the number of revertants corrected for cell survival , therefore, does not exceed 20%. The dose-response curves for greater doses (see Fig.2) could be mathematically described under an assumption that the number of mutations is related linearly to the dose multiplied by the survival fraction by the equation

$$m(D) = (q.D + m_o)*e^{-D/D_o} \qquad (1)$$

Owing to the exponential function in Eq.1, where D_o=60 Gy and q is the low-dose mutation rate, the linearity of dose response curve is a good criterion of the influence of cell lethality.

The number of spontaneous revertants was about 30-40 per plate for TA 98, 140-170 for Ta100, and 200-300 for Ta 102. The dose doubling the number of spontaneous revertants was about 10-15 Gy for TA 98 and TA 100, 2-3 Gy for TA 102. In the case of the TA 102 strain the variations shown above depended on the time for which the plates had been kept before irradiation (the time of irradiation couldn't be set precisely). The number of spontaneous revertants (m_o) was subtracted from mutation rates (m) and the slope of dose-response curves was determined:

$$q = (m-m_o)/D \qquad (2)$$

where D is the dose. The values of RBE were determined as RBE = q/q_γ, where q_γ is the slope for γ-radiation from the simultaneous experiment. All dose-response curves from heavy ion irradiation were linear up to 15 Gy. The values of q_γ were about q_γ=3-4 mutations/plate/Gy for TA 98, q_γ=12-15 for Ta 100, and q_γ=50-70 for TA 102.

A typical result of heavy ion irradiation is shown in Fig.3. The effectiveness of helium ions to induce revertant colonies sharply decreases with increasing LET in the case of the TA 98 strain. On the other hand, the TA 100 and TA 102 strains do not exhibit any marked changes for higher LET. The values of RBE are summarized in the Table.

To confirm decreasing effectiveness of He ions with increasing LET in the case of the TA 98 strain, we used α-particles with the average initial LET≅115 keV/μm. We also expected decreasing RBE for TA 102 strain. The results of this experiment are shown in Fig.4. Owing to the fact that the conditions of the experiments could be well controlled absolute values of mutation rates could be well reproduced. Therefore,

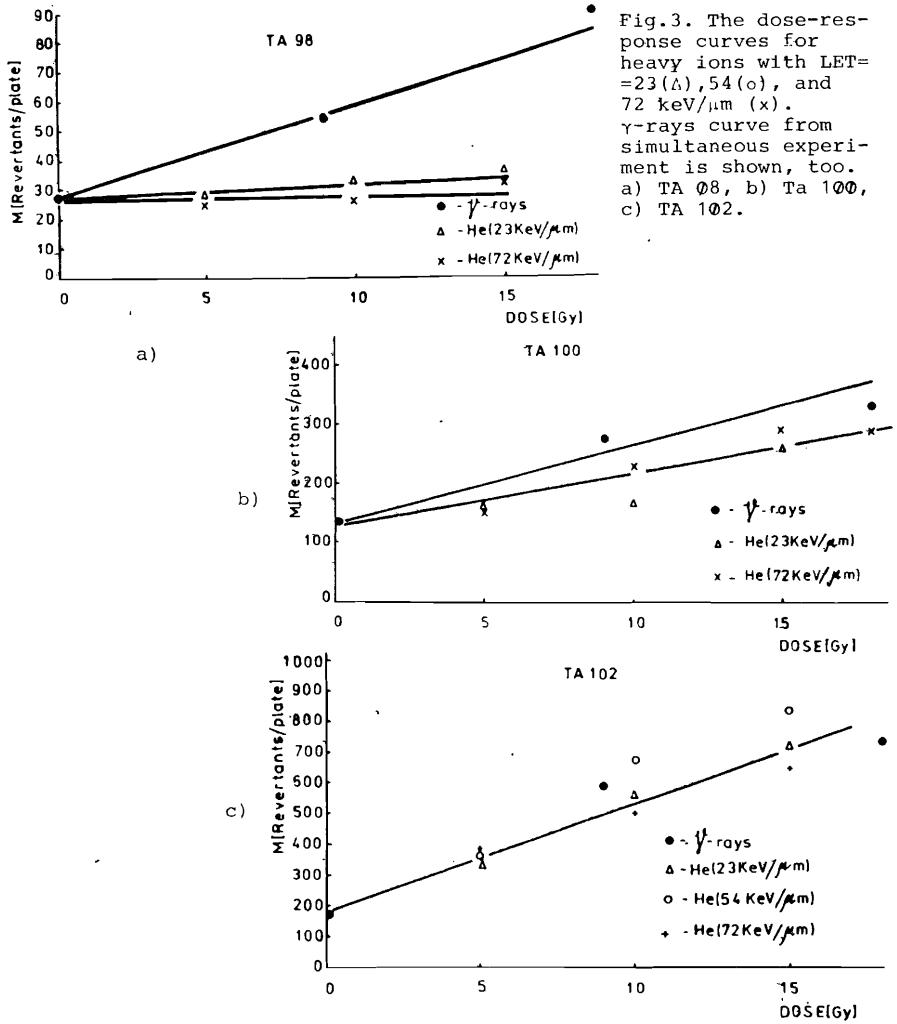

Fig.3. The dose-response curves for heavy ions with LET= =23(Δ),54(o), and 72 keV/μm (x). γ-rays curve from simultaneous experiment is shown, too. a) TA 98, b) Ta 100, c) TA 102.

the points are shown with standard deviations. We found that there were no revertants induced by α-particles in the TA 98 strain. On the other hand, the TA 102 strain responded very efficiently with the RBE near to 1.

In order to compare γ-rays with heavy particles in the low LET region, fast protons were used for further experiments (data not shown). The values of RBE (see the Table) are near to 1.

There are probably no essential differences in mutagenic effectiveness of γ-rays and fast protons.

TABLE. The values of RBE for various types of particles in the case of three Salmonella tester strains.

STRAIN	RELATIVE BIOLOGICAL EFFECTIVENESS				
	He(23)*	He(54)	He(72)	p(0.73)	α-par.(115)
TA 98	0.35±0.06	0.58±0.05	0.18±0.08	1.02±0.04	0.04±0.07
TA100	0.70±0.11	-	0.75±0.10	0.98±0.05	-
TA102	1.05±0.05	1.31±0.12	0.81±0.11	0.86±0.09	0.86±0.05

* - in the parantheses the values of LET.

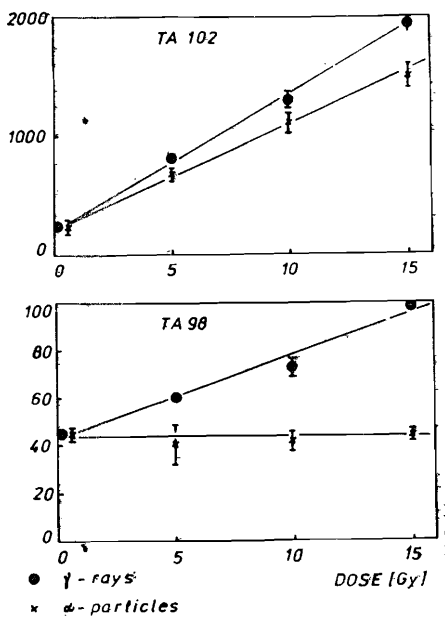

Fig.4. The dose-response curves for α-particles with initial LET≅ ≅115 keV/μm (x). The γ-rays curve from the simultaneous experiment (●) is shown, too. a) TA 102, b) TA 98.

DISCUSSION

We have investigated mutagenic action of low doses of different radiations in three Salmonella tester strains: the TA 98 strain (reversions after frameshift mutation), the TA 100 and TA 102 strains (reversions after substitutions).

The levels of spontaneous revertants differed in the three strains and we have also found the dependence of the reversion rates on the type of agar and on the amount of agar per plate similar to that in [10]. The variations are smallest for the TA 98 and greatest for the TA 102 strain. The increasing time between plating of cell suspension and adding to the top agar also increased spontaneous mutation rates in TA 102. Therefore, the number of spontaneous revertants is not a good quantity to which the induced mutation rates could be related as it was done in previous papers [6,7]. On the other hand, the number of induced revertants should not depend on mutagenic impurities in agar although the state of cells is important, of course. Therefore, we subtracted the number of spontaneous revertants from the whole number of mutant colonies in all experiments.

Several factors affect the mutation rates after γ-irradiation Increased doses give exponential cell killing, which leads to a local maximum in the dependence of the mutation rate (m) on the dose (D). If the mutation events arise independently of lethal events, the initial slopes of this curves should give actual mutation rates.

The difference in mutation rates between TA 98 and TA 100 can be explained by different molecular events leading to the reversions: frameshift in TA 98 and substitution in TA 100. The difference between TA 100 and TA 102 possibly corresponds to greater production of thymin injuries (t'-products) in comparison with other base damages [11], which could lead to TA 102 reversions (TAA nonsense codon is the target). Multiple pAQ1 plasmid increases the response of TA 102 cells to irradiation. TA 102 cells are proficient in excision repair.

Therefore, some injuries which could lead to cell death in TA 100 can be transformed to mutations in TA 102.

The mutation rate if measured as the number of mutant colonies per plate depends, however, on the number of cells per plate. We have also observed the increased mutation rates if the plates were incubated for 4-5 hours and then irradiated (data not shown). The number of viable cells can be smaller after keeping the plates in a refrigerator for a long time. Another important factor is the status of cells - stationary phase cells give less revertants (data not shown). It can be expected that the response will be greater or smaller both for heavy ions and for γ-rays. Therefore, the ratios of mutation rates have been measured in simultaneous experiments with γ-rays and heavy particles.

Sharply decreasing effectiveness of ionizing radiation with greater LET in producing mutations can be explained by two factors. Firstly, the production of pre-mutational injuries can be decreasing as LET increases (single strand breaks or base damages). Such injuries should be simple and should arise from the depositions of a small portion of energy to DNA or near to it (on the other hand, complex injuries arising from multiple events of energy deposition will be produced more efficiently by heavy ions). Secondly, the fluctuations of energy absorbed in different cells makes the population of hit cells smaller as LET increases. The surviving cells are surviving not because of their resistance but because they were not irradiated (hit by a particle). The cells which were hit by one particle could be damaged to such an extent that their survival is much lower than one and so the normalisation to actually surviving cells does not reflect the intrinsic mutation rate.

The induced substitutions detected in the TA 100 and TA 102 strains are probably due to a different mechanism. The evidence is provided by different dependence of the initial slope of m(D) curves on LET. No substantial decrease of the effectiveness of α-particles in comparison with γ-rays could be observed in the

TA 102 strain (with LET more than 100 keV/μm). On considering the fluctuations of energy in the cell population (some part of cells is not hit at all), we can do the conclusion that the effectiveness of α-particles in the induction of TA 102 revertants is much higher if related to the subpopulation of hit cells. Therefore, some kind of DNA injuries of complex form should be involved. Such injuries which are more efficiently produced by high LET particles are double strand breaks or multiply damaged single strands. The first type of DNA injuries should lead to lethal events. So some kind of complex SSB should be the cause of mutation events. The linearity of M(D) dependence suggests that the induction of SOS repair is not involved. Therefore, the breaks are probably the pre-mutational injuries. Simple calculations quantitatively confirm such a possibility.

The number of base damages is much greater than that of SSB or even SSB with a complex structure. Nevertheless, they probably lead to frameshifts with a probability much less than one. Complex SSB should lead to substitutions with a probability near to one.

It can be expected that in the case of direct mutations arising mainly as a result of substitutions in some of many sites the effectiveness of high LET particles will be greater in comparison with γ-rays. In the case of quadratic m(D) dependences when the induction of SOS repair is probably included, the increased LET should lead to even more increased mutation rates - the SOS system is induced by heavy ions more efficiently than by γ-rays.

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Received by Publishing Department

on December 9, 1987

Козубек С. и др. E19-87-858

Индукция ревертантов в тест-штаммах бактерий Салмонеллы при воздействии тяжелых ионов и γ-лучей

Была изучена эффективность действия разных типов ионизирующих излучений на тест-штаммах Салмонеллы. Наблюдалось резкое падение биологической эффективности ионизирующих излучений с ростом линейной передачи энергии /ЛПЭ/ у TA 98 штамма. У других двух штаммов такой зависимости не наблюдалось. Делается заключение о том, что существуют по крайней мере два типа премутационных повреждений ДНК - первый тип индуцируется γ-излучением и приводит к сдвигу рамки считывания, второй тип индуцируется тяжелыми частицами и приводит к замене оснований.

Работа выполнена в Лаборатории ядерных проблем ОИЯИ.

Препринт Объединенного института ядерных исследований. Дубна 1987

Kozubek S. et al. E19-87-858

The Induction of Revertants by Heavy Particles and γ-Rays in Salmonella Tester Strains

Mutagenic action of different types of ionizing radiations has been investigated in Salmonella tester strains. The effectiveness of ionizing radiation decreased with increasin LET in the TA 98 strain. No marked decrease could be detected in the TA 100 and TA 102 strains. The results suggest that at least two different types of premutational DNA injuries are produced by ionizing radiation - the first is produced by γ-rays and leads to frameshifts, the second is produced by heavy particles and leads to substitutions.

The investigation has been performed at the Laboratory of Nuclear Problems, JINR.

Preprint of the Joint Institute for Nuclear Research. Dubna 1987