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IMMUNE RESPONSE TO SYNTHETIC POLYSACCHARIDE–PROTEIN CONJUGATE

T. A. Makarenko, N. A. Kocharova, Yu. E. Tsvetkov, L. S. Edvabnaya, Yu. A. Knirel', L. V. Bakinovskii, E. V. Kholodkova, E. S. Stanislavsbii, and N. K. Kochetkov UDC 012.017.1.014.46:[615.37:547.458+547.96

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The first attempt to create an artificial antigen by modifying natural carrier proteins by simple chemical compounds (haptens) was undertaken by Landsteiner in the 1930s [3]. Artificial antigens of carbohydrate nature were obtained by conjugation of natural antigenic polysaccharides, their synthetic fragments, or fragments obtained by their partial depolymerization, with proteins, and it was found that they are immunogenic, and that many of them possess protective properties [2, 5-7]. In the investigation described below a conjugate of the synthetic polysaccharide α-1,6-mannan with protein was obtained for the first time and the immune response of rabbits to this artificial antigen studied.

EXPERIMENTAL METHOD

A synthetic polysaccharide (SPS) containing an aglycone spacer with a free amino group, was α-1,6-mannan with $C_n \sim 10$, the reducing end of which was fixed in the form of 6-aminohexyl-glucopyranoside. The SPS was synthesized as described previously [1]. It was converted into the isothiocyanate derivative by treatment with thiophosgene and introduced into the reaction with amino groups of lysine residues of bovine serum albumin (BSA) [7]. According to the results of analysis the conjugate thus obtained contained 23% of carbohydrates and 71% of protein, corresponding to the addition of 11 moles of SPS to one mole of BSA. The BSA was obtained from "Serva" the thiophosgene from "Aldrich" and the SPS as described in [2]. The protein concentration was determined as in [4]. To determine the content of carbohydrates the conjugate was hydrolyzed (2 M CF_3COOH, 1 h, 120°C) and analyzed with the aid of a carbohydrate analyzer. Gel chromatography was carried out on a column (1.5 × 82 cm) with TSK HW-50S ("Merck," West Germany). Solution was carried out with 0.1 M phosphate buffer containing 0.15 M NaCl (pH 9.0). The elution curves were obtained with the aid of a UV-detector (280 nm) and a "Technicon" carbohydrate analyzer.

Chinchilla rabbits weighing 2-2.5 kg were immunized with SPS-BSA antigen (anti-SPS-BSA): first injection — 1 mg of antigen subcutaneously with Freund's complete adjuvant, followed one month later by the second injection — 1 mg of antigen intravenously. Bleeding was carried out on the 7th day after the second immunization, and the serum prepared from the blood was kept at −30°C or lyophilized. Antibodies to SPS were determined by the indirect method of enzyme immunoassay (EIA), at the end point of the reaction. Optimal doses of antigen were established by titration of the anti-SPS-BSA using different doses of antigen. The solid-phase carrier for immobilization of the antigen consisted of polystyrene plates ("Linbo," USA). All the operations were performed at room temperature. The antigen was dissolved in carbonate–bicarbonate buffer (pH 9.0) in a dose

N. I. Mechnikov Research Institute of Vaccines and Sera, Academy of Medical Sciences of the USSR. N. D. Zalinskii Institute of Organic Chemistry, Academy of Sciences of the USSR, Moscow. Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 110, No. 9, pp. 293-294, September, 1990. Original article submitted December 29. 1989.

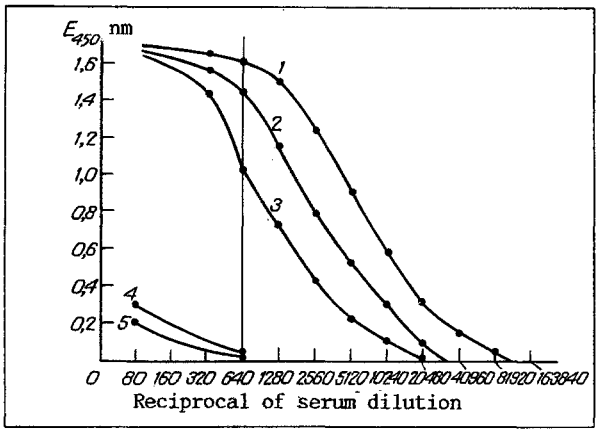

Fig. 1. Results of study of artificial antigen (S-PS-BSA) by enzyme immunoassay. Abscissa: reciprocal of serum dilution. 1) anti-S-PS-BSA; 2) anti-3-PS-B5A + inhibition by mannan; 3) anti-5-PS-BSA + inhibition by BSA; 4) normal rabbit serum; 5) BSA + inhibition by S-PS-BSA.

of 10 μg/ml and applied to plates which were incubated for 18 h. The antisera were diluted with 0.15 M phosphate buffer (pH 7.2) and incubated for 2.5 h. The plates were washed to remove excess of the reagents with the same buffer. Antibodies against rabbit immunoglobulins, labeled with peroxidase (produced by the N. F. Gamaleya Research Institute of Immunology, Epidemiology, and Microbiology) in a dilution of 1:1000, were incubated for 18 h. The substrate-indicator mixture, containing 0.4% of orthophenylenediamine ("Sigma") in 0.5 M citrate-phosphate buffer (pH 6.0) and 0.042% H_2O_2, was incubated for 30 min. The reaction was stopped by the addition of 3 N H_2SO_4. The result was read at a wavelength of 450 nm on the "Titertek Multiscan" eximeter.

Inhibition of EIA was carried out by antigens in a dose of 100 μg/ml, for which purpose the antigen was mixed with successive dilutions of antiserum in different volumes and incubated at constant temperature of 37°C for 2 h. Successive dilutions of antiserum were introduced into wells in the plate with adsorbed antigens, and serum mixed with antigens in the same dilutions, into wells of the parallel row. EIA was then carried out in the same way as by the direct reaction.

EXPERIMENTAL RESULTS

The results of the study of anti-SPS-BSA-serum by EIA are given in Fig. 1. The antiserum was found to react with the conjugate (homologous antigen) in dilutions of up to 1:81920 (Fig. 1:1). The reaction was inhibited by homologous antigen to a dilution of 1:640 (Fig. 1:5), and in this case the optical density even of the minimal dilution of the serum (1:80) was under 0.2. Adsorption of specific antibodies thus takes place almost to the level of "natural" antibodies in normal serum (Fig. 1:4). SPS appreciably inhibited the reaction (Fig. 1:2): the optical density of each dilution of serum to an artificial antigen was 1.5-2 times lower in the inhibition test than in the direct test, evidence that the antiserum studied contained specific antibodies to S-PS. Meanwhile, antibodies to BSA must also be present in the antiserum. For instance, the protein specifically inhibits EIA (Fig. 1:3). Comparison of the results of inhibition of EIA with the aid of S-PS-BSA (Fig. 1:5) and with BSA or S-PS alone (Figs. 1:2 and 1:3) clearly shows that the conjugate possesses much stronger inhibitory properties than BSA or S-PS separately. This is evidently due to the presence of antibodies specific for regions close to binding of polysaccharides to protein and/or to "conformation determinants" appearing as a result of conjugation, which are not present in the original components, in the anti-S-PS-BSA serum.

Thus synthetic α-1-6-mannan, conjugated with BSA, stimulates specific antibody formation in rabbits. The synthetic polysaccharide α-1-6-mannan was conjugated with a natural protein (BSA). The resulting conjugate induces the formation in rabbits of specific antipolysaccharide antibodies, detected by EIA.

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IMMUNOCHEMICAL STUDY OF WATER-SOLUBLE CORNEAL ANTIGENS

T. V. Belikova, S. M. Ziborov, and V. E. Formazyuk

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Corneal antigens are found in considerable isolation from the immunogenesis system and they exhibit organ-specificity. Injury to the cornea as a result of exposure to infectious agents, trauma, or surgery creates conditions for the resorption of the corneal proteins and promotes the development of autosensitization [4, 6]. There is no doubt that the development of effective methods of prevention and treatment of cases of keratitis with an autoimmune component, and also of severe postoperative complications such as uveitis, epithelial-endothelial corneal dystrophy, or rejection of a graft depends on our knowledge of the localization, specificity, and physicochemical properties of the corneal antigens.

Nine antigens were found in a corneal extract by the immunodiffusion method [3]. However, the immunochemical properties of these proteins remained virtually unstudied.

The aim of this investigation was a detailed study of water-soluble antigens of the bovine cornea.

EXPERIMENTAL METHOD

Tissues of the eyes and other organs of persons dying from trauma and from the corresponding bovine organs were used. The tissues were minced mechanically, ground with quartz sand, treated with 3-4 volumes of Tris-glycine buffer, pH 8.3, and additionally homogenized. The resulting homogenate was frozen 3 times to −10°C and thawed at room temperature, after which it was centrifuged at 8000 rpm for 30 min. The supernatant was used to isolate and study the antigens. A specific antiserum was obtained by immunizing rabbits with tissue extract from bovine cornea with the addition of Freund's complete adjuvant by the method of intradermal injections at 10 to 12 sites on the trunk, at the rate of 1 ml of extract per kilogram body weight, 4 times with intervals of 9 days between the injections. The protein concentration in the extracts, measured by Lowry's method, was 20-30 mg/ml. Reimmunization was carried out 1 month after the last immunization. Blood was taken form the marginal vein of the animals' ear on the 7th, 9th, and 11th days after reimmunization. Serum obtained from the blood was exhausted with dried human blood plasma. Precipitating antibodies, corresponding to the corneal antigens, were detected in the immune sera in a microversion of the immunodiffusion method in a layer of gel [10]: immunodiffusion was carried out in 1.5% agar gel for 24 h, the volume of the sample being 10 μl. The organ-specificity of the corneal antigens thus found was tested by the same method

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