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BLOOD SERUM PROTEINS AS POSSIBLE INDICATORS OF BURN TOXINS

B. E. Movshev and V. I. Averchenko

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The state of the blood proteins in thermal burns has often been investigated [5, 7, 8]. Changes in the relative concentrations of individual serum proteins [12], qualitative characteristics of protein fractions detectable by electro- and immunoelectrophoresis [1, 6, 11], the level of the so-called acute phase proteins [10], and other parameters have been regarded as prognostic signs in burns. On the whole, however, data on the pathogenetic significance and specificity of disturbances of the blood protein system in burns are highly contradictory. In previous studies of burned skin as the most likely source of autoimmunization and autointoxication the formation of a specific burn toxin was demonstrated and its important role in the pathogenesis of the initial period of burn toxemia was established [3, 4].

This paper describes the results of a comprehensive analysis of serum proteins corresponding in certain physicochemical characteristics to the high-molecular-weight toxin of burned skin.

EXPERIMENTAL METHOD

A thermal burn of the skin was inflicted on female Wistar rats weighing 100-120 g (anesthetized with 1 ml of 0.3% pentobarbital solution, flame burn from cotton soaked in alcohol, area of burn 15-20% of body surface, exposure 30 sec). Blood was taken from the animals' heart 48 h after burning under ether anesthesia and, after clot formation, the serum was separated by centrifugation. Serum was obtained under similar conditions from normal, unburned rats.

Fractionation of the serum proteins by two-stage precipitation with ammonium sulfate (fractions corresponding to 60-75 and 63-70% saturation) and gel-filtration on Sephadex G-200 was carried out as described previously [4]. All operations corresponded exactly to the isolation of toxic fractions from a saline extract of burned skin.

The gel permeation coefficient (K_{av}) was calculated by the equation:

$$K_{av} = \frac{V_e - V_0}{V_t - V_0},$$

where V_e is the elution volume of the corresponding fraction; V_0 the outer bed volume, deter-

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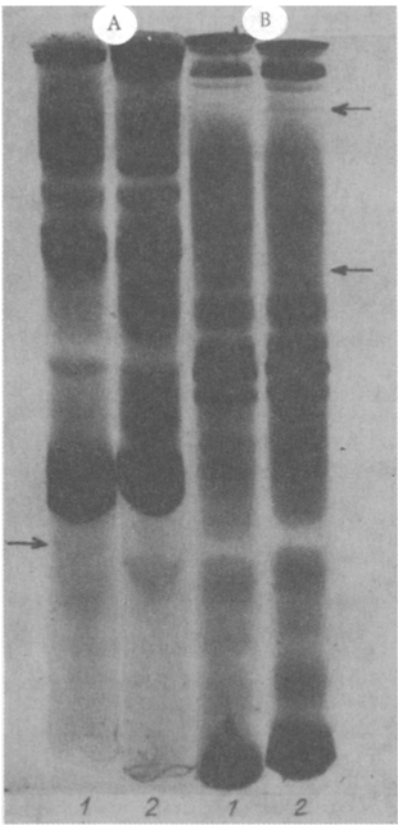

Fig. 1. Disc electrophoresis of blood serum from burned (1) and normal (2) rats. A) Duration of fractionation 45 min, current 4 mA; B) duration of fractionation 105 min, current 10 mA. Anode below. Arrows indicate lines found only in burned rats.

TABLE 1. Protein Yield and Toxicity before and after Fractionation of Serum from Normal (control) and Burned (experiment) Rats

Object	Method of fractionation	Control			Experiment		
		protein		toxicity	protein		toxicity
		mg	%		mg	%	
Original serum	—	29 400	100	—	51 700	100	4/5
60-75% fraction	Precipitation with ammonium sulfate	23 300	79,2	4/5	11 300	21,9	0/3*
63-70% fraction	The same	10 500	35,7	3/5	8 200	15,9	3,5
K_{av} = 0.13-0.25 fraction	Gel filtration	75,5	0,26	—	76,5	0,15	0/5

Legend. Numerator gives number of mice dying within 72 h after injection of preparation in a dose of 0.3 mg protein; denominator gives number of mice in experiment; asterisk dose of preparation 0.18 mg protein.

mined from elution of blue dextran (2×10^6 daltons, from Ferrak, Berlin); V_t denotes the volume of the column.

Disc electrophoresis in polyacrylamide gel (PAG) was carried out in system I [2]. After staining in 0.25% Coomassie blue solution (Ferrak) and washing with acetic acid the gels were photographed on lined plates and the optical density of the lines measured on a DMK-2 densitometer (Toyo, Japan). The relative electrophoretic mobility (REM) of the lines was expressed in conventional units, the mobility of the buffer or bromphenol blue front being taken as 1.

The protein concentration in the preparations was determined by Lowry's method or from the optical density at 280 nm.

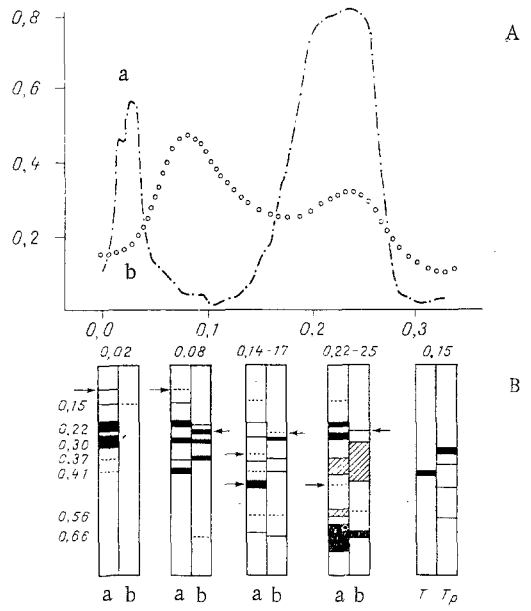

Fig. 2. Fractionation and composition of protein fractions 63-70 from serum of normal (a) and burned (b) rats. A) Gel-filtration. Abscissa, value of K_{av}. Ordinate, optical density (E_{280}); B) disc electrophoresis in 7.5% PAG. Anode below. Numbers above columns give K_{av} in gel filtration experiments; figures below give REM. Arrows indicate noncoinciding lines. T) High-molecular-weight toxin from burned skin; T_p) degradation products of toxin.

EXPERIMENTAL RESULTS

Electrophoresis in PAG revealed at least 25 components reflecting the complex structure of the blood protein system (Fig. 1). The number of components and their distribution by zones in normal rat serum did not differ on the whole from those described in the literature for serum proteins in man and experimental animals [2, 9]. With a change in the duration of electrophoresis, the concentration of the gel, or pH of the buffer system, most fractions were found to consist not of single proteins but of mixtures of proteins with closely similar characteristics.

The content of components 4, 14, 15, 16, 22, and 25 in the rats' serum 48 h after burning was 30-75% higher than normal, but the albumin level (24) was 19% lower and the γ-globulin level 20% lower. The last of these components was determined densitometrically from the decrease in optical density of a diffuse band occupying the region from the start line to the α_2-globulins.

Quantitative features of the burned rats' serum distinguishing it from normal serum include the appearance of additional lines 26 and 27 in the region of slow α_2-globulins and of a line 28 in the prealbumin zone. This agrees with data in the literature on hypermacroglobulinemia [5, 8] and on an anomaly of the serum α_2-globulins and prealbumins in burns [8, 6, 11].

Despite the high resolving power of electrophoresis in PAG, no anomaly which could be ascribed to burn toxin could be found in whole blood serum by this method. Further analysis was therefore carried out on protein preparations obtained by fractional precipitation with ammonium sulfate and by gel filtration on Sephadex G-200. Serum from normal rats fractionated under analogous conditions served as the control. Data on the yield of protein and toxicity of the preparations are given in Table 1.

The results of biotesting did not reveal what was responsible for the toxic properties of the preparations, especially the fractions of normal serum. This contradicts the results of experiments carried out in the writers' laboratory showing that the serum of healthy animals is completely harmless for mice with blockade of the reticuloendothelial system.

After salting out with ammonium sulfate a much higher yield of protein was obtained from normal serum than from the serum of burned rats. This was evidently on account of proteins with a comparatively low molecular weight, for after isolation of high-molecular-weight proteins by gel filtration the yield of the end product was practically identical whether the source was serum from burned or normal rats.

According to the results of electrophoresis in PAG both high-molecular-weight fractions remained highly heterogeneous and contained 12-15 components, only some of which can be regarded as a distinguishing feature between normal serum and serum in burns (Fig. 2). For instance, the high-molecular-weight protein fraction from serum of burned rats was characterized by loss of the lines with REM values of 0.10, 0.35 and, possibly, 0.46 present in normal serum, and by the appearance of a line with REM of 0.26, not present in normal serum. The importance of differences of this kind requires further study.

Although on the whole for many of the components studied a higher value of K_{av} corresponds to greater mobility of the protein in PAG, this rule does not apply in reverse. Some serum proteins from normal and burned rats corresponded to each other in their electrophoretic mobility (for example, proteins with REM of 0.30, 0.37, and 0.41), but differed in their K_{av} value on gel filtration. Migration of these proteins during electrophoresis was evidently determined only by the value of the charge and not by the molecular sieve effect. It can be concluded on theoretical grounds [2] that one of the measurements of the molecule of these proteins cannot exceed 5 nm.

Analysis of high-molecular-weight serum proteins from normal and burned animals showed that in their physicochemical characteristics none corresponded to the toxin from burned skin used in parallel experiments as marker. As the writers showed previously [3], high-molecular-weight burn toxin is precipitated by ammonium sulfate at 63-70% saturation, is eluted from a column with Sephadex G-200 at $K_{av} = 0.15$, and has an REM value of 0.43 on electrophoresis in 7.5% PAG. None of the serum components investigated possessed these properties.

It is a noteworthy fact that components with REM values of 0.35, 0.40, 0.49, and 0.59 were present in serum. The discovery of the principles governing their appearance and identification on the basis of size of molecules is difficult because of their relatively low concentration. The components were formed separately from each other or in different combinations, both in serum from burned rats and in serum from normal rats. The writers showed previously that breakdown products of the high-molecular-weight toxin of burned skin possess this mobility.

The considerable quantitative and qualitative changes in the blood protein system under the influence of burn trauma are thus highly complex in character and take the form of fluctuations in the relative content of individual fractions and also of the appearance of additional fractions not present in normal serum.

Meanwhile, neither in whole serum of burned rats nor in its fractionation products could a component be found which, in its physicochemical characteristics, would correspond to the high-molecular-weight toxin of burned skin. It can be tentatively suggested that, since it possesses extremely strong biological activity, burn toxin is present in the blood in very small quantities and (or) it is bound with other proteins. Under the conditions of fractionation and analysis (salting out, gel filtration, disc electrophoresis) which were used in the present investigation, the possibility of the appearance of breakdown products of burn toxin in the blood cannot be ruled out.

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ISOLATION AND STUDY OF THE PROPERTIES OF THE REGULATORY SUBUNIT OF cAMP-DEPENDENT PROTEIN KINASE

V. A. Yurkiv, E. S. Severin,
S. P. Petukhov, and T. V. Bulargina

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Cyclic AMP (cAMP) exerts its activating action on cAMP-dependent protein kinase through its regulatory subunit (R). Under the influence of cAMP the holoenzyme protein kinase dissociates according to the scheme:

$$R_2C_2 \xrightarrow{2cAMP} (R\cdot cAMP)_2 + 2C,$$

which leads to the appearance of the active catalytic subunit (C) [4].

It is also known that the regulatory subunit of type II cAMP-dependent protein kinase (R-II) contains four functional regions responsible for binding cAMP, for contact with C, and for forming the R=R bond in the holoenzyme, and an autophosphorylation site [6, 14, 15]. According to some workers, R is a single polypeptide chain, readily broken down by proteolytic enzymes into fragments, one of which preserves the cAMP binding center [13].

The investigation described below was a continuation of the study of protein kinases in the mucosa of the rabbit's small intestine [3]. To study the cAMP binding site the method of inhibitor analysis was used, with analogs of cAMP containing substitutents in different parts of the molecule.

EXPERIMENTAL METHOD

Binding of [^{3}H]-cAMP with the regulatory subunit was determined by the method in [7] in 100 µl of incubation medium containing 50 mM potassium phosphate buffer, pH 6.5, 2 mM NaCl, 5 mM theophylline, bovine serum albumin in a concentration of 1 mg/ml, and 4-7 µM of [^{3}H]-cAMP (2×10^5 to 4×10^5 cpm).

Electrophoresis in 12.5% polyacrylamide gel in the presence of sodium dodecylsulfate was carried out [9] in cylindrical (5 × 70 mm) gels with a g-250 and scanned at 600 nm in a Carl Zeiss (East Germany) densitometer.

The protein concentration was determined by the method in [12]. Protein solutions were concentrated by dialysis against 20% polyethyleneglycol solution (mol. wt. 40,000 daltons).

The 8-(2-hydroxyethylthio)-cAMP was immobilized on epoxy-activated sepharose by the method in [16]. The cAMP analogs were generously provided by N. N. Gulyaev.

EXPERIMENTAL RESULTS

The small intestine of a rabbit was removed and washed with cold physiological saline. The mucosa was curetted, frozen, and kept at −70°C. To obtain a cytosol, the frozen tissue

Laboratory of the Molecular Basis of the Pathogenesis of Infectious Diseases, Central Research Institute of Epidemiology, Ministry of Health of the USSR, Moscow. Department of Biochemistry, Biological Faculty, M. V. Lomonosov Moscow State University. (Presented by Academician of the Academy of Medical Sciences of the USSR S. E. Severin.) Translated from Byulleten' Eksperimental'noi Biologii i Meditsiny, Vol. 94, No. 12, pp. 36-38, December, 1982. Original article submitted July 2, 1982.

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