Purification of Genus-Specific Chlamydial Antigen and Its Separation into Several Components by Ion-Exchange Chromatography

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Sodium deoxycholate-extracted genus-specific chlamydial antigen was purified from contaminating substances by ion-exchange chromatography with DEAE-Sephacel, resulting in a decrease in the complement-fixing activity of the antigen, whereas the enzyme-linked immunosorbent assay activity increased. By successive elution with 0.5, 1.0, and 1.5 M acetate buffer at least three clearly separated components were consistently recovered in 14 trials. The identity of these components as genus-specific chlamydial antigen was demonstrated in enzyme-linked immunosorbent assay tests with specific antisera. The antigenic activity of these components was not diminished by prior treatment of the chlamydial particles with pronase. Antiserum prepared by immunization of rabbits with the antigenic component I of an egg-propagated antigen reacted predominantly with the antigenic components I and II of a cell culture-propagated antigen.

Both species of the genus *Chlamydia*, *Chlamydia trachomatis* and *C. psittaci*, possess a common complement-fixing antigen which can be extracted by mild ionic detergents such as sodium dodecyl sulfate (1) and sodium deoxycholate (5). Dhir et al. (3) proved the lipid-carbohydrate nature of this antigen by extracting a "crude lipid antigen" with apolar organic solvents and separating the polysaccharide component by alkaline saponification. Whereas the complete antigen was active in the complement fixation (CF) test, the isolated polysaccharide reacted only in the CF inhibition test. The immunodominant group of the polysaccharide was characterized by Dhir et al. (2) as an acidic carbohydrate with eight carbon atoms (2-keto-3-deoxyoctanoic acid). Preparation of this purified antigen required a complicated procedure, including a series of different steps of extraction and ion-exchange and gel chromatography; moreover, part of the original antigenicity was lost during the purification procedure.

Sensitive immunoassay systems like the enzyme-linked immunosorbent assay (ELISA) or radioimmunoassay require reagents of high immunological specificity and purity (9). It was the aim of this investigation to develop a simple procedure for the preparation of a stable, purified, genus-specific chlamydial antigen which could be used for immunological, diagnostic, and analytical purposes.

MATERIALS AND METHODS

Preparation of antigen. Strain 8583 of *C. psittaci* (enzootic abortion of ewes, 165th egg passage) was propagated in the allantoic cavity of 11-day-old chicken embryos (egg antigen), and in McCoy cell cultures (TC antigen) by using Roux bottles (8), but without centrifugation of the inoculum onto the monolayers. Allantoic fluids and cell culture suspensions with high chlamydial yields were stored at -70°C . For purification from soluble substances, chlamydial suspensions were centrifuged 3 times for 1 h at $30,000 \times g$ (4°C), and the pellets were suspended in phosphate-buffered saline (pH 7.2).

Enzymatic treatment. Pronase P from *Streptomyces griseus* (Boehringer Mannheim Corp.) was used in a concentration of $100 \mu\text{g/ml}$. Enzymatic treatment was performed at 40°C for 6 h with constant stirring of the chlamydial suspension.

DO extraction. Sedimented particles were suspended $\geq 1:6$ (wt/vol) with 1% sodium deoxycholate (DO), and the suspension was kept at 45°C for 4 h with constant stirring (6). The suspension was centrifuged at $23,700 \times g$ for 1 h at 24°C . The extraction procedure was repeated once or twice, and the supernatant fluids (CF titer, $\geq 1:32$) were pooled and dialyzed 3 times for 12 h against 1 liter of 0.05 M sodium acetate buffer (pH 5) at 4°C . For use in the CF test the supernatant fluid was dialyzed against phosphate-buffered saline under the same conditions. Negative egg and TC antigen was produced by DO extraction of homogenized chorioallantoic membranes and McCoy cells not infected with chlamydiae.

Ion-exchange chromatography. Preswollen DEAE-cellulose (DEAE-Sephacel; Pharmacia, Freiburg) was

suspended in 5 volumes of 0.05 M sodium acetate buffer (pH 5) and kept overnight. The sediment was washed 5 times with an equal volume of buffer, suspended in 1 volume of buffer, degassed, and mixed, and 60 ml was filled into a 2- by 15-cm glass column at a rate of $7.5 \text{ ml cm}^{-2} \text{ h}^{-1}$. The absorbance was measured at 280 nm with a LKB Uvicord-S photometer and recorded with an LKB potentiometric recorder. The eluate was collected in 2.6-ml fractions with LKB Multirac equipment. After equilibration of the column with 5 volumes of the buffer described above, 5 ml of DO-extracted antigen was layered at a flow rate of $4.5 \text{ ml cm}^{-2} \text{ h}^{-1}$ onto the column and eluted successively with 26 to 34 ml of 0.05, 0.5, 1.0, 1.5, and 2.0 M sodium acetate buffer (pH 5).

CF test. For CF tests, as well as for the ELISA, a chlamydial antiserum from a naturally infected sheep was used. CF tests were performed in a microtiter system by using exactly 2 U of complement and overnight incubation.

ELISA. ELISAs were performed in Immulon microtiter plates (Flow Laboratories, Bonn) by the method of Voller et al. (11). Antigen was diluted with coating buffer (0.05 M sodium carbonate-bicarbonate, containing 0.2 g of sodium azide, pH 9.6) 1:100 (vol/vol) and adsorbed to the wells at 37°C during overnight incubation in a humid chamber. A protein A-Sepharose-purified immunoglobulin G fraction of a rabbit-anti-sheep immunoglobulin G serum (Nordic, Dietzenbach) was conjugated with horseradish peroxidase (250 U/mg; Boehringer Mannheim) by the method of Wilson and Nakane (12). The conjugate was used in a dilution of 1:400, and the antichlamydial sheep serum was used at 1:100. Additionally, the specific immunoglobulin G fraction of a rabbit-anti pigeon immunoglobulin G serum purified by affinity chromatography was conjugated with horseradish peroxidase, and ELISA tests were performed by using a pool of 32 chlamydia-positive pigeon sera (CF titer, 1:32). When the positive serum pool was diluted 1:100 this conjugate could be used at a dilution of 1:10,000. As the substrate and indicator solution 0.2 M 2,2-azino-di-(3-ethyl-benzthiazoline-sulfonic acid) (Boehringer Mannheim), and 2 mM H_2O_2 in 0.05 M sodium citrate buffer (pH 4.25) was used. ELISA activity was read with a Titertek multiscan photometer (Dynatech, Denkendorf), and documented as ELISA units (absorbance at 405 nm $\times$ 1,000).

Protein A was prepared from strain 2227 of *Staphylococcus aureus* (kindly supplied by W. Schaeg, Department of Bacteriology, University of Giessen) by means of lysozyme (10), purified by affinity chromatography (7) on a rabbit IgG-Sepharose column, and conjugated with alkaline phosphatase (400 U/mg; Boehringer Mannheim) by the two-step glutaraldehyde method of Engvall (4). As the substrate and indicator solution, *p*-nitrophenylphosphate (Boehringer Mannheim) was used. For testing column fractions with rabbit immune serum against purified chlamydial antigen, the conjugate was diluted 1:25,000, and the immune serum was diluted 1:10.

ELISA-active fractions from chromatographic peaks were pooled, dialyzed against phosphate-buffered saline, and either concentrated by using an Amicon system 52 with an XM50 filter (Amicon Corp., Witten) or lyophilized and suspended in phosphate-buffered saline to the desired concentration.

The effect of elution buffers on the affinity of the antigen to the microtiter plates was investigated by testing collected fractions before and after dialysis against coating buffer.

Immunization with purified genus-specific antigen. Fractions 14 through 21 of a DO-extracted, chromatographically purified chlamydial egg antigen (Fig. 1) were pooled, dialyzed against 0.15 M NaCl, concentrated to about 1 ml, filter sterilized, and stored in 300- μ l portions at -70°C . Each portion was emulsified with an equal volume of incomplete Freund adjuvant, and six 100- μ l injections were given intracutaneously to each rabbit. Second and third series of injections were given at 3-week intervals. Animals were bled 10 days after the third series. These antisera were used to test chromatographically purified antigen originating from cell cultures in an indirect ELISA using the protein-A-alkaline phosphatase conjugate.

RESULTS AND DISCUSSION

Typical elution profiles of DO-extracted genus-specific chlamydial antigen chromatographed on DEAE-Sephacel are shown in Fig. 1 and 2. At least three antigenically active fractions clearly separate from each other (antigenic components I, II, III, etc.) eluted from the column with buffers at 0.5, 1.0, and 1.5 M. There were no distinct differences in the chromatographic patterns of egg and TC antigen. From 14 different antigen pools fractions of chlamydial antigen eluted in the pattern described, with the main antigenic activity usually appearing with 1 M acetate buffer (Fig. 2). Whereas the active fractions had a low absorbancy, a large fraction with high absorbancy but without antigenic activity eluted when 2 M acetate buffer was used. Here, a significant difference in chromatographic profiles of pronase-treated and untreated antigens existed.

This contaminating material lacking antigenic activity was significantly reduced by pronase treatment. Since peaks with antigenic activity were almost identical in experiments with and without pronase treatment, it can be assumed that this enzyme does not affect the chlamydial antigen. However, since separation of the chlamydial group antigen from contaminating substances could be obtained by ion-exchange chromatography alone, later trials were performed without pronase treatment of the chlamydial suspensions.

The results of these experiments suggest that protein does not participate in the antigenic activity of the genus-specific chlamydial antigen complex.

In Fig. 1 the reactivity of the pooled pigeon serum with the chromatographically separated antigenic components of the chlamydial group antigen is compared with that of the sheep serum. Both sera did not react with negative DO antigens in the ELISA and consequently could be used for the specific detection of chlamydial

FIG. 1. Chromatography of an egg-propagated, DO-extracted chlamydial antigen on DEAE-Sephacel. Antigenic activity was monitored by horseradish peroxidase ELISA with antichlamydial pigeon serum (○) or with antichlamydial sheep serum (●). —, Absorbance at 280 nm. Arrows indicate elution at the given concentration (molar) of sodium acetate buffer (pH 5). The shaded area indicates the pooled fraction used for immunization of rabbits. *, Absorbance range changed from 0.1 to 0.5.

FIG. 2. Chromatography of a cell culture-propagated, DO-extracted chlamydial antigen on DEAE-Sephacel. Antigenic activity was monitored by horseradish peroxidase ELISA with antichlamydial pigeon serum (○) or by protein A ELISA with rabbit antiserum against peak I shown in Fig. 1 (shaded area). —, Absorbance at 280 nm. Arrows indicate elution at the given concentration (molar) of sodium acetate buffer (pH 5). *, Absorbance range changed from 0.1 to 0.5.

TABLE 1. CF and ELISA activity of an egg-propagated chlamydial antigen before and after different steps of purification

Purification step	Sediment (g)	Volume (ml)	CF titer	ELISA ^a	
				Units	Units × volume
Chlamydial suspension	1.59	130	1:2	152	19,760
After pronase treatment	0.60	20.6	1:32	998	20,559
After washing	0.32	20.0	1:32	1,102	22,040
After DO extraction	0.15	10.5 ^b	1:64	674	7,077
After chromatography					
Peak I		30.0	1:2	218	6,540
Peak II		33.6	1:4	231	7,762
Peak III		20.2		146	2,949
Pooled and concentrated		20.4	1:8	693	14,137

^a ELISA activity is expressed as ELISA units with antichlamydial sheep serum.

^b Supernatant fluid.

antigens. The pooled pigeon sera and the sheep serum might be expected to contain different spectra of antibody specificities, but the pigeon serum pool reacted in exactly the same manner as the sheep serum with the three components of the antigen. This indicates the identity of these components with the chlamydial group-specific antigen first described by Jenkin et al. (5) and characterized by Dhir et al. (2), which is common to all chlamydiae.

The rabbit antiserum produced by immunization with antigenic component I of the group antigen (Fig. 1) reacted with all antigenic components (Fig. 2), but predominantly with the first two peaks. The more intense reaction of the rabbit immune serum with the first antigen peak as compared with the reaction of the pigeon serum with this peak may indicate that the substance which eluted there showed the closest relationship to the inoculum used for immunization of the rabbits. The fact that all antigenic components reacted with the rabbit immune serum produced by immunization with only one component would indicate the existence of closely related cross-reacting antigenic structures or contamination of the inoculum with the other antigenic components.

The activities of materials from different steps of purification of the genus-specific chlamydial antigen are shown in Table 1. Pronase treatment and subsequent washing resulted in a weight reduction of about 80%, with a slight rise in antichlamydial CF and ELISA activity. After DO extraction the group-specific antigenic activity was found in the supernatant fluid, apparently without loss of specific CF activity, but accompanied by a drop of ELISA activity to 1/3 of the titer of the original suspension. However, chromatographic purification with DEAE-Sephacel increased the ELISA activity twofold, whereas the CF activity decreased sharply. Consequently, for the production of complement-fixing, genus-specific chlamydial antigen for di-

agnostic purposes, DO extraction combined with or without pronase treatment seems to be the method of choice, whereas for sensitive ELISA antigen, chromatographic purification with DEAE-Sephacel may be recommended, which results in a highly purified antigen. With this simple method, the complete antigen complex can be eluted with 1.5 M acetate buffer. Stored at -70°C, the concentrated antigen complex can be used as soluble, genus-specific chlamydial antigen for at least 6 months without loss of antigenic activity in ELISA tests.

Due to its high sensitivity, the ELISA proved to be an excellent method for testing single chromatographic fractions. At low dilutions, adherence of the antigenic components to the plastic surface of the Immulon plates was affected slightly by the high molarity and the acidic pH of the chromatographic buffers. However, by testing the collected peaks before and after dialysis it could be shown that adherence of the antigen to the plates was not affected in any way when a 100-fold dilution of the fractions in coating buffer was used. The CF test appeared to be unsuitable for testing chromatographic fractions due to its insensitivity and to hemolysis caused by the chromatographic buffer.

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