Different deoxyribonucleases in human lymphocytes

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ABSTRACT

The distribution pattern of deoxyribonuclease activities in human lymphocytes has been examined by micro-disc-electrophoresis. Four groups of deoxyribonuclease activities, differing in their electrophoretic mobility, in the nature of their optimal substrate and in their optimal incubation conditions, are characterized. There are two alkaline DNase-activities. One corresponds to DNase I (EC 3.1.4.5), the other having pH optimum of about pH 9.0, prefers denatured DNA as substrate and is not dependent on divalent cations. The fractions with an acid pH optimum can be subdivided into two groups, which differ in their activity towards native DNA, towards denatured DNA, in their activity when succinate is present and in their pH optimum.

INTRODUCTION

There are only few observations on DNases in human lymphocytes. An acid, lysosomal DNase (EC 3.1.4.6) was demonstrated in lymphocytes of peripheral blood in children with acute bacterial and viral infections, in children suffering from acute leukemia and in normal children (1,2). Slor et al. (3) demonstrated alkaline DNase activity attacking primarily heat denatured DNA and to a lesser extent ultraviolet-irradiated DNA in both normal and Xeroderma pigmentosum lymphocytes.

Mammalian cells from other tissues obviously show several differing DNase activities, usually grouped as DNase I (EC 3.1.4.5) associated with the mitochondria and the nucleus (4) and DNase II (EC 3.1.4.6) associated with the lysosomes (5).

From nuclear preparations of HeLa cells four distinct enzymes with an alkaline pH optimum could be separated by CM-Sephadex column chromatography (6). They differ in resistance to thermal inactivation and in relative activity with different substrates. Additionally an alkaline DNase from rat liver non-histone chromatin proteins with a preference for native DNA and another from nucleoplasm proteins with preference for denatured DNA have been described by O'Connor (7). In

nuclear preparations (8) from mice and rat liver alkaline DNase, requiring different divalent cations were found. Acid DNase activities were observed in histochemical studies in rat liver nuclei (9) and in purified calf thymus nuclei (10). Moreover there are some results concerning DNases specific for UV-irradiated DNA, DNA with heat induced lesions and DNA after ionising radiation (11-16).

In this paper we characterize the distribution pattern of some DNases from human lymphocytes.

MATERIALS AND METHODS

Materials were obtained as follows: DNase I (2400 U/mg) from Worthington, Freehold (N.J.); E. coli ^{14}C DNA (spec. act. : 43 $\mu\text{Ci/mg}$) from the Radiochemical Centre, Amersham (England); Ronpacon 75% from Cilag Chemie GmbH, Alsbach (Germany); Thrombovetren (4000 IE Heparin/2 ml) Promota, Hamburg (Germany); Ficoll from Pharmacia, Fine Chemicals AB, Uppsala (Sweden); Galloctanine from Fluka AG (Switzerland); Chromalaune from Merck, Darmstadt (Germany); Herring sperm DNA, prepared according to Zahn et al. (17), was a gift from H. Mack, Illertissen (Germany).

Heat denatured DNA was prepared by heating native herring sperm DNA or E. coli ^{14}C -DNA for 10 minutes to 100°C in a boiling water bath and chilling in ice.

Lymphocytes were isolated from normal donors essentially according to Böyum (18) with modifications. The lymphocytes, which after the centrifugation step formed a layer between the upper plasma fraction and the lower Ficoll layer, were removed, washed three times with cold 0.9% NaCl and then suspended in 0.25 M sucrose 1.5 mM CaCl_2 . The concentration was adjusted using a Coulter Counter to $40 \cdot 10^6$ cells/ml, before freezing the samples to -20°C . Before use, the cells were disrupted by freezing and thawing three times. They were then incubated at 4°C in 1 volume of 0.2% Triton X-100 in the spacer gel buffer (0.176 M Tris- H_3PO_4 pH 6.7).

The electrophoretic run for in situ detection of DNases was essentially performed according to Neuhoﬀ (19) with some modifications. We used a 5% acrylamide spacer gel pH 6.7, 0.176 M Tris- H_3PO_4 and a separation gel 13.4% acrylamide, pH 8.8, 0.177 M Tris- H_2SO_4 . The electrophoresis was performed at

700 μ A/gel for 80 minutes with the anode at the bottom. The DNA concentration in the small-pore gel was 0.3 mg herring sperm DNA per ml. All operations from gel formation to incubation were carried out at 4°C. A final sample volume of 8 μ l per gel was applied. After the electrophoretic run the gels were developed as described by Zöllner et al. (20). The gels were incubated in different standard incubation mixtures, (A): 0.1 M Na-acetate pH 5, 0.5 mM EDTA, (B): 0.1 M Tris-HCl pH 7.4, 10 mM $MgCl_2$, 1 mM $CaCl_2$. They were then stained with gallo-cyanine-chromalaune. After destaining with water the optical density was recorded with a densitometer (Kipp and Zonen). The densitometer tracings were normalized to equally treated crystalized DNase I.

In vitro assays of total DNase activity were carried out in a total volume of 120 μ l containing: 20 μ l enzyme sample; 0.08 μ g E. coli ^{14}C -DNA and 20 μ g herring sperm DNA in buffer A or B at 37°C. After 0 h, 4 h and 8 h 50 μ l samples were withdrawn and applied on filter discs (Whatman no 1). The acid precipitable radioactivity was determined as described by Bollum (21). The acid-soluble radioactivity was used as function of DNase activity.

Protein was estimated according to the method of Lowry et al. (22).

RESULTS

In vitro determination. In human lymphocytes isolated from the peripheral blood DNase activities were detectable under different conditions using the in vitro assay. Table 1 summarizes the values for total activity obtained from three different preparations studied. These results show, that at least two different DNase activities in mammalian cells are existing, one in the acid and another one in the alkaline pH range. As known from previous studies (23,24), the alkaline DNase usually has a smaller activity. When 10^{-2} M $MgCl_2$ and 10^{-3} M $CaCl_2$ were added to the acid incubation mixture small activities were detectable, if however instead of the divalent cations EDTA, $5 \cdot 10^{-3}$ M was added to the alkaline incubation mixture no activity at all was found. This dependence on EDTA or on divalent cations is in accordance with the literature (5,25). In both DNases the activity with denatured DNA was found to be lower than with native substrate, as described in literature (5,23,24,25).

In situ determination. Using the techniques of the micro-disc-electrophoretic DNase-test seven different DNase-bands are detectable (Fig. 1). At pH 7.4 with

Table 1: DNase activities in human lymphocytes from three different males with acid and alkaline incubation using native and denatured DNA as substrate. The activity is expressed as acid soluble radioactivity (in cpm) released from 0.04 μ g E. coli DNA (2700 ^{14} C cpm) per mg enzyme fraction per 4 hours.

	native DNA	denatured DNA
0.1 M Na-acetat pH 5.0 + $5 \cdot 10^{-3}$ M EDTA	500	68
	530	95
	540	57
0.1 M Tris-HCl pH 7.4 + 10^{-2} M $MgCl_2$ + 10^{-3} M $CaCl_2$	26	9
	57	35
	65	36

divalent cations and native DNA as substrate in the gel only one distinct activity band No 1 is visible (Fig. 1 A). With the same incubation mixture and with denatured DNA in the gel this activity band No 1 is diminished; however on the top of the gel an additional, slowly migrating DNase band No 7 is detectable (Fig. 1 B). Under acid incubation conditions with EDTA (buffer A) we found a broad nucleolytic activity with five peaks (Fig. 1 C (No 2-6)). Their number and their relative positions did not change with denatured DNA; however the activities of the bands No 4-6 were diminished by about 60%, whereas the bands 2 and 3 did not alter their activity with this substrate (Fig. 1 D).

In another set of experiments some DNase bands were characterized in more detail: Band 1 and 7 and the two groups with band 2 and 3 and band 4, 5 and 6.

Fig. 1: Densitometer tracings of DNA-containing polyacrylamide gels after electrophoresis of freeze-thawed lymphocytes. The gels contained 0.3 mg native or denatured herring sperm DNA/ml in 13.4% acrylamide separation gel at pH 8.8. On each gel 8 μ l of an equivalent of $20 \cdot 10^6$ lymphocytes per ml in spacer gel buffer was applied. For experimental conditions, see Materials and Methods. A: native DNA and incubation in alkaline buffer B for 4 hours; B: denatured DNA and incubation in alkaline buffer B for 4 hours; C: native DNA and incubation in acid buffer A for 4 hours; D: denatured DNA and incubation in acid buffer A for 4 hours.

Figures refer to bands.

Figure 2: pH-activity curves of the different DNase bands in human lymphocytes.
 o — o band 1 with native DNA and Tris-HCl buffer 10^{-2} M $MgCl_2$, 10^{-3} M $CaCl_2$
 x ---- x bands 2, 3 with native DNA and acetate buffer + $5 \cdot 10^{-3}$ M EDTA
 ● - - - ● bands 4, 5, 6 with native DNA and acetate buffer + $5 \cdot 10^{-3}$ M EDTA
 ■ ..... ■ band 7 with denatured DNA and Tris-HCl-buffer + 10^{-2} M $MgCl_2$ + 10^{-3} M $CaCl_2$

Figure 2 shows the pH activity curves for these different DNase groups. The alkaline DNase, detectable with native DNA in the gel (band 1), has a pH optimum of about 7.0, whereas the enzyme active with denatured DNA (band 7), has its highest pH optimum at 9.0. In the case of the acid DNases the pH optimum of the bands 4, 5 and 6 was about 5.5, while the bands 2 and 3 have their maximum activity at pH 5.0.

The acid activities have similar thermal inactivation curves. Heating for 60 minutes to $56^{\circ}C$ brings no significant loss of activity, whereas the alkaline activi-

ty (band 1) drops drastically after 10 min (Fig. 3). After 10 minutes the alkaline DNase activity (band 7) is already completely gone.

Figure 3: Thermal inactivation of the acid DNases (bands 4-6) (● — ●) and the alkaline DNase (band 1) (○ -- ○) activity with native DNA in the polyacrylamide gel. The samples were heated for the specified time to 56°C before determination of the activity in the DNA-polyacrylamide-gels as described under Materials and Methods.

The different fractions were also tested in their standard incubation mixtures with the addition of N-bromosuccinimide, NaF, Na₂SO₄, iodoacetic acid, iodoacetamide, CuCl₂, 2-mercaptoethanol and p-chlormercuribenzoate at concentrations between $2 \cdot 10^{-2}$ M and $4 \cdot 10^{-4}$ M. The results are summarized in Table II.

Table II shows the results for the bands 4-6. If 10^{-2} M MgCl₂ + 10^{-3} M CaCl₂ was instead of EDTA in the acid incubation mixture, no activity could be detected. The bands 2 and 3 differ only with respect to p-chlormercuribenzoate

Table II: Activity of the acid DNases (bands 4, 5, 6) in the standard buffer A (0.1 M Na-acetate pH 5.0, 0.5 mM EDTA) supplemented with several components and activity of the alkaline DNase (band 1) in standard buffer B (0.1 M Tris-HCl pH 7.4, 10 mM $MgCl_2$, 1 mM $CaCl_2$) supplemented with several components (control = 100%).

component	pH of standard buffer	concentration of the component in standard buffer A respectively B			
		$4 \cdot 10^{-4}$ M	$2 \cdot 10^{-3}$ M	10^{-2} M	$2 \cdot 10^{-2}$ M
N-bromsuccinimide in	5.0	100%	< 50%		
	7.4	< 50%			
NaF in	5.0			100%	
	7.4			100%	
Na_2SO_4 in	5.0		100%	90-50%	< 50%
	7.4			100%	90-50%
Jodoacetic acid in	5.0	< 50%			
	7.4		100%		< 50%
Jodoacetamide in	5.0				100%
	7.4	100%	< 50%		
$CuCl_2$ in	5.0		100%	< 50%	
	7.4	100%	< 50%		
2-Mercaptoethanol in	5.0			100%	
	7.4			100%	
p-Chlomercuribenzoate in	5.0		100%	90-50%	
	7.4		100%	90-50%	
Na-succinate in	5.0			100%	90-50%
	7.4				

and sodiumsuccinate; these agents did not inhibit these enzyme activities.

Table II also summarizes the results for the alkaline band 1. When MgCl_2 and CaCl_2 are omitted in the standard buffer B or if in addition $5 \cdot 10^{-2}$ M EDTA is present, the activity disappears. 10^{-2} M 2-mercaptoethanol inhibits with 10^{-2} M MgCl_2 in the incubation mixture. In contrast, the alkaline DNase activity, band 7, specific for denatured DNA was not altered by EDTA.

DISCUSSION

Evidence has been presented that there are four groups of DNases in human lymphocytes. An alkaline one with preference for native DNA as substrate (band 1), corresponds in all properties to the one known from pancreas named DNase I (4,25). Besides the substrate preference this activity is characterized by a pH optimum of 7.0, dependency on divalent cations and inhibition by EDTA, N-bromosuccinimide, iodoacetamide and CuCl_2 in low concentrations. Also the fact that 2-mercaptoethanol only inhibits without calcium is in accordance to the characteristics of DNase I (4,25). The second alkaline DNase (band 7) is quite different with respect to pH, substrate preference, divalent cation requirement and electrophoretic mobility. It seems similar to the activities described by Slor (3) and Lindahl (14) possibly involved in repair processes.

The acid DNases are characterized as members of two groups. One, from bands 4-6, seems to be related to the acid DNase II by its substrate preference, inhibition by divalent cations, by Na_2SO_4 , by iodoacetic acid, by N-bromosuccinimide and to a smaller extent by iodoacetamide (5). Besides their different electrophoretic mobility, the group from bands 2 and 3 differs from the other acid activities by its sharp optimum at pH 5.0 and its missing discrimination between native and denatured DNA as substrate. In addition the activity is not altered by succinate in the incubation mixture.

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