EXPERIMENTAL GENETICS

BLOOD LEVELS OF EXTRACELLULAR DNA IN IRRADIATED RATS

V. G. Vladimirov, A. S. Belokhvostov, S. S. Sherlina, I. N. Vasil'eva, and A. M. Voskresenskii

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At the end of the 1970s, with the appearance of reliable methods of investigation, there appeared well-substantiated reports that the blood plasma of animals and man contains certain quantities of DNA [3, 6, 8]. Subsequent tests showed that in some pathological states this DNA level may rise. In particular, this happens during the development of certain types of tumors in man and animals, in systemic lupus erythematosis, and also in animals exposed to ionizing radiation [9-11]. It has even been suggested that there are prospects for the use of this phenomenon in the diagnosis of radiation damage [10]. Attempts to identify and study the structure of this extracellular DNA, in the pathological conditions mentioned above, led to the conclusion that its composition is heterogeneous: high-molecular-weight and low-molecular-weight fractions were distinguished [1, 11, 12]. The role and physiological importance of the extracellular DNA are by no means clear. It has been suggested only that it has a functional connection with the development of responses to extremal factors and its participation in the formation of the adaptation syndrome.

The aim of the present investigation was to study the time course of changes in the plasma DNA level of rats irradiated in various doses, to attempt to establish dose—time relationships, and to determine certain of its molecular characteristics.

EXPERIMENTAL METHOD

Experiments were carried out on male albino rats weighing 180-220 g, kept on the ordinary standard diet and animal house schedule. The rats were subjected to a single dose of whole-body irradiation on the IGUR-1 apparatus with ^{137}C γ-quanta, within the dose range from 1 to 100 Gy and with a dose rate of 1.9 Gy/min.

Blood was collected from the animals at various times (from 1 h to 7 days) after irradiation. To obtain EDTA-plasma, a 0.5 M solution of EDTA (pH 7.35) was added in the ratio of 70:1. Blood cells were carefully removed by successive centrifugation, first at 810*g* and later at 2200*g*.

Nucleic acids were isolated from the plasma by phenolic deproteinization. For this purpose, nuclease inhibitors, detergents, and phenol were quickly added to the blood plasma thus obtained (at the rate of 10 μl of 0.1% potassium polyvinyl sulfate, 10 μl of a 3% suspension of bentonite, 1 ml of a 10% solution of sodium dodecylsulfate, and 330 μl of a 3 M Na-acetate buffer, pH 5.75, to 10 ml of plasma); after mixing, 10 ml of water-saturated phenol was added to the mixture. During the mixing with phenol, care was taken to avoid the formation of a colloidal mass. The resulting mixture was shaken for 10 min on a rotary shaker, under "mild" conditions, to prevent degradation of the DNA molecules. This procedure was repeated after the addition of 1 ml chloroform. The samples were then centrifuged for 15 min at 12,000*g* and 4°C. The aqueous phase thus obtained was treated with half its volume of 80% phenol, agitated, and again shaken for 10 min at room temperature, after which it was centrifuged in the cold for 10 min at 12,000*g*. Deproteinization of the aqueous phase with phenol was repeated 3 times, after which the nucleic acids were precipitated from the aqueous phase with the aid of ethanol, cooled to −20°C. The samples were kept for 12 h at −20°C and recentrifuged at 12,000*g* in the cold. The resulting residue was dried in vacuo and then dissolved in 10-20 μl of buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA).

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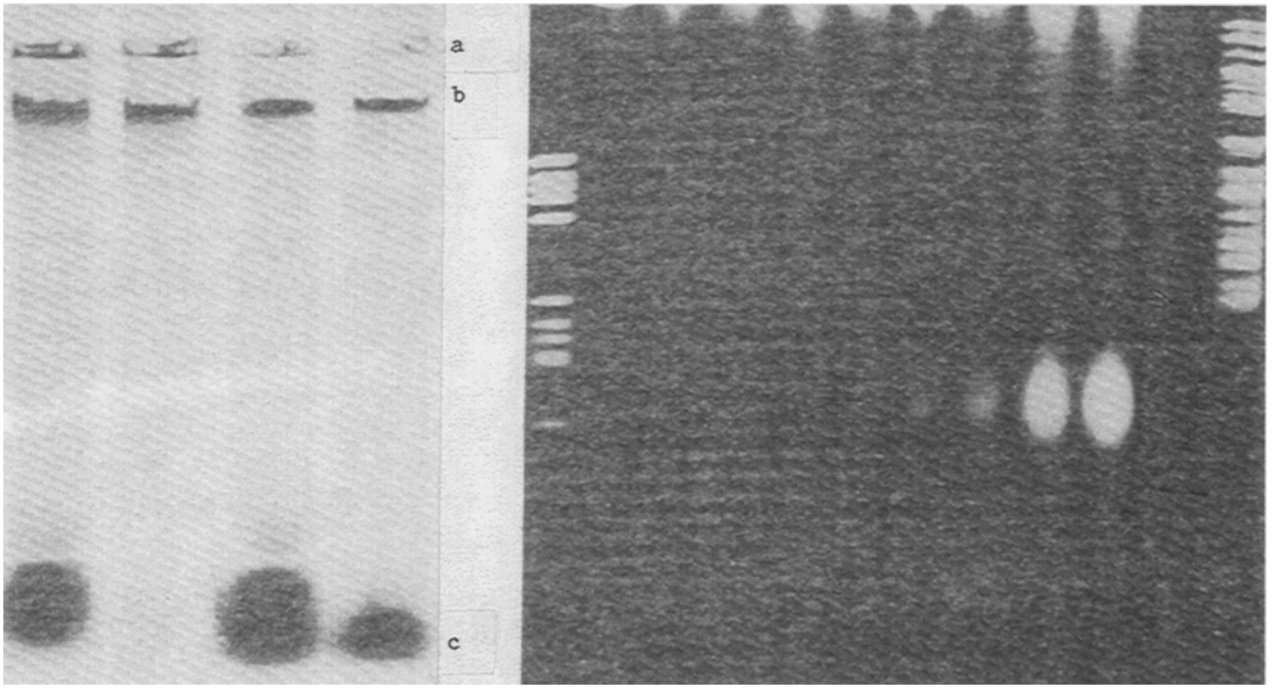

Fig. 1. Electrophoretic tests. Electrophoresis in combined 0.5-2% agarose gel of blood plasma nucleic acid preparations from intact rats (1, 2) and rats irradiated in a dose of 8 Gy (3, 4) (A). 1, 3) Without treatment of preparations with enzymes (control); 2, 4) after treatment with pancreatic RNase: a) starting wells; b) fraction of high-molecular-weight DNA; c) fraction of low-molecular-weight DNA. Electrophoresis in 2/16% gradient polyacrylamide gel of rat blood plasma DNA preparations (B). 1) Marker enzymes: DNA of plasmid pBR322, treated with restriction endonuclease BspR I; 2) plasma DNA from intact animals 2 h after irradiation; 3) in a dose of 1 Gy, 4) 2 Gy, 5) 4 Gy, 6) 8 Gy, 5 h after exposure: 7) to a dose of 1 Gy, 8) 2 Gy, 9) 4 Gy, 10) 8 Gy; 11) marker enzymes of DNA of phage λ, treated with restriction endonuclease Alu I.

DNA was determined spectrofluorometrically with diaminobenzoic acid dihydrochloride [4].

The fractional composition of the DNA was analyzed by electrophoresis in a polyacrylamide gradient or in 0.85% agarose gel in 0.04 M Tris-acetate buffer, pH 7.7, with 0.01 M EDTA [2].

Pieces of the polyacrylamide gel, containing the low-molecular-weight fraction of DNA, were cut out, eluted with 0.5 M ammonium-acetate buffer, pH 5.5, and labeled with ^{32}P by the nick translation method, followed by blot hybridization with genomic DNA [2].

EXPERIMENTAL RESULTS

The experiments showed that the quantity of DNA in the blood plasma of intact rats is 26.3 ng/ml. We could find reliable information on the plasma nucleic acid level only in the case of man. Judging by reports of individual authors, who used different methods to isolate the nucleic acids, the extracellular DNA content in human blood varies from 10 to 25 ng/ml, agreeing in principle with the results we obtained and with data in the literature [6, 10].

During electrophoresis in agarose gel the DNA tested behaved like a sufficiently homogeneous fraction, with mobility comparable with that of the DNA of phage λ, which has a molecular weight of the order of 31 megadaltons (Fig. 1A).

TABLE 1. Effect of Irradiation in Various Doses on Blood Plasma DNA Concentration (in ng/ml) in Albino Rats (M ± m)

Dose of irradiation, Gy	Time after irradiation						
	1 h	2 h	5 h	1 day	2 days	3 days	7 days
2	25,5±3,4 (11)	35,7±3,1* (9)	34,8±5,7 (8)	20,1±5,0 (5)	19,2±4,0 (6)	21,0±3,0 (5)	21,0±1,5 (4)
4	30,3±5,1 (1)	54,8±5,7* (10)	38,5±5,6* (6)	31,8±6,3 (6)	22,1±6,2 (6)	24,8±4,0 (6)	22,6±3,6 (2)
8	34,9±1,8 (5)	51,2±11,0* (10)	73,8±13,6* (7)	21,2±1,9 (4)	34,6±12,8 (5)	35,2±4,9 (5)	36,5±17,9 (3)
100	52,8±19,4* (3)	109,6±28,8* (6)	394,0±74,4* (5)	111,0±19,3* (6)	114,4±31,4* (3)	93,8±63,2* (3)	—

Legend. Number of experiments indicated in parentheses; six rats took part in each experiment. DNA concentration in intact animals was 26.3 ± 1.8 (17). *$p < 0.05$: difference compared with normal significant.

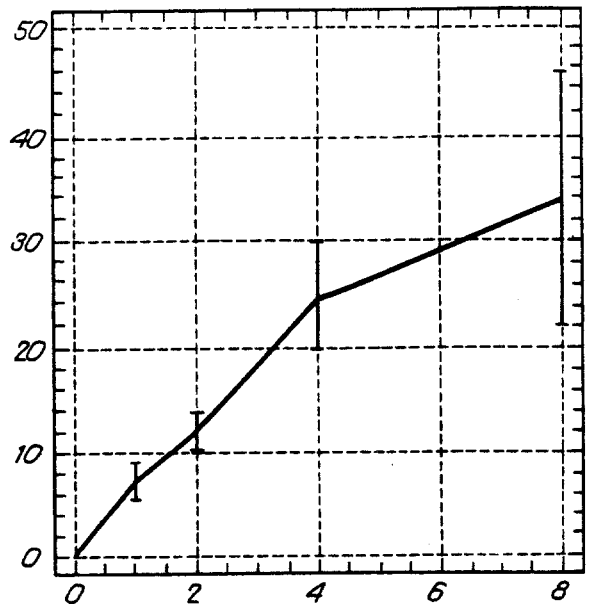

Fig. 2. Effect of irradiation in different doses after exposure for 5 h on blood plasma concentration of low-molecular-weight DNA. Abscissa, dose of irradiation (in Gy); ordinate, DNA content (in ng/ml).

Irradiation of the rats led to an increase in the plasma DNA concentration. This was observed initially after exposure to a dose of 2 Gy, when after 2 h the DNA concentration was significantly higher than normally (Table 1). With a further increase in the dose of irradiation, the concentration of this DNA rose progressively. The greatest increase took place after exposure to ionizing radiation in a superlethal dose (100 Gy). In that case, after only 1 h the DNA concentration was twice the normal level, and after 5 h it was more than 15 times higher. The raised extracellular DNA level in the blood in this series of experiments persisted until death of the animals (3 days). Thus the duration and the degree of the postirradiation increase in the DNA concentration in the cell-free part of the blood depends primarily on the dose of irradiation: the higher the dose, the greater the increase in the DNA level, and the longer it persists.

It was shown by electrophoresis in agarose and in gradient polyacrylamide gel that irradiation causes the appearance in the blood plasma of a normally absent low-molecular-weight DNA fraction, similar in molecular weight to DNA of nucleosomes (Fig. 1A and B). Treatment with RNase, as will be clear from Fig. 1A (fraction a) confirms that the high-molecular-weight fraction is in fact DNA. After irradiation this fraction persists, but a new DNA fraction appears, and judging

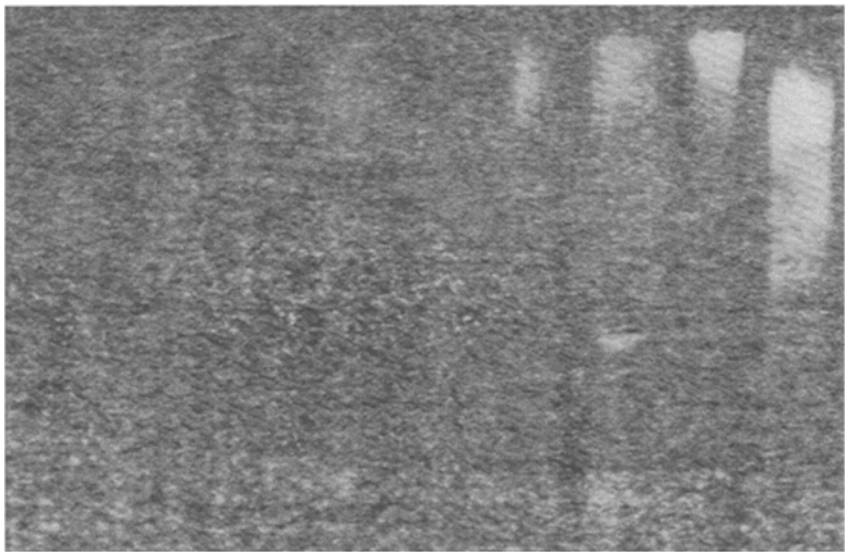

Fig. 3. Blot-hybridization of blood plasma low-molecular-weight DNA from irradiated rats, labeled with ^{32}P, with nucleic acid preparations. 1) Absence of hybridization with fragments of DNA of phage λ, treated with restriction endonuclease HindIII (negative control); 2) spontaneous hybridization with DNA preparations in blood plasma from intact rats, 3-8) hybridization with DNA in blood plasma of irradiated rats, 2 h after exposure in dose of: 3) 2 Gy, 4) 8 Gy, 5) 100 Gy; 5 h after exposure in a dose of: 6) 2 Gy, 7) 8 Gy, 8) 100 Gy; 10) hybridization with rat liver DNA, fragmented by restriction endonuclease EcoR I.

by its electrophoretic mobility, it has low molecular weight. That it is DNA by nature is shown by its resistance to RNase and, conversely, its sensitivity to DNase (fraction b in Fig. 1A). The low-molecular-weight DNA could be determined quantitatively after exposure for 2 h, but its most significant increase was observed 5 h after exposure to ionizing radiation. With an increase in the dose of irradiation the low-molecular-weight DNA fraction increased (Fig. 2). Comparison of the time course of the increase in total and low-molecular-weight DNA leads to the conclusion that nearly all the increase in the plasma DNA concentration took place on account of the low-molecular-weight fraction.

The low-molecular-weight DNA fraction appearing in blood plasma almost does not hybridize with high-molecular-weight DNA isolated from blood plasma of unirradiated rats (Fig. 3). This last finding suggests that the high-molecular-weight DNA has mainly unique nucleotide sequences. It reassociates with genomic DNA weakly, possible evidence of its origin from unique sequences of the genome of those with a small number of copies. Blot-hybridization showed that isolated plasma low-molecular-weight DNA hybridizes well with many fragments of genomic DNA (Fig. 3). All these facts may evidently be used as evidence of the genomic origin of the low-molecular-weight DNA (for example, due to fragmentation of chromatin). However, the presence of a large number of certain repeating sequences, characteristic of extrachromosomal DNA, suggests rather that it is extrachromosomal in its origin.

Extrachromosomal DNA appears in certain extremal situations [5]. It may perhaps be the source of the low-molecular-weight DNA which accumulates in blood plasma after irradiation. An oligonucleotide sequence, associated with increased recombining ability of DNA, has been found in a cloned fragment of extrachromosomal DNA [7]. The appearance of low-molecular-weight RNA (containing about 180 base pairs) in blood plasma during tumor development was demonstrated previously in rats [3] and in man [12]. Exposure to strong extremal factors (including ionizing radiation), activating structural changes within the genome, evidently activate synthesis of extrachromosomal DNA followed by fragmentation, which in our case was recorded before the appearance of the low-molecular-weight DNA. This suggests that this response may be universal and possibly protective in nature. The fact that the dose of γ-irradiation was directly proportional to the increase in the concentration of low-molecular-weight DNA indicates that the method of determining

low-molecular-weight DNA has potential value as a basis for laboratory methods of analysis in connection with the diagnosis of moderately severe radiation damage.

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EFFECT OF HEPATOPROTECTIVE AGENTS THIOCTACID AND FLAVOBION ON HISTONES IN THE INTACT AND REGENERATING LIVER OF IRRADIATED RATS

M. Kožurkova, K. Kropačova, E. Mišurova, and R. Rexa

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As the principal protein components of eukaryotic chromosomes histones are of fundamental importance in the stabilization of DNA structure in chromatin at different phases of the cell cycle and they play an important role in the regulation of gene expression [1].

Changes induced in histones by irradiation in vivo in different organs [2-5] or induced by partial hepatectomy [6, 7] have received comparatively little study. There is only sporadic information on the effect of irradiation on histones in the regenerating liver [8], and in combination with the use of hepatoprotective agents flavobion and thioctacid, we have no data as yet. Flavobion has many positive effects on the damaged liver: it stabilizes membranes, stimulates synthesis of ribosomal RNA [9], and, according to some data [10], its use leads to a reduced risk of development of toxic liver damage in patients. Thioctacid is a coenzyme of the Krebs cycle and is recommended as a preparation for use against liver damage in radiation sickness [11]. The aim of the investigation was to discover changes in some cytological parameters and in nucleic acid levels, the total content of extractable histones, and relative replacement of histone fractions after exposure to radiation and correction by hepatoprotective agents.

Department of Cellular and Molecular Biology, Faculty of Science, P. J. Šafarik University, Košice, Czechoslovakia.

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