IMMUNOSORBENTS FOR THE PREPARATION OF *Mycobacterium bovis* BCG ANTIGEN

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The preparation of immunosorbents for the isolation of *Mycobacterium bovis* BCG antigen is described. Polyclonal rabbit antibodies are attached both by oriented immobilization following oxidation with periodate to a cellulose and agarose support with hydrazide groups, and directly to agarose activated with cyanogen bromide. The properties of sorbents in relation to the method of preparation and to the support used are compared. The best properties were exhibited by immunosorbent attached to cellulose beads.

Key words: Immunosorbent; *Mycobacterium bovis* BCG antigen; Oriented immobilization.

The genus *Mycobacterium* includes causative agents of human and animal tuberculosis highly detrimental to the organism[1]. Mycobacterial antigens with a role in serum diagnostics (tuberculin) and in the study of immunity response to mycobacterial infections have been in the center of practical interest world-wide. It is expected that, in addition to obtaining a more specific tuberculin, the purified antigens could contribute to a better understanding of the pathogenesis of tuberculosis and to examining immunity toward mycobacteria, in connection with the stimulation of T lymphocytes and macrophages, as well as to the development of new vaccines, etc. The conditions of purification are still a topical problem, in spite of the first experiments dating back nearly a hundred years. Classical physico-chemical methods of separation (precipitation, gel filtration, ion-exchange chromatography and preparative electrophoresis) have been used for the isolation of antigens with molar masses in the range of 12–85 kDa

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from culture filtrates or *Mycobacterium bovis*[2–4] and *M. tuberculosis*[5,6] cell extracts. At present isolations are carried out even more frequently with the aid of bioaffinity chromatography using immobilized antibodies interacting with antigens[7,8]. These methods provide a high specific yield in a single step, the key prerequisite being an immunosorbent with an antibody (optimally monospecific or monoclonal, but most frequently polyclonal) attached to a suitable support.

To prepare the antigen with enhanced specific activity we used immunoaffinity chromatography on a sorbent with immobilized polyclonal antibodies of rabbit hyperimmune serum against an antigen from the cell mass of *M. bovis* BCG. The immunosorbent prepared by the most frequently employed attachment to CNBr-activated agarose was compared with immunosorbents prepared by oriented immobilization of the antibody through its selectively oxidized sugar moieties to supports based on agarose (Affi-Gel HZ) or bead cellulose containing hydrazide groups.

EXPERIMENTAL

Preparation of bacterial mass. Mycobacterium bovis BCG (strain 198, Moscow) and *M. kansasii* (strain ATCC 12478) were grown in a surface culture on Sauton's synthetic medium at 37 °C for 6–8 weeks. Then the bacteria were separated from the medium by centrifugation and three-fold washing with 10 mM Tris-HCl (pH 7.8) and kept in separate small samples at –20 °C.

Preparation of complex sonicated antigen of M. bovis BCG. Suspension of wet bacterial mass in 2 M Tris-HCl at pH 7.8 was disintegrated by sonication in ice (20 min, 20 kHz; MSE Scientific Instruments, Sussex, Great Britain). The disintegrated mass was centrifuged for 45 min at 20 000 g and 4 °C. The supernatant was filtered through the sequence of Millipore filters with membrane pores of 1.2, 0.6, 0.45 and 0.22 μm in diameter. The clear filtrate was dialyzed against distilled water. The dialyzate was again filtered through Millipore filters with 0.45 and 0.22 μm pores and then freeze-dried. The protein content was determined by Kjeldahl's method as described by Chibnall *et al.*[9].

Preparation of M. kansasii cell mass for adsorption of rabbit polyclonal serum. The material was prepared from *M. kansasii* by diluting the wet mass with physiological saline at pH 7.4 (phosphate-buffered saline, PBS, containing 10 mM phosphate and 0.145 M NaCl) and was sonicated under the same conditions as above. This disintegrated bacterial mass, including the supernatant, was used for serum adsorption.

Preparation of polyclonal hyperimmune rabbit serum. Rabbits were immunized four times in sequence at weekly intervals, using the *M. bovis* BCG antigen applied in four 200 μl subcutaneous injections in the trunk and one in the foot pad. The first immunization dose was prepared by diluting 2 mg antigen in 1 ml physiological saline, the following immunization doses were prepared in incomplete Freund's adjuvant. Two months after the last immunization a subcutaneous booster dose was applied (2 mg antigen dissolved in 1 ml physiological saline). A week later the animals were bled. Sera containing (on the basis of a qualitative immunodiffusion test) antibodies against *M. kansasii* were combined and kept in aliquots at –20 °C.

Isolation of the immunoglobulin fraction. Serum (10 ml) was diluted with PBS four times whereupon the same volume of precooled (to 4 °C) saturated solution of ammonium sulfate was added dropwise and the mixture was left to stand for 1 h at 4 °C. The precipitate was centrifuged at 12 000 g for 30 min at 5 °C (Prepsin, MSE Scientific Instruments). The sediment was washed twice with cold 50% ammonium sulfate and recentrifuged. The precipitate was then dissolved in a minimum amount

of water and dialyzed against a 1 : 3 dilution of PBS. Any lipid precipitate was removed by centrifugation. The immunoglobulin was stored at –20 °C.

Serum saturation. Isolated immunoglobulins were mixed with the antigen mass of *M. kansasii* (3 : 1), stabilized with NaN_3 (0.01%) and stirred on a rotary stirrer for 24 h at room temperature. The antigen mass was centrifuged and the procedure repeated twice. After the last centrifugation the opacity was removed by filtration through a Millipore filter with 0.2 μm pores.

Immobilization of antibodies on CNBr-Sepharose 4B. Immunoglobulins were transferred by dialysis into 0.1 M carbonate buffer (pH 9.0) with 0.5 M NaCl. The solution (containing 5.6 mg protein per ml) was then combined with the same volume of solid CNBr-Sepharose 4B (Pharmacia-LKB) pre-equilibrated in the same buffer. The binding was left to proceed for 16 h at 4 °C. After immobilization the sorbent was washed alternately in five cycles with 0.1 M borate buffer (pH 9) and 0.1 M acetate buffer (pH 5.0). The last washing was done with borate buffer.

Oriented immobilization of antibodies through the oxidized sugar moiety. To immobilize the antibodies using hydrazide derivatives of agarose and cellulose, use of the aldehyde groups originating by the selective oxidation of the sugar moieties with periodate[10] was made. The isolated immunoglobulins were first dialyzed against a 0.1 M acetate buffer (pH 5.5). The antibody solution (2 mg/ml) was combined with 10% volume of 0.1 M $NaIO_4$. Oxidation took place at 4 °C for 20 min on a rotary stirrer in the dark. The excessive periodate was then removed by a 10-min reaction with ethylene glycol added to the reaction mixture to a concentration 20 mmol/l. The low-molecular-weight components were then removed on a column of Sephadex G-25 fine (1.6 × 30 cm), equilibrated with 0.1 M acetate buffer (pH 4), containing 0.5 M NaCl, or by dialysis overnight at 4 °C using the same buffer. The oxidized antibodies (12–25 ml) diluted with 0.1 M acetate buffer containing 0.5 M NaCl (pH 4.8) to a concentration of 2 mg *per* ml were combined with the same volume of a hydrazide support. The agarose support was Affi-Gel HZ (Bio-Rad), the cellulose support was Perloza MT 500 (Lovochemie, Czech Republic) with attached adipic dihydrazide. The binding proceeded on a rotary shaker for 24 h at 4 °C. The sorbents were then washed with the acetate buffer until absorbance dropped to zero.

Preparation of hydrazide bead cellulose HZ. The hydrazide cellulose was prepared by a modification of the procedure of Benes *et al.*[11].

Activation of cellulose: 5 g of Perloza MT 500 filtered from an aqueous suspension was transferred to aqueous acetone by treating it with acetone. The mixture was stirred for 30 min with 10 ml acetone, then a 5-ml aliquot of the liquid was removed, mixed with 5 ml acetone and again a 5-ml aliquot was withdrawn. Then 66.2 μl 10% NaOH was added, the mixture was stirred for 1 h at 5 °C and finally 35 mg 2,4,6-trichloro-1,3,5-triazine was added. Stirring was continued for 45 min and the product was washed in a column with 30 ml acetone and 30 ml water (both cooled to 0 °C).

Reaction with adipic dihydrazide: Activated cellulose in 4.2 ml 0.05 M borate buffer (pH 8.5) was mixed with 58.3 mg adipic dihydrazide (Sigma, pure) at room temperature for 3.5 h. The pH was maintained throughout with NaOH at 8.8–9.0. The product was washed with water and stored at 4 °C. The content of hydrazide groups was 10.1 mmol *per* ml (determined with the aid of trinitrobenzenesulfonic acid by a method modified for primary amino groups[12]). This corresponds to 83% of the value calculated from the Cl^- content formed in the reaction of the activated cellulose with hydrazide in the eluate after the reaction.

Determination of the immobilized protein content was done by a direct method based on amino acid analysis[13,14] (amino acid analyzer Durrum D-500, Palo Alto, CA, U.S.A.) in an aliquot sample of the solution obtained by hydrolysis of the support (6 M HCl, 20 h, 110 °C) and correction was made for a blank. Indirect determination was based on a spectrophotometric determination at 280 nm (using a Pye–Unicam PU 8610 or a Beckman 1200 spectrophotometer) from the difference between the protein amount introduced into the immobilization reaction and that found in the eluate after the reaction.

Immunoaffinity chromatography was done in columns of 1 cm diameter and 5–27 cm height at room temperature using UV detection at 280 nm (Uvicord 4701A, LKB). The column was equilibrated with physiological saline at pH 7.4. The antigen adsorption (1–20 mg dissolved in 1–27 ml equilibration buffer) was done at a flow rate of 6–24 ml/h while washing and elution were done at a rate of 24 ml/h. The elution agents are summarized in Table II. The individual fractions (2 ml) were collected in a fraction collector Ultrorac 7000 (LKB) into test tubes with a neutralizing solution. This solution was 1 M $NaHCO_3$ for acid eluates and 0.1 M boric acid for alkaline eluates. The eluted fractions with an absorbance of less than 0.01 were combined, dialyzed for 36 h at 4 °C against distilled water and freeze-dried (if a gravimetric determination of the percentage of eluted antigen was intended) or simply concentrated on a membrane filter with molar mass threshold of 10 kDa. Rechromatography was done in the same column by the same procedure, except that an antigen concentrated by freeze-drying from the first or second chromatography. The column was regenerated either after each experiment or after 3–5 experiments, using 0.2 M boric acid and 50 mM sodium tetraborate, 60% ethanol in 0.1 M acetic acid and PBS with 1% Tween 20.

Analysis of antigens by electrophoresis on polyacrylamide gel (SDS-PAGE)[15] was done in 12.5% separation and 4% resolution gel under reducing conditions in a discontinuous buffer system at 140 V for 3 h. The gel was used directly for placing on a nitrocellulose membrane or visualization was achieved with Coomassie Brilliant Blue-R-250.

Immunoblots. The protein components separated in SDS-PAGE were transferred electrophoretically to a nitrocellulose membrane (BA 83 with 0.22 μm pores, Schleicher–Schuell) and subsequently immunodetected with the corresponding antisera using a second antibody tagged with peroxidase, and then stained with the substrate[16].

Direct ELISA. The ELISA technique was used in a modification for detecting serum antibodies by a procedure described by Wisingerova *et al*.[17].

RESULTS AND DISCUSSION

The activity and stability of immobilized biologically active agents depend fully on the selection of the solid support and the immobilization technique[18]. At one time the most frequently used activated support was CNBr-Sepharose in spite of the low stability of the attached immobilized substances[19]. Macroporous spherical cellulose[20] when used with a corresponding immobilization technique appears to be a suitable alternative to agarose supports for a number of applications. Comparison of the properties of an immunosorbent for the isolation of the *Mycobacterium bovis* BCG antigen with cellulose and agarose supports is the subject of the present publication.

To prepare the immunosorbent we used polyclonal rabbit antibodies with or without adsorption, the point in adsorption being immunoglobulin purification when antibodies against some minority components of the *M. bovis* antigen are removed by using the antigen mass of *M. kansasii*. To activate the sugar moieties of the immunoglobulin we used oxidation with sodium periodate. Both immunoblotting and ELISA tests showed that the oxidation of the sugar moieties does not damage the antibody as such.

The antibodies were immobilized via the protein part to a CNBr-activated agarose and *via* their periodate-oxidized sugar components to supports with hydrazide groups of agarose or cellulose type. The immobilization was followed throughout its course by absorbance at 280 nm and after hydrolysis of the product by amino acid analysis. Using

the supports with hydrazide groups we also followed the binding of an oxidized and a nonoxidized antibody. The course of immobilization for bead cellulose is shown in Fig. 1. With the agarose type the course is analogous. It appears that the major part of the antibodies are attached *via* the oxidized groups. However, nonspecific interactions, even if much lower, may take place also with the nonoxidized antibody. The amount of immobilized antibody bound to different supports is shown in Table I.

The total concentration of immobilized immunoglobulin is slightly higher when using the hydrazide cellulose than with the Affi-Gel HZ and CNBr-Sepharose, the percent binding being practically the same with both hydrazide supports and higher than with the CNBr-Sepharose. Both the adsorbed and nonadsorbed immunoglobulin are attached in much the same manner. For antigen chromatoghraphy we used immunosorbents with immunoglobulins after adsorption, immobilized on a hydrazide support of

TABLE I
Immobilization of immunoglobulin on different supports

Support	IgG, mg/ml of sorbent		Bond
	applicated	bound	%
CNBr-Sepharose 4B	1.32[a]	1.02	77
Affi-Gel HZ	1.26[b]	1.03	81.5
Cellulose HZ	1.38[a]	1.15	83
	1.34[b]	1.10	82.1

[a] Antibody without saturation; [b] antibody after saturation.

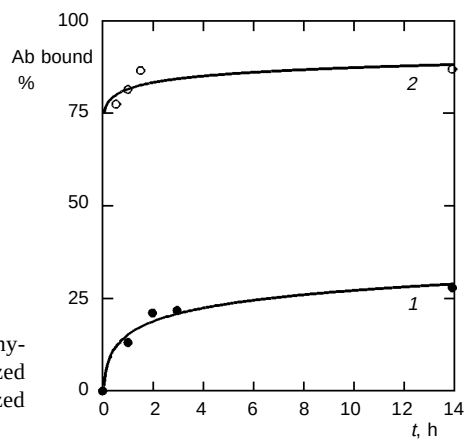

FIG. 1
Time dependence of antibody (Ab) binding to hydrazide-containing bead cellulose. *1* Nonoxidized antibody, *2* antibody with sugar moiety oxidized with $NaIO_4$

cellulose type. Adsorption of the antigen to the immunosorbent was tested with three buffers, the highest sorption being achieved with 0.1 M PBS (pH 7.0–7.5) as shown in Fig. 2. The presence of the detergent (0.05% Tween 20) in the buffer had no effect, just like the application of 0.1 M Tris-HCl at pH 8.0. There was no difference between sorption at 4 °C and at room temperature.

The results of studying the effects of flow rate on the adsorption of antigen are shown in Fig. 3. At flow rates exceeding 22 ml/h the amount of adsorbed antigen decreased.

The immunocomplex formed during adsorption of mycobacterial antigens to the three supports studied is very stable. The amount of eluted antigen expressed as weight percentage of protein obtained from the applied sample by different elution agents is shown in Table II.

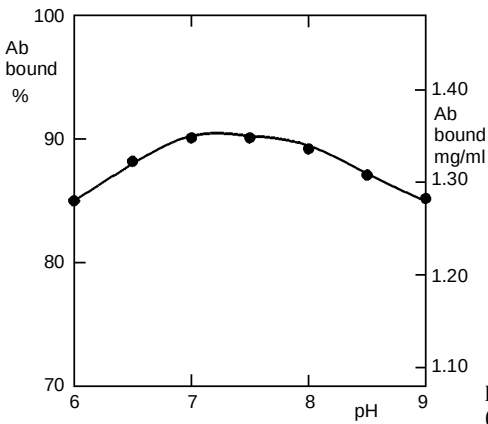

FIG. 2
pH dependence of binding of oxidized antibody (Ab) to hydrazide-containing bead cellulose

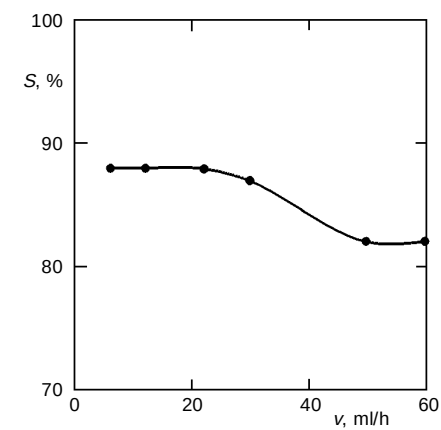

FIG. 3
Dependence of antigen adsorption (*S*) to a cellulose support with immobilized antibody on flow rate

The stability of immunosorbents after antigen desorption using a variety of elution agents is defined as the number of cycles required to decrease the adsorption capacity to 75% of the original, the results being shown in Table III.

TABLE II
Desorption of antigen from immunosorbent – cycling

Elution agent	Protein eluted from immunosorbent, %[a]					
	CNBr-Sepharose		Affi-Gel HZ		Cellulose HZ	
	A	B	A	B	A	B
4 M urea, pH 9	8	–	4	4	10	7
4 M urea, pH 9	8	–	4	4	10	7
1 M NaCl, pH 7.4	3	–	4	4	4	3
4 M urea, pH 9 (after 1 M NaCl)	7	–	3	3	8	5
0.1 M glycine-HCl, pH 2.8 (after 1 M NaCl)	3	–	6	6	6	6
0.1 M glycine-HCl, pH 2.8 (separately)	4	–	8	8	6	6
1 M NaSCN, pH 7.4 (separately)	5	–	5	4	5	5
0.1 M $MgCl_2$, pH 7.4	4	–	2	2	2	2

[a] Eluted protein is given in % of sample applied to column (12 mg antigen in 1 ml PBS/8 ml sorbent); A Ab bound without saturation; B Ab bound after saturation.

TABLE III
Stabilization of immunosorbents during influence of elution agents

Elution agent	Number of cycles, binding of antigen/desorption[a]		
	CNBr-Sepharose	Affi-Gel HZ	Cellulose HZ
1 M NaCl, pH 7.4	12	25	23
4 M urea, pH 9	8	10	21
6 M urea, pH 9	8	10	21
0.1 M glycine-HCl, pH 2.5	8	21	19
1 M NaSCN, pH 7.4	12	25	23
0.5 M $MgCl_2$, pH 7.4	11	23	23

[a] Number of cycles required to decrease the sorption capacity to 75% of the original.

The results obtained indicate a lower stability of protein binding to polysaccharide supports activated with CNBr. It is clear that it is advantageous to bind glycoproteins via their oxidized sugar moieties on supports with hydrazide groups[21]. Comparative experiments using immunosorbents prepared by binding periodate-oxidized sugars of the antibodies against mycobacterial antigens to the most frequently used commercial agarose derivative Affi-Gel HZ and to a hydrazide derivative prepared by binding adipic dihydrazide to bead cellulose (Perloza) document the fine properties of the latter for use in affinity chromatography.

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