Antigens of *Bordetella pertussis*

V. Separation of Agglutinogen 1 and Mouse-Protective Antigen

R. F. ROSS* AND J. MUNOZ

Rocky Mountain Laboratory, National Institute of Allergy and Infectious Diseases, Hamilton, Montana 59840

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Agglutinogen 1 of *Bordetella pertussis* strain 353/Z (serotype 1) was separated from protective antigen and histamine-sensitizing factor by starch-block electrophoresis. Most of the agglutinogen 1 migrated towards the cathode in starch-block electrophoresis, although some remained near the origin. Fractions containing most of the agglutinogen 1 were free of detectable mouse-protecting or histamine-sensitizing activities. Agglutinogen 1 from a serotype 1,3 *B. pertussis* strain (J20) migrated similarly to the agglutinogen 1 from strain 353/Z. All agglutinogen 3 activity was found at the point of application in the starch block. No clear relationship was found between agglutinogen 1 and mouse-protecting antigen or histamine-sensitizing factor.

Eight different agglutinogens have been described for *Bordetella pertussis* (1, 5, 6). Six of these (1, 2, 3, 4, 5, 6) are species specific, and two (7, 13) are shared with other species of the genus *Bordetella*. Agglutinogen 1 is common to all strains of *B. pertussis*, whereas agglutinogens 2, 3, 4, 5, and 6 are found in various combinations as strain-specific antigens. Agglutinogens 2, 3, and 5 resist 100 C for 2.5 hr, but 1, 6, and 7 are destroyed by this treatment (4).

The relationship of these agglutinogens to immunity induced by pertussis vaccines is not clear. Work with purified fractions of *B. pertussis* has indicated that at least some of the agglutinogens are separated from the antigen that is responsible for immunity in the mouse protection test of Kendrick et al. (10). Moreover, no difference in mouse-protective activity has been demonstrated among vaccines prepared from cultures possessing different combinations of agglutinogens (2, 7, 8, 24). These observations indicate that the specific agglutinogens 2, 3, 4, 5, and 6 do not play a significant role in active immunization of mice. This cannot be said with certainty of agglutinogen 1, because this antigen is present in all smooth strains of *B. pertussis* (5), and definite separation of this agglutinogen and mouse-protective antigen (PA) has not been reported.

Recent epidemiological findings (3, 17) and results of passive mouse protection tests (18, 19) have suggested to the authors that type-specific immunity to *B. pertussis* may exist. These studies have emphasized the need for more precise definition of the role of the agglutinogens in immunity to *B. pertussis*.

We previously showed that the histamine-sensitizing factor (HSF) and PA can be found in the same fractions obtained from starch-block electrophoresis (15), and that the physical and chemical properties of PA and HSF are similar if not identical. However, PA or HSF has not been obtained in a pure form. Because of the uncertainty regarding the role of agglutinogen 1 in protection and the unavailability of a pure preparation of PA, it was important to determine whether these antigens could be separated.

**MATERIALS AND METHODS**

Serotype 1 *B. pertussis* strain 353/Z and serotype 1, 3 strain J20 were obtained from N. Preston, University of Manchester, Manchester, England (17). Strain 04965, another serotype 1 strain, was obtained from Parke Davis, Detroit, Mich., through the courtesy of Henry Devlin. *B. pertussis* strain 2753, serotypes 1, 2, 3, 4, and 5, which was used to challenge mice, was obtained from Merck & Co., West Point, Pa. It was originally obtained from P. Kendrick as strain 18-323. *B. bronchiseptica* strain 22067 (serotype 7, 8, 9, 12, 13) was obtained from G. Eldering, Michigan Department of Public Health, Grand Rapids, Mich.

*B. pertussis* cells were grown in and harvested from liquid medium (23) as previously described (15). Dried whole cells for agglutinin absorption were
prepared by dialyzing freshly grown cells against cold deionized water and lyophilizing them.

Antigens used for immunization of rabbits and for agglutination tests were saline suspensions of cells grown on Bordet-Gengou agar with 15% horse blood and preserved in 1:10,000 Merthiolate. In some cases, 0.3% Formalin was used instead of Merthiolate for cell suspensions used in agglutination tests.

The plate agglutination test was performed by mixing one drop of antigen (40 billion cells per ml) with one drop of each serum dilution; the plate was rocked several times, incubated for 5 min in a partially closed box to prevent evaporation, rocked again several times, and read for agglutination over a fluorescent light-illuminated viewer.

Antiserum specific for agglutinogen I was produced by immunizing rabbits with a series of intravenous (iv) and subcutaneous (sc) injections of Merthiolate-killed B. pertussis 353/Z cells. The antisera was made monospecific by absorption with 40 mg of dried B. bronchiseptica 22067 cells and 5 mg of autoclaved B. pertussis 120 cells per ml of serum. A sample of agglutinin I serum was also supplied by G. Eldering.

Alkaline saline extract (SE) from B. pertussis strains 353/Z, 04965, and J20 and from B. bronchiseptica strain 22067 were prepared by a procedure previously described (15). The SE from strain 353/Z had a histamine-sensitizing dose (SD50) of <5 μg per mouse and a protecting dose (PD50) of 5 μg per mouse.

Acetone-dried cells, SE, and MgSO4-precipitated HSF were prepared as previously described (16a). The sediments after extraction of SE and after precipitation of HSF by MgSO4 were dialyzed against distilled water and lyophilized.

Endotoxin was prepared by the trichloroacetic acid extraction method of Boivin as described by Kabat (9).

Mouse protection tests were performed as previously described (16), by using intracranial (ic) challenge with B. pertussis 2753. Histamine sensitization tests were performed by giving the sensitizing materials iv and challenging the mice intraperitoneally (ip) 24 hr later with 0.5 mg of histamine base given as histamine diphosphate (14).

Agglutinin absorption tests to determine amounts of agglutinin present in various fractions were performed as follows. Saline dilutions of antigen were mixed with an equal volume of antiserum diluted to a concentration twice its plate agglutination titer, and the mixture was incubated for 15 min at room temperature, mixed again, and allowed to incubate for an additional 15 min. The mixture was centrifuged, and the absorbed serum was tested for agglutinins as described above. Nonspecific absorption of antibodies was controlled by absorbing the sera with SE prepared from B. bronchiseptica strain 22067, and specific absorption was controlled by absorbing with SE from strain 353/Z. Consistently, 0.25 to 0.5 mg of SE from strain 353/Z absorbed antibodies from 1 ml of diluted serum.

Starch-block electrophoresis was performed as described previously (15). The starch block (50 by 9.5 by 1.2 cm) was made in phosphate buffer (pH 6.2; μ = 0.02). SE (100 mg) was applied at the center of the block. Electrophoresis, performed at 6 to 10 ma and 200 v, was carried out at 4°C for 40 hr. The block was cut into 25, 2-cm strips, each strip was then eluted with the phosphate buffer, and the eluates were dialyzed against water and lyophilized.

RESULTS

SE from B. pertussis 353/Z was fractionated by starch-block electrophoresis, and the fractions were subsequently tested by agar diffusion. This SE exhibited an antigenic complexity similar to that of SE prepared from another strain of B. pertussis (15). Sixteen different bands developed with the anti-B. pertussis serum (Fig. 1). Only four bands developed when factor I antiserum was employed (Fig. 2). One band was located at fractions 7 and 8, a second in fraction 10, and a third in fractions 9 to 15 which corresponded closely to the distribution of agglutinin I. A fourth was a weak band close to the sample application in fractions 14 and 15 (this may be due to protective antigen).

The relationships of optical density, agglutinin I activity, mouse-protecting activity, and histamine-sensitizing activity of the various starch-block electrophoresis fractions are shown in Fig. 3.

The HSF and PA activities remained near the point of application in fractions 13 and 14. Some PA was also found in fraction 15. Most of the factor I agglutinin-absorbing activity migrated toward the cathode and was found in fractions 8 to 11. Also, some of this activity was usually found close to the origin (fractions 13 to 16; Fig. 3). This distribution of agglutinin I, HSF, and PA occurred in many different electrophoretic runs; only once was all of the agglutinin activity found in the region of fractions 8 to 11. Fractions 9 to 15 produced a line of precipitate which coincided with agglutinin I activity. Fraction 10 had, in addition, another unidentified band (Fig. 2).

Starch-block electrophoresis was also done with SE from B. pertussis strains 04965 (serotype I) and J20 (serotypes 1, 3). SE from strain 04965 gave similar results to those obtained with strain 353/Z SE. With J20, it was found that agglutinin 3 did not move from the point of application, whereas agglutinin 1 had a distribution similar to that found with SE from strains 353/Z and 04965.

Agglutinin I activity of various preparations of B. pertussis 353/Z was compared with the PA and HSF of each preparation. Levels of
PA and HSF did not correlate with content of agglutinogen I (Table 1). For example, 0.125 mg of SE absorbed all of the agglutinins from antiagglutinogen I serum and had a PD₉₀ of 14.7 μg/mouse, whereas 0.5 mg of MgSO₄-precipitated HSF was required to absorb the same amount of agglutinin, yet it had a PD₉₀ virtually the same as the parent SE. The lack of correlation of agglutinogen and HSF activity is even more striking. Endotoxin from 353/Z did not contain agglutinogen, HSF, or PA.

DISCUSSION

Since the development of the mouse protection test by Kendrick and co-workers (10) for the evaluation of pertussis vaccines, efforts have been made to determine which component of the B. pertussis cell is responsible for the induction of immunity in children. Heat-labile toxin (22), agglutinogens (17), and hemagglutinin (11) were considered important in induction of specific immunity to whooping cough. Studies published by the British Medical Research Council (12) showed a direct correlation between PA activity of pertussis vaccines and their effectiveness in production of immunity to whooping cough. Since this report, it has been assumed that the antigen measured by the mouse protection test is identical to that responsible for immunization of children. With this mouse protection test, heat-labile toxin, heat-stable toxin, some “agglutinogen” preparations, and hemagglutinin have failed to produce active immunity (13, 20).

The substance responsible for mouse protection has been suspected to be a lipoprotein, the activity of which is destroyed by heating at 80°C for 30 min; it is polydispersed and can be...
isolated in a soluble form by various means (13, 14). This substance is different from some agglutinogens, heat-labile toxin, hemagglutinin, and endotoxin (13). The results presented here show that mouse-protective activity of purified fractions is not due to agglutinin 1. Most agglutinin 1 can be separated by starch-block electrophoresis from HSF and protective antigen. This isolated agglutinin was not serologically related to PA, HSF, or endotoxin from B. pertussis and did not protect mice or sensitize them to histamine. Preston has indicated that agglutinogens 1, 2, and 3 are important in protecting children against whooping cough (17–19). His conclusions were based on results obtained with passive mouse protection tests. Antisera specific for these factors passively protected mice better against B. pertussis strains containing homologous agglutinogens than against other strains. Indirect evidence also suggested to Preston that vaccines employed in England suppressed infections with strains homologous to the vaccine but not those due to other serological types (17). However, Eldering et al. (7, 8) were not able to detect a significant difference in active or passive immunization studies with strains of different serotypes, and we, in the present report, could not show mouse protection with agglutinin 1. However, these observations do not explain the role of these agglutinin factors in the prophylaxis of whooping cough. Before this question is answered, it will be necessary to determine the role of the various purified antigens in protection against whooping cough. Intracerebral challenge with B. pertussis is not a close approximation of infection in man. It is known that, in the mouse, depending on the route of challenge (ic or intranasal), two distinct antigens, and perhaps also mechanisms, are involved in protection against

Fig. 2. Immunodiffusion test of eluates from starch-block electrophoresis fractions of SE from B. pertussis 353/2 developed with specific agglutinin 1 antiserum (F1). The fractions were labeled starting at the most cathodal portion of the block. The well labeled Endo contained endotoxin from B. pertussis 353/2.
FIG. 3. Relationship of optical density, agglutinogen 1, HSF, and PA of eluates from starch-block electrophoresis fractions from SE of B. pertussis 353/Z. The sample of SE was applied at fraction 13. (Cathode on left; anode on right.) The slight difference in distribution noted for HSF and PA may be due only to the different doses employed for the protection test and the histamine sensitization test.

TABLE 1. Agglutinogen content, compared to protective and histamine-sensitizing potency of various preparations from 353/Z Bordetella pertussis

<table>
<thead>
<tr>
<th>Prepn</th>
<th>Amt of material used to absorb 1 ml of diluted antiserum</th>
<th>PD50b</th>
<th>SD50c</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.125</td>
<td>0.25</td>
<td>0.5</td>
</tr>
<tr>
<td>Whole cells</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Acetone-dried cells</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Alkaline saline extract</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Sediment from SE prep.</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MgSO4 ppt from SE</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Supernatant after removal of HSF by MgSO4</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Trichloroacetic acid extract (endotoxin)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

a The specific antiagglutinogen 1 serum had a titer of 1:80. Values are expressed as milligrams per milliliter. Symbols: +, agglutination (insufficient absorption); −, no agglutination (complete absorption).

b PD50 = 50% protective dose. Values expressed as micrograms per mouse.

c SD50 = 50% histamine sensitizing dose. Values expressed as micrograms per mouse.

infection by B. pertussis (21). Thus, it is premature to state that agglutinogens do or do not play a role in immunity to whooping cough. It seems well established, however, that agglutinogens, endotoxin, heat-labile toxin, and hemagglutinins are not involved in the active immunization of mice challenged ic with virulent B. pertussis.
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