AN IMMUNO-ELECTRON MICROSCOPY STUDY
OF THE SLIME LAYER ANTIGEN OF
PSEUDOMONAS AERUGINOSA

APPROVED:

[Signatures]

Major Professor

Minor Professor

Director of the Department of Biology

Dean of the Graduate School

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This investigation was concerned with the relationship of the slime layer material of Pseudomonas aeruginosa, Verder and Evans strain 1369, to the presumably somatic "O" type of antigen used by these authors as the base for their serological schema.

Antisera against a highly purified slime layer antigen from this strain, as well as against whole cells, was prepared by inoculation of New Zealand white rabbits. Ferritin-labeled gamma globulin from the antiserum specific for the slime layer antigen were used to visualize the slime layer by electron microscopy. Suspensions of the organism, unwashed or washed 1 to 6 times, were examined by this technique as well as by ruthenium red staining to facilitate visualization of the slime material.

By these techniques it was evident that the simple washing procedures removed the slime layer material. Cells
washed 6 or more times were found to have lost essentially all the slime material, but retained cell wall integrity and viability.

Washed and unwashed preparations were also tested by standard tube agglutination techniques against the antiserum specific for the purified slime layer antigen, and antiserum prepared against whole cells. Agglutinability was progressively lost in proportion to the number of washings when the cells were tested against the anti-slime antigen, but remained relatively constant when tested against antiserum to whole cells. These findings clearly indicate that the slime layer antigen is serologically distinct from the antigenic substances found in the cell wall.

Cross reactions between unwashed and washed cell preparations of two different serotypes (Verder and Evans; strains 1369 and 2108) showed no detectable cross reactivity with antiserum against the heterologous strain. This indicates that both the slime layer and cell wall materials from these two strains are serologically distinct. The findings indicate the necessity of re-examining the serological schemata based supposedly
on somatic antigens in an attempt to determine whether the established methods of serotyping are actually based on the specificity of the slime layer, the cell wall, or both.
AN IMMUNO-ELECTRON MICROSCOPY STUDY
OF THE SLIME LAYER ANTIGEN OF
PSEUDOMONAS AERUGINOSA.

THESIS

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Robert L. Pardue, B. S.
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INTRODUCTION

Several serological schemata have been proposed for typing strains of *Pseudomonas aeruginosa*. Of these, the most reliable and widely recognized are those of Habs (25) in Europe and Verder and Evans (57) in this country. Although the latter investigators have used flagellar antigens for strain differentiation, the main basis for their schema, as well as that of Habs, is the occurrence of a heat stable (100°C for 2 1/2 hr.), presumably somatic antigen.

Alms and Bass (2, 3), Alexander, et al. (1), and others have isolated a protection-inducing antigen from the slime layer of *Pseudomonas aeruginosa*. A highly purified preparation can be obtained by treatment of the ethanol precipitated slime material with trichloroacetic acid to remove extraneous protein, followed by Pronase treatment to digest away the major portion of the residual protein. In this form the antigenic preparation will induce a protective response in mice when given in amounts less than 1 microgram per mouse. The major
portion of this material consists of polysaccharide, with only about 4 per cent consisting of protein. The relative heat stability of the protective antigen, as well as the fact that absorption of antisera against whole cells (heated for 2 1/2 hours at 100°C to destroy flagellar antigens) will remove the protective component as well as the agglutinating antibody suggests that the so-called slime layer antigen may well be an extension of the cell wall material on which the serological schemata cited above are based. Furthermore, Bass and McCoy (6) have shown that a close correlation exists between protection against challenge and agglutination titers against the Verder and Evans 'O' serotypes.

The cell walls of Gram-negative bacteria are multilayered structures, as reported by Kellenberger and Ryter (30), Mergenhagen (36), and Wardlaw (58). The outer most protein-lipid layer lies over a middle lipopolysaccharide and a rigid "R" layer lying adjacent to the cell membrane. The mucopeptide "R" layer is responsible for the endotoxic activity associated with Gram-negative bacteria (14). The extraneous capsular
material found in smooth bacteria may arise from one of these underlying layers of the cell wall and may be an extension of its base layer. This extraneous slime or capsular material may limit or inhibit the bactericidal action of antibody and complement (48).

The current studies were undertaken in an attempt to determine if the protective slime layer of Alms and Bass (2, 3) was, in fact, an extension of the underlying lipopolysaccharide or was, in fact, a separate slime or capsular layer exhibiting serological specificity independent of the specificity of underlying structures.
MATERIALS AND METHODS

Immunization of Animals. The animals used for the production of antisera were New Zealand rabbits ten to twelve weeks old, weighing between four and five pounds. Each rabbit was bled prior to inoculation to check for any naturally occurring antibodies against various strains of Pseudomonas.

The antigen used for production of anti-slime antiserum was a pronase-treated alcohol-precipitated fraction (APF), as defined by Cha, Bass, and Shetlar (13). One hundred mg of the freeze-dried APF, prepared from the slime layer of a Verder and Evans strain 1369 (Group II), was provided by Dr. Joseph A. Bass, Department of Biological Sciences, North Texas State University. The freeze-dried APF was dissolved in ten ml of sterile distilled water. Two and one-half ml of the solution was then mixed with an equal volume of Freund's complete adjuvant (Difco). This suspension was then injected subcutaneously at five different sites, one ml per site, all on the same day.
Antisera were also produced against whole, heat-killed cells by the use of cell suspensions prepared in the same manner as was used for preparation of test antigen for agglutination tests using heat-killed organisms. The only exception was that cell suspensions were heated for 2 1/2 hours at 100°C to insure complete destruction of flagellar antigens. The final suspension, which corresponded in density to a number three MacFarland barium sulfate standard, was mixed with an equal volume of Freund's complete adjuvant. A total of 5 ml of this mixture was inoculated subcutaneously on the same date into New Zealand white rabbits in divided doses at 5 different sites.

After 6-7 days, samples of serum were obtained from each rabbit and the titers were checked by tube agglutination. If the titer was found to be less than 1:320, a second inoculation of a similar amount of antigen was carried out 10 days after the first injection, and the serum again tested. A second booster injection of 2 ml was given intravenously fourteen days after the first injection. Six days following the final injection, if the titer was
found to be 1:640 or higher, the rabbits were exsanguinated by cardiac puncture. The antisera were pooled, labelled, and stored in small aliquots at -20°C.

Rabbits were bled at 9, 10, 14, 21, and 28 days after primary immunization, and the serum was tested for agglutination titer as noted below. Serum was stored in 30 ml sterile capped test tubes at -20°C.

**Organisms and Media.** *Pseudomonas aeruginosa* Verder and Evans strains 1369 (Group II), and 2108 (Group III) were obtained from Dr. Bass. Organisms were streaked on the surface of three Brain-heart Infusion (BHI) agar (Difco) slants, and allowed to incubate at 27°C for 18-24 hours. To the BHI slants, 5 ml of sterile 0.85 per cent saline were added. Growth was harvested by gentle scraping and then agitated on a Vortex, Jr. tube agitator for 10-15 seconds. The cell suspension was transferred to 1500 ml of BHI broth in a three liter Erlenmeyer flask which was placed in a shaking water bath at 37°C for 18-24 hours. The cells were harvested by centrifugation in a Sorvall RC2-B Ultracentrifuge. The broth culture was divided into 250 ml aliquots and placed in sterile plastic centrifuge bottles.
Tube Agglutination. A standard two-fold dilution tube agglutination test was used to titrate the rabbit antisera. The antigen consisted of a suspension of live cells of *Pseudomonas aeruginosa*, grown 18-24 hours in BHI broth on a shaking water bath at 37°C. The cell mass was separated by centrifugation and diluted to correspond to the O.D. of a number three Barium sulfate MacFarland standard. One half ml of the antigen suspension was added to appropriate dilutions of antiserum and the mixture incubated at 50°C for four hours, then placed in the refrigerator overnight. Appropriate saline and normal serum controls were included in all tests.

Adsorption of Sera. Absorptions were carried out by mixing one volume of packed cells with an equal volume of 1:5 dilution of serum, and incubating the mixture as described above. The absorbed serum was collected by centrifugation and stored at -20°C. When heat-killed cells were used in agglutination tubes, the suspension was prepared as previously described and then was heated for one hour at 100°C. Immediately prior to use the cells were sedimented by centrifugation and resuspended in fresh saline to the desired turbidity.
Electron Microscopy. All electron microscopy was carried out on an RCA EMU-3G electron microscope. Grids for whole mount specimens were first coated with a thin layer of formvar, then placed in a vacuum evaporator and carbon-coated. The bacterial specimens were gently suspended, from growth on BH agar slants, in physiological saline and diluted to an optical density equal to a number 3 MacFarland standard. A small drop of the suspension was placed directly on the grid and allowed to air dry prior to shadowing with chromium.

Fixation, dehydration and embedding were carried out according to Kellenberger and Ryter (30) using 3.6 per cent glutaraldehyde and 1 per cent osmium tetroxide at pH 7.4. After dehydration by treatment with increasing concentrations of ethanol, a small sample of the cells was taken and placed in a 50 per cent Epon 812, 50 per cent propylene oxide mixture and allowed to stand for 30 minutes. The specimens were placed in a plastic embedding capsule half-full or 100 per cent Epon 812 and sufficient 100 per cent Epon added to fill the capsule. The filled capsules were left at room temperature for 24 to 48 hours, then transferred
to a 60°C oven for a 72 hour period to facilitate polymerization.

Sections were cut at eight µm on a Sorvall MT-2 Ultramicrotome and sections were stained for two minutes with a saturated uranyl acetate solution followed by a one minute staining with lead citrate (43).

**Ruthenium Red.** Ruthenium red staining was carried out according to the procedures described by Pate and Ordel (42) as modified from Luft (34). Cells harvested directly from broth culture or washed as noted below were packed by centrifugation. The cell mass was then subdivided and small portions fixed for one hour in cracked ice in the following solution: 3.6 per cent glutaraldehyde, 0.5 ml; 0.2 molar cacodylate buffer, pH 7.3, 0.5 ml; ruthenium red stock solution, 1,500 ppm in distilled water, 0.5 ml. The clumps were then rinsed in 0.15 M cacodylate buffer for ten minutes and fixed for three hours at room temperature in the following solution: 1 per cent osmium tetroxide in distilled water, 0.5 ml; 0.2M cacodylate buffer, pH 7.3, 0.5 ml. The cells were then rinsed briefly in buffer, dehydrated
in a graded series of alcohols, and embedded in Epon 812 (41). Ruthenium source was Alfa Inorganics, incorporated, Beverly, Massachusetts. Sections were cut on a Sorvall MT-2 ultramicrotome using glass knives.

After drying, whole mounts of cells on grids were inverted on a drop of ruthenium red for fifteen minutes, then rinsed in distilled water and air-dried.

**Gamma Globulin Fractionation.** Gamma Globulin fractionation was carried out according to Weir (60) using a 33 per cent ammonium sulfate precipitation. To two volumes of whole serum, one volume of saturated ammonium sulfate (SAS) was added dropwise with constant stirring with a magnetic stirrer. The 33 per cent SAS mixture was allowed to stand for 45 minutes or longer at 4-5°C before centrifugation. Centrifugation was carried out at 5,000 rpm for ten minutes and 4-5°C. The sediment was resuspended in the original volume of 0.9 per cent sodium chloride. The gamma globulin fraction was precipitated by 33 per cent SAS for fifteen minutes at room temperature. The second precipitate was washed twice in the original volume of 40 per cent SAS.
replicat a third time by 33 per cent SAS for fifteen minutes at room temperature. The precipitate was washed twice in 40 per cent SAS and redissolved in 0.9 per cent sodium chloride, dialyzed against 0.9 per cent NaCl with three changes of saline over a 48 hour period. Dialyzed gamma globulin was stored in sterile capped pyrex tubes at 0°C.

**Ferritin-Antibody Conjugation.** The ferritin used was 2X crystalized (Nutritional Biochemical, Cleveland, Ohio). P, P\'-difluoro-m, m\'-dinitrophenyl sulfone (FNPS) (53, 54, 55) was chosen as the coupling agent to obtain a higher yield and greater retention of antibody activity. To a mixture of 160 mg of rabbit globulin (RGG) and 460 mg of ferritin, dissolved in sufficient 4 per cent aqueous sodium carbonate to constitute a 4 per cent protein solution, was added 1 ml of chilled acetone containing 5 mg of FNPS. After stirring in the cold (2-3°C) for 24 hours, the mixture was exhaustively dialyzed (48 hours) against normal saline and then centrifuged to remove small amounts of precipitate which tended to form. The remaining mixture contained unconjugated
antibody, ferritin-conjugated antibody and unconjugated ferritin.

Removal of unconjugated antibody was accomplished in a Beckman Model L Ultracentrifuge using cellulose nitrate tubes, by three centrifugations at 100,000 g (35,000 rpm) for 4 1/2 hours each time. After each run, the conjugated protein and free ferritin formed a pellet which was resuspended in 13.5 ml of cold 0.05 M phosphate buffer at pH 7.5. The final pellet was resuspended in 1-2 ml of 0.05 M phosphate buffer and stored in a sterile vial at 4-5°C until used. Removal of unconjugated ferritin by starch block electrophoresis, used by Borek and Silverstein (8) was not done, since Weir (60) deems this to be an unnecessary step. Final ferritin-antibody conjugate was 40 per cent less reactive than native gamma globulin yet more reactive than the conjugate prepared by using the toluene 2,3-diisocynate method of Singer (49, 50, 51).

Washing and Labeling. A 1,500 ml, 24 hour, BHI broth culture was divided into six 250 ml aliquots, placed in 300 ml plastic bottles and centrifuged in
a Sorvall RC2-B at 5,000 rpm for fifteen minutes at 4-5°C. The supernatant was discarded and samples taken. This first centrifugation was considered as a packing stage only and not as a wash. Cells from each of the remaining six bottles were resuspended in 225 ml of 0.85 per cent saline and centrifuged at 5,000 rpm, for fifteen minutes at 4-5°C. This procedure was repeated six times. The samples taken from each washing stage were diluted with 0.85 per cent saline to a turbidity equal to a MacFarland number 3 BaSO₄ standard. This suspension was used for tube agglutination tests and for reaction with ferritin labeled antibody.

Following each washing stage 2 ml of diluted washed cells were removed and 4 to 6 drops of ferritin conjugate added dropwise. The mixture was incubated in the cold (4-5°C) for twelve hours, then centrifuged at 3,000 rpm for ten minutes. The resulting pellet was washed twice in Earl's basal salt solution and fixed by the method of Kellenberger and Rycer (30). Sections were cut at a thickness of 8 μm.
RESULTS

Normal serum samples were taken from each of the five rabbits (A, B, C, D, E). At time zero, all five animals showed no detectable titer against suspensions of either heat-killed or live P. aeruginosa, strain 1369. Ten days after the initial series of immunizations the serum of 4 of the 5 reacted positively against both live and heat-killed organisms. Titers against the heat-killed organisms ranged from 1:20 to 1:80 but were considerably higher against the live organism suspensions (1:320 to 1:5120, figure 1).

This striking difference in titer raised the possibility that heat-treatment of the cells might be destroying an antigenic configuration present in the live organism. Accordingly, aliquots of the antislime antiserum were absorbed with heat-killed cells and with live cells. In both instances the titer against live cells was markedly reduced from 1:10,240 to 1:320 by single absorption with a live suspension and to less than 1:10 following absorption with the heat-killed suspension.
Table I. Agglutin production in 5 rabbits immunized with a single injection of purified slime layer (25mg/rabbit). Titers were determined against live suspensions of *P. aeruginosa*
These findings indicate that the antiserum produced against the purified slime material possesses no antibodies specific only for live cells since heat-killed cells removed essentially all the agglutins against the live organism.

The slime layer antigen used herein was prepared by a simple washing procedure (13). When cell suspensions were washed several times, this material continued to be released into the supernatant, but the yield per wash progressively decreased until essentially no further material was obtained (Bass, unpublished results). Nevertheless (on the basis of these observations) it is not clear whether the slime layer moiety remains as a substantial part of the antigenic mosaic typical of the cell wall of Gram-negative rods. If this were the case, cells denuded of the surrounding slime cover should still be agglutinated by anti-slime layer antibody. If, on the other hand, the cell wall of thoroughly washed organisms was devoid of the slime antigen, it would be reasonable to postulate that the slime layer was more probably an accumulation of exudative material
and did not truly represent a portion of the integral
cell wall. Antiserum prepared against the purified
slime antigen was therefore tested against live suspensions
of the homologous organism which had been washed 1-6 times
to remove the slime layer. The results, shown in Table 1,
clearly indicate a marked loss of agglutinability of live
cells, suggesting an almost complete loss of slime antigen
following exhaustive washing.

When cells similarly treated were reacted with
antiserum prepared against heated, whole cells, the
results were strikingly different (Table 2). Thus,
the repeat titration against antislime antiserum (column
1) was essentially identical to the results in Table 1.
On the other hand, there was no significant decrease in
titer when the washed cells were tested against antiserum
to the whole heat-killed organism (column 2). These
results were not surprising, in that antiserum against
whole cells should contain antibodies directed against
cell wall antigens in addition to those against slime
material. That the former antibodies were in fact present
is indicated by the fact that cells washed free of slime
<table>
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<th>ANTIGEN(^+) TREATMENT</th>
<th>RECIPROCAL OF AGGLUTINATION TITER</th>
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<tr>
<td>Unwashed</td>
<td>10,480</td>
</tr>
<tr>
<td>Washed 1x</td>
<td>10,240</td>
</tr>
<tr>
<td>Washed 2x</td>
<td>1,280</td>
</tr>
<tr>
<td>Washed 3x</td>
<td>320</td>
</tr>
<tr>
<td>Washed 4x</td>
<td>160</td>
</tr>
<tr>
<td>Washed 5x</td>
<td>40</td>
</tr>
<tr>
<td>Washed 6x</td>
<td>&lt;5</td>
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\(^+\)Live organisms Verder and Evans, strain 1369.
TABLE III. A Comparison of the Successive Washings of Live *P. Aeruginosa* on Agglutinability by Antisera against Whole Cells as Opposed to Antisera Against Purified Slime Layer Antigen.

<table>
<thead>
<tr>
<th>Antigen† Treatment</th>
<th>Antiserum</th>
<th>anti-whole heat-killed++ cells (Verder and Evans 1369)</th>
<th>anti-slime unabsorbed absorbed with live cells washed 6X</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>column 1</td>
<td>column 2</td>
<td>column 3</td>
</tr>
<tr>
<td>Unwashed</td>
<td>20,480</td>
<td>540</td>
<td>520</td>
</tr>
<tr>
<td>Washed 1X</td>
<td>5,120</td>
<td>640</td>
<td>160</td>
</tr>
<tr>
<td>Washed 2X</td>
<td>2,560</td>
<td>640</td>
<td>80</td>
</tr>
<tr>
<td>Washed 3X</td>
<td>320</td>
<td>320</td>
<td>40</td>
</tr>
<tr>
<td>Washed 4X</td>
<td>40</td>
<td>320</td>
<td>20</td>
</tr>
<tr>
<td>Washed 5X</td>
<td>&lt;5</td>
<td>320</td>
<td>20</td>
</tr>
<tr>
<td>Washed 6X</td>
<td>5</td>
<td>320</td>
<td>10</td>
</tr>
</tbody>
</table>

†Live organisms Verder and Evans, strain 1369.

++Heat-killed, 2 1/2 hours, 100°C.
antigen were still agglutinated to a high titer. When this same antiserum was absorbed with live cells washed 6x, and was then tested against unwashed live cells, they were agglutinated at essentially the same titer as obtained with the unabsorbed antiserum. However, when suspensions of live cells washed 1-6 times were tested as before, a decrease in titer similar to that previously obtained with anti-slime antiserum was observed. Thus, absorption with the cells denuded of the slime layer, removed the antibodies directed against the cell wall antigens but did not absorb out those antibodies specific for the slime antigen. When antiserum against whole cells of a different serotype was tested against unwashed and washed homologous cells (Verder and Evans, strain 2108), essentially the same pattern as noted in column 2, Table 2 was observed.

To test the possibility that the cell wall antigen(s) were shared by different serotypes, antisera against whole cells of strains 1369 and 2108 were reacted with 6x washed suspensions of the two strains. In both instances, the test organism reacted positively only with
its homologous antiserum, indicating that, at least in these two strains, no common cell wall antigen existed. It should be noted that unwashed preparations also failed to react with the heterologous antiserum.

**Electron Microscopy.** Electron photomicorgraphs at varying magnifications of dry mounted, chromium shadowed, whole cells showed an intact rod structure apparently surrounded by a less dense layer (Figure 1.1). When the slime layer was made electron opaque by the use of ruthenium red, a large undisturbed capsular layer was visible in unwashed cell preparations (Figure 1.2) with successive washings a decreasing amount of extracellular material was seen (Figures 2.1, 3.1, 4.1, 5.1, 6.1, and 7.1). After the sixth and final washing there was little or no ruthenium red-stained extraneous mucopolysaccharide.

When these same preparations of washed and unwashed cells were treated with ferritin labeled immuno-globulin from antiserum against the purified slime antigen, a similar sequence was observed.

Photomicrographs of ferritin-antibody labeled cells revealed a massive labeling in the mucopolysaccharide
Figure 1.1. *Pseudomonas aeruginosa*, Strain 1369, 24 hour culture (unwashed), chromium shadowed, X 21,500.

Figure 1.2. *Pseudomonas aeruginosa*, Strain 1369, 24 hour culture (unwashed), ruthenium red stained, X 24,500.
Figure 1.3. *Pseudomonas aeruginosa*, Strain 1369, ferritin labeled, $\times 24,500$. 
Figure 2.1. *Pseudomonas aeruginosa*, washed x1, X 27,250.

Figure 2.2. *Pseudomonas aeruginosa*, washed x1, X 21,500.
Figure 3.1. *Pseudomonas aeruginosa*, washed X2, X 24,500.

Figure 3.2. *Pseudomonas aeruginosa*, washed X2, ferritin labeled, X 24,500.
Figure 4.1. Pseudomonas aeruginosa, washed X3, X 21,250.

Figure 4.2. Pseudomonas aeruginosa, washed X3, ferritin labeled, X 24,500.
Figure 5.1. *Pseudomonas aeruginosa*, washed x4, X 18,500.

Figure 5.2. *Pseudomonas aeruginosa*, washed x4, ferritin labeled, X 24,250.
Figure 6.1. *Pseudomonas aeruginosa*, washed $\times 5$, $\times 27,500$.

Figure 6.2. *Pseudomonas aeruginosa*, washed $\times 5$, ferritin labeled, $\times 27,500$. 
Figure 7.1. *Pseudomonas aeruginosa*, washed x6, x 27,500.

Figure 7.2. *Pseudomonas aeruginosa*, washed x6, ferritin labeled, x 32,400.
Figure 8.1. *Pseudomonas aeruginosa*, washed x6, thin sectioned, ferritin labeled, x 14,500.
slime coat of the unwashed cells (Figure 1.3). Again successive washings reduced the amount of ferritin labeled substances (Figures 2.2, 3.2, 4.2, 5.2, 6.2, 7.2). Following the sixth washing procedure, essentially no labeling was evident. Thin sections of the latter preparation showed intact cells with no labeling except in small patches of extraneous slime material (Figure 8.1). There was no labeling of cell wall or cell membrane material.
D I S C U S S I O N

Wardlaw (58), on the basis of his own observations, as well as those of Kallenberger and Rytén (30) and of Weidel and co-workers (59), has suggested that the cell wall of Escherichia coli consists of an outer layer of lipoprotein, a middle layer of lipopolysaccharide, and an inner layer of mucoprotein. He suggested, however, that the outer lipoprotein layer did not constitute a solid, unbroken envelope, but rather, that the lipopolysaccharide layer extruded periodically through the lipoprotein layer. Viewed from the surface, rather than a cross-section, the cell wall thus presented a mosaic in which areas of lipoprotein and lipopolysaccharide are adjacent.

Similar studies have not been done of P. aeruginosa, although a number of reports in the literature deal with the chemical composition of the cell wall (9, 10) and of the slime layer (6, 13). No similarity in the chemical make-up of these two structures has been noted, and no attempt has been made by investigators in this field to
equate the two substances. Nonetheless, the more widely known serological schemata of Verder and Evans (57) and of Habs (25) have been based on antibodies which have been induced by immunization of rabbits with heat-killed, (100°C for 2 1/2 hours), smooth organisms. On the basis of the heat stability of the immunizing substances, the antigens responsible have been termed somatic or 'O' type antigens. The fact that the slime layer materials have been disregarded as the antigens on which these typing schemata are based may in part be due to the well-known fact that similar capsular antigens such as the Vi antigen of Salmonella typhosa and the K antigens of E. coli are destroyed by heating at 100°C for 2 1/2 hours.

Alms and Bass (2, 3), however, have isolated a protective antigen from the slime layer which is stable when treated in the above fashion, and Bass and McCoy (6) have demonstrated that protection in experimental infections correlates well with the serological schemata referred to above. The question arising from such observations then, is whether the serological schemata of Verder and Evans and of Habs are in fact based on the
heat stable slime layer antigen, rather than on the antigens found on the underlying cell wall. The experiments undertaken in the present study represent an initial step in the elucidation of this problem. Thus, if the slime layer were simply an extension of the lipopolysaccharide layer, repeated washings should remove the extraneous slime capsule, but should leave the basic antigenic mosaic extant. In such a case, agglutination of washed cells with antiserum against the purified slime antigen should continue to occur, unless the lipopolysaccharide material was completely leached from the outer layers of the cell wall. In such an instance a break in the integrity of the wall of repeatedly washed cells should be evident by electron microscopy.

The alternative hypothesis, e.g., that the slime material is not related to the underlying lipopolysaccharide layer, appears more tenable, in view of the above cited differences in chemical composition of cell walls and slime antigen. Additionally, it should be noted that Alms and Bass (2, 3) have demonstrated that the partially purified slime layer antigen does not behave in a manner
consistent with that of classical endotoxin from Gram-negative organisms, a substance closely associated, if not identical, with the lipopolysaccharide layer.

The present findings support this latter hypothesis. Live cells washed repeatedly to remove the soluble slime layer progressively lost their agglutinability by antiserum prepared against the purified slime layer antigen. Simultaneously, as determined by ruthenium red staining, the slime layer was seen to disappear until, following the sixth wash, only occasional patches of stained material were observed. In some instances these were loosely attached to the cell, but apparently were not an integral portion of the cell wall. When ferritin-labeled antibody against the purified slime antigen was used to detect the presence of this substance, a similar picture emerged. There was no indication of reaction of the labeled antibody with the cell wall proper, but only with material which was previously extraneous to the cell wall. Finally, it should be noted that the surface of cells denuded of the slime material by repeated washings presented an intact cell wall structure. Furthermore,
these cells remained viable, as determined by counts on suspensions of washed and unwashed cells of comparable density.

It is also of interest to note that cells washed free of the slime layer, readily absorbed antibody directed at the underlying structures, but failed to remove agglutinins which were specific for the slime material.

Quite obviously, the present data are insufficient to clarify the relationship of the Verder and Evans and Habs systems of classification to the slime antigens. It should be noted, however, that the above workers have stressed the need for use of "smooth" strains such as used in the present experiments to demonstrate the presence of a slime layer.

On the other hand, when washed organisms of strain 1369 were tested with antiserum prepared against whole cells of strain 2108, no agglutination was observed. Conversely, washed cells of the latter strain failed to agglutinate in the presence of high-titered antiserum against whole cells of strain 1369. This would indicate
that in addition to possessing antigenically dissimilar slime layer antigens, these two strains also fail to share common cell wall antigens. This presents the possibility that strains of P. aeruginosa may be differentiated by two separate antigenic systems. It is also interesting to speculate on the possibility that a given 'O' or cell wall antigen is found only in association with a certain slime layer antigen. Precedence for this exists in fairly constant pairing of certain 'O' and 'K' (capsular) antigens in the enteropathogenic strains of E. coli.
SUMMARY

Antiserum was prepared in rabbits against a highly purified antigen from the slime layer of a strain of Pseudomonas aeruginosa. Gamma globulin preparations from this antiserum were labeled with ferritin, and the labeled reagent used to demonstrate the presence of a slime layer by electron microscopy. Ruthenium red staining of the slime material was used as a corollary technique. Gentle washings of cells (from 1 to 6 times) resulted in loss of the slime material until, following the sixth wash, the cells were seen to be essentially denuded of this substance. However, the basic cell structure appeared intact. Such cells failed to be agglutinated by high-titered antiserum against the purified slime antigen. On the other hand, they continued to be agglutinated by antiserum prepared against whole cells. These findings, together with the failure of washed cells of this strain to react against antiserum against whole cells of another serotype suggest that
serotyping of *P. aeruginosa* may be dependent not only on the antigenic specificity of the slime material, but also on specific antigens associated with the cell wall proper.
BIBLIOGRAPHY


