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Alkaline endonuclease(s) activity in the thymus and spleen of normal and irradiated mice

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Evidence has been found of an alkaline endonuclease activity in the cytoplasmic and nuclear fraction isolated from the thymus and spleen of mice. The chromatin-associated endonuclease activity was identified only in the spleen. The enzyme(s) was active on both single- and double-stranded DNA, but the reaction was faster if single-stranded DNA was used as a substrate. Maximum activity was found in the pH range of 7·9 to 8·1 in the presence of 10 mM Mg^{2+} and 1 mM Ca^{2+}. The enzyme(s) splits DNA, yielding 3′-hydroxyl terminated polynucleotides. It is suggested that this alkaline endonuclease(s) is responsible for the formation of deoxyribopolynucleotides in the thymus and spleen of irradiated mice.

1. Introduction

A few hours after whole-body irradiation, a representative fraction of deoxyribopolynucleotides (DPN) can be extracted from thymus and spleen by homogenization in saline solutions (Cole and Ellis 1957, Skalka and Matyášová 1964, Pierucci 1967, Okada 1970, Suciu, Uray and Abraham 1975). DPN are susceptible to the action of venom phosphodiesterase (VPDE) and resistant to spleen phosphodiesterase (SPDE), which is in agreement with the presence of 3′-hydroxyl terminal groups and the absence of 5′-hydroxyl terminal groups (Swingle and Cole 1967). It was suggested that the endonuclease responsible for the formation of DPN is the pancreatic DNase or DNase I (Swingle and Cole 1967). On the other hand, it was shown that both the thymus (Lindberg and Skoog 1970, Lindberg and Eriksson 1971) and spleen (Lindberg 1964, 1966, 1967 a, b) contain large amounts of DNase I inhibitors. It therefore seems improbable that DNase I is implicated in the formation of DPN. We report here the presence of an endonuclease with the maximum activity at pH 7·9 to 8·1 in both thymus and spleen of normal and irradiated mice. As determined by the action of VPDE and SPDE, this alkaline endonuclease(s) yields 3′-hydroxyl terminal groups. This enzyme(s) is probably responsible for the formation of DPN in the thymus and spleen of irradiated mice.

2. Materials and methods

2.1. *Irradiation*

Male mice of the Swiss strain, weighing 27 ± 3 g, were given whole-body irradiation in plastic cages using a ^{60}Co therapeutic unit (Theratron 80) (FSD = 80 cm; 80 rad/min). The dose-rate was measured with a Siemens universal dosimeter. The irradiated and control mice received a standard diet.

2.2. *Tissue fractionation*

Nuclei were isolated from the thymus and spleen using 0·25 M sucrose, 0·3 mM $CaCl_2$ solution with or without the addition of 0·1 per cent Triton X–100, as indicated by Bauer, Oliphant and Reeve (1975) and Haynes, Wolff and Till (1966). The supernatant fluid obtained after the first centrifugation (600 × g) was used for the assay of endonuclease activity in the cytoplasmic fraction. The isolated nuclei were homogenized in 0·14 M NaCl, 1 mM $MgCl_2$, 10 mM Tris-HCl buffer pH 7·4 (1 : 5, weight per volume). The homogenate was used for the determination of endonuclease activity in the nuclear fraction. The chromatin was, as suggested by Zubay and Doty (1959), isolated by homogenization and washing the nuclear fraction six times in 0·14 M NaCl, 1 mM $MgCl_2$, 10 mM Tris-HCl buffer pH 7·4 (1 : 5, weight per volume). The suspension obtained after the last homogenization was used for the assay of endonuclease activity. The DNA content of the tissue homogenates was determined as previously described (Suciu, Uray and Maniu 1976).

2.3. *Determination of deoxyribonuclease activity*

The usual procedure for the determination of deoxyribonuclease activity was adopted (Kunitz 1950). The reaction mixture contained 200 to 400 μg calf-thymus single- or double-stranded DNA (NBC, Cleveland, Ohio), 100 mM Tris-HCl buffer pH 6·9 to 9·0, 10 mM $MgCl_2$, 1 mM $CaCl_2$ in 1·85 ml of solution and 0·1 ml tissue homogenate. Blank samples were prepared by the addition of EDTA to a final concentration of 10 mM; and $MgCl_2$ and $CaCl_2$ were omitted. The samples were incubated for 1 to 4 hours at 37°C. The reaction was stopped by cooling and addition of 50 μl $HClO_4$ 70 per cent solution. The samples were centrifuged at 1400 × g, and the U.V.-absorption at 260 nm was measured in the supernatant fraction. One unit of endonuclease activity was assumed to be that activity which caused an increase in absorbancy of 0·001 per min per ml (cuvette 1 cm) under the given conditions. The results were expressed as units per mg thymus or spleen DNA (figures 1, 6 and 7).

2.4. *Determination of endodeoxyribonuclease activity*

The products of DNA digestion obtained as described in § 2.3. were analysed by DEAE-cellulose chromatography as suggested by Tomlinson and Tener (1962). The column (20 × 1 cm) was equilibrated with 7 M urea, and the mixture of nucleotides was separated by stepwise elution. Fractions of 20 ml containing increasing concentrations of sodium acetate pH 7·5 (0·05 to 0·5 M) and 7 M urea were run with a flow-rate of 15 ml per hour.

2.5. *Susceptibility to exonuclease of the DNA partially degraded by alkaline endonuclease*

A solution of 400 μg per ml double-stranded DNA, 20 mM Tris-HCl buffer pH 8·1, 10 mM $MgCl_2$ and 1 mM $CaCl_2$ was first partially digested by the action of thymic and splenic endonuclease (see § 2.3). The proteins were removed by extraction with chloroform : *n*-octanol (24 : 1), as previously described (Suciu and Mustea 1970). The pH was then adjusted to 8·7 by addition of solid Tris, and portions of 2 ml were treated with five units of venom phosphodiesterase (VPDE) (Calbiochem AG, Lucerne, Switzerland) in 0·1 ml of 90 per cent

glycerol. The OD_{260} was recorded over a period of 5 hours at 37°C. The direct VPDE reaction with undigested double-stranded DNA was determined in similar conditions.

The action of SPDE on the partially-digested DNA by alkaline endonuclease, obtained as described above, was determined as indicated by Swingle and Cole (1967). A Sephadex G-25 (medium) column (5 × 1 cm) was used to transfer the partially-degraded DNA into a 0·1 M Tris-acetic acid buffer pH 6·5, containing 0·5 mM EDTA. Fractions of 1 ml were treated with 0·1 units of spleen phosphodiesterase (SPDE) (Worthington Biochemical Corporation, Freehold, New Jersey U.S.A.) in 0·05 ml of 90 per cent glycerol. The OD_{260} was recorded over a period of 5 hours at 37°C. The control sample contained undigested double-stranded DNA.

The activity of both VPDE and SPDE was checked on DNA samples partially digested by the action of DNase I and DNase II, as indicated by Swingle and Cole (1967).

3. Results

3.1. *Alkaline endonuclease(s) activity in the thymus and spleen of normal mice*

The maximum deoxyribonuclease activity was found in both the cytoplasmic and nuclear fraction within the pH range of 7·9 to 8·1 (figure 1). The chromatin-associated deoxyribonuclease activity was evident only in spleen. The enzyme degrades both single- and double-stranded DNA, but for all cell fractions the reaction was faster if single-stranded DNA was used as substrate (results partially included in figure 1). The deoxyribonuclease was more active in the spleen.

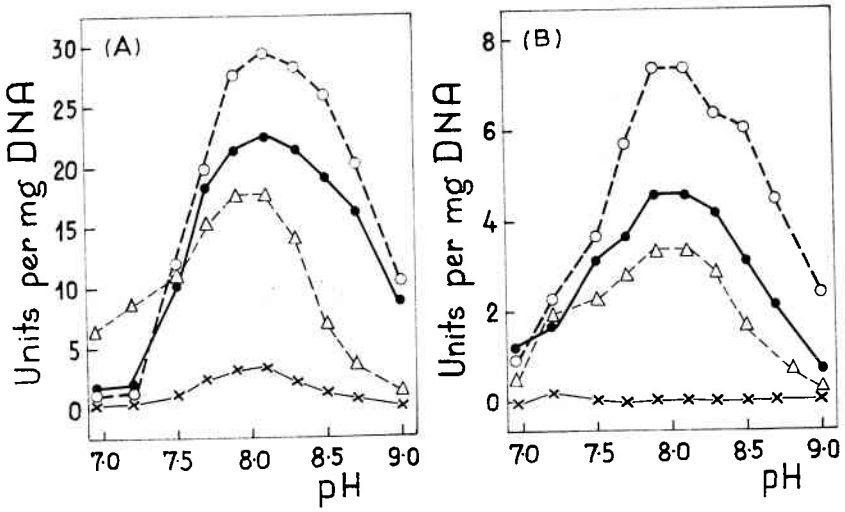

Figure 1. Alkaline deoxyribonuclease activity in the spleen (A) and thymus (B) of mice. Cytoplasmic fraction (△), nuclear fraction (●) and chromatin fraction (×) using double-stranded DNA as substrate. Nuclear fraction (○) using single-stranded DNA as substrate. Conditions of assay were described in § 2.3.

The enzyme was activated by the presence of 4 to 20 mM Mg^{2+} and 1 mM Ca^{2+} (figure 2). The absence of Mg^{2+} or its presence in concentration of 30 to 80 mM induced the inhibition of deoxyribonuclease activity.

Evidence of endodeoxyribonuclease activity was obtained by DEAE-cellulose chromatography (see § 2.4). The chromatographic patterns (figure 3) indicate that the products of digestion of both thymic and splenic deoxyribonucleases

contain large amounts of oligonucleotides and representative fractions of mononucleotides. It was determined that a significant fraction of mononucleotides was present in the tissue extract. On the other hand, it has been demonstrated that the endonucleolytic character is often associated with an exonucleolytic activity (Laskowski 1971). However, the possibility that both thymus and spleen contain exonuclease(s), with optimum activity in the alkaline pH range, cannot be ruled out (Lennartz, Coquerelle and Hagen 1975). For comparison, the chromatographic patterns corresponding to an endonuclease (DNase I) and an exonuclease (SPDE) have been included in figure 3.

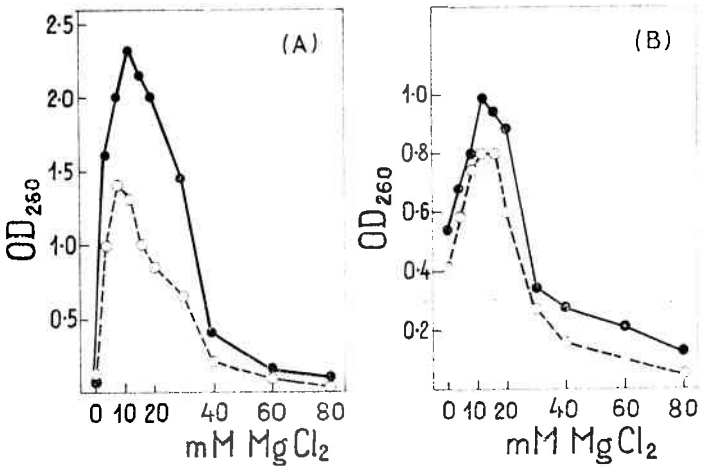

Figure 2. Alkaline deoxyribonuclease activity (pH 8·1) in the nuclear fraction from spleen (A) and thymus (B) in the presence of increasing concentrations of Mg^{2+}. In the absence (○) and in the presence (●) of 1 mM Ca^{2+}. The assay conditions were described in § 2.3. Double-stranded DNA as substrate (200 μg per ml).

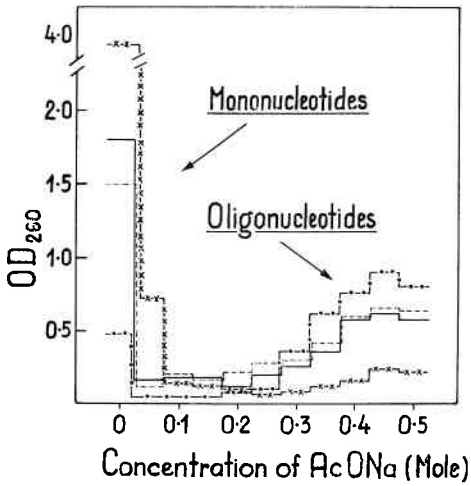

Figure 3. DEAE-cellulose chromatography of DNA digested by DNase I (●–●), thymic alkaline endonuclease (nuclear fraction) (– – –), splenic alkaline endonuclease (nuclear fraction) (——) and DNase II followed by exonuclease SPDE (×–×). Conditions of fractionation were described in § 2.4.

Determination of the site of splitting of the DNA chain by alkaline endonuclease was performed, according to the method of Swingle and Cole (1967), using VPDE and SPDE. The partially-digested double-stranded DNA obtained by incubation of DNA, with the nuclear fraction isolated from thymus and spleen (see § 2.2) was then subjected to the action of VPDE and SPDE (figure 4).

The completion of hydrolysis was attained only for VPDE. This finding indicates that the products of DNA digestion obtained by the action of alkaline endonuclease contain 3′-hydroxyl terminal groups.

The incubation for 10 to 30 min at 56°C of the nuclear fraction isolated from thymus and spleen (see § 2.2) was associated with a decrease in alkaline endonuclease activity (figure 5). However, 2 to 4 min of incubation at 56°C produced an increase in activity. These data suggest that, *in vivo* alkaline endonuclease activity may be partially checked by some inhibitors which are probably rapidly degraded at 56°C. It is possible that the DNase I inhibitors that have been found in both thymus and spleen (Lindberg and Skoog 1970, Lindberg and Eriksson 1971, Lindberg 1964, 1966, 1967 a, b) are responsible for this effect.

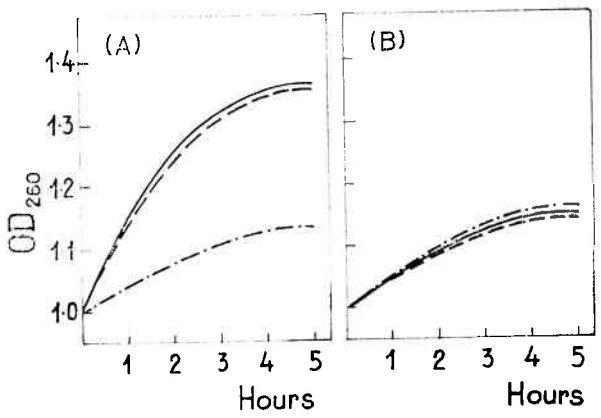

Figure 4. Action of VPDE (A) and SPDE (B) on the DNA previously digested by alkaline endonuclease from spleen (nuclear fraction) (——) and thymus (nuclear fraction) (– – –). Control DNA in the absence of alkaline endonuclease (.–.). Conditions of assay were described in § 2.5.

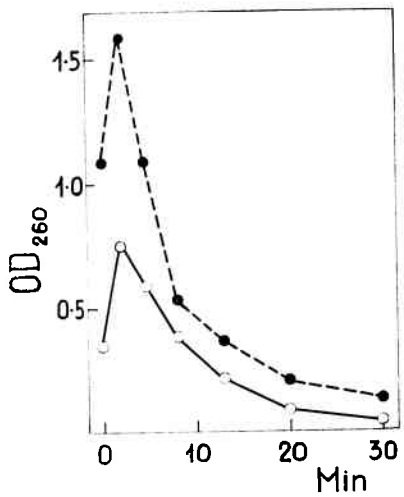

Figure 5. Thermal inactivation at 56°C of alkaline endonuclease (pH 8·1) in the nuclear fraction isolated from spleen (●) and thymus (○). The assay conditions were described in § 2.3. Double-stranded DNA as substrate.

3.2. *Alkaline endonuclease*(s) *activity in the thymus and spleen of irradiated mice*

Four hours after whole-body irradiation with 300 rad, the maximum activity of endonuclease was found in the same pH range of 7·9 to 8·1 as in normal mice (figure 6). However, irradiation depressed the alkaline endonuclease activity (figure 7) up to a dose of about 800 rad. Whole-body irradiation causes a decrease in protein synthetic rate (Richmond, Ord and Stocken 1957,

Herranen 1964, Smit and Stocken 1964) and a general inhibition of the transcription machinery in lymphoid tissues undergoing interphase death (Patil, Pradhan and Sreenivasan 1975, Chetty, Netrawali, Pradhan and Sreenivasan 1976). Accordingly, the decrease in alkaline endonuclease activity (figure 7) may be associated with the general inhibition of protein synthesis.

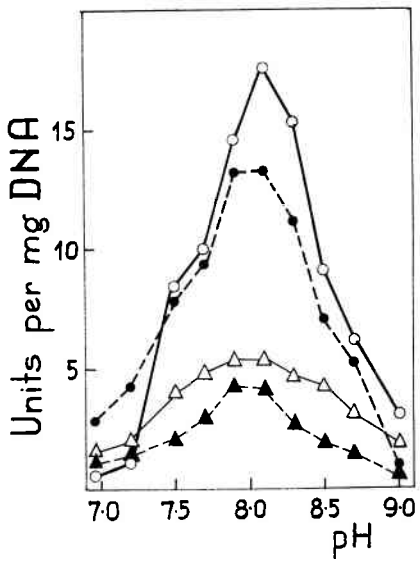

Figure 6. Alkaline endonuclease activity in the thymus and spleen of mice 4 hours after whole-body irradiation with 300 rad. Activity determined for the whole tissue homogenate obtained in 0·14 M NaCl, 10 mM Tris-HCl buffer pH 7·4 and 1 mM $MgCl_2$ solution. Conditions of assay were described in § 2.3. Normal spleen (○), irradiated spleen (●), normal thymus (△) and irradiated thymus (▲). Double-stranded DNA as substrate.

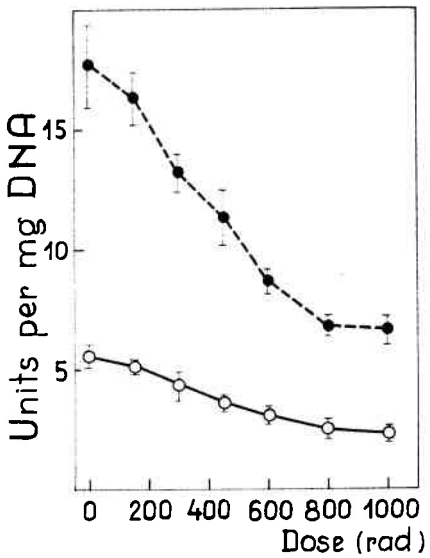

Figure 7. Alkaline endonuclease activity (pH 8·1) in the spleen (●) and thymus (○) of mice 4 hours after whole-body irradiation. Conditions of assay were the same as in figure 6. The values are means of the results obtained in three separate experiments (± SEM), each group consisting of eight mice.

4. Discussion

The analysis of deoxyribonucleotides (DPN) reported by Swingle and Cole (1967) indicates that the enzyme responsible for radiation-induced DNA degradation in lymphoid organs must be an endonuclease, yielding 3′-hydroxyl terminated oligonucleotides. The activity of alkaline endonuclease(s) observed

in both thymus and spleen (figure 1) corresponds to these requirements (figures 3 and 4). The enzyme(s) presents similar properties with the liver Mg^{2+}/Ca^{2+}-dependent endonuclease (Hewish and Burgoyne 1973, Burgoyne, Hewish and Mobbs 1974, Burgoyne and Mobbs 1975). Since the liver, a radioresistant organ, and the radiosensitive organs, thymus and spleen, contain endogeneous endonuclease(s) with maximum activity at pH 8·0, and the formation of DPN can be observed only in the radiosensitive tissues (Skalka, Matyášová and Chlumecká 1965), it is probable that the sequence of events leading to the DNA degradation involves other preliminary determinant steps. The formation of DPN in lymphoid organs is dependent on time (Swingle and Cole 1967), the presence of oxygen (Honjo, Takeda and Maeda 1962) and probably the loss of histones (Ernst 1962, Bauer *et al.* 1975). These requirements must be associated with some biochemical changes related to nuclear disorganization, leading finally to interphase death (Okada 1970).

It has been shown that the accessibility of DNA in chromatin to nuclease digestion is restricted, and for some DNases, at the limit of digestion, most of the DNA that remains is in the form of relatively homogeneous small fragments, which vary from about 40 to 200 nucleotide pairs in length (Itzhaki 1971, 1974, Bostock, Christie and Hatch 1976, Simpson and Whitlock 1976, Burgoyne *et al.* 1974, 1975, Lohr and Van Holde 1975, Felsenfeld 1975). The DNA of irradiated lymphoid tissues is digested *in vivo* into regular fragments of about the same length (Swingle and Cole 1967, Skalka, Matyášová and Cejkova 1976). Immediately after irradiation with 140 to 520 krad, owing to the direct and indirect action of radiation (Okada 1970), the end-group analysis of DNA isolated from thymocytes revealed the presence of 3′-hydroxyl, 5′-hydroxyl, 5′-phosphate and other unknown strand-break terminals (Lennartz *et al.* 1975). On the other hand, the analysis of DPN isolated from spleen 4 hours after irradiation with 600 or 1000 rad indicated the absence of 5′-hydroxyl terminals and the presence of 3′-hydroxyl terminal groups (Swingle and Cole 1967). All these data show that, after whole-body irradiation with doses up to 1000 rad, the thymic and splenic DNA presents specific sites susceptible to alkaline endonuclease digestion, which can be different from the sites of damage induced by radiation.

The observations reported suggest that alkaline endonuclease is implicated in the process of DNA degradation accompaning the interphase death. However, the possibility that alkaline endonuclease is active in the repair of the DNA single-strand breaks (Lennartz *et al.* 1975) can not be ruled out.

Since alkaline endonuclease activity was identified in thymus and spleen as well as in the liver (Hewish and Burgoyne 1973), further investigations are necessary for the isolation and characterization of this enzyme(s).

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On a mis en évidence, chez les souris, une activité endonucléasique alcaline dans la fraction cytoplasmique et nucléaire isolée du thymus et de la rate. L'activité endonucléasique, associée à la chromatine, a été identifiée uniquement dans la rate. L'enzyme(s) est active envers l'ADN mono- et dicaténaire, mais la réaction est plus rapide si on utilise comme substrat l'ADN mono-caténaire. L'activité maximale a été trouvée dans le domaine du pH 7,9–8,1 en présence de 10 mM Mg^{2+} et 1 mM Ca^{2+}. L'enzyme(s) sépare

l'ADN, en formant des polynucléotides terminées avec des groupes 3′-hydroxyles. On suggère que cette endonucléase(s) alcaline est responsable de la formation des désoxyribopolynucléotides dans le thymus et la rate des souris irradiées.

In zytoplasmatischen und nuklearen Fraktionen, die aus dem Thymus und der Milz von Mäusen isoliert worden waren, wurde eine alkalische Endonukleaseaktivität gefunden. Die Chromatingebundene Aktivität wurde nur in der Milz festgestellt. Das bzw. die Enzyme waren sowohl gegenüber einsträngiger als auch doppelsträngiger DNS aktiv, die Reaktion war mit einsträngiger DNS als Substrat jedoch schneller. Das Maximum der Aktivität wurde im pH-Bereich von 7·9–8·1 in Gegenwart von 10 mMMg^{2+} und 1 mM Ca^{2+} gefunden. Bei der Spaltung der DNS entstehen 3′-Hydroxyl-terminale Polynukleotide. Es wird vorgeschlagen, daß diese alkalischen Endonukleasen für die Bildung von Desoxypolynukleotiden im Thymus und der Milz bestrahlter Mäuse verantwortlich sind.

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