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# Immunotherapeutic Potential of Monoclonal Antibodies Against *Pseudomonas aeruginosa* Protein F

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To unambiguously demonstrate the immunotherapeutic potential of outer membrane porin protein F from *Pseudomonas aeruginosa*, a series of monoclonal antibodies have been isolated and demonstrated to be specific for protein F by Western blotting procedures. The antibodies recognize a surface-exposed antigenic site that is conserved on all *Pseudomonas aeruginosa* strains tested to date. One of these monoclonal antibodies named MA4-4 resulted in passive protection against subsequent infections by *Pseudomonas aeruginosa* in two different mouse infection models. In vitro studies using human polymorphonuclear leukocytes suggested that this antibody opsonized *Pseudomonas aeruginosa* for phagocytosis. The data suggest that immunotherapy based on porin protein F has definite potential for success.

Immunotherapy against a bacterium generally involves one of two types of approaches: active vaccination with an immunogenic component from the bacterial cell surface or passive administration of antibodies directed against a cell surface component. Alternatively, immunotherapy can be directed against extracellular toxins or proteases, although such an approach has limitations (with the possible exceptions of diphtheria and tetanus toxoid vaccines) because it does not directly attack or suppress the growth of the organism producing the toxins.

The ideal cell surface component vaccine should have the following properties: the ability to induce a specific immune response, surface exposed antigenic sites, strong conservation among a wide range of strains of the bacterial species which are the target of immunotherapy, lack of toxicity, ability to elicit a memory (IgG) response, ease of purification, and it must, of course, result in protection of the vaccinated animal or human against subsequent infection. Unfortunately, many of the vaccine candidates examined to date lack one or more of these properties. For example lipopolysaccharides (LPS), pili and flagellae in *Pseudomonas aeruginosa* strains are relatively poorly conserved (1, 2, 3). In contrast, outer membrane proteins have been demonstrated to have all of the above properties and thus have been considered potential vaccine candidates against a number of bacteria including *Neisseria gonorrhoeae*, *Salmonella typhimurium* and *Shigella* species (see reference 4 for discussion).

Our laboratory has been interested in the potential of *Pseudomonas aeruginosa* outer membrane proteins as targets for immunotherapy. To clearly and unambiguously define this potential in active vaccination experiments would require a homogeneous purified preparation of the outer membrane protein of interest; however, in our experience it is extremely difficult to purify outer membrane proteins to homogeneity. The major contaminant is often LPS which has many immunological properties, including adjuvanticity, toxicity, high immunogenicity, mitogenicity etc., which considerably complicate the situation. Even when these contaminants are removed (something that can be quite difficult in the case of LPS; see reference 5), antigenic modulation may occur due to structural alterations in the protein in question. Therefore, passive immunotherapy was chosen to demonstrate the potential of specific outer membrane proteins as targets for immunotherapy. In particular, monoclonal antibodies specific for outer membrane protein F were used to demonstrate the many features of this protein which make it an excellent vaccine candidate.

## Immunogenicity of Protein F

Two techniques have been used to demonstrate that protein F is immunogenic (i.e. capable of eliciting a specific antibody response) in animals and humans. Both of these techniques, Western blotting and crossed immunoelectrophoresis using purified protein F as an antigen (6, 7), have allowed precise identification of antibodies directed against protein F in the sera of patients who have had *Pseudomonas aeruginosa* infections. The ability to measure anti-protein F antibodies in the sera of such patients strongly indicates that protein F is immunogenic.

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Two hundred and thirty-nine sera from 52 patients with cystic fibrosis complicated by *Pseudomonas aeruginosa* lung infections have been examined (6, 7). Each of these sera had antibodies to protein F. In addition, such antibodies were observed in the sera of convalescent patients recovering from *Pseudomonas aeruginosa* bacteremia and in the sera of rats with chronic lung infections (7). These data demonstrated that regardless of the type of infection or the serotype of the infecting organism, serum antibodies were reactive with protein F from our laboratory wild type strain *Pseudomonas aeruginosa* PA01. This in turn provided evidence for the hypothesis that protein F was antigenically conserved, a concept strongly supported experimentally by the demonstration that polyclonal antibodies to the protein F of our wild type strain cross-reacted with the protein F of all 17 of the International Antigen Typing Scheme type strains (4).

A further implication of these data was that the conserved antigenic sites of protein F included some sites localized on the bacterial surface. To probe this further at the epitope (single antigenic site) level, monoclonal antibodies were made to *Pseudomonas aeruginosa* protein F.

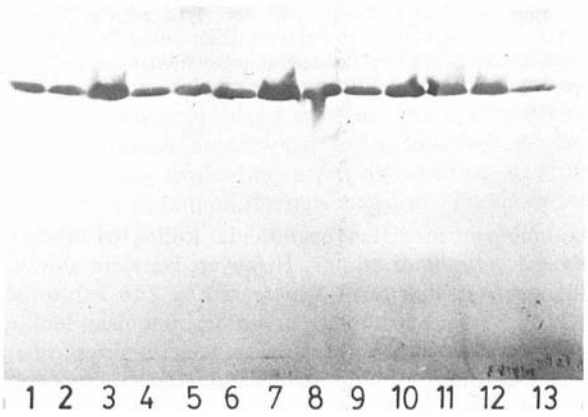


**Figure 1:** Western electrophoretic blot of outer membranes of *Pseudomonas aeruginosa* strains after interaction with a protein F specific monoclonal antibody MA5-8. The blot was made by electrophoretic transfer of separated outer membrane proteins from sodium dodecyl sulfate polyacrylamide gels onto nitrocellulose paper. The electrophoretic blot was then treated with the monoclonal antibody MA5-8 followed by a goat-anti-mouse alkaline phosphatase conjugated antibody and addition of the substrate (Naphthol AS-MX phosphoric acid and Fast Red TR salt). The outer membrane samples are Lane 1 – *Pseudomonas aeruginosa* strain H103; Lane 2 – strain CF Plnm; Lane 3 – CF C81m; Lane 4 – CF C81nm; Lane 5 – CF C96m; Lane 6 – CF C96nm; Lane 7 – CF C20m; Lane 8 – CF C20nm; Lane 9 – CF C21m; Lane 10 – CF C2nm; Lane 11 – CF C91m; Lane 12 – CF C91nm; Lane 13 – purified protein F from strain H103. M denotes a mucoid strain, nm denotes the nonmucoid spontaneous revertant and CF denotes *Pseudomonas aeruginosa* strains isolated from cystic fibrosis patients.

### *Conservation of Surface Localized Epitopes of* Pseudomonas aeruginosa *Protein F*

A series of monoclonal antibodies were made against *Pseudomonas aeruginosa* protein F and four of these were characterized in some detail. Each antibody interacted with protein F from all strains of *Pseudomonas aeruginosa* tested regardless of the source, serotype, mucoidy or colony morphology of the strains. In all, the four antibodies each interacted with 60 different *Pseudomonas aeruginosa* strains when examined by enzyme-linked immunosorbent assay (8), Western blotting (8, 9) (see Figure 1), indirect immunofluorescence (9) or colony blotting (10).

It was of some interest to determine if these four antibodies recognized a single antigenic epitope or four distinct epitopes. Five separate criteria were found which suggested that there were two classes of monoclonal antibodies with distinct specificities (10). These criteria included reactivity with proteolytic or cyanogen bromide fragments, stability of the epitope to 2 mercaptoethanol, ability of the monoclonal antibody to recognize oligomeric associations of protein F on sodium dodecyl sulfate gels and reactivity with the protein F equivalent from *Pseudomonas syringae* and *Pseudomonas putida* (but not with other Pseudomonads).

### *Surface Localization of Protein F Epitopes*

To demonstrate that the conserved protein F epitopes were localized at the bacterial cell surface, two methods were employed, indirect immunofluorescence

**Table 1:** Immunoprotection of mice by the protein F-specific monoclonal antibody MA4-4 against subsequent challenge with *Pseudomonas aeruginosa* strain M2.[a]

| | Survival | |
|---|---|---|
| | Untreated mice | Burned mice |
| Controls | 1/10 | 0/10 |
| MA4-4 injected | 9/10 | 9/10 |

[a] Mice of the B6/D2 subline were given 0.1 or 1 mg of monoclonal antibody MA4-4 in the tail vein. The untreated mice were challenged 2 h later with an intraperitoneal injection of 2 × LD50 (4 × $10^6$ live organisms) of a virulent *Pseudomonas aeruginosa* strain M2. Control mice received either saline or a monoclonal antibody MA1-6 specific for a conserved, non-surface located epitope (9), in the tail vein. To increase the pathogenic potential of strain M2, anaesthetized mice were subjected to a 10 s alcohol burn (see column 3) over a 1 square inch area of their preshaved backs and immediately afterwards (2 h after the injection of monoclonal antibody) challenged subcutaneously at the burn site with 8 × LD50 (100 live organisms) of strain M2. Survival was followed for eight days.

(9) and colony immunoblotting (10). The former technique allowed unambiguous demonstration that the epitopes of protein F recognized by the four monoclonal antibodies were surface localized in a small number of *Pseudomonas aeruginosa* strains (9; see Table 1). The latter technique allowed simple screening of a wider variety of strains (10). In all, these data demonstrated that both of the conserved antigenic epitopes on outer membrane protein F were exposed and available to the immune system. Thus, the monoclonal antibodies recognizing these epitopes seemed to provide an excellent model system for examining the immunotherapeutic potential of protein F.

### *Passive Protection with Monoclonal Antibodies to Protein F*

Two mouse protection models were used to examine the ability of the protein F-specific monoclonal antibody MA4-4 to protect mice against subsequent infections. The first model involved intravenous injection of monoclonal antibodies and subsequent (2 h later) intraperitoneal challenge with live *Pseudomonas aeruginosa* organisms. While clear evidence of protection was observed using this model, after challenge with either strain M2 (Table 1) or another virulent strain PA103 (data not shown), only low levels of protection were observed, and the LD50 of immunized mice increased only 2.5 to 3.5 times that of non-immunized mice. It could be argued, however, that this model was suboptimal since control mice died very rapidly (2–12 h), and it required a massive challenge of bacteria (leading to a low antibody: bacteria ratio) to kill the mice. Consistent with this, immunoprotection of mice in this model, even with type specific LPS, rarely exceeds 3 x LD50 (11, 12).

The second protection model involved slight thermal injury to the mice as described by Stieritz and Holder (13). In this model the LD50 for control mice was reduced five orders of magnitude and the control mice which succumbed to infection survived at least 2–3 days. Preliminary experiments (Table 1) again suggested that monoclonal antibody MA4-4 given intravenously, passively protected mice against subcutaneous challenge with 8 x LD50 of strain M2.

### *Opsonophagocytosis with a Protein F-Specific Monoclonal Antibody*

In collaboration with D.P. Speert (Children's Hospital, Vancouver), we examined the underlying mechanism giving rise to passive protection with monoclonal antibody MA4-4. Despite repeated attempts we were unable to demonstrate that MA4-4 could promote complement-mediated bactericidal killing of *Pseudomonas aeruginosa* strains. However, we were able to demonstrate that MA4-4 increased by 2 to 7 fold the phagocytosis by human polymorphonuclear leukocytes of strain M2 (Table 2) as well as three other serum-resistant *Pseudomonas aeruginosa* strains (data not shown). In each case the opsonization was independent of the presence of complement. These data strongly suggest that monoclonal antibody MA4-4 is an opsonizing antibody. This conclusion is consistent with the IgG2a subtype of MA4-4.

**Table 2:** Opsonization of *Pseudomonas aeruginosa* strain M2 for phagocytosis by human polymorphonuclear leukocytes.[a]

| Antibody source | Complement source | Phagocytic uptake (% of input) |
|---|---|---|
| None | none | 22 ± 17 |
| None | guinea pig | 29 ± 19 |
| Monoclonal antibody MA4-4 | none | 61 ± 25 |
| Monoclonal antibody MA4-4 | guinea pig | 50 ± 21 |
| Heated anti-whole outer membrane serum | none | 60 ± 27 |
| Heated anti-whole outer membrane serum | guinea pig | 46 ± 20 |
| Heated pooled human serum | none | 45 ± 22 |
| Heated pooled human serum | guinea pig | 36 ± 18 |

[a] Human polymorphonuclear leukocytes from healthy volunteers were purified as previously described (14). *Pseudomonas aeruginosa* strain M2 was added at a bacteria to leukocyte ratio of 20:1. In addition, an antibody source [5 % of an affinity purified antibody MA4-4 preparation, or a rabbit anti-whole outer membrane serum (4) or pooled human serum – heated at 56 °C for 30 min, where indicated, to inactivate complement] and/or complement source were added. The 3.26 % suspension of commercial guinea pig complement had a complement hemolytic activity for red blood cells equivalent to pooled human serum. The phagocytosis mediated by heated pooled human serum presumably reflects the presence of natural opsonins in the serum.

### Passive or Active Immunotherapy with Protein F

These results suggest that monoclonal antibodies themselves might be excellent immunotherapeutic agents against *Pseudomonas aeruginosa* infections. Alternatively, Gilleland and colleagues (12) have recently demonstrated protection by active vaccination of mice using purified protein F. At present it is impossible to conclude with conviction which

of these approaches will ultimately yield success, for both have advantages and disadvantages.

As mentioned above, it is extremely difficult to purify protein F so that it is free of LPS (5), and thus possible contamination of protein F preparations with endotoxin must always be considered. Perhaps the major disadvantage of active immunotherapy, however, is related to the etiology of *Pseudomonas aeruginosa* bacteremia. *Pseudomonas aeruginosa* often infects immunocompromized patients such as those with cancer and leukemias (who receive immunosuppressive therapy) or with major burns and wounds. It is questionable whether active therapy will be successful in such patients. In addition the onset of *Pseudomonas aeruginosa* bacteremia is often rapid and the time until death short, thus restricting the time for induction of specific antibodies in response to vaccination (the alternative would seem to be vaccination of the entire population).

In contrast, passive therapy with monoclonal antibodies lends itself to the treatment of even rapidly developing infections (see Table 2) and may, in combination with granulocyte infusion therapy, overcome *Pseudomonas aeruginosa* infections even in highly immunocompromized patients. In addition, monoclonal antibodies offer the substantial advantages of ease of purification, high specificity, and day-to-day reproducibility. Unfortunately such a therapeutic approach is not without problems. Many researchers are worried about serum sickness and induced immunoregulatory defects as a consequence of injection of large amounts of antibody, although some of these problems may be solved through the use of human monoclonal antibodies. Nevertheless, monoclonal antibodies offer an exciting new approach to immunotherapy against *Pseudomonas aeruginosa*.

## Acknowledgements

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