Data on inhibition of KO_2-induced chemiluminescnce by ionol derivatives, taken in equal concentration (10^{-5} M), are given in Table 1. Clearly the ionol derivatives used are weak inhibitors of chemiluminescence, induced by oxygen radicals. No correlation likewise was found between the effectiveness of inhibition of LPO in microsomes and the effectiveness of inhibition of KO_2-induced chemiluminescence by ionol derivatives.

From the results as a whole it can be concluded that inhibition of LPO by the hydrophobic ionol derivatives used is realized predominantly by interaction with lipid radicals, and chemiluminescence induced in a microsomal suspension arises mainly through interaction of luminol with lipid radicals (and not with oxygen radicals).

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IDENTIFICATION OF GLUCOCORTICOID-ACTIVATED Ca^{2+}-DEPENDENT NUCLEASES IN RAT THYMOCYTE NUCLEI

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KEY WORDS: programmed death, glucocorticoids, Ca^{2+}-dependent nucleases, poly(ADP-ribosylation)

Cell death in the course of embryo- and morphogenesis and of specific T-killing, as well as death of lymphoid cells under the influence of radiation and glucocorticoid hormones are examples of programmed cell death. The biochemical manifestation of programmed cell death is accumulation of chromatin fragments which are multiples of mononucleosomes [14]. These fragments are formed by activation of endogenous nucleases. A key in chromatin degradation is most probably played by Ca/Mg-dependent endonuclease [1, 3]. However, it is not yet clear whether endonucleolysis takes place as a result of the action of pre-existing enzymes or whether it depends on induction of synthesis of new proteins.

This paper describes an attempt to identify nucleases activated by glucocorticoids in thymocyte nuclei and to explain some mechanisms of the activation of these enzymes.

EXPERIMENTAL METHOD

Noninbred male albino rats were subjected to bilateral adrenalectomy and kept on a saline diet for 5-10 days. The animals were given dexamethasone ("Cadila," India) by intraperitoneal injection in a dose of 5 mg/kg. Thymocyte nuclei were isolated by the method in [2]. 0.6M NaCl-extracts of the nuclei were obtained by homogenization in buffer containing 0.6 M NaCl, 0.05 M Tris-HCl, pH 7.5, 0.5 mM PMSF, and 1 μM pepstatin A, and were subsequently centrifuged at 200,000 g for 30 min on an L8-70 ultracentrifuge ("Beckman," USA). Histones were removed from the extracts by ion-exchange chromatography on a column with CM-Sephadex C-50 ("Pharmacia," Sweden) [12].

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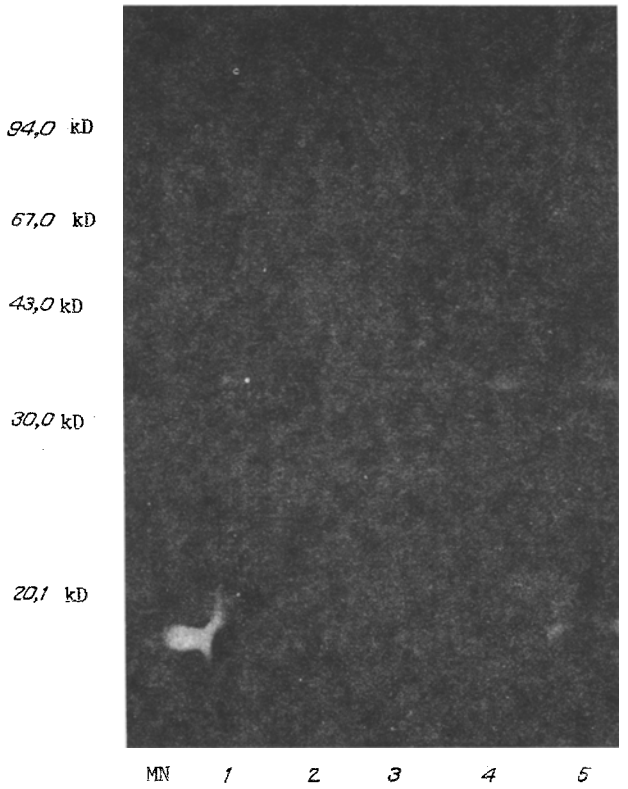

Fig. 1. Fluorogram of nucleases extracted with 0.6M NaCl from thymocyte nuclei 1, 2, 4, 6, and) h after injection of dexamethasone into rats. Here and in Figs. 2 and 3, lanes are arranged from left to right, respectively; below) micrococcal nuclease (MN).

The substrate used to determine nuclease activity was ^{3}H-thymidine-labeled DNA from E. coli (strain ONI) with specific activity of 93,000 cpm/μg.

Electrophoresis of proteins in polyacrylamide gel (PAG) containing polymerized ^{3}H-DNA was carried out in the presence of SDS [11]. Calibration by molecular weight was carried out with a mixture of standards from 14 to 94 kilodaltons ("Pharmacia").

Nuclease activity was detected as described in [7, 13]. Micrococcal nuclease (10 pg) was used as the positive control in these experiments. For quantitative determination of nuclease activity the gels were soaked in the fluorographic reagent) Amplify ("Amersham," England) and dried in vacuo at +80°C. The dried gels were exposed with ORWO HS II x-ray film (East Germany) for 7-10 days. The fluorograms were subjected to densitometry by the Gel Scanner 1310 instrument (ISCO, USA), by integrating the area beneath the peak.

To detect poly(ADP-ribose) in the protein fractions we used polyclonal antibodies generously provided by I. P. Beletskii (Institue of Biological Physics, Academy of Sciences of the USSR). The antibodies were labeled with Na^{125}I by the method in [8]. After electrophoresis the proteins were transferred to PVDF paper ("Millipore," USA) by the method in [10]. The filters were treated as described in [9], using ^{125}I-labeled antibodies, and the immunoblots were exposed with x-ray film.

EXPERIMENTAL RESULTS

The 0.6M NaCl extracts of nuclei obtained 0, 1, 2, 4, and 6 h after the addition of dexamethasone were fractionated by SDS-PAG electrophoresis with polymerized ^{3}H-DNA of E. coli. To detect nuclease activity of the protein fractions, after rinsing to remove SDS the gels were incubated in ion-free medium or in the presence of Ca^{2+} and Mg^{2+} and subjected to fluorography. Nuclease activity was detected only on fluorograms of gels incubated in the presence of both cations, and corresponded to a protein with mol. mass of 35 kDa. The level of nuclease activity did not change during the period of testing (Fig. 1). The enzyme was not detected on the fluorograms if the animal received injections of dexamethasone together with cycloheximide (CHI), an inhibitor of protein synthesis. Simultaneously with disappearance of the enzyme under the influence of CHI, endonucleolysis of thymocyte nuclei incubated under optimal conditions for the action of Ca/Mg-dependent endonuclease also was blocked.

Previously a similar technique was used to characterize nucleases responsible for hormone-induced degradation of DNA by thymocytes [4]. The authors cited showed that injection

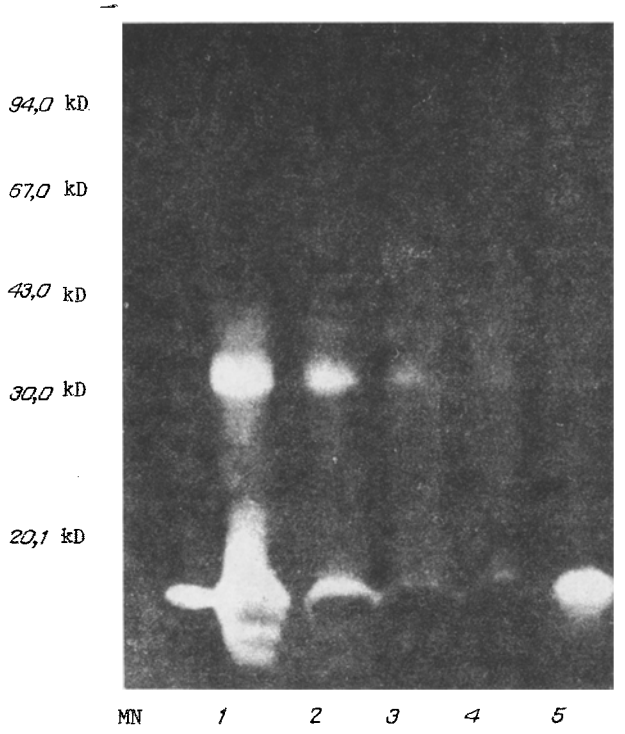

Fig. 2

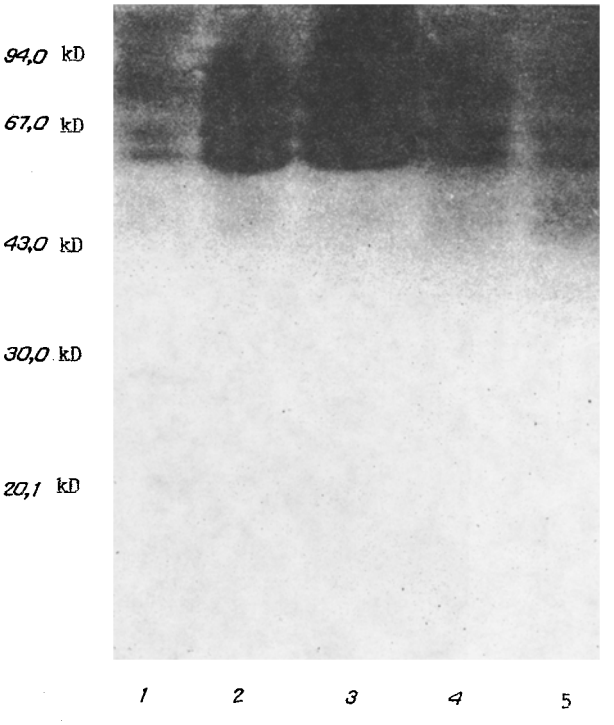

Fig. 3

Fig. 2. Fluorogram of nucleases extracted with 0.6M NaCl from thymocyte nuclei 1, 2, 4, 6, and 0 h after injection of dexamethasone into rats.

Fig. 3. Autoradiogram of immunoblots of poly(ADP-ribosylated) proteins, isolated from thymocyte nuclei 1, 2, 4, 6, and 0 h after exposure to dexamethasone.

of dexamethasone into the animals promoted synthesis of two principal families of proteins: a duplex of 30-32 kDa and three or four proteins with mass of 12-19 kDa, possessing DNA activity. Under these circumstances synthesis of nucleases of the group with lower molecular mass increased, parallel with the increased intensity of DNA degradation. The location of the nucleases in the gel was established from disappearance of fluoroscence of DNA stained with ethidium bromide. Unlike the results in [4], we found only one protein fraction, possessing nuclease activity. Accordingly, we postulate the possible screening of fluorescence of DNA by histones present in the extracts. This was shown by the fact that histones possessed electrophoretic activity similar to that of the "nucleases" discovered by the author cited.

To remove histones we used ion-exchange chromatography on a column with CM-Sepharose C-50. Nuclear extracts purified from histones were analyzed as mentioned above. In this case, a series of nucleases corresponding to proteins with mol. masses of 32, 17.7, 17.0, and 16.4 kDa were identified on the fluorograms. The nuclease activity of these proteins increased fourfold 1 h after injection of the glucocorticoid into the animals, and fell to the control level 4 h after the beginning of observation (Fig. 2). Incidentally, activity of the enzymes discovered depended on the presence of Ca^{2+}. Addition of both Ca^{2+} and Mg^{2+} to the incubation medium had a synergic effect only relative to the low-molecular-weight group of nucleases. Participation of Ca^{2+}-dependent nucleases in internucleosomal fragmentation of chromatin also was proved in [3, 5].

The problem of the mechanism of activation of endogenous nucleases during programmed cell death has not yet been solved. We know that poly(ADP-ribosylation) depresses activity of Ca/Mg-dependent endonuclease [15]. It was therefore interesting to determine the content of poly(ADP-ribose) in the nucleases discovered. On autoradiographs of immunoblots of nuclear extracts purified from histones we were able to find poly(ADP-ribosylated) proteins in zones corresponding to the nucleases with mol. mass of 17.7, 17.0, and 16.4 kDa (Fig. 3). Incidentally, the quantity of poly(ADP-ribose) in fractions possessing nuclease activity was proportional to their protein content. Consequently, the conclusion that activation of nucleases can be explained by a disturbance of poly(ADP-ribosylation) was not confirmed. The authors of [6] reached a similar conclusion.

It can be postulated on the basis of our findings that a relatively broad spectrum of nucleases is involved in the process of glucocorticoid-induced death of lymphoid cells. Poly(ADP-ribosylation) has no effect on activity of the identified nucleases.

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MECHANISMS OF THE ANTIOXIDATIVE ACTION OF SCREENED PHENOLS IN BIOMEMBRANES: EFFECTS OF IONOL AND ITS DERIVATIVES

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The development of oxidative stress induces a deficiency of natural antioxidants in the body, with the results that lipid peroxiation (LPO) becomes uncontrollable [9, 15]. To maintain the balance between pro- and antioxidant systems, an additional quantity of antioxidants must be introduced. Emergency replenishment of deficient antioxidants with the aid of vitamin E, a natural inhibitor of free-radical oxidation, is limited by the mechanisms of its transport (tocopherol-carrying proteins) [5, 6], and as a result, it is insufficiently effective. This difficulty can be overcome with the aid of synthetic antioxidants, for which specific transport mechanisms do not exist in the body [2].

One of the most widely used synthetic antioxidants is ionol (4-methyl-2,6-di-tert-butylphenol), permitted in the USSR and many other countries as a food preservative [17]. However, it has recently been shown that ionol possesses a direct membrane-destabilizing action in experiments in vitro, causing a sharp increase in membrane permeability [7]. The toxicity of ionol in doses in excess of 500 mg/kg has been demonstrated in vivo, and is manifested as hyperplasia of the lungs and changes in the cell components in rats [10, 16]. It is considered that these toxic effects of ionol are due to the action of intermediates of its metabolic activation in the cytochrome P-450 system [11, 12].

The study of the characteristics of screened phenols (and, in particular, of ionol derivatives) have been included in the program of the joint Bulgarian–Soviet "Biostab" Project with the ultimate aim of selecting homologs: 1) possessing sufficiently high antioxidative activity in biomembranes, 2) not exhibiting direct membrane-disturbing effects, and 3) not

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