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TITLE

DNA-repair after irradiation of cells with gamma-rays and neutrons

FINAL REPORT FOR THE PERIOD

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FINAL REPORT

Research Agreement: RC/1331-RB

Title of Project: DNA-repair after irradiation of cells with gamma-rays and neutrons.

Research Institution: Institut für Biologie, Forschungszentrum Seibersdorf.

Principal Scientific Investigator: Dr. Hans Altmann

Time period covered: December 1st 1972 - October 1st 1975

Scientific Background and Scope of Project:

Recently, there has been an increasing amount of interest in the use of neutrons for radiotherapy, mainly because of the reduced oxygen enhancement ratio (OER) of high LET radiation compared with radiation of lower LET. Thus neutrons seem to cause more damage to hypoxic cells than do X- or gamma rays at the same degree of injury to the normal tissue. The effect of neutron irradiation seems to be less modifyied by pre- and post-irradiation conditions than the damage produced by X- or gamma rays.

Though research has focused on DNA as the main target for radiation damage and loss of cellular proliferative capacity, yet very little is known about the effects of neutron irradiation on mammalian DNA. Because of the importance of repair processes in radiotherapy and in incidental exposure to radiation we have concentrated our efforts on the elucidation of DNA repair processes following neutron irradiation of cells.

PART A: Structural modifications of DNA produced by neutron or gamma irradiation.

Experimental methods:

All experiments were carried out on calf thymus DNA (Sigma, St. Louis, sodium salt of DNA, type I). DNA was dissolved in 10^{-2}m NaCl, final concentration was $5 \cdot 10^{-5}$(P)m. All neutron irradiations were done in the SNIF of the Austrian Astra reactor (see ref. 1) in position E-6 (D_n = 34 rad/s; D_γ = 5,7 rad/s) and position E-6 (D_n = 6,7 rad/s; D_γ = 1 rad/s). Gamma irradiation was performed in a 6 kCi Co^{60}-gamma cell (dose rate = 260 rad/s).

The structural alterations of irradiated DNA were observed by measurements of absorption spectra, sedimentation rate and viscosity. The extent of single and double strand production by neutron and gamma irradiation was investigated by ultracentrifugations in alkaline respective neutral sucrose gradients. Absorption measurements were performed in a Zeiss PMQ II photometer at a wave length of 250 nm, viscosity was measured in a rotation-viscosimeter according to Crothers and Zimm (2).

Results obtained:

T_m determinations of aqueous DNA solutions irradiated by gamma respective neutron-gamma radiation revealed a $RBE_{n-\gamma}$ of $0,38 \pm 0,03$. Calculations at the assumption of an additive effect of neutrons and contaminating gamma irradiation showed an RBE_n of 0,28. These results correlate very well with the calculated value of $RBE_{n-\gamma}$ for hyperchromicity, which was $0,35 \pm 0,03$. Though it was impossible to perform irradiation within the SNIF and our Co^{60}-facility at the same dose rate we were, nevertheless, able to do RBE calculations, since T_m measurements proved to be independent of the gamma dose rate.

The slightly decreased effect of mixed neutron-gamma irradiation was also demonstrated by gradient centrifugations: Neutron-gamma irradiation produced fewer single and double strand breaks compared with pure gamma irradiation. The same fact applies to

viscosity determinations: The effect of mixed neutron-gamma irradiation was less than the effect of gamma rays (alone). All RBE values for mixed neutron-gamma radiation are <1 and are modifyied by the neutron dose rate: The greater the dose rate, the smaller the amount of DNA damage produced within the SNIF.

Experiments carried out on dry DNA showed the same qualitative effect of both kinds of radiation, but RBE values for neutron-gamma radiation were slightly increased. This increase may be due to an increased radiosensitivity of DNA caused by the raise of temperature during irradiation.

PART B: Determinations of unscheduled DNA synthesis and re-joining of single and double strand breaks.

Experimental methods:

Repair processes of DNA occurring after irradiation can be measured by unscheduled DNA synthesis, i.e. repair replication of non-S-phase cells. The cell systems used were mainly mouse spleen suspensions (Strain Swiss), and human peripheral lymphocytes. A few experiments were also performed on spleen cells of a patient suffering from Morbus Hodgkin and on rat spleen suspensions (Strain: Sprague Dawley).

Spleen cell suspensions were prepared by gently treatment of the tissue in a Potter glass tube and filtering the cell suspension through miracloth. Peripheral lymphocytes were obtained after steile collection of venous blood and separation of lymphocytes, red cells and polymorphonuclear leukocytes by Ficoll-Urografin gradient centrifugation. All cell suspensions were adjusted to about 10^8 cells/ml.

a. Autoradiogrpahic studies: To suppress semiconservative DNA synthesis cells were preincubated in PBS containing $5 \cdot 10^{-3}$ mol hydroxyurea for 30 min. For irradiation cells were washed in Tris-Ac ($p_H = 7,4$) and resuspended in polyethylene

tubes containing 2 ml icecold Tris-buffer. Irradiations within the SNIF were performed in a special lucite container (fig. 1) which maintained a temperature of 4°C during irradiation. Immediately after irradiation cells were referred to PBS containing 10 µCi ^{3}H-TdR/ml and $2 \cdot 10^{-3}$ mol hydroxyurea and incubated at 37°C for 50 min. After incubation cells were washed in cold TdR, fixed and mounted on gelatine coated slides. Autoradiograms were done with Kodak NTB 3 Nuclear Emulsion, slides exposed for 13 days. Grain counting was performed in a double blind manner; for each determination 600 cells were evaluated, the number of grains per labeled cell and the percentage of labeled cells was determined.

Uptake of ^{3}H-TdR was also measured by liquid scintillation counting after extracting DNA according to the method of Schneider (3)

b. Gradient centrifugations. Mouse spleen cells were preincubated in Hank's BSS containing 10 µCi/ml ^{3}H-thymidine for 1 h, washed and irradiated in Tris-Ac. Aliquot samples were taken immediately after irradiation and after 30 min incubation at 37°C. After washing cells were treated with 80% ethyl-alcohol and lysed in 0,25 mol NaOH at ice temperature. Lysates were layered on top of a 5-20% alkaline sucrose gradient and centrifuged in a Beckmann Spinco SW 40 Ti-Rotor at 35.000 rpm for 300 min. For the estimation of double strand breaks, cells were homogenized in NaCl, RNA digested by RNase and protein by pronase. After addition of chloroform, samples were centrifuged, decanted and chloroform extracted by ether. The neutral gradients were centrifuged at 180.000 g for 180 min.

Results obtained:

All experiments showed a significantly reduced number of labeled cells after mixed neutron-gamma irradiation, compared with gamma irradiation, though the number of grains per labeled cell was the same for both types of irradiation (fig. 2, 3, 4).

Gradient centrifugations on alkaline sucrose demonstrated more single strand breaks produced by 7.5, 15 and 30 krad gamma irradiation compared with the same doses of mixed neutron-gamma irradiation (fig. 5). These strand breaks were very efficiently rejoined after gamma irradiation; after mixed neutron-gamma irradiation rejoining was only observed up to doses of 15 krad, at 30 krad no rejoining at all was observed. Gradient centrifugations on neutral sucrose showed the same results: Neutron-gamma irradiation produced a smaller amount of double-strand breaks, but rejoining after neutron irradiation was only observed at 7.5 and 15 krad (fig. 6). Studies on the inhibition of semiconservative DNA synthesis again showed a smaller effect of neutron irradiation compared with gamma rays (fig. 7).

PART C: Effect of repair inhibition

A possible effect of the detergent Tween 80 and Nonident P 40 on unscheduled DNA synthesis was studied by autoradiographic methods after mixed neutron-gamma irradiation (D_n = 5 krad). No repair incorporation could be observed after application of Tween 80 (0,002%) and Nonident P 40 (0,002%) (Table 1).

Table 1

		grains/cell	% labeled cells
Exp. 1	NP 40 (0,002%)	0	0
	T 80 (0,002%)	0	0
	control	12,13 ± 1,0	10,6 ± 3,1
Exp. 2	NP 40 (0,002%)	0	0
	T 80 (0,002%)	0	0
	control	11,99 ± 0,6	17,0 ± 5,0
Exp. 3	NP 40 (0,002%)	0	0
	T 80 (0,002%)	0	0
	control	12,65 ± 1,0	11,5 ± 3,3
Exp. 4	NP 40 (0,001%)	9,47 ± 0,9	12,3 ± 1,1
	control	13,10 ± 1,9	12,8 ± 3,8

In previous studies we were also able to demonstrate an inhibition of gamma induced unscheduled DNA synthesis by Tween 80 and Nonident P 40, though to a lesser extent than after mixed neutron-gamma irradiation (4).

Table 2

	dpm/ugm DNA	
	+ HU	- HU
n-γ irradiated + NP 40 (0,001%)	724,8	2098,8
n-γ irradiated control	1159,6	2649,9

Autoradiographic studies were also parallel by measurements of ^{3}H-thymidine incorporation in the acid-soluble DNA fraction by liquid scintillation counting. At 40 min incubation after 5 krad n-γ irradiation the results presented in Tab. 2 were obtained.

Besides studying unscheduled DNA synthesis investigations were also conducted on cell survival after application of Tween 80. The survival of E.coli BrT^{-} was investigated after gamma and mixed neutron-gamma irradiation. Both survival curves demonstrate a significant shoulder, which was reduced by Tween 80. Again the effect of Tween 80 observed after mixed neutron-gamma irradiation was more significant than its effect after gamma irradiation (fig. 8).

Conclusions:

All presented data indicate less damage of DNA after mixed neutron-gamma compared with pure gamma irradiation at the dose-rates used: The number of single and double strand breaks was reduced both in DNA irradiated in a dry stage and in aqueous solution. The extent of DNA break production in lymphoid cells was less after neutron-gamma irradiation with respect to gamma irradiation. Neutrons were also shown to be less effective in the suppression of semiconservative DNA synthesis. Repair processes of neutron induced damage, i.e. unscheduled DNA synthesis

and DNA strand break rejoining, could be observed, though to a smaller extent in comparison with pure gamma irradiation.

Since most data available on the effect of neutrons on various biological systems are indicating RBE values >1, we have to take into account targets different from DNA, as e.g. nuclear and cellular membranes. The theory of O- and N-effects, first suggested by Alper (5), could have validity for our studies, too. Since our experimental data again support the findings of Neary et al (6) and Christensen et al (7) who could not show much increase in effectiveness of neutrons with increasing LET up to 1000 MeV-cm^2/gm, they seem to favor the theory of an interaction between DNA and lesions in the membrane as it was also suggested by Cramp and Bryant (8). Studies of Berliner et al (9) on unscheduled DNA synthesis revealed that about 70% of repair sites occur in areas adjacent to the nuclear membrane, and though these studies were performed after UV-irradiation, they again emphasize the importance of integrity of the nuclear membrane for DNA repair synthesis.

To summarize the results of the present investigations we can conclude that mixed neutron gamma irradiation did not lead to an increase in DNA damage, and repair processes similar to those occurring after low LET irradiation could be observed. But these repair processes seem to be arrested at higher doses, perhaps attributable to an increase of damage to a second target within the cell.

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9. BERLINER, J. et al.: Radiat.Res. 63, 1975, 544.

Legends to figures:

Fig. 1.: Irradiation container: The sample was sealed within a polyethylene tube put into an ice-filled inner lucite container. The latter was placed into a second lucite container supplied with styropor. The third container consisting of aluminium walls and a lead cap remained within the reactor and was only used to introduce the sample into the SNTF.

Fig. 2: Unscheduled DNA synthesis measured autoradiographically after 30 krad mixed neutron-gamma and 30 krad gamma irradiation.

Fig. 3: Autoradiogram of peripheral lymphocytes after 30 krad gamma irradiation.

Fig. 4: Autoradiogram of peripheral lymphocytes after 30 krad mixed neutron-gamma irradiation.

Fig. 5: Sedimentation-profiles of DNA of mouse spleen cells in alkaline sucrose. Left side: mixed neutron-gamma irradiation, right side: gamma irradiation.

Fig. 6: Sedimentation-profiles of DNA of mouse spleen cells in neutral sucrose after A = 7,5 krad, B = 15 krad and C = 30 krad mixed neutron-gamma irradiation. D = unirradiated control.

Fig. 7: Inhibition of semiconservative DNA synthesis after gamma and mixed neutron-gamma irradiation.

Fig. 8: Survival of E.coli BrT^- after gamma resp. neutron-gamma x RBE 10 irradiation.
o —— o Sample without T 80
● —— ● Sample with 0,002% T 80

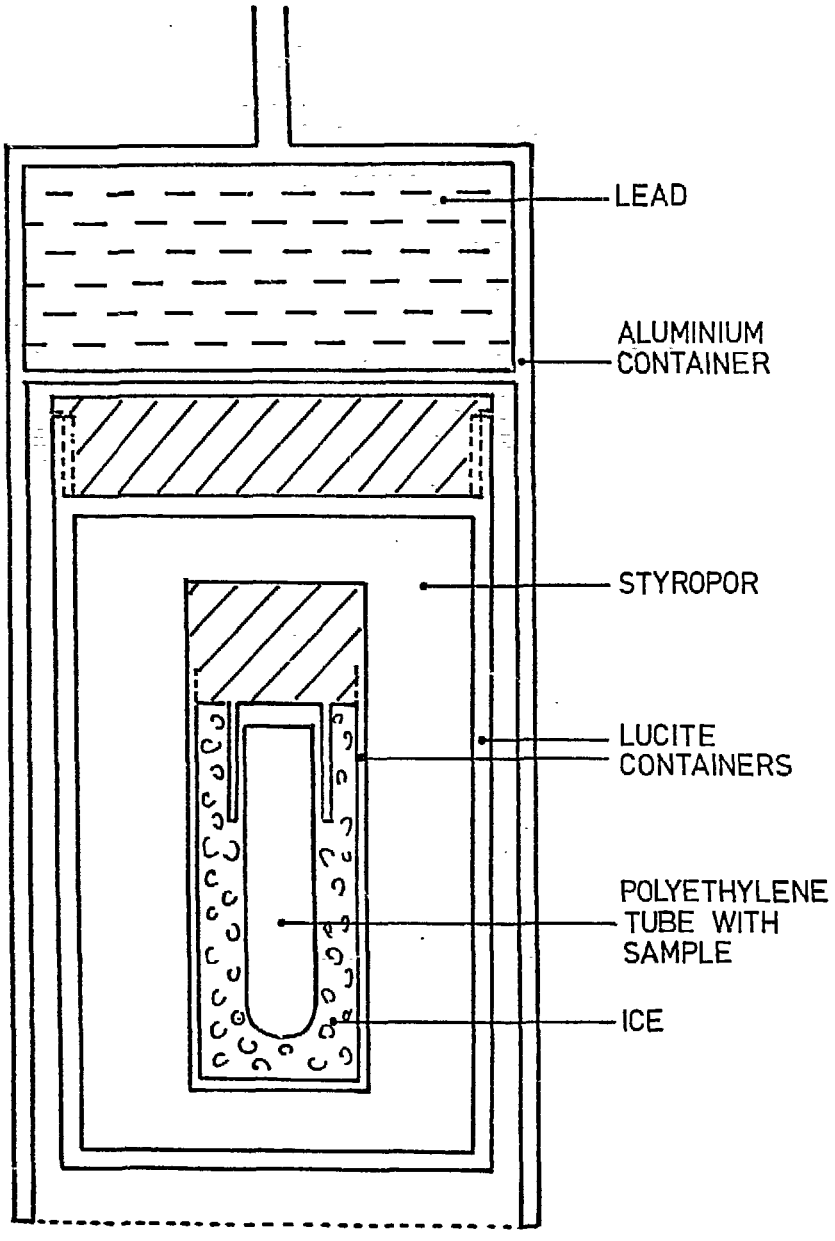

LEAD
ALUMINIUM CONTAINER
STYROPOR
LUCITE CONTAINERS
POLYETHYLENE TUBE WITH SAMPLE
ICE

Figure 1

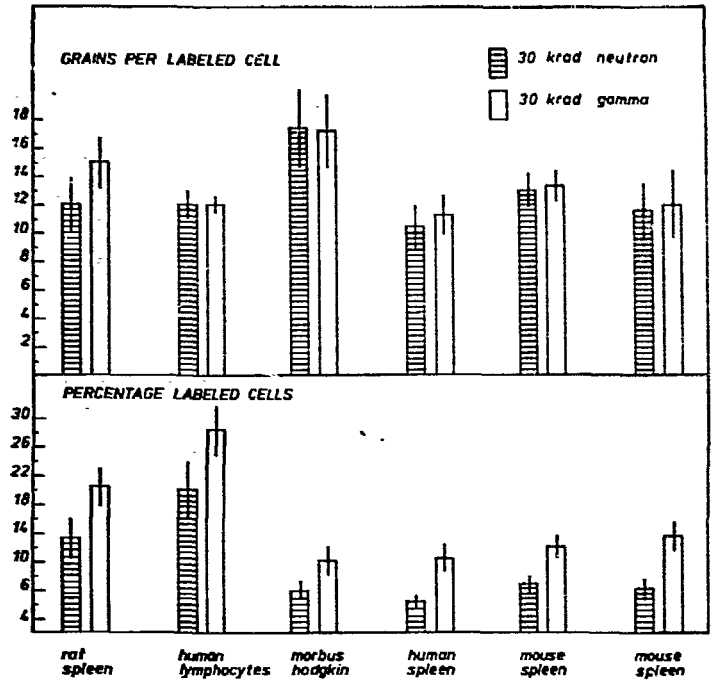


Figure 2

Figure 3

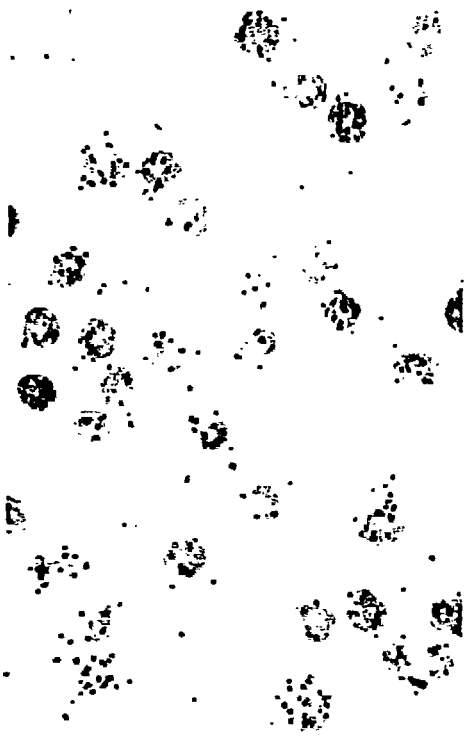


Figure 4

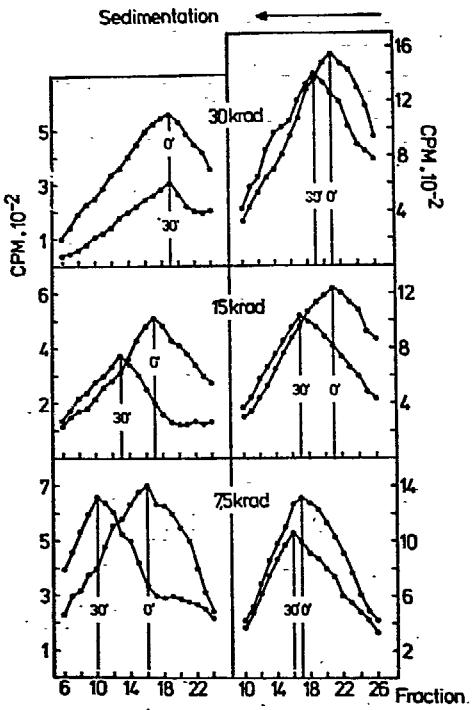


Figure 5

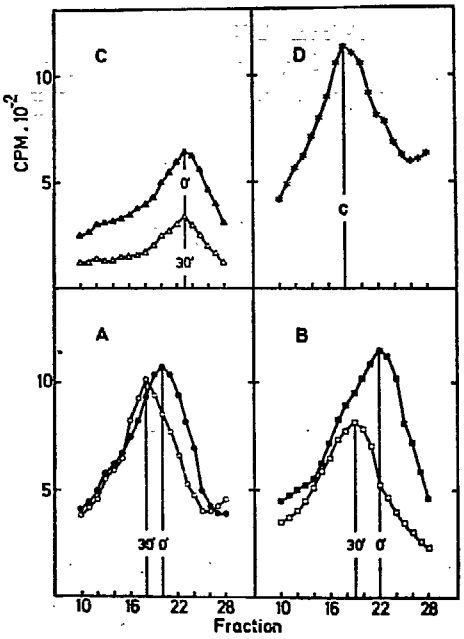


Figure 6

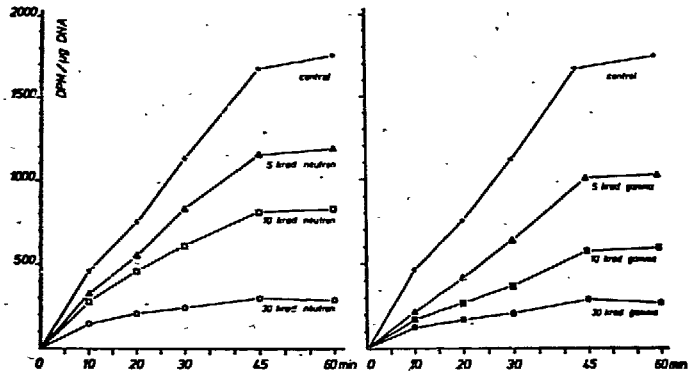
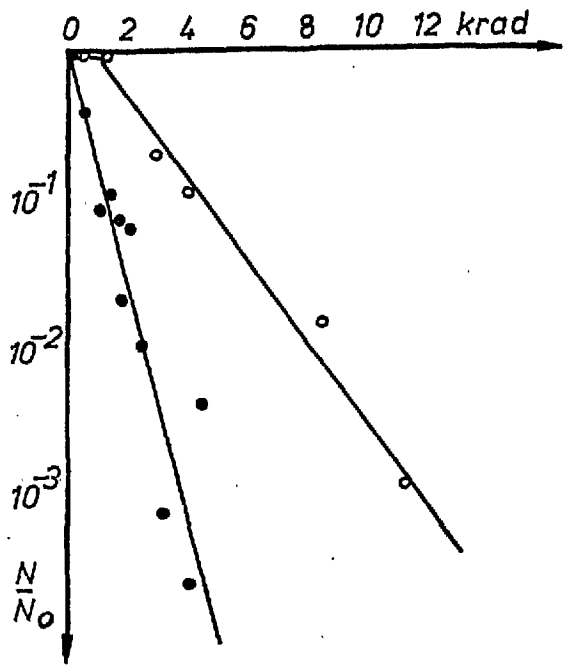
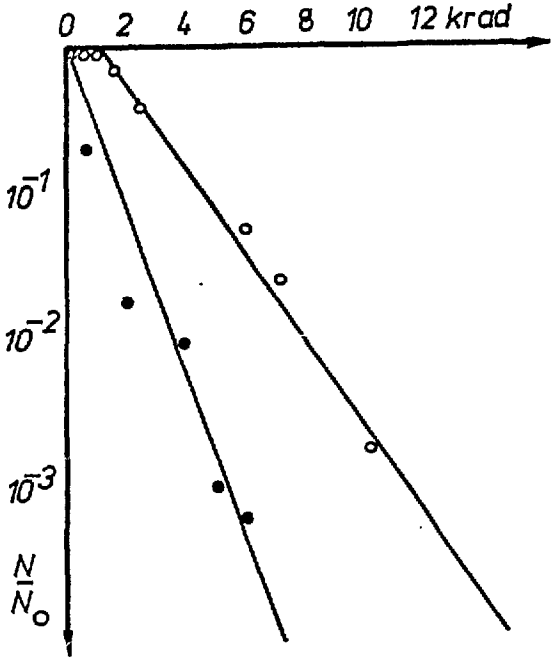


Figure 7



Neutron-gamma irradiation



gamma irradiation

Figure 8