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Evidence for reproductive death of dividing cells in thymus and spleen following whole-body gamma-irradiation of mice

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Mice were given whole-body gamma-irradiation with 300 and 800 rad 1 hour or 1 day after the administration of ^{3}H-thymidine. The time-course of the specific activity of DNA in thymus and spleen and the release of radioactivity into the fraction of DNA soluble in 0·14 N NaCl were determined for 28 hours after exposure. The results indicate that on average the loss of mitotically-active cells was delayed compared with the loss of a representative fraction of non-dividing cells. This suggested that different mechanisms might be implicated in the dying process of the dividing and non-dividing lymphoid cells. Using a dose-fractionation method, the total activity of DNA was determined in thymus and spleen at 24 hours after the first exposure and 25 hours after the *in vivo* labelling of DNA. A parallelism has been found between the results of this experiment and the recovery effect of the Elkind–Sutton type. It may be concluded that the dying process responsible for the loss of the dividing cells from thymus and spleen after irradiation probably involves reproductive death.

1. Introduction

Based on cell diameter, the lymphoid population from thymus and spleen was divided into large, medium and small lymphocytes. The medium and large lymphocytes represent the mitotically-active cells responsible for the rapid rate of cell renewal in these organs. Most small lymphocytes are non-dividing and represent about 90 per cent of the whole lymphoid population (Borum 1973, Fabrikant 1968, Fliedner, Kesse, Cronkite and Robertson 1964, Hanna 1964, Metcalf and Wiadrowski 1966). Attempts to demonstrate the actual occurrence of reproductive death in the lymphoid population from thymus and spleen after exposure to radiation failed (Puck 1966, Jackson, Christensen and Forkey 1968). It was established that after irradiation the lymphoid cells die of interphase death (for review see Okada 1970, Gerber and Altman 1970).

In the present studies, the time course of the release and retention of labelled DNA in thymus and spleen was determined during the first day after irradiation of mice. As ^{3}H-thymidine is incorporated only into the dividing fraction of lymphoid cells (Borum 1973, Fabrikant 1968, Metcalf and Wiadrowski 1966), the results indicate the mean response to radiation of medium and large lymphocytes. The data provide evidence which support the assumption that after irradiation the dividing cells die of reproductive death.

2. Materials and methods

2.1. *Radioactive labelling*

Male mice of the A2G strain weighing 25 $\pm$ 2 g were injected intraperitoneally with 25 μCi of ^{3}H-thymidine (5 Ci per mmole, Amersham, England).

2.2. *Irradiation conditions*

The animals were whole-body irradiated in plastic cages at 120 rad/min. The dose-rate was measured by a Siemens universal dosimeter. The irradiated and control mice were given a standard diet.

2.3. *DNA determination*

Mice were killed by cervical dislocation, and the thymus and spleen were homogenized in cold 0·14 N NaCl with a Potter–Elvehjem homogenizer. The whole homogenate was treated with a solution of KOH to a final concentration of 0·2 N, as indicated by Skalka, Matyášová and Chlumecká (1965) and the samples were incubated 24 hours at 37°C. Perchloric acid was added to a final concentration of 0·2 N, and the samples were centrifuged for 10 min at 1400 g. The sediment was hydrolysed in 0·5 N $HClO_4$ for 15 min at 70°C. The hydrolysate was used for the diphenylamine colour reaction (DeFrance and LePecq 1961) and for the determination of ^{3}H-thymidine radioactivity.

2.4. *Determination of soluble and insoluble DNA fractions*

The method was essentially that of Skalka *et al.* (1965). The homogenized thymus and spleen samples were centrifuged at 2500 × g for 15 min at 4°C. The supernatant fluid was treated with cold perchloric acid to a final concentration of 0·2 N, and the samples were centrifuged for 10 min at 1400 × g. The supernatant fluid was discarded; and the sediment was hydrolysed in 0·5 N $HClO_4$ for 15 min at 70°C. Fractions of hydrolysate were used for the diphenylamine colour reaction (DeFrance and LePecq 1961) and the determination of the ^{3}H-thymidine radioactivity. This fraction of DNA soluble in 0·14 N NaCl and insoluble in 0·2 N $HClO_4$ was assumed to contain the soluble DNA. To the pellet obtained after the first centrifugation containing the fraction of insoluble DNA, a solution of KOH was added to a final concentration of 0·2 N, and the determination was continued as described above.

2.5. *Radioactive measurement*

The radioactivity was counted in a liquid scintillation spectrometer (Betaszint BF–5003). The counting vial contained 0·5 ml of hydrolysate and 5·5 ml of solution of 60 g naphthalene, 4 g p-terphenyl, 0·2 g POPOP, 880 ml dioxane, 120 ml toluene, 100 ml methanol and 20 ml ethyleneglycol.

The radioactive measurement allowed the determination of the total activity (c.p.m. per DNA per organ) and specific activity (c.p.m. per mg DNA) of DNA in thymus and spleen. Each value given in figures represents an average of the results obtained from six to eight animals (± standard error of the mean).

3. Results

3.1. *Retention of labelled DNA in thymus and spleen after irradiation*

Mice were given whole-body irradiation 1 hour after the administration of ^{3}H-thymidine. Investigation of the time dependence showed that the specific activity of DNA in thymus (figure 1) and spleen (figure 2) increased significantly ($p < 0·05$) at 10 hours after exposure. It appears that a fraction of unlabelled cells was lost from these organs within 7 to 10 hours after irradiation. On the contrary, at 10 hours after irradiation the specific activity of DNA decreased compared with that found in normal animals (figures 1 and 2) if the radiation dose

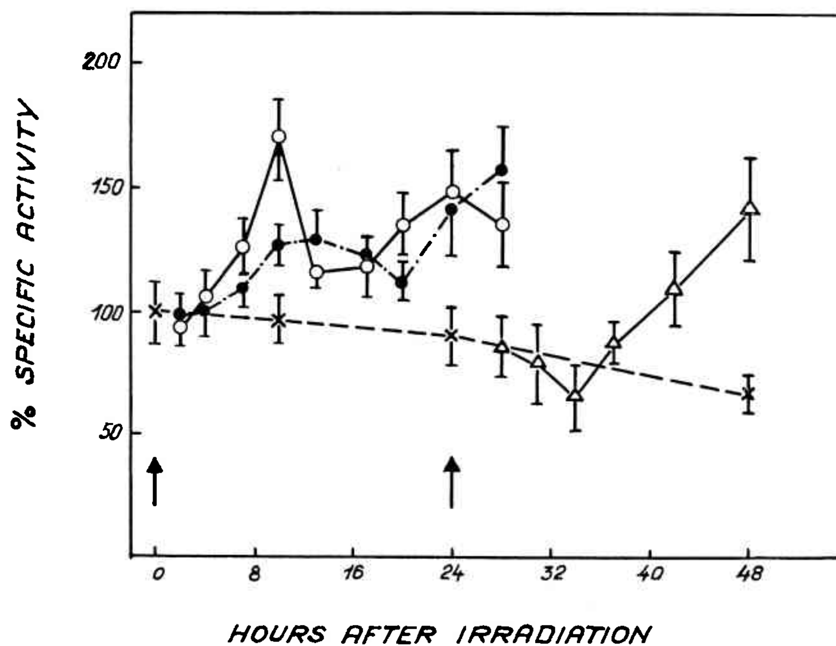

Figure 1. Specific activity of DNA in thymus. ^{3}H-thymidine administered 1 hour before irradiation with 300 (○) and 800 (●) rad, or 1 day before exposure with 300 rad (△). Results expressed as percentage of specific activity values of unirradiated controls (×) at 1 hour or 1 day after administration of the label.

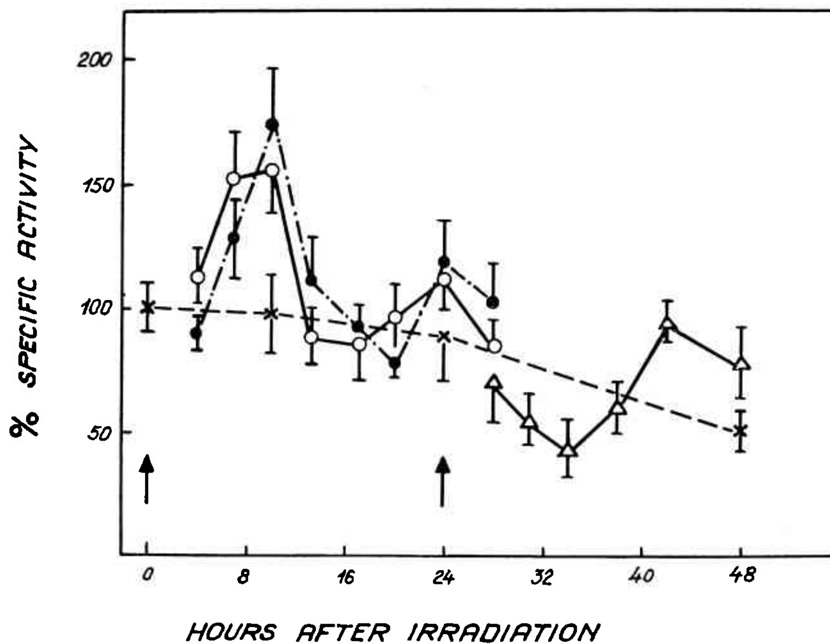

Figure 2. Specific activity of DNA in spleen. The same experiment as in figure 1. Symbols as in figure 1.

was given at 1 day after administration of the label. The results suggest that, in the course of 1 day, a large fraction of labelled cells changed their response to radiation and were lost from thymus and spleen up to 10 hours after exposure.

3.2. *Release and retention of labelled DNA in thymus and spleen after irradiation*

After irradiation, a representative fraction of DNA can be extracted from lymphoid tissues using 0·14 N NaCl solution (Skalka *et al.* 1965, Pierucci 1967, Suciu, Uray and Banu 1974, Suciu, Uray and Abraham 1975). Figure 3 shows

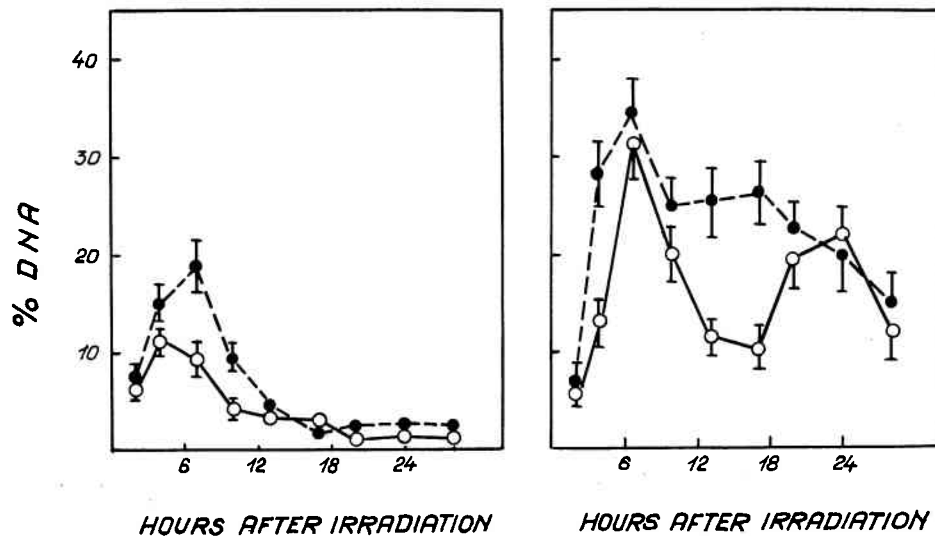

Figure 3. Time course of the release of soluble DNA in spleen (left) and thymus (right) after irradiation with 300 (○) and 800 (●) rad. Results expressed as percentage of soluble DNA in the whole DNA.

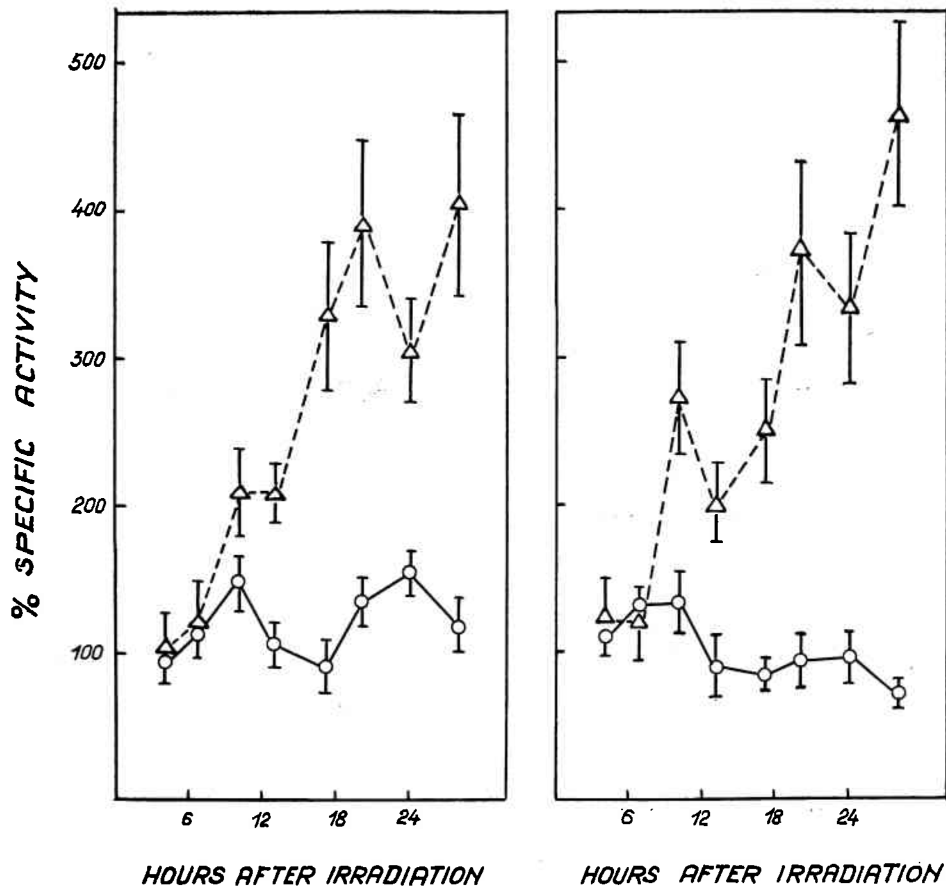

Figure 4. Specific activity of soluble DNA (△) and unsoluble DNA (○) in thymus (left) and spleen (right) after irradiation with 300 rad. Results expressed as percentage of specific activity of unirradiated controls at 1 hour after administration of ^{3}H-thymidine.

the time course of the release of soluble DNA in thymus and spleen after whole-body, irradiation with 300 and 800 rad. Four to seven hours after exposure the specific activity of DNA was about the same in the fraction of soluble and insoluble DNA in both thymus and spleen (figure 4). Then, a marked increase of the specific activity of soluble DNA was found up to 28 hours after exposure. Similar results were obtained after irradiation with 800 rad, but for clarity they were omitted from figure 4. These findings indicate that the loss of labelled DNA exceeded the loss of unlabelled DNA within 10 to 28 hours after exposure. Accordingly, the specific activity of whole DNA decreased from 10 to 20 hours after irradiation, if the label was administered 1 hour before exposure (figures 1 and 2).

3.3. *Two-dose fractionation experiment*

The experiments described above suggest that different mechanisms could be involved in the death of dividing and non-dividing lymphoid cells. As the non-dividing lymphocytes die in interphase (Okada 1970), it is conceivable for the mitotically-active lymphocytes to die of reproductive death. The later possibility was tested by measuring the total activity of DNA in spleen and thymus after irradiation with fractionated doses of 135 rad (figure 5). ^{3}H-Thymidine was administered 1 hour before the first dose of 135 rad. The second irradiation was performed at different intervals after the first exposure. The total activity of DNA in thymus and spleen was determined at 24 hours after the first dose of radiation. The results reported in figure 5 are similar to those indicating a

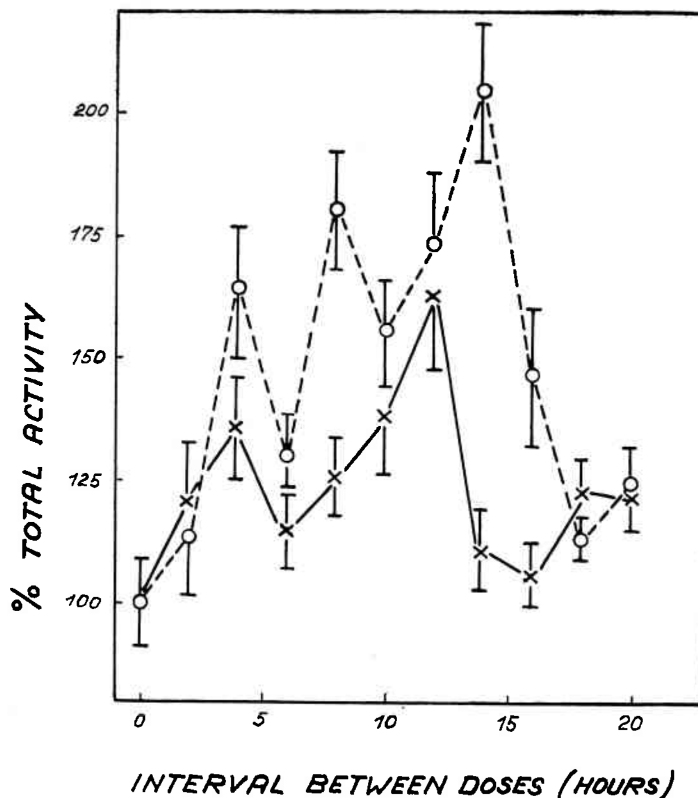

Figure 5. Total activity of DNA in thymus (×) and spleen (○) after irradiation with fractionated doses. ^{3}H-thymidine administered 1 hour before the first dose of 135 rad. Total activity of DNA determined at 24 hours after the first exposure. Results expressed as percentage of total activity of DNA at 1 day after irradiation with 270 rad.

recovery process of the Elkind–Sutton type (Elkind and Sutton 1959, Sinclair and Morton 1964).

4. Discussion

After the administration of a single dose of ^{3}H-thymidine, the labelled cells represent an approximately synchronized group among the mitotically-active cells of the tissue (Metcalf and Wiadrowski 1966). In earlier studies on the retention of DNA in thymus, spleen and small intestine after irradiation, the loss of previously labelled DNA has been equated with the loss of labelled cells from the tissue (Nygaard and Potter 1960, Gerber, Gerber, Altman and Hempelmann 1963, Sanders, Dalrymple and Robinette 1964, Suciu *et al.* 1975). The limits of this interpretation have been discussed by Nygaard and Potter (1960). The data on the retention of labelled DNA in thymus and spleen have shown that, 24 hours after irradiation, the surviving fraction of labelled cells was higher than the surviving fraction of unlabelled cells (Suciu, Uray and Abraham, in the press). As ^{3}H-thymidine is incorporated only into the dividing fraction of medium and large lymphocytes (Borum 1973, Fabrikant 1968, Metcalf and Wiadrowski 1966), it appears that small lymphocytes are more sensitive to radiation. This finding is supported by the observation that in the thymus of X-irradiated mice the number of small cells decreased to a greater extent after doses larger than 200 R (Blomgren and Révész 1968). The experiments reported here also indicate that after irradiation the loss of the previously-labelled cells from thymus and spleen was delayed compared with the loss of unlabelled cells (figures 1 and 2). If irradiation was performed at 1 day after administration of the radioactive precursor, the time course of the specific activities of DNA retained in thymus and spleen failed to reveal the delayed loss of labelled DNA. The results suggest that 1 day after incorporation of ^{3}H-thymidine the labelled cells change their response to radiation. This observation appears to parallel the rapid transformation of medium and large lymphocytes into non-dividing small lymphocytes (Borum 1973, Metcalf and Wiadrowski 1966, Fabrikant 1968, Hanna 1964, Fliedner *et al.* 1964).

The release of labelled DNA into the fraction of soluble DNA (figure 4) was delayed compared with the release of unlabelled DNA (figure 3) when ^{3}H-thymidine was administered 1 hour before irradiation. The increased solubility of DNA is produced by the disorganization of nuclear structures in the dying process of lymphoid cells after irradiation (Okada 1970). It is therefore probable that the dying process of labelled cells was somewhat delayed.

It has been reported that the limiting rate of depletion of nucleated cells from thymus and spleen was achieved within the range of 200 to 300 rad (Puck 1966, Jackson *et al.* 1968), and that the death of the whole lymphoid population is due mainly to interphase death followed by cell lysis (Jackson *et al.* 1968, Jackson, Christensen and Bistline 1969, Jackson and Christensen 1972, Sato and Sakka 1969). However, reproductive death of dividing bone-marrow lymphocytes has been demonstrated by Elkind–Sutton split-dose survival experiments (Fabrikant 1972). Our results obtained by labelling the mitotically-active cells before irradiation indicate that, with respect to the loss of cells from thymus and spleen, the bulk of dividing cells appear to die after a large fraction of non-dividing cells. These findings suggest that the mechanism implicated in the dying process of the relatively small fraction of dividing lymphocytes could be

different from that of non-dividing cells. Since the total activity of the previously-labelled DNA retained in thymus and spleen after irradiation reflects the retention of labelled cells (Nygaard and Potter 1960, Sanders *et al.* 1964), we used this parameter in a two-dose fractionation experiment. The results indicate that, within the period of 24 hours after irradiation, the labelled cells exhibit different degrees of radio-sensitivity (figure 5). These data are similar to the Elkind–Sutton split-dose survival curves (Okada 1970), which suggests that a representative fraction of dividing cells from thymus and spleen die of reproductive death. However, the results in figure 5 do not reflect the survival of lymphoid cells, but more probably the sterilization effect of radiation (Fabrikant 1972). The mean cell-cycle times for medium and large thymocytes were established as being in the range of 7 to 8 hours (Metcalf and Wiadrowski 1966, Fabrikant 1968). In agreement with these data, within the period of 12 hours after the first exposure, we found two maxima of recovery (figure 5). The first post-irradiation mitotic peak of thymus lymphocytes has been observed at 6 to 8 hours after whole-body irradiation with 75 rad (Jackson and Christensen 1972). As a reflection of the period when the cell population reached the radiosensitive stages G_2 and M (Sinclair and Morton 1964), this is the time which corresponds to the position of the first minimum reported in figure 5.

The split-dose experiment show that, during the period of 14 hours after the first exposure, the dividing cells from spleen exhibit three maxima of recovery (figure 5). The cell-cycle times for lymph-node germinal-centre primitive cells were estimated in the range of 5 to 7 hours (Fliedner *et al.* 1964, Hanna 1964). It therefore seems unlikely that the recovery maxima correspond to the same cell-population. This assumption is in agreement with earlier data, which suggest that the dividing spleen cells consist of two fractions with different levels of radiosensitivity (Gerber and Altman 1970, Nygaard and Potter 1960).

Finally, it must be pointed out that in previous studies the dose fractionation method was used exclusively in cell-survival experiments (Okada 1970). Thus, the analogy between the Elkind–Sutton split-dose survival results and our data (figure 5) may be fortuitous. Further investigations are therefore necessary to extend our knowledge of the relationship between the survival of the previously-labelled cells in thymus and spleen and the total activity of DNA retained in these organs after irradiation.

Des souris ont été gamma-irradiées avec 300–800 rad une heure ou un jour après l'administration de ^{3}H-thymidine. Pendant les 28 heures après l'irradiation on a déterminé l'évolution avec le temps de l'activité spécifique de l'ADN dans le thymus et la rate, de même que la mise en liberté de la radioactivité dans la fraction de l'ADN soluble en 0,14 N NaCl. Les résultats montrent qu'en moyenne la perte des cellules mitotiquement actives est retardée par rapport à la perte d'une fraction représentative des cellules mitotiquement inactives.

Les résultats suggèrent que dans le processus de mort des cellules lymphoides mitotiquement actives et mitotiquement inactives des mécanismes différents peuvent être impliqués. Utilisant une méthode de fractionnement de la dose on a déterminé l'activité totale de l'ADN dans le thymus et la rate 24 heures après l'exposition et 25 heures après marquage *in vivo* de l'ADN. On a trouvé un parallélisme entre les résultats de cette expérience et les effets de redressement de type ‘ Elkind–Sutton ’. On peut conclure que dans le processus de la mort, responsable de la perte des cellules mitotiquement actives du thymus et de la rate, après irradiation, est probablement impliquée la mort reproductive.

Mäuse wurden eine Stunde oder einen Tag nach der Verabreichung von ^{3}H-Thymidin mit 300 und 800 rad gamma-bestrahlt. Während 28 Stunden nach der Bestrahlung wurden die zeitliche Veränderung der spezifischen DNS-Aktivität in Thymus und Milz sowie die Freisetzung der Radioaktivität in die in 0,14 N NaCl-Lösung lösliche Fraktion bestimmt. Die Ergebnisse zeigen, daß der Verlust von mitotisch aktiven Zellen im Durchschnitt gegenüber dem Verlust einer repräsentativen Fraktion von mitotisch inaktiven Zellen verzögert ist. Diese Tatsache weist darauf hin, daß der Tod der lymphoiden, mitotisch aktiven und das Absterben der mitotisch inaktiven Zellen nach verschiedenen Mechanismen ablaufen kann. Mit Hilfe einer Dosisfraktionierungstechnik wurde 24 Stunden nach der ersten Bestrahlung und 25 Stunden nach der Markierung der DNS *in vivo* die Gesamtaktivität der DNS in Thymus und Milz bestimmt. Dabei wurde ein Parallelismus zwischen den Resultaten dieses Experiments und dem Elkind–Sutton–Erholungsprozeß gefunden. Daraus kann gefolgert werden, daß an dem Absterbeprozeß der mitotisch aktiven Zellen, der nach Bestrahlung zum Verlust dieser Zellen aus Thymus und Milz führt, der reproduktive Tod beteiligt sein könnte.

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