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Flagellar Preparations from *Pseudomonas aeruginosa*: Isolation and Characterization

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Flagella from various strains of *Pseudomonas aeruginosa* were isolated by shearing the flagella followed by differential centrifugation to obtain typical filaments as viewed through an electron microscope. Electrophoretic analysis showed a major protein band corresponding to a flagellin with a molecular weight of 53,000. Among the strains tested, flagellar antigen (FAg) preparations isolated from strains 1244 and 1210 routinely gave the highest percentage of flagellin, with the least amount of protein impurities, when grown on succinate-mineral salts medium. All FAg preparations contained 3 to 10 μg of 2-keto-3-deoxyoctonate-positive material per mg of protein. Strain PA-103 lacked flagella and exhibited no flagellin band, and preparations from PA-103 had a relatively higher content of 2-keto-3-deoxyoctonate. The isolation of a highly purified, single-banded flagellin could be accomplished by elution of the 53,000-molecular weight gel band. Amino acid analysis showed 16 amino acids, but no proline. Antisera to FAg preparations were used to demonstrate inhibition of motility of strains RM-46 and M-2. Heated RM-46 FAg antisera and PA-103 antisera did not inhibit motility.

Studies on the opportunistic infection of a debilitated host by strains of *Pseudomonas aeruginosa* have indicated that certain bacterial components, such as proteases and exotoxin A (6, 9, 12, 16, 20, 25), may play a role in infection and fatality, particularly in bacteremic cases (6, 25). However, other factors, such as attachment (10, 23, 24) and motility, appear to be involved when the important aspects of initial entry and subsequent colonization are visualized. It is interesting in this respect that McManus et al. (14) and Craven and Montie (5) have suggested the importance of motility and chemotaxis in the pathogenesis of *P. aeruginosa* in burned rodents.

Recently, we reported the use of flagellar antigen (FAg) preparations in protecting burned mice (T. C. Montie, R. C. Craven, R. Wheeler, and I. A. Holder, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, B17, p. 17). This work further describes the method of preparation and characterization of the *P. aeruginosa* FAg preparations. These preparations have been used to demonstrate the protective capacity of the FAg in infected burned mice (9).

MATERIALS AND METHODS

Organisms and growth media. Several *P. aeruginosa* strains were used to isolate flagellin. These included strains M-2, WR-5, and PA-103, which have been used for the examination of virulence in burned mice (8, 22), and strain RM-46, which was initially used for chemotaxis and motility studies (15). Other strains used were 1244 and 1210 which, along with strain RM-46, are very virulent (50% lethal dose, <100 colony-forming units [CFU]) in burned mice. Strains 1410 and 1431 were isolated from the sputa of cystic fibrosis patients. Strain 5933 was obtained from R. Ansorg (2).

Stock cultures were maintained at 4°C as a dilute suspension in Luria broth (1% NaCl-1% tryptone-0.5% yeast extract, pH 7.0). This medium was also used with 2% agar for overnight culture for FAg preparations. A mineral salts (MS) medium adjusted to pH 7.0 was used as previously described (15), with sodium succinate (0.4%) as the carbon source.

Motility studies were conducted by using capillary tubes essentially as described by Adler (1) but modified for *P. aeruginosa* (15), except that serum was added both to the well and to the capillary tube. In some experiments, α-aminoisobutyrate was added to the capillary tube. For bacterial plate counts, cells were diluted in tryptone broth and plated on tryptone plates containing 1.6% agar.

All chemicals were those available commercially as certified reagent grade. Electrophoresis reagents were purchased from Bio-Rad Laboratories (Richmond, Calif.).

Isolation of FAg preparations. FAg was prepared by using a differential centrifugation technique. Overnight liquid cultures (20 ml each) grown in Luria broth or on MS were used to inoculate the surface of the agar medium in a large, stainless steel pan. After 17 to 24 h of growth at room temperature or 30°C, the cultures were gently scraped from the agar surface with a glass rod, and potassium phosphate buffer (0.01 M, pH 7.0)

was added to facilitate removal. The cell suspension was centrifuged for 15 min at 5,000 × *g* and 4°C. The resulting pellet was resuspended in phosphate buffer at a ratio of 100 ml of buffer per 6 g of wet weight cells. This suspension was blended in a commercial blender for 3 min to shear off the flagella. The suspension was centrifuged for 15 min at 16,000 × *g* and 4°C, and the resulting supernatant was centrifuged again for 3 h at 40,000 × *g* in a model L5-75 Beckman centrifuge with a 42.1 rotor. The pellets were carefully removed, suspended in a small amount of phosphate buffer, dialyzed against distilled water, and lyophilized. The dry preparations were stored desiccated at −20°C.

FAg preparation refers to lyophilized preparations obtained at this stage of isolation. As detailed below, preparations contained some lipopolysaccharide (LPS) (2-keto-3-deoxyoctonate [KDO]-positive material) and an additional minor component(s) when assayed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The content varied with the strain and method of preparation used. For immunological experiments (9), an effort was made to select preparations that were 90 to 98% pure, with regard to contaminating protein.

Analytical methods. Protein determinations of the lyophilized preparations were made with the Coomassie blue technique of Bradford (4), with crystalline bovine serum albumin as a standard, and Bio-Rad dye reagent (Bio-Rad Laboratories). The detection of KDO was carried out by the method of Karkhanis et al. (11).

For electron microscopic examinations, bacteria and FAg preparations were stained with 2% phosphotungstic acid neutralized to pH 7.0 with KOH.

FAg preparations were routinely compared, and we determined molecular weights by SDS-PAGE, using a Bio-Rad slab gel apparatus (Bio-Rad Laboratories) and following a modification of the procedure of Laemmli (13). The FAg preparation was boiled for 1 min in 2% SDS and a sample buffer before 20 to 25 μg of the preparation was applied to the sample gel. The stacking gel contained 5% acrylamide, and the running gel contained 10% acrylamide. Protein was routinely detected by staining with Coomassie blue, according to the method of Fairbanks et al. (7).

Amino acids were determined with a Beckman amino acid analyzer (model 116). A strain 1244 or 5933 FAg sample was digested for 20 h at 110°C with 3 N mercaptoethane-sulfonic acid (18). The 5933 FAg preparation was further purified by elution of the pure flagellin band with sodium phosphate buffer (0.01 M, pH 7.0) and 0.1% SDS. The area containing flagellin was located by comparison with an indicator strip of stained standard proteins from the same gel.

RESULTS

Electron microscopy of bacteria and FAg preparations. During our studies of the comparative chemotactic capacities of three *Pseudomonas* strains (5), we examined the strains for the presence of flagella by negative straining (Fig. 1). Strains M-2 and WR-5 were typically flagellated and exhibited the single polar flagellum that is characteristic of *P. aeruginosa*. Strain PA-103, however, lacked flagella. The latter

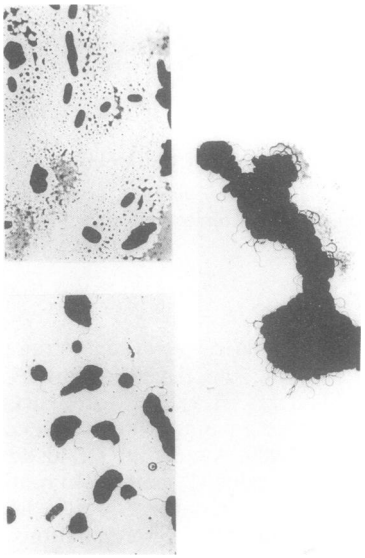

FIG. 1. Electron microscopic comparison of three strains of *P. aeruginosa*: strain PA-103 (upper left), strain WR-5 (lower left), and strain M-2 (right). ×1,800.

observation has been recently confirmed (B. Iglewski, personal communication). FAg preparations from strain RM-46 were also examined by negative staining (Fig. 2). These preparations showed numerous flagellar filaments and some debris.

Polyacrylamide gel assays of isolated samples and molecular weight determinations. The protein profiles of some representative FAg preparations were obtained by SDS-PAGE (Fig. 3). The flagellin subunit appeared as a single major band. A similar band has been identified in strains 1244 and 1210 and in a series of both mucoid and nonmucoid strains isolated from cystic fibrosis patients. An estimation of the molecular weight gave a value of approximately 53,000 (Fig. 4). The number of minor bands varied considerably among experiments and with the strain used. The major band of strain WR-5 had an apparent molecular weight 10,000 lower than those of strains M-2 and RM-46. Strain WR-5 exhibits a deficiency in chemotactic response (5). The protein corresponding to the flagellin bands of strains M-2, RM-46, or WR-5 was not present in samples from the nonflagellated strain PA-103 (Fig. 3).

It is noteworthy that we obtained FAg samples of higher purity by selecting the appropriate strain for growth. Although yields varied considerably (Table 1), strains 1210 and 1244 gave dry weight yields generally higher than the yields of other strains. Also, fewer protein impurities were observed in preparations from these strains (Fig. 5). Preliminary amino acid analysis of FAg

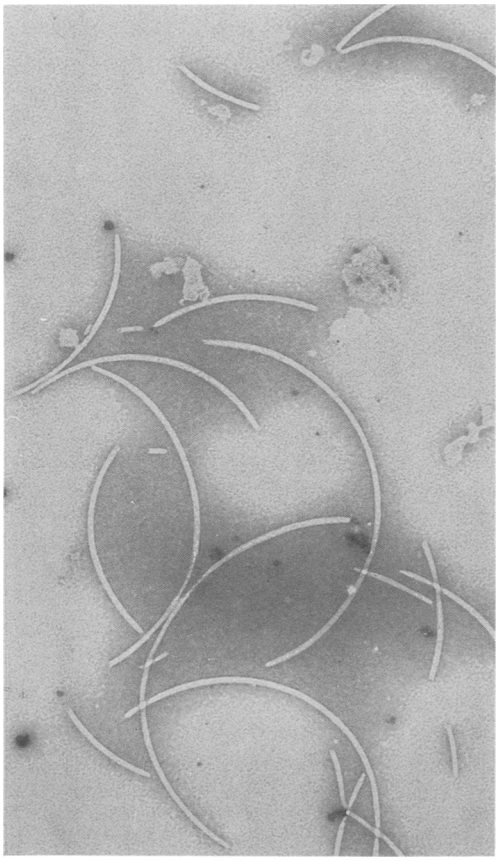

FIG. 2. Electron microscopic visualization of an FAg preparation from strain RM-46. ×65,000.

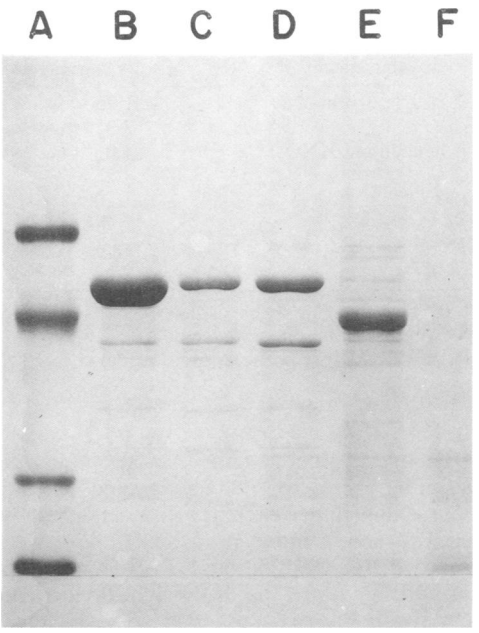

FIG. 3. SDS-PAGE of *P. aeruginosa* FAg preparations. (Lane A) Standard proteins and their molecular weights. From top, bovine serum albumin (66,000), ovalbumin (45,000), trypsinogen (24,000), and β-lactoglobulin (18,400). (Lanes B and C) Major band (M-2 flagellin; molecular weight, 53,000). (Lane D) Strain RM-46. (Lane E) Strain WR-5. (Lane F) Strain PA-103.

preparations from either strain 1244, which exhibited a single band in the gel assay, or strain 5933, which was eluted from a gel as described below, showed that at least 16 amino acids were present. These included lysine, histidine, arginine, aspartic acid, glutamic acid, glycine, alanine, valine, threonine, serine, methionine, cysteine, isoleucine, leucine, tyrosine, and phenylalanine. No proline was detected.

Recently, we demonstrated that flagellins can be isolated from SDS slab gels by elution with 0.1% SDS and phosphate buffer. After we dialyzed and lyophilized the eluted material, an electrophoretic assay showed a single band of flagellin protein (Fig. 6).

Effects of growth substrate on FAg yield. The FAg preparation used for protection studies in burned mice was prepared by using a succinate-MS-agar medium. Although the total dry weight of the final flagellar pellet increased up to three to four times when a rich medium was used (Table 1), the amount of FAg preparation relative to the amount of total protein decreased as indicated by the gel assay (Fig. 7). The gel assay showed numerous protein bands appearing in the preparations from bacteria flagellin grown on rich medium and relatively small amounts of stained flagellin as compared with FAg from MS preparations (Fig. 7).

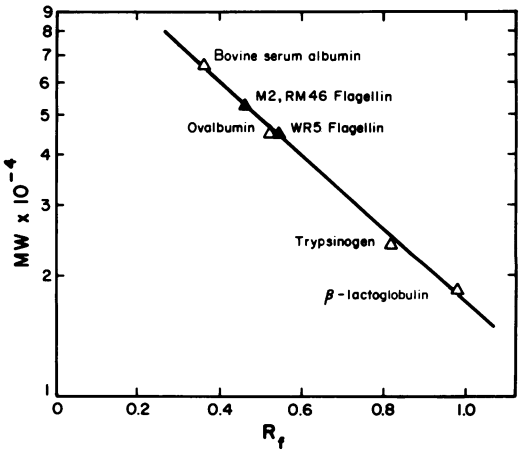

FIG. 4. Molecular weight (MW) determinations with SDS-PAGE. Standard proteins were as described in the legend to Fig. 3.

TABLE 1. Analyses of various FAg preparations[a]

Strain (growth medium)[b]	FAg yield (mg of dry wt)	Protein content (mg/mg of FAg dry wt)	KDO[c] (μg/mg of protein)
M-2 (Luria broth-agar)	21.75	0.83	3.3
M-2	3.34	0.15	1.6
M-2 (Succinate KNO_3)	4.06	0.28	9.2
M-2	4.22	0.37	4.3
WR-5	1.20	0.15	8.0
1244	7.48	0.39	10.2
1410	5.08	0.47	10.2
PA-103	1.61	0.10	36.0

[a] FAg preparations were isolated as described in the text.
[b] The growth medium was succinate-ammonium-sulfate-MS medium, except where indicated.
[c] Assay sensitive at the 1- to 10-μg level (see reference 11).

The overall yield of the PA-103 preparation was generally decreased, as expected, since this strain is flagellum deficient (Table 1). There was also a threefold increase in KDO content relative to FAg preparations. LPS content was therefore higher in the dry weight final fraction from strain PA-103. Although some KDO was present in all of the fractions, the relative amounts were small (2 to 10 μg/mg of protein) and apparently generally insignificant in contributing to the protection of mice (9).

Effect of FAg antisera on motility of strain M-2. Experiments were carried out to quantitate by capillary assay the antimotility activity of various FAg antisera. Sera from mice immunized with 50 or 1 μg of FAg were used (9). Results showed that serum samples from mice immunized with 50 μg of FAg from RM-46 or M-2 clearly inhibited the migration of strain M-2 cells into capillary tubes at all serum dilutions (Fig. 8). For example, at 1:40 serum dilutions, RM-46 and M-2 sera inhibited 80 and 66%, respectively, as compared with the control immunized only with alum. At this same serum dilution, PA-103 serum or *Escherichia coli* LPS control serum affected motility 24 to 34%, as compared with the control immunized only with alum. At some serum dilutions, alum, LPS, and PA-103 sera caused an apparent stimulation of motility over the buffer control (equivalent to the base-line value of 6.3×10^{-4} CFU per capillary). Viability counts from the slide wells (bactericidal activity) did not show any consistent decreases, except that there was some reduction in the CFU of both strain M-2 and the no-serum control wells.

Two experiments in which 1 μg of an FAg preparation was the immunogen are shown (Fig. 9). The mice were immunized with a single injection of an FAg preparation, and after 14 days, antisera were collected. Controls were mice injected with saline alone (saline control

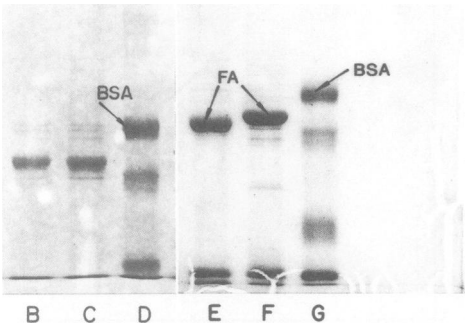

FIG. 5. FAg (FA) preparations of high flagellin content from strains 1244 and 1210. (Lanes B, C, and E) Three different 1210 FAg preparations. (Lanes D and G) Standard proteins (see Fig. 3). (Lane F) 1244 FAg preparation. BSA, bovine serum albumin.

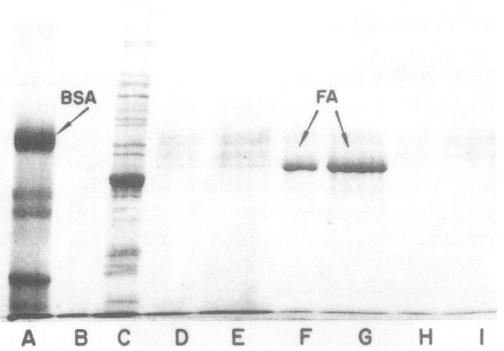

FIG. 6. Protein bands of *P. aeruginosa* flagellin rerun after elution from unstained SDS-polyacrylamide gel. (Lane A) Standard proteins; from top, bovine serum albumin (BSA), ovalbumin, trypsinogen, and lactoglobulin. (Lane C) 1410 FAg (FA) preparation (nonmucoid strain from a cystic fibrosis patient). (Lanes D to I) Samples eluted from previous gel run for strain 1244. (Lanes D and E) Eluted protein with an electrophoretic mobility greater than that of FAg. (Lanes F and G) Eluted FAg. (Lanes H and I) Eluted protein that was less mobile than FAg.

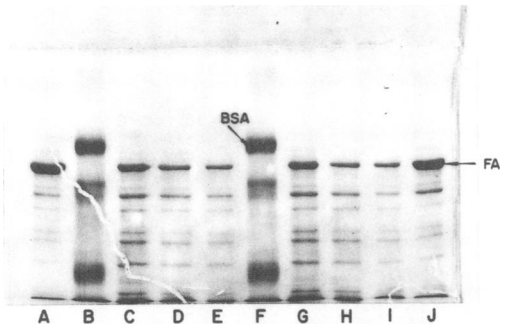

FIG. 7. Comparison of M-2 FAg (FA) preparations from organisms grown on MS or Luria broth-agar medium. (Lanes A and J) 25 μg of FAg preparations from two different experiments, with nitrate substituted for ammonium in MS medium. (Lanes B and F) Standard proteins, as shown in Fig. 3. (Lanes C, D, and E or G, H, and I) 125, 50, and 25 μg of FAg preparations, respectively, from cells grown on Luria broth-agar medium in two different experiments. BSA, Bovine serum albumin.

serum). In one experiment, the test serum was heated to 56°C for 30 min before use. In this experiment, RM-46, M-2, and 1244 sera all inhibited motility 19 to 25%, as compared with saline control serum. There was a slight inhibition by PA-103 serum, but it actually stimulated motility, as compared with the no-serum control. Strain RM-46(H) (serum heated at 90°C for 30 min) produced no inhibition. Bactericidal activity was only observed in the PA-103 serum well.

For comparison, another experiment (see Fig. 9) was done with immune serum made by using 1 μg of FAg, but the test sera were not preheated. Results showed a similar pattern of inhibition, with strain M-2 and 1244 sera producing the highest relative inhibition, 33 and 35%, respectively, as compared with the saline control serum. In this case, PA-103 and RM-46(H) samples apparently stimulated motility. Although some bactericidal activity was noted, no consistent correlations were observed.

A qualitative confirmation of the capillary results was further obtained by comparing the effect of mouse FAg antisera on colony spreading on motility plates (Fig. 10). Sterile paper disks containing antisera were placed over strain M-2 stabs made with M-2 cells. Spreading was inhibited by M-2 or RM-46 antisera, but not by PA-103 antisera.

We have concluded from these experiments and from results showing a lack of migration of *P. aeruginosa* from the general site of inoculation in the skin of FAg-immunized mice (9) that protection against burn infection in mice results from immobilization by an antiflagellar antibody.

DISCUSSION

FAg preparations for use as a vaccine have been prepared from various strains of *P. aeruginosa*. A molecular weight of 53,000 for flagellin from strains 1244, RM-46, M-2, and several other strains has been estimated by using SDS-PAGE. This weight for flagellin is in a range corresponding to that reported for *E. coli* and salmonellae (21), and, recently, for *P. aeruginosa* (3). Preparations from strain PA-103 showed

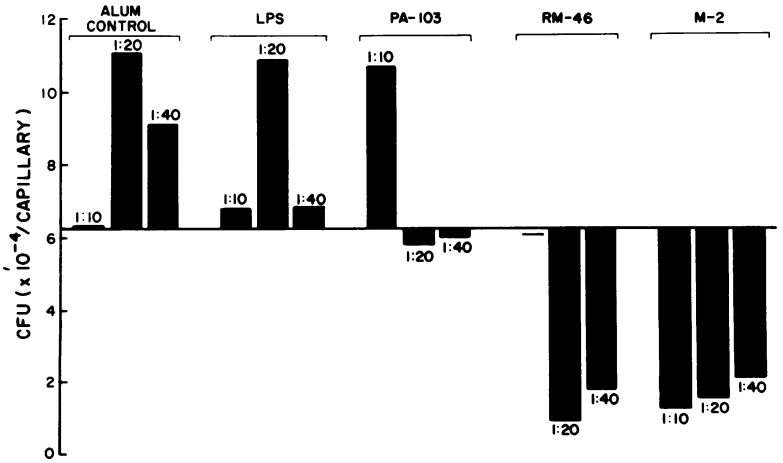

FIG. 8. Effect of various mouse FAg antisera on motility of *P. aeruginosa* strains. Mice were immunized with 50 μg of FAg from the strains indicated, and after 14 days, antisera were prepared. Controls consisted of mice immunized with alum alone or 50 μg of *E. coli* LPS. Inhibition is indicated by a decrease in the number of *P. aeruginosa* CFU per capillary. The base line represents the number of bacteria migrating in the capillary containing only buffer (equal to 6.3×10^4 CFU).

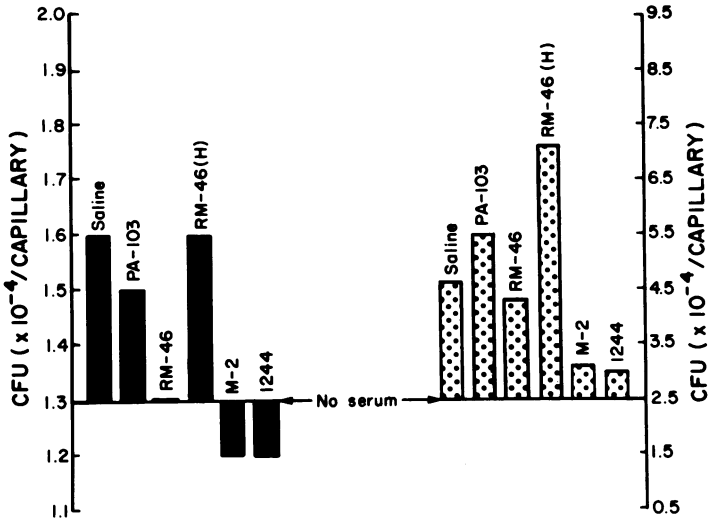

FIG. 9. Effect of various mouse FAg antisera on motility of *P. aeruginosa* strains. Experiments were as described in the legend to Fig. 8, except that 1 μg of immunogen was used. Sera were diluted 1:10. Controls were saline and strain RM-46(H) (antiserum heated to 90°C for 1 h). The base line (no serum) represents the capillary containing only buffer. The dark bars represent sera heated to 56°C for 30 min just before testing; the stippled bars represent sera that were not heated.

no apparent flagellin band. This observation is consistent both with the observed lack of motility and chemotaxis (5) and with the absence of flagella demonstrated by electron microscopic observations. Strain WR-5 also exhibited reduced chemotaxis, but had flagella; however, the molecular weight of the flagellin protein was 10,000 smaller than that of the other strains. This difference in molecular weight may reflect some aberration in the flagellum, which could account for the decrease in the chemotactic response of strain WR-5 to amino acids (5). Some slight variation in the apparent molecular weights of flagellins from strains of the two different H antigen types a and b (3) recently has been observed (T. C. Montie, unpublished data). These apparent differences are so far detected only by variations in the electrophoretic mobilities of the flagellins in SDS-PAGE. Our preliminary hypothesis is that these variations are indicative of differences in that portion of the primary structure of the proteins responsible for antigen specificity.

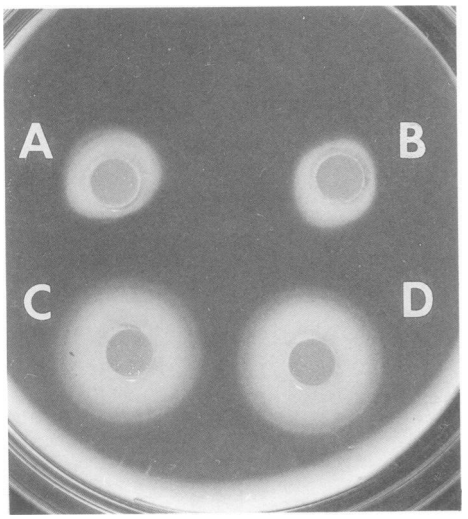

FIG. 10. Effect of mouse FAg antisera on colony spreading. Four stabs of M-2 cultures were made into motility agar (1% tryptone–0.3% yeast extract–0.5% NaCl–0.3% agar). The stabs were immediately covered with a filter paper disk which was dipped into the appropriate serum from mice immunized with 1 μg of FAg. The plate was incubated for 24 h at room temperature. (A) RM-46 FAg serum. (B) M-2 FAg serum. (C) Saline-immunized control. (D) PA-103 non-FAg serum.

Assays were made of FAg preparations isolated from agar medium by using succinate-MS medium or a rich medium. A comparison of the data indicated that although the yield increased with the rich medium, the ratio of impure proteins to flagellins also increased relative to MS medium. The increase in nonflagellin protein in rich medium very likely reflects a partial suppression of flagellin biosynthesis relative to total cell protein biosynthesis. When comparing a number of experiments, we saw no major differences between flagellin levels in MS medium FAg preparations when KNO_3 was substituted for ammonium salts in the medium. It is suggested from these data that the stimulation of che-

motaxis in nitrate-grown cells as compared with ammonium-grown cells as recently reported (R. C. Craven and T. C. Montie, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, I69, p. 98) is not explained by a variation in flagellar content.

Electrophoretic analyses of FAg preparations from various *P. aeruginosa* strains revealed that, from certain strains, for example strains 1244 and 1210, flagellin can be isolated in a fairly pure state (see Fig. 5), simply by the differential centrifugation technique employed. This may reflect either a higher flagellar content in these strains, or a more resilient cell surface, which would prevent release of contaminating proteins during shearing, or both.

It is interesting that the flagellin lacks proline, since it is found in very significant quantities (11 to 12 residues) in both strain PAK and strain PAO pilus subunits (17). These data indicate that pili could not be a significant contaminant in the FAg preparations. Also, no 17,000-molecular-weight band corresponding to pilin (17) is routinely seen in the electrophoretic assays.

It is difficult to calculate the precise amount of LPS in a given sample of flagellin. Samples selected for immunization (9) contained less than 5 μg of KDO-positive material per mg of protein. Pure LPS contains about 2% KDO, but this percentage varies with the method of isolation, the strain used, and the purity of a given preparation of LPS. Extrapolating from KDO content to determine the amount of LPS in an unknown sample adds additional difficulty. Substances such as bacterial glycolipid may contribute 12 to 13% KDO-positive material (11), since this fraction probably contains primarily the KDO-rich portion of the LPS molecule.

The flagellin samples contained some LPS and, therefore, were used at 1-μg (dry weight) levels for immunization studies. At the 1-μg level, any biological effects of LPS were sufficiently diluted out. The PA-103 extracts containing LPS in higher percentages were used as controls. As alluded to below, we observed an in vivo (9) lack of protection by the PA-103 preparation, a correlation of protection with H antigen content, and the loss of cross-agglutination of antisera with heat-killed organisms except with the PA-103 preparation. These results are consistent with the assumption that *P. aeruginosa* LPS is insignificant in protection studies (9).

One microgram of FAg, as isolated from strains M-2 and RM-46, served to protect burned mice against challenge with various *P. aeruginosa* strains (9) and, along with strain 1244 FAg, generated antimotility antibody (see Fig. 9). The FAg preparations from strains M-2 and RM-46 in an agglutination assay (9) and the latter FAg preparation in the motility assay (see Fig. 9) were inactivated by high temperatures, whereas in the control strain PA-103, some of the isolated material was not heat sensitive (9). These results are consistent with the analytical data and indicate that a relatively higher portion of the PA-103 preparation is LPS. The lack of cross-reactivity of PA-103 preparation with M-2 or RM-46 FAg preparations (9), the failure to protect the burned mice (9), and the lack of significant effects of PA-103 antiserum on motility are results which correlate with the lack of flagella in strain PA-103. The above data underscore the specificity of FAg preparations as protective immunogens.

The mechanism by which the antiflagellar serum prevents the migration of *Pseudomonas* into capillary tubes is not known. Our results, however, are in agreement with those of Resnick et al. (19), showing no correlation between the bactericidal activity and the immobilizing activity of cholera FAg antisera. In fact, in almost all tests, no bactericidal activity was seen. This was particularly apparent in the sera inactivated for complement. One might visualize the immobilization taking place by aggregation of antibody at the surface of the flagellum, causing inefficient flagellar rotation. Eventually, bacterial aggregation may occur as the concentration of antibody molecules per flagellum increases. Further studies are needed to verify such proposals.

We can conclude that FAg preparations can be readily obtained from various strains of *P. aeruginosa* by shearing and by differential centrifugation. The purity of the initial preparation can be improved by varying the culture conditions and by strain selection. A pure FAg preparation can be obtained by elution of the 53,000-molecular-weight band. This protein is an effective immunogen (9) and apparently acts by immobilizing the invading *P. aeruginosa*, thus offering clinical potential for a protective vaccine.

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