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Visualization of *Pseudomonas aeruginosa* O Antigens by Using a Protein A-Dextran-Colloidal Gold Conjugate with Both Immunoglobulin G and Immunoglobulin M Monoclonal Antibodies

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Two lipopolysaccharide O-antigen-specific monoclonal antibodies, MA1-8 (an immunoglobulin G1 [IgG1]) and MF15-4 (an IgM), were used to localize the O antigen of the lipopolysaccharide of *Pseudomonas aeruginosa* PAO1. A protein A-dextran-gold conjugate with an average particle diameter of 12.5 nm was used to label bacterial cells treated with MA1-8, while a second antibody (goat anti-mouse IgM) was required before the same probe could interact with cells treated with the IgM antibody MF15-4. Both antibodies resulted in exclusive labeling of the surface of *P. aeruginosa* PAO1 but not that of an isogenic O-antigen-lacking rough mutant. When the monoclonal antibodies became attached to the cell surface of *P. aeruginosa* PAO1, resulting in an even coating, the foldings and other topographic details could not be discerned by negative staining. In thin sections of monoclonal-antibody-treated bacteria, a 20- and a 30- to 40-nm thick amorphous layer was observed around the outside of the outer membrane when MA1-8 (IgG) and MF15-4 (IgM) plus goat anti-mouse IgM antibodies were used, respectively. This amorphous layer presumably resulted from the stabilization of the lipopolysaccharide structure by the monoclonal antibodies which prevented the long O-antigen chains from collapsing owing to dehydration.

Bacterial cell surface studies were initiated to generate knowledge of how microorganisms function as well as how they interact with an animal host or with the environment. In the past, much effort has been spent to improve high-resolution electron microscopy (20) and the techniques for surface antigen localization. Some of these localization methods for studying microorganisms involve the use of an enzyme and its end-product depositions (1, 28) or the use of polyclonal antibodies conjugated to an electron-dense marker, such as ferritin (28, 34, 40, 41). More recently, monodispersed colloidal gold has emerged as the most versatile marker for cell-labeling studies. The advantages of using colloidal gold versus the use of other markers have been described in detail in several recent reviews (14, 21, 38). With the development of hybridoma technology by Kohler and Milstein (23), monoclonal antibodies have been rapidly replacing polyclonal antibodies as the immunological reagent in in situ antigen detection studies because of their qualities of specificity, availability, and reproducibility.

Lipopolysaccharide (LPS) plays an important role as a structural component of the cell walls of gram-negative bacteria. This macromolecule has also been implicated as a potential virulence factor of some organisms (9). In the past two decades, many LPS localization studies involving *Escherichia coli* and *Salmonella typhimurium* have been reported (3, 32–34, 40). Although data from these studies provided information on the distribution of LPS in the outer membrane, the architectural relationship between the LPS molecules and the cell surface has not been thoroughly examined. We produced monoclonal antibodies of both immunoglobulin G (IgG) and IgM isotypes that are specific against the O antigen of *P. aeruginosa* PAO1. Using these antibodies and protein A-dextran-gold (protein A-DexAu) as immune probes, we attempted to shed some light on the extent to which the O antigen can extend away from the bacterial cell surface.

MATERIALS AND METHODS

Bacterial strain and growth conditions. *P. aeruginosa* PAO1 H103 of O serogroup 5 of the international antigen typing scheme was used in all labeling studies. Strain AK1414, a mutant of *P. aeruginosa* PAO1 which lacks some outer core sugar residues and is completely devoid of O side chains (5), was used as a negative control in the labeling experiments. *P. aeruginosa* cultures were maintained on 2% (wt/vol) Proteose Peptone (PP2) agar (Difco Laboratories, Detroit, Mich.). Several colonies were inoculated into 50 ml of 2% PP2 broth and incubated at 37°C with shaking for 4 h or until the optical density at 600 nm was approximately 1.0. This culture was then used immediately for labeling.

Monoclonal antibody production. The production of monoclonal antibodies directed against *P. aeruginosa* PAO1 LPS has been previously described (17). MA1-8 (IgG1 class), a mouse monoclonal antibody specific for the O antigen of *P. aeruginosa* PAO1 LPS, was described by Hancock et al. (17). The mouse monoclonal antibody MF15-4 (IgM class) was produced as described in detail by Lam et al. (25). Large quantities of antibodies were produced by injecting 10^6 cells of pure hybridoma clones into pristane (2,6,10,14-tetramethylpentadecane; Sigma Chemical Co., St. Louis, Mo.)-primed BALB/c BYJ mice. Titration of monoclonal antibodies was performed by an enzyme-linked immunosorbent assay (ELISA) (35) by using microtiter plates (Immulon 2 U plates; Dynatech Laboratories, Inc., Alexandria, Va.) coated with either 0.5 μg of LPS per well or 10^7 Formalin-fixed whole cells per well, with the titer being read as the highest antibody dilution giving an optical density at 405 nm of ≥0.1 by using an alkaline phosphatase enzyme conjugate system. The ELISA reader used was a Titertek Multiscan model (Flow Laboratories, Inc., Mississauga, Ontario, Can-

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ada). The immunoglobulin class was determined by using a Mouse-Typer Sub-Isotyping Kit (Bio-Rad Laboratories, Richmond, Calif.).

Characterization of monoclonal antibodies. The O-antigen specificity of both monoclonal antibodies was examined by using Western immunoblotting (42). Briefly, the LPS of *P. aeruginosa* PAO1 was separated by discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis by the method of Darveau and Hancock (10) and silver stained by the method of Tsai and Frasch (43). The gels used were 0.75 mm thick and consisted of a 4% stacking gel and a 14% separating gel containing 4 M urea. Subsequent electrophoretic transfer of LPS from the gels to nitrocellulose was performed by the method of Towbin et al. (42). The antibody incubation procedure followed was that described by Mutharia and Hancock (35), except that a peroxidase-conjugated second antibody and 4-chloro-1-naphthol were the enzyme and substrate used.

Preparation of gold markers. (i) DE-DexAu. The preparation of diaminoethane-derivatized dextran (DE-Dex) was as described by Hicks and Molday (18). Colloidal gold particles 12.5 nm in diameter (Au125) were prepared by a method modified from Frens (13) and Bendayan (4) in which 1% (wt/vol) sodium citrate was added to a solution of 0.01% (wt/vol) tetrachloroauric acid (Sigma). DE-DexAu125 particles used subsequently as negative control markers were prepared by dissolving 25 mg of DE-Dex in 2.5 ml of 2 mM sodium phosphate buffer (pH 7) and adding this solution to 25 ml of freshly prepared gold (18). After being stirred for 30 min, the DE-DexAu solution was centrifuged at 34,800 × *g* for 45 min. The pellet was suspended in 12 ml of Tris-buffered saline (0.15 M NaCl, 0.02 M Tris chloride [pH 7.4]), and the solution was centrifuged as described above. The final pellet was redissolved in 1 ml of Tris-buffered saline containing 0.1% bovine serum albumin (Sigma) and 0.1% NaN_3. Bovine serum albumin was added to further stabilize the gold preparation against precipitation, and NaN_3 is a preservative. The DE-DexAu solution was stored at 4°C in sterile centrifuge tubes.

(ii) Protein A-DexAu. Protein A-DexAu125 markers were prepared by dissolving 50 mg of DE-Dex in 50 ml of gold solution. This mixture was activated with 0.6 ml of 10% glutaraldehyde, washed, and reacted with 2 ml of a solution (1 mg/ml) of protein A (Boehringer Mannheim, Dorval, Quebec, Canada) as described by Hicks and Molday (19). The gold marker was stored at 4°C in sterile centrifuge tubes.

Transmission electron microscopy. (i) Cell labeling with gold markers. A 2-ml sample of the 4-h culture, as described above, was sedimented by centrifugation (480 × *g*, 10 min), suspended in 2 ml of 2% PP2 broth, and divided into two samples so that one could serve as a control. MF15-4 antibody (60 μl) was added to each tube, mixed, and left to stand at room temperature for 30 min to allow for antigen-antibody binding. The samples were centrifuged, washed once in PP2 broth to remove unreacted antibody, and suspended in 2 ml of fresh broth. All washing steps for electron microscopy were carried out with PP2 broth to ensure that the cells maintained their wetting properties, which was shown to be critical in negative-staining procedures. A second antibody (60 μl), goat anti-mouse IgM (Cappel Worthington Biochemicals, Malvern, Pa.), was then added to each sample and allowed to react for 30 min at room temperature. Following the incubation with the second antibody, the samples were centrifuged and washed as before and resuspended in 2 ml of fresh broth. To one of the tubes, 80 μl of protein A-DexAu125 was added, and to the other tube, being used as a control, 80 μl of DexAu125 was added. Both tubes were then incubated for 30 min at room temperature. After incubation, the samples were centrifuged and the pellets were washed in fresh PP2 broth to remove unbound marker. Pellets were suspended in 2 ml of fresh broth, and half the volume of each sample was removed for thin-section preparation. Labeling with MA1-8 antibody was carried out as described above, but the second antibody step was omitted, since this monoclonal antibody is of IgG class and thus can react directly with the protein A-DexAu125 marker.

In addition to the control sample which was treated with DexAu125 lacking protein A, several other controls were included to ensure that labeling was due to specific antigen-antibody interaction and antibody-protein A interaction. These controls were as follows: (i) a sample with monoclonal antibody treatment omitted; (ii) a sample with the second antibody omitted when IgM monoclonal antibody was used; and (iii) a sample in which the rough mutant, strain AK1414, was used in place of strain PAO1.

(ii) Negative staining of labeled cells. Carbon-Formvar-coated 200-mesh copper grids were prewetted with 2% PP2 broth and then floated on 1 drop of sample for 5 min. Excess sample was drawn off with a wedge of filter paper. The grids were then floated on 1 drop of 0.5% ammonium molybdate (pH 7) for 5 s. Excess stain was removed with filter paper as before, and grids were allowed to air dry for at least 15 min before examination in a Philips 300 transmission electron microscope operating at an accelerating voltage of 60 kV.

(iii) Thin-section preparation. The method of thin-section preparation was as described by Chan et al. (7) with slight modification. Each of the labeled samples was fixed in 1 ml of 0.5% glutaraldehyde buffered with 0.1 M cacodylate at pH 7.4 for 3 h at 4°C. After fixation, samples were enrobed in 4% noble agar and washed in buffer. The cores were then dehydrated through a stepwise alcohol series and infiltrated in propylene oxide. The samples were then embedded in an Epon resin (Epon 812) and cured for 2 days at 60°C. Ultrathin sections were obtained by cutting the embedded material with an ultramicrotome (Reichert Jung Ultracut E). Subsequently, the sections were stained with 2% uranyl acetate and 2% lead citrate and examined in a Philips 300 transmission electron microscope operating at an accelerating voltage of 60 kV.

RESULTS

Monoclonal antibodies. The specificity of MA1-8 against the O antigen of *P. aeruginosa* PAO1 has been reported previously (17, 26). This monoclonal antibody was subtyped by using the mouse typing kit produced by Bio-Rad and was shown to be an IgG1. Monoclonal antibody MF15-4 agglutinated both live and Formalin-fixed whole cells of *P. aeruginosa* PAO1 and reacted with both Formalin-fixed whole cells and purified LPS of *P. aeruginosa* PAO1 in an ELISA. In a Western immunoblotting experiment, MF15-4 bound to a ladder of bands which comigrated with the heterogeneous smooth O-antigen-containing LPS bands revealed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 1). This blotting pattern was identical to the one produced by MA1-8. By using the Bio-Rad subtyping kit, MF15-4 was confirmed to be an IgM. Since both of these monoclonal antibodies reacted with international antigen typing scheme serotype 5 (Lanyi type 3a,d) but not with international typing scheme serotype 16 (Lanyi type 3a,b), which have identical sugar chains and differ only in one linkage (22), we conclude

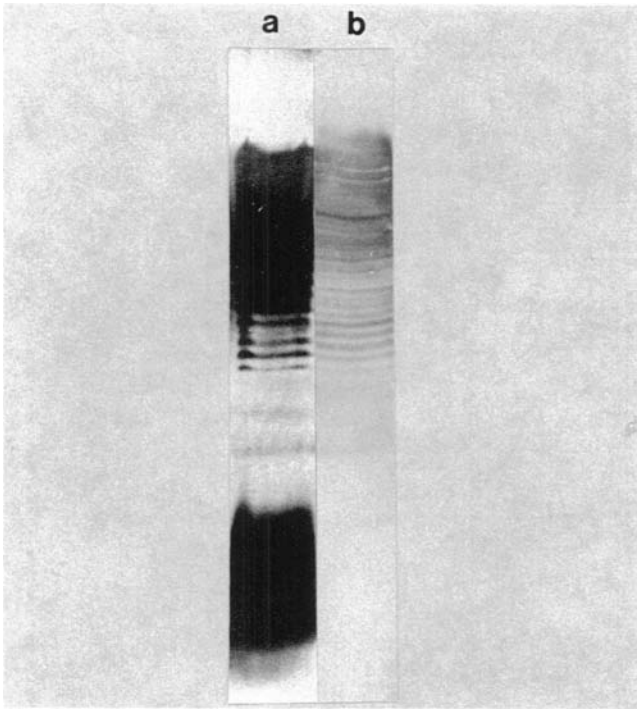

FIG. 1. Demonstration of the O-antigen-specific reactivity of MF15-4. (a) LPS as revealed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver staining. LPS (5 μg) was added to the lane. Notice the ladderlike banding pattern of the O-antigen polysaccharide. (b) An immunoblot of the LPS on nitrocellulose revealing the reactivity of monoclonal antibody MF15-4 toward the O-antigen bands.

that they must have nearly identical specificities which include this single unique linkage. The titer of the MA1-8 preparation was found to be 10^6 against both LPS and whole cells, while MF15-4 had a titer of 10^7 against both antigens.

Gold markers. The addition of 3 ml of 1% sodium citrate to 50 ml of 0.01% tetrachloroauric acid generated a monodispersed colloidal gold suspension with an average particle diameter of 12.5 nm. There were no discernible differences under the electron microscope among the untreated gold (Au125), the DexAu125, and protein A-DexAu125 even though other investigators described a halo around the gold markers when protein A was adsorbed (37). The gold marker suspensions were usually made fresh immediately before being used for the immunolabeling work, although they were found to be stable when stored at 4°C over a 3-month period.

Immunolabeling of cells. During the labeling procedures, both monoclonal antibodies MA1-8 and MF15-4 were found to agglutinate the bacteria. Upon sedimentation by centrifugation, all samples treated with protein A-DexAu125 produced red-colored cell pellets, while samples incubated with the negative control marker DexAu125 produced a light-pink cell pellet (characteristic of *P. aeruginosa*) and a red-colored supernatant. In addition, when either of the monoclonal antibodies was omitted, even the addition of protein A-DexAu125 did not produce red-colored cell pellets. These observations served to confirm the specific interaction between protein A and the IgG antibodies.

Treatment of *P. aeruginosa* PAO1 cells with the IgG antibody MA1-8 and protein A-DexAu125 resulted in specific labeling of the O antigens on the surface of cells (Fig. 2b). Bacterial appendages such as flagella and pili were not labeled. When a rough mutant strain, AK1414, devoid of O antigen was used and treated with MA1-8 and protein A-DexAu125, no specific cell surface labeling was observed (Fig. 2a). Saturation of *P. aeruginosa* PAO1 with MA1-8 was achieved when 1 ml of mid-log-phase cells was treated with 60 μl of the ascitic fluid of this antibody. Under this condition, the gold particles were seen to label the O antigens of the LPS on at least 50% of the cell surface (Fig. 2c). The average distance between the gold probes and the surface of the cell appeared to be approximately 20 nm (Fig. 2c). Nonspecific background staining by the protein A-DexAu125 was not discernible. The distribution of the gold probe on the bacterial cell surface was also found to be random, with no distinctive area with a higher density than others.

The use of a second antibody, an affinity-purified IgG (goat anti-mouse IgM), to interact with the IgM antibody MF15-4 enabled us to label *P. aeruginosa* PAO1 with the same protein A-DexAu125 (Fig. 3). An increased density of gold labels per cell was immediately apparent. This was presumably due to the fact that each IgM monoclonal antibody bound to the cell surface could bind more than one IgG goat anti-mouse IgM antibody, thus amplifying the number of protein A-binding antibody molecules on the cell surface. In addition, gold particles were observed to localize O antigens at an average distance of 30 to 40 nm away from the cell surface (Fig. 3, arrow). Again, nonspecific background staining was not seen.

The coating of the bacterial cell surface with the extra protein layers, including the monoclonal antibodies, the second antibody, and the protein A of the gold probe, had distinctive effects on the cell surface details observed after the negative-staining technique. The topographic details of strain PAO1 became progressively more blurred in negatively stained preparations when the cells were treated with either MA1-8 or MF15-4 and the second antibody (Fig. 2b and c and 3). When the rough strain AK1414 was used as a control, no gold labels were observed (Fig. 2a). In addition, neither of the monoclonal antibodies caused agglutination of the rough strain (data not shown). Negatively stained samples of the treated AK1414 cells appeared not to be different in terms of surface features when compared with untreated AK1414 or the smooth strain PAO1. In these cells, the surface details, including the folding of the membrane, a typical dehydration artifact of bacterial cells under the electron microscope, could easily be discerned (Fig. 2a).

The specificity of the interaction between the protein A-DexAu markers and the monoclonal antibodies were further illustrated in thin sections of the preembedding labeled cells. Gold markers were seen to specifically localize the O antigen beyond the periphery of the outer membrane. A gap of approximately 20 nm could be seen in some areas between the outer membrane and the gold marker. This gap was usually filled with a fuzzy amorphous layer (Fig. 4a) not characteristically seen on thin-section preparations of *P. aeruginosa*. Under saturating monoclonal antibody conditions, the distance between the gold markers and the outer membrane surface of the bacteria became more obvious (Fig. 4b). Instead of appearing as patches of amorphous strands on certain areas of the cell surface (Fig. 4a), a 20-nm thick layer composed of an amorphous structure was seen around each cell in thin-section preparations (Fig. 4b). We feel that the observation of a layer appearance despite the apparently incomplete coverage of the cell surface by gold particles was due either to removal of gold particles after

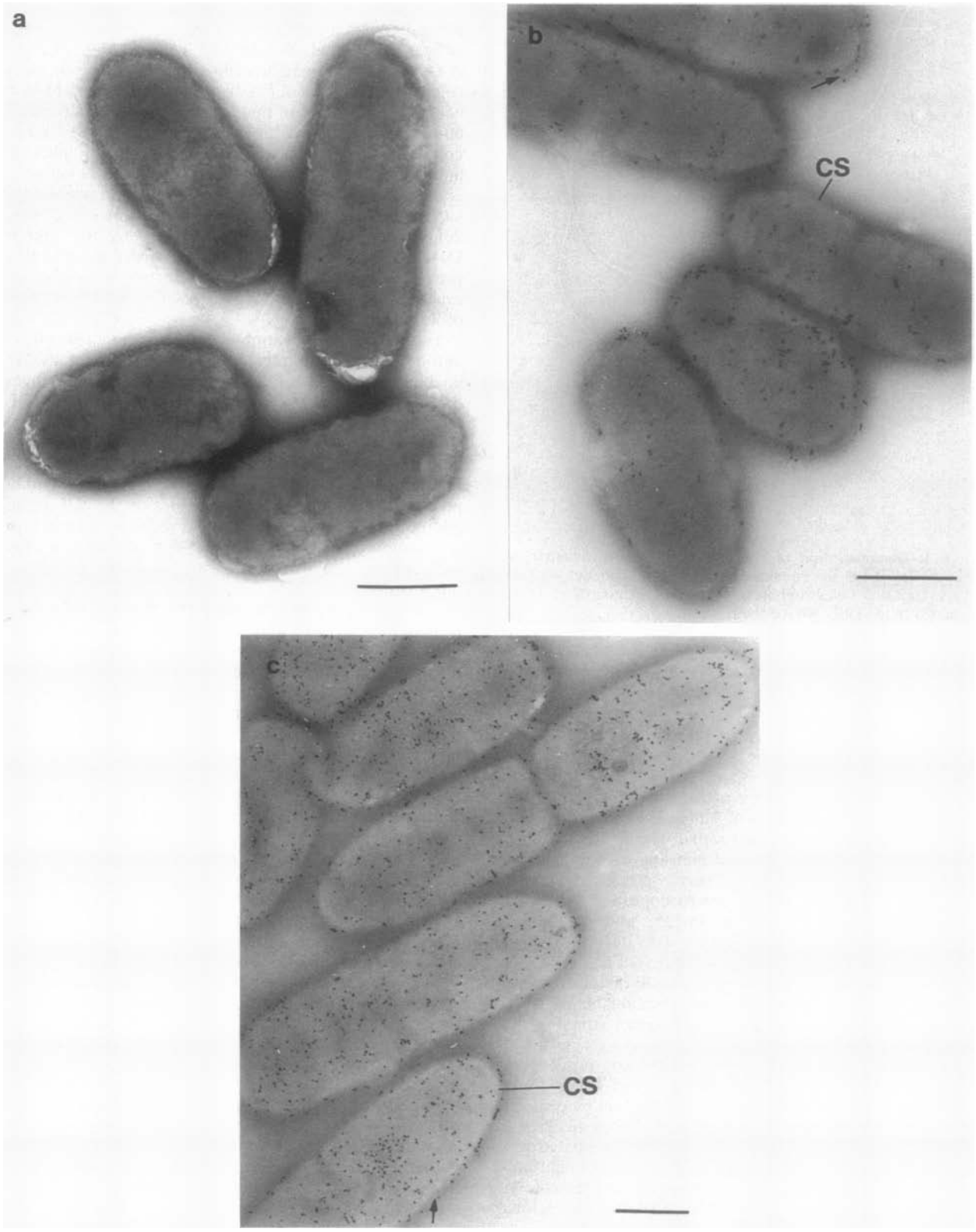

FIG. 2. Negative staining of *P. aeruginosa* treated with MA1-8 (IgG) and protein A-DexAu125. (a) Control. The rough mutant, strain AK1414, showed no specific labeling, although one or two gold particles were seen owing to nonspecific trapping. (b) Strain PAO1 reacted with a suboptimal concentration of MA1-8 (5 μl). Specific labeling was achieved with no background gold labels. (c) Strain PAO1 reacted with a saturating concentration of MA1-8 (60 μl); the density of gold labels was greatly increased with no discernible background labeling. CS, Cell surface. Arrows show the space between the gold particles and the cell surface. ——, 0.5 μm.

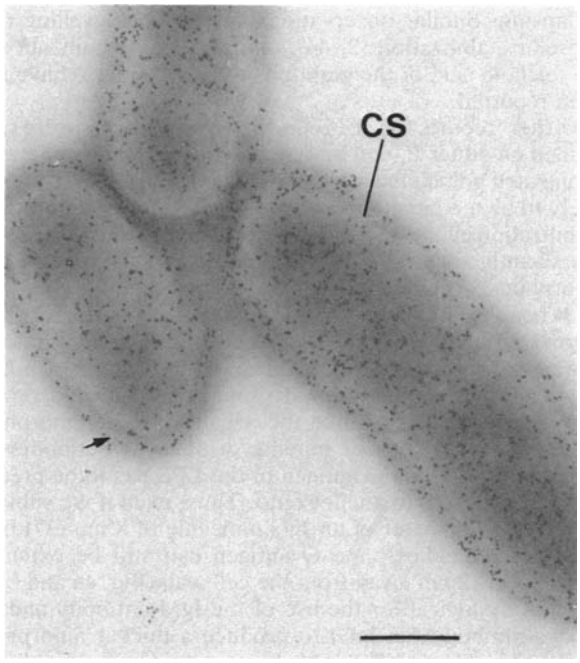

FIG. 3. Electron micrograph of *P. aeruginosa* PAO1 treated with protein A-DexAu125 and a saturating concentration of MF15-4 (IgM; 60 μl) plus the second antibody, goat anti-mouse IgM. Note the density and specificity of the labeling. The space (arrow) between the gold particles and the cell surface (CS) of the bacterium can be observed to be approximately 30 to 40 nm. ——, 0.5 μm.

embedding and sectioning by the thorough washing procedures used or to antibodies in adjacent sections of the cells stabilizing the layer in the sections shown.

In thin sections of cells treated with the IgM antibody MF15-4, a greater intensity of labeling around each cell was apparently consistent with the improved surface coverage observed in intact cell experiments (Fig. 3). In addition, we could also observe an amorphous structure with a thickness approaching 40 nm on the surface of each cell (Fig. 5).

DISCUSSION

In this study, mid-log-phase bacterial cells were treated with monoclonal antibodies before they were incubated with the indirect gold marker protein A-DexAu125. Protein A, a cell wall component of *Staphylococcus aureus*, has the characteristic property of binding to the Fc region of IgG with high affinity (37). On the basis of this property, we had no trouble labeling cells treated with the IgG antibody MA1-8, by using the protein A-DexAu125 probe (Fig. 2b and c and 4a and b). Although it has been reported in some (29) but not all studies (27) that protein A interacts relatively poorly with the IgG1 subclass of mouse immunoglobulins, we experienced no difficulty in obtaining interaction of MA1-8 with protein A. However, in order for the protein A of the protein A-DexAu125 probe to interact with the IgM antibody MF15-4, we used a second antibody which was an affinity-purified, IgG isotype goat anti-mouse IgM antibody. The second antibody in this case would interact with the mu chain of the IgM, while the Fc region would be available to subsquently interact with protein A-gold. Whenever an IgM antibody reacted with the O antigen, there would be at least

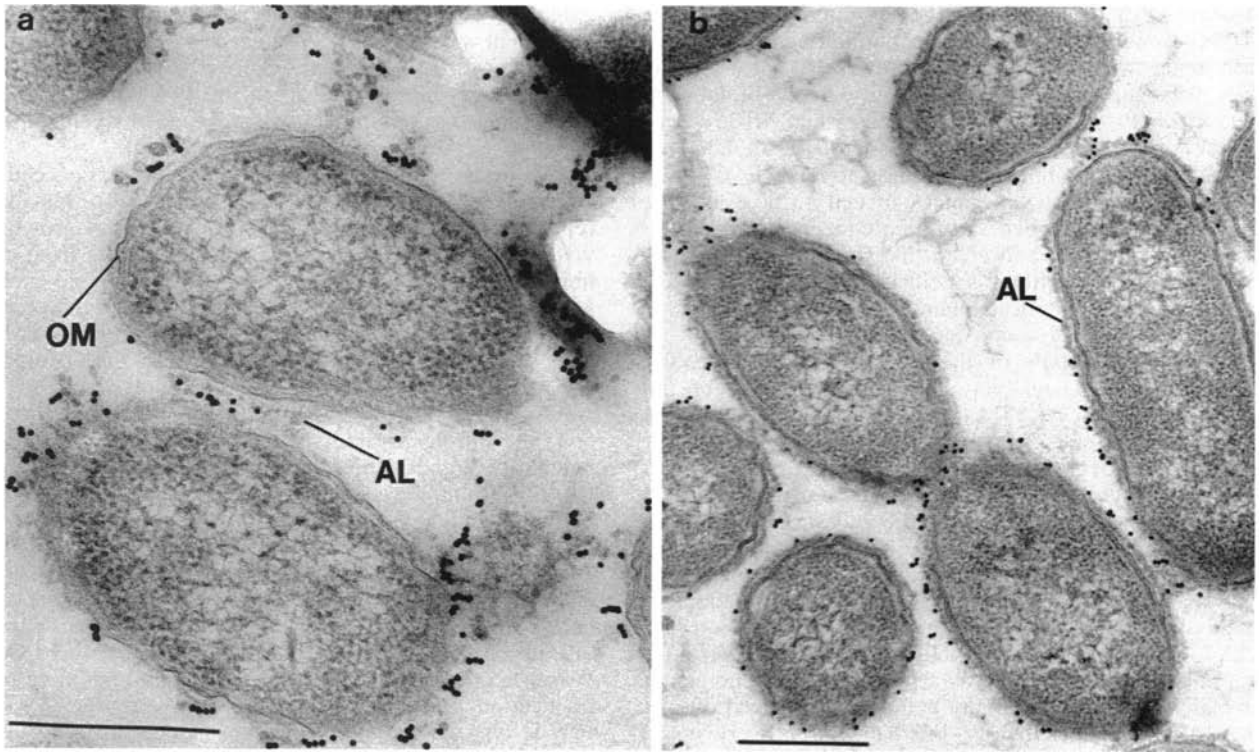

FIG. 4. Electron micrographs of thin sections of *P. aeruginosa* PAO1 treated with MA1-8 (IgG) and protein A-DexAu125. (a) At a suboptimal concentration of MA1-8 (5 μl), specific labeling was achieved and the amorphous layer (AL) became apparent. (b) At a saturating concentration of MA1-8 (60 μl), the gap between the colloidal gold and the outer membrane (OM) was unmistakable because of the presence of an amorphous layer (AL). The gold labels were found to be excluded to the outside of the amorphous layer, which formed a consistent layer around each cell. ——, 0.5 μm.

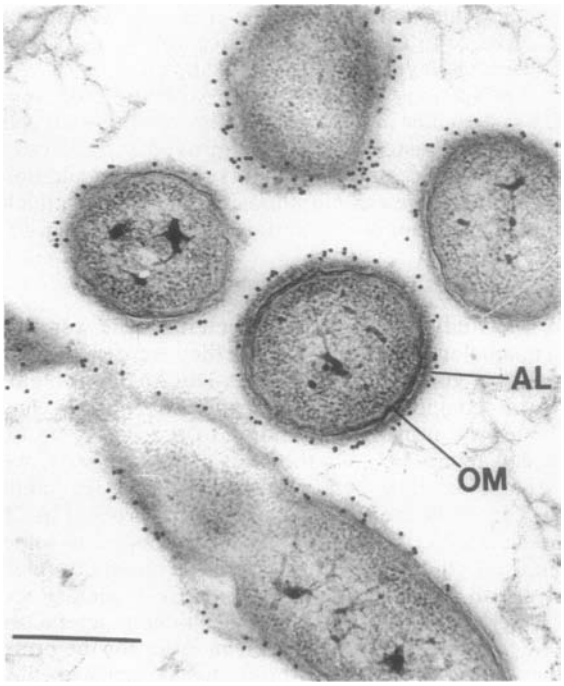

FIG. 5. Electron micrographs of thin sections of *P. aeruginosa* PAO1 treated with protein A-DexAu125 and a saturating concentration of MF15-4 (IgM; 60 μl). Note the thickness of the amorphous layer (AL) around the outer membrane (OM) of each cell. Arrows show a gap between the label and the outer membrane. ——, 0.5 μm.

two or more of the second antibodies interacting with the IgM. Subsequently, each of the second-antibody molecules would react with one protein A-DexAu125 probe. This theory was supported by the higher density of labeling observed on *P. aeruginosa* PAO1 cells when MF15-4 was used. We increased the concentrations of the monoclonal antibodies until optimum and saturating antibody levels were determined. Since the differences in cell surface labeling were observed even when saturating concentrations of the antibodies were used, and given that the antibodies apparently had the same epitopic specificity (see Results), differences in affinity cannot explain the different degree of labeling.

Topographical details of the cell surface were readily discernible in the negatively stained preparation of the control cells, strain AK1414 (Fig. 2a). When we compared antibody-treated cells with control cells, we were most intrigued to discover that these bacterial cells appeared to be covered by a layer of material such that the membrane folds and other topographic details on the cell surface could no longer be discerned (Fig. 2b and c and 3). These observations were further confirmed when the fuzzy amorphous layer was also observed around each cell in thin sections of antibody-treated cells (Fig. 4b and 5).

The formation of this amorphous layer outside the outer membrane can be explained as a stabilization phenomenon in which the monoclonal antibodies cross-linked the O antigen of the LPS. Such cross-linking apparently prevented the collapse of the LPS during the dehydration steps of the embedding procedure. This observation was similar to results in one of our earlier studies in which the use of type-specific antibody helped to stabilize the capsular material of *Streptococcus* group B type Ia (30) and keep it from collapsing. Similar observations of capsular swelling (31), capsular stabilization (2, 44), and antibody stabilization of the teichoic acid of the gram-positive cell wall (6) have also been reported.

Earlier reports on LPS localization studies were performed on either *E. coli* or *S. typhimurium* by using ferritin-conjugated polyclonal antibodies. This type of conjugate was likely to be too large to penetrate the LPS layer, and thus no stabilization effect was reported. From our results, the use of the O-antigen-specific IgG antibody MA1-8 revealed an amorphous structure with a thickness of 20 nm (Fig. 4a and b). When the O-antigen-specific IgM antibody MF15-4 plus a second antibody was used, the amorphous layer approached a thickness of approximately 30 to 40 nm (Fig. 5). In both instances, the protein A-gold probe was excluded on the outside of the layer. While this electron-dense amorphous structure may represent binding of stain to antibodies, it would seem that the O antigen of the LPS has to be present for the antibodies to anchor onto. Thus, even if we subtract the average diameter of an IgG molecule of 8 nm (37) from the 20-nm thickness, the O antigen can still be extended about 10 to 12 nm away from the cell wall (Fig. 4a and b). It is perfectly logical for the use of the IgM antibody and the second antibody (an IgG) to produce a thicker amorphous layer (30 to 40 nm). The IgM, being a pentamer of immunoglobulins, can be estimated to have an average diameter of approximately 10 to 20 nm, depending on which way the IgM is oriented on the bacterial cell surface (12). When 8 nm is also subtracted for the IgG molecule of the second antibody used, we can again predict that the O antigen of *P. aeruginosa* PAO1 is capable of extending 10 to 12 nm away from the cell wall. In a more recent localization study by Bayer et al. (3), they observed that the O antigen of encapsulated *E. coli* extended 40 to 50 nm into the capsular region, in partial agreement with the results obtained in our study. However, we cannot directly compare our results with theirs because of differences in bacterial organism, type of antibody used (polyclonal versus monoclonal), and type of labeling method used (postembedding versus preembedding).

The amorphous layer observed outside *E. coli* cell surfaces in a study published by Shands (40) was clearly reported as a capsular layer when the cells were grown in in vivo conditions. *P. aeruginosa* clinical isolates from cystic fibrosis patients often produce alginatelike, mucoid exopolysaccharides (11). This alginate material is not a tight capsule (8) and can be described as a loose slime which is easily sloughed off into the growth medium or the surrounding environment (24). Even when this exopolysaccharide was stabilized by polyclonal antibodies in our previous study (24), the fibrous structure observed extended as irregular patches of strands. These strands can extend from one cell to the next in a microcolony of *P. aeruginosa*, and these antibody-stabilized strands do not form a consistent layer of equal thickness around the outer membrane of each cell. Therefore, the amorphous layer observed here (Fig. 4a and b and 5) is unique. In addition, the specificities of our monoclonal antibodies MA1-8 and MF15-4 against the O antigen of *P. aeruginosa* PAO1 have been confirmed by ELISA and Western immunoblotting studies (Fig. 1) and in previous publications (17, 26).

Wild-type *P. aeruginosa* strains are semirough, in that only 15% of the LPS molecules are capped by an O-antigen side chain (15, 45; see also the large amount of rough LPS in Fig. 1, lane A). However, the average length of these O-antigen side chains would appear to be 60 to 80 sugars in length, assuming that, like *Salmonella* LPS (36), the adjacent

bands of *P. aeruginosa* LPS (Fig. 1) differ by one repeating tri- to tetrasaccharide unit. Thus, despite the rather low capping frequency of LPS by O-antigen side chains, there appears to be a potential for coverage of the bacterial surface by these side chains. Consistent with this, Sadoff et al. (39) demonstrated that an LPS rough-core-specific monoclonal antibody, 19.22.1, is not protective in mouse intraperitoneal-challenge studies. In contrast, LPS O-antigen-specific monoclonal antibodies described in the same study were found to be protective (39). Therefore, the existence of an LPS O-antigen layer, demonstrated in the present study, would provide an explanation for these results, since such a layer would be expected to inhibit the binding of or exclude rough-core-specific antibodies. It should be noted, however, that monoclonal antibodies to outer membrane porin protein F of *P. aeruginosa* are capable of binding to the surface of intact *P. aeruginosa* cells (35) and providing passive protection against *P. aeruginosa* infections in mice (16). Therefore, either protein F-specific antibodies must be able to pass through the postulated LPS O-antigen coating of the bacterial surface or else the surface coverage by LPS O antigens is incomplete.

ACKNOWLEDGMENTS

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