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# TOXICITY OF HIGH-MOLECULAR-WEIGHT PROTEIN FROM BURNED SKIN REVEALED BY BIOLOGICAL TESTS *in vivo* AND *in vitro*

B. E. Movshev, R. V. Nedoshivina, and I. K. Koryakina

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The study of burn toxemia — an important stage in the pathogenesis of burns — has for a long time been hindered by the lack of adequate methods for the isolation of toxic substances. Attempts to purify burn toxin have frequently been carried out and have been partly successful [13, 14]. Contrary to expectation, however, investigations of "Rosenthal's glycopeptide" and "Schoenenberger's lipoprotein" have not been pursued further, possibly because the methods used to isolate the toxins were laborious and, more important, nonspecific.

In the researches of Fedorov and co-workers attention was concentrated on the immunologic specificity of factors of burn toxemia [1, 3, 5, 7, 10, 11]. A detailed study of products from burned skin served as the basis for development of a direct immunochemical method of isolation of the toxin [12], a high-molecular-weight protein toxin was isolated for the first time [6], and its high pathogenicity was demonstrated [8].

The object of this investigation was to determine the comparative characteristics of activity of the high-molecular-weight protein toxin of burned skin on the basis of the results of biological testing by two independent methods, used in the writers' laboratory to study burn toxemia.

## EXPERIMENTAL METHOD

The skin of 207 burned Wistar rats was used as the source of the toxic material. A burn affecting 15-20% of the body surface was inflicted by the flame of a cotton swab soaked in alcohol for an exposure of 45 sec. Extracts of burned skin (EBS) were prepared by the method in [4].

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The toxin was isolated from EBS with the aid of immunosorbents based on activated CNBr-Sepharose 4B (Pharmacia Fine Chemicals) [5]. Immunosorbents of two types, differing in the specificity of their fixed antibodies, were used. By means of the type I immunosorbent, containing antibodies only against the specific component of the toxin, pure burned skin toxin could be obtained free from impurities (conventionally described as T toxin). The type II immunosorbent contained a wider spectrum of antibodies and could be used to isolate all burn-specific components, both toxic and nontoxic, from the EBS (burn antigens). These preparations were conventionally described as "OT toxin."

Eluates of the toxin, pooled from two to four cycles of use of the immunosorbent, were concentrated by ultrafiltration through Diaflo XM-50 membranes (Amicon, USA) and dialyzed against 0.14 M NaCl solution. Protein was determined by Lowry's method.

Biological testing *in vivo* was carried out on 347 mice with their reticuloendothelial system (RES) blocked [9] by a single intravenous injection of C-11/1431a ink (Günter Wagner, West Germany) in a dose of 24 mg/100 g body weight. After 30-40 min the experimental mice were given an intraperitoneal injection of 1 ml of the test preparation. Toxicity was evaluated as the number of mice dying in the course of 72 h.

For *in vitro* bioassay the method of blood cultures was used, with determination of the migration index of healthy human leukocytes [2]. The test preparations were added to a film of leukocytes and, after incubation for 18 h at 37°C, the migrating capacity of the cells was assessed planimetrically.

Four groups of preparations were subjected to bioassay: 1) freshly prepared EBS in doses of 0.2 to 5 mg protein per mouse or in dilutions of 1:100 and 1:200 for addition to culture fluid; 2) EBS after passing through an immunosorbent (exhausted EBS) in the same doses and dilutions; 3) T toxin in doses of 0.01 to 0.05 mg protein per mouse; 4) OT toxin in doses of 0.20 to 0.75 mg protein per mouse.

The results were subjected to statistical analysis by the t-test and determination of the coefficient of correlation.

## EXPERIMENTAL RESULTS

Bioassay *in vivo* (Table 1). The results of assay of EBS were consistent and confirmed previous findings: All 27 preparations of EBS had a strong toxic action on mice with their RES blocked. During the first few hours after injection of the preparations into the animals signs of toxic effects appeared (loss of mobility, rapid respiration, paralysis). In some animals the disorders increased in severity and proved fatal. In this group 45.1% of the total number of mice taken for the experiment died ($LD_{50}$ = 5 mg protein of EBS).

Convincing results were obtained when "exhausted EBS" was tested. Injection of these preparations had no toxic action on mice with their RES blocked. Of 63 mice used in the experiment in this group only one animal died, evidently from accidental causes. Loss of toxic properties after passage of the EBS through the specific immunosorbent is a significant fact, and the group as a whole can serve as an example of successful biological verification. EBS was freed from its accompanying toxic activity under mild conditions, preventing any injury to the material, simply through interaction between the toxin and antibodies fixed to the insoluble substrate. The value of this biological control is confirmed by the fact that the difference between the EBS and the "exhausted EBS" was the result of one feature alone, namely the presence or absence of toxin.

On injection of preparations of T toxin an effect was given even by the smallest doses. Toxic manifestations after injection of these preparations corresponded on the whole to the characteristic features of EBS, and $LD_{50}$, which was 0.027 mg protein, coincided with activity of the previous batches of the preparation, namely 0.016-0.025 mg.

The action of OT toxin in general followed exactly the same principles as the action of the pure toxin. A marked toxic effect (35.6% of mice died) was observed during the first few hours after injection of the preparations. In this group, just as in the others, the toxic effect was clearly dependent on the protein content in the preparation, and on that basis its $LD_{50}$ was calculated to be 0.75 mg.

Comparison of the characteristics of activity of the T toxin and the OT toxin indicates that the toxic effect, manifested as death of experimental mice with their RES blocked, was due to the action of the high-molecular-weight protein toxin from burned skin and not to other burn-specific antigens.

TABLE 1. Determination of Activity of Preparations by Bioassay Methods

| Batch of preparation | Statistical index | Bioassay in vivo 1 | | Biossay in vitro 2 |
|---|---|---|---|---|
| | | absolute | % | |
| 1) EBS | $\bar{x}\pm m$ | 59/131 | 45,1±4,4 | −8±3,1 |
| | $n$ | | 27 | 20 |
| 2) exhausted EBS | $\bar{x}\pm m$ | 1/63 | 1,6±1,6 | 6±2,7 |
| | $n$ | | 19 | 19 |
| | $t_1$ | | 9,4* | 3,41* |
| 3) T toxin | $\bar{x}\pm m$ | 27/63 | 42,9±6,2 | −14±3,5 |
| | $n$ | | 12 | 8 |
| | $t_1$ | | 0,289 | 1,282 |
| | $t_2$ | | 6,42* | 4,52* |
| 4) OT toxin | $\bar{x}\pm m$ | 32/90 | 35,6±5,0 | −10±2,8 |
| | $n$ | | 4 | 15 |
| | $t_1$ | | 1,426 | 0,478 |
| | $t_2$ | | 6,42* | 4,11* |
| | $t_3$ | | 0,91 | 0,892 |

*Difference statistically significant: $P \leq 0.05$.

Note: 1. Numerator gives number of mice which died, denominator total number of mice used in experiments. $t_1$, $t_2$, $t_3$ — Compared with batches 1, 2, and 3 of preparation, respectively. 2. Changes in area of migration of leukocytes in conventional units.

Bioassay *in vitro*. Investigation of the toxic properties by the blood culture method is based on determination of the migrating power of healthy human leukocytes under the influence of a test preparation or medium compared with a control (Ringer's solution). The migration power in the control group is taken to be 100. The toxic effect (inhibition of migration) is assessed as the difference between the experiment and control.

Of the 20 EBS preparations tested in blood cultures, 16 were found to give a toxic effect. Inhibition of migration was characterized by the mean index for the group (−8 ± 3). On the other hand, preparations of "exhausted EBS" gave no toxic action (6 ± 3) in any of 19 experiments. The difference between the groups is highly significant ($P < 0.01$).

On the addition of preparations of the toxin to a leukocytic film they were found to have a marked toxic action. The level of activity of T toxin and OT toxin was roughly the same, according to the leukocyte migration inhibition index (−14 ± 3.5 and −10 ± 2.8, respectively).

Statistical analysis of the results obtained by the two independent bioassay methods revealed correlation between them ($P = -0.156$).

Despite the standard conditions under which the preparations were obtained, the level of their biological activity did not remain unchanged. One reason for its variability may have been the bioassay system itself. The advantage of bioassay on mice with a blocked RES is that toxicity can be evaluated in its most general form. The effect is assessed according to the "all or nothing" principle, and in order to allow for individual variations in reactivity, several animals in a group must be studied. The blood culture method enables the toxic effect to be assessed quantitatively in tissue culture. Previously, both methods have been used to determine the toxicity of sera in burns [2, 9].

The investigation thus showed that EBS and, in particular, preparations of burn toxin isolated from it have a clear toxic action when tested both *in vivo* and *in vitro*. After passage through a specific immunosorbent, the EBS lose their toxic properties because of reversible andspecific binding of the toxin by antibodies fixed to the insoluble matrix.

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# ELASTASE ACTIVITY IN EXPERIMENTAL PANCREATITIS

V. A. Kubyshkin, I. I. Musin, R. G. Ambartsumyan, L. V. Mikrikova, A. A. Aleksandrov, and A. A. Izotova

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A most important role in the pathogenesis of acute pancreatitis is played by the pancreatic enzyme elastase, which occupies a special place among tissue proteolytic enzymes of animals and man in its ability to hydrolyze one of the most inert tissues of the body, the scleroprotein elastin.

Since the discovery of elastase [5], the pathobiochemical spectrum of its action has been widely studied. Elastase is characterized not only by a marked elastolytic effect [16] and a proteolytic action on hemoglobin, fibrin, and albumin [6], but also by a certain lipolytic activity [9, 13]. Accordingly, elastase may have a much more traumatic effect than trypsin, carboxypeptidase, and lipase [2, 6]. However, the results of investigation of the elastolytic activity of the blood in acute pancreatitis are contradictory, evidently because different methods were used to produce the pancreatitis and to determine elastase [6, 11, 12, 15]. Meanwhile, the problem of the inhibitory property of the blood relative to elastase and the mechanism of production of its inhibitor still remains unsolved [13, 16]. In particular, investigations [8, 10, 14] have revealed a marked fall in the elastase concentration in the general circulation compared with its concentration in the portal blood flow and an unchanged level of inhibitor in these systems. Furthermore, elastase is not inactivated in the pancreas when its concentration rises. The explanation of these phenomena may lie in the active role of the liver in the production of inhibitor and reduction of the elastolytic activity of the blood.

The object of this investigation was to study relations of elastase with its inhibitor in the blood and also with lactate dehydrogenase (LDH) isozymes, which reflect the state of the liver function during the development of acute pancreatitis.

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S. I. Spasokukotskii Professorial Surgical Clinic, and Laboratory of Clinical Biochemistry, Central Research Laboratory, N. I. Pirogov Second Moscow Medical Institute. (Presented by Academician of the Academy of Medical Sciences of the USSR V. S. Savel'ev.) Translated from Byulleten' Eksperimental'noi Biologii i Meditsiny, Vol. 90, No. 10, pp. 425-427, October, 1980. Original article submitted June 20, 1979.

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