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Structure and Biological Properties of Solubilized Envelope Proteins of *Bordetella pertussis*

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The structure and biological properties of solubilized envelope proteins of *Bordetella pertussis* have been examined. Several envelope proteins were found to be specific for phase I strains of *B. pertussis* and could be isolated by selective detergent extraction. These proteins had molecular weights of 90,000, 86,000, 81,000, 33,000, 31,000, and 30,000 and were reduced or absent in envelope preparations from *Bordetella bronchiseptica, Bordetella parapertussis*, or phase IV strains of *B. pertussis*. When the envelope preparations from phase I *B. pertussis* were assayed in the mouse intracerebral protection test they were found to be highly protective, and there was a strong correlation between the protective potency and the lymphocytosis-promoting factor (LPF) content of different preparations. Treatment with glutaraldehyde reduced the LPF activity, toxicity, and protective potency of the envelope extracts. Similarly affinity chromatography of envelope proteins on columns of haptoglobin coupled to Sepharose 4B reduced both the LPF content and the protective potency. The addition of a small amount of purified LPF to the haptoglobin-treated proteins restored the protective potency. The LPF by itself was nonprotective, indicating a potentiating role of LPF in the mouse intracerebral challenge test.

Extensive research has been carried out over the past 20 years into the isolation and characterization of the immunologically and pharmacologically active components of *Bordetella pertussis*. The nature of the antigens involved in the protection of mice against intracerebral challenge with *B. pertussis* or of children against whooping cough, however, is still a matter of discussion. With a view to resolving some of these problems a study of the envelope proteins of *B. pertussis* has been undertaken. Parton and Wardlaw (24) showed that the envelope protein profile of *B. pertussis*, when examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), was dependent on the phenotypic mode of the organism: X-mode virulent organisms possessed two proteins in the 30,000-molecular-weight region which were not found in their avirulent C-mode counterparts. Similar changes were observed between the phase I (virulent) and phase IV (avirulent) genotypes. These differences have been subsequently confirmed, and additional differences in the higher-molecular-weight region have been observed (7, 8, 26). The X-mode or phase I-specific proteins have been further examined in this study.

The proteins isolated from envelopes of *B. pertussis* using the dipolar ionic detergent Empigen BB or sodium deoxycholate (DOC) were found to be highly protective in mice against intracerebral infection (25, 26) and in rabbits against respiratory infection with *B. pertussis* (3). Histamine-sensitizing factor (HSF) and lymphocytosis promoting factor (LPF) activities were also found in the envelope extracts. Munoz et al. (19, 20) claimed that a single substance, termed pertussigen, which possesses the activities of and is equivalent to HSF, LPF, and islets-activating protein is the most important antigen for the immunization of mice against intracerebral infection with *B. pertussis*. Purified LPF, however, is toxic to mice and, at doses tolerated by the mice, nonprotective (13, 28). The protective activity of LPF can be expressed after treatment with glutaraldehyde to reduce the toxic activities (19, 20). In an attempt to resolve some of these problems the biological manifestations of pertussigen (sensitization to histamine and induced lymphocytosis) and the mouse protective activities of various preparations derived from the envelopes of *B. pertussis* have been examined. Various treatments, such as glutaraldehyde and high pH, and purification proce-

dures have been employed to try and dissociate the biological activities of the envelope preparations.

MATERIALS AND METHODS

Bacterial strains and cultural procedures. *B. pertussis* strain Wellcome 28 (kindly donated by P. Novotny, Wellcome Research Laboratory, Beckenham, Kent) was used predominantly throughout, except where indicated (Fig. 1). The cultures were maintained as freeze-dried ampoules and recovered by growth on charcoal agar plates containing 10% (vol/vol) defibrinated horse blood. Cells from plates were inoculated into 100 ml of medium in 250-ml conical flasks and then incubated at 35°C for 24 h on an orbital shaker (180 rpm). Medium (300 ml) in Thompson bottles was inoculated with 10 ml of primary culture and shaken on a gently reciprocating shaker at 35°C for 16 to 24 h for *Bordetella bronchiseptica* and *Bordetella parapertussis* or for 36 to 40 h for *B. pertussis* and *B. parapertussis*. Occasionally cultures of *B. pertussis* were grown without shaking for 5 days at 35°C. The medium of Sato et al. (27) was mainly used, but that of Lane (17) or Stainer and Scholte (29) was occasionally used. After growth, cells were harvested by centrifugation and stored at −20°C for subsequent envelope preparation.

Preparation of envelopes and extracts. Envelopes were prepared by Braun homogenization and differential centrifugation and then extracted with Empigen BB (Marchon Division, Albright & Wilson, Whitehaven) for 90 min at 37°C as described previously (25, 26) or with 0.05 M Tris-hydrochloride–1 M NaCl, pH 8.0 (Tris-NaCl buffer), for 30 min at 4°C (25). Outer membrane proteins were prepared as follows: a suspension of envelopes (approximately 10 mg of protein per ml) was mixed with an equal volume of 1% (wt/vol) sodium lauryl sarcosinate (SLS) in 0.1 M Tris-hydrochloride, pH 8.0, and incubated at room temperature for 1 h. The suspension was then centrifuged at 17,500 × g for 30 min at 4°C. Approximately 30% of the protein was solubilized by the SLS. The pellet was dispersed in water to about 80% the original volume of envelope suspension and extracted, by shaking at 37°C for 90 min, with an equal volume of 1.2% (vol/vol) Empigen BB in 0.1 M phosphate buffer, pH 8.0. This extracted about 20 to 30% of the remaining envelope proteins, and the extract obtained by centrifugation was enriched in the phase I-specific proteins. Alternatively, direct Empigen BB extracts of envelopes were dialyzed at 4°C against approximately 100 volumes of 0.05 M phosphate–0.5 M NaCl, pH 6.5. After usually about 24 h of dialysis (two to three changes), a precipitate was formed which, when recovered by centrifugation and dispersed in 0.05 M phosphate–0.5 M NaCl, pH 6.5, accounted for approximately 20% of the original Empigen BB extract protein. The precipitated proteins were also enriched in the phase I-specific proteins.

Column chromatography. To separate the protein and lipopolysaccharide (LPS) components of Empigen BB extracts the detergent had first to be changed to DOC as the components were not satisfactorily resolved by chromatography in the presence of Empigen BB. The extracts were thus pressure dialyzed overnight against water and then dispersed to approximately half the original volume in 1% (wt/vol) DOC in 0.05 M phosphate buffer, pH 8.0, and dialyzed against this buffer for 2 to 3 h. A sample (5 ml; 3 to 5 mg of protein per ml) was run on a Sephadex G75 or G100 column (45 by 2.5 cm) equilibrated in 0.05 M phosphate, pH 8.0, containing 0.5% (wt/vol) DOC. The fractions collected were analyzed for absorption at 280 nm and protein and LPS concentration and also by SDS-PAGE. The protein and LPS peaks were pooled and concentrated by pressure dialysis.

The LPF content of envelope protein preparations was reduced by affinity chromatography on haptoglobin columns. The envelope proteins were prepared by Empigen BB extraction followed by chromatography on Sephadex G75 in the presence of DOC. The protein was then concentrated by pressure dialysis and dispersed in 0.6% (vol/vol) Empigen BB in 0.05 M phosphate, pH 8.0, to about 5 mg of protein per ml. The preparation was dialyzed overnight against 0.05 M phosphate–0.5 M NaCl, pH 6.5. Any precipitated protein (less than 10% total) was removed by centrifugation (17,500 × g, 30 min, 4°C). Approximately 3 ml of supernatant was loaded onto a column (10-ml glass pipette) of human haptoglobin coupled to CNBr-activated Sepharose 4B (14) equilibrated with 0.05 M phosphate–0.5 M NaCl, pH 6.5. The protein was allowed to remain on the column for 1 h and then eluted. The fractions absorbing at 280 nm were pooled, concentrated by pressure dialysis, and dialyzed against phosphate-buffered saline. The eluted protein was occasionally passed through a second haptoglobin–Sepharose 4B column. LPF could be eluted from the column with 0.05 M Tris-hydrochloride–1 M potassium thiocyanate, pH 10.

Purification of LPF. LPF was purified from cell disintegrates (Dynomill or Braun homogenization) of *B. pertussis* strain Wellcome 28 by the haptoglobin affinity method of Irons and MacLennan (14).

Glutaraldehyde treatment of envelope preparations. The technique of Munoz et al. (20) for the glutaraldehyde detoxification of pertussigen (LPF) was basically employed. Tris-NaCl extracts of *B. pertussis* envelopes were dialyzed against 0.02 M phosphate–0.5 M NaCl, pH 7.4, for 18 h, and 1.7 ml of 0.3% (wt/vol) glutaraldehyde in 0.02 M phosphate–0.5 M NaCl, pH 7.4, was added to 5 ml of dialyzed extract (200 to 400 μg of protein per ml). After incubation at room temperature for 2 h, 0.7 ml of 0.3 M L-lysine in the same buffer was added, and the preparation was incubated for a further 2 h at room temperature before dialysis at 4°C four times against 250 ml of 0.02 M phosphate–0.5 M NaCl, pH 7.4, containing 0.02 M L-lysine and then once against 250 ml of the buffer without L-lysine.

Similarly envelope proteins extracted with Empigen BB were also treated with glutaraldehyde. The envelope proteins were separated from LPS by Sephadex G75 chromatography and dialyzed for 24 h against 0.02 M phosphate–0.5 M NaCl, pH 7.4. A 4-ml sample of the protein suspension (0.73 mg of protein per ml) was treated with 20 μl of 25% glutaraldehyde for 2 h at room temperature. A 0.5-ml amount of 0.2 M L-lysine in 0.02 M phosphate–0.5 M NaCl, pH 7.4, was added, and the preparation was incubated for a further 2 h at room temperature before dialysis against 0.02 M phosphate–0.5 M NaCl–0.02 M L-lysine, pH 7.4, for 48 h.

Analytical techniques. Analytical SDS-PAGE was performed as described previously (25), with the fol-

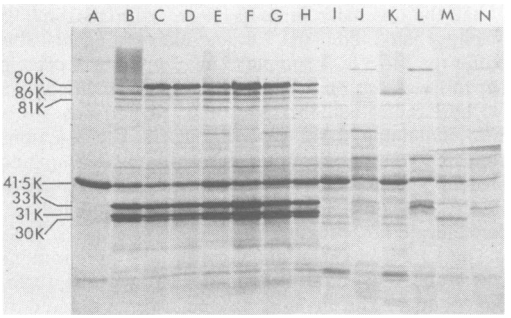

FIG. 1. SDS-PAGE of outer membrane proteins from various strains of *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*. Empigen BB extracts of SLS-insoluble envelopes were treated with SDS and run on SDS-PAGE as described in the text. Lanes: (A) *B. pertussis* 134 phase IV, (B) *B. pertussis* Wellcome 28, (C) *B. pertussis* 360E, (D) *B. pertussis* Tohama, (E) *B. pertussis* D6229, (F) *B. pertussis* M2, (G) *B. pertussis* 134 phase I, (H) *B. pertussis* NIH 114, (I) *B. bronchiseptica* NCTC 8344, (J) *B. parapertussis* NCTC 8250, (K) *B. bronchiseptica* APM 21 (isolated from grivet monkey), (L) *B. parapertussis* NCTC 7385, (M) *B. bronchiseptica* NCTC 452, (N) *B. parapertussis* NCTC 10522. The molecular weights of the major proteins are shown on the left.

lowing modifications. (i) The samples were not dialyzed against Tris-NaCl buffer before electrophoresis, but treated directly with SDS at 100°C. (ii) The concentration of acrylamide in the resolving gel was adjusted to 12% (wt/vol), and that of bisacrylamide was adjusted to 0.1% (wt/vol) to increase the resolution in the 30,000-molecular-weight region. Low-molecular-weight standard proteins (Bio-Rad Laboratories) were used as molecular weight markers. Gels were stained with Coomassie blue R250 (9).

Protein concentrations of envelope suspension were estimated by the biuret method (12), and protein concentrations of extracts were estimated by an automated Lowry method. The LPS content of extracts and column fractions was determined by using an inhibition of hemagglutination assay as follows. Doubling dilutions (0.1 ml) in phosphate-buffered saline of the preparation to be tested were mixed with a 1:50 dilution of rabbit antiserum raised against whole cells of *B. pertussis* strain 360E and incubated at room temperature for 1 h. A 0.2-ml sample of a 0.5% (vol/vol) suspension of goose erythrocytes sensitized with hot phenol-extracted *B. pertussis* LPS (31) was added. After 16 h of incubation at 4°C the tests were read, and the hemagglutination was scored at 0 to 4. Inhibition titers are expressed as the reciprocals of the final dilutions of the preparation causing inhibition of hemagglutination.

Biological assays. The mouse intracerebral challenge test (15) was used as described previously (26) to determine the mouse 50% protective dose values of the various preparations after adsorption onto Alhydrogel (Superfos Export Company A/S).

LPF activities of extracts were determined in CF-1 mice as described by Morse and Morse (18), and the amount of protein required to double the leukocyte count after 3 days (compared with phosphate-buffered saline-vaccinated controls) was calculated.

HSF activities of envelope extracts were determined in NIH (Dubby) mice. Extracts (0.5 ml), diluted in phosphate-buffered saline, were injected intraperitoneally into groups of 20 mice. After 3 days the mice were challenged intraperitoneally with 1 mg of histamine-free base as described by Morse and Morse (18). The amount of protein required to sensitize 50% of mice to a lethal challenge of 1 mg of histamine was determined.

The toxicity of extracts was determined by injecting 0.5 ml of suitable dilutions of the extracts in phosphate-buffered saline intraperitoneally into groups of 20 NIH mice. Deaths were recorded after 3 days, and 50% lethal dose values were calculated.

RESULTS

Preparation of solubilized envelope protein extracts. Cell envelopes were prepared from several strains of *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*. The envelope proteins were usually solubilized directly with Empigen BB, which extracts mainly the phase I-specific proteins (25, 26). Alternatively, the envelopes were extracted with SLS, and then the insoluble material was extracted with Empigen BB. By analogy with other gram-negative bacteria, SLS solubilized principally the cytoplasmic membrane

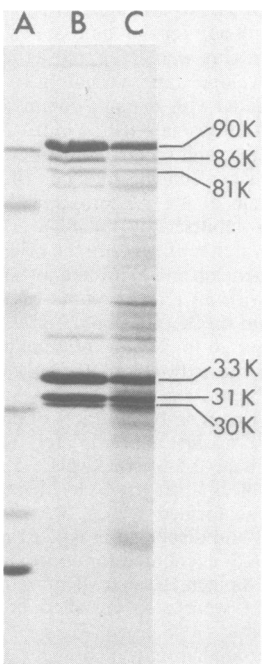

FIG. 2. SDS-PAGE of *B. pertussis* phase I-specific outer membrane proteins. Lanes: (A) molecular weight standards, (B) precipitate obtained by dialysis of Empigen BB envelope extract against phosphate-NaCl buffer, (C) Empigen BB envelope extract.

TABLE 1. Biological activities of envelope protein preparations of *B. pertussis*

Envelope prepn	μg of protein/mouse ± SEM (*n*)				% of protein solubilized
	PD_{50}[a]	HD_{50}[b]	WBC × 2[c]	LD_{50}[d]	
Empigen BB extract	2.1 ± 1.2 (42)	15.8 ± 1.4 (9)	11.1 ± 1.9 (5)	371 ± 1.1 (4)	30–40
Empigen BB protein[e]	2.4 ± 1.3 (11)	8.4 ± 1.4 (7)	24.2 ± 7.8 (8)	761 ± 1.1 (5)	
Tris-NaCl buffer extract	0.67 ± 1.4 (12)	0.91 ± 1.5 (7)	1.7 ± 0.4 (3)	31.6 ± 1.9 (2)	3–5

[a] PD_{50}, Dose that protected 50% of the mice challenged intracerebrally.
[b] HD_{50}, Dose that sensitized 50% of the mice to lethal challenge of 1 mg of histamine.
[c] WBC × 2, Dose required to double the leukocyte count of the mice.
[d] LD_{50}, Dose lethal to 50% of the mice.
[e] Protein prepared from Empigen BB extracts by Sephadex G75 chromatography in the presence of DOC.

proteins (10, 11); the phase I-specific outer membrane proteins were then extracted with Empigen BB. In our hands, as has been found by others (8), the separation of *B. pertussis* outer and inner membranes by sucrose density gradient techniques proved unsatisfactory. Figure 1 shows the SDS-PAGE profiles of SLS-insoluble, Empigen BB-soluble proteins of envelopes from seven phase I strains of *B. pertussis*, one phase IV strain of *B. pertussis*, three *B. parapertussis* strains, and three *B. bronchiseptica* strains. The main points to observe from Fig. 1 are as follows. (i) Certain peptides are specific to phase I *B. pertussis* when compared with phase IV strains. These include three major proteins with molecular weights of 33,000, 31,000, and 30,000 and also three slower-migrating proteins with molecular weights of 90,000, 86,000, and 81,000. (ii) The phase I-specific proteins have constant molecular weights irrespective of the strain of *B. pertussis*. In fact, for 18 strains of phase I *B. pertussis* the molecular weights of these proteins, as determined by this gel electrophoresis system, were constant (data not shown). (iii) Other proteins also have constant molecular weights irrespective of strain. The major envelope protein of *B. pertussis* had a molecular weight of 41,500, except in one phase IV strain where the molecular weight was 43,000. (This protein is poorly extracted by Empigen BB, except in phase IV cell envelopes, and in Fig. 1 appears as a minor protein. The molecular weight estimations of this protein were made on total envelope proteins in SDS.) (iv) *B. parapertussis* and *B. bronchiseptica* have envelopes with distinct protein profiles. The region around 30,000 molecular weight showed only weak protein components. Bands equivalent to the *B. pertussis* phase I-specific proteins have been reported for *B. bronchiseptica* and *B. parapertussis*, but in lower amounts (8). In Fig. 1 the *B. bronchiseptica* and *B. parapertussis* extracts were more similar to phase IV than to phase I *B. pertussis* extracts. The major envelope protein had a molecular weight of 42,000 for *B. parapertussis* and 43,000 for *B. bronchiseptica*, which was slightly higher than that for the *B. pertussis* major envelope protein. (v) Certain proteins were common to all preparations from *Bordetella* envelopes (for example, proteins with molecular weights of 77,000, 63,000, and 49,000).

An alternative method of preparing phase I-specific proteins is illustrated in Fig. 2. Envelopes were extracted with Empigen BB, and the extracts were dialyzed at 4°C against 0.05 M phosphate–0.5M NaCl, pH 6.5, to selectively precipitate proteins by gradual removal of detergent. A precipitate appeared after overnight dialysis which may account for 20% of the total protein. The precipitate (Fig. 2B) was strongly

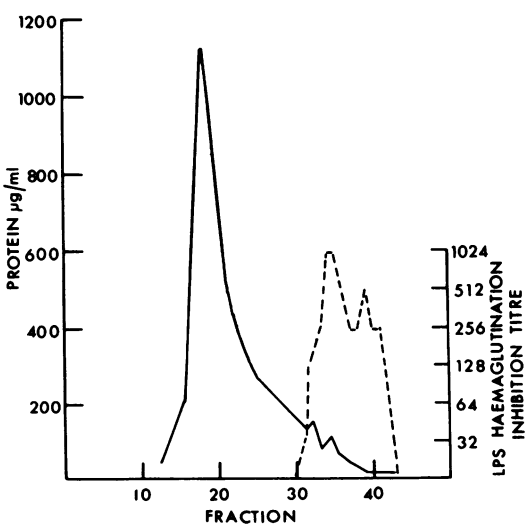

FIG. 3. Separation of protein and LPS components of Empigen BB extracts of *B. pertussis* on Sephadex G100 in 0.5% sodium deoxycholate. Symbols: ——, protein; ------, LPS hemagglutination inhibition titer.

TABLE 2. Effect of glutaraldehyde treatment on the biological activities of *B. pertussis* envelope proteins

Prepn	μg of protein/mouse (mean ± SEM)		Deaths/total[a]
	PD_{50}[b]	WBC × 2[c]	
Tris-NaCl extract	1.7 ± 2.1	0.94 ± 0.23	10/10
Tris-NaCl extract-glutaraldehyde	12.4 ± 1.4	>15	0/10
Empigen BB-extracted protein	1.3	2.8	0/10
Empigen BB-extracted protein–glutaraldehyde	16.7	>100	0/10

[a] Deaths per total of mice injected with 15 μg of protein; deaths were recorded after 3 days.
[b] PD_{50}, Dose that protected 50% of the mice challenged intracerebrally.
[c] WBC × 2, Dose required to double the leukocyte count of the mice.

enriched in the phase I-specific proteins and resembled typical outer membrane protein preparations from other gram-negative bacteria. The supernatant, however, consisted of a more heterogeneous mixture of proteins probably arising from the cytoplasmic membrane.

Biological activities of solubilized envelope proteins. As found previously, the Empigen BB extracts of phase I strains of *B. pertussis* had high mouse protective activity and were equally protective whether from static or shaken cultures (25, 26). The extracts also had considerable HSF and LPF activities (Table 1). The LPS content of Empigen BB extracts can be reduced by column chromatography on Sephadex G75 or G100 in the presence of DOC (Fig. 3). The isolated protein component is protective and possesses HSF and LPF activities, but has a reduced toxicity (Table 1). The LPS component was nonprotective and toxic to mice (data not shown).

Treatment of envelopes with Tris buffer containing 1 M NaCl extracted only 3 to 5% of the envelope proteins, but the material obtained was highly active in the mouse protection test, had high HSF and LPF activities, and was considerably toxic to mice (Table 1).

Attempts to resolve the biological activities of envelope extracts. The data in Table 1 indicate a strong correlation between the mouse protective activity and the HSF and LPF activities of extracts. In general envelope extracts which had high HSF or LPF activities (for example, extracts made with Tris-NaCl buffer or Tris-NaCl buffer containing Empigen BB or DOC) were highly potent in the mouse protection test. In contrast, extracts made with SLS had low LPF and mouse protective activities. Empigen BB extracts dialyzed against phosphate buffer, pH 9.0, had reduced LPF and mouse protective activities. Chromatography of Empigen BB-extracted envelope proteins on media such as Sepharose 6B, DEAE-Sepharose 6B, or Sephacryl S300 failed to satisfactorily resolve the activities. Partial separation of LPF activity and protective potency did, however, occur after dialysis of extracts against 0.05 M phosphate–0.5 M NaCl, pH 6.5, which, as mentioned above (Fig. 2), produced a precipitate enriched in phase I-specific proteins. The precipitate had a 50% protective dose value of 2 μg of protein compared with 0.9 μg protein for the supernatant. The amount of protein required to double the leukocyte count of mice was, however, only 23 μg for supernatant compared with over five times this value for the precipitate.

Glutaraldehyde treatment. Munoz et al. (20) reported that treatment of purified pertussigen with glutaraldehyde dramatically reduced the LPF activity and toxicity of the preparation and allowed the protective activity to be expressed.

TABLE 3. Effect of affinity chromatography with haptoglobin-Sepharose 4B on the biological activities of *B. pertussis* envelope proteins

Prepn	μg of protein/mouse ± SEM (*n*)	
	PD_{50}[a]	WBC × 2[b]
Envelope protein	3.1 ± 1.3 (5)	23.3 ± 5.8 (5)
Envelope protein after haptoglobin chromatography	24.6 ± 2.1 (5)	>300 (5)
Envelope protein after haptoglobin chromatography with LPF added[c]	1.6 ± 1.4 (4)	15.2 ± 1.4 (2)
LPF	NP[d]	0.1 ± 0.02 (3)

[a] PD_{50}, Dose that protected 50% of the mice challenged intracerebrally.
[b] WBC × 2, Dose required to double the leukocyte count of the mice.
[c] A 0.1- to 0.2-μg amount of LPF was added to 50 μg of envelope protein and diluted with the envelope proteins for the tests.
[d] NP, No protective activity detected at the top dose tested (1 μg).

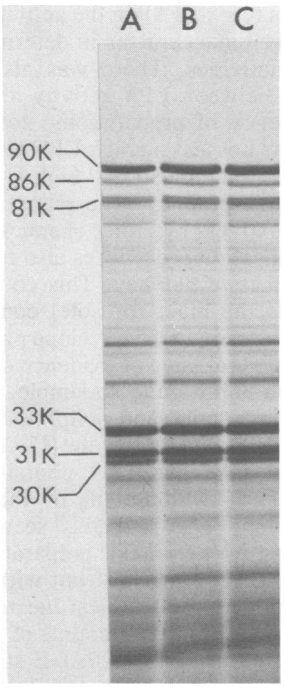

FIG. 4. SDS-PAGE of *B. pertussis* envelope protein preparations. Lanes: (A) Empigen BB-extracted protein, (B) Empigen BB-extracted protein after affinity chromatography on haptoglobin-Sepharose 4B, (C) Empigen BB-extracted protein after affinity chromatography on haptoglobin-Sepharose 4B with LPF readded (0.1 μg of LPF per 50 μg of envelope protein).

If this is the case then treatment of extracts of *B. pertussis* envelopes with glutaraldehyde might be expected to reduce the LPF activity and toxicity, but maintain (or perhaps enhance) the protective activity. Tris-NaCl buffer extracts of envelopes or envelope proteins isolated with Empigen BB and purified of LPS were thus treated with glutaraldehyde after dialysis against 0.02 M phosphate–0.5 M NaCl, pH 7.4 (Table 2). Glutaraldehyde reduced both the protective and LPF activities of the preparations, but there may be a greater reduction in the lymphocytosis promoting activity. The toxicity of the Tris-NaCl extracts was also reduced. When examined by SDS-PAGE the glutaraldehyde-treated preparations appeared mainly as aggregates which did not migrate into the gel.

Affinity chromatography. It is possible to remove virtually all of the LPF activity from Empigen BB extracts by affinity chromatography with haptoglobin coupled to Sepharose 4B (13). For these experiments Empigen BB extracts with LPS removed by Sephadex G75 chromatography were used. The results of LPF and mouse protective activities of the preparations before and after haptoglobin chromatography are shown in Table 3. Clearly the affinity chromatography reduces both activities. (In some experiments there was no detectable lymphocytosis when mice were injected with 300 μg of haptoglobin-treated protein.) The addition of a small amount of purified LPF to the envelope proteins after haptoglobin chromatography restored both activities. (LPF was added to the level of 0.1 to 0.2 μg of LPF to 50 μg of envelope protein and was diluted with the envelope proteins for the protection test. The highest dose of LPF was thus 0.1 to 0.2 μg per mouse.) The purified LPF by itself was nonprotective. The preparations before and after affinity chromatography (with and without additional LPF) were examined by SDS-PAGE. The results (Fig. 4) showed the preparations to be indistinguishable. The levels of LPF (subunits of 27,200, 22,400, 21,100, and 12,600 molecular weight [14]) were thus too low to be detected by this technique.

DISCUSSION

The complex biological and structural properties of *B. pertussis* envelope proteins have been examined in this study. At least six envelope proteins were found to be specific to phase I *B. pertussis* when compared with phase IV organisms. The results thus basically confirm those of others (8, 24, 26), but slight differences are evident in the reported number of phase I-specific proteins and their molecular weights. The 33,000-, 31,000-, and 30,000-molecular-weight, phase I-specific proteins reported here are undoubtedly equivalent to the two major X-mode or phase I-specific proteins referred to by Parton and Wardlaw (24), Robinson and Manchee (26), and Ezzell et al. (8). The electrophoresis system employed here resolves the smaller of these two proteins into the 31,000- and 30,000-molecular-weight components, provided that a low concentration of bisacrylamide was used in the gels. Ezzell et al. (8) also reported two phase I-specific proteins in the 80,000- to 100,000-molecular-weight region compared with three reported here. Such discrepancies may be due to differences in the methods of growth of the organisms or envelope preparation or to the use of different detergents to extract the proteins. The phase I-specific proteins reported here were found to have constant molecular weights irrespective of the strain of *B. pertussis* from which they were isolated. In contrast other gram-negative pathogens such as *Haemophilus influenzae* (4), *Neisseria gonorrhoeae* (16), and *Neisseria meningitidis* (30) have major outer membrane proteins which were found to vary with strain and serotype.

The major envelope protein from *B. parapertussis* or *B. bronchiseptica* was found to have a slightly higher molecular weight than that from

B. pertussis. This has also been reported by Ezzell et al. (8), who found, in addition, proteins in the 30,000-molecular-weight region which were specific to phase I *B. bronchiseptica* or C-mode *B. parapertussis*, although in low amounts when compared with the equivalent phase I-specific proteins from *B. pertussis*. In the present study these proteins were virtually absent from *B. bronchiseptica* or *B. parapertussis* envelope extracts. This may again be due to differences in the methods of envelope preparation in the two laboratories, or the strains of *B. parapertussis* and *B. bronchiseptica* employed here may be partially degraded.

We found, as did Ezzell et al. (8), that the cytoplasmic and outer membrane components of *B. pertussis* envelopes could not be resolved by standard procedures of discontinuous sucrose gradient centrifugation. The proteins extracted from envelopes with Empigen BB cannot thus be definitely referred to as outer membrane proteins. However, the 30,000-molecular-weight, phase I-specific proteins are probably outer membrane components, as they have several properties in common with the major outer membrane proteins of other gram-negative bacteria. These include (i) their molecular weights and relative abundance in the envelopes (4, 16, 23, 30), (ii) their relative insolubility in Triton X-100 (8) and SLS (10, 11), and (iii) their altered electrophoretic mobilities depending on the temperature of solubilization in SDS (5, 24). Empigen BB also solubilizes a number of minor proteins which are probably associated with the cytoplasmic membrane. These proteins can be separated from the phase I-specific proteins by dialysis against 0.05 M phosphate–0.5 M NaCl, pH 6.5 (Fig. 2, lane B).

The precise role of the *B. pertussis* phase I-specific proteins is difficult to assess. The components responsible for the phase I-specific biological activities of *B. pertussis*, when isolated from membranes or cells, have molecular weights distinct from those of the phase I-specific envelope proteins when examined by SDS-PAGE. For example, filamentous hemagglutinin has several high (up to 220,000)-molecular-weight components (2, 13, 25); LPF has several low-molecular-weight subunits (2, 13, 18), and agglutinogen 2 has a molecular weight of about 22,000 (13). The phase I-specific proteins may have additional, as yet unknown, virulence functions.

In general the LPF activity of envelope extracts correlated well with HSF activity. This is expected since a single component has been reported as being responsible for both activities (19, 20). However slight differences recorded in the HSF/LPF ratios of extracts (Table 1) were probably due to the effects of other envelope components (e.g., LPS) on the activities and the acknowledged inaccuracies in determining such biological activities. There was also a strong correlation between LPF activity and the protective potency of preparations, so that envelope extracts possessing high LPF activity (e.g., Tris-NaCl extracts) also had high HSF activity and protective potency. The reduction in LPF activity of extracts by either chemical modification or purification procedures also resulted in a loss in protective potency. This could indicate that LPF is the main (or sole) contributor to protective potency in the envelope preparations. However, several lines of evidence suggest that the situation is not quite so simple. (i) Purified LPF is toxic to mice and nonprotective unless detoxified with glutaraldehyde (13, 19, 20, 28). The envelope preparations contained native LPF. (ii) The LPF content of the envelope extracts is low. This is readily seen in Fig. 4, which shows three envelope preparations identical on SDS-PAGE analysis, but with markedly different protective potencies due to the presence or absence of small amounts of LPF below the detection level of SDS-PAGE staining with Coomassie blue. (In this case the electrophoretic profile is not a good indicator of protective potency.) Similarly, very low levels of LPF are needed in purified preparations of filamentous hemagglutinin to ensure protective potency of the preparations (6, 20). The LPF content of the envelope protein preparations in this study is approximately 0.5%, which would mean a 50% protective dose value for the LPF in the preparations, assuming it to be the sole protective antigen, of 0.01 μg. LPF toxoid in our hands (unpublished data) and others (19, 20) has a 50% protective dose value 200-fold higher than this. (iii) There is not a direct linear relationship between LPF activity and protective potency of the envelope preparations. For example, the outer membrane protein precipitate formed by dialysis of Empigen BB extracts against phosphate-NaCl buffer has far less LPF activity than the supernatant, but has about half the protective potency. Similarly, glutaraldehyde treatment of envelope proteins or affinity chromatography with haptoglobin-Sepharose 4B reduced the LPF activity more than the protective potency (Tables 2 and 3).

Thus, if LPF is the major protective antigen in the envelope preparations, then its protective potency must be considerably enhanced by other envelope proteins and its toxicity must be modified to allow its protective activity to be expressed. A more feasible alternative explanation of the results is the converse, i.e., that LPF has a pronounced potentiating effect on the protective activities of envelope antigens in addition to any protective activity of LPF itself.

The adjuvant properties of *B. pertussis* and LPF in particular have been reported (19, 20), and Nakase and Doi (22) found mixtures of LPF and filamentous hemagglutinin to be highly potent in the mouse intracerebral challenge protection test. The nature of other protective antigens in the envelope preparations is uncertain. Filamentous hemagglutinin is unlikely to be involved, as most of the preparations were obtained from shaken cultures which do not express this antigen (1, 25). The serotype-specific agglutinogens which were found to be present at a low level in the extracts by an inhibition of agglutination assay (data not shown) and also the outer membrane proteins themselves may contribute to protection. We have recent results from our laboratory (A. Robinson and L. Irons, submitted for publication) which indicate a strong potentiating effect of LPF on the activities of envelope preparations from phase IV *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* protecting against murine intracerebral infections of *B. pertussis*. These effects may be due not to an increase in levels of circulating antibody, but to a pharmacological effect of LPF specific to intracerebral infections which allows access of antigen or antibody to the site of infection (21). Thus, although LPF is an essential component for protecting mice against intracerebral infection with *B. pertussis*, it may not be of primary importance in protecting children against whooping cough.

The results in this paper indicate the need to consider outer membrane proteins as constituents of an acellular pertussis vaccine in addition to purified filamentous hemagglutinin and toxoided LPF. The potentiating role of native (toxic) LPF is a problem for any future pertussis vaccine formulations, and studies are planned to determine whether this is due to a pharmacological effect of LPF specific to intracerebral infections.

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