Secretion of Neutral and Acid DNases in Cultivated Human Lymphocytes after Incubation with DNA; Possible Consequences for Inhalation Anesthesia

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After incubation with DNA human lymphocytes release neutral and acid DNase activities into the culture medium; the release depends on DNA concentration and time of cultivation. The electrophoretic mobility of the released neutral DNase activity is in accordance with DNase I and the electrophoretic mobility of the released acid DNase activity with DNase II. The released DNase activities do not originate from dead cells and are not influenced by blast cell formation. The anesthetic halothane can inhibit the released neutral and acid DNase activities. Inhalation anesthesia can possibly disturb the correlation between DNA and DNases in human blood.

Introduction

A correlation exists in blood plasma between free DNA concentration and DNase activity. Species with a high concentration of DNA have little DNase activity and *vice versa* (Cox and Gokcen, 1976). In plasma from cats there is no DNA detectable and so the activity of DNase is very high (Cox and Gokcen, 1976). In humans increased DNA concentrations can occur in the plasma in connection to various diseases (Raptis and Menard, 1980; Lippmann *et al.*, 1982; Shapiro *et al.*, 1983; Stroun *et al.*, 1987), while in other diseases the DNase activity increases (Kowlessar and McEvoy, 1956; Gavosto *et al.*, 1959; Tournut *et al.*, 1978; Taper *et al.*, 1981). The physiological implications of this correlation between the DNA and the DNases are not completely clear. Plasma DNase can split exogenous DNA from bacteria and viruses and can support the resistance against infections (Cox and Gokcen, 1977; Nishiyama *et al.*, 1982). In some diseases antibodies against DNA can be found in the plasma (Raptis and Menard, 1980; Rochnis *et al.*, 1974; Steinman, 1975).

The neutral plasma DNase shows a strong biochemical identity with DNase I from the pancreas (Love and Hewitt, 1979; Funakoshi *et al.*, 1979) and the acid plasma DNase with DNase II from the spleen (Sierakowska and Shugar, 1977). However, it is not clear whether these organs contribute to the regulation of the correlation between DNA and DNase in the plasma. The white blood cells can possibly be directly involved in these regulation processes.

For this reason we examined cultures of peripheral human lymphocytes whether neutral and acid DNases are released after incubation with DNA. The correlation between DNA and DNase in the plasma could actively be regulated in this way. In addition an examination was made to prove whether halothane, an inhalation anesthetic, can inhibit the activities of released DNases. An inhibition could explain a correlation between anesthesia and an often observed increased susceptibility to infections of patients. An inhibition prevents the splitting of exogenous DNA from bacteria or viruses, so that the course of an infection would be intensified. Isolated DNase I can be inhibited by halothane in a cell-free system (Reitz *et al.*, 1982).

Materials and Methods

Chemicals

The sources were as follows: Ficoll solution, penicillin, streptomycin and tissue culture (TC) medium 199, Biochrom, Berlin (Germany), fetal

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calf serum and DNase I (2000 units/mg) from bovine pancreas, Boehringer Mannheim, Mannheim (Germany), DNase II (200 units/mg) from bovine spleen, Serva, Heidelberg (Germany), G actin from rabbit muscle, Sigma Chemical, St. Louis (U.S.A.), halothane, Hoechst, Frankfurt (Germany), gallocyanine, Fluka, Buchs (Switzerland), potassium chromium(III)sulfate, Merck, Darmstadt (Germany); all other chemicals were from Merck, Darmstadt (Germany) and Serva, Heidelberg (Germany).

DNA was isolated from holothurias *(Holothuria tubulosa)* according to Zahn *et al.*, 1962; a stock solution of the holothuria DNA in 0.01 M Tris-(hydroxymethyl)-aminomethane-HCl (Tris-HCl), pH 7.4 + 1 mM EDTA + 50 mM NaCl was prepared prior to the investigation and diluted before use with the DNase assay buffer.

Isolation and cultivation of lymphocytes

The lymphocytes were isolated in a density gradient from heparinized venous blood (Böyum, 1968) and cultivated; culture medium: 80% TC medium 199, pH 7.4, 15% fetal calf serum, 5% penicillin-streptomycin solution; 10^6 cells/ml culture medium (2 ml cultures); DNA was added in different concentrations to the culture medium; time of cultivation without shaking: 0 h, 12 h, 24 h, 36 h, 48 h, 60 h and 72 h, 37 °C, 5% CO_2 v/v, water-saturated atmosphere. The supernatant was examined for DNase activities after cultivation. The vitality of the cells was determined with trypan blue and for blast cell determination the cells were stained with May + Grünwald/Giemsa solution according to a handbook of the Merck pharmaceutical company 1974.

Exposing supernatant to halothane

After centrifugation of the lymphocytes the supernatant of some cultures was filled into bowls (0.5 ml) and placed in a desiccator at 37 °C and 100% humidity. Halothane was added in 1.5 vol.-% to a water vapor saturated flow of air (4 l/min) entering the desiccator during the exposure time of 1 h. The halothane concentration was regulated by a halothane vaporizer (Dräger, Lübeck, Germany). At the end of halothane exposure, volume changes due to evaporation and pH changes were found to be without any statistical significance. Controls were exposed to ambient air as well as to a 4 l/min flow of air. The dissolved halothane was determined as described (Reitz *et al.*, 1982).

Determination of DNase activities

The neutral and the acid DNase activity were determined in the supernatant of the lymphocyte cultures after centrifugation of the cells with a modified method of Allfrey and Mirsky (1952).

a) Neutral DNase activity assay: microreaction vessels with 100 µl supernatant and 300 µl DNA solution (0.2 mg/ml DNA in 0.1 M Tris-HCl, pH 7.4 + 10 mM $MgCl_2$ + 1 mM $CaCl_2$), incubation at 37 °C for 4 h. Thereafter, 300 µl of ice-cold 15% perchloric acid were added. The samples were cooled for 20 min at 0 °C. The precipitate was centrifuged at 17,000×*g* for 4 min and the absorbance of the supernatant was measured at 260 nm. The absorbance of a non-incubated, parallel test was subtracted as background. For inhibiting DNase activity the supernatant of the lymphocyte cultures was treated with G actin or exposed to halothane (Reitz *et al.*, 1982). Mean values and S.D. of the results were determined.

b) Acid DNase activity assay: microreaction vessels with 100 µl supernatant and 300 µl DNA solution (0.2 mg/ml DNA in 0.1 M Na acetate, pH 5.0 + 5 mM EDTA), incubation and measurement of the absorbance as described above. For inhibiting DNase activity the supernatant of the lymphocyte cultures was exposed to halothane (Reitz *et al.*, 1982). Mean values and S.D. of the results were determined.

Determination of DNase activity after electrophoresis

The determination of DNase activity was performed using the *in situ* detection of DNases in DNA-containing polyacrylamide gels following electrophoretic separation (Reitz and Gutjahr, 1992). A 5% acrylamide spacer gel and a 13.4% acrylamide separation gel were used; the DNA concentration in the small pore gel was 0.3 mg/ml; neutral DNase activity: spacer gel – Tris–H_3PO_4, pH 6.7, separation gel – Tris–H_2SO_4, pH 8.8 (Maurer, 1971); acid DNase activity: spacer gel – KOH–CH_3COOH, pH 5.9, separation gel – KOH–CH_3COOH, pH 4.3 (Maurer, 1971). The

electrophoresis was performed at 700 μA/gel for 80 min with the anode at the bottom (neutral DNase activity) and for 120 min with the anode at the top (acid DNase activity). A final sample volume of 15 μl supernatant (lymphocyte cultures) per gel was applied, in control experiments DNase I (neutral DNase) and DNase II (acid DNase). After electrophoresis the gels were incubated for 4 h at 37 °C in 0.1 M Tris-HCl, pH 7.4 + 10 mM $MgCl_2$ + 1 mM $CaCl_2$ (neutral DNase activity) and in 0.1 M Na acetate, pH 5.0 + 5 mM EDTA (acid DNase activity). The gels were stained with gallocyanine–potassium chromium(III)sulfate (0.15% gallocyanine, 5% potassium chromium(III)-sulfate) for 12 h. After destaining with water the optical density of the gels were recorded with a densitometer. Unstained gel regions represent places of hydrolyzed DNA and zones of DNase activity.

Statistical analysis

For statistical analysis t-test was used. All experiments were repeated for ten times with lymphocytes from different healthy persons.

Results

Human lymphocytes *in vitro* release neutral and acid DNase after contact with isolated DNA into the culture medium (Tables I, II). The release of the DNases depends on the DNA concentration in the culture medium and also on the time of cultivation. A high DNA concentration in the culture medium reaches the maximum release of neutral and acid DNases after 12 h and then reduces continuously if the culture is continued. By contrast, a low DNA concentration in the culture medium reaches the maximum release of neutral and acid DNases only after 24 h. It also continuously reduces activity thereafter.

The released neutral DNase activity has an electrophoretic mobility that conforms with the DNase I from bovine pancreas and in the case of the released acid DNase activity the electrophoretic mobility conforms with that of the DNase II from bovine spleen (Fig. 1). The released neutral DNase activity can be inhibited by G actin, as well, which also indicates an identity with DNase I.

The activity of released neutral and released acid DNases can be inhibited by halothane; the inhibition is statistically significant (Table III).

Table I. Secretion of neutral DNase activity in cultivated human lymphocytes after incubation with DNA. (Mean values of the results are shown.)

Time of cultivation [h]	DNA concentration					
	0 g/ml	2×10^{-4} g/ml	2×10^{-5} g/ml	2×10^{-6} g/ml	2×10^{-8} g/ml	2×10^{-10} g/ml
0	0.069	0.065	0.067	0.063	0.057	0.061
12	0.113	0.312	0.278	0.157	0.108	0.105
24	0.147	0.218	0.203	0.214	0.205	0.159
48	0.107	0.120	0.110	0.134	0.127	0.161
72	0.028	0.034	0.040	0.039	0.044	0.031
	Absorbance at 260 nm					

Table II. Secretion of acid DNase activity in cultivated human lymphocytes after incubation with DNA. (Mean values of the results are shown.)

Time of cultivation [h]	DNA concentration					
	0 g/ml	2×10^{-4} g/ml	2×10^{-5} g/ml	2×10^{-6} g/ml	2×10^{-8} g/ml	2×10^{-10} g/ml
0	0.030	0.029	0.033	0.032	0.022	0.024
12	0.076	0.281	0.221	0.164	0.069	0.070
24	0.126	0.195	0.146	0.165	0.139	0.117
48	0.069	0.094	0.080	0.088	0.073	0.082
72	0.035	0.029	0.020	0.034	0.029	0.027
	Absorbance at 260 nm					

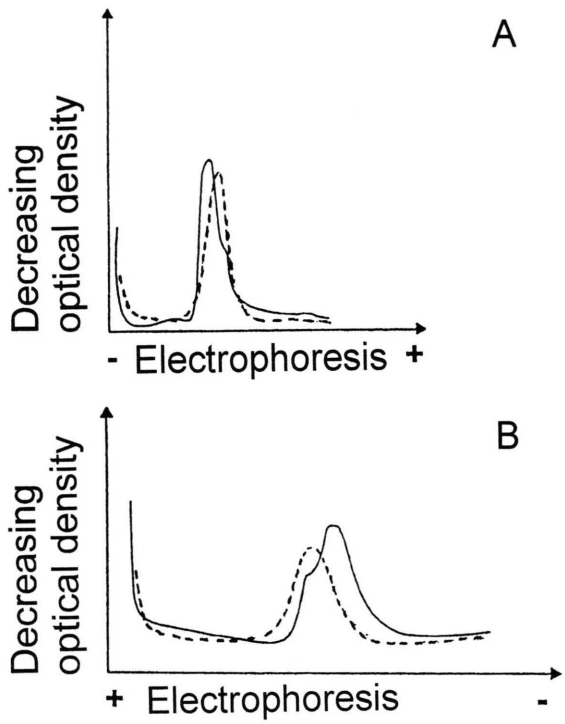

Fig. 1. Densitometer tracings of DNA-containing polyacrylamide gels after electrophoresis of: A) 5 µl DNase I (10^{-6} g/ml) and 15 µl supernatant of lymphocyte cultures; B) 5 µl DNase II (10^{-5} g/ml) and 15 µl supernatant of lymphocyte cultures. Representative samples are shown. The neutral DNase activity in the culture medium of human lymphocytes after incubation with DNA is comparable in its electrophoretic mobility with DNase I and the acid DNase activity with DNase II; details see Materials and Methods. ——: A) DNase I, B) DNase II; – – – –: supernatant.

After 1 h of halothane exposure (1.5 vol.-%) an average amount of 0.42 mM dissolved halothane can be measured in the supernatant of the lymphocyte cultures.

The released DNases do not originate from dead cells that disintegrate in the culture medium. Compared with control experiments, the vitality of the cells, after the DNA addition into the culture medium, is not influenced at the time of the highest DNase secretion (Table IV). Also, the increased DNase secretion cannot be seen in connection to an unspecific lymphocyte stimulation by the DNA, as the development of blast cells was only slightly increased at the time of the highest DNase secretion compared to control experiments (Table V). The release of the neutral and acid DNase occurred independently from death and proliferation rate of the cultivated lymphocytes.

Discussion

In human blood plasma DNA is found in a concentration between 3 and 11 ng DNA/ml (Dennin, 1979). The concentration determination is dependent on the method used, as there are also examinations in which the proof of DNA in plasma from healthy humans did not succeed (Stroun *et al.*, 1987). The DNA in human plasma can be traced in free and in bound form and varies in its molecular length between less than 0.2 µm and about 13 µm (Dennin, 1979). In cancer patients (Shapiro *et al.*, 1983; Stroun *et al.*, 1987), patients with lupus erythematosis (Raptis and Menard, 1980), viral hepatitis (Neurath *et al.*, 1984) or lung embolism (Lippmann *et al.*, 1982) the DNA concentration is in part greatly increased. The DNA concentration in the serum of cancer patients reduces during therapy (Leon *et al.*, 1977).

The origin of the free DNA in the plasma is unknown. The activity of DNases in plasma corresponds with the DNA concentration and is reduced in patients with lupus erythematosis and in some cancer patients (Cox and Gokcen, 1977; Funakoshi *et al.*, 1979). In pancreas carcinoma the DNA concentration in the blood is especially high and the activity of the DNases especially low (Funakoshi *et al.*, 1979). It can be assumed that the DNases of the plasma are involved in the regulation of the DNA concentration. Moreover, they play a role in preventing infections and split exogenous DNA from bacteria and viruses (Cox and Gokcen, 1977; Nishiyama *et al.*, 1982). If mice are treated with bacterial toxins then the concentration of the DNA in their blood increases (Fournie *et al.*, 1974), at the same time DNases are activated (Cox and Gokcen, 1976). High DNA concentrations in the blood of mice lead to the development of DNA antibodies (Fournie *et al.*, 1974).

The neutral plasma DNase shows a strong biochemical identity with the DNase I from the pancreas (Love and Hewitt, 1979; Funakoshi *et al.*, 1979) and the acid plasma DNase with the DNase II from the spleen (Sierakowska and Shugar, 1977); it is therefore assumed that both these organs regulate the activity of the DNases in plasma. This examination shows, however, that

Table III. Inhibition of neutral and acid DNase activities in the supernatant of cultivated human lymphocytes after exposing to 1.5 vol.-% halothane for 1 h. (Mean values of the results are shown.)

Neutral DNase activity						
	Control:			Halothane exposure:		
Time of	DNA concentration			DNA concentration		
cultivation [h]	0 g/ml	2×10^{-4} g/ml	2×10^{-5} g/ml	0 g/ml	2×10^{-4} g/ml	2×10^{-5} g/ml
0	0.069	0.065	0.067	0.064	0.066	0.063
12	0.113	0.321	0.278	0.079	0.098	0.105
24	0.147	0.218	0.203	0.081	0.103	0.089
48	0.107	0.120	0.110	0.059	0.070	0.062
72	0.028	0.034	0.040	0.032	0.030	0.029
			Absorbance at 260 nm			
Acid DNase activity						
	Control:			Halothane exposure:		
Time of	DNA concentration			DNA concentration		
cultivation [h]	0 g/ml	2×10^{-4} g/ml	2×10^{-5} g/ml	0 g/ml	2×10^{-4} g/ml	2×10^{-5} g/ml
0	0.030	0.029	0.033	0.031	0.032	0.028
12	0.076	0.281	0.221	0.049	0.085	0.079
24	0.126	0.195	0.146	0.038	0.055	0.063
48	0.069	0.094	0.080	0.033	0.029	0.031
72	0.035	0.029	0.020	0.030	0.032	0.028
			Absorbance at 260 nm			

Table IV. Vitality of cultivated human lymphocytes after incubation with DNA. (Mean values of the results are shown.)

Time of	DNA concentration		
cultivation [h]	0 g/ml [%]	2×10^{-4} g/ml [%]	2×10^{-5} g/ml [%]
0	98.9	98.9	98.9
12	95.3	93.8	94.2
24	92.5	91.0	89.8
48	79.9	78.2	80.1
72	69.1	68.4	69.3

Table V. Blast cell formation of cultivated human lymphocytes after incubation with DNA. (Mean values of the results are shown.)

Time of	DNA concentration		
cultivation [h]	0 g/ml [%]	2×10^{-4} g/ml [%]	2×10^{-5} g/ml [%]
0	0.0	0.0	0.0
12	3.1	4.2	4.5
24	6.9	9.1	8.8
48	14.2	18.3	18.0
72	26.4	29.2	30.9

also the lymphocytes of the peripheral blood could contribute to the regulation of the balance between DNA and DNases in the plasma. In lymphocyte cultures high DNA concentrations induce the secretion of neutral and acid DNase activities from the cells. The secreted neutral and acid DNases show the same electrophoretic mobility as the neutral DNase I and the acid DNase II; whether or not they are identical to DNase I or DNase II is not proved.

Halothane inhibits both the activities of the secreted neutral DNase and the secreted acid DNase from the lymphocytes. The inhibition is probably a result of interactions between the hydrophobic halothane molecules and the hydrophobic amino acids of the enzymes, so that changes in conformation and loss of activity occurs (Reitz *et al.*, 1982). Anesthetics can react themselves directly with proteins (Schoenborn, 1968). It is not known whether halothane also *in vivo* disturbs the balance of the activity of the DNases between the DNA and the DNases of the plasma. Clinical observations certainly allow interesting conclusions: A high DNA concentration is found in the plasma of patients with viral hepatitis (Neu-

rath *et al.*, 1984) and these patients also have a bad prognosis for an operation (Harville and Summerskill, 1963). It is possible that DNase activities are inhibited during anesthesia so that no DNA splitting can take place, resulting in the patient's health becoming worse. Mice that are given an anesthesia with halothane and at the same time an injection of hepatitis virus have an increased death rate (Moudgil, 1873). Interactions between anesthesia and an increased susceptibility to infections could be explained by the inhibition of DNase activities that prevent the exogenous DNA from bacteria or viruses; here *in vivo* examination are necessary.

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