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Application of Monoclonal Antibodies to the Study of the Surface Antigens in *Pseudomonas aeruginosa*

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I. INTRODUCTION

The ability of bacteria to establish an infection in the susceptible host involves in part the interaction of their surface antigens with the host immune system. In

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addition, *Pseudomonas aeruginosa* elaborates a large repertoire of virulence factors (28) whose roles in the disease process are not yet fully understood. The high intrinsic resistance to antibiotic therapy and the adverse prognosis (3,30) associated with *Pseudomonas* infections have triggered interest in the development of immunotherapeutic measures to augment the available antibiotic regimes (21,29).

Owing to its surface location, relative ease of purification, and immunogenicity, lipopolysaccharide (LPS) has been the major macromolecule studied for its potential in vaccine development. The Parke-Davis heptavalent vaccine known as "Pseudogen," described by Hanessian *et al.* (7), and PEV-01, prepared by Miller *et al.* (16) at the Wellcome Research Laboratories, are both LPS-based vaccines. This type of vaccine is far from perfect because of adverse side effects caused by the endotoxic (lipid A) portion of LPS. In addition, it usually provides limited serotype-specific protection (21). Thus, a search for less toxic immunizing preparations is warranted.

Major outer-membrane proteins are surface located and often highly conserved among a given species of bacteria (6,10,20). Therefore common antigens that can provide immunoprotection against infection by a species of bacteria should exist among these proteins. In this context, the immunogenicity of outer-membrane antigens has been studied in organisms such as *Haemophilus influenzae* (8,13), *Neisseria* spp. (2), *Shigella* spp. (1), and *Salmonella typhimurim* (12). All of these studies used partially purified proteins or polyclonal sera and thus suffered from lack of purity and low specificity of the protective antibodies.

We have identified some common antigens of *P. aeruginosa* using monoclonal antibodies (19). In this chapter, we will describe progress made using such highly specific antibodies in the analysis and understanding of the complex antigenic moieties of the outer membrane of *P. aeruginosa*.

II. BACKGROUND

Pseudomonas aeruginosa has emerged in recent years as one of the major causes of gram-negative septicemia in North America (21,29). In addition, it is frequently associated with terminal lung disease in patients with cystic fibrosis, which is the most common fatal autosomal recessive disease in Caucasian society (21).

Two basic therapeutic approaches are currently being utilized against pathogenic bacteria, antibiotics and immunotherapy. At present, the treatment of *P. aeruginosa* with antibiotics can be difficult due to the high intrinsic resistance of this bacteria to most common antibiotics (30). This has led medical researchers to consider the possibility of immunotherapy (21,29). To facilitate such studies it is necessary to identify cellular components with surface-localized antigenic sites

that are both immunogenic and available to the host's immune system. We describe here the advantages of monoclonal antibodies in such studies.

III. RESULTS AND DISCUSSION

A. Production of the Monoclonal Antibodies and Analytical Methods

The production of monoclonal antibodies directed against *P. aeruginosa* outer-membrane antigens has been described (6,17). Hybridomas secreting specific antibodies were obtained by the fusion of spleen cells from BALB/c mice immunized with outer membranes or purified outer-membrane proteins of *P. aeruginosa* with cells of the NS/1 myeloma cell line. The resultant hybrid cells were cloned to obtain individual cells producing monoclonal antibodies specific for the immunizing antigens as tested in the enzyme-linked immunosorbent assay (ELISA) using antigen-coated polyvinyl plates (17,22). Antibody production was enhanced by growth of the hybrid cells in ascites in the peritoneal cavities of mice previously primed with Pristane.

Electrophoretic blotting procedures were carried out for precise determination of the specificity of individual monoclonal antibodies. In this procedure, outer membranes or purified outer-membrane proteins of *P. aeruginosa* (4) separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis were transferred to nitrocellulose by the Western blotting method described by Towbin *et al.* (25). Subsequent immunostaining was as described by Mutharia and Hancock (17). The protein blots were developed by blocking nonspecific protein binding sites on the nitrocellulose with 3% bovine serum albumin, followed by incubation, in turn, with the monoclonal antibody, then an anti-mouse IgG antibody conjugated to alkaline phosphatase, and, finally, a histochemical substrate for alkaline phosphatase. The deposition of the histochemical substrate on the nitrocellulose occurs only in the region(s) where the monoclonal antibody has reacted with its electrophoretically transferred substrate. For protein-specific monoclonal antibodies this usually occurs in a single band (see later).

B. The Use of Monoclonal Antibodies in Crossed Immunoelectrophoresis Analysis of *Pseudomonas aeruginosa* Antigens

In crossed immunoelectrophoresis (27), soluble antigens of interest are electrophoretically separated in agarose in the first dimension. These separated antigens are then electrophoresed at right angles through an intermediate gel into a second-dimension agarose gel which contains antibody. The antigens react with

the antibody to form characteristic rocket-shaped precipitins in the second-dimension gel. A complex overlapping reference pattern of rockets is observed when a mixture of antibodies is used in the second-dimension gel due to the electrophoretic separation of individual antigens (which form rockets only with their specific antibodies) in the first dimension. Highly specific reagents such as monoclonal antibodies can be incorporated into the intermediate gel. These monoclonal antibodies will then react with a single antigen before the antigens enter the second-dimension gel, thus removing a specific precipitin from the reference pattern.

A reference pattern of 36 rocket-shaped precipitins was routinely observed using a pooled *P. aeruginosa* PAO-1 antigen and a homologous polyclonal antibody preparation (Fig. 1A). Among the precipitins, antigen 31 was identified as the LPS because (i) it was the antigen that did not cross-react when standard antigen, a heterologous pool of antigens from *P. aeruginosa* strains of serotypes 3, 5, 6, and 11 of the Habs typing scheme, was added to the intermediate gel; (ii) it disappeared from the reference pattern when rabbit antibodies against the outer membranes of *P. aeruginosa* were included in the intermediate gel; and (iii) it reacted to form a precipitin line of identity in crossed line immunoelectrophoresis with purified LPS in the intermediate gel (14).

When monoclonal antibody MA1-8 (LPS O antigen specific) was used in the intermediate gel, antigen 31 reacted with this antibody and was displaced from the reference pattern. However, a precipitin that may have derived from antigen 5 now formed a tail to antigen 31 (Fig. 1B). This particular tail-like precipitin was identical to the antigen reacting with the LPS-rough core-specific monoclonal antibody MA3-8 (Fig. 1C). Therefore, by using monoclonal antibodies from two hybridoma lines in crossed immunoelectrophoresis, we clearly demonstrated the existence of subpopulations of LPS (represented by antigens 5 and 31).

Porin protein F, the major outer-membrane protein of *P. aeruginosa,* was shown to be immunogenic because anti-protein F antibodies were found in patients either acutely or chronically infected with *P. aeruginosa* (5,14). In a rat lung infection model, the production of anti-protein F antibodies was detected as early as 7 days postinfection (14). Outer-membrane proteins are known to be closely associated with LPS. Therefore, when assessing the immunogenicity of such proteins, a question may arise as to whether the antibody response in patients and animal model infections was indeed elicited by the protein. Crossed immunoelectrophoresis of protein F against polyclonal anti-PAO-1 antibody revealed the association of this protein with LPS as overlapping precipitin peaks (Fig. 2A). When monoclonal antibody MA4-4 (protein F specific) was used instead of the anti-PAO-1 antibody, the protein F precipitin was clearly identified (Fig. 2B), and the darkly stained LPS precipitin that resembled antigen 31 disappeared. This precipitin formed by protein F and the monoclonal antibody

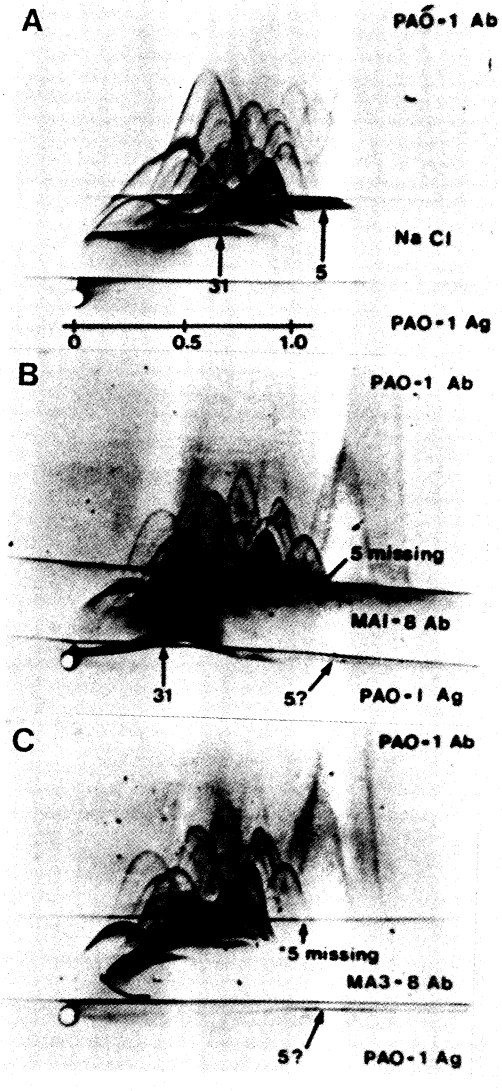

Fig. 1. Identification of LPS from PAO-1 antigen (Ag) by crossed immunoelectrophoresis with specific antibodies (Ab) added to the intermediate gel, and crossed line immunoelectrophoresis with other antigens added to the intermediate gel PAO-1 antigen (2 μl) and PAO-1 antibody (16.7 μl/cm^2) were used. Materials added to the intermediate gels were as follows. (A) Saline, as control. (B) Monoclonal antibody (MA1-8) against the smooth LPS of PAO-1; antigen 31 clearly has dropped away from the reference pattern. (C) Monoclonal antibody (MA3-8) against the rough LPS of strain AK1160. Antigen 31 seems to be unaffected, but a new precipitin line is formed (arrow), and antigen 5 is missing from the precipitin pattern.

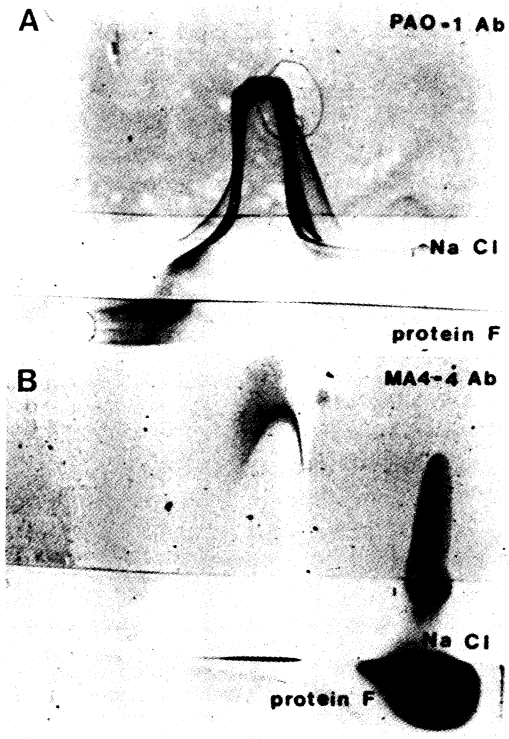

Fig. 2. Crossed immunoelectrophoresis with intermediate gel and crossed line electrophoresis of protein F. (A) Purified protein F (16 μl) was used as antigen (Ag). Its tight association with LPS was obvious, and overlapping precipitins occurred. (B) Monoclonal antibody (Ab) (MA4-4) was added to the second-dimension gel at 8.4 μl/cm².

MA4-4 was identical to the precipitin formed when patient or rat sera were used as the antibody and purified protein F was the antigen (14).

C. Monoclonal Antibodies as Immune Probes to Study Cell Surface Immunochemistry and Surface Antigen Localization

The accessibility of an immunogenic protein on the surface of intact bacterial cells is a characteristic of great importance, especially when such a protein is being considered for its potential use in clinical identification, in epidemiological studies and in the development of both active and passive vaccines. Surface localization of outer-membrane proteins has been indicated in studies using the lactoperoxidase radioiodination method (15) and a dansylation procedure (23).

Kandler, *J. Immunol.* 132, 883–887 (1984), by permission.

However, these methods have been shown to label non-outer-membrane proteins as well, perhaps as a result of disruption of the outer cell surface integrity (24). Furthermore, they do not reveal if the surface accessible portions of the protein are immunogenic.

We were interested in studying the topography of the major outer-membrane proteins on the cell surface of *P. aeruginosa*. As probes we used monoclonal antibodies to porin protein F and to lipoprotein H2. Monoclonal antibody MA1-8 specific for the O antigen of the LPS of serotype 5 (International Antigen Typing Scheme) was used as a positive control. In these studies, intact *P. aeruginosa* cells were interacted in suspension with dilutions of the monoclonal antibody and then with a fluorescein isothiocyanate-conjugated anti-mouse antibody (17). The labeled cells were then examined for fluorescence with a fluorescence microscope with a halogen lamp and appropriate filters.

The results of these studies showed that all strains of *P. aeruginosa* tested including isolates from cystic fibrosis patients interacted with the three porin protein F-specific monoclonal antibodies, MA2-10, MA4-4, and MA4-10 (17). None of these monoclonal antibodies interacted with strain H283, a protein F-deficient mutant, as demonstrated by the lack of fluorescence on the H283 cells (Table I).

The serotype 5 LPS-specific monoclonal antibody MA1-8 interacted with both strain H103 and its protein F-deficient derivative H283, but not with strain ATCC33348 (serotype 1) or an O antigen-deficient (rough) mutant, strain H233 (also derived from strain H103) (Table I). Monoclonal antibody MA1-6, specific for protein H2, interacted only with strain H233, the rough mutant, but not with H103, H283, or the serotype 1 strains (Table I). These results suggested that the lipoprotein H2 is either not exposed on the surface or its accessibility on the cell surface is masked by LPS O side chains in wild-type (smooth) *P. aeruginosa* strains. The binding of the monoclonal antibody MA1-6 with only the rough *P. aeruginosa* strain may be due to either unmasking of this protein because of the lack of O antigen on the LPS of the rough mutant or to rearrangement of macromolecules in the outer membranes of the rough strain.

The procedure of using indirect immunofluorescence in conjunction with monoclonal antibodies offers several advantages over the previously described chemical labeling techniques in the study of cell surface antigens, including speed, specificity, and maintenance of cell surface integrity.

D. Colony Blotting Analysis of Accessibility of Specific Antigenic Determinants on Bacterial Colonies

Colony blotting immunoassay facilitated rapid screening of a variety of *P. aeruginosa* strains for the expression of the specific antigenic determinants recognized by the monoclonal antibodies used. The method we used was a modifi-

TABLE I

Demonstration of Cell Surface Localization of Outer Membrane Components of *P. aeruginosa* by Using Immunofluorescence

		Cell surface binding of antibodies against outer membrane					
		Protein F			Protein H2	LSP O antigen	Components
	Phenotype	MA2-10	MA4-4	MA4-10	MA1-6	MA1-8	Whole outer-membrane rabbit antisera
Bacterial strain							
H103[a]	Wild-type serotype 5	+[b]	+	+	−	+	+
H283[a]	Protein F deficient	−	−	−	−	+	+
H223[a]	Rough	+	+	+	+	−	ND[c]
ATCC 33352	Serotype 5	+	+	ND	−	+	+
ATCC 33364	Serotype 17	+	+	ND	+	+	+
ATCC 33348	Serotype 1	+	+	ND	−	−	+
CF isolates							
CF 221[d]	Rough	+	+	ND	+	−	+
CF C46	Mucoid	+	+	ND	−	−	+
	Nonmucoid revertant	+	+	ND	−	−	+
CF C47[e]	Mucoid	+	+	ND	+	−	+
	Nonmucoid revertant	+	+	ND	+	−	+

[a] Strains were isogenic.
[b] +, Positive fluorescence; −, no fluorescence.
[c] Not determined.
[d] Thirteen other *P. aeruginosa* cystic fibrosis isolates gave results similar to CF 221.
[e] Nine other mucoid and nonmucoid revertant pairs gave results identical to CF C47.

cation of one developed by Henning *et al.* (9) in that we employed enzymatic immunostaining instead of radioimmunostaining after the blotting step. In these studies, colonies of the bacterial strain were replica plated on agar plates and then transferred onto nitrocellulose filters by direct contact. These filters were then immunostained using the same procedure as described earlier for the Western blotting technique. The enzyme used was horseradish peroxidase and the substrate was 4-chloro-1-naphthol in Tris-buffered saline with 1% methanol containing 0.05% hydrogen peroxide (v/v). The development of a blue color represented a positive result.

TABLE II

Binding of Monoclonal Antibodies Directed against *P. aeruginosa* Outer-Membrane Antigens to Colony Blots[a]

	Protein F			Protein H2	LPS O antigen
	MA2-10	MA4-4	MA5-8	MA1-6	MA1-8
Bacterial strains					
H103	+	+	+	−	+
H188	+	+	+	−	+
H223 LPS deficient	+	+	+	+	−
Serotype 5	+	+	+	−	+
Serotype 1	+	+	+	−	−
Cystic fibrosis isolates					
CF 221[b] rough	+	+	+	+	−
CF C46 mucoid	+	+	+	−	−
Nonmucoid revertant	+	+	+	−	−
CF C47[c] mucoid	+	+	+	+	−
Nonmucoid revertant	+	+	+	+	−
Other *Pseudomonads*					
P. syringae	+	+	−	+	−
P. fluorescens[d] ATCC13525	−	−	−	+	−

[a] +. Positive color development on colony; −, no color development on colony.

[b,c] Twelve[b] cystic fibrosis strains and eight[c] mucoid strains and nonmucoid revertants gave results similar to CF 221[b] and CFC47, mucoid and nonmucoid pairs,[c] respectively.

[d] Strains *P. fluorescens, P. stutzeri, P. chlororaphis,* and *P. aureofaciens* only gave positive results with antibody MA1-6. Other strains, *S. typhimurium, Escherichia coli, P. pseudomallei,* and *P. cepacia,* all gave negative results on colony blots with all the monoclonal antibodies. In the test, colonies were transferred to washed nitrocellulose filters from agar plates by contact. The filters were blocked in 3% gelatin in Tris-buffered saline (TBS) (Tris-HCl, 20 m*M;* 1 m*M* NaCl, pH 7.5) for 30 min at 37°C. Subsequent steps to process the blots were incubation in 1% gelatin–TBS containing (1) the test monoclonal antibody and (2) an anti-mouse IgG coupled to horseradish peroxidase. The color developing reagent was 4-chloro-1-naphthol in TBS containing 16.7% methanol and 0.015% H_2O_2. Blue color on colonies denoted a positive reaction.

[a] Strains were isogenic.
[b] +, Positive fluorescence; −, no fluorescence.
[c] Not determined.
[d] Thirteen other *P. aeruginosa* cystic fibrosis isolates gave results similar to CF 221.
[e] Nine other mucoid and nonmucoid revertant pairs gave results identical to CF C47.

When porin protein F-specific monoclonal antibodies, MA2-10, MA4-4, and MA4-10 were used, colonies from all the *P. aeruginosa* strains tested except strain H283 (protein F deficient) reacted to give a blue color (18). In addition, the presence or absence of the mucoid exopolysaccharide from mucoid and non-mucoid strains of *P. aeruginosa* did not apparently affect the accessibility to antibodies of protein F (Table II). The accessibility of LPS was also found to be unaffected by the mucoid exopolysaccharide when the LPS O antigen-specific monoclonal antibody MA1-8 was used in this study.

Using the lipoprotein H2-specific monoclonal antibody MA1-6 in colony blot immunoassays, we obtained similar results to the immunofluorescent staining studies described earlier. Only blots from colonies of rough LPS O antigen-deficient mutants reacted to give a positive blue color (Table II).

The use of monoclonal antibodies in the colony blot analysis enabled rapid screening of *P. aeruginosa* strains for the surface exposure of single antigens such as porin protein F. The results obtained correlated well with those of the immunofluorescence staining analysis. This method may well prove to be a powerful tool for screening for variants and mutants in genetic studies.

E. Characterization of Antigenic Domains of Porin Protein F

Owing to their high specificity, monoclonal antibodies can be used to reveal specific antigenic domains within a protein (11,26). Therefore, a study of the specific interaction of protein F with our library of protein F-specific monoclonal antibodies should throw considerable light on the structure and function of this protein (18). Peptide fragments of protein F were derived by either chemical cleavage using cyanogen bromide or by enzymatic digestion using papain or trypsin. Papain and trypsin digestion yielded a 31,000-dalton peptide which interacted with the protein F-specific monoclonal antibodies MA2-10, MA4-4, and MA4-10 (Fig. 3). However, the same peptide, when reduced by 2-mercaptoethanol treatment, did not interact with any of the monoclonal antibodies.

Cyanogen bromide cleavage yielded six fragments of protein F. Only one of the protein F-specific monoclonal antibodies, MA5-8, interacted with any of these fragments. Monoclonal antibody MA5-8 was also found to be unique in that it interacted with the oligomeric form of protein F on Western blots (17), and it did not interact with any of the protein F peptides derived from enzymatic digestion using trypsin, papain, or pronase (Fig. 3).

We interpret these preliminary studies to mean that monoclonal antibody MA5-8 interacted with a different antigenic site (epitope) than those interacting with other proten F-specific antibodies. This epitope is not affected by 2-mercaptoethanol treatment, but is sensitive to papain or trypsin digestion. The epitopes recognized by the other monoclonal antibodies, MA2-10, MA4-4, and MA4-10, are present on the same 31,000-dalton peptide fragment which apparently con-

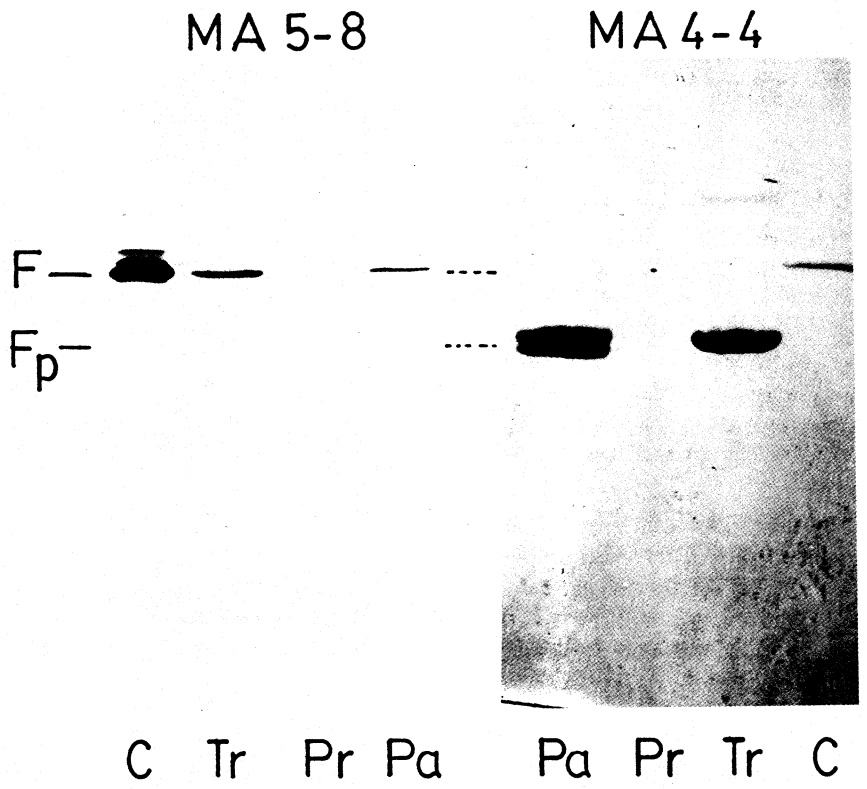

Fig. 3. Interaction of monoclonal antibodies MA4-4 and MA5-8 on Western Immunoblots with uncleaved protein F and protein F fragments derived by enzymatic digestion using pronase (Pr), papain (PA), and trypsin (Tr). (C), Control, uncleaved protein F.

tains at least one of the internal disulfide groups of protein F. Presumably they recognize a tertiary conformation of protein F, since these monoclonal antibodies do not react with this peptide or the intact protein after reduction of the disulfide groups of protein F. We do not yet know if distinct or identical epitopes are recognized by these monoclonal antibodies.

F. Demonstration of Surface Location of Porin Protein F *in Vivo* by Passive Protection Studies Using Monoclonal Antibody MA4-4

We have initiated a study of the passive protectiveness of protein F-specific monoclonal antibodies for two major reasons. First, we are interested in discovering if these monoclonal antibodies can confer protection against *Pseudomo-*

TABLE III

Protective Effect of Monoclonal Antibody MA4-4 (Protein F Specific) in B6D2F1 Mice against Challenge by *P. aeruginosa* Strains PA103 and M2

Group	Bacterial challenge (dose)[a]	Number of mice		
		Challenged	Survivors	Survival (%)
Control	Strain PA103 (2×10^6)	28	5	18
MA4-4 injected[b]	Strain PA103 (2×10^6)	27	16	59
Control	Strain M2 (4×10^6)	10	1	10
MA4-4 injected[b]	Strain M2 (4×10^6)	10	9	90

[a] Dose of bacteria is expressed in colony-forming units (CFU). The results are taken from five separate experiments of *P. aeruginosa* PA103 and two separate experiments for *P. aeruginosa* M2.

[b] 0.1 mg (protein) of the purified monoclonal antibody MA4-4 was injected intravenously into each mouse 2 hr before the bacterial challenge.

nas bacteremia and is thus potentially useful for immunotherapy. Second, we feel that positive protection by these antibodies against *Pseudomonas* infections will provide excellent *prima facie* evidence that protein F is surface exposed *in vivo*.

In these studies, groups of B6D2F1 mice (an inbred F1 strain) were challenged with one of two virulent *P. aeruginosa* strains, PA103 and M2 (Table III). The survival rate for saline-injected control animals was 18% for mice injected with 2×10^6 colony-forming units of strain PA103, and 10% for mice injected with 4×10^6 colony-forming units of strain M2. However, when 0.1 mg (protein) of affinity-purified monoclonal antibody MA4-4 (specific for protein F) was injected intravenously into mice 2 hr before the peritoneal bacterial challenge, the survival rate was increased to 59% for those challenged by strain PA103 and 90% for those challenged by strain M2 ($p < .001$ by the Fisher Exact test).

Therefore, a significant level of protection was conferred by the interaction of monoclonal antibody MA4-4 with the bacteria *in vivo*. This led us to believe that protein F is expressed and is accessible to the monoclonal antibody *in vivo*. More work is warranted to determine if other monoclonal antibodies can also confer protection or if monoclonal antibodies in general have good immunotherapeutic potential.

IV. CONCLUSIONS

The specificity, availability, and reproducibility of monoclonal antibodies has made these antibodies highly versatile and potent reagents for the analysis of bacterial antigens. By using monoclonal antibodies, we have greatly improved

the resolution of crossed immunoelectrophoresis and have identified the existence of subpopulations of LPS within one strain of *P. aeruginosa*. When used in an indirect immunofluorescence labeling study, we confirmed the surface accessibility of porin protein F and LPS molecules. In the same studies, we have also discovered that lipoprotein H2 was only accessible on the cell surface of *P. aeruginosa* strains deficient in the O-antigen region of LPS. In the colony blot study, monoclonal antibodies facilitated the rapid screening of the expression of a single antigen such as porin protein F in various strains of *P. aeruginosa*. Using this assay, we could identify a mutant, such as strain H283 (porin protein F deficient), because it did not express protein F during growth. The full potential of monoclonal antibodes was demonstrated in the peptide analysis of protein F where specific antigenic domains were identified. From these studies, we are gaining a better understanding of the molecular architecture of the *P. aeruginosa* cell surface.

V. PROSPECTS FOR THE FUTURE

With their clear advantages over polyclonal sera, we feel that monoclonal antibodies will be increasingly used to map and identify antigenic epitopes on bacterial cell surface antigens. This will have two major applications. First, since it is extremely difficult at present to solve the three-dimensional structures of membrane proteins (despite the relative ease of primary amino acid sequence determination), the mapping of antigenic determinants recognized by monoclonal antibodies should assist greatly in definition of complex structures. Second, monoclonal antibodies are the best possible reagents for unambiguously demonstrating conserved proteins. Such proteins, once identified, can be investigated for vaccine potential. An offshoot of these studies is the possibility that the monoclonal antibodies used to demonstrate these common antigens will themselves have immunotherapeutic value.

VI. SUMMARY

Monoclonal antibodies were used in conjunction with a variety of immunochemical techniques to analyze the cell surface antigens. The use of two different lipopolysaccharide-specific monoclonal antibodies in crossed immunoelectrophoresis revealed the existence of subpopulations of LPS within one strain of *P. aeruginosa*. To determine the surface localization of the porin protein F, four monoclonal antibodies specific for this protein were shown to interact with intact cells of *P. aeruginosa* and gave high-intensity fluorescence in indirect immunofluorescence experiments. The *in vivo* expression of protein F was also

shown when one of the protein F-specific monoclonal antibodies successfully mediated protection against *P. aeruginosa* infection in mice. Also by immunofluorescence, a monoclonal antibody specific for lipoprotein H2 was shown to interact with this protein only in O antigen-deficient mutants of *P. aeruginosa*. The usefulness of monoclonal antibodies as specific reagents for antigen analysis was best demonstrated by the interaction of the protein F-specific monoclonal antibodies with protein F peptides derived by cyanogen bromide chemical cleavage or by trypsin and pronase digestions. From such peptide analyses, the chemistry of the unique antigenic domains of protein F can be determined.

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