DNA ASSAY IN HUMAN BLOOD PLASMA

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The plasma concentration of DNA, its properties, and its origin are important in the light of data on transfection, the transforming action of fragments of cellular DNA, and on the mobile multiple dispersed genes, which are structurally similar to proviral genes and, finally, on the DNA excreted by human lymphocytes [3, 13] during culture, and on the existence of extracellular RNA-containing DNA-synthesizing complexes [5]. When different methods are used to determine the DNA concentration, the results may vary from negative values to 40 μg/ml of plasma [1, 4, 7, 9, 11, 12, 14]. The nonreproducibility of data obtained by different authors is due, above all, to the unusually high protein-DNA ratio in blood plasma compared with that observed in cellular material.

In the investigation described below the DNA level in blood plasma was determined by fluorometry of a complex of DNA with bis-benzimide. This dye interacts only with DNA and enables it to be determined in concentrations as low as 10 ng/ml [2, 6, 10].

EXPERIMENTAL METHODS

Blood was taken from donors with the use of heparin (final concentration 25 U/ml, from Spofa, Czechoslovakia). The blood was centrifuged at 3000 rpm for 30 min on a K70 centrifuge at 4°C. The plasma was removed and used for further tests. No traces of disintegrated leukocytes were present in the plasma samples. For deproteinization, an equal volume of a 20% solution of NaCl was added to samples of plasma 0.1-0.2 ml in volume, and they were placed in a boiling water bath for 2-3 min. After cooling at room temperature the samples were centrifuged at 3000 rpm for 30 min. To verify the completeness of transfer of the plasma DNA

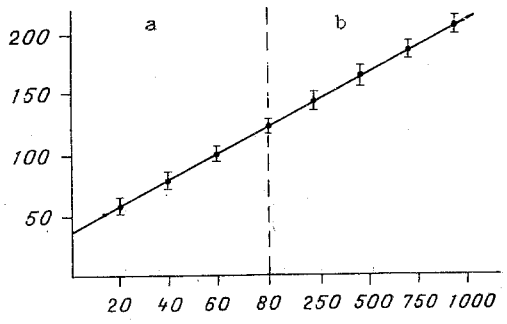

Fig. 1. Dependence of fluorescence level on volume of supernatant of blood plasma (a) and of DNA standard (b). Abscissa, volume of supernatant and DNA (in μl); ordinate, fluorescence of DNA with bis-benzimide (in relative units). Concentration of standard DNA solution was 12.5 μg/ml.

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TABLE 1. Transfer (in %) of Endogenous and Exogenous DNA from Human Blood Plasma into Supernatant after heating for 2-3 min in 10% NaCl

No. of donor	DNA concentration, μg/ml				Transfer, %
	initially	added	total measured		
			with bis-benzimide	with DABA	
1	5,6	10	15,0	17,0	95,6
2	6,1	10	13,1	10,2	81,3
3	5,0	10	11,2	15,0	74,7
4	23,4	20	42,5	45,2	95,6
5	27,7	22	50,4	53,1	101,1
6	15,0	30	44,9	47,1	99,8
7	18,5	30	44,2	45,0	91,1
8	9,5	30	38,5	41,2	97,6
$M \pm m$	—	—	—	—	92,1±3,3

TABLE 2. Hydrolysis (in %) of Endogenous and Exogenous DNA in Supernatant of Human Blood Plasma Containing 10% NaCl and Heated for 2-3 min at 100°C

No. of donor	DNA concentration, μg/ml		Hydrolysis of DNA, %	DNA concentration, μg/ml			Hydrolysis of DNA, %
	before treatment with DNase	after treatment with DNase		added	total before treatment with DNase	after treatment with DNase	
1	5,4	0,3	95,1	10	13,0	1,0	91,9
2	9,1	0,2	97,9	10	15,0	0,6	96,1
3	13,4	1,0	92,7	20	30,9	1,3	95,8
4	20,6	0,6	97,1	20	46,2	1,5	96,8
5	25,4	1,3	94,7	20	43,2	3,0	93,2
$(M \pm m)$	—	—	95,5±0,84	—	—	—	94,7±0,94

into the supernatant, commercial calf thymus DNA (from Serva, West Germany) was added to some samples of plasma with NaCl, at the rate of 10, 20, and 30 μg/ml plasma, before heat treatment.

The DNA concentration was measured as fluorescence of the DNA–bis-benzimide complex (X33258, Serva) at 470 nm on a "Jobin Ivon" fluorometer. Fluorescence was excited at 365 nm. Assay buffer, consisting of 0.05 M Na-phosphate buffer, pH 7.4, containing 1.3 M NaCl and 25 μl of a solution of bis-benzimide with a concentration of 60 μg/ml were introduced into a cuvette, after which 20 μl of supernatant of the test plasmas was added several times and the reading of the fluorescence recorded. Next a preparation of commercial calf thymus DNA (250 ng) was added to the same cuvette and fluorescence measured at the same wavelength. The commercial DNA, used as standard, was heated beforehand to 100°C in 10% NaCl for 2-3 min. A graph showing the intensity of fluorescence as a function of the quantity of supernatant and of commercial DNA is given in Fig. 1.

The DNA concentration was calculated by a formula worked out as follows: a level of fluorescence C corresponds to an unknown quantity of DNA in supernatant A, but since a known quantity of calf thymus DNA B was added to the same cuvette as that in which the samples were analyzed, the total DNA concentration B + A will correspond to a level of fluorescence D. Consequently:

$$\frac{A}{C} = \frac{B+A}{D}.$$

To assess the specificity of this method, deproteinized samples of plasma, after precipitation with alcohol and lyophilization, were treated with DNase and the concentration of DNA with bis-benzimiide and with 3,5-diaminobenzoic acid (DABA) in them was measured [11].

EXPERIMENTAL RESULTS

Our attempts to determine the DNA concentration in native blood plasma with bis-benzimide, with ethidium bromide, DABA, or by the methods of Burton and Schmidt and Thannhauser were un-

successful. The most simple and effective way of overcoming these difficulties was to heat the samples of plasma containing 10% NaCl for 2-3 min. During treatment in this way, not less than 92% of DNA was found to cross into the supernatant fraction, and its concentration was determined in parallel tests with bis-benzimide and DABA (Table 1). Since fluorescence of the complex of bis-benzimide with denatured DNA is only half of that with active DNA, a series of determinations of fluorescence of the DNA samples was undertaken before and after heating at 100°C in 10% NaCl for 2-3 min. The level of fluorescence before and after heating was the same, indicating the absence of any significant denaturation changes, reducing fluorescence of the DNA–bis-benzimide complex. Endogenous and commercial DNA contained in the supernatant fraction was precipitated by more than 70% by 2 volumes of alcohol. The DNA residue, dissolved in 0.05 M Tris-HCl buffer, pH 7.4, was treated with DNase. As a result of this at least 95% of the DNA was removed – both endogenous plasma DNA and exogenous commercial (Table 2). Consequently, fluorescence of the samples of supernatant, obtained after heating the plasma with 10% NaCl, with bis-benzimide and DABA enabled the DNA concentration in human blood plasma to be determined with an accuracy of about 95%. DNA concentrations measured by this method in the blood plasma of 20 healthy donors were found to be virtually identical for both fluorophores, but preference must be awarded to bis-benzimide, as being more specific and not requiring acid hydrolysis of the DNA.

Blood plasma of 30% of the donors contained DNA in a concentration of 5-10 μg/ml, 21% in a concentration of 10-15 μg/ml, 25% – 15-20 μg/ml, 10% – 20-25 μg/ml, and 20% – 25-30 μg/ml. Since the average DNA content per leukocyte is 7-8 pg, and since tne mean number of leukocytes in 1 ml of blood is $5 \cdot 10^6$, the total quantity of cellular DNA in 1 ml blood will be 35-40 μg. Since 50% of the blood volume is accounted for by erythrocytes, the extracellular DNA content in 1 ml of blood will vary from 3-5 to 12-15 μg, or from 10 to 50% of the cellular DNA.

It can be concluded from these results that the DNA concentration in plasma is 10-50% of its level in the cells contained in the same volume of plasma; this DNA, moreover, is inaccessible not only for determination by the usual methods, but also for the action of endogenous and exogenous DNases. Our experiments in which blood plasma was treated with DNase showed that only 20% of the DNA is destroyed by it.

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