PURIFICATION AND CHARACTERISATION OF A FIMBRIAL HAEMAGGLUTININ FROM *BORDETELLA PERTUSSIS* FOR USE IN AN ENZYME-LINKED IMMUNOSORBENT ASSAY

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SUMMARY. The fimbrial haemagglutinin (F-HA) of *Bordetella pertussis* grown on solid medium was extracted with 1M sodium acetate for 72 h at 20°C, and partially purified by Sephacryl S-300 gel chromatography. A pooled fraction with fimbrial haemagglutinating activity was shown to contain fimbriae of the expected morphology by electron microscopy. Chemical and biological assays showed that the F-HA fraction contained some heat-labile agglutininogen and lipopolysaccharide but no measurable lymphocytosis-promoting factor or heat-labile toxin. The F-HA fraction used as antigen in an enzyme-linked immunosorbent assay (ELISA) permitted the detection of antibodies in convalescent serum from a patient with whooping cough. The impurities, heat-labile agglutinogens and lipopolysaccharide, did not contribute to the ELISA activity. The method for preparation of the F-HA antigen is simple, reproducible and gives a high yield.

INTRODUCTION

The isolation of *Bordetella pertussis* from patients with whooping cough is unreliable, especially after the first 2 weeks of infection, and attempts have been made to establish the diagnosis by immunological techniques. Agglutination, complement fixation, haemagglutination inhibition and immunofluorescence have been used to detect antibodies to *B. pertussis* and either whole bacteria or crude supernates from liquid cultures have been used as antigen (see Granström *et al.*, 1982).

The use of whole bacteria has made it impossible to develop a specific assay because different antigens, which are not all unique to *B. pertussis*, are either associated with or released from the bacteria (Pittman, 1979). These include (i) fimbriae, which agglutinate erythrocytes and have been named the fimbrial haemagglutinin (F-HA), (ii) heat-labile agglutinogens, types 1–3, which are believed to be outer-membrane proteins and form the basis for serotyping *B. pertussis*, (iii) lipopolysaccharide, a structural component of the outer membrane, (iv) heat-labile toxin, a dermonecrotic protein toxin, and (v) the

lymphocytosis-promoting factor, identical to the histamine-sensitising factor and the islet-activating protein.

We report here the isolation and partial purification of a fimbrial fraction from *B. pertussis* and its use as an antigen in an enzyme-linked immunosorbent assay (ELISA).

MATERIALS AND METHODS

Bacterial strains. *Bordetella pertussis* strains GL353 (serotype 1), 360E (serotype 1,2), M2 (serotype 1,3), and 18323 (serotype 1,2,3), *B. parapertussis* strain ATCC15237, and *B. bronchiseptica* strain ATCC19385 were from the National Bacteriological Laboratory, Stockholm, Sweden. They were held as lyophilised cultures.

Preparation of sodium acetate extracts. Bacteria were grown on Bordet-Gengou Agar (Difco Laboratories, Detroit, Mich., USA) containing 30% horse blood. After one subculture of the lyophilised bacteria, 24-h cultures were harvested and suspended in 1M sodium acetate (Masry, 1952) to a density of about 10^{12} bacteria/ml. After extraction for 3 days at room temperature with occasional gentle shaking, the bacteria were centrifuged at 5000 *g* for 30 min. The supernates were filtered through membrane filters of pore size 0.20 μ m (Nalgene Co., New York, USA).

Column chromatography. The extracts were filtered in 10-ml portions through a Sephacryl S-300 gel column (28 $\times$ 360 mm), equilibrated and eluted with phosphate-buffered saline (PBS), pH 7.2, containing 0.5M NaCl. Fractions of 5 or 7 ml were collected (figs. 1 and 2).

Determination of protein and sugar content. The protein content of the fractions was determined by the method of Bradford (1976) with bovine serum albumin as standard. Sugar content was determined by the colorimetric method of Dubois *et al.* (1951) with glucose as standard. Samples were assayed in triplicate.

Test for heat-labile toxin (HLT). The presence of HLT was determined by intracutaneous injection of 0.1 ml of the test fraction into the shaved back of rabbits. The size of the lesions was recorded after 24 and 48 h.

Test for heat-labile agglutinogens. Groups of five male mice (NMRI, Eklund, Sweden) were given subcutaneous injection of 0.5 ml of the test fraction. A second dose was given 2 weeks later. After a further 2 weeks the mice were bled and sera from each group were pooled (Munoz and Bergman, 1977). The serum pools were titrated in microplates (see below) for agglutinating activity against strains GL353 serotype 1, 360E serotype 1,2, and M2 serotype 1,3 before and after absorption with autoclaved bacteria. Fractions containing HLT were inactivated by heating at 56°C for 10 min before injection.

Preparation and assay of lipopolysaccharide (LPS). The amount of LPS was estimated by a limulus assay (Pyrostat Reagent Kit, Millipore, Freehold, New Jersey, USA). LPS was extracted by the method of Westphal, Lüderitz and Bister (1952). The water phase was ultracentrifuged at 100 000 *g* and the pellet was resuspended, washed twice with distilled water, and freeze-dried. About 50% of the dry weight of the extract was LPS as estimated by the limulus assay.

Preparation and assay of lymphocytosis-promoting factor (LPF). LPF was assayed by intravenous injection of 0.2 ml of the test fraction into each of five mice. After 3 days, the mice were bled and blood pooled within each group. White blood cells were counted in a Coulter Counter (Coulter Electronics Ltd, Harpenden, Herts.) LPF activity was also measured by a haemagglutination test (see below).

LPF binds to sialic acid-containing proteins, and Irons and MacLennan (1979) used haptoglobin conjugated to Sepharose to purify it. We used fetuin (Sigma Chemical Co., St Louis, Miss, USA), which is rich in sialic acid, coupled to a CNBr-activated Sepharose gel (Pharmacia, Uppsala, Sweden). Culture filtrates from bacteria grown in a liquid medium (Cohen and Wheeler, 1946), were applied to a column equilibrated with PBS containing 0.5M NaCl. After washing of the column, LPF was eluted by raising the pH to 11.5 and then immediately dialysed against PBS containing 0.5M NaCl. Purified LPF had a specific haemagglutinating (HA) activity (see below) of 5120 HA units/mg of protein. Details of the preparation are to be described by us (manuscript in preparation).

Sera. Monospecific sera against agglutinogens 2 and 3 were prepared by absorption of a human pertussis immunoglobulin preparation (National Bacteriological Laboratory, Stockholm, Sweden) and rabbit hyperimmune serum containing antibodies to all three agglutinogens. Sera were absorbed with either strain 360E (serotype 1,2) or strain M2 (serotype 1,3) leaving antibodies against agglutinogens 3 and 2 respectively, by incubating for 3 h at 37°C with bacteria at a concentration of 2×10^{12} bacteria/ml. Absorption was continued overnight at 4°C and the bacteria were removed by centrifugation. The procedure was repeated with autoclaved cells of *B. pertussis* to remove antibodies against common heat-stable antigens, e.g., LPS. The specificity of the antisera was assayed by titration in microplates with a bacterial suspension standardised at 3×10^{10} bacteria/ml. The plates were incubated at room temperature for 3 h and kept at 4°C overnight before reading. A serum specific for agglutininogen 1 was raised in rabbits hyperimmunised with strain GL353 (serotype 1).

Two human sera were used in the ELISA assays: one obtained from a volunteer hyperimmunised with a *B. pertussis* 1,2,3 strain and one convalescent-phase serum from a patient with whooping cough verified by positive culture.

Haemagglutination test. Serial twofold dilutions of fractions in PBS were made in 0.025-ml volumes in V-shaped microtitration plates (Sterilin, 43 Broad Street, Teddington, Middlesex). An equal volume of 0.5% (v/v) fresh chicken erythrocytes was added to each well. The plates were agitated thoroughly and incubated at room temperature for 1 h. One haemagglutination unit was defined as the highest dilution of the sample that caused complete haemagglutination visible to the naked eye. The two haemagglutinins, F-HA and LPF were distinguished by the addition of 0.5M NaCl to the diluent, which inhibits the fimbrial haemagglutination, but does not affect the LPF haemagglutination.

Haemagglutinating activity of a fraction was removed by mixing 0.1 ml of the fraction with 0.3 ml 10% (v/v) chicken erythrocytes. After incubation for 30 min at 4°C the erythrocytes were removed by centrifugation and the supernate was used in further tests.

ELISA assay was done by the microplate modification of Voller *et al.* (1974) of the method of Engvall and Perlmann (1972). Fractions from the Sephacryl S-300 gel chromatography were diluted 50-fold in PBS and 0.1-ml samples were used to coat the microplate wells by overnight incubation at room temperature. After the plates had been washed three times with PBS containing 0.05% (v/v) Tween 20, 0.1 ml of an appropriate dilution of serum was added to duplicate wells and the plates were incubated at room temperature for 1 h (IgG antibody determination) or at 37°C for 2 h (IgM). The hyperimmune serum was diluted 10 000-fold and the convalescent-phase serum 1000-fold. The plates were washed as above and 0.1 ml of alkaline phosphatase-conjugated swine anti-human IgG or IgM (Orion Diagnostica, Helsinki, Finland) or sheep-anti-rabbit Ig prepared by the methods of Carlsson, Lindberg and Hammarström (1972) was added and left at room temperature for 3 h. After a final washing, 0.1 ml of the *p*-nitrophenylphosphate substrate was added and the plates were incubated at 37°C for 60 min. Readings were done automatically in a Titretek Multiscan (Flow Laboratories Svenska AB, Solna, Sweden).

Determination of mouse-protective activity (MPA). The technique recommended by the World Health Authority for evaluating the potency of vaccines was used (WHO Expert Committee on Biological Standardization, 1979). Groups of 10 mice were given subcutaneous injection of 0.5 ml of the sample. After 14 days the mice were challenged intracerebrally with 50 000 bacteria/mouse ($100\text{--}200 \times \text{LD}_{50}$). Deaths were recorded during the next 14 days and results were expressed as percentage survival.

Electronmicroscopy. One drop of the fimbrial haemagglutinating fraction (no. 2 in table I) was applied to a carbon-coated grid. A 2% (w/v) solution of sodium tungstic silicate was used for negative staining and the grids were examined in a Philips EM 200 electron microscope operated at 60 kV.

RESULTS

The protein content of the sodium acetate extracts from *B. pertussis* strain 18323 was in the range 1–2 mg/ml and the sugar concentration was approximately 0.2 mg/ml. F-HA titres were 256–1024. The extracts also

contained, as expected, heat-labile agglutinogens 1, 2 and 3, and HLT. LPF was detected in some extracts, at a concentration of 0.2–0.5 $\mu\text{g/ml}$ (0.2 $\mu\text{g/ml}$ was the lower limit of detection of purified LPF in the two assay systems used). The concentration of LPS in the extracts was 0.5 mg/ml . The crude extracts could be used as an antigen in the ELISA test with convalescent phase sera at a dilution of 100–200.

Sephacryl S-300 gel chromatography

Five distinct peaks (I–V) were always observed at an absorbance of 280 nm (figs. 1 and 2). Protein analysis showed a high protein content in peaks II and III whereas peaks I and V contained mainly sugar (fig. 1). The F-HA activity did not coincide with any of the major protein or sugar peaks but appeared as a distinct peak between peaks I and II. The degree of purification of the five fractions constituting the F-HA peak is summarised in the table. The protein content of the haemagglutinating fractions 1–5 increased at the same time as the sugar content decreased and neither the protein nor the sugar content correlated with the HA activity of the fractions. The purity of the F-HA

TABLE
Purification of fimbrial haemagglutinin by Sephacryl S-300 gel chromatography

Preparation	Volume (ml)	Haemagglutination titre	Protein ($\mu\text{g/ml}$)	Sugar ($\mu\text{g/ml}$)	Percentage yield	Purification factor
Sodium acetate extract	10	512	1700	220	100	1
Fraction: no 1	5	32	5	5.4	3	21
no 2	5	256	13	4.5	25	65
no 3	5	256	25	2.8	25	34
no 4	5	128	37	2.0	13	11
no 5	5	64	53	1.1	6	4

fractions varied but fraction no. 2 at the top of the HA peak had a specific activity of around 20 000 HA-units/mg of protein. An electron micrograph of this fraction shows the fimbria-like structure typical of the F-HA of *B. pertussis* (fig. 3).

The purity of the F-HA fractions with regard to other antigens was carefully studied. HLT always eluted immediately after the F-HA activity (fig. 1) and was not present in the haemagglutinating peak. The heat-labile agglutinin 1 appeared as a distinct peak preceding and slightly overlapping the haemagglutinating activity (fig. 1). Agglutinin 2 could not be detected in any of the fractions. Agglutinin-3 activity was found in every fraction without any distinct peak. The titres of agglutinin 3 in fractions were in the range 32–128. LPS was also demonstrated in the fractions that contained the F-HA. However, the amount of LPS in these fractions was 10–20 $\mu\text{g/ml}$, which was <10% of the LPS content of the crude extract. LPF was not detected in any of the fractions by haemagglutination or by the lymphocytosis test. The MPA of the fractions coincided with the haemagglutinating activity (fig. 2), and hence also with the ELISA activity (see below).

FIG. 1.—Sephacryl S-300 gel chromatography of sodium acetate extract. $\circ$ — $\circ$ = fimbrial haemagglutinating (F-HA) titre; $\bullet$ — $\bullet$ = agglutinin-1 titre. Fractions of 5 ml were collected.

ELISA activity

Fractions corresponding to peak I and II (fig. 2) showed the highest activity as antigen in ELISA. The positions of the maximum ELISA activity could vary slightly from one chromatographic run to another (2–3 fractions). Also, the fall in the IgG activity in convalescent serum (one fraction) has also been observed in the IgM activity, but was not regularly present.

The specificity of the F-HA fraction as antigen in ELISA was further studied because contaminating substances had been found in the peak. To exclude the possibility that antibodies against the heat-labile agglutinogens contributed to the ELISA activity, extracts of different serotypes were purified by Sephacryl S-300 gel chromatography. Extracts from strains GL353 (serotype 1), 360E (serotype 1,2) and M2 (serotype 1,3) gave the same elution profiles for F-HA and ELISA activity as the extract from the test strain 18323 (serotype 1,2,3). Furthermore, monospecific human hyperimmune sera against agglutinogens 2 and 3 did not give ELISA titres above the background level when the F-HA fraction was the antigen. Monospecific rabbit antiserum against agglutinogens 1, 2 and 3 was assayed in the same way. Although all sera had high titres (> 512) in the agglutination test, they had very low activity that was not above the background level in the ELISA test.

The significance of the LPS contaminant was studied in an absorption experiment. A hyperimmune serum with a high ELISA titre ($> 200\,000$) against the F-HA preparation, was absorbed with autoclaved *B. pertussis* strain 18323. A reduction of $< 10\%$ of the ELISA activity was obtained. This loss in activity was no higher than that observed when the serum was treated in the same way with PBS. Furthermore, when an LPS preparation of 0.05 – $100\ \mu\text{g/ml}$ was used as antigen in the ELISA test, very low titres were recorded; at a concentration of $0.5\ \mu\text{g/ml}$ (the highest concentration in the working dilution of the F-HA fraction) the activity was equal to the background level.

Because we found, by using purified LPF at 0.004 – $4\ \mu\text{g/ml}$ as antigen in ELISA, that concentrations of LPF below the detection level, *i.e.*, $< 0.2\ \mu\text{g/ml}$, could contribute to the ELISA activity, the F-HA peak fraction was filtered through a fetuin-conjugated Sepharose column to remove non-detectable LPF. The column could bind $> 1\ \text{mg}$ of LPF without leakage but 80% of the ELISA and the F-HA activities were recovered. The loss of activity was the same as that obtained with a plain Sepharose gel. Furthermore, an LPF preparation was added to a sodium acetate extract to raise the LPF-haemagglutinating titre of the extract from zero to 128, and then filtered through a Sephacryl S-300 gel. LPF-haemagglutinating activity was recovered as two peaks in the position of peaks III and IV, and was therefore distinct from the F-HA activity. Finally, preparation of antigen from *B. parapertussis* and *B. bronchiseptica*, both of which lack LPF, gave a similar elution pattern for F-HA and ELISA activity as that from *B. pertussis*. Activities were lower than that obtained with *B. pertussis* strain 18323 but were comparable with those obtained from some other strains of *B. pertussis* studied during the course of this work.

Removal of F-HA by adsorption on to chicken erythrocytes was used to

FIG. 2.—Sephacryl S-300 gel chromatography of sodium acetate extract: ○—○ = fimbrial haemagglutinating (F-HA) titre; ●—● = mouse protective activity (MPA); ELISA activity, — = IgG in human hyperimmune serum, - - - = IgG in convalescent phase serum, . . . = IgM in convalescent phase serum. Fractions of 7 ml were collected.

FIG. 3.—Electron micrograph of purified fimbrial haemagglutinin (F-HA) from fraction no. 2 shown in the table. The bar on the micrograph represents 100 nm.

prove that the ELISA test measured antibodies directed against the F-HA. After one treatment of a F-HA peak with chicken erythrocytes, < 10% of the ELISA and none of the haemagglutinating activity remained. A second adsorption abolished virtually all the ELISA activity.

Antibody determination by ELISA

The fractions with haemagglutinating activity were pooled for use as antigen in the ELISA test. The pool contained only one quarter of the total fractions with antigen activity in the ELISA test but exclusion of other fractions ensured a higher purity of the antigen preparation. The antigen was stable at 4°C for 3–4 months but there was a slow decrease of activity on further storage. Antigen-coated microplates could be kept, covered with parafilm, for at least 4 weeks at 4°C without loss of activity. Rapid freezing to –20°C and thawing did not noticeably reduce the activity of the antigen but repeated freezing and thawing cycles resulted in a progressive loss. ELISA activity was

FIG. 4.—Coating curve of a representative F-HA fraction against a human hyperimmune serum (see *Methods*).

lost after prolonged storage at -20°C . However, the ELISA activity of the preparation was more stable than the haemagglutinating activity.

The coating efficiency of the F-HA antigen was tested on different types of microplate. Either polyvinyl or polystyrene microplates could be used; the binding to polyvinyl plates was twice as efficient as to polystyrene plates but because polyvinyl plates would not fit into the Titretrek Multiscan apparatus polystyrene plates (M 129B, Flow Laboratories) were used. The pH dependence of antigen binding was studied in the range pH 6.5–10.5 and was found to be pH independent within this range. At very low antigen concentrations, however, a higher degree of binding was obtained at physiological pH . Therefore, PBS, pH 7.2–7.3, was used as the coating buffer. The optimal coating dose with different preparations in these conditions was 0.5–1 μg protein/ml. A representative coating curve is shown in fig. 4. When coating curves were fitted by linear regression to the equation $\text{absorbance} = a \times [\text{antigen}]^b$ and the correlation coefficient was calculated, the correlation coefficients were 0.98–1.00.

DISCUSSION

The F-HA obtained after sodium acetate extraction of agar-grown bacteria was subjected to only one purification step, gel chromatography on Sephacryl S-300 (fig. 1 and table I). The specificity of the F-HA preparation was based

on the following criteria: (i) electron micrographs showed homogeneous filamentous structures about 2×60 nm in size (fig. 3); (ii) the specific activity of the F-HA peak fraction ($\sim 20,000$ units/mg of protein) was similar to that reported by Nakase, Doi and Kasuga (1975), Arai and Sato (1976) and Irons and MacLennan (1979); (iii) the haemagglutinating titres with chicken erythrocytes were the same as those reported by Nakase *et al.* (1975); (iv) the F-HA fraction had the same mouse-protective activity (fig. 2) as that reported for purified F-HA by Sato *et al.* (1979) and Nakase and Doi (1979). The PD50 of purified F-HA was 1–2 μ g protein.

The procedure gives a high yield of F-HA. More than 70% of the F-HA activity of the crude extract was regularly recovered, occasionally more than 100% but this was probably due to separation of haemagglutination inhibitors in the crude extract. It is simple and less time consuming than methods previously used, which have involved repeated chromatographic steps (Nakase *et al.*, 1975), starch-block electrophoresis, sucrose density-gradient centrifugation, electrophoresis and chromatography procedures (Arai and Sato, 1976), or affinity chromatography (Irons and MacLennan, 1979).

The F-HA fraction was not, however, pure F-HA. It contained heat-labile agglutinogens and LPS but HLT or LPF activity was not detected.

When the F-HA fraction was used as antigen in an ELISA assay (fig. 2) antibody was detected in the two human sera used. The ELISA activity of the antigen was parallel with the haemagglutinating activity. The observed variations could be due either to differences in coating efficiency or in exposure of accessible antigen sites of the fimbriae or both. The ELISA assay is intended to be a specific assay for estimating the humoral antibody response after whooping cough and control experiments were performed to investigate the influence of the heat-labile agglutinogens and LPS contaminants on the F-HA ELISA. Neither the agglutinogens nor the LPS, in the concentrations present in the F-HA fraction, contributed to the ELISA activity. Assays were also done to eliminate the possibility that LPF, if present in the F-HA fraction in amounts < 0.2 μ g/ml that could not be detected, could contribute. Passage of the fraction through a fetuin-conjugated column, which specifically binds LPF, did not reduce the ELISA activity when compared with a plain Sephacryl gel. Our results show that the F-HA fraction used as antigen in ELISA specifically estimates antibodies directed against the fimbrial haemagglutinin. The use of the F-HA ELISA assay for the diagnosis of whooping cough is reported in the accompanying paper (Granström *et al.*, 1982).

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