Production of Different Antibodies after Simultaneous Immunization of Animals with Two Antigens

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 143, No. 6, pp. 718-720, June, 2007
Original article submitted March 26, 2007

We propose a method of simultaneous immunization with two different antigens for isolation of two types of antibodies from the same antiserum. Bacterial proteins (*Bacillus stearothermophilus* glyceraldehyde-3-phosphate dehydrogenase and *Escherichia coli* GroEL chaperonin) served as the antigens. Affinity purification of antibodies was carried out using two columns: with covalently immobilized glyceraldehyde-3-phosphate dehydrogenase or GroEL chaperonin. During stage I, the antiserum was applied onto the column with immobilized glyceraldehyde-3-phosphate dehydrogenase, after which antibodies to glyceraldehyde-3-phosphate dehydrogenase were eluted. During the next stage, the antiserum without antibodies to glyceraldehyde-3-phosphate dehydrogenase was passed through the column with immobilized GroEL and antibodies to chaperonin were isolated. Antibodies to glyceraldehyde-3-phosphate dehydrogenase and to GroEL had high titers and exhibited no cross-reaction.

Key Words: *antibodies; immunization; antigen; immunoadsorbents; chaperonin*

In order to prepare antiserum (AS) containing antibodies (AB) only to the needed antigen, the animals are usually immunized with this antigen [3]. This approach appreciably facilitates further work with AS, which is often used without isolation of specific AB from it (for example, for detection of protein in immunoblotting). However, only highly purified proteins should be used for this immunization, in order to rule out the appearance of AB to admixture proteins in AS. Because of these limitations, the scientists now prefer working with AB purified on immobilized antigens [2]. The existence of effective methods for purification of the needed AB from the serum suggests the possibility of immunization of animals with 2 and more antigens simultaneously. However, the so-called "antigen competition" is possible in this case. After injection of a mixture of 2 antigens to animals, AB to both antigens are detected in the serum, but their levels are different: the higher is the level of AB to one antigen, the lower is the level of AB to the other antigen. Nevertheless, the phenomenon of antigen competition and other side effects of simultaneous immunization with antigens do not preclude attempts at stimulating the immune response by injection of various adjuvants.

The aim of this study was to develop a method for simultaneous immunization of rabbits with two different proteins with subsequent purification of AB on immunoaffinity columns containing immobilized antigens.

MATERIALS AND METHODS

In order to obtain polyclonal AB to glyceraldehyde-3-phosphate dehydrogenase (GAPD) and GroEL chaperonin, 3 Chinchilla rabbits aged 2-3 months were immunized with a mixture of native GAPD and GroEL with Freund's adjuvant by the standard method [5]. The first immunization was carried out

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0007-4888/07/1436-0771

subcutaneously with a mixture of these proteins (0.5 mg/ml each) with complete Freund's adjuvant in 1:1 volume ratio. Since injection of bacterial proteins induces potent immune response, repeated immunizations were carried out intravenously with a mixture of proteins, but without adjuvant.

The IgG fraction from AS was isolated by ammonium sulfate precipitation. Two AB types (to GAPD and GroEL) were isolated from the same AS using a previously suggested affinity chromatography procedure [1]. In order to obtain immunoadsorbents, the antigens were isolated and immobilized on bromocyan-activated 4B sepharose.

Bacillus stearothermophilus GAPD was isolated from *E. coli* (strain GM-109) cells transformed with pBluescript II plasmid containing *B. stearothermophilus gap* gene under lac promotor [6]. GroEL chaperonin was isolated from *E. coli* (strain W3110) cells transformed with pOF39 plasmid containing *GroEL* gene. Chaperonin was purified as described previously [4]. Immunoadsorbents were prepared by immobilization of GAPD and GroEL on BrCN-activated sepharose [1] (sepharose was activated with 50 mg BrCN/ml packed gel). After immobilization the preparations were treated with 25% glutaraldehyde solution for the formation of cross-links between protein subunits. Antibodies were eluted with 50 mM glycine-HCl buffer (pH 2.3) containing 0.15 M NaCl.

Protein concentrations were measured by the method of Bradford. Immunodiffusion on agar-coated glass plates was carried out by the method of Ouchterlony. Electrophoresis in 12% PAAG in the presence of sodium dodecyl sulfate was carried out after Laemmli. Serum titers of AB to GAPD and GroEL were evaluated by classical indirect ELISA.

Immunoblotting after protein separation by PAAG electrophoresis with sodium dodecyl sulfate was carried out as follows. Electrophoretic separation of proteins was followed by electrotransfer onto the nitrocellulose membrane in a Mini-Trans-Blot device (Bio-Rad). After washout and incubation of the membranes in blocking buffer the material was incubated with the first AB (to GAPD or chaperonin) and then with second AB. Horseradish immunoperoxidase-conjugated goat AB to rabbit immunoglobulins served as second AB (1:10,000 dilution; Pierce). The presence of proteins was verified using diaminobenzidine.

RESULTS

Antibodies to both antigens were present in AS obtained from 3 rabbits immunized with two different antigens simultaneously. This was demonstrated by the method of immunodiffusion in agar after Ouchterloney. The results of immunoblotting carried out after electrophoresis of GAPD and chaperonin preparations in the presence of sodium dodecylsulfate also indicate the presence of AB to both antigens (Figs. 1, 2). Antibody titer in this experiment was determined as the last dilution at which AS reacted with the antigen on the blot. The data indicate that the titer of AB to GAPD was 1:600 (Fig. 1), to GroEL 1:1200 (Fig. 2).

These data were confirmed by evaluation of AB titer by ELISA: AB titers were 1:1,000,000 for GAPD and 1:300,000 for GroEL. These high AB titers indicate high immunogenic activities of these proteins, despite possible competition between the antigens, and make their use for simultaneous immunization of rabbits possible and convenient.

Antibodies to different antigens were isolated from polyclonal serum by affinity chromatography on immobilized antigens (GAPD and GroEL chaperonin). Protein preparations immobilized on bromocyan-activated sepharose and additionally treated with cross-linking reagent (glutaraldehyde) were used for isolation of AB. Glutaraldehyde treatment fixed the protein in the oligomer state and pre-

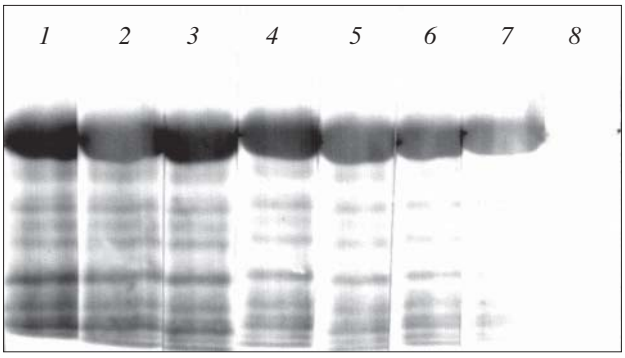

Fig. 1. GAPD immunoblotting. Nitrocellulose membranes, onto which GAPD was transferred after PAAG electrophoresis with sodium dodecylsulfate, were treated with immunoglobulins in the following dilutions: track *1*) 1:10; *2*) 1:100; *3*) 1:200; *4*) 1:300; *5*) 1:400; *6*) 1:500; *7*) 1:600; *8*) 1:800. Staining of proteolytic fragments of lower molecular weight is seen under the main GAPD track.

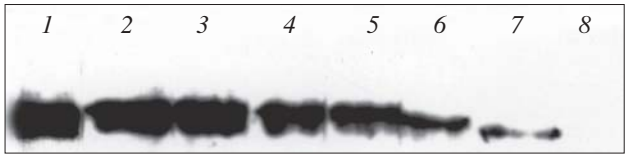

Fig. 2. GroEL chaperonin immunoblotting. Nitrocellulose membranes, onto which GroEL chaperonin was transferred after PAAG electrophoresis with sodium dodecylsulfate, were treated with immunoglobulins in the following dilutions: track *1*) 1:100; *2*) 1:200; *3*) 1:400; *4*) 1:600; *5*) 1:800; *6*) 1:1000; *7*) 1:1200; *8*) 1:2000.

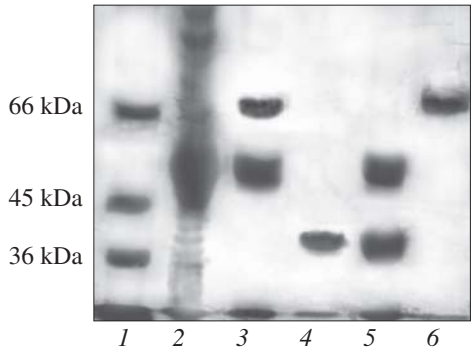

Fig. 3. Electrophoretic separation of proteins adsorbed on sepharose-bound antigens, in PAAG in the presence of sodium dodecylsulfate. *1*) marker proteins; *2*) IgG fraction from AS; *3*) immobilized GroEL (66 kDa) after incubation with AB to GroEL (band with mol. weight 55 kDa: AB heavy chains; light chains of AB molecules not shown); *4*) immobilized GAPD (36 kDa) after incubation with anti-GroEL; *5*) immobilized GAPD (36 kDa) after incubation with AB to GAPD (band with mol. weight 55 kDa — AB molecule heavy chains); *6*) immobilized GroEL after incubation with anti-GAPD.

vented its dissociation into subunits during AB elution with low-pH buffer.

In order to isolate AB to two proteins, the IgG fraction was incubated for 1 h with immobilized GAPD and free AB were eluted for their further utilization for isolation of AB to chaperonin. The column with immobilized GAPD was washed with buffer (pH 2.3) for elution of anti-GAPD. Proteins left free in column 1 were applied onto the column with immobilized GroEL, after which the column was washed and AB to chaperonin were eluted with buffer (pH 2.3).

Cross reaction of the resultant AB with the target antigens was analyzed by the fixed partner method. Samples of covalently immobilized GAPD and GroEL were incubated with equimolar concentrations of isolated AB, washed, and analyzed by PAAG electrophoresis with sodium dodecyl sulfate. After incubation of immobilized GroEL with isolated antibodies to chaperonin, the protein bands corresponding to GroEL monomer (66 kDa) and AB heavy chains (50 kDa; Fig. 3, track *3*) were detected in the sample. After incubation of immobilized GAPD with AB to GroEL, only GAPD monomers were detected (36 kDa), this indicating the absence of reaction between anti-GroEL and this antigen (Fig. 3, track *4*). On the other hand, AB to GAPD effectively bound immobilized enzyme (Fig. 3, track *5*: 2 bands with mol. weights 36 and 50 kDa). Immobilized GroEL did not react with AB to GAPD (Fig. 3, *6*). Hence, two types of isolated AB did not cross-react.

The study demonstrated the possibility of obtaining AS containing high titers of AB to two different antigens by simultaneous immunization of rabbits with these different antigens. Antibodies isolated from AS by successive affinity chromatography on immobilized antigens are characterized by high affinity to the antigen and do not cross-react.

Our results indicate the possibility of injecting new antigens to previously immunized animals. The possibility of immunization with several antigens and repeated use of animals makes this approach convenient for small laboratories, when it is necessary to obtain low amounts of polyclonal AB to different antigens.

The study was supported by grants from the Russian Foundation for Basic Research (No. 05-04-48955 and 06-04-48240-a).

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