SYMPOSIUM ON RELATIONSHIP OF STRUCTURE OF MICROORGANISMS TO THEIR IMMUNOLOGICAL PROPERTIES

I. IMMUNOLOGICAL AND OTHER BIOLOGICAL ACTIVITIES OF BORDETELLA PERTUSSIS ANTIGENS

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INTRODUCTION

The complexity of bacterial cells, as well as their ability to mutate to different morphological, antigenic, and metabolic forms, is well known to microbiologists. Bordetella pertussis is no exception. This gram-negative coccobacillus, which does not possess flagella or form spores, is among the smallest bacteria in size but not in importance. Morphological features change quite readily through mutations, especially in sub-optimal media. Usually, long filamentous forms develop which possess only few of the characteristics of the smooth form. Growth on solid media, such as Bordet-Gengou agar, normally gives rise to very small coccobacillary forms; growth in many liquid media gives rise to larger bacilli with properties otherwise identical to those of agar-grown cells (99).

Soon after becoming familiar with this organism, one learns that the cell morphology is extremely important in the study of various serological, biochemical, and other biological activities. To find typical properties, one must employ smooth organisms. The present report deals entirely with some of the serological and biological activities of selected smooth strains of B. pertussis. It is now known that there are various serological types of this organism, differentiated by agglutination in monospecific sera (1, 18, 19). For the most part, the similarity in biological activity among the types is outstanding. A comprehensive review on B. pertussis antigens has recently been published by Schweinsberg (89).

CELL STRUCTURE

Typical B. pertussis cells are coccobacillary in shape (0.5 to 1.0 × 0.3 to 0.5 μ) and have a capsule (57, 90) which is not seen unless a special staining procedure is used. The physical structure of these cells, as shown by electron microscopy, is rather simple (Fig. 1) even at this magnification. The cell can be separated easily into two different elements: the well-defined structure known as cell wall (Fig. 2), and the amorphous material contained inside the cell wall which is called protoplasm (69). Each of these two morphological entities is complex and has substances with biological activities of great practical and theoretical interest.

A more complex, but still greatly simplified, picture of the composition of the B. pertussis cell can be seen in Fig. 3, which depicts an agar diffusion test with antiserum to whole cells in the center and sonically disrupted cells of different strains in the outside wells. This picture gives a much better idea of the complexity of the cell. However, it indicates only the minimal
number of the substances of immediate interest, i.e., the antigens of *B. pertussis*. Depending on the antiserum used, the number of antigens detected can vary considerably (67). It is clear, however, that there are many antigens, and the main problem has been to separate these substances from one another in their biologically active form.

**BIOLOGICAL ACTIVITIES**

Many biological activities of importance have been described for *B. pertussis*. The following biological activities or substances have been recognized: (i) the so-called agglutinogen (9, 38), (ii) the heat-labile toxin (8, 22, 39), (iii) the heat-stable toxin (endotoxin; 16, 38, 39), (iv)
Fig. 2. Cell walls prepared from Bordetella pertussis (from reference 69).

hemagglutinin (34, 35, 51, 52), (v) the histamine-sensitizing substance (74, 75), and (vi) the protective antigen (50, 59, 86).

Figure 4 gives a diagrammatic distribution of these substances in the cell. As can be seen, the locations of agglutinogen and hemagglutinin are not too well-determined, but they are surface antigens which are easily removed from the cell (39, 86). As indicated above, there are many more antigens in the cell (85), but no specific activity other than antigenicity is known for them. Many of these antigens must be cellular enzymes and other functional substances in the cell.

Agglutinogen

Before discussing "agglutinogen," it should be made clear that there are many surface substances
in the cell that can produce agglutinins. At least 14 different antigens were recognized among various strains of *B. pertussis* by Eldering (18, 20). The so-called “agglutinogen” is most probably one of these substances, which is highly antigenic and easily extractable from the cells. This is the substance that will be discussed here.

Although agglutinogen has not been identified as capsular material, it appears to be a surface antigen and must be only loosely associated with the cell wall of *B. pertussis*. The agglutinogen, as stated, is easily extractable from cells (16, 38, 39), and detectable amounts are found in culture supernatants, even in young cultures. When cells are disrupted ultrasonically (39) or by other means, the agglutinogen is released almost entirely into the soluble portions of the cell (38). It can also be extracted by repeatedly washing the cells (104). From these supernatants, agglutinogen can easily be purified by means of isoelectric precipitation, ammonium sulfate and picric acid precipitation (40, 91), or by sodium sulfate fractionation (88). The early studies on purified agglutinogen (47, 107) showed this sub-

stance to be a nucleoprotein but, according to the recent work of Onoue et al. (73) who purified agglutinogen by means of diethylaminoethyl (DEAE)-cellulose column chromatography, it is a simple protein with no ribonucleic acid or carbohydrate. These workers found the molecular weight to be 10,000, which seems rather low for an antigenic substance. Agglutinogen, as purified by Smolens and Mudd (91), was found to be nontoxic, since 5 mg given intraperitoneally did not kill mice. When agglutinogen purified by the sodium sulfate method was tested for antigenicity, it was found to be fully antigenic, as had been found with other preparations (40, 91, 107). It produced agglutinins in rabbits and mice (Table 1; 88), and it was capable of removing agglutinins from an antiserum against smooth cells of *B. pertussis* (Table 2). Purified agglutinogen was not capable of protecting mice against intracranial challenge with virulent *B. pertussis*, nor was it capable of sensitizing mice to histamine (88). It is now well-accepted that this agglutinogen is not concerned with protection (102). Since most smooth cultures contain agglutinogen, it is to be expected that agglutinin formation will be correlated with immunity in animals or children that have been immunized with whole-culture vaccines (23, 24).

Although it is commonly accepted that most strains of smooth *B. pertussis* contain a serologically similar agglutinogen (9, 102), there are other factors in the cell which produce agglutinins. By agglutination tests, both Eldering (18, 19, 20) and Andersen (1) found different serological types among various strains of *B. pertussis*. 

![Diagram of typical Bordetella pertussis cell](http://mmbr.asm.org/)
TABLE 1. Agglutinin production in mice after injection of purified agglutinogen

<table>
<thead>
<tr>
<th>Group no.*</th>
<th>Dilution of serum†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
</tr>
<tr>
<td>Normal mouse serum</td>
<td>-</td>
</tr>
</tbody>
</table>

* Each group represents sera from five mice.
† Figures give reciprocal of serum dilution. Symbols: + = detectable agglutination; - = no agglutination. Data taken from reference 88.

Andersen (1) found one common thermolabile and one thermostable O antigen in all strains studied, and Eldering et al. (20) found at least three common antigens in the smooth strains tested. The substance called agglutinogen, which is highly antigenic (40, 88, 91, 107), must be one of these common factors, and should be found in most if not all smooth cultures of B. pertussis. The recent findings of Preston and Evans (80) that antisera prepared to the various agglutinogens can protect mice from intracranial challenge with strains containing the specific agglutinogen are extremely interesting. Previously, no protective activity had been associated with the agglutinogens except perhaps with factor 1 of Eldering (18).

Agglutinogen has been used as a skin-test antigen to detect susceptibility to whooping cough in children (29, 30, 31, 37, 91), to evaluate the efficacy of vaccines (29, 30, 37, 46, 65, 87, 91), and to determine the extent of protection induced by vaccines (31, 65). Flosdorf et al. (37) found that skin test in children acted as a booster and increased agglutinin titers in the blood; they reported that 99% of children from 6 to 14 months of age who had no history of exposure to whooping cough had negative skin tests, whereas 75% of those with a history of whooping cough had positive skin tests. Children with negative skin tests developed agglutinins in their blood after three intracutaneous tests (30, 37). Positive skin test was correlated with agglutinin titers in blood (31). Although these studies were encouraging and interesting, it was soon reported that the skin test was not reliable (55, 65), and it never was adopted in public health practice.

Evans (21) found a good correlation between agglutinin titer and protective effect of antibacterial sera but, as pointed out above, this is to be expected when whole-cell vaccines are employed. Actually, no relationship has been found between the protective antigen and agglutinogen (22, 48, 84, 88), and purified protective antigen does not produce high titers of agglutinins although it does produce good immunity (25, 78).

HEMAGGLUTININ

Hemagglutinin is a substance capable of agglutinating red blood cells of chickens and other animals (51, 52). This substance is produced by most, but not all, freshly isolated strains of B. pertussis. It can be found in the supernatant liquid cultures, and can be extracted easily from young agar-grown cells with 2 M sodium chloride or 1 M sodium acetate (63). The hemagglutinin is found in the early phase of growth in liquid media, and the concentration falls when the cells are still in the logarithmic-growth phase (63, 106). For these reasons, it appears that hemagglutinin is either a surface substance or a material excreted from the cell. Hemagglutinin can be removed from cell extracts by chicken red cells or red-cell stroma (97). Keogh and North (51) and Rankin and Fisher (82) believed that this substance was responsible for the protection of mice and children from B. pertussis, but this has been shown to be erroneous (63, 97). It was also thought by Keogh and North (51), as well as by

TABLE 2. Agglutinin-absorption capacity of purified agglutinogen

<table>
<thead>
<tr>
<th>Preparation*</th>
<th>Agglutinogen unit/</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starting cell suspension</td>
<td>12,800</td>
</tr>
<tr>
<td>Agglutinogen (once precipitated)</td>
<td>12,800</td>
</tr>
<tr>
<td>Agglutinogen (reprecipitated)</td>
<td>6400</td>
</tr>
</tbody>
</table>

* Agglutinogen was purified by Na sulfate fractionation as described in reference 88.
† Agglutinogen unit is defined as the amount of agglutinogen which completely absorbs the agglutinins from 0.1 ml of a 1:20 dilution of a standard antiserum (from reference 88).
Ungar (98), that hemagglutinin was related to virulence of \textit{B. pertussis}, as measured by the intranasal test, but Standfast (93, 94) could not confirm this relationship.

**Heat-Labile Toxin**

The protoplasm of the cell is a complex material which has many antigens. One of them, the heat-labile toxin, has marked biological activity (3, 8). This extremely labile substance is released upon lysis of the cells (69, 103). In practice, this substance might be responsible for the toxicity of fresh whole-cell vaccines. Since it can be destroyed by heat at temperatures which do not destroy the protective antigen (22, 69), its activity should be easily removed from these vaccines. The toxin is apparently protected from destruction when inside the cells, because toxic activity persists for some time after cells are harvested and killed with Merthiolate. Once the toxin is released from the cells by lysis, it is destroyed rapidly and is difficult to maintain in an active form. Not much is known about the nature of the destruction of the toxin, except that there is a reduction in molecular weight as the activity is reduced (Palmstierna and Milner, \textit{personal communication}). The toxin has been purified considerably by means of DEAE-cellulose column chromatography (Table 3). Although the toxin is considered by some (105) to be deoxyribonucleoprotein, Banerjea and Munoz (3) found it to be a protein, since it gives a protein ultraviolet absorption (Fig. 5), has a high N content, and is destroyed by the proteolytic enzyme trypsin but not by deoxyribonuclease or ribonuclease (Table 4; 6, 69, 96). The purified preparations do not sensitize mice to histamine, and the toxin does not protect mice against intracranial challenge with \textit{B. pertussis} (2, 69, 100). The role of the toxins (heat-labile or heat stable toxin) in immunization of mice against intranasal challenge, however, should be carefully considered. It has been the experience of some workers that two different antigens may be involved in the immunization of mice by intracranial and intranasal challenge (12, 13), and that the antigen involved in protection against intracranial challenge is heat-labile, whereas that involved in intranasal-challenge protection is

**TABLE 3. Mouse toxicity of fractions obtained from DEAE-cellulose columns**

<table>
<thead>
<tr>
<th>Fraction*</th>
<th>Toxicity†</th>
</tr>
</thead>
<tbody>
<tr>
<td>First eluate</td>
<td>0/15</td>
</tr>
<tr>
<td>Washings with buffer</td>
<td>0/15</td>
</tr>
<tr>
<td>I</td>
<td>0/15</td>
</tr>
<tr>
<td>II</td>
<td>0/15</td>
</tr>
<tr>
<td>III</td>
<td>0/15</td>
</tr>
<tr>
<td>IV</td>
<td>0/15</td>
</tr>
<tr>
<td>Fractions with NaCl gradient</td>
<td>0/15</td>
</tr>
<tr>
<td>1</td>
<td>0/15</td>
</tr>
<tr>
<td>2</td>
<td>0/15</td>
</tr>
<tr>
<td>3</td>
<td>0/15</td>
</tr>
<tr>
<td>4</td>
<td>0/15</td>
</tr>
<tr>
<td>5</td>
<td>12/15</td>
</tr>
<tr>
<td>6</td>
<td>14/15</td>
</tr>
<tr>
<td>7</td>
<td>13/15</td>
</tr>
<tr>
<td>8</td>
<td>9/15</td>
</tr>
<tr>
<td>9</td>
<td>3/9</td>
</tr>
<tr>
<td>10</td>
<td>0/9</td>
</tr>
</tbody>
</table>

* A portion (0.5 ml) of fraction was given intraperitoneally to each mouse.
† Expressed as no. of deaths/total no. tested.
Data taken from reference 3.

\textbf{FIG. 5. Spectrophotometric readings of protoplasm and purified heat-labile toxins from \textit{Bordetella pertussis} (from reference 3).}
TABLE 4. Effect of trypsin, deoxyribonuclease, and ribonuclease on toxicity of whole protoplasm of Bordetella pertussis

<table>
<thead>
<tr>
<th>Protoplasm</th>
<th>Enzyme</th>
<th>Toxicity of mixture†</th>
</tr>
</thead>
<tbody>
<tr>
<td>µg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>Trypsin</td>
<td>8/9</td>
</tr>
<tr>
<td>100</td>
<td>Trypsin control</td>
<td>0/0</td>
</tr>
<tr>
<td>100</td>
<td>Deoxyribonuclease</td>
<td>8/9</td>
</tr>
<tr>
<td>100</td>
<td>Deoxyribonuclease control</td>
<td>0/3</td>
</tr>
<tr>
<td>100</td>
<td>Ribonuclease</td>
<td>8/9</td>
</tr>
<tr>
<td>100</td>
<td>Ribonuclease control</td>
<td>0/3</td>
</tr>
</tbody>
</table>

* Each mouse received intraperitoneally 0.5 ml of test material containing 100 µg of protoplasm and either 14 µg of trypsin or 7 µg of deoxyribonuclease or ribonuclease. Controls received either protoplasm alone or enzyme alone in the same volume.

† Expressed as no. of deaths/total no. tested. Data taken from reference 3.

heat-stable (95). Large doses of B. pertussis are usually required to kill mice challenged by the intranasal route (14), and it is probable that during growth within the animal these bacteria produce enough toxins to kill the mice. However, no relationship was found by Standfast between intranasal virulence and heat-labile toxin production (93, 94).

It is conceivable that this very toxic heat-labile substance may play an important role in the symptomatology of whooping cough (8, 15, 42, 92), and that antitoxin in the blood of children may protect them from the ill effects of the toxin released during infection. The toxin, however, most likely does not play any significant role in the prophylaxis of whooping cough.

HEAT-STABLE TOXIN (ENDOTOXIN)

Substances which have similar properties to the Boivin lipopolysaccharide-protein complexes isolated from the enteric group of bacteria have also been isolated from B. pertussis (11, 16, 17, 26, 27, 36, 40, 58).

Cruickshank and Freeman (11) isolated a polysaccharide from B. pertussis that protected mice challenged intraperitoneally. Eldering (17) isolated lipopolysaccharides from various strains of Bordetella and obtained products similar to the endotoxins of other gram-negative bacteria. She employed the trichloroacetic acid method of Boivin et al. (7) or the hydrochloric acid or trypic-digestion method of Felton and Kauffman (28). MacLennan (58) recently described the isolation and characterization of endotoxin from Bordetella species, including B. pertussis. The lipopolysaccharide from this species was extracted by the phenol method of Westphal et al. (101). It was found that B. pertussis endotoxin contained 8.2% nitrogen, 2.6% phosphorus, 0.6% nucleic acid, 18% aldehydose, 16.5% hexosamine, and 5% hexose. The lipopolysaccharide constituted approximately 3.4% of the cell. These preparations reacted with specific immune serum and were not toxic to rabbits and mice in 1-mg amounts. The preparation was pyrogenic, despite its relative lack of toxicity; the pyrogenic dose in rabbits was in the order of 2.5 µg/kg of body weight.

Farthing (26) and Farthing and Holt (27) recently studied the adjuvant activity of lipopolysaccharide preparations from B. pertussis. It is well-known that endotoxins from other gram-negative bacteria have a pronounced stimulatory effect on antibody production (10, 49). It is also known that whole B. pertussis cells have similar activity (43, 44, 53, 62, 68). The endotoxin preparations of Farthing and co-workers were made by the phenol method of Westphal et al. (101), and were active in stimulating antibody response in doses of from 0.5 to 5 µg, which compares favorably with the results obtained by others with endotoxin preparations from other microorganisms (49). Lipid A prepared by acid hydrolysis of the lipopolysaccharide was also active, but to a much lesser extent. Even 150 µg of lipid A did not produce as good a response as 0.5 µg of lipopolysaccharide (26).

Although MacLennan (58) found that the lipopolysaccharide from the strain of B. pertussis that he employed was not toxic for mice in 1-mg amounts, in the present tests endotoxin from another strain was prepared which was toxic to mice in doses of 200 to 300 µg given intraperitoneally. The method used to make these preparations was the trichloroacetic acid method of Boivin et al. (7). In addition, it was found that this preparation did not protect mice from intracranial challenge with virulent B. pertussis nor sensitize mice to histamine (Munoz and Schuchardt, unpublished data). Malmgren and Ribi (personal communication) obtained endotoxins from B. pertussis by the phenol method of Westphal et al. (101), and found them to be toxic.
and pyrogenic. Their preparations also failed to protect mice from intracranial challenge with *B. pertussis* and to sensitize mice to histamine. The chemical composition was very similar to that of other endotoxins from gram-negative bacteria. In addition, they found that the toxicity of their preparations depended to a large extent on the strain of *B. pertussis* used and on the medium in which the cells were grown.

**Protective Antigen and Histamine-Sensitizing Factor (HSF)**

The most important, and thus far the most elusive, antigen in *B. pertussis* is the protective antigen. This substance is now known to be a cell-wall component (6, 69, 106). The antigen can be obtained in solution by various methods (4, 13, 32, 41, 61, 71, 78), from either cell walls or whole cells. Pillemer et al. (78) prepared the protective antigen free from toxic material and with little or no agglutininogen. This material represented only a minute proportion of the organism. The antigen was prepared from sonically disrupted cells; these cells liberated the protective antigen as a "soluble" (?) material which could then be selectively adsorbed onto human red-cell stroma. This adsorption was irreversible. The stroma-adsorbed protective antigen (SPA) was found to be highly effective in protecting mice (25, 78). It was also found to possess histamine-sensitizing activity (60, 78). In attempts to reproduce the findings of Pillemer et al., with the strain of *B. pertussis* used, satisfactory results could not be obtained. The prolonged sonic treatment was deleterious to the protective activity; the centrifugation of sonically disrupted cells at 1935 × g for 2 hr did not clear the preparations of cell fragments as judged by the turbidity of supernatants; and the sedimented material contained most of the protective activity, especially when the supernatants were completely cleared by faster centrifugation. As Pillemer et al. (78) stated, however, the described "...optimal conditions for production of P. A. relate entirely to strain 134; if other strains are used, the optimal conditions may be different." Since it has been shown that the protective antigen and the HSF are located in the cell wall (6, 69, 106), one wonders whether the preparations of SPA did not consist mainly of cell-wall fragments absorbed or trapped by the red-cell stroma. At best, the method of Pillemer et al. (78) would not be suitable for the identification of the protective antigen, since the absorbed antigen could not be easily dissociated from the red-cell stroma.

It was, then, desirable to find other methods of obtaining soluble preparations from which to purify the protective antigen. As stated above, many workers have obtained preparations of the protective antigen in solution (5, 32, 41, 45, 61, 66, 77), but the methods have usually been inefficient (66). Recently, Barta (4) reported a method, employing sodium deoxycholate, to dissolve the *B. pertussis* cells, which in his hands seems to have produced complete dissolution of the protective antigen. In 1956, we (Sagin, Tulis, and Munoz, *unpublished data*) tried an almost identical method with some degree of success; however, recovery of protective antigen was at times not encouraging. These soluble preparations were also active in sensitizing mice to histamine. Millman et al. (66) found this method unsuitable, however, since in their hands the protective antigen was destroyed.

Whole live cells (1000 billion/ml) + 3 volumes of acetone, filtered

Cell paste, 3 volumes of acetone (same volume as before) added, filtered

Cell paste, dried and finely ground

To 15 g of dried cells, 500 ml of saline added, homogenized and pressurized, 500 ml of saline again added, pH adjusted to 8.5, incubated overnight at 2 to 5 C, centrifuged at 27,000 × g for 40 min

Supernatant is saline Sediment re-extracted two more times, and supernatants pooled with SE

**FIG. 6. Outline of procedure used for preparing saline extracts (SE) from Bordetella pertussis.**
Recently it has been possible to extract from acetone-dried cells considerable amounts of the protective antigen in what appears to be true solution (70, 71). Owing to the importance of this antigen, details will be given of the methods which have been employed in its extraction and purification.

Preparation of Saline Extract

The procedure followed is outlined in Fig. 6. Cells were extracted twice with 3 volumes of acetone at room temperature and then freed of acetone. These acetone-dried cells were suspended in saline, homogenized, and ruptured in the pressure cell of Ribi et al. (83). The ruptured-cell suspension was adjusted to pH 8.5, kept cold and constantly stirred for 1 to 2 hr, and stored overnight at 2 to 5 C. It was then centrifuged for 40 min at 27,000 × g in a Servall refrigerated centrifuge. The clear amber supernatant fluid was dialyzed against repeated changes of cold distilled water, and was lyophilized. This dried saline-extracted material (SE) was used as starting material for further fractionation. The sediment after removal of the SE still contained considerable amounts of protective and HSF activity. Re-extraction of this sediment twice, with saline at pH 8.5, removed most of the protective and HSF activity from the cells. The SE from the first extraction was analyzed for some of its chemical constituents. It was found to contain 14.6% nitrogen, 4.5% hexose, 0.4% hexosamine, 1.8% phosphorus, and 28.4% fatty acid esters plus fatty acid amides (as palmitic acid).

The SE can be further purified by the use of starch block electrophoresis, curtain electrophoresis, or cellulose column chromatography. Starch block electrophoresis was the most promising of these methods. Unfortunately, however, the fractions obtained by this method were contaminated with material from the starch, making the chemical identification of the substance rather difficult. The results obtained from the starch block electrophoresis were, nevertheless, interesting, because they showed the com
plexity of the SE and the possibility of separating the protective antigen from other cell components. Figure 7 illustrates the results obtained with starch block electrophoresis in pH 6.2 phosphate buffer (µ = 0.024) at 10 ma and 310 v. The curve shows seven tyrosine peaks. HSF and protective activities were found in fractions 12, 13, 14, and 15. No activity was found in fractions 1 through 11 or 18 through 26.

The results obtained with gel diffusion tests on all these starch block fractions are illustrated in Fig. 8. At least 12 antigenic materials were found in these fractions. Only one band seems to correlate with protective and HSF activities, but no absolute proof that this band is due to the protective antigen has yet been obtained. It was concluded that considerable purification was achieved by electrophoresis in starch.

When electrophoresis was performed at a higher pH, the HSF moved toward the anode and spread through a rather broad zone. The protective antigen behaved in identical fashion (Fig. 9).

As the protective antigen was purified, it seemed to become somewhat insoluble and perhaps also unstable. It was extremely interesting that protective antigen and the HSF could not be dissociated from each other. Every fraction in which HSF activity was demonstrated also contained protective activity. In fact, by studying the purification of HSF, it was found that the two activities were in the same fraction of the B. pertussis cell. Both had the same electrophoretic mobility, could be precipitated at pH 4.9, and were precipitated by the same concentration of acetone or alcohol. Both are found in the cell walls, and the potency of vaccines is correlated with the HSF activity. Differences in stability of the two factors have been reported by various investigators (13, 56, 61, 79).

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**FIG. 8.** Ouchterlony-type gel diffusion test with various starch block electrophoresis fractions. Anti-Bordetella pertussis rabbit serum in center row of wells and different fractions in outside wells (from reference 71).
ACTIVITIES OF B. PERTUSSIS ANTIGENS

The partial separation obtained by some workers (13) might be explained by differences in stability of the two activities in the same complex molecule, or by a dependence of one activity on a certain degree of polymerization or rearrangement of the molecular species involved. The two activities are lost by a variety of treatments, and some treatments seem to affect one activity more readily than the other (13, 79, 81). The two activities apparently can be separated by preferential destruction of one, while leaving the other intact or only slightly affected. It should be emphasized that the histamine-sensitization phenomenon is not due to the production of sensitizing antibodies (33, 60), but rather to some still not clearly understood physiological effect on the susceptible mouse. Protective activity, on the other hand, is dependent on the stimulation of the immune mechanisms, and can be demonstrated in various strains of mice whether they are susceptible to HSF or not.

HSF activity is destroyed by heating the preparations (54, 60, 72) at 75 C for 0.5 hr. The two activities apparently can be separated by preferential destruction of one, while leaving the other intact or only slightly affected. It should be emphasized that the histamine-sensitization phenomenon is not due to the production of sensitizing antibodies (33, 60), but rather to some still not clearly understood physiological effect on the susceptible mouse. Protective activity, on the other hand, is dependent on the stimulation of the immune mechanisms, and can be demonstrated in various strains of mice whether they are susceptible to HSF or not.

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The table below shows the effect of heat on protective and HSF activity of saline-extractable material (SE) from acetone-dried cells.

<table>
<thead>
<tr>
<th>Temp °C</th>
<th>Protective activity</th>
<th>HSF activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dose (in μg)</td>
<td>Dose (in μg)</td>
</tr>
<tr>
<td>20</td>
<td>6/15</td>
<td>6/14</td>
</tr>
<tr>
<td>70</td>
<td>11/14</td>
<td>10/15</td>
</tr>
<tr>
<td>80</td>
<td>14/15</td>
<td>13/15</td>
</tr>
<tr>
<td>Unheated</td>
<td>2/13</td>
<td>3/14</td>
</tr>
</tbody>
</table>

* Preparations were heated for 0.5 hr at indicated temperatures.
* Mice received intraperitoneally the dose indicated, and 14 days later were challenged intracranially with 40,000 virulent Bordetella pertussis cells. Results were recorded 14 days later.
* Mice received intraperitoneally the dose indicated, and 4 days later were challenged intraperitoneally with 0.5 mg of histamine. Results were recorded 2 hr later.
* No. of deaths/total no. tested.
With SE, the heat susceptibility of both activities was found to be similar (Table 5). Formalin treatment at 37°C also destroys the HSF activity (64) and reduces the protective activity (Table 6).

The toxicity of the protective antigen is not great, and it may actually be rather nontoxic. Although no data have been obtained on the direct toxicity of this substance, it is known that the materials from which it is extracted are only slightly toxic, when compared with the toxicity of whole cells. A comparison of the toxicity of various materials obtained during the preparation of SE is given in Table 7. The simple treatment of the cells with acetone is sufficient to destroy most of the toxic factor in the whole cells. This factor is most likely the heat-labile toxin, which has been shown before to be detoxified by various solvents (such as alcohol) which detoxify cell extracts even in the cold (76). There was some toxicity left in the cells, and this toxic material seems to become soluble as the extractions are increased. It is suspected that this toxicity is due to the heat-stable toxin which is also found in the B. pertussis cell (16, 26, 27, 36, 40, 58).

Preparations of purified HSF or protective antigen possess many of the biological activities described for the whole cells. These preparations possess the ability to sensitize mice to anaphylaxis (both actively and passively induced), to increase the permeability of the capillaries to Evans Blue and to increase the antibody response to antigens administered with it. It remains to be seen whether on further purification these biological activities can be dissociated.

**Summary**

*B. pertussis* has many antigens distributed in its various morphological elements. Some of these substances have important biological activities which have long been recognized. The most external antigens are the so-called agglutinogen and the hemagglutinin. The cell walls seem to contain the heat-stable toxin, the protective antigen, and the histamine-sensitizing factor, while the heat-labile toxin and various other unknown antigens are present in the protoplasm. It appears that all of these antigens are distinct entities, most of which have been obtained in a purified form. The so-called histamine-sensitizing factor, appears to be closely associated with, if not identical to, the protective antigen.

**Literature Cited**


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**Table 7. Toxicity of *Bordetella pertussis* cells and various fractions**

<table>
<thead>
<tr>
<th>Material</th>
<th>Toxicity (LD&lt;sub&gt;50&lt;/sub&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole cells</td>
<td>45</td>
</tr>
<tr>
<td>Acetone-dried cells</td>
<td>500</td>
</tr>
<tr>
<td>Saline extract, 1st</td>
<td>519</td>
</tr>
<tr>
<td>Saline extract, 2nd</td>
<td>283</td>
</tr>
<tr>
<td>Saline extract, 3rd</td>
<td>283</td>
</tr>
<tr>
<td>Sediment</td>
<td>&gt;800</td>
</tr>
</tbody>
</table>

* Mice received intraperitoneally 0.2 ml of the material dissolved in buffered saline (pH 7.2).

---

**Table 6. Effect of formalin and Merthiolate on HSF and protective activity of SE<sup>a</sup>**

<table>
<thead>
<tr>
<th>Dose (μg/mouse)</th>
<th>HSF activity&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Protective activity&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Merthiolate</td>
<td>Formalin</td>
</tr>
<tr>
<td>20</td>
<td>8/10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0/10</td>
</tr>
<tr>
<td>40</td>
<td>10/10</td>
<td>1/9</td>
</tr>
<tr>
<td>80</td>
<td>8/10</td>
<td>1/10</td>
</tr>
<tr>
<td>160</td>
<td>9/10</td>
<td>0/8</td>
</tr>
<tr>
<td>Control</td>
<td>0/9</td>
<td>13/13</td>
</tr>
</tbody>
</table>

*<sup>a</sup> Saline extract (400 μg/ml) was incubated in presence either of 0.5% formalin or 1:10,000 Merthiolate (Eli Lilly & Co., Indianapolis, Ind.) for 7 days at 37°C.

*<sup>b</sup> Mice received intraperitoneally the dose indicated, and 4 days later were challenged intraperitoneally with 0.5 mg of histamine. Results were recorded 2 hr later.

*<sup>c</sup> Mice received intraperitoneally the dose indicated, and 14 days later were challenged intracranially with 40,000 virulent *Bordetella pertussis* cells. Results were recorded 14 days later.

*<sup>d</sup> No. of deaths/total no. tested.


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