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IMMUNOCHEMICAL STUDY OF ANTIGENIC DETERMINANTS OF RECOMBINANT HBsAg PRODUCED BY *Bombyx mori* LARVAE

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Antibodies to various protein fractions isolated from larvae of mulberry silkworm containing recombinant HBsAg, to plasma HBV, and to healthy human serum were obtained. The cross reactivity of the antibodies was studied using enzyme-linked immunosorbent assay (ELISA). The antigenic determinants of recombinant HBsAg are identical to those in plasma and have practically no common antigenic determinants with healthy human blood serum.

Key words: *Bombyx mori*, recombinant, HBsAg.

The widespread infection caused by hepatitis B virus (HBV) and the variety and severity of clinical forms of this disease and its consequences thrust hepatitis B (HB) into the list of most important medical problems. Numerous so-called symptomless carriers of HBsAg are a principal reservoir of the infection [1, 2].

Several methods for detecting HBsAg have been developed. The most common in developed countries is solid-phase immunoanalysis as radioimmune and immuno-enzyme constructs [3]. The anti-HBs used in diagnostic constructs are primarily blood serum of animals that have been subjected to multiple immunization by purified HBsAg. Monoclonal antibodies to HBsAg are used too. The source of purified HBsAg can be plasma of a carrier of this antigen or recombinant HBsAg obtained using various expression systems [4]. Considering the complexity of isolating and purifying HBsAg from human blood plasma [5-6], the use of recombinant HBsAg is more promising.

Our goal was to prepare a monospecific antiserum to recombinant HBsAg, which is produced by mulberry silkworm (*Bombyx mori*) larvae, and to analyze comparatively antigenic determinants of recombinant HBsAg with plasma HBV.

A method for preparing genetically engineered HBsAg from *B. mori* larvae was developed at the Institute of Genetics and Experimental Plant Biology of the Academy of Sciences of the Republic of Uzbekistan in the laboratory of molecular genetics [7]. A homogenate of *B. mori* larvae (I) was prepared previously by a method developed there [7] from larvae of silkworms, which produce a protein with antigenic determinants to HBV. Precipitation by alcohol (20%) and salt (30%, ammonium sulfate) isolated fractions II and III, respectively, which were enriched in HBsAg. The resulting fractions were used as immunogen for preparing antiserum and antigen for studying the antigenic determinants.

Protein preparations I, II, and III in addition to human plasma HBV were used to immunize BALB/c mice. One week after the last immunization murine blood serum was collected. IgG was isolated from it by double precipitation by ammonium sulfate with subsequent desalting on a Sephadex G-25 column. It was further purified on a DEAE-cellulose column.

Enzyme-linked immunosorbent assay (ELISA) was used to study the comparative immunogenicity of recombinant HBsAg with human plasma HBV. Thus, flexible polystyrene planchets were sensitized with human plasma HBV, which was used as antigen in 100 µl at 20 µg/ml. Then, the resulting antibodies to protein preparations I, II, and III and human plasma HBV were placed. The resulting antigen—antibody (AG—AB) complexes were identified using antimurine IgG conjugate from rabbit at working dilution 1:1000.

The results of the immunogenicity of protein preparation containing recombinant HBsAg and blood serum infected with HBV containing plasma HBV are presented below:

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Specimen	SP index, D = 492 nm
Negative control	0.014
Positive control	0.625
Antiserum to blood serum infected with HBV	0.3
Antiserum to fractions I, II, and III	0.28

The results show that protein preparations containing recombinant HBsAg and human plasma HBV give identical immune responses.

The presence of common antigenic determinants of protein preparations I, II, and III with healthy human blood serum was determined by the following experiment. BALB/c mice were immunized with healthy human blood (HHB) serum. The reactivity of the resulting antiserum to HHB (AB) with preparations I, II, and III and human plasma HBV (AG) was studied by cross immunochemical analysis. It was found that fraction II, which contains recombinant HBsAg, has practically no common antigenic determinants with HHB serum (<5%) whereas human plasma HBV contains ~20% of common antigenic determinants.

We prepared the conjugate of IgG with horseradish peroxidase (Sigma) according to the Nakane and Kawaio method in order to identify the AG—AB complexes that were formed. According to the literature, performing the reaction under optimal conditions preserves 68% of the enzymatic and 99% of the immunological activity [8]. The results of incorporating the peroxidase tracer into the immunoglobulins using this method indicate that the actual yield of immunoreactive conjugates is substantially lower and less than 30-40% [9]. Taking these data into account, we preparaed conjugates of antibodies to horseradish peroxidase according to the Nakane and Kawaio method with a slight modification. Thus, the excess of sodium periodate was removed by desalting on a Sephadex G-25 column. The Schiff bases were reduced by treatment of the conjugates with sodium borohydride and then fractionated on a Sephadex G-25 column. The active fraction was collected. The working concentration (sensitivity) was determined by titration. For this, human plasma HBV was adsorbed in wells of polystyrene trays, incubated first with conjugate, washed, and incubated with substrate. The conjugate titer was determined using successive dilutions to the final point of reaction. The conjugates obtained by us exhibited a sensitivity upon 1:10,000 dilution.

Thus, we isolated a protein fraction of *B. mori* larva homogenate containing recombinant HBsAg that has high immunogenicity and the lowest cross reactivity with HHB serum and that can be used to formulate a diagnostic agent for HBsAg.

EXPERIMENTAL

Homogenate of *B. mori* larvae (I) and fractions II and III were prepared from larvae of mulberry silkworm, which produces a protein with antigenic determinants to HBV, as described previously [7]. Human plasma HBV and HHB were supplied by the Institute of Epidemiology (Tashkent).

All fractions were checked for the presence of antigenic determinants of HBsAg using a diagnostic test consisting of systems for HBsAg based on solid-phase ELISA (ORTHO).

Mice (BALB/c) were immunized by protein fractions I, II, and III isolated from *B. mori*, human plasma HBV, and HHB serum. Protein samples (0.5 ml, 200 µg/ml) were mixed with an equal volume of whole Freund's adjuvant to produce a homogeneous mass and were injected ip into mice. The procedure was performed four times at one-week intervals. Seven days after the last immunization blood was collected, incubated for 1 h at 37°C, and centrifuged. Serum was collected for subsequent study.

Antibodies were isolated from murine blood serum by precipitation twice with $(NH_4)_2SO_4$. An equal volume of saturated ammonium sulfate was added to the serum. The mixture was stirred for 1 h at room temperature and centrifuged at 10,000 rpm for 10 min. The resulting precipitate was dissolved in the minimal volume of phosphate-salt buffer at pH 6.8 (PSB). Saturated ammonium sulfate (2/3 of the volume) was added to the solution. The mixture was stirred and centrifuged. The solid was dissolved in sodium phosphate buffer (5 mM) at pH 8.0 and placed on a Sephadex G-25 column equilibrated with this same buffer. Protein fractions were collected from the column using a Uvicord instrument operating at 280 nm (LKB, Sweden) and placed on a DEAE-cellulose column equilibrated with this same buffer. Antibodies were collected in a gradient from 0 to 0.25 M NaCl, dialyzed against PSB at pH 6.8, and used in further work.

Binding of antigen to antibody (AG—AB) was determined using enzyme-linked immunosorbent assay (ELISA).

A 96-well planchet (Nunclon, Denmark) was charged with PSB (100 µl) at pH 6.8 containing 20-30 µg/ml protein and held for 12 h at 4°C. Unbound antigen was removed. Protein fractions I-III isolated from *B. mori*, plasma HBV, and HHB were used as antigens. The planchets were treated for 1 h with PSB containing 0.1% Tween-20 (Sigma, USA) or 0.5% casein and then washed of PSB. The washed planchets were treated with antibody (50 µl), incubated for 1 h at 37°C, and washed with PSB containing 0.1% Tween-20. An AG—AB reaction was revealed using murine anti-IgG conjugate with horseradish peroxidase. Binding was determined by a color reaction in the presence of *o*-phenylenediamine in 0.1 M sodium citrate at pH 4.5. The reaction was stopped by adding H_2SO_4 (50 µl, 10%) to the wells.

Averages of the resulting data were obtained by determining optical densities spectrophotometrically at 492 nm. The positive control was serum infected with HB. The negative control was homogenate of *B. mori* larvae that did not contain recombinant HBsAg and PSB, the optical densities of which were considered as background. The ELISA results were considered positive if they were more than twice the negative control.

Preparation of Murine Anti-IgG. Antibodies from murine blood serum were obtained and purified by the method described above. A rabbit was immunized with the resulting antibodies. Protein (6 mg) was emulsified with whole Freund adjuvant and injected sc at several points. The injection was repeated after 10-14 days using incomplete Freund's adjuvant. The antibody titer was determined after two immunization cycles. If it was greater than 1:2000, ~20 ml of rabbit blood containing murine IgG was collected. The antibodies were further purified as described above. The murine anti-IgG from rabbit obtained in this manner was used for preparing conjugate.

The titer of the antibodies was determined using successive dilutions to the final point of reaction. Antigen in this instance was murine blood serum.

Preparation of Horseradish Peroxidase—IgG Conjugate. Murine anti-IgG that was isolated as described above was dialyzed against NaCl (10 mM). Carbonate buffer at pH 9.5 was added until the final concentration was 0.2 M. Horseradish peroxidase (Sigma, USA, 10 mg/ml) was dissolved in doubly distilled water and treated dropwise with sodium periodate until the final concentration was 0.02 M. The mixture was incubated for 20 min at room temperature in the dark with constant shaking. Unbound sodium peroidate was removed on a Sephadex G-25 column. The collected peroxidase solution was poured into carbonate buffer at pH 9.5, treated with anti-IgG in a 1:2 ratio, and incubated for 2 h. The reaction was stopped by sodium borohydride at 40 µl per 1 ml of conjugate (concentration = 4 mg/ml). The mixture was incubated for 30 min. The resulting conjugate was dialyzed against PSB. Glycerine was added until the final concentration was 50%. The mixture was stored at -20°C.

The conjugates prepared by us exhibited sensitivity upon 1:10,000 dilution.

The protein content was determined by the Lowry method [10].

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