Immuno-electronmicroscopy of fimbriae-like structures on *Bordetella pertussis* serotype 1.3

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Summary. Fimbriae-like filaments were demonstrated on the surface of *Bordetella pertussis*, serotype 1.3, by negative staining and electronmicroscopy. Immuno-electronmicroscopy with a monoclonal antibody specific for strains possessing agglutinin 3, and colloidal gold, gave strong labelling of these structures. However, incubation with adsorbed polyclonal anti-agglutinin 3 serum gave only weak labelling of the distal parts of the filaments and of the bacterial surface. The different binding patterns of the two antisera suggested that the epitopes involved were dissimilar. Thus, agglutinin 3, as defined by conventional adsorbed sera, appeared to be associated with the fimbriae-like structures but was not necessarily identical to the fimbrial subunit protein. The monoclonal antibody, however, was more likely directed against the subunits of the fimbriae-like structures on serotype 1.3 bacteria.

Introduction

Agglutinogens of *B. pertussis* are surface substances, defined serologically, of which agglutinogens 2 and 3 may have a role in bacterial adhesion (Preston, 1985; Robinson *et al.*, 1985; Cowell *et al.*, 1986). These serotype antigens are thus considered to be candidates for inclusion in acellular vaccines (Carter and Preston, 1984; Robinson *et al.*, 1985; Miller, 1986). Based on epidemiological data, it is recommended that whole-cell vaccines should contain the three major agglutinogens, 1, 2 and 3 (Preston, 1963; World Health Organization, 1979). The nature of the species-specific antigen, agglutinin 1, is not known (Robinson *et al.*, 1985). Some investigators have described both agglutinogens 2 and 3 as fimbriae by immuno-electronmicroscopy with monoclonal antibodies (Ashworth *et al.*, 1982, 1985). The fimbrial nature of agglutinin 2 seems to be generally accepted, but the nature of agglutinin 3 is still debatable (Preston, 1985; Cowell *et al.*, 1986; Weiss and Hewlett, 1986). In contrast to the immuno-electronmicroscopic evidence (Ashworth *et al.*, 1985), electronmicroscopy of a large number of serotyped strains revealed no fimbriae on serotype 1.3 strains whereas strains of serotypes 1.2 and 1.2.3 were all fimbriated (Carter and Preston, 1984).

In the present study negatively stained samples of three well-characterised strains possessing agglutinin 3 were examined by electronmicroscopy. Serotype 1.3 bacteria were also examined by

immuno-electronmicroscopy and gold-labelling with adsorbed anti-agglutinin 3 serum and a monoclonal antibody specific for strains possessing agglutinin 3 (Ashworth *et al.*, 1985).

Materials and methods

Bacteria

B. pertussis strain M 2 (serotype 1.3) was obtained from Dr N. W. Preston, Pertussis Reference Laboratory, Department of Bacteriology and Virology, University Medical School, Manchester. Strains CN 5476 (serotype 1.3) and 28 (serotype 1.2.3) were obtained from Dr P. Novotny, Department of Bacteriology, Wellcome Research Laboratories, Beckenham, Kent. The strains were stored and cultivated as described previously (Lothe *et al.*, 1985; Fredriksen *et al.*, 1985, 1987).

Electronmicroscopy

The bacteria, grown on Bordet-Gengou (BG) plates for 60 h at 33–34°C, were suspended in distilled water, centrifuged at 2300 *g* for 20 min, washed once and resuspended in distilled water. Bacteria from shaken cultures, grown in a modified Stainer and Scholte medium (Lothe *et al.*, 1985) for 60 h at 33–34°C, were harvested by centrifugation, then washed and resuspended as above. Droplets (5 μ l) of the suspensions were applied to carbon-filmed grids which had previously been glow-discharged in air (Namork and Johansen, 1982). The grids were blotted almost dry after 1 min and then stained with a drop of sodium phosphotungstate 0.25% w/v, pH 7.0, for another 1 min. Electronmicroscopy was performed on a JEM 100B operated at 80 keV. Liquid

nitrogen anti-contaminant devices were in use when examining all specimens.

Sera

The anti-agglutinin 3 (factor 3) serum was rendered specific for agglutinin 3 by adsorption of rabbit hyperimmune serum against whole *B. pertussis* cells, strain M 2, with bacteria of heterologous serotype (Fredriksen *et al.*, 1985, 1987). Control (factor 0) serum was made by absorption of the same anti-M 2 serum with autologous cells (*i.e.*, M 2). This gave a reduction in agglutinating activity from a titre of 1280 to <5 (Fredriksen *et al.*, 1987). The monoclonal antibodies AG2A and AG3A (Ashworth *et al.*, 1985), specific for cells possessing agglutinin 2 and 3, respectively, were obtained from L. A. E. Ashworth, Public Health Laboratory Service, Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire. The monoclonal antibodies were of the IgM class and were supplied as culture supernates. In a micro-agglutination assay (Fredriksen *et al.*, 1987) the culture supernates gave a titre of 32.

Immuno-electronmicroscopy

Bacterial suspensions were applied to untreated carbon-filmed grids, left for 2 min and blotted almost dry. Grids were then incubated face down on drops of a suitable antibody dilution, then washed on several drops of 0.1 M ammonium acetate, pH 7.0, and blotted almost dry, before incubation with the appropriate gold probe (see below). Finally, after several washings with ammonium acetate buffer, the specimens were stained with sodium phosphotungstate 0.25% w/v, pH 7.0, for 30 s.

The bacteria were incubated for 2 min with a 1000-fold dilution of the polyclonal sera and for 2 min with a 10-fold dilution of staphylococcal protein A conjugated to 10-nm colloidal gold particles (Polaron, Bio-Rad, Richmond, VA, USA). When applying the monoclonal sera, the specimens were incubated for 10 min with an 8-fold dilution and incubated for 2 min with a 10-fold dilution of goat anti-mouse IgM conjugated to 5-nm gold particles (Janssen Pharmaceutica, Bersee, Belgium). All incubations were at room temperature. The dilutions of sera and gold probes were made in 0.1 M ammonium acetate, pH 7.0.

Results

Electronmicroscopy of bacteria revealed thin fimbriae-like filaments on all three strains examined. Fig. 1 shows a micrograph of strain M 2, serotype 1.3, from BG plates. The extending structures were short and rigid with an irregular outline, 4.5–5 nm in width and 100–250 nm in length. In liquid cultures, the number and length of the filaments were increased. Examinations of the

Fig. 1. Electronmicrograph showing fimbriae-like structures on *B. pertussis* strain M2, serotype 1.3, grown on BG agar, negatively-stained with sodium phosphotungstate 0.25% w/v, pH 7.0 (150 000 $\times$).

other strains showed that strain CN 5476 (serotype 1.3) had more filaments than strain M 2, but less than strain 28 (serotype 1.2.3) (micrographs not shown).

Immuno-electronmicroscopy of strain M 2, with the monoclonal antibody AG3A gave a clear specific labelling of the fimbriae-like structures (fig. 2c), whereas after incubation with the monoclonal antibody AG2A (fig. 2d), no labelling of these structures was seen. The factor-3 serum gave weak labelling of the distal parts of the filaments (fig. 2a) and some labelling of the bacterial surface (not shown). Bacteria incubated with factor-0 serum (fig. 2b), gave no specific labelling. After incubation with the monoclonal antibodies, the extending filaments were more difficult to visualise because of antibody binding and, presumably, to other material in the monoclonal preparation (figs. 2c and 2d). The use of higher concentrations of sera and gold probes or longer incubation times gave higher background labelling. The specificities of the probes were checked by incubating the bacteria with the probes only; no binding was seen.

Discussion

The serotype-1.3 strains in this study clearly carried fimbriae-like appendages, as did the serotype 1.2.3 strain. The filaments were short, thin and with an irregular outline, which may have been due to partial disintegration. Their diameter (4.5–5 nm)

Fig. 2. Immuno-electronmicroscopy of *B. pertussis* strain M2, serotype 1.3, grown in liquid culture. Bacteria were incubated with anti-agglutinin 3 serum (a) and factor 0 serum (b) as primary antibodies, then labelled with protein A-gold (10 nm). In (c) and (d) bacteria were incubated with AG3A or AG2A, respectively, then labelled with anti-mouse IgM-gold (5 nm). (150 000 $\times$).

was smaller than that reported for the *B. pertussis* fimbriae (5–6 nm) studied by Steven *et al.* (1986).

The observation that liquid culture seemed to increase the number and length of the filaments, and that the fimbriae-like filaments on the serotype-1.2.3 strain were more numerous, are in accordance with other data (Ashworth *et al.*, 1985). The finding that the serotype-1.3 strains examined by Carter and Preston (1984) did not carry fimbriae-like structures could have been due to the washing procedures used or to the fact that most of their strains were fresh isolates, whereas we used labo-

ratory strains. Furthermore, expression of the filaments may have been inhibited by the use of Charcoal Blood Agar by Carter and Preston (1984) or as suggested above, the filaments of serotype-1.3 strains may be particularly fragile. Nevertheless, Carter and Preston (1984) were able to agglutinate serotype-1.3 strains, on which no filaments were seen, with factor 3-specific serum. Thus, their results give strong support to the view that agglutinin 3 is not synonymous with fimbriae-like structures.

The use of different nomenclatures for *B. pertussis*

agglutinogens seems to complicate the identification of the serotype antigens (Weiss and Hewlett, 1986). Purified fimbriae from strain 114, which possesses agglutinin 3, have been reported not to induce antibodies to agglutinin 3. These purified fimbriae are instead stated to represent agglutinin 6 (Cowell *et al.*, 1986). We have been using the typing schemes of Preston, which does not recognise agglutinin 6 (Preston *et al.*, 1982). Anti-agglutinin 6 reagents were not available and thus we have not tested the present strains for agglutinin 6 properties.

The strong labelling of the fimbriae-like structures on serotype-1.3 strains with the monoclonal antibody AG3A is similar to the picture presented by Ashworth *et al.* (1985) with the same monoclonal antibody. However, immuno-electronmicroscopy of strain M 2 with polyclonal anti-agglutinin 3

serum, did not show such strong binding to one single visible structure. This may suggest that there are differences in the specificities of the polyclonal anti-agglutinin 3 serum and the AG3A monoclonal antibody. We believe AG3A to be specific for an epitope of the fimbriae-like structures found on cells of serotypes 1.3 and 1.2.3, and we suggest that our anti-agglutinin 3 serum has its main activity directed towards other antigenic determinants, probably associated with both the fimbriae-like structures and the cell body. The different binding patterns indicate that the term agglutinin 3 covers more than one structure; or, possibly, that not all agglutinin 3 is assembled into fimbriae-like structures.

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