

Characterization of Different Deoxyribonucleases in Human Lymphocytes

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Four groups of deoxyribonuclease activities from human lymphocytes have been characterized by deoxyribonuclease assay in DNA-containing polyacrylamide gels following their separation by disc-electrophoresis. All activities hydrolyse DNA endonucleolytically. One neutral deoxyribonuclease found in the cytoplasmic fraction prefers native or UV-irradiated DNA over denatured DNA as substrate and is a 5'-monoester former. Two groups of acid deoxyribonuclease activities are detectable in the nuclear fraction. Both are 3'-monoester formers. One is as well active with denatured DNA as with native DNA, the other one shows the same activity with native and UV-irradiated DNA but lower activity with denatured DNA. An alkaline deoxyribonuclease activity, also localized in the nucleus, is a 5'-monoester former, and prefers denatured or UV-irradiated DNA as substrate.

Different deoxyribonuclease (DNase)-activities have been described in human^{1–3} and avian lymphocytes or lymphoblasts^{4, 5}. Two groups of DNases, usually called DNase I (EC 3.1.4.5) associated with mitochondria and the nucleus⁶ and DNase II (EC 3.1.4.6) associated with the lysosomes⁷ are well characterized. Additional activities have been described in mammalian cells. Thus distinct DNases have been found in HeLa cells⁸, in liver cells^{9, 10} and in calf thymus nuclei¹¹. There are reports about DNases possibly involved in DNA replication^{8, 12, 13} and DNA repair^{14–16}. Recently we reported the occurrence of four groups of DNases in human lymphocytes¹⁷. In the present paper these enzyme activities are further characterized in respect to their substrate specificity, mode of action and intracellular localization.

Materials and Methods

Materials were obtained as follows: Ronpacon 75% from Cilag Chemie GmbH, Alsbach (Germany); Thrombovetren (4000 IE Heparin/2 ml) from Promota, Hamburg (Germany); Ficoll from Pharmacia, Fine Chemicals AB, Uppsala (Sweden); Gallocyanine from Fluka AG (Switzerland); Chromalaun from Merck, Darmstadt (Germany); Aquasol from NEN Chemicals GmbH, Dreieichenhain (Germany); Herring sperm DNA was a gift from H. Mack, Illertissen (Germany); DNase I (EC 3.1.4.5)

from Worthington, Freehold, New Jersey (USA); phosphodiesterase from snake venom (EC 3.1.4.1) (1.5 U/mg), phosphodiesterase from calf spleen (EC 3.1.4.18) (2 U/mg), and lysozyme (EC 3.2.1.17) (22 000 U/mg) from Boehringer Mannheim GmbH (Germany); [³H-methyl]thymine (spec. act.: 5.0 Ci/mmol) from the Radiochemical Centre, Amersham (England). All other reagents were from the highest analytical grade as supplied by Serva, Heidelberg (Germany) and Merck, Darmstadt (Germany).

[³H]DNA was labeled in a thymine-deficient mutant of *Escherichia coli* (CR 34/K 416 thy[–]) in a synthetic medium containing a total of 3 µg thymine/ml supplemented with [³H-methyl]thymine at 0.2 mCi/l for 6 hours. The harvested bacteria were lysed with lysozyme (5 mg/ml) in a buffer pH 8.5 containing *p*-aminosalicylate 10% and EDTA 0.2%. The DNA was prepared and purified by repeated fractional precipitation using the cationic detergent cetyltrimethylammonium bromide¹⁸. The final product had a specific activity of 5.8 µCi/mg DNA.

Heat denatured DNA was prepared by heating native herring sperm DNA or *E. coli* [³H]DNA for 10 min at 100 °C in a boiling water bath and chilling in ice. Irradiation of DNA was performed according to Weinblum *et al.*¹⁹. The thymine dimer content of the irradiated DNA was 22% determined after formic acid hydrolysis by high-pressure liquid cation-exchange chromatography.

Lymphocytes were isolated as described¹⁷. Isolations of nuclei were performed by homogenization of the cells in 0.88 M sucrose, 1.5 mM CaCl₂ followed by centrifugation 24 000 rpm for 90 min in a SW 50 rotor (Spinco L 50) in a discontinuous density gradient in which the upper phase is the

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homogenate and the lower phase is 2.2 M sucrose, 0.5 mM CaCl_2 ²⁰.

The determination of DNase-activities was performed using the *in situ* detection of DNases in DNA-containing polyacrylamide gels following electrophoretic separation ^{17,21}. Before use, the cells were disrupted by freezing and thawing three times. We used a 5% acrylamide spacer gel pH 6.7, 0.176 M $\text{Tris-H}_3\text{PO}_4$ and a separation gel 13.4% acrylamide, pH 8.8, 0.177 M $\text{Tris-H}_2\text{SO}_4$. The electrophoresis was performed at 700 $\mu\text{A/gel}$ for 80 min with the anode at the bottom. The DNA concentration in the small-pore gel was 0.3 mg native or denatured herring sperm DNA per ml or 0.3 mg native, denatured, or UV-irradiated [³H]DNA from *E. coli*. All operations from gel formation to incubation were carried out at 4 °C. A final sample volume of 8 μl per gel corresponding an equivalent of 1.6×10^5 lymphocytes in spacer gel buffer was applied. After the electrophoretic run the gels with herring sperm DNA were incubated in different incubation mixtures for 4 hours at 37 °C and stained with galloxyaniline-chromalaune. After destaining with water the optical density was recorded with a densitometer.

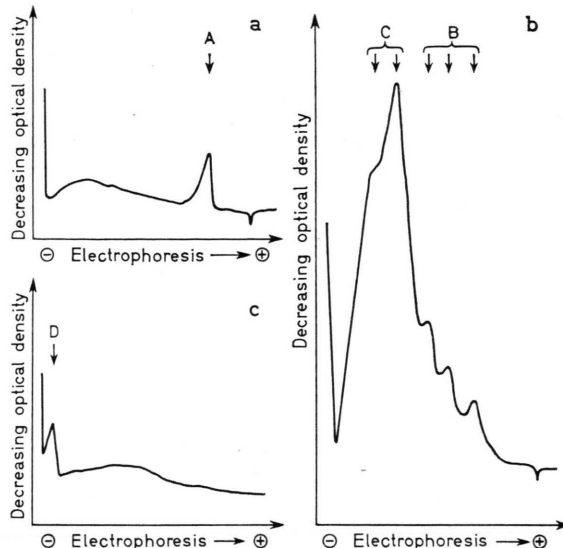
The gels with radioactive DNA were cut immediately after the run into 1.5 μl slices. These were incubated each in 100 μl of a suitable incubation mixture for 4 hours. The incubation mixture was then transferred into polyethylene counting vials with 10 ml Aquasol and the radioactivity determined in a liquid scintillation spectrometer.

For the characterization of the DNase-activities as exo- or endonucleases the products of the DNase-assay using [³H]DNA containing gels were analyzed directly by thin-layer chromatography ²². The position of the terminal phosphate in DNA fragments after hydrolysis by the different DNases from lymphocytes was determined by chromatography of the products of further cleavage of DNA-oligonucleotides by phosphodiesterase I and II. DNase-activities in lymphocytes were assayed following disc-electrophoretic separation in [³H]DNA containing gels as described above. Each incubation mixture containing DNA fragments was then divided in four aliquots and the same volume of one of the following solutions was added: a. 50 mM Tris-HCl pH 8.5, 10 mM MgCl_2 ; b. 5 μg phosphodiesterase I per ml solution a; c. 0.3 M Na-succinate pH 6.5; d. 0.5 mg phosphodiesterase II per ml solution c.

These samples were incubated for 5 hours at 37 °C and then the chromatographic analysis of fragments after hydrolysis with phosphodiesterases was carried out according to Pataki ²².

Results and Discussion

In human lymphocytes four groups of DNase-activities are detectable (Fig. 1) using the DNase-assay in DNA containing polyacrylamide gels. One activity migrating farthest towards the anode has a



Figs 1 a, b, c. Densitometer tracings of DNA-containing polyacrylamide gels after electrophoresis of freeze-thawed lymphocytes. The gels contained 0.3 mg native (a and b) or denatured (c) herring sperm DNA/ml in 13.4% acrylamide separation gel at pH 8.8. On each gel 8 μl of an equivalent of 20×10^6 lymphocytes per ml in spacer gel buffer was applied. The gels were incubated for 4 hours: a in 0.1 M Tris-HCl pH 7.4, 10 mM MgCl_2 , 1 mM CaCl_2 ; b in 0.1 M Na-acetate pH 5.0, 1.5 mM EDTA ; c in 25 mM Tris-HCl pH 8.5, 10 mM MgCl_2 . For experimental conditions see Materials and Methods. The letters A, B, C and D refer to the different DNase-activities (see Results and Discussion).

pH optimum of 7.0 (A), two additional groups of acid DNases are discernible, one with a pH optimum of 5.0 (B) and another with a maximum activity at pH 5.5 (C). In addition there is a DNase-activity showing optimal activity in the range of pH 9.0 (D). They differ also in their dependency of divalent cations and activity in the presence of substances as N-bromsuccinimide, Na_2SO_4 , iodoacetic acid, iodoacetamide, CuCl_2 , 2-mercaptoethanol and Na-succinate ¹⁷. As shown in Fig. 2 the relation of the activities with native, denatured or UV-irradiated DNA as substrate is different between the DNase-activities. DNase A is as well active with UV-irradiated DNA as with native DNA as substrate, but much less with denatured DNA. The group B shows less activity with UV-irradiated DNA

in contrast to C, where only denatured DNA is a poor substrate. Completely different is the substrate preference of DNase D, which acts as well on denatured DNA as on UV-irradiated DNA but not on native DNA.

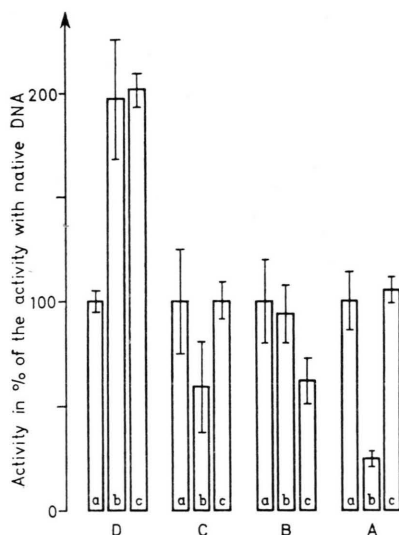


Fig. 2. The activity of DNases A, B, C and D from human lymphocytes with native (a), denatured (b), or UV-irradiated (c) DNA as substrate. The activity was determined with [^3H]DNA-containing polyacrylamide gels as described in Materials and Methods. Incubation: A in 0.1 M Tris-HCl pH 7.4, 10 mM MgCl₂, 1 mM CaCl₂; B and C in 0.1 M Na-acetate pH 8.5, 1.5 mM EDTA; D in 25 mM Tris-HCl pH 8.5, 10 mM MgCl₂. The activity is expressed in % of the activity with native DNA as substrate.

If the DNA fragments released by each DNase are analyzed (Table I), only a small percentage of mononucleotides could be detected, suggesting that all activities hydrolyse DNA endonucleolytically.

Table I. DNA fragments formed by the different DNases from human lymphocytes. % Oligonucleotides from the total amount of DNA fragments formed by the different DNase-activities were determined by thin-layer chromatography.

	DNA-fragments formed by DNase-activity:			
	D	C	B	A
% Oligonucleotides	64	82	71	77

From the chromatographic analysis of oligonucleotides formed by hydrolysis with DNase A and D and exposed to further enzymatic cleavage with phosphodiesterase I and with phosphodiesterase II follows that they are 5'-monoester former (Table II). The incubation of fragments formed

Table II. Determination of the terminal phosphate in DNA-fragments after hydrolysis by different DNase-activities from lymphocytes. The DNA-fragments of the different DNases were further incubated: a with 50 mM Tris-HCl pH 8.5, 10 mM MgCl₂; b with 5 μg phosphodiesterase I per ml a; c with 0.3 M Na-succinate pH 6.5; d with 0.5 mg phosphodiesterase II per ml c. The products were analyzed by thin-layer chromatography (see Materials and Methods).

DNA fragments of DNase:	% of DNA-fragments resistant to further incubation with:			
	a	b	c	d
A	84	3	100	97
B	100	79	96	15
C	90	82	100	14
D	96	8	100	100

by these enzymes with phosphodiesterase I resulted in further profound cleavage which may be seen from the disappearance of oligonucleotides at the application point and total resistance to the action of phosphodiesterase II. An opposite situation is found for the acidic DNases C and B. The DNA fragments of these enzymes are resistant to phosphodiesterase I but susceptible to phosphodiesterase II (Table II). It can be followed that they are 3'-monoester former.

Table III. DNase-activities in the nuclear and cytoplasmic fraction of human lymphocytes. The DNase-activities were determined by the *in situ* detection of DNases in DNA-containing polyacrylamide gels (see Materials and Methods).

Cell fraction	Activities of the different DNases in % of the activity in whole cells			
	D	C	B	A
Cytoplasm	25	13	16	88
Nucleus	70	49	59	3

The determination of DNase-activities of lymphocytes in the nuclear and cytoplasmic fraction shows that only the DNase A is localized in the cytoplasm (Table III). The small activities of the DNases B, C and D in the cytoplasm are probably released from the nucleus into the cytoplasm during homogenization of the cells. The DNase A corresponds in all properties to the DNase from bovine pancreas named DNase I⁶. This was suggested by Zöllner *et al.*¹⁷ from the pH optimum and dependence on divalent cations, and is now confirmed by the endonucleolytic mode of action, the substrate specificity and the fact that this enzyme is a 5'-monoester former. Also the cytoplasmic localization is in accordance with this assumption.

The group of acid DNase-activities (B) with a pH optimum of 5.0 represents endonucleases and 3'-monoester formers. The equal activity with both denatured and native DNA as substrates distinguishes these activities from the well characterized acid spleen DNase named DNase II⁷.

A different situation is found for the group of acid DNase-activities (C) with a pH optimum of 5.5. Besides the endonucleolytic mode of action and the characteristic as a 3'-monoester former also the preference of native DNA over denatured DNA is in accordance with the DNase II from spleen⁷. Only the nuclear localization seems to contradict the lysosomal origin of the DNase II^{7, 23}. However comparable DNase-activities were described in mammalian nuclei by several authors^{10, 11, 24-26}.

The alkaline DNase-activity D is characterized by its preference of denatured and UV-irradiated DNA as substrate. The endonucleolytically formed DNA fragments are terminated with 5'-phosphate. There

is some correspondence with the UV-specific nuclease from HeLa cells described by Burt and Brent¹⁶ and Brent¹⁵. In addition Slor and Lev³ showed that this DNase-activity is also active with denatured DNA, but in contrast to alkaline DNase D this DNase is an exonuclease. An endonuclease specific for UV-irradiated DNA was described by Bacchetti *et al.*¹⁴ in HeLa cells. This endonucleolytic activity is not acting on dimers but will be able to recognize other forms of UV-damage resulting in mispaired regions in the DNA. So also denatured DNA, as in the case of DNase D from human lymphocytes, will be an adequate substrate, but there are no details concerning the activity with denatured DNA or the nature of the DNA fragments in Bacchetti's paper.

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