PREPARATION OF ANTITOXIN FRAGMENTS FROM "DIAFERM-3 IÉM" ANTITETANUS SERUM

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Fragments of antibodies not reacting in the agar diffusion test with tetanus toxin and giving 3 peaks by fractionation on Sephadex G-100 were obtained from "Diaferm-3 IÉM" purified proteolyzed antitetanus horse serum by reduction with mercaptoethanol and alkylation with monoiodoacetic acid. Only the peak II fractions possessed antitoxic activity and delayed precipitation in agar in a test system of tetanus toxin and the original "Diaferm-3 IÉM" serum and had electrophoretic mobility similar to that of γG-globulin. The molecular weight of the fractions of peak II, determined by gel-filtration, was 45,000 and the sedimentation constant was about 3S. The specific antitoxic activity of the peak II fractions was 3-5 a.u./mg protein (compared with 6-7 a.u./mg protein for the original serum).

Antibodies against tetanus toxin in antitoxic horse sera are bound with two protein fractions: the γ_2- and T-globulins [3, 8, 13, 15-17, 20, 21]. The γ_2-globulin of horse antitoxic serum can be broken down to 3.5 S fragments by treatment with proteolytic enzymes (pepsin, papain, etc.), reduction, and alkylation of the disulfide bonds [5, 19]. The preparation of monovalent fragments of antibodies from the T-globulins of horse serum is particularly interesting. Results [9, 11, 12] have shown that the T-globulin of horse antitoxic serum can be split only into bivalent 5-2S fragments. Schultze and co-workers [19] obtained monovalent 3.5S fragments of the T-globulin of horse antitetanus serum. The view has been put forward [22] that the difference between the digestion of γ_2-and T-globulins by proteolytic enzymes is due to the presence of and additional disulfide bond in the Fd-fragment of T-globulin. This is characteristic of the T-globulins and is not found in any of the classes of γ-globulin. According to other writers [6] this difference between the digestion of γ_2- and T-globulins is due to the presence of an additional peptide in the active center of the T-globulin.

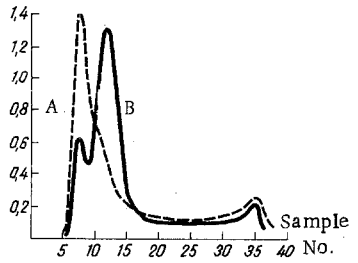

Fig. 1. Fractionation of original "Diaferm-3 IÉM" antitetanus serum (A) and its fragments (B) on Sephadex G-100. Abscissa, No. of sample; ordinate, optical density at 280 nm.

The object of this investigation was to obtain monovalent antibodies from "Diaferm-3 IÉM" antitetanus serum, which is a purified proteolyzed antitoxin belonging mainly to the T-globulin fraction [2-4, 8, 10].

EXPERIMENTAL METHOD

"Diaferm-3 IÉM" antitetanus serum, batch No. 599, from the I. I. Mechnikov Moscow Institute of Vaccines and Sera was used. The antitetanus serum (1.6% solution of protein) was treated with 0.1 M mercaptoethanol in 0.1 M phosphate buffer, pH 7.5, for 66 h at 20°C to reduce the disulfide bonds. The production was then alkylated with 0.2 M monoiodoacetic acid at pH 7.5-9.0 for 2-2.5 h and dialyzed against 0.01 M phosphate buffer, pH 7.5, for 24 h. After dialysis, the proteolytic digest was concentrated to its initial volume and fractionated on a column with Sephadex G-100 (60 × 1.8 cm) in 0.01 M phosphate buffer, pH 7.5.

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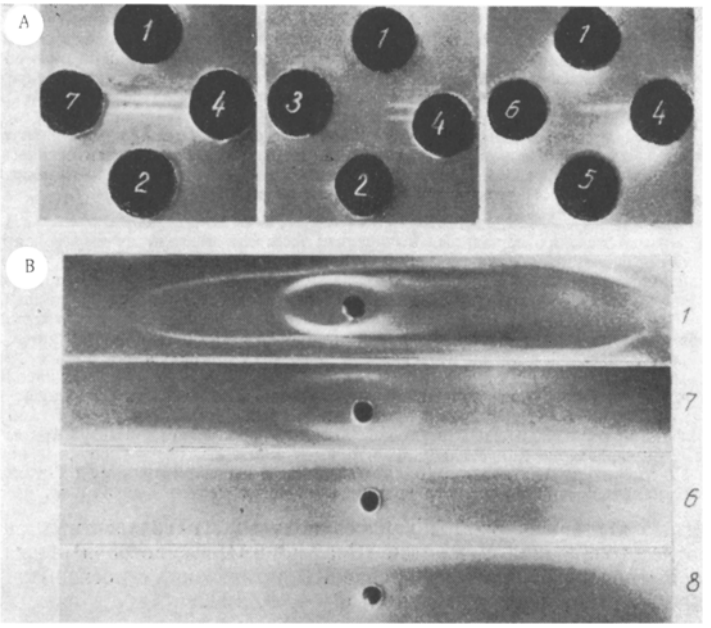

Fig. 2. Precipitation test (A) and immunoelectrophoresis (B) in agar of tetanus toxin with Diaferm-3 IÉM serum and its fragments: 1) original Diaferm-3 IÉM serum; 2) tetanus toxin; 3) digested serum, not fractionated; 4) physiological saline; 5) purified tetanus toxin; 6) peak II of digested serum; 7) peak I of digested serum; 8) peak III of digested serum. Gutters contain rabbit antihorse serum.

The optical density of the fractions was determined with a type SF-4A spectrophotomer at 280 nm. The protein concentration in the combined fractions was determined by the method of Lowry et al. [14]. The antitoxin was titrated biologically in mice. The molecular weight of the fragments was determined by Whitaker's method [23] by gel-filtration through Sephadex G-100, equilibrated with 0.05 M phosphate buffer, pH 7.5, containing 0.1 M KCl. A calibration curve was plotted for protein with a molecular weight of 22,500-65,000. The sedimentation constant was determined by ultracentrifugation on the Spinco model E analytical ultracentrifuge at a mean rotor speed of 56,094 rpm, using 0.01 M phosphate buffer, pH 5, as the solvent. The immunochemical properties of the antitoxin fragments were determined in the double agar diffusion test, by immunoelectrophoresis in agar in the micromodification [1], and in the delay of precipitation test. The following materials were used in the tests: rabbit antiserum against horse serum proteins, tetanus toxin from Leningrad Research Institute of Vaccines and Sera, batch No. 21 and purified tetanus toxin prepared by O. P. Sakharova, working in the authors' laboratory.

EXPERIMENTAL RESULTS

On fractionation of the original serum (Diaferm-3 IÉM) on Sephadex two peaks were obtained (Fig. 1A). Antitoxic activity was concentrated in the fractions of peak I and amounted to 6-7 a.u./mg protein. The fractions of peak II contained no antitoxic activity, and they did not react in the precipitation test with antiserum against horse serum proteins or with tetanus toxin. The fractions of peak II were evidently the low-molecular weight product of proteolytic digestion of the serum, in agreement with data in the literature [7] on the existence of various products of proteolysis in the Diaferm-3 IÉM serum. The fractions of peak I gave a precipitation test with purified tetanus toxin. From the antigenic point of view the peak I fractions consisted of proteins with the mobility of IgG-globulin, as well as α_1- and α_2- globulins (results of immunoelectrophoresis with rabbit antiserum against horse serum proteins).

On fractionation of the digested serum on Sephadex three peaks were obtained (Fig. 1B). Fraction III consisted of salts remaining after dialysis. Antitoxic activity was concentrated in fraction II and amounted to 3-5 a.u./mg protein. The unfractionated hydrolyzed serum did not give a precipitation reaction in agar with tetanus toxin, but delayed this reaction in a homologous test system of tetanus toxin and the original

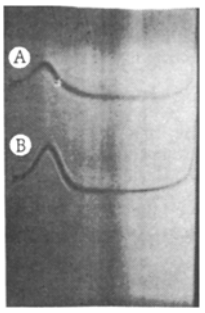

Fig. 3. Sedimentation diagram of digested, unfractionated Diaferm-3 IÉM serum (A) and of peak II fractions (B); 56,094 rpm for 1 h from time of reaching top speed.

Diaferm-3 IÉM serum (Fig. 2A). Fractions I and II likewise did not give a precipitation reaction in agar with tetanus toxin. Delayed precipitation was given only by fraction II. Purified tetanus toxin was used as an additional control of the specificity of delay of precipitation. Under these conditions also, precipitation in agar was delayed only by fraction II of the digested serum (see Fig. 2A). The fragments of antitoxin retained their antigenicity. Immunoelectrophoretic analysis showed that fraction I consists mainly of protein with the mobility of γ_1- and γ_2-globulins, and fraction II of protein with the mobility of IgG-globulins. Fraction III did not contain antigens (Fig. 2B).

The molecular weight of the peak II fragments possessing antitoxic activity was 45,000. The elution volume of the antitoxin fragments was the same as the elution volume of ovalbumin, with a molecular weight of 45,000. The molecular weight of the original Diaferm-3 IÉM serum was between 80,000 and 100,000.

The sedimentation constant of the antitoxin fragments, as shown by ultracentrifugation, was 3S (Fig. 3). Fraction II of the digested serum also contained a small quantity of fragments with lower molecular weight, possibly L- and H-chains of the antitoxin molecule. Besides the main components with a sedimentation constant of 3S the digested, unfractionated serum also contained about 10% of molecules with sedimentation constants of 6S or more.

The investigation thus showed that antitoxin fragments with specific antitoxic activity, having a sedimentation constant of about 3S and a molecular weight of 45,000, can be obtained from "Diaferm-3 IÉM" antitetanus serum.

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