Rabbit Immunoglobulin Responses to the Flagella, Somatic, and Protective Antigens of a Highly Protective Strain of *Clostridium chauvoei*

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The immunoglobulin response of rabbits to the flagella (H), somatic (O), and protective antigens of a highly protective strain of *Clostridium chauvoei* was studied using antisera that had been fractionated by Sephadex G-200 chromatography. The H antigen elicited the characteristic agglutinin response to a protein antigen—early production of 19S globulin followed by persistent 7S globulin production. The O antigen stimulated a transient agglutinin response which was detected in both the 19S and 7S serum fractions. Protective antibody was assayed by passive protection tests in mice. Using these tests the protective activity of the rabbit sera was found to be confined exclusively to the 7S serum fractions. Purified immunoglobulin G, prepared by DEAE-cellulose chromatography of the above sera, was also tested and found to confer considerable passive protection on mice. It is considered that either the protective antigen fails to stimulate an immunoglobulin M response or that immunoglobulin M is relatively ineffective in conferring protection against infection in the mouse passive protection tests.

Relatively little research has been reported on the bacteriology and immunology of *Clostridium chauvoei* although the fatal disease "Blackleg" of sheep and cattle caused by this organism is of considerable economic importance (11, 12). In laboratory animals *C. chauvoei* is pathogenic to mice and guinea pigs but not rabbits. Protection against this disease may be afforded by vaccination, but, unlike other clostridial infections in which antitoxic immunity plays the predominant role in protection, protective immunity against *C. chauvoei* is considered to be largely antibacterial (3, 12).

The vegetative cells of *C. chauvoei* possess two known agglutinogens, a heat-stable somatic O antigen common to all strains, and a heat-labile flagella H antigen of which there are two types (9). The H antigen has been shown to be unimportant as a protective antigen (3), whereas, for many strains, the heat stable O antigen appears to be the main protective antigen (3, 8).

I am studying the properties, including immunological properties, of the protective antigen of a highly protective strain of *C. chauvoei*, strain CH3. In previous papers (2, 3, 4a) the main protective antigen of this strain was shown to be a heat-labile cell wall antigen which in guinea pigs and rabbits stimulates the production of nonagglutinating protective antibody. This paper reports a study of the immunoglobulin response of a group of rabbits to the main protective antigen of strain CH3 as well as its less protective O antigen and nonprotective H antigen. Rabbits were chosen in preference to guinea pigs for these experiments as the latter fail to measurably respond to *C. chauvoei* O antigen (2).

**MATERIALS AND METHODS**

**Strains used.** *C. chauvoei* strain CH3 was used for the immunization of rabbits and for agglutination tests. For challenging mice in the passive protection tests, the highly virulent challenge strain CH4 was used. These strains are described in previous publications (2, 3).

**Preparation of immune rabbit sera.** Four English albino male rabbits, each weighing 1.2 to 1.8 kg, were given successive intravenous doses of 0.2, 0.4, 0.8, 1.6, and 3.2 ml of a formalin-killed strain CH3 cell suspension on days 1, 4, 7, 11, and 14, respectively. On days 4, 7, 11, 14, 18, 21, 26, 32, 53, 67, and 81 each rabbit was bled by cardiac puncture, the sera for that day were pooled, and the quantity and type of antibody present were established for each serum pool. Before use all rabbits were tested and found to be negative for the presence of specific agglutinins.

**Fractionation of immune rabbit sera.** A Wright chromatographic column (3.2 by 90 cm) packed with Sephadex G-200 (Pharmacia, Sweden) was used for gel filtration. The gel was equilibrated with 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride...
buffer, pH 8.0, containing 1 M NaCl, sodium azide (0.01% wt/vol), and thimerosal (0.01% wt/vol).

Undiluted serum (2 ml) was applied to the column, and an upward flow of 12.5 ml/h was maintained by adjusting the hydrostatic pressure. The column was run at room temperature (20 °C), and the absorption of the effluent at 280 nm was continuously recorded using an LKB Uvicord II adsorptiometer and recorder. Fractions (5 ml) were collected from the column in an LKB RadiRac fraction collector.

All the fractions from the first and second protein peaks of the fractionated serum sample were tested for the presence of agglutinating antibody, and fractions of a peak having agglutinins were pooled and concentrated to the original serum volume through an ultrafiltration membrane (Diaflo PM 10, Amicon Corp.). The concentrated serum was again tested for the presence of antibodies.

DEAE-cellulose fractionation of sera. Serum globulins were precipitated by 40% saturation with (NH₄)₂SO₄, and the precipitate was dissolved in physiological saline and dialyzed exhaustively against 0.03 M phosphate buffer, pH 6.3.

The globulins were then added to a column (1.6 by 70 cm) containing DEAE-cellulose (Whatman, DE 32) equilibrated with 0.03 M phosphate buffer, pH 6.3. The column was eluted with the same buffer, and the combined fractions from the one 280 nm absorbing peak which emerged were concentrated to the original serum volume through an ultrafiltration membrane (Diaflo PM 10).

2-ME treatment of sera. Serum samples were treated with 0.1 M 2-mercaptopethanol (2-ME) by the method of Adler (1). Control samples were subjected to the same method except that they were not treated with 2-ME.

Immunoelectrophoresis. Immunelectrophoresis of serum fractions, at original concentration, was carried out in 1.5% Ionagar with barbital buffer, pH 8.6 (I = 0.075), for 60 to 80 min at a potential drop of 3 V/cm (4). Commercial goat anti-whole rabbit serum (Hyland), anti-rabbit immunoglobulin G (IgG) (Hyland), and anti-rabbit immunoglobulin M (IgM) (Miles Laboratories, Israel) sera were added to the antiserum trough for development of the precipitin arcs.

Antigens for agglutinating suspensions. The preparation of these suspensions has been described previously (2).

Tube agglutination tests. Serial twofold dilutions of sera were prepared in phosphate buffered 0.42% (wt/vol) saline (pH 7) containing thimerosal (0.01% wt/vol). For undiluted sera, or column peak fractions concentrated to the original serum volume, a starting serum dilution of 1/10 was used. A volume of 0.25 ml of serum dilution was then mixed with 0.25 ml of antigen.

For H agglutination tests, Dreyer tubes were used, and the mixtures were incubated in a water bath at 50 C. Loose floccular agglutination occurring within 4 h was read as H agglutination. Wasserman tubes were used for O agglutination tests; the mixtures were incubated at 37 C for 2 h and then left for 20 h at 4 C before reading. The end point of these tests was chosen as the last tube showing definite agglutination when compared with a control tube containing anti-

gen and buffered saline only. For sera, concentrated peaks, and fractions from the column, the reported titer represents the reciprocal of the final dilution showing this end point.

Mouse passive protection test. Protective antibody in sera or peaks from the G-200 column was assayed using two different passive protection test methods in mice, the difference being in the route of administration of the immunoglobulin.

(i) Intraperitoneal immunoglobulin administration. Swiss white mice (18 to 21 g) were given a single intraperitoneal dose of 0.5, 0.4, 0.3, 0.2, 0.1 or 0.05 ml of serum or serum fraction using four mice per dose. After 6 h these mice were challenged intramuscularly (hind leg) with 0.25 ml of a strain CH4 spore suspension containing approximately 250 spores in 2.5% (wt/vol) CaCl₂. Mice surviving the observation period of 4 days were regarded as being fully protected. Control mice dosed with similar volumes of isotonic saline or normal rabbit serum were also challenged and always died within 30 h of challenge.

(ii) Intramuscular immunoglobulin administration into the site of challenge. Swiss white mice (18 to 21 g) were first given an intramuscular spore challenge identical to that in (i) above. After 4 h the challenged mice were given graded doses of immunoglobulin directly into the infected muscle. Control-challenged mice injected with isotonic saline or normal rabbit serum instead of immunoglobulin were also included. As in the first test, mice surviving the observation period of 4 days were regarded as being fully protected.

The effective dose giving 50% protection to a group of mice (ED₅₀) was calculated for each serum or serum fraction by the Spearman-Karber method (6) and the degree of protection in the serum or fraction is expressed as 1/ED₅₀.

RESULTS

Rabbit agglutinin response to cells of C. chauvoei strain CH3. Intravenous inoculation of a group of four rabbits with formalin-killed cells of C. chauvoei strain CH3 produced the agglutinin response shown in Fig. 1. Both O and

FIG. 1. Rabbit agglutinin response to cells of C. chauvoei strain CH3. Rabbits dosed on days 1, 4, 7, 11, and 14 with 0.2, 0.4, 0.8, 1.6, and 3.2 ml of cell suspension, respectively.
H agglutinating activity were at a maximum between days 11 to 21, and thereafter both decreased.

**Rabbit protective antibody response to cells of C. chauvoei strain CH3.** The rabbit sera were assayed for protective antibody using the mouse passive protection test method (i) with intraperitoneal serum dosage and subsequent intramuscular challenge. The results are shown in Fig. 2. These show that protective antibody was detectable after day 11, at a maximum between days 14 to 32, and almost undetectable by day 67.

**Gel filtration of sera on Sephadex G-200: O and H agglutinin response.** Serum from each bleed was fractionated into three protein peaks (Fig. 3) by gel filtration through Sephadex G-200. The fractions from the first protein peak, corresponding to 19S serum components, and the second peak, corresponding to 7S components, were separately concentrated to the volume of serum originally placed on the gel and tested for O and H agglutinins (Fig. 4). The H response involved the early production of 19S antibody which subsided and was followed by a strong persistent 7S response. The O response differed in that the 19S peak was earlier and more persistent than the 7S peak, the latter being only detectable between days 14 to 53.

When the samples of the concentrated 19S and 7S peaks were treated with 2-mercaptoethanol, the agglutinating activity of the 19S peaks was completely destroyed. The 7S agglutination titer was generally reduced slightly.

**The protective antibody response.** Sephadex G-200 19S and 7S peaks, after concentration to the original serum volume, were assayed for protective antibody in the mouse passive protection test method (i) using intraperitoneal dosing.

Using this test there was no evidence of any protection in the 19S peak of any bleed; all dosed mice died within the same period as the controls.

For each bleed, the protective antibody was found to be in the 7S peak, being first detected at day 11, at a maximum between days 18 to 21, and disappearing between days 39 to 53 (Fig. 5). Because it was suspected that the failure of the 19S peak to protect may be due to the inability of the larger immunoglobulin molecule to be
absorbed rapidly from the intraperitoneal cavity and reach the site of infection, a passive protection test method was developed in which the immunoglobulin was administered directly into the infected muscle, i.e., passive protection test method (ii). The results of this test are also shown in Fig. 5.

It will be observed that these results closely resemble those obtained with the passive protection test using intraperitoneal dosage. As with that test, the administration of 19S immunoglobulin had no measurable effect on the infection, whereas the 7S fraction of sera taken between days 11 to 39 was capable of stopping the infection and conferring full protection.

**Immunoelectrophoresis of the 19S and 7S peaks from the gel filtration column.** To identify the serum proteins in the 19S and 7S peaks from the gel filtration column, representative samples from the column were concentrated to original serum volume and examined by immunoelectrophoresis using goat antisera against rabbit whole serum, rabbit IgG, and rabbit IgM.

The 19S peak contained IgM, α2 macroglobulin, and lipoprotein. The IgM precipitin line was not present in sera previously treated with 2-ME. No lines developed when this peak was run against anti-rabbit IgG serum.

The 7S peak contained several protein lines, the IgG line being very strong. No IgM was detectable in this peak.

**The protective activity of purified IgG.** Six milliliters of sera from bleeds taken at days 21 and 26 were pooled, and the globulins from a 6-ml portion of this pool were fractionated on DEAE-cellulose. The material eluted, after concentration to the original serum volume (6 ml), had a protein concentration of 4 mg/ml. When examined by immunoelectrophoresis, one strong line was obtained against anti-whole rabbit and anti-rabbit IgG sera, and no line was detectable against the anti-IgM serum.

The protective activity of the purified IgG was then determined and compared with that of the original serum pool using the mouse passive protection test method (i), i.e., intraperitoneal administration. The IgG was found to have an 1/ED50 of 7.1, whereas that of the original serum pool was 13.3.

**DISCUSSION**

This study was undertaken as part of a wider investigation of the properties of the main protective antigen of a highly protective strain of *C. chauvoei*, strain CH3. This antigen has previously been shown to be a heat-labile cell-wall antigen that does not stimulate agglutinating antibody in guinea pigs or rabbits (3, 4a).

The primary aim of the work reported in the present paper was to obtain information about the antibody producing properties of the main protective antigen, the O and H antigen responses being of secondary interest only.

The nonprotective H antigen (3) stimulated an early 19S antibody response which was followed by a strong, persistent 7S response—the characteristic response to a protein antigen (11). The heat-stable O antigen of *C. chauvoei*, although relatively unimportant as a protective antigen of strain CH3, appears to be the only protective antigen of many other strains (3, 8). In this study the O antigen elicited transient 19S and 7S globulin responses, the 19S being slightly more persistent.

In the studies of *C. chauvoei* at these laboratories, we have been unable to find an in vitro test that accurately reflects the protective antibody level of an immune serum (2, 3; unpublished data). An in vivo test was, therefore, developed in mice. Two mouse passive protection test methods were adopted, one using intraperitoneal injection of immunoglobulin before intramuscular challenge and the other using administration of immunoglobulin directly into the infected muscle.

These tests showed that the 19S fractions of the immune rabbit sera (containing IgM as the only detectable immunoglobulin) had no measurable protective activity in mice. As many of these fractions had a considerable agglutinin titer that was 2-ME sensitive, it is unlikely that
the absence of protective activity was due to the denaturation of the IgM.

All the measurable protective activity of the different sera was found to be in the 7S fractions; these fractions contained no IgM detectable by immunoelectrophoresis. The 7S protective antibody response of the rabbits paralleled the protective antibody response measured in their whole sera, the difference in level of response probably being accounted for by the experimental losses incurred during fractionation and recombination of the fractions.

Purified IgG was found to have a considerable capacity to passively protect mice against infection. It would appear, therefore, that IgG is the main immunoglobulin stimulated by C. chauvoei strain CH3 that is capable of protecting mice against infection with C. chauvoei. IgM protective antibody was either not stimulated by the protective antigen or was not capable of conferring passive protection on the mice. A similar finding was made by Dolby and Dolby (5), who noted that the 7S globulin fractions of rabbit Bordetella pertussis antisera were at least 100 times as effective as the 19S fractions in passively protecting mice against intracerebral challenge with B. pertussis.

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LITERATURE CITED