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Ultrastructural Localization of Capsules, Cell Wall Polysaccharide, Cell Wall Proteins, and F Antigen in Pneumococci

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The localization of pneumococcal capsular and cell wall antigens was examined by immunoelectron microscopy. C polysaccharide (C-Ps), a common component of all pneumococci, was uniformly distributed on both the inside and outside of the cell walls. The thickness of the C-Ps varied with the strain. Encapsulated strains were covered by varied amounts of capsular polysaccharide concealing the C-Ps of the bacteria so as to render it inaccessible to anti-C-Ps antibodies. In addition to C-Ps, protein antigens were demonstrable on the surface of nonencapsulated pneumococci. The proteins were not masked by the C-Ps layer. An extra layer on the cell walls was conspicuous on electron micrographs of both rough and encapsulated pneumococci. The nature of this extra layer has not been disclosed. F antigen, another common antigen of pneumococci, was uniformly distributed on the surface of the plasma membranes. During the course of the experimental work a reproducible method of gold labeling immunoglobulins was developed.

Streptococcus pneumoniae may cause serious infections such as pneumonia, otitis media, meningitis, and bacteremia in humans. The pathogenesis is but poorly understood. It is known that the presence of a polysaccharide capsule promotes the virulence of the pneumococcus by virtue of its antiphagocytic properties and that antibody to the capsular material alters its surface properties and facilitates phagocytosis. A phosphorylcholin containing teichoic acid, designated C polysaccharide (C-Ps), is covalently linked to the peptidoglycan layer of the pneumococcal cell wall (5, 22). C-Ps is known to be able to activate the alternative complement pathway and to participate in the action of the pneumococcal autolysin (22). Since C-Ps is common to all pneumococci (18), it has been suggested that antibodies against this antigen might confer species-specific protection against pneumococcal infections (8, 21). In addition to the cell-wall-linked C-Ps, pneumococci possess another common polysaccharide antigen called F antigen due to its "Forssman reactivity." This antigen apparently consists of C-Ps covalently linked to a lipid moiety; i.e., it is a lipoteichoic acid (4). Proteins associated with the cell wall of the pneumococcus have also been described (1, 12). In the present study the localization of these different surface and cell wall antigens of the pneumococcus was examined by immunoelectron microscopy.

MATERIALS AND METHODS

Bacterial strains. Six encapsulated strains of *S. pneumoniae* were type reference strains from the collection of the World Health Organization Collaborating Centre for Reference and Research on Pneumococci of types 1, 3, 6A, 6B, 25, and 27. Five additional strains were used: a pneumococcal C mutant strain (CSR, SCS-2, clone 1) with a C-Ps capsule (17) and four noncapsulated strains, three from the American Type Culture Collection (Rockville, Md.) (ATCC 11733, 12213, and 27336) and one (Jena 1) isolate from a patient with conjunctivitis.

Antisera. (i) Rabbit antisera against *S. pneumoniae*. Antisera against types 1 and 3, group 6 (i.e., types 6A and 6B), types 25 and 27, and both the C mutant strain (anti-C-Ps antiserum) and one of the rough strains (ATCC 12213) were raised in rabbits by repeated intravenous injections of Formalin-treated bacteria as described previously (11). Normal sera from unimmunized rabbits were used as controls. The titers of the type and group antisera, determined by the capsular reaction test as described elsewhere (10), were 16 or higher. For use in immunoelectrophoresis and for labeling with colloidal gold, immunoglobulin fractions were prepared from the sera by precipitation with ammonium sulfate (1.8 M at pH 6.5) and ion-exchange chromatography (acetate buffer [pH 5.0]; ionic strength, 0.05) on a DEAE-Sephadex A50 column (Pharmacia, Uppsala, Sweden) (9).

(ii) Monoclonal anti-phosphorylcholine antibody. Mouse-mouse hybridomas were made by standard procedures, and the monoclonal anti-phosphatidylcholine antibody, designated HAS, was prepared from ascitic fluid and characterized as previously described (16).

(iii) Anti-antibodies. Rabbit anti-mouse immunoglobulin and peroxidase-conjugated swine anti-rabbit immunoglobulin were purchased from Dakopatts (Glostrup, Denmark). Ferritin-labeled goat anti-rabbit immunoglobulin was from Miles Scientific, Div. Miles Laboratories, Inc. (Naperville, Ill.).

Preparation of colloidal gold and labeling of immunoglobulin. Utensils were cleaned by treatment overnight with dichromate sulfuric acid or in 2% Extran AP12 (E. Merck AG, Darmstadt, Federal Republic of Germany) and rinsed repeatedly with distilled water. Centrifuge tubes were coated with a 0.1% (wt/vol) solution of silicone grease in *n*-hexane and air dried. With minor modifications the technique of Frens (6) for the preparation of a sol of 15-nm gold particles was used. At room temperature 1.0 ml of 1% chlorauric acid and 3 ml of 1% (wt/vol) sodium citrate were added during magnetic stirring to 100 ml of redistilled water in a 250-ml heavy-wall Erlenmeyer flask. The mixture was heated under reflux and boiled for 15 min and then cooled to room temperature (20°C). The final color of the suspension was deep, brilliant red. An exact adjustment of the pH is critical for labeling of proteins with colloidal gold. Normally a pH meter cannot be used, since the electrodes will be coated

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with a layer of gold. We adjusted the pH of the gold sol to 8.7 as follows. Drop by drop 0.1 M Na_2CO_3 was added to the sol, and the pH was simultaneously monitored with indicator strips (Universal indikator pH 0-14; Merck). After the addition of approximately 1.5 ml of Na_2CO_3, a pH close to the desired value was reached, and by means of vacuum a small volume of the sol was ultrafiltered through a collodion bag with a pore size of 10 nm (SM 13200; Sartorius, Göttingen, Federal Republic of Germany). The pH of the gold-free ultrafiltrate was then measured with a pH meter with glass electrodes. When necessary a few drops of 0.1 M Na_2CO_3 or 0.1 N HCl were added to the gold suspension, and the pH was checked again as described above. The pH of the immunoglobulin solutions was also adjusted to pH 8.7 with 0.1 M Na_2CO_3 or 0.1 N HCl. The amount of immunoglobulin required to stabilize the gold sol was assessed by examining the resistance to flocculation (induced by the addition of 100 μl of 9% NaCl) obtained after the addition of serial dilutions of the protein to 500-μl samples of the sol as described previously (7). The calculated amount of immunoglobulin (approximately 1 mg), with an excess of 10%, was added to 100 ml of gold sol under magnetic stirring at room temperature (20°C). After 15 min 800 μl of a 1% polyethylene glycol (molecular weight, 20,000) solution was added, and after a further 15 min 2 ml of 5% bovine serum albumin in distilled water was added for additional stabilization. The immunogold markers were kept overnight in a refrigerator and then concentrated by centrifugation at 10,000 × *g* for 20 min. The clear supernatant was removed, except for approximately 2.5 ml in which the loose precipitate of immunogold was gently suspended. The concentrated suspension of immunogold markers was filtered through a 0.22-μm Millex microfilter (Millipore S.A., Velizy, France) and stored until use at 4°C. Aggregated gold adhering to the wall of the centrifuge tubes was discarded. Two batches of immunogold markers were prepared, one from the immunoglobulin fraction of anti-C-Ps antiserum and one from that of normal rabbit serum.

Antigens. (i) Autolysate was prepared as described previously (17). Briefly, the cells harvested by centrifugation of 1,000 ml of trypsin broth culture of the C-mutant strain, after 6 h of incubation at 37°C, were lysed in 20 ml of saline containing 0.1% sodium deoxycholate. Cell debris was removed by centrifugation, and the supernatant, containing soluble proteins and C-Ps, was used as the autolysate. (ii) Purified C-Ps was prepared from the autolysate (17) as follows. Proteins and nucleic acids were removed from the autolysate by chloroform-butanol treatment and precipitation with 25% (vol/vol) ethanol containing 1% (wt/vol) $CaCl_2$. After centrifugation the crude C-Ps was precipitated from the supernatant with 80% (vol/vol) ethanol at pH 5.0, dissolved in saline, and fractionated by gel filtration on a Sepharose 4B column (Pharmacia). The high-molecular-weight fraction (peak I) of the purified C-Ps was precipitated with ethanol, washed with acetone, and dried in vacuum; this fraction is referred to as C-Ps(I). Since the C-Ps(I) was prepared from autolysed bacteria it may conceivably contain some fragments of peptidoglycan in addition to the chemically defined C-Ps.

Preparation of derivatives of the C-Ps(I). C-Ps(I) was activated with CNBr as follows: 10 μl of a 4% (wt/vol) solution of CNBr in dimethyl formamide was added to 1 ml of a 0.5% solution of C-Ps(I) in redistilled water. The pH was kept at 11 for 2 min by adding drops of 1 N NaOH and then quickly lowered to around 8 by adding one or two drops of 1 N HCl. The activated C-Ps(I) was then immediately mixed with equal amounts of the spacer to be linked to it, and the pH was adjusted with 0.1 N HCl or 0.1 N NaOH to 8.75 and kept constant for 2 h. Two spacers were used: 6-aminohexanoic acid and 2-phenylethylamine. Before use, the 6-aminohexanoic acid–C-Ps(I) was dialyzed against distilled water, and 2-phenylethylamine–C-Ps(I) was precipitated with 4 volumes of ethanol and dissolved in distilled water to a concentration of 1 mg/ml.

Affinity chromatography. Anti-C-Ps antibodies were removed from serum by affinity chromatography. A C-Ps affinity column was prepared by covalently linking 6-aminohexanoic acid–C-Ps(I) to Sepharose 4B. First, Sepharose was activated by 1,4-butanediol diglycidyl ether and washed with distilled water and acetone as described previously (20). The 10 g (wet weight) of activated gel was mixed with 40 ml of 1 M ethylenediamine (pH 10, adjusted with HCl) and shaken overnight at room temperature. The ethylenediamine-Sepharose was washed extensively with distilled water on a sintered glass filter (Büchner funnel; pore size, 16 to 40 μm), drained, and transferred to a flask containing 20 ml of 6-aminohexanoic acid–C-Ps(I) solution [5 mg of 6-aminohexanoic acid–C-Ps(I)/ml]. The coupling reagent 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide hydrochloride (20 mg; Sigma Chemical Co., St. Louis, Mo.) was added, and the pH was maintained at 6.8 by adding 0.1 N NaOH. After 2 to 3 h the pH was constant, and the flask was shaken overnight at room temperature. The gel was then washed extensively with distilled water, 3 M $MgCl_2$, and saline, successively, on the filter.

Absorption of anti-C-Ps antibodies was performed on a column (1 by 10 cm) packed with the C-Ps(I)-Sepharose. Two milliliters of the antiserum raised against the rough pneumococcal strain, ATCC 12213, was applied to the column and eluted slowly with saline. The eluate was monitored at 280 nm; the protein peak was collected, passed through the column once again (after regeneration of the gel), and finally checked for traces of anti-C-Ps antibodies by an enzyme-linked immunosorbent assay. For regeneration of the C-Ps(I)–Sepharose, 3 M $MgCl_2$ followed by saline was used.

Anti-C-Ps enzyme-linked immunosorbent assay. Enzyme-linked immunosorbent assay for the detection of anti-C-Ps antibodies in sera was carried out by using the procedure for screening of hybridomas (16), with the following modifications. The microdilution plates were coated directly with a solution of 2-phenylethylamine–C-Ps(I) (1 μg/ml of saline, 50 μl per well). Peroxidase-conjugated swine anti-rabbit immunoglobulin was used as detection antibody. The substrate used was *ortho*-phenylenediamine (Fluka AG, Buchs, Switzerland), and the results were expressed as optical densities read at 490 nm.

Immunoelectrophoresis. Crossed immunoelectrophoresis of pneumococcal autolysate and of purified C-Ps(I) was carried out with 1% agarose in Tris-Veronal buffer (pH 8.6) with an ionic strength of 0.02 as described elsewhere (17, 18).

Preparation of pneumococcal cells for electron microscopy. Trypsin broth (100 ml; see below) was inoculated with 2 ml of an overnight 5% serum broth culture (Statens Seruminstitut) and incubated at 37°C for 6 h. Glutaraldehyde (12.5% glutaraldehyde in 0.1 M cacodylate buffer [pH 7.3]) was added to a final concentration of 1.25%, and the suspension was kept in a refrigerator overnight. The bacterial cells were collected by centrifugation and suspended in 0.1 volume of 0.1 M ethanolamine (pH 8.0) for 1 h at room temperature to block the remaining aldehyde groups. The cells were then

washed twice with phosphate-buffered saline (PBS; 0.1 M NaCl, 0.05 M phosphate buffer [pH 7.3]). After the last centrifugation the bacterial cells were carefully suspended in 2.5 ml of PBS. The fixed cells were used in the procedures described below.

Pneumococcal cell walls. Cell wall material for use in three experiments was prepared as follows. Formalin-fixed cells of the rough strain ATCC 12213 prepared from 4.5-liter cultures were centrifuged and suspended in a few milliliters of PBS. The creamy, heavy cell suspension was passed through a French pressure cell (American Instruments Co., Silver Spring, Md.) exposed to a pressure of 12,000 lb/in^2. Five passages were required before no gram-positive cells could be found by microscopy. The broken cells were suspended in 20 ml of 2% sodium dodecyl sulfate and boiled in a water bath for 30 min, treated with RNase, DNase II (Sigma), and trypsin (Trypure Novo; NOVO Industri, Copenhagen, Denmark), and then boiled again in 2% sodium dodecyl sulfate (24). The cell walls were washed several times in PBS before use. Cell walls prepared from the rough pneumococcal strain ATCC 11733 (100 mg) grown in a synthetic ethanolamine-medium free of choline (24) were kindly supplied by Alexander Tomasz, the Rockefeller University, New York. The lyophilized choline-free cell walls were suspended in 20 ml of PBS and stored for 3 days in a refrigerator before use.

Preparation of specimens for electron microscopy. The following specimens were prepared and examined. (i) Untreated cells and cell walls without labeling were used. (ii) For the one-layer technique cells or cell walls were incubated with normal or specific rabbit serum. (iii) For the double-layer technique, cells or cell walls were incubated with normal or specific rabbit serum followed by incubation with ferritin-labeled goat anti-rabbit immunoglobulin. In one experiment cells were trypsinized before use as follows. The cells were suspended in 2 ml of 0.1 M Tris hydrochloride buffer (pH 8.0) containing 2 mg of trypsin (Trypure Novo) per ml and incubated at 37°C for 1 h, and trypsin was then inhibited by washing the cells with a ml of 1 mM phenylmethylsulfonyl fluoride (Sigma) in PBS. (iv) For the triple-layer technique, cells or cell walls were first incubated with monoclonal anti-PC antibody, then with rabbit anti-mouse immunoglobulin, and finally with ferritin-labeled goat anti-rabbit immunoglobulin. (v) For labeling with immunogold markers, cells were incubated with normal or anti-C-Ps antiserum labeled with colloidal gold. Capsulated strains were afterward incubated with type-specific rabbit antiserum to stabilize the capsules.

In general, cells from 100 ml of cell walls from 1.5 liters of culture were used for each experiment. After two washings the bacterial material was suspended in 2.5 ml of PBS, and an equal volume of antibody (or anti-antibody) was added. If not otherwise stated the following dilutions in PBS were used because preliminary experiments had shown them to give the most clear-cut results: normal serum, 1:2; type-specific sera and anti-C-Ps antiserum, 1:6; monoclonal anti-PC antibody, 1:5; anti-rough antiserum (absorbed for anti-C-Ps antibody), 1:2.5; rabbit anti-mouse immunoglobulin, 1:12.5; ferritin-labeled goat anti-rabbit immunoglobulin, 1:12.5. The immunogold markers were used undiluted (2.5 ml of markers was prepared from 100 ml of gold sol). The incubation steps were carried out in tubes rotated end over end (20 rpm) at 37°C for 1 h followed by two washings with 40 ml of PBS. After the last washing the bacterial material was packed by centrifugation at 1,500 × *g* and enrobed in melted (45°C) 1.5% agar (Noble Agar; Difco Laboratories, Detroit, Mich.) in 0.1 M cacodylate buffer (pH 7.3) with 0.01 M $CaCl_2$. After hardening of the agar, blocks of about 1 mm^3 were cut and fixed overnight in a refrigerator in 3% glutaraldehyde in 0.1 M cacodylate buffer with 0.01 M $CaCl_2$, followed by postfixation in 1% osmium tetroxide in the same buffer and 1 h of en bloc treatment with 2% barbiturate buffered uranyl acetate (pH 7.3) (3). Dehydration, Vestopal W (Martin Jaeger, Geneva, Switzerland) embedding, and further preparation for thin sectioning and electron microscopy were performed as previously described (3). Electron microscopy was carried out on Philips EM 300 or 201 C electron microscopes at 60 kV by using primary magnifications of ×9,000. Exposures were made on Kodak Fine Grain Release Positive Film type 5302. For this study about 1,100 micrographs were taken. Suitable fields were photographically enlarged as desired (generally ×10). Cells, cell walls, capsules, and membranes were measured directly on the photographs by means of a ruler or a magnifying lens (seven times) equipped with a built-in scale with 0.1-mm subdivisions.

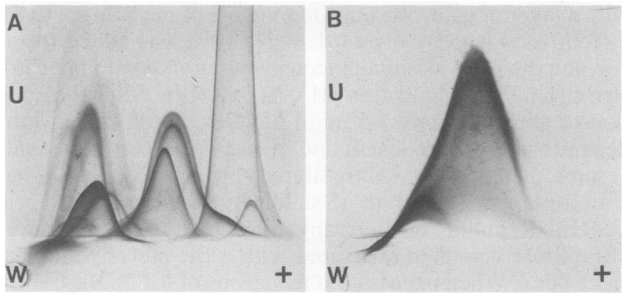

FIG. 1. Specificities of anti-C-Ps antiserum, raised against the C mutant strain, and of anti-rough antiserum, raised against the rough strain ATCC 12213, examined by cross immunoelectrophoresis. Upper gel (U): A, 10 μl of anti-ATCC 12213 antiserum; B, 3 μl of anti-C-Ps antiserum per cm^2 of gel area; 10 μl of pneumococcal autolysate was applied to wells (W). The plates were 5 by 7 cm. See the text for details.

Trypsin broth was prepared essentially as described by Pope and Smith (15); the only modification was that the trypsin digestion was discontinued when an analysis had shown the aminonitrogen content to be at least 1 mg/ml.

Chemicals. Unless otherwise stated, chemicals used were of the highest quality available from commercial sources.

RESULTS

Specificity of antisera. The specificities of the anti-C-Ps antiserum and the anti-rough antiserum were examined by crossed immunoelectrophoresis of pneumococcal autolysate and of C-Ps(I). The anti-C-Ps antiserum (16, 17) only precipitated C-Ps(I) (Fig. 1B). The anti-rough antiserum on the other hand did not precipitate C-Ps(I) (data not shown), but precipitated a number of proteins from the autolysate (Fig. 1A). However, by the enzyme-linked immunosorbent assay some anti-C-Ps antibody was demonstrated to be present in the anti-rough antiserum (dilution, 1/1,000; optical density, 1.062), but this antibody was completely removed by affinity chromatography on C-Ps(I)–Sepharose (dilution, 1/100; optical density, 0.021). The normal rabbit serum did not contain anti-C-Ps antibody (dilution, 1/100; optical density, 0.010), and electron microscopy controls performed both by the double-layer technique and by the colloidal gold technique showed that immunoglobulin prepared from normal rabbit serum did not bind to any part of the bacteria (Fig. 2D)

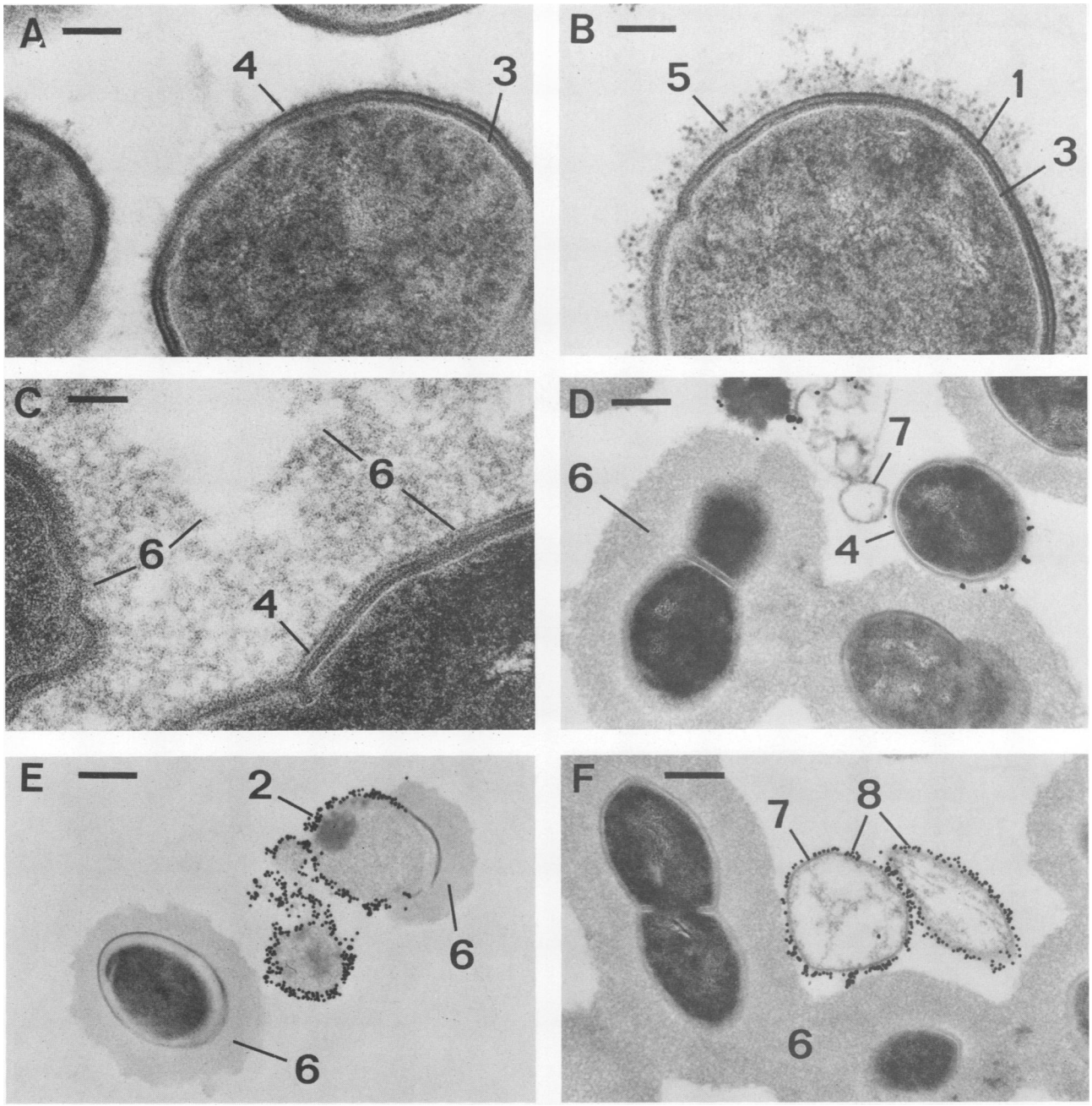

FIG. 2. Visualization of pneumococcal capsules and different cell wall and plasma membrane components by immunoelectron microscopy. The strains used were (A and B) rough strain ATCC 12213, (C) type 1, (D and F) type 6A, and (E) type 25. Initially the cells were incubated with (A) trypsin, (D) normal rabbit immunoglobulin labeled with colloidal gold, or (E and F) anti-C-Ps antibody labeled with colloidal gold. Then the specimens were tested by (A and B) the double-layer technique with C-Ps(I) absorbed rough serum (protein specific) in the first layer or (C to F) the single-layer technique with homologous typing antisera. For details, see the text. The following substructures can be identified on the micrographs: 1, peptidoglycan layer of the cell wall; 2, C-Ps layer; 3, middle electron lucent layer of plasma membrane; 4, extra layer; 5, protein layer; 6, capsular layer; 7, plasma membrane; 8, F-antigen. Bars, 0.1 μm (A to C) and 0.3 μm (D to F).

or to isolated cell walls (Fig. 3A). In contrast, anti-C-Ps antibody labeled with colloidal gold was found to bind to the cell walls of rough pneumococci (positive control; data not shown).

General ultrastructural features. Pneumococci examined by electron microscopy showed the following general features. The different strains of pneumococci were typical gram-positive cocci lying as single cells or in short chains. Many cells were in the process of division (Fig. 2B, C, D, and F), a sign of exponential growth. The plasma membrane was difficult to distinguish in intact pneumococci because its outer layer coalesced with the inner layer of the cell wall and its inner layer coalesced with the cytoplasm (Fig. 2A, B, and C). Free plasma membranes released as a result of spontaneous cell lysis (Fig. 2D and F) were triple-layered unit membranes about 9 nm thick.

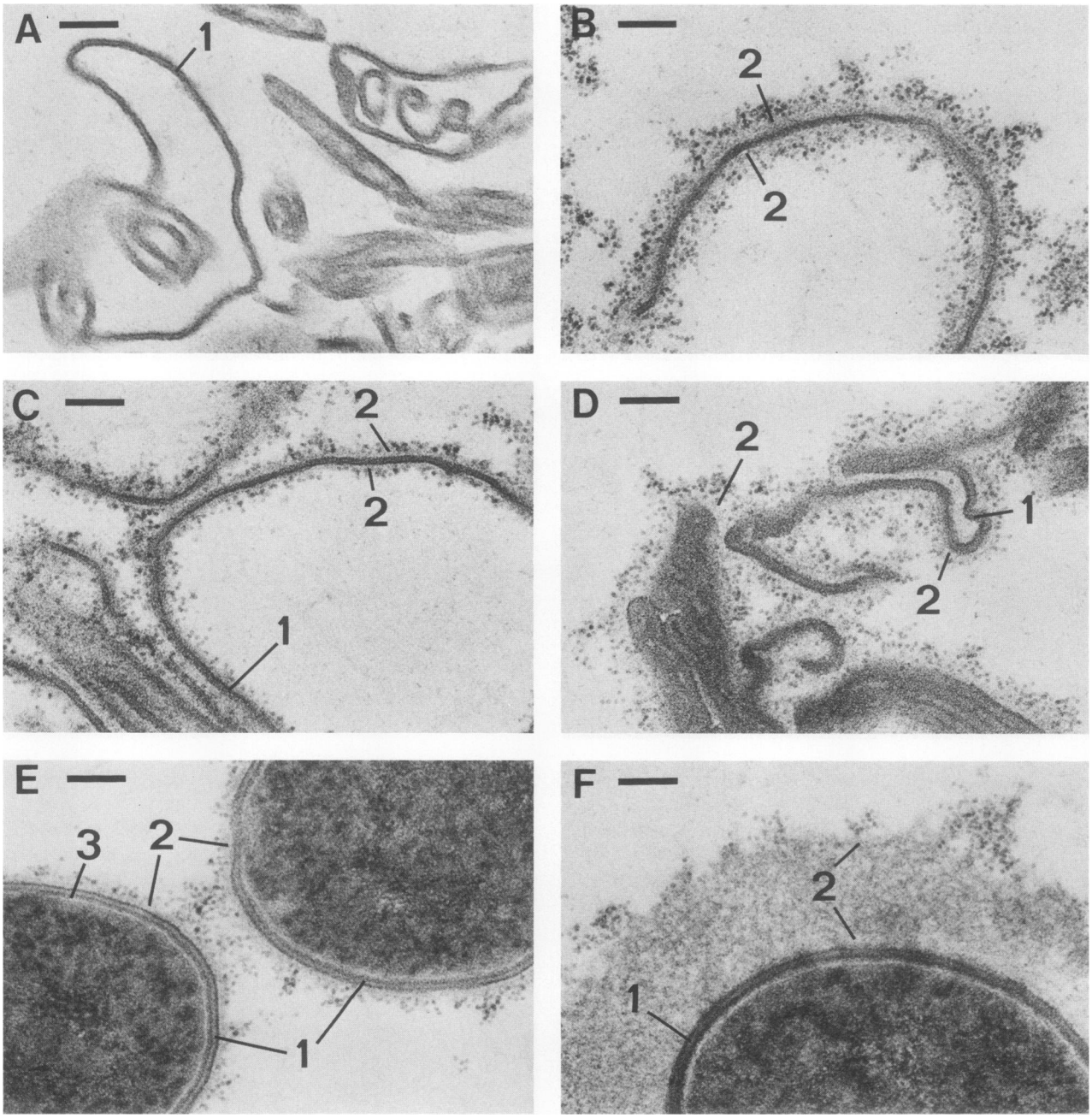

FIG. 3. Localization of C-Ps on pneumococcal cell walls examined by immunoelectron microscopy. The strains used were (A to C) rough strain ATCC 12213, (D) choline-free cell walls received from A. Tomasz, (E) rough strain ATCC 11733, and (F) the C mutant strain. Initially the preparations were incubated with (A) normal rabbit immunoglobulin, (B) monoclonal anti-PC antibody, or (C to F) anti-C-Ps antiserum. Then the specimens were tested by (A and C to F) the double-layer technique or (B) the triple-layer technique. For details, see the text. The following substructures can be seen on the micrographs: 1, peptidoglycan layer of the cell wall; 2, C-Ps layer; 3, middle electron lucent layer of plasma membrane. Bars, 0.1 μm.

Isolated cell walls were triple layered, limited by dense layers, with a thickness around 15 nm (Fig. 3A). In intact cells the thickness of the cell walls varied from 16 nm in most strains up to 25 nm in the type 3 strains.

An extra layer of fluffy material was often seen adherent to the outer surface of the cell walls both in rough cells (Fig. 2A) and in encapsulated pneumococci after they had lost their capsules (Fig. 2D). The extra layer was also found beneath the capsular layer (Fig. 2C). It was still present after trypsinization of the cells (Fig. 2A) but could not be found on isolated cell walls (Fig. 3A).

Protein antigens were uniformly distributed on the surfaces of rough pneumococci (Fig. 2B), and these antigens were not masked by the C-Ps layer (see above). The protein antigens were visualized by incubating the cells with the protein-specific anti-rough antiserum (absorbed for anti-C-Ps

antibody) with the double-layer technique and ferritin markers (Fig. 2B). Trypsinized control cells did not react with this antiserum (Fig. 2A).

C-Ps was demonstrated on the inner and outer surfaces of isolated cell walls both by the double-layer technique with polyclonal anti-C-Ps antiserum (Fig. 3C and D) and by the triple-layer technique with monoclonal anti-PC antibody (Fig. 3B). The C-Ps layers on the inner and the outer side looked alike (Fig. 3B, C, and D), but the width of the layers differed according to whether the double-layer technique (Fig. 3C) or the triple-layer technique (Fig. 3B) had been used for the visualization. Identical results were obtained when normal (Fig. 3C) and choline-free (Fig. 3D) cell walls were examined with the polyclonal anti-C-Ps antiserum. However, the monoclonal anti-PC antibody only bound to the normal cell walls (Fig. 3B) and not to the choline-free cell walls (data not shown).

Considerable variations were seen in the thickness of the C-Ps layers on the surfaces of different strains of pneumococci. The C mutant strain had a thick and capsule-like C-Ps layer (Fig. 3F), whereas only a thin layer was seen on the rough strain ATCC 11733 (Fig. 3E). In the three other rough strains examined (ATCC 12213 and 27336 and the Jena strain) the C-Ps layers varied between these extremes. The C-Ps layers on the surfaces of rough pneumococci were also demonstrated by incubation of the cells with anti-C-Ps antiserum labeled with colloidal gold (data not shown). When encapsulated bacteria were incubated first with the gold-labeled anti-C-Ps antiserum and then with unlabeled type-specific antiserum (to stabilize the capsules), the gold particles were located only on free plasma membranes (Fig. 2F) and on bacterial cells that had lost their capsules (Fig. 2E). The anti-C-Ps antiserum did not bind at all to the surfaces of encapsulated bacteria (Fig. 2E and F), demonstrating that C-Ps was fully covered by the capsular material. The binding of anti-C-Ps antiserum to plasma membranes (Fig. 2F) showed that the C-Ps part of the F antigen is exposed on the surfaces of the membranes and that the F antigen is uniformly distributed in these membranes.

Capsules. To visualize polysaccharide capsules on bacterial cells, special precautions have to be taken to stabilize these structures. In control preparations incubated with normal rabbit serum diluted 1:1, the capsules had collapsed during the dehydration steps of the embedding procedure, and the polysaccharide appeared as condensed structures of low electron density on the cell surfaces (data not shown). By adding type-specific antisera to the pneumococci the capsules were stabilized by formation of immunocomplexes between the antibodies and the capsular polysaccharides (Fig. 2D, E, and F). The degree of stabilization was dependent on the amount of antibody used. In a series of experiments group 6 serum was added to type 6A and type 6B pneumococci. Antiserum diluted 1:150 did not stabilize the capsules, and they appeared as fluffy material loosely associated with the cell surfaces. Antiserum diluted 1:30 did preserve the structures, but the most well-defined capsules were seen after the addition of antiserum diluted only 1:6 (Fig. 2D and F). The width of both type 6A and 6B capsules was around 300 nm. Considerable variation of capsular thickness between different types of pneumococci was found.

Even though the cultures were grown under optimal conditions and fixed with Formalin in the early log phase, some cells, most probably from the inoculum, that had lost or partly lost their capsules coexisted with fully encapsulated cells (Fig. 2D and F). By examining overview fields enlarged ×10,000 (data not shown) we found that less than 0.1% of the pneumococci did not possess a well-developed capsule.

DISCUSSION

Type-specific anti-pneumococcal capsular antibodies confer protective immunity. During recent years conflicting reports about the possible protective role of antibodies to C-Ps have been published (21, 26). Further, it has been suggested that pneumococcal cell wall components, rather than the capsules, induce inflammatory reactions (24).

We have studied the localization of different pneumococcal antigens—with special emphasis on polysaccharides—by means of immunoelectron microscopy to possibly achieve a better understanding of the course of pneumococcal infections.

The general morphology of pneumococci displayed in the present study was in agreement with the features reported by others (22, 23). The cells were typical oval-shaped cocci. The cytoplasm of the cells was encompassed by triple-layered 16- to 25-nm-wide cell walls with a plasma unit membrane closely associated with the inner side. Free plasma membranes consisted of three layers that were each around 3 nm wide, but seen in intact cells the outer layer was around 5 nm wide, and the plasma membrane appeared to be asymmetrical as previously reported (23).

The electron density of polysaccharides is generally low, and some kind of pretreatment is needed to disclose the localization of these substances by electron microscopy (2). Moreover, because they are hydrophilic, the dehydration process necessary for embedding and sectioning of the specimens causes the capsules to collapse (2). Incubation of the bacteria with anti-capsular antibody before embedding preserved their morphology (2). The amount of antibody added was critical for the preservation. As reported by others, the capsules appeared as homogenous layers between 200 and 400 nm in thickness, depending on the type and the strain (13, 14, 19).

Below the capsules and on nonencapsulated bacteria a 15-nm extra layer was often seen on the surfaces of the cell walls. This extra layer was not removed by trypsin treatment but was absent from isolated cell walls. The nature of the extra layer has not been disclosed. Its existence has not been reported previously, as far as we know.

Proteins were exposed and uniformly distributed on the surfaces of rough pneumococci and were removed by trypsin treatment of the cells as also reported by others (1, 12).

C-Ps, a common antigen of pneumococci (18), is covalently linked to the peptidoglycan of the bacterial cell walls (5, 22, 24). Since anti-C-Ps antibody can be removed from pneumococcal antiserum by absorption with whole cells of rough pneumococci, and since such cells can be agglutinated by anti-C-Ps antibody, C-Ps must be exposed on the surfaces of nonencapsulated pneumococci. However, the precise localization of C-Ps and its relation to other cellular constituents has not previously been disclosed. In the present study, C-Ps was found to be localized not only on the surface of nonencapsulated whole cells but also on both the outer and the inner sides of isolated cell walls. Identical results were obtained with choline-containing and choline-free cell walls examined with rabbit anti-C-Ps antiserum. Because this antiserum is known to react with peptidoglycan fragments (17, 25), the experiments were repeated with monoclonal antibody specific for the phosphoryl-choline part of the C-Ps (16). In agreement with the previous results the

monoclonal antibody bound to both sides of choline-containing cell walls but, as expected, not to the choline-free cell walls. In some strains only a thin rim of C-Ps was seen on the cells, whereas cells of other strains had a thicker layer of C-Ps on their surfaces.

The C-Ps of encapsulated bacteria was concealed by the capsular material. This observation is in agreement with the finding that anti-C-Ps antibodies fail to protect mice challenged intraperitoneally with a lethal dose of encapsulated pneumococci (21). We found that less than 0.1% of the cells in early-log-phase cultures of encapsulated pneumococci had lost their capsule. The observation by Yother et al. (26), that the cells prepared by them from culture of encapsulated pneumococci absorbed about 0.3% of the amount of anti-C-Ps antibody absorbed by a rough strain, probably shows that their culture contained a slightly higher percentage of nonencapsulated cells than ours.

Similarly, antibodies to F antigen, another antigen common to all types of pneumococci, are not protective (18). The F antigen was found to be uniformly distributed in the plasma membranes and located with the C-Ps parts of the molecules exposed on the surface and the lipid part anchored in the lipid bilayers of the plasma membranes.

The results of our study lead us to suggest that anti-C-Ps antibody may contribute to the removal of pneumococci with no or partially disintegrated capsules during the course of pneumococcal infections, thereby diminishing inflammatory reactions. However, it does not seem likely that anti-C-Ps antibody facilitates the phagocytosis of exponentially growing pneumococci, because they have well-developed, intact, capsules that completely conceal the C-Ps.

During the course of the experimental work a reproducible method of gold labeling of immunoglobulins was developed. Gold-labeled antibodies were found to be useful for the precise localization of cell wall and surface antigens of pneumococci.

LITERATURE CITED

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