18. Gallin, E. K., and J. I. Gallin. 1977. Interaction of chemotactic factors with human macrophages induction of transmembrane potential changes. *J. Cell Biol.* 75:277-289.

19. Korchak, H. M., and G. Weissmann. 1978. Changes in membrane potential of human granulocytes antecede the metabolic responses to surface stimulation. *Proc Natl Acad. Sci. USA* 75:3818-3822.

20. Seligman, B. E., and J. I. Gallin. 1980. Use of lipophilic probes of membrane potential to assess human neutrophil activation. *J. Clin. Invest.* 66:493-503.

21. Whitin, J. C., C. E. Chapman, E. R. Simons, M. E. Chovaniec, and H. J. Cohen. 1980. Correlation between membrane potential changes and superoxide production in human granulocytes stimulated by phorbal myristate acetate. *J. Biol. Chem.* 255:1874-1878.

22. Young, J. D.-E., J. C. Unkeless, H. R. Kaback, and Z. A. Cohn. 1983. Macrophage membrane potential changes associated with Fc receptor ligand binding. *Proc. Natl. Acad. Sci. USA* 80:1357-1361.

23. Schleicher, M., A. Noegel, D. M. Watterson, and E. C. Gotschlich. 1983. Calmodulin, a eukaryotic regulator protein, binds in vitro to outer membrane proteins from *Neisseria* species. *Fed. Proc.* 42:861.

24. Rosenbusch, J. P. 1974. Characterization of the major envelope protein from *Escherichia coli*. *J. Biol. Chem.* 249:8019-8029.

25. Tokunaga, H., M. Tokunaga, and T. Nakae. 1979. Characterization of porins from the outer membrane of *Salmonella typhimurium* 1. Chemical Analysis. *Eur. J. Biochem.* 95:433-439.

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The Outer Membrane Proteins of *Pseudomonas aeruginosa:* Immunotherapeutic Potential

R.E.W. Hancock, E.C.A. Mouat, S.L. Butters and D.P. Speert

Gram negative bacteria, like *Pseudomonas aeruginosa*, contain as one of their major surface-located structures, the outer membrane. This membrane has an unusual asymmetric composition compared to many biological membranes since it contains a glycolipid lipopolysaccharide (LPS) in its outer (surface) monolayer, whereas the inner monolayer apparently contains phospholipids, but no LPS. In addition, the outer membrane contains a limited number of "major" proteins present in very high copy number (2×10^4 to 3×10^5 per cell).[1] Functionally, the outer membrane is involved in maintenance of the structural integrity of the cell, in exclusion of hydrophobic substances and resistance to detergents, and as a major permeability barrier, with a size-dependent exclusion limit for hydrophilic compounds.[1] Additionally, since the outer membrane is surface-exposed and available to the host immune system,[2] it probably plays a significant role in the pathogenesis of gram negative bacterial infections.

At least two classes of molecules are present on the surface of *P. aeruginosa*, LPS and outer membrane proteins. Although LPS is capable of eliciting a substantial immune response that will give type-specific protection,[3] its variability within the organism (there are 17 different LPS O antigen types[4]) has somewhat limited its usefulness as a target for immunotherapy. Similar variability has been observed for other surface structures such as pili[5] and flagella.[6] Therefore, we have considered the possibility that outer membrane

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proteins will contain strongly conserved, surface-located antigenic sites that will serve as targets for immunotherapy.

IMMUNOGENICITY OF OUTER MEMBRANE PROTEINS

To determine whether outer membrane proteins were surface exposed and immunogenic in vivo, we screened the sera of specific patient populations for antibodies to outer membrane proteins.[7,8] In particular, we examined patients with cystic fibrosis who often suffer from chronic *P. aeruginosa* lung infections. Patients who had been colonized with *P. aeruginosa* for 18 months to 9 years had significantly higher ELISA antibody titers against outer membrane proteins ($p < 0.001$ by contingency table analysis) than patients who had no history of *P. aeruginosa* infections.[7] The mean serum antibody titer of the colonized patients was 1.3×10^5, equivalent to the serum antibody titer of rabbits hyperimmunized with purified outer membranes;[9] in contrast, uncolonized patients had average titers of only 2.5×10^2.

The nature of the antibodies in these and other sera was examined by Western immunoblotting[7] and crossed immunoelectrophoresis.[8] A total of 239 sera from 52 patients with cystic fibrosis was examined for the presence of antibodies to the major outer membrane porin protein F; each of these sera contained anti-protein F antibodies[7,8] (e.g., Fig. 29.1). The sera from 19 of these patients were interacted with Western electrophoretic blots of separated outer membrane proteins.[7] In addition to the anti-protein F antibodies, the sera often contained antibodies to proteins E, H_2, I, and a variety of minor outer membrane proteins.

Antibodies to outer membrane protein F were also observed in the sera of convalescent patients who had recovered from *P. aeruginosa* bacteremia, and in the sera of rats with chronic lung infection.[8] In each case described above, the protein F antigen, used to demonstrate the presence of antibodies in the sera, was derived from our laboratory wild type strain. Thus, this data was consistent with the concept that protein F was antigenically conserved, especially since the *P. aeruginosa* strains eliciting the anti-protein F antibodies usually differed from the laboratory wild type strain in a number of characteristics, including serotype. A further suggestion that arose from this data was that these conserved antigenic sites on protein F were immunogenic and possibly surface exposed. In order to probe this further at the epitope (antigenic site) level, monoclonal antibodies were made against *P aeruginosa* protein F.

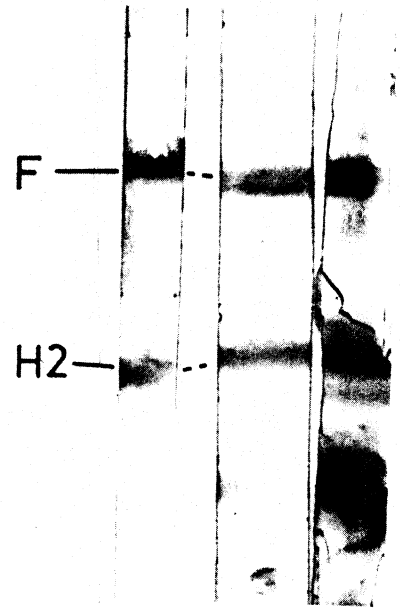

FIGURE 29.1. Western electrophoretic blots of separated outer-membrane components of *P. aeruginosa* strain H103 after interaction first with the sera from cystic fibrosis patients with a history of *P. aeruginosa* lung infections and then with goat antiserum to human immunoglobulin conjugated to alkaline phosphatase and finally a histochemical stain for alkaline phosphatase. The specificity of the antibodies in the patient sera was revealed by the site of deposition of the histochemical stain. Many weaker bands were lost during photographic reproduction due to the wide range of antibodies in the sera which resulted in a high background. The location of the outer membrane proteins indicated on the left hand side of the gel was determined using monoclonal antibodies to these antigens.

CONSERVATION OF ANTIGENIC EPITOPES IN *P. AERUGINOSA*

A series of monoclonal antibodies was made against protein F, and four of these were characterized in some detail.[10] The antibodies fell into two groups on the basis of their reactivity with: 1) the protein F equivalent from two other strains of *Pseudomonas*, *P. putida* and *P. syringae*; 2) reduced protein F; and 3) cyanogen bromide or proteolytic peptides (Table 29.1).[10,11] Three separate tests, ELISA,[10] Western immunoblotting,[10] and a newly developed test, colony immuno-

TABLE 29.1. Features Differentiating Two Classes of Monoclonal Antibodies Against Protein F

Property	Monoclonal Antibody	
	MA5-8	MA2-10, MA4-4, MA4-10
Reactivity with purified protein F	+	+
Surface labeling of intact *P. aeruginosa* cells	+	+
Reactivity with the protein F equivalent from *P. putida* and *P. syringae*	-	+
Reactivity with a 29 kD papain and with a 31 kD trypsin proteolytic fragment of protein F	-	+
Reactivity with cyanogen bromide fragments (23,28 kD) of protein F	+	-
Antigenic reactivity stable to 2 mercaptoethanol	+	-
Binding to oligomeric associations of protein F on SDS polyacrylamide gels	+	-

Source: Data from refs. 10, 11.

blotting[11] (see below), were used to demonstrate the ability of each of these monoclonal antibodies to interact with all tested *P. aeruginosa* strains (about 70 to date), including all 17 serotypes of *P. aeruginosa* and a wide variety of clinical isolates (Fig. 29.2). Thus, these monoclonal antibodies define at least two separate protein F epitopes which are conserved in the species *P. aeruginosa*.

Using similar methods, we have also demonstrated that monoclonal antibodies MA1-6[12] and 5E4[13] recognized conserved epitopes on outer membrane lipoprotein H2 and lipid A, respectively. The lipid A-specific monoclonal antibody 5E4 reacted not only with *P. aeruginosa*, but also with 97 percent of the tested species of gram negative bacteria.[13] In contrast, monoclonal antibodies against the rough core or O antigen of LPS recognized epitopes present in only a limited subset of *P. aeruginosa* strains.[10,12]

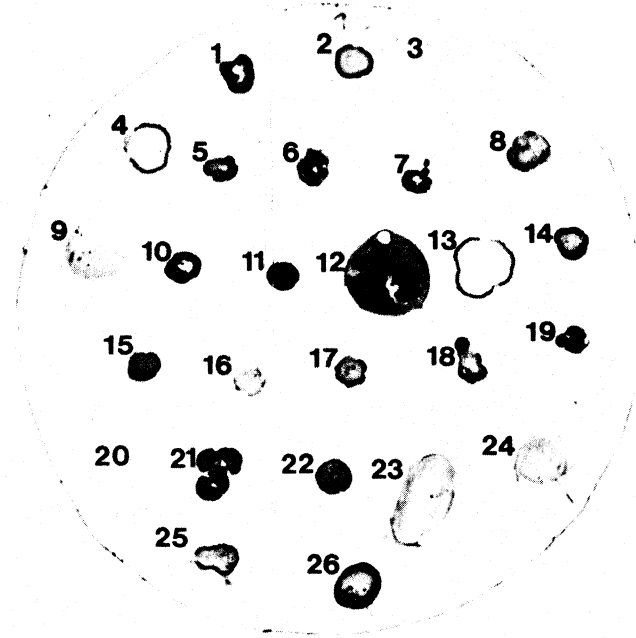

FIGURE 29.2. A colony immunoblot showing a *P. aeruginosa* protein F specific monoclonal antibody MA4-10 with the following strains (unless specified the strains are all *P. aeruginosa*) 1 - CF832; 2 - PA01; 3 - *P. stutzeri* ATCC17588; 4 - CF6094; 5 - CF3660-1; 6 - CF4522; 7 - CF2314; 8 - CF1452; 9 - CF3790; 10 - CF284; 11 - CF221; 12 - CF1278; 13 - CF4349; 14 - L; 15 - CF21nm; 16 - CF21m; 17 - CFC1nm; 18 - CFC1m; 19 - CF9490; 20 - *P. pseudomallei*; 21 - CFC81; 22 - CFC6nm; 23 - CFC6m; 24 - CFC20; 25 - CFC47; 26 - CFC91. The strains with the prefix CF are cystic fibrosis patient isolates. The suffixes "m" and "nm" refer to mucoid strains and their spontaneous nonmucoid derivatives, respectively. Strains PA01 and L were originally blood isolates. In some of the positive reactions (e.g., 1, 2, 4, and 13) a clear center is surrounded by a ring demonstrating a positive reaction. The reason for this is probably that bacteria in the center of the colony were transferred to the nitrocellulose in greater numbers. Those bacteria from the center of the colony, which bound monoclonal antibodies, were associated with other bacteria rather than being bound directly to nitrocellulose and thus were lost during the immunostaining procedure (which involved many washing steps).

SURFACE LOCALIZATION OF ANTIGENIC EPITOPES IN *P. AERUGINOSA*

Two methods were used to demonstrate that some of our monoclonal antibodies interacted with surface located epitopes—i.e., indirect immunofluorescence[10] and colony immunoblotting.[11] In the former technique, intact cells were interacted with monoclonal antibodies and then, in turn, with a rabbit anti-mouse Ig antibody and a fluorescently tagged goat anti-rabbit Ig antibody. Positive fluorescence indicated that the first (mouse monoclonal) antibody had bound to a surface epitope. The second method was simpler and allowed screening of a large number of strains (Fig. 29.2), although we considered it reliable only when the appropriate controls were performed. Bacterial cultures were patched onto nutrient agar plates and grown until visible growth could be seen. The bacteria were then transferred to nitrocellulose by contact, non-specific binding sites blocked by incubation with 3 percent BSA, and then the colony blot incubated with the monoclonal antibody of interest. The blot was subsequently incubated with a peroxidase-tagged goat anti-mouse Ig antibody followed by a histochemical stain for peroxidase. Positive color indicated that the monoclonal antibody had bound to a surface localized epitope.

The results of these two assays were identical when the same strains were tested. It was demonstrated that both of the protein F epitopes described above were surface localized; in contrast, the conserved protein H2 epitope, recognized by antibody MA1-6, was only surface localized in rough, LPS O antigen-deficient variants of *Pseudomonas*. Controls demonstrated that strains lacking proteins F and H2 failed to interact with monoclonals specific for the missing proteins.[10,11] Neither LPS O side chains nor mucoid exopolysaccharide influenced adversely the interaction of the protein F-specific monoclonal antibodies with their respective surface localized epitopes.[11]

OPSONOPHAGOCYTOSIS WITH A MONOCLONAL ANTIBODY SPECIFIC FOR PROTEIN F

Because the protein F-specific monoclonal antibodies recognized conserved surface localized epitopes, we wished to determine whether they could opsonize *P. aeruginosa* cells. Therefore, antibody MA4-4 was chosen for further studies. Despite repeated attempts, we were unable to show opsonization of complement-mediated bactericidal killing by MA4-4 (L. M. Mutharia and R. E. W. Hancock, unpublished observations). However, we were able to demonstrate that antibody MA4-4 increased by six-fold the phagocytosis of *P. aeruginosa* as measured by the number of cells which associated with human polymorphonuclear leukocytes in the presence or absence of monoclonal antibodies (Table 29.2). This opsonic effect was relatively independent of the presence of complement. The slight increase in opsonization in the presence of complement could be ascribed to the effects of complement alone. Opsonization by monoclonal antibody MA4-4 was observed with four separate *P. aeruginosa* strains including strain M2 (see below).

TABLE 29.2. Enhancement of the Association of *P. aeruginosa* Strain 112 with Human Polymorphonuclear Leukocytes U[illegible] Monoclonal Antibody MA4-4

Antibodies added	Complement added	Number of bacteria per polymorphonuclear leukocyte	Percent uptake
-	-	2.2 ± 1.9	16
-	+	3.8 ± 3.8	27
MA4-4	-	9.6 ± 5.1	68
MA4-4	+	11.1 ± 6.5	79
αOM	-	4.6 ± 3.6	33
αOM	+	7.6 ± 3.4	54
PHS	-	12.1 ± 5.3	86

Notes: Human polymorphonuclear leukocytes from healthy volunteers were purified as previously described.[17] *P. aeruginosa* strain 112 was added at a bacteria to leukocyte ratio of 14:1. In addition, where indicated, an antibody and/or a complement source was included. The antibodies used were either affinity purified monoclonal antibody MA4-4, a rabbit anti-whole outer membrane sera (αOM) prepared as described previously,[9] or pooled human serum (PHS). All were used at 5 percent. The guinea pig complement was used as a 3.26 percent suspension of commercial guinea pig complement.

PASSIVE IMMUNOTHERAPY WITH MONOCLONAL ANTIBODY MA4-4

Monoclonal antibody MA4-4 had a series of properties which suggested that it was an excellent candidate for immunotherapy. Therefore, we examined the protective capacity of this monoclonal antibody using two mouse infection models. In the first model, D6/B2 F1 mice were injected in the tail vein with 0.1 mg of affinity purified antibody MA4-4. Two hours

later th[illegible]eceived an intraperitoneal challenge with one of two virulent *P. aeruginosa* strains, PA103 or M2. In each case, animals which had received monoclonal antibody prior to challenge demonstrated significantly ($p < 0.001$ by the Fisher Exact test) better survival during subsequent challenge. The LD_{50} for monoclonal antibody-protected mice was increased about three-fold over saline-injected mice (Table 29.3). Injection with the monoclonal antibody MA1-6, which was specific for a conserved, non-surface-located site on protein H2, did not result in protection.

Although the protection mediated by antibody MA4-4 in this animal model was perhaps marginal, it should be noted that the animal model is sub-optimal since the mice that died in this model generally lived only 2-12 hours and it required massive challenges of bacteria to result in lethality. Indeed, immunoprotection of mice in this model, even using type specific LPS as an immunogen, rarely exceeds three LD_{50}s.[14,15] Therefore, we have more recently turned to the mouse burn model of Stieritz and Holder.[16] The slight thermal injury induced for this model reduces the LD_{50} for unimmunized mice about four to five orders of magnitude. Using this model, we could demonstrate 80-100 percent protection against a challenge of eight LD_{50}s (Table 29.4).

In conclusion, we have demonstrated that outer membrane proteins contain antigenic epitopes which are conserved throughout the species *P. aeruginosa*. In the case of *P. aeruginosa* protein F, two of these conserved epitopes are surface localized. The specificity, reproducibility, and availability of

TABLE 29.3. Immunoprotection by Monoclonal Antibody MA4-4 of Mice Against Subsequent Challenge with *P. aeruginosa* Strains

	LD_{50}	
Strains	Controls	MA4-4 Protected
PA103	1.4×10^6	4.8×10^6
M2	2.8×10^6	7.0×10^6

Notes: B6D2 F1 mice were injected in the tail vein with 0.1 mg of affinity purified monoclonal antibody MA4-4 in saline. Two hours later they were given 1 to 8×10^6 bacteria in saline intraperitoneally and their survival recorded after three days. At least eight animals were used for each dose of bacteria. The LD_{50}s were calculated by the method of Reed and Muench.[18]

BLE 29.4. Passive Protection in the Mouse Burn Model of Stieritz and Holder by Preinjection of a Monoclonal Antibody MA4-4

Amount of MA4-4 Administered	Survival After 8 Days
0 (saline control)	0/10
0.1 mg	4/5
1.0 mg	5/5

Notes: Mice of the B6/D2 subline were given 0.01, or 1 mg of monoclonal antibody MA4-4 intravenously in the tail vein. Two hours later a 10 sec alcohol burn was given to the anaesthetized mice over a one square inch area of their preshaved backs [this burn was sufficient to cause a slight reddening of the skin but no obvious tissue damage]. One hundred organisms of strain M2 in saline [equivalent to eight LD_{50}s] were administered subcutaneously and survival followed over time.

such monoclonal antibodies has led us to consider them for immunotherapy. Injection of mice with one of our protein F-specific monoclonal antibodies can protect against subsequent lethal challenge with *P. aeruginosa*. In vitro experiments have suggested that the mechanism of protection is through the opsonization for phagocytosis of the *P. aeruginosa* cells.

REFERENCES

1. Nikaido H, Nakae T: The outer membrane of gram-negative bacteria. *Adv Microb Physiol* 19:163-250, 1979.
2. Taplits M, Michael JG: Immune response to *E. coli* B surface antigens. *Infect Immun* 25:943-945, 1979.
3. Crowder JG, Fisher MW, White A: Type-specific immunity in *Pseudomonas* disease. *J Lab Clin Med* 79:42-54, 1972.
4. Lanyi B, Bergan T: Serological characterization of *P. aeruginosa*. *Methods Microbiol* 10:93-168, 1978.
5. Bradley DE: *Pseudomonas aeruginosa* pili pp. 319-338. In: Bradley DE, Raizen E, Fives-Taylor P, Ou J (eds): *Pili*. Washington, DC, 1978.
6. Ansorg R: Flagella specific H antigenic schema of *P. aeruginosa*. *Zbl Bakt Hyg I Abt Orig* A 242:228-238, 1978.

7. Hancock REW, Mouat ECA, Speert DP: Quantitation and identification of antibodies to the outer membrane proteins of *P. aeruginosa* in the sera of cystic fibrosis patients. *J Infect Dis* 149:220-226, 1984.

8. Lam JS, Mutharia LM, Hancock REW, Hoiby N, Lam K, Baek L, Costerton JW: Immunogenicity of *Pseudomonas aeruginosa* outer membrane proteins examined by crossed immunoelectrophoresis. *Infect Immun* 42:88-98, 1983.

9. Mutharia LM, Nicas TI, Hancock REW: Outer membrane proteins of *Pseudomonas aeruginosa* serotype strains. *J Infect Dis* 146:770-779, 1982.

10. Mutharia LM, Hancock REW: Surface localization of *Pseudomonas aeruginosa* outer membrane protein F using monoclonal antibodies. *Infect Immun* 42:1027-1033, 1983.

11. Mutharia LM, Hancock REW: Characterization of two surface-localized antigenic sites of porin protein F of *Pseudomonas aeruginosa*. *Canad J Microbiol* (in press).

12. Hancock REW, Wieczorek AA, Mutharia LM, Poole K: Monoclonal antibodies against *Pseudomonas aeruginosa* outer membrane antigens: Isolation and characterization. *Infect Immun* 37:166-171, 1982.

13. Mutharia LM, Crockford G, Bogard WC, Hancock REW: Monoclonal antibodies specific for *E. coli* J-5 lipopolysaccharide: Cross reaction with other gram negative bacterial species. *Infect Immun* 45: (in press).

14. Gilleland HE Jr, Parker MG, Matthews JM, Berg RD: Use of a purified outer membrane protein F (porin) preparation of *Pseudomonas aeruginosa* as a protective vaccine in mice. *Infect Immun* 44:49-54, 1984.

15. Sourek J, Vymola F, Zelenkova L: Mouse-protection experience with monovalent *Pseudomonas aeruginosa* vaccine. *Zbl Bakt Hyg I Abt Orig A* 246:353-362, 1980.

16. Sieritz DD, Holder IA: Experimental studies of the pathogenesis of infections due to *Pseudomonas aeruginosa*: description of a burned mouse model. *J Infect Dis* 131: 688-691.

17. Speert DP, Eftekhar F, Puterman ML: Nonopsonic phagagocytosis of strains of *Pseudomonas aeruginosa* from cystic fibrosis patients. *Infect Immun* 43:1006-1011, 1984.

18. Reed LG, Muench H: A simple method for estimating fifty percent end-points. *Amer J Hyg* 27:493-497, 1938.

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The Low Calcium Response and Virulence in the *Yersiniae*

Jon D. Goguen and Janet Yother

The three species of the genus *Yersinia* pathogenic for man share an unusual, essential, and highly complex plasmid encoded virulence determinant. Although under active investigation since its discovery in the 1950s, the role of this determinant in pathogenesis is incompletely understood. Here, we briefly present our current view of some recent data provided by other laboratories, as well as some of our own recent observations.

Virulent strains of *Yersinia pestis*, the causative agent of bubonic plague, require millimolar concentrations of Ca^{2+} for growth at 37°C but not 26°C (9,11). When shifted from 26°C to 37°C in Ca^{2+}-free media, such strains cease growth over a period of one to two generations as the result of an ordered shutdown of net macromolecular synthesis (25). The conditions which arrest growth also result in induction of two proteins known as the V and W antigens (2,3). In temperature-shift experiments, these proteins appear about one hour after the change to 37°C and are synthesized until growth of the cells and net protein synthesis stop (4,24). A similar response occurs in virulent strains of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* (3,4). We refer to this phenomenon—cessation of growth at 37°C and coordinate expression of V and W—as the low calcium response (LCR). Other investigators have designated LCR^+ strains as either Vwa^+, for production of

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