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Panreactive Monoclonal Antibody to Porin (Protein F) of *Pseudomonas aeruginosa* : Passive Immunotherapy in Mice

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled, "Panreactive Monoclonal Antibody to Porin (Protein F) of Pseudomonas aeruginosa: Passive Immunotherapy in Mice" submitted by Kurt E. Williams in partial fulfillment of the requirements for the degree of Master of Science.

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ABSTRACT

Pseudomonas aeruginosa (PA) is an important nosocomial pathogen which presents a continuing therapeutic dilemma due to its intrinsic and acquired antibiotic resistance. Immunotherapy may offer a valuable adjunctive mode of treatment. In this thesis, passive immunotherapy using murine monoclonal antibodies (Mabs) directed against outer membrane antigens of PA was investigated.

Mab 48H.3 was found to be specific for outer membrane protein (OMP) F of PA by Western blot analysis. It recognized an epitope conserved in all 13 Homma PA serotype strains and all laboratory strains of PA used, but in no other species nor genera tested. Mab 48H.1, which was specific for protein H complex, also recognized only PA strains. The immunogen for both Mab's was the Homma serotype M strain.

Passive administration of Mab 48H.3 caused a 60-to-300-fold increase over control LD_{50} values for three PA strains of different serotypes in a mouse burn wound model of PA sepsis. Protection was dose-dependent. A lesser degree of protection was observed for two of these strains, PADG1 (Homma G) and PAO503 (Homma B), in a cyclophosphamide-treated mouse model of PA sepsis. No protection was demonstrable against PA strains M-2 and Ps388 in the mouse burn wound model.

Protection did not correlate with virulence nor with serotype. Studies *in vitro* showed no significant difference in the amount of surface exposed protein F among strains or in different media. Binding of Mab 48H.3 was similar for all strains in whole-cell and OMP ELISAs.

ELISA binding of Mab 48H.3 was markedly enhanced by heating of whole-cells or OMPs. Electron microscopy using colloidal-gold-labelled protein A showed that unheated, live, whole cells did not bind Mab 48H.3 and only subpopulations of heated cells bound avidly. Mab 48H.1 did bind to live PADG1 cells but showed no protective capability against PADG1 in the mouse burn wound model.

An anti-LPS antibody Mab 43_b provided B-serotype-specific protection against PAO503 at concentrations 100- to 10,000-fold less than the concentration of Mab 48H.3 used. Co-administration of Mab 48H.3 with Mab 43_b did not improve the protection achieved with Mab 43_b alone in the mouse burn wound model.

These data demonstrate that despite panreactivity *in vitro*, protection by anti-protein F Mab 48H.3 did not extend to all strains of PA. Protective efficacy could not be correlated to bacterial binding *in vitro* and further investigations of protein F expression *in vivo* are needed to clarify this situation. The data also confirm the protective superiority of anti-LPS antibodies.

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

I. INTRODUCTION AND REVIEW OF LITERATURE

A. Epidemiologic Aspects of Pseudomonas aeruginosa Infections

Pseudomonas aeruginosa (PA) continues to be an opportunistic nosocomial pathogen of major medical importance. Despite the presence of multiple cellular and excreted virulence factors, this ubiquitous organism rarely infects individuals with intact anti-bacterial defenses; only 3% to 6% of community-acquired infections are caused by PA (14), and few of these result in fatalities. In the hospital environment, however, with the availability of a patient population made susceptible either by virtue of their underlying disease process, the application of vigorous chemotherapy or invasive procedures, PA can be a lethal pathogen.

The incidence of nosocomial infection by PA varies markedly among hospitals and even within a single institution (26). Large multi-centre studies reveal some general trends. Both the incidence and the relative frequency of PA infection have been gradually increasing (26), with data from individual medical centres, published reports and the National Nosocomial Infections Study (NNIS), demonstrating a rise in the relative frequency of PA infection from 6.6% of all nosocomial infections in 1976 to 8.6% in 1980 (76). NNIS figures for 1983 (76)

show a further increase to 10.6%, exceeded only by Escherichia coli (18.6%), and narrowly by Staphylococcus aureus (10.8%) and Enterococci (10.7%).

Despite improvements in antibacterial prophylaxis and therapy, 3% to 15% of hospitalized patients still develop nosocomial infections (168). The persistently high rate of mortality and morbidity and added costs of health care, particularly due to nosocomial bacteremia (99) emphasize the need for alternate modes of therapy. Stamm et al. (168) have suggested that passive and/or active immunotherapy effective against the six most common gram-negative nosocomial pathogens would have a major beneficial medical and financial impact. Certain epidemiologic factors presented in their study and others demonstrate that PA may be the most promising candidate for immunotherapeutic intervention.

Fatality due to PA infection is higher than that due to other gram-negatives. Stamm et al. (168) report similar fatality ratios for all gram-negative pathogens. A close analysis of the data shows that while the differences are not statistically significant for urinary tract infections, surgical wound infections and pneumonias there was a trend for higher fatality ratios (FR) for PA than for the other five gram-negative bacilli. The FR for bacteremia due to PA (49%) was significantly higher than the FR for bacteremia due to the others (26-38%) except

for Serratia (44%). Kreger et al. (82) have published similar findings, but attribute the difference to a higher proportion of patients with more serious underlying illnesses developing septicemia due to PA.

Unlike E. coli, PA is not part of the normal flora of human beings. Surveys of healthy populations and of patients entering the hospital show that only 4% to 12% are colonized with PA in their feces (14). In surgical patients, fecal colonization rises to 25% after 10 days of hospitalization, and 43% after 15 days (51). Fecal carriage is also increased by the administration of systemic antibiotics (163). The potential benefits of prevention of fecal carriage are demonstrated by a study by Schimpff et al. (157) in which 30% of cancer patients colonized with PA developed PA septicemia, while only 7% of patients who were not colonized were similarly infected.

Conventional methods of prevention of PA infection are frustrated by the organisms's ability to survive and flourish in many environmental situations. Due primarily to its ability to survive in most environments such as infusion fluids, ventilators, sink traps and even disinfectants (26), PA is an increasingly important cause of both endemic and epidemic nosocomial bacteremia (99). Despite the development of newer anti-Pseudomonal beta-lactams, conventional treatment of PA septicemia is also

inadequate. In part, this is due to the well-known intrinsic and acquired antibiotic resistance of the organism. NNIS figures show that PA and Serratia are the most commonly resistant to aminoglycoside antibiotics and that the percentage of resistant isolates has increased between 1980 and 1983 (76). PA is also the most commonly resistant to both cefotaxime and moxalactam (76).

Even in vitro sensitivity to antibiotics does not guarantee clinical efficacy. Davis et al. (35) and Flick and Cluff (45) argue that there is no documented scientific proof of the efficacy of antibiotics in PA sepsis. In patients with gram-negative sepsis, Kreger et al. (82) noted better survival of patients treated with bacteriostatic agents than of patients who received bactericidal antibiotics. This may have been due to release of overwhelming amounts of endotoxin during massive destruction of bacteria (72,73,162) as has been demonstrated in animal models of sepsis. Endotoxin release in vivo has been shown recently to depend on antibiotic class, rather than the amount of bacterial killing (162). The human clinical ramifications of this finding are unproven, but the implication is that conventional treatment with high doses of certain antibiotics may have an adverse effect on the course of PA sepsis (72).

The efficacy of immunotherapy, particularly prophylactic passive and active immunotherapy, is enhanced by the early identification of those patients who are at risk for the disease in question. Although PA has been isolated from nearly every anatomic site of infection and in recent years has appeared in healthy, non-hospitalized hosts with increasing frequency (27), it is still primarily associated with four groups of immunocompromised patients: patients with 1) malignancy, especially during courses of chemotherapy; 2) cystic fibrosis; 3) burns; and 4) trauma requiring treatment in an intensive care unit (ICU). These classical associations and a tendency toward endemicity greatly facilitate the identification of patients at risk for PA infection.

PA remains a prominent pathogen in patients with either quantitative abnormalities of neutrophils (due to chemotherapy, immune suppression or malignancy) or qualitative extrinsic defects due to sub-optimal T-cell and B-cell interaction (166,167) (such as in CF or immune suppression with steroids) (10). Bacteremia with this organism is seen in patients with reticuloendothelial malignancy (158) or with solid tumors (9), due to increasingly aggressive chemotherapy. In one study, PA was the leading cause of documented pneumonia and bacteremia in patients with non-Hodgkin's lymphoma (12). While PA was responsible for approximately 10% of all

infections in another study of cancer patients, it was isolated in 21% of cases of bacteremia and had a case fatality rate of 65% (26). The degree of granulocytopenia correlated most strongly with the development of bacteremia in those patients and both granulocytes and tissue macrophages play a crucial role in host defenses against PA (14).

Patients with cystic fibrosis (CF) are frequently chronically colonized with PA. Frank PA pneumonia is a frequent, recurrent and serious complication, though septicemia is uncommon, possibly due to the presence of high levels of circulating anti-PA antibodies, and an apparently competent immune response to PA (41,61). Still, death for most CF patients is caused by overwhelming PA infection and resultant pulmonary failure (136). The defects in anti-bacterial defenses which predispose CF patients to PA colonization and infection have been the subject of intensive investigation. Among the factors frequently cited are: 1) prior infection with S. aureus resulting in damage to lung tissue (100); 2) increased use of antibiotics to treat prior infections with S. aureus or H. influenzae (83); 3) defects in mucocillary transport (150); 4) impairment of T-lymphocyte responses to PA antigens (166) and to antigens of some other gram-negative bacteria (167); and 5) a primary defect in the structure and function of IgG (42,103). It

is generally felt that many, if not all, of these abnormalities may be secondary to infection rather than primary predisposing factors, although it is likely that they contribute to recurrent and chronic infection (172). The abnormal volume and consistency of mucus and the unusual avidity of PA for mucins and tracheal epithelial cells (144) mediated by either pili (145) or by extracellular mucoid exopolysaccharide (146) may be sufficient to explain the association between PA and CF (136).

With the advent of effective anti-Staphylococcal therapy, 60% to 90% of septicemic episodes following significant burn wounds are now caused by PA (34). Though the number of septicemic episodes has decreased with the use of topical antibacterial prophylaxis (123), PA retains its relative importance (142). The deleterious effects of thermal injury on host defenses are complex and will be dealt with in detail in later sections.

The fourth group of patients predisposed to infection with PA includes a heterogenous collection of underlying disorders with the common factors of extreme severity of illness and multiple invasive procedures. Cross (26) notes the emergence of PA as an important pathogen complicating trauma due to armed conflict. In his review of PA infection, Bodey (14) notes that hospital-acquired PA pneumonia is most common in patients with hematologic

malignancies, CF, diabetes, chronic lung or heart disease and surgical patients. Tracheostomy and ventilatory support were additional predisposing factors in many of these patients. Mortality varied from 50% to 80% (14).

B. Immunotherapy of Pseudomonas aeruginosa Infection

The current status of anti-Pseudomonal vaccines that are now in clinical trial as well as potential future vaccines being evaluated in animal models has been reviewed by Cryz (31). The earliest published investigations of vaccine efficacy involved whole-cell vaccines with undefined antigenic composition. Feller and Pierson (40), confronted by a 51% incidence of PA septicemia (with a 62% fatality ratio) among severely burned patients in their burn unit, developed a heat-killed and phenol-preserved vaccine from a single clinical isolate. Using a combination of active and passive immunotherapy they first demonstrated the practicality of immunotherapy against PA septicemia in rabbits (39) and in humans (40). Analysis of their results is complicated by their use of a historical control group. Marked improvements in other modalities of burn-wound patient care were also introduced during that period. The failure of active immunotherapy alone to protect burn-wound patients in early phases of the study is attributed to the inability of some severely burned patients to respond

quickly enough with high titres of anti-PA antibodies (4). In a second published report, Sachs (151) used six different PA strains, two Staphylococcus pyogenes strains (all clinical isolates from his burn unit) and a Staphylococcal toxoid to immunize 39 burn-wound patients. Their small number of patients and the maldistribution of patients in terms of severity of burns between the control and the immunized group made statistical analysis difficult. However, the immunized group demonstrated a trend toward a decreased incidence of PA septicemia and even in ultimately fatal cases of PA septicemia, prolonged survival. No data were given in either study regarding toxicity.

While monovalent vaccines produced locally may have a place in endemic infection, a vaccine with a wider spectrum of activity is required before embarking on a multicentre clinical trial. Attempts to use the serotype strains developed by Verder and Evans (177) in multivalent vaccines to protect mice from experimental PA infection yielded variable protection. Fisher et al. (43) identified seven prevailing immunotypes which provided cross-protection against all commonly isolated serotypes with minimal interference when administered together. Extracts from these immunotypes were then used to prepare a heptavalent vaccine, composed primarily of lipopoly-

saccharide (LPS) (63). Extensive clinical evaluation has followed.

Alexander and Fisher (5) compared a historic control group of unvaccinated burn-wound patients with smaller groups vaccinated by various routes and with various doses of heptavalent Pseudomonas vaccine (Pseudogen: Parke-Davis) over a 5-year period. Protection was correlated with levels of anti-PA antibodies and was generally dose-dependent. Active immunization at maximal doses reduced mortality of PA infection from 14.1% in the historical control group to 3.1% in those vaccinated. Mortality from other infections was comparable in all groups. Of particular note is that the adjunctive use of PA-hyperimmune globulin (from healthy volunteers immunized with Pseudogen) in eight maximally immunized patients who did develop PA septicemia reduced the mortality to 0%.

A total of 176 vaccinated cancer patients were compared with 185 concurrent control patients in a randomized, but not blinded prospective study (185). Protection against fatality associated with PA infection was statistically significant ($p < 0.01$) and there was a trend toward reduced mortality from PA bacteremia as well as reduced incidence of PA infections in the vaccinated group. Of the patients who developed PA bacteremia, 4 of 14 survived in the vaccinated group while all 19 in the control group died.

Two smaller studies involving patients with leukemia (53,127), trials involving CF patients previously colonized with PA (127) and one study of surgical intensive care unit patients receiving ventilatory support (137) all showed no benefit from Pseudogen vaccination. Clinical trials with Pseudogen have been halted primarily due to the unacceptable incidence of local and systemic toxicity, particularly in cancer patients (185).

Several valuable findings have emerged from these and related studies. Though toxicity limited the dose, protection from PA infection was evident in some patient subgroups. This effect could be augmented by the administration of hyperimmune globulin, which was non-toxic in these studies. As more has been learned about virulence factors of PA, the importance of the production of antibodies against LPS has been emphasized. Cryz and his colleagues showed that rabbit anti-LPS IgG provided passive protection from a homologous strain of PA in a cyclophosphamide-treated mouse model (30). They also demonstrated that IgG directed against exotoxin A or elastase was unable to provide similar protection. Pollack et al. (139) have shown that a favorable outcome of PA septicemia correlates best with high anti-core IgM and type-specific anti-LPS antibody levels. Anti-exotoxin-A and anti-core IgG levels correlated individually with survival, but did not increase the

prognostic value of the combination of anti-core IgM and type-specific antibodies. These factors have stimulated a search for other methods of eliciting non-toxic protective anti-LPS antibodies.

Seid and Sadoff (161) have chemically removed ester-linked fatty acids from type-5 LPS to produce a 100-1000 fold less toxic antigen (D-LPS). D-LPS alone was not immunogenic, but when linked to a protein carrier it did elicit type-specific antibodies in mice. The protective capability of D-LPS is unknown. Similarly, Tsay and Collins (175) have employed acid treatment of immunotype 1 LPS to generate a low molecular weight polysaccharide which was then covalently conjugated to bovine serum albumin. This protein-polysaccharide conjugate was 4000-fold less toxic than native LPS but retained significant immunogenicity. Mice actively or passively immunized with the conjugate or mouse anti-conjugate antiserum respectively were protected from fatal infection in both a model of intra-peritoneal infection and a mouse burn-wound model of PA sepsis.

Pier and his colleagues have approached the problem by using high molecular weight polysaccharide (PS) purified from the culture supernatant of growing PA as an immunogen. They have found that PS induces type-specific antibodies in animals (131) and in humans (133). Though the PS vaccine is not as potent an immunogen as LPS, it is

non-toxic and protective in animal models against homologous Fisher immunotypes (131) and specific heterologous immunotypes (134,135). PS from Fisher immunotype 3 is structurally identical to the immunotype 3 LPS O-sidechain, but the basis for cross-protection for immunotype 5 is unknown (135).

Bryan et al. (18) have attempted to avoid toxicity by changing the route of administration. LPS delivered by short periods of aerosolization or ingested in drinking water protected mice from sepsis due to PA of the homologous serotype. Protection was achieved in a mouse burn-wound model and an "iron-loaded" mouse model of PA sepsis. No absolute change in survival was demonstrable in a cyclophosphamide-treated leukopenic mouse model, but there was a significant delay in time of death in the immunized animals. Bivalent protection was achieved by concurrent administration of 2 different serotypes of LPS, but sequential immunization gave suboptimal protection for the second serotype administered. No protection was evident for one of the four serotypes used, possibly because of an insufficient dose or duration of administration (18). Toxicity was limited to a temperature elevation of 0.5°C to 0.8°C early during the immunization process. Phase 1 clinical trials are planned.

Another vaccine which has reached clinical trials is the polyvalent extract vaccine (PEV-01) described by Miler et al. (105). It contains sterile EDTA-glycine extracts of 16 different PA serotypes selected from among 150 strains for their virulence. A variable proportion of each component has been found to be outer membrane protein (106), but the antigenic composition of PEV-01 is largely unknown since LPS and other virulence factors have not been directly determined. Chemical analysis showed 20% lipids and 13% each of protein and carbohydrate (105). Clinical trials were commenced in burn patients in 1977. Preliminary results showed that patients responded to vaccination with low titres of specific antibodies to all 16 of the vaccine components administered together (78,79). Two study populations were used. For the English population there were no deaths reported from PA sepsis in either the control or the vaccinated group and no difference in overall mortality between the two groups (79). In the Indian study population, death due to PA was common because of a lack of topical burn-wound therapy effective against PA. Here, mortality in vaccinated adults was reduced to 6.6% from the 40.6% in the unvaccinated control. Vaccinated children had 4.8% mortality while mortality in the control group was 20.8%.

A later published report of the Indian study population (148) included 746 patients, 35.7% of whom

received no immunotherapy, while 39.8% got PEV-01 alone. A total of 16.5% were passively immunized with immunoglobulin from healthy volunteers vaccinated with PEV-01 and 8% were treated with a combination of PEV-01 and immunoglobulin. Adult patients had approximately the same reduction in mortality with all modalities (from 42.5% in the control to 11.8% - 14.7% in the treated groups). Children benefitted more from immunoglobulin alone (5.6% mortality) than from the other regimens (10.75% and 11.5% mortality). PA was replaced by Klebsiella as the most common opportunistic pathogen in vaccinated patients. Vaccine toxicity was limited to a slight rise in body temperature in some patients at 6 h post-vaccination. To date, the trials with PEV-01 have involved only burn-wound patients.

Original endotoxin protein (OEP) is a proteinaceous extract derived from PA-N10 (Homma serotype 5) which consists primarily of a protein with a molecular weight of 22,000 d (2). OEP possesses an antigen common to 14 Homma serotypes (1) and vaccination of mice with OEP protects them against intraperitoneal infection by all seven of Fisher's immunotype strains (2). Recently, Homma and his colleagues found that a multicomponent vaccine composed of alkaline protease toxoid, elastase toxoid and OEP provided significantly better protection in a mink model of PA pneumonia than OEP alone (70). Neither vaccine provides

protection against Homma serotype 16 strains M2 and PF2243, which are apparently devoid of the common antigen (121). Preliminary results of clinical trials with the multicomponent vaccine involving burn patients and patients with chronic lung disease are promising (as reviewed in Homma, 1980) (69).

A non-Pseudomonal immunogen, E. coli J5 (J5) presents the potential for broad spectrum immunotherapy. The J5 mutant of E. coli 0111 lacks uridine 5'-diphosphate-galactose 4-epimerase and therefore the ability to add serotype-determining O-specific side-chains to its LPS (37). This exposes the core region of LPS, the lipid A moiety of which is chemically similar and antigenically cross-reactive in a wide variety of gram-negative genera including PA (93,115). Early animal studies demonstrated significant passive protection of rabbits from fatal sepsis due to E. coli, Klebsiella and PA (187,188). A double-blind randomized clinical trial in which PA was the second most common clinical isolate showed a reduction of mortality in patients with documented gram-negative sepsis from 36% in the control to 22% in those given J5 antiserum ($p = 0.001$). The protective effects in the most severely ill patients and those in profound shock were more convincing with a reduction of mortality from 77% in unimmunized patients to 44% in J5 serum recipients ($p = 0.003$) (189).

Although the protective effects of J5 are significant, type-specific immunity appears to offer superior protection. Sadoff et al. (153) found that active immunization with J5 offered no protection against fatal PA burn-wound sepsis whereas immunization with purified LPS did. Pennington and Menkes (129) used a guinea pig model of hemorrhagic pneumonia to show that vaccination with Pseudogen was significantly more protective than active immunization with J5 (85% survival for Pseudogen, 42% for J5, $p = 0.001$; and 15% survival for unvaccinated controls). Cross-reactivity with all serotype strains and 14/14 clinical isolates of PA of mouse monoclonal antibodies to the lipid A portion of J5 has been demonstrated (116). Recently, Teng and his colleagues produced a human monoclonal IgM antibody specifically directed against J5 lipid A. This antibody afforded passive protection against fatal sepsis caused by strains of Klebsiella, E. coli and PA in a mouse model of intraperitoneal infection (173). Cryz et al. (32) were able to obtain protection against only one of five serotype strains of PA using active immunization with O-side-chain deficient LPS antigens of PA, E. coli J5 or Salmonella typhi.

Exotoxin A is a secreted product of PA originally described by Liu (90) that inhibits protein synthesis in mammalian cells by adenosine diphosphate ribosylation of

elongation factor 2 (74). It is toxic or lethal for a wide variety of mammals (90,91). The level of anti-exotoxin A titres in humans correlates with survival of PA bacteremia (25,138). Passive administration of anti-exotoxin A antibodies or immunization with toxoid plus an adjuvant protected burned mice from fatal PA sepsis due to toxigenic but not non-toxigenic strains (125,126). Passive transfer of rabbit anti-toxin A IgG failed to protect neutropenic mice from PA sepsis with a toxigenic strain (30). All toxoid preparations to date have either reverted during storage or require potent adjuvants for immunogenicity, making them unfit for clinical trials (reviewed in Cryz) (31). One exception is the mutant derived by Cryz and his coworkers which produces a toxoid designated CRM66 (28). CRM66 is a non-toxic immunogen, antigenically indistinguishable from exotoxin A. Immuno-protection experiments are pending (182).

Bacterial motility is an important virulence factor for PA in the mouse burn-wound model (24). Active immunization with flagellar preparations protects mice against homologous PA sepsis in this model apparently by immobilizing the organisms at the site of the wound infection (66).

Adherence to mucosal cells via pili may be an important step in the pathogenesis of PA infections (145,180). The feasibility of blocking this initial step

with pilus serotype specific antibodies has been established but *in vivo* trials have not been attempted (180).

The alginic acid of PA may act as both an anti-phagocytic factor (122,166) and an organelle for adherence (146). Antibodies to alginate have been found in the sera of CF patients and are easily induced when alginate is injected into experimental animals (17). Woods and Bryan (183) have reported recently that immunization of rats with highly purified alginate resulted in the generation of high titres of specific antibodies which promoted clearance of PA from the lungs of chronically infected rats. However, they also noted immune complex deposition in immunized animals only. The clinical consequences are unclear, yet the implication is that the vaccine in its present form may be inappropriate for human use.

Proteases, such as alkaline protease and elastase are generally accepted as virulence factors (182). Though Cryz *et al*. (30) showed that no protection resulted from passive administration of anti-elastase antibodies to leukopenic mice, Homma *et al*. (70) reported increased efficacy of their OEP vaccine in mink if detoxified elastase and alkaline protease were added. Toxicity of these proteoids is unknown.

A stable temperature-sensitive mutant of the Fisher immunotype I strain was produced by Hooke and her

colleagues for the development of a live PA vaccine (71). Intraperitoneal or aerosol immunization with this strain protected against intraperitoneal or aerosol infection by its wild-type parent (71,165). No comment was made regarding specificity of protection or toxicity.

Ribosomal vaccines have been developed by Lieberman (86,87,88). The presence of polysaccharide moieties in the vaccines, the serotype-specificity of the protection against intraperitoneal challenge in mice, and the retention of protective capability after trypsin and ribonuclease treatment, all suggest that LPS or polysaccharide contaminants are the major antigens.

It is clear that the optimally protective antigen or combination of antigens have not yet been formulated. As is evident from the foregoing discussion, antibodies directed against serotype-specific antigens of PA correlate well with protection in human and experimental sepsis. Many patient groups, especially CF patients and leukopenic cancer patients apparently do not benefit from attempts to elicit these antibodies. Direct vaccination with LPS has been discontinued due to unacceptable toxicity. Non-toxic, antigenically similar substitutes are less immunogenic and usually have unknown protective capabilities.

Passive immunization with immunoglobulin elicited by this type of vaccine or with monoclonal antibodies to

serotype-specific LPS (81) are less toxic and equally efficacious. Both active and passive regimens require the presence of multiple antigens or antibodies. This means a vaccine or immune globulin mixture of some complexity with demonstrated potential for immune interference and suboptimal protection against some serotypes.

Though the majority of isolates belong to one of the seven Fisher immunotypes, systems based on the same O-side chain antigens with as many as 17 distinct serotypes have been described. The correlation among these has been reviewed (69). However, from 1% to 25% of isolates may not be typable (31), in some cases due to a severe decrease in the amount of O-side-chain-containing LPS (60). Therefore no vaccine based on this system has even a theoretical chance of protecting against all strains of PA.

The other candidates for immunotherapy mentioned previously correlate less well with protection and are even less likely than LPS to cover all PA isolates. Significant proportions of PA isolates do not have OEP, exotoxin A, elastase, alkaline protease and alginate. The pathogenic significance of flagellae and pili is limited to only some types of infections and the serology of pili is largely unknown.

C. Outer Membrane Proteins of Pseudomonas aeruginosa

The ideal immunotherapeutic candidate would be a single antigen which is conserved throughout all PA serotypes, non-toxic and highly immunogenic when administered alone, and able to elicit highly protective antibodies which do not form immune complexes.

It has been approximately a decade since Eagon et al. (36) first suggested the use of OMPs of PA as immunotherapeutic antigens. Since then, several interesting candidates, led by porin protein F, have emerged among the OMPs. Immunologic investigation of these proteins has been made possible by advances in laboratory methods which have allowed their classification, biochemical characterization, and isolation in functional forms.

Several schema have been proposed for the classification of major OMPs of PA (15,54,103,109,170). The nomenclature which has found the widest usage is a modification of the alphabetical system of Hancock and Nikaido (54) proposed by Hancock and Carey (56). The number and molecular weight of several of the proteins varies depending on the conditions of solubilization and electrophoresis employed. Maximal resolution of OMPs was obtained by solubilizing in SDS and electrophoresing on a 14% polyacrylamide gel (54). Eight major OMPs with the following molecular weights were identified: 1) D1, 46,000 d; 2) D2, 45,500 d; 3) E, 44,000 d; 4) F, 39,000 d;

5) G, 25,000 d; 6) H1, 21,000 d; 7) H2, 20,500 d; and 8) I, 9,000 to 12,000 d.

The most extensively investigated of these OMPs is protein F. Protein F is the most numerous OMP at 1 to 3 x 10^5 copies per cell (118). Its apparent molecular weight is variously reported as 33,000 d to 45,000 d, depending upon the technique of solubilization and the conditions of electrophoresis (8,109). Heating at 100°C for 30 min or more, solubilization in 2-mercaptoethanol, and pretreatment with trichloroacetic acid all increase the apparent molecular weight (M_r) of protein F. Similarly heat-modifiable OMPs of E. coli have extensive B- (pleated-sheet) polypeptide structure which undergoes a gross conformational change to alpha-helical structure when heated in sodium dodecyl sulfate (117). Mizumo and Kageyama (109) suggest the same mechanism is involved for protein F.

Protein F is the major hydrophilic channel through the outer membrane of PA, hence its designation as a porin (55,118). Investigation of this important function was made possible by the development of a method for separating outer from inner membrane without using EDTA, to which the OMPs of PA are very sensitive. The OMPs thus isolated were used to reconstitute a permeability barrier in LPS-phospholipid vesicles (54). Separation and purification of isolated outer membranes revealed protein

F to be constitutive, and the major porin (11,55). Protein D1 and protein P were later identified as minor inducible porins (57,58). Protein F was found to have an exclusion limit for saccharides of approximately 6000 to 9000 d, inferring a pore radius of about 2.3 nM (11). Pores *in vivo* are probably composed of trimers of protein F (118).

Apart from being a channel for the ingress of nutritional substances, protein F may function as a virulence factor. Nicas and Hancock (118) used a protein F-deficient mutant, its wild-type parent, three revertants, and an antibiotic super-susceptible mutant to provide *in vivo* confirmation of the importance of F as the major hydrophilic channel for the uptake of beta-lactam antibiotics. Their results suggested that only 1 in 400 of the porin molecules present was actually forming an active functional channel. Calculations of OMP permeability coefficients revealed that *E. coli* was more than 10 times more permeable to a chromogenic beta-lactam than wild-type PA, which might explain in part the high intrinsic resistance of PA to beta-lactam antibiotics in general. The protein F-deficient mutant was 1/5 as permeable as its parent and its revertants. However, permeability to beta-lactams is a multifactorial process involving, in addition, LPS and its effect on the open-closed state of pores (7) or its influence on the

hydrophobicity of the external surface of PA (48). The close association of porin and LPS has made the acquisition of LPS-free purified porin very difficult (47,56).

Immunologic investigations have helped to establish the feasibility of using protein F as an immunotherapeutic antigen. Mutharia et al. (113) reported that three of the major OMPs, F, H2 and I, are antigenically closely related in all serotype strains and several clinical isolates of PA. Monoclonal antibodies specific for H2 interact with OMPs of all 17 serotypes of PA, two P. fluorescens strains, two P. putida, a P. anguilliseptica strain and an Azotobacter vinelandii strain, but not with a strain of P. acidovorans, two Aeromonas strains, a Vibrio, nor an Edwardsiella (59). However, H2 appears not to be surface-exposed since no binding to live, intact, wild-type PA strains was demonstrated by immunofluorescent staining using anti-H2 Mab (114). The Mab did bind to a rough, LPS-deficient mutant. Mab's to protein F, on the other hand, showed fluorescence with live cells representing all 17 serotype strains and all 15 available clinical isolates of PA, and one strain of P. putida. Surface exposure of protein I has not been assessed.

Protein F, H, and I also appear to be B-cell mitogens (21). Picomole amounts of each protein had a mitogenic effect on T-cell depleted spleen-cell preparations from

mice genetically unable to respond to LPS, thereby controlling for the well-known mitogenic effect of LPS (21).

The immunogenicity and surface exposure of OMPs has been investigated *in vivo*. Fernandes *et al*. (41) found that chronic lung infection in CF patients with PA produced antibodies to OMPs with MWs of 58,500 d, 37,500 d and sometimes to a third OMP with MW 34,000 d. Because rabbits immunized with formalin-killed PA responded only to the 58,500 d OMP it was concluded that this was the only surface-exposed OMP. Hedstrom *et al*. (65) found that experimental subcutaneous PA abscesses in mice provoked antibodies to proteins F, H2, I and a 16,000 d protein which was probably pilin. In a second study of 19 CF patients with chronic lung infection with PA, Hancock *et al*. (61) found antibodies against protein F in all cases. Antibodies to E, H2, I and minor OMPs were frequently, but not invariably present. No evidence for the protective capability of these anti-OMP antibodies was given in any of these studies.

While experimental work outlined in this thesis was in progress, Gilleland *et al*. (47) offered proof of the protective nature of anti-porin antibodies. Mice actively immunized with two 10 ug doses of partially purified porin were protected from intraperitoneal challenge with homologous or heterologous serotype strains of PA.

Antibody response to contaminant LPS accounted for a significant proportion of the protection from homologous challenge, but could not explain the heterologous protection. Significant protection was seen after challenge with up to 3 times, but not 4 times, the LD_{50} dose of a heterologous strain. Passive protection in mice using multiple doses of heterologous rabbit anti-porin antibodies was also demonstrated. Protection was significant against challenge with twice the LD_{50} dose of a heterologous strain.

Protein F has many characteristics desirable in the ideal immunotherapeutic antigen for PA. It is surface-exposed, immunogenic in a variety of mammals including humans, non-toxic in mice and rabbits, and evokes a protective response in mice. Many questions remain regarding optimal exploitation of the immunotherapeutic potential of protein F. One important approach is the use of monoclonal antibodies against protein F in passive immunotherapy.

Mab's to OMPs of PA were developed at the University of Calgary by Mackie et al. (98). Their preliminary studies revealed an increased propensity of PA over Neisseria meningitidis (NM) to provoke species-specific antibodies in response to immunization with surface antigen extracts. Several Mab's were identified as being species-specific or panreactive for PA; that is, in an

enzyme-linked immunosorbent assay (ELISA) system they recognized OMPs of all 13 Homma serotype strains, but not OMPs of other Pseudomonas species nor those of other gram-negative or gram positive organisms. Three of these Mab's were selected for further study. One of the three, designated 48H.3, was subsequently found to be specific for protein F. 48H.3 was also found to be panreactive while 48H.4, 48H.6, 48H.9 and 48H.10 recognized only Homma M - serotype PA on OMP ELISAs (96) Determination of the specificities of these Mab's was part of this thesis work.

The advantages of using a passive immunotherapeutic approach extends to both the investigational and the theoretical clinical aspects of this study. Highly specific Mab's provide unequivocal proof of the identity of the antigen involved in protection studies. Use of Mab's to identify an optimally protective antigen at the epitope level might assist in the development of a synthetic vaccine. LPS contamination of purified porin preparations might interfere with assessment of the protective capacities of a protein F vaccine by: 1) providing serotype-specific protection (47); 2) providing protection against other serotypes or eliciting cross-reacting antibodies specific for the lipid A portion of LPS (115); 3) initiating B-lymphocyte mitogenesis and polyclonal stimulation to produce a variety of unrelated antibodies, including autoantibodies (33,46). Passively

administered antibodies developed after immunization with a porin preparation would be subject to the same factors.

Clinical use of Mab's combines the advantages of passive immunotherapy and the unique advantages of Mab's. Mab's represent an unlimited source of immunoglobulin of reproducible specificity and affinity whereas immunization and periodic harvesting of immune serum from humans or animals is an expensive and cumbersome process (173) and the antibody preparations obtained vary widely in composition and efficacy (152). Since malignant cells are involved in the manufacture of Mab's, the theoretical possibility of transmission of oncogenes exists. Infection of hybridomas with Epstein-Barr virus (EBV) and subsequent transmission is also a consideration. The natural loss of chromosomal material from hybridomas can allow the selection of clones lacking the EBV-carrying genome and reverse transcriptase activity (173). Transmission of hepatitis, cytomegalovirus and HTLV III are serious disadvantages associated with the use of polyclonal sera (173).

Active immunotherapy is advantageous if long-term immunity is desired. For instance, CF patients might benefit from this approach if immunized against PA before pulmonary colonization has taken place. However, in the majority of patients at risk for PA infection, such as burn patients, ICU patients and cancer patients undergoing

chemotherapy, immediate short-term protection is required. Passive immunotherapy can be commenced as soon as the patient has been identified as being at risk, and continued for the duration of the period of increased risk. The onset of protection by vaccination depends on the rapidity of the recipient's immune response, whereas intravenous passive immunotherapy provides immediately high levels of antibodies.

Even with early identification and initiation of active immunotherapy, protection depends upon the patient's ability to respond adequately to the antigen(s) used. Administration of cytotoxic chemotherapy is associated with a declining antibody response to vaccines against PA and Streptococcus pneumoniae (108,127). Patients whose underlying malignancies involve defective antibody production (chronic lymphocytic leukemia and multiple myeloma) have an increased incidence of infection even when they are not neutropenic, due to a decreased antibody response to pathogenic bacteria (176). Burn wounds and trauma provoke multiple immunologic defects including depressed immunoglobulin levels due to catabolism, leakage into injured tissue, and decreased synthesis (112). Failure of anti-Pseudomonal vaccines to protect burn patients from death due to PA sepsis has been associated with their inability to make adequate antibody response to the vaccine (4). Clearly, it is inadvisable

to depend upon the immune responses of these patients to protect them against PA sepsis.

Passive immunotherapy may also be used therapeutically after infection has occurred whereas there is no rational basis for the use of active immunotherapy in this situation. Administration of antibodies as treatment or prophylaxis may be regarded as physiologic replacement.

Animal models to assess the protective capabilities of our anti-protein F Mab were selected on the basis of their similarity to human infection. Mice were chosen as experimental subjects because the Mab was of mouse origin. The first model utilized was the mouse burn-wound model of Stieritz and Holder (169). In this model mice are subjected to a non-lethal, 30% body surface area, full thickness thermal injury, then infection is established in the burn wound. The second model was the cyclophosphamide-treated mouse model of Cryz et al. (29) which involves induction of leukopenia with cyclophosphamide followed by dermal infection with PA. These models were designed to mimic patient populations at high risk for PA sepsis, both in terms of the underlying disease process and the route of infection. Both models also demonstrate the increased susceptibility to PA which accompanies human burn wounds and chemotherapy in that the LD_{50} for PA strains is reduced by as much as 10^5 to 10^6 below that of untreated mice. Death as the final outcome

in both models provides an unambiguous parameter for the assessment of treatment.

Other models of PA infection were considered. The rat model of chronic respiratory infection of Cash et al., (19) involves instillation of PA-impregnated agar beads into rat lungs. Assessment of results in this model requires histologic analysis of the infected lungs and technical difficulties have prevented the use of mice in this model system. (D.E. Woods, personal communication). Intraperitoneal challenge, though widely used in the literature, bears little resemblance to PA infection in humans. The LD_{50} doses are very high (10^7 to 10^8 cfu/mL) predisposing mice to an early endotoxic mode of death rather than an infective illness. LD_{50}'s can be lowered in this model by using artificial enhancers of virulence such as hog mucin (44) or by 'iron-loading' organisms grown under conditions of iron depletion (18) neither of which mimic clinical situations.

Models involving larger animals have been used in the past to assess the protective capabilities of anti-LPS antibody and to monitor the physiologic changes during PA sepsis. Harvath and Andersen (64) immunized dogs with specific LPS, rendered them granulocytopenic, and found that they were protected against challenge by a homologous strain of PA. Pennington (128) found similar protection in guinea pigs against acute PA pneumonia. A model of

localized infection in the cornea of mice has been used to assess contributions to virulence of elastase and toxin A (120) and iron acquisition and its effect on toxin A regulation and virulence (181). Welsh et al., (179) used a rabbit model of corneal infection to prove the beneficial effect of topical therapy with equine anti-LPS hyperimmune serum. Though ophthalmologic infections of PA are important and potentially serious, they represent a specialized interest.

At present, serotype-specific anti-LPS antibodies clearly dominate immunoprotective considerations for PA sepsis. During the course of work for this thesis, Bryan et al., (unpublished data) developed an Mab directed against the O-side chain of PAO503, a Homma serotype B organism. It therefore became possible for us to compare the degree of protection obtained with our anti-porin F Mab and this anti-LPS Mab and to assess the effects of combined immunotherapy using the two Mab's together. These data would enable a rough estimate of the relative potency of the antibodies and their interaction in treating PA sepsis. Immunotherapeutic potential of the uncharacterized anti-OMP Mab's was also be to assessed.

Antibodies may exert an immunotherapeutic effect by more than one mechanism. Antibodies to excreted virulence factors such as exotoxin A, alkaline protease, elastase, and LPS might bind to these antigens and neutralize their

toxic effects. It has been shown that OMPs can also be excreted during normal growth (110), but whether protein F is released in significant amounts and what effect this has on virulence is not known. In addition, antibodies directed against antigens in structural components such as LPS and OMPs might bind to intact bacterial cells and either opsonize the cells for more effective phagocytosis or exert a lethal effect via binding of complement. A third mode of action for anti-protein F antibodies might be to interfere with the porin function of protein F. If antibody binding leads to a decrease in permeability, this might nutritionally compromise the cell. A decrease in permeability might also slow the entry of B-lactam antibiotics into the cell, showing an antagonistic interaction with this standard mode of therapy. If an increase in permeability results, anti-porin Mab's may prove to be synergistic with B-lactam antibiotics. To investigate the parameters of Mab binding to PA and its consequences whole-cell ELISA assays, binding of radioactively labelled Mab's, electron microscopy of the binding of gold-labelled Mab's, and an assay of cell permeability were employed. These methods, along with polyacrylamide gel electrophoresis of OMPs or surface radiolabelled whole cells were used to look for strain variability to account for any differences in the effect of the Mab's among strains.

II. MATERIALS AND METHODS

A. Monoclonal Antibodies

Monoclonal antibodies (Mab's) against outer membrane proteins (OMPs) were prepared by E.B. Mackie and B.M. Longenecker (97). Panreactive Mab's 48H.1, 48H.3 and 48H.5 as well as 48H.4, 48H.6, 48H.9 and 48H.10 are described in more detail above. Monoclonal antibody 43b, against LPS, was the generous gift of L.E. Bryan (University of Calgary). Mab's against OMPs of Neisseria meningitidis used as control injections in the same experiments were kindly supplied by E.B. Mackie (University of Calgery). All Mab's were supplied and used as a crude mouse ascites unless otherwise indicated.

B. Purification of Monoclonal Antibodies

Standard methods were followed (159). Briefly, the ascites was first centrifuged at 100,000 x g for 30 min (Beckman L5-50B ultracentrifuge; Beckman Instruments, Palo Alto, CA 94304 USA) and then the Mab was precipitated from the supernatant by 50% ammonium sulfate. After dialysis to remove the ammonium sulfate, the supernatant was cleared by centrifugation at 15,000 x g for 10 min (Beckman J2-21 centrifuge) and fractionated on a DEAE cellulose anion exchange column from which the Ig was eluted with a linear NaCl gradient. Column fractions were assayed for Mab by ELISA.

C. Bacterial Strains and Growth Conditions

PADG1 is a P. aeruginosa clinical isolate from the sputum of a patient with cystic fibrosis (19). P. aeruginosa PAO was first described by Haas and Holloway (52). PADG1 and PAO were kindly supplied by D.E. Woods (University of Calgary). Pseudomonas aeruginosa strains M-2 (164) and Ps 388, originally isolated by B. Minshew, Seattle, WA (75), were generously supplied by P.A. Sokol (University of Calgary). PAO503 (met-9011) a methionine-requiring P. aeruginosa mutant originally obtained from B.W. Holloway, Monash University, Australia, was a kind gift from A.J. Godfrey (University of Calgary). Strains representing the 13 Homma serotypes of PA, designated alphabetically from 'A' through 'M', and their corresponding heterologous rabbit antisera were obtained from J.Y. Homma (67,68).

For routine culturing, the bacteria were maintained on tryptic soy agar (Gibco Diagnostics, Madison, WI, 53713, USA). Various growth media were used as indicated below. These included: tryptic soy broth (TSB; Gibco Diagnostics), brain heart infusion (BHI; Difco Laboratories, Detroit, MI, USA) and two specially prepared low-iron media (TSB-DC and S-medium). TSB-DC was prepared as previously described by Ohman et al. (119). Thirty g of TSB powder and 5 g of Chelex-100 (Chelex; Bio-Rad

Laboratories, Richmond, CA, 94804, USA) were stirred in 90 mL of deionized water for 4-6 h at room temperature. This mixture was then dialyzed against 1000 mL of deionized water overnight at 40°C. The dialysate was made up to 960 mL with deionized water and autoclaved. When cool, 20 mL of filter-sterilized solutions of each of 50% glycerol (Sigma, St. Louis, MO, USA) and 2.5 M L-monosodium glutamate (MSG; Sigma) were added to make up a final volume of 1000 mL.

S-medium is a chemically defined medium based on M-9 medium (143). To 940 mL of deionized water were added 1 g of NH_4Cl, 11.4 g of $Na_2HPO_4.7H_20$, 3 g of KH_2PO_4, 5 g of NaCl, 0.2 g $MgSO_4.7H_20$, 1.68 g of EDTA (disodium dihydrate) and 27 g of disodium succinate dihydrate. After autoclaving, volume was brought up to 1000 mL by addition of 20 mL of filter-sterilized 50% glycerol and 40 mL of filter-sterilized 2.5 M MSG.

All chemicals were purchased from Fisher Scientific Co., Fairlawn, NJ 07410, USA or J.T. Baker Chemical Co., Phillipsburg, NJ 08865, USA unless otherwise indicated.

D. Isolation of Outer Membrane Proteins

The method of Hancock and Nikaido (54) was used with minor modifications. Bacteria were grown overnight at 37°C in 2 L volumes of BHI in a rotary shaking incubator (Controlled Environment Incubator Shaker, New

Brunswick Scientific Co., Inc., Edison, NJ, USA). Cells were then collected by centrifugation in 250 mL plastic bottles (Nalgene; Sybaron, Rochester, NY 14002, USA) at 5000 rpm (Beckman J2-21 centrifuge, JA-14 rotor head, Beckman Instruments) for 20 min. Cells were suspended in 30 mM Tris (Sigma) at pH 8.0 and recentrifuged as above. Sedimented cells were then resuspended in 20-40 mL of 20% (w/v) sucrose (Fisher) in 50 mM Tris with 0.2 mM dithiothreitol (DTT; Sigma) pH 7.9 at 40C. Cells were disrupted by two passages through a French Press (French Pressure Cell Press, American Instrument Co. Inc., Silver Spring, MD, 20910, USA) at 15,000 psi followed by sonication for 30-45 sec at power 7 (Sonifier Cell Disruptor 350; Branson Sonic Power Co., Danbury, CO, USA). Cellular debris were removed by centrifugation at 1000 x g for 10 min. Supernatants were layered onto a discontinuous sucrose gradient with 3 mL of each of 70%, 64% and 58% sucrose layered from bottom to top. After centrifugation at 46,000 rpm for 16 h, (Ti50 rotor, L5-50B ultracentrifuge; Beckman), outer membrane fractions I and II were collected by aspiration with a Pasteur pipette, mixed with 6-8 mL of Tris-DTT and centrifuged at 46,000 rpm for 1 h. Pellets were resuspended in 0.1 M phosphate buffer (pH 7.0) with 0.2 mM DTT and stored at -20°C until used.

Protein content of OMP preparations was determined using the method of Lowry (92).

E. Enzyme-Linked Immunosorbent Assays

Detection of antibody to outer membrane proteins: Isolated OMPs were diluted in coating buffer (0.5 M Tris, pH 9.6) to a protein concentration of 5-10 ug/mL, then heated at 100°C for 10 min. Titertek 96-well microtitration plates (Flow Laboratories, Mississauga, Ontario) received 200 ul/well of either the heated or unheated OMP mixture and were incubated at 40°C overnight. Wells were then evacuated by suction and washed four times with 200 uL of PBS-Tween (96) and stored at -70°C until used. Serum or ascites containing anti-OMP antibody was appropriately diluted in PBS-Tween and 200 ul/well were incubated for 1 h at 37°C in the previously prepared plates. The plates were washed as above and 200 ul/well of a 1:1000 dilution of peroxidase-conjugated rabbit anti-mouse IgG (Miles-Yeda Ltd., Kiryat Weizmann, Rehovot, Israel) in PBS-Tween were then applied and incubated for 1 h at 37°C. After again washing as above, 200 ul/well of purified 5-aminosalicylic acid substrate (38) were applied and color allowed to develop for 30 min. Reactions were assessed visually and at 450 nM by an automated ELISA plate reader (MR 580; Dynatech Instruments Inc., Santa Monica, CA).

Whole-cell ELISAs: Whole bacterial cells were grown overnight in TSB or S-medium, sedimented at 10,000 x g (Beckman J2-21 centrifuge) and diluted to 10^7 or 10^8 cells/mL in PBS. Bacteria were killed either by boiling for 10 min or by addition of formalin to 0.3%. After a single wash in PBS, the killed cells were resuspended in coating buffer and applied to microtitre plates as above. All subsequent steps were as above for OMP ELISA's.

Detection of antibody to lipid-A: Purified lipid-A derived from PAO grown in BHI was kindly supplied by G.D. Campbell (University of Calgary). Lipid-A was diluted to a concentration of 5.0 ug/mL in carbonate buffer (pH 9.6) and coated onto microtitre plates as above. All other steps in this ELISA were as above.

Isotyping of monoclonal antibodies: Standard Ouchterlony double-diffusion immunoprecipitation assays (124) failed to yield lines of precipitation with any of our Mab's. Pooled rabbit anti-isotype antibodies specific for IgG1, IgG2a, IgG2b, or IgG3 (Miles-Yeda) were used as the "antibody" in this and all other attempted methods for isotyping. Next, a variety of "dot-blot" methods were tried, which involved initial application of one of OMP preparation, anti-OMP Mab, or anti-isotype antibody, followed by blocking, washing and incubation as in our protocol for Western blotting, using anti-OMP Mab followed by anti-isotype antibody, anti-isotype antibody alone, or

anti-OMP Mab alone respectively. These methods gave positive reactions, but extensive cross-reaction was observed.

Therefore, a rapid, more specific ELISA method was devised. Plates were coated with OMP preparations and incubated with Mab's as above. Before color development, however, 200 uL of a 1:200 dilution of anti-isotype antibody was incubated in appropriate wells for 1 h at 37°C. A goat anti-rabbit IgG peroxidase conjugate (Miles-Yeda) was applied in the final step before color development. This method eliminated cross-reactions with IgG1 and IgG2a, but some cross-reaction was still observed between IgG2b and IgG3.

F. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The method of Laemmli and Favre (85) was followed with minor modifications. A 6% stacking gel was used in all cases, with 11%, 12.5% or 14% running gels. Ultrapure acrylamide (Bio-Rad) and bisacrylamide (International Technologies Inc., New Haven, CO, USA) were used without charcoal filtering to make up stock solutions. Samples were run through both the stacking and the running gels at a constant current of 25-30 mA using a Heathkit Regulated Power Supply, model IP 2717A (Heathkit). Molecular weight standards were run with each gel. These were phosphorylase b, 94,000; bovine serum albumin fraction V,

68,000; pyruvate kinase, 58,000; lactate dehydrogenase, 35,000; soybean trypsin inhibitor, 21,000; and lysozyme, 14,300 (all Sigma). Gel size, number of lanes per gel and running time varied. Generally, 11% gels were run until the dye front reached the bottom of the gel sandwich (11 cm for the more commonly used small gels) while 12.5% and 14% gels were run for 1.4 times the time that it took for the dye front to reach the bottom.

G. Protein Staining

Gels were stained with a solution of 0.1% (v/v) Coomassie Brilliant Blue R (Sigma) in a solution of 50% methanol and 10% acetic acid in ddH20 overnight at room temperature. Two changes of a solution of 5% methanol and 10% acetic acid in ddH20 were used to destain gels. Protein-stained gels were generally photographed wet.

H. Western (Protein) Blotting of Outer Membrane Proteins

The method of Towbin et al. (174) was used with some modifications. Gels were blotted for 30 min at a constant voltage (90V) using a TE 50 Transphor Electrophoresis Unit (Hoefer Scientific Instruments, San Francisco, CA, 94107, USA). Protein transfer to Trans-Blot nitrocellulose paper (Bio-Rad) was confirmed by

Coomassie-staining the gel and amido black staining of the nitrocellulose after blotting.

Immunologic detection of OMPs was accomplished as follows. Nitrocellulose paper was first rinsed twice in Tris-saline (0.9% NaCl and 10 mM Tris, pH 7.4) then blocked for 1 h at 37°C and left overnight at 40°C in Tris-saline with 3% HiPure fish gelatin (Norland Products Inc., North Brunswick, NJ, USA). This and all subsequent steps were followed by 5 washes with Tris-saline. The blocked paper was then incubated for 1 h at 37°C in Tris-saline/3% HiPure with an appropriate dilution of Mab:ascites (1:2000 for 48H.1 and 48H.3, and 1:50 for 48H.9 and 48H.7). After washing as above the paper was again incubated at 37°C for 1 h in Tris-saline/3% HiPure with either a 1:1000 dilution of horse-radish peroxidase-conjugated rabbit anti-mouse IgG (H + L) (Miles-Yeda Ltd., Rehovot, Israel) or horse-radish peroxidase-conjugated Staphylococcal protein-A (Sigma) diluted to a concentration of 2 ug/mL in Tris-saline. The final wash was followed by 10-15 min exposure to either 25 ug/mL o-dianisidine (Sigma) in Tris-saline or 0.5 mg HRP color reagent/mL (Bio-Rad) in 16.7% methanol (v/v) in Tris-saline. Hydrogen peroxide, prediluted approximately 1:6 in ddH20 was added to a final concentration of 0.01% (v/v) to each of the above reagents. Color reactions were stopped by multiple rinses with ddH20.

I. Animal Models: the Mouse Burn-Wound Model

This and all other models employed female Swiss-Webster mice purchased from Simonsen Laboratories (1180C Day Road, Gilroy, CA 95020, USA). The mouse burn-wound model of Stieritz and Holder (169) was slightly modified. Mice weighing 18-20 g were anaesthetized with methoxyflurane (Metofane, MTC Pharmaceuticals, Mississauga, Ontario) and subjected to a 15 sec thermal injury using 0.5 mL of 95% ethanol over a 2.5 x 2.5 cm area on the back. Immediately afterward, 0.5 mL of an appropriate dilution of organisms in phosphate-buffered saline (PBS), pH 7.3 (Oxoid Ltd., Nepean, Ontario) were injected subcutaneously into the burn-wound. Mortality was monitored over 96 h.

J. Animal Models: Cyclophosphamide-Treated Mouse Model

A modification of the model of Cryz et al. (29) was used. Mice weighing 18-22 g were given intra-peritoneal injections of cyclophosphamide (Procytox; Horner Inc., Montreal, Quebec) prepared according to the package instructions, in the following fashion. On day 0 each mouse received 200 ug of cyclophosphamide per g of mouse weight, followed by 150 ug/g on each of days 2 and

4. On day 5, the mice were anaesthetized as above and a 2 cm x 2 cm area shaved on the back (model A-5 Animal Grooming Clipper, Oster, Sunbeam Corp., Milwaukee, WI, 53217, USA). A 0.5 - 1.0 cm incision was made with a scalpel blade in the shaved area through skin only, and 10 uL of appropriate dilutions of organisms in PBS were injected into the wound using a sterile-tipped micro-pipette. Mortality was followed over 96 h.

Neutropenia was confirmed by white blood cell counts on an automated counter with the kind cooperation of the Department of Laboratories, Hematology Division, Foothills Provincial General Hospital. Blood for counts was collected by cardiac puncture or tail-venipuncture in capillary whole blood collectors with K_2EDTA (Microtainer, Becton Dickenson and Co., Rutherford, NJ, 07070,USA).

K. Animal Models: Administration of Mab's

For animal protection studies and assessment of antibody kinetics, crude ascites containing Mab (Mab:ascites) was used. A single dose of 0.2 mL of appropriate dilutions of Mab's in PBS was given by tail-vein injection 10 h before infection in the mouse burn-wound model and at 1-3 h before infection in the cyclophosphamide-treated mouse model. Control animals were injected with identical amounts of either sterile PBS

or similar titres of Mab's against Neisseria meningitidis to rule out immunoadjuvant effect.

Mab kinetics were studied as follows. Mice were injected with either 48H.1 or 48H.3 then sacrificed and serum obtained by exsanguinating cardiac puncture at 15 min, and 1, 4, 12, 24, 36, 48, 60, 72, 84 and 96 h after injection. In some experiments, mice were thermally injured as above, or thermally injured and infected 10 h after injection and sampled as above. Each point represents an average of individual determinations from three or more mice. Mab titres were determined by ELISA as above, with preimmune mouse serum as the negative control.

L. Statistical Methods

Efficacy of passive immunization with Mab's was assessed by comparing the dose of organism at which 50% of infected mice would die (LD_{50}) for control and immunized groups. LD_{50} values were calculated by the method of Reed and Muench (147). Ten-fold dilutions of organisms with five mice per dilution group were used in most experiments.

An alternative experimental format employed a single dose of organisms and compared per cent survival at various times after infection by chi-square analysis (149). Generally 15-20 mice per group were required to

achieve statistical significance. In cases where a difference in absolute survival over a reasonable period of time could not be demonstrated, life-table analysis (22) was used to assess the significance of any perceived delay in time of death.

M. Radioiodination of Cell Surface-Exposed and Isolated Proteins

Labelling of surface-exposed OMPs with ^{125}Iodine (^{125}I; Amersham Corp., Oakville, Ontario) was according to the method of Sullivan and Williams (171) with slight modifications. Briefly, acid-washed glass scintillation vials were coated by evaporation with 0.5 mL of a stock solution of Iodo-Gen (101) (Pierce Chemical Co., Rockford, Illinois). Bacterial suspensions of 5 x 10^9 cells in 2.0 mL of PBS were added to the vials followed by 50 uL of ^{125}I in PBS (2.0 mCi/mL). The labelling reaction was stopped after 1 min by decanting the mixture and diluting it in 8 mL of 10 mM sodium iodide in PBS. Following two more washing/centrifugation steps with NaI/PBS as above, and two washing/centrifugation steps with 1 mL of 0.5 M Tris (pH 6.8) the remaining pellet was resuspended in lysing buffer. A small sample was counted in a gamma counter (1282 Compugamma Universal Gamma Counter, LKB-Wallac, Turku, Finland) and 10^5 cpm of sample in a final volume of 25 uL lysing buffer was boiled for 10 min and

proteins separated by 12.5% SDS-PAGE. After electrophoresis, the gel was dried (2003 Slab Gel Dryer, LKB-Hoefer Scientific Instruments, San Francisco, CA, USA) and overlaid with x-ray film (Kodak X-Omat AR-5 X-Ray Film; Eastman Kodak Co., Rochester, NY, USA) which was developed after 12 h exposure.

Labelling of isolated proteins was accomplished with ^{125}I (0.10 mCi/100 ug protein) using Iodo-Beads (Pierce Chemical Co.) as the catalyst (101). Molecular weight markers and Mab's were labelled in this way.

N. Binding of Monoclonal Antibodies to Bacterial Cells

Direct visualization of Mab binding by electron microscopy was accomplished using the method of Godfrey (49). Bacterial cells were grown overnight in TSB, TSB-DC, or S-medium and collected by centrifugation at 2000 rpm x 10 min. Cells were resuspended to an optical density of 1.0 at 550 nM (approximately 10^9 cells per mL) in PBS, and 0.1 mL was taken and pelleted in a Beckman Microfuge B. The pellet was resuspended in 50 uL of a 1:1000 dilution of Protein A/gold, EM grade, 15 nM size (Janssen Life Sciences/SPI Supplies, Toronto, Ontario) and incubated at 37°C for 1 h with gentle rotation. The suspension was then pelleted and washed twice with 50 uL of 0.05 M HEPES buffer, pH 7.45. One drop of the HEPES

suspension was placed on a sheet of Parafilm "M" (American Can Co., Greenwich, CT, 06830, USA) and a Formvar (J.B. EM, Pointe-Claire, Dorval, PQ) coated grid was floated on the surface for 3 min. The grid was dried then washed in ddH20 by flotation, redried and examined using a Phillips EM 300 electron microscope.

Fields for photography were selected at random although an attempt was made to include all parts of any cells visible within a field. Cells which were not completely visible in the photographs were omitted from analysis. The number of particles per cell was estimated by counting the number of particles present on all cells of the specified type which were completely visible in each photograph then dividing by the number of cells counted.

III. RESULTS

A. Specificity of MAB 48H.3 and 48H.5

Two monoclonal antibodies, Mab 48H.3 and Mab 48H.5 were among those reported as panreactive by Mackie et al. (97), that is they recognized an epitope common to all Pseudomonas aeruginosa but absent from other bacteria. Figure 1 demonstrates that 48H.3 recognizes a band of the same molecular weight in all 13 Homma serotype strains of PA and three of our test organisms, PADG1, PAO1, and PA0503. The apparent molecular weight (M_r) of 38 kd. is consistent with that of the heat or 2-Me-modified form of porin protein F.

Heat and 2-Me-modification of this protein band is demonstrated in Figure 2. Appearance of silver stain suggests that this band represents the major component of the outer membrane preparation and that this is porin protein F. M_r in the unheated form and in the absence of 2-Me is 33-34 kd. Addition of 2-Me raises the M_r to 38 kd. Immunoperoxidase staining with Mab 48H.3 as the specific antibody demonstrates affinity of Mab 48H.3 for all forms of protein F but for no other protein bands. Mab 48H.5 showed a pattern identical to that shown by Mab 48H.3 in Figure 2 (data not shown).

When gels were overloaded with higher concentrations of OMP's, Mab's 48H.3 and 48H.5 recognized higher molecular weight bands in the 45-60 kd range. Some of

these bands were heat and 2-Me-modifiable. The identities of these bands are unknown.

An ELISA system was developed for the detection and quantitation by titre of our monoclonal antibodies. The simplest system, using live organisms as the solid phase antigen layer gave low, uninterpretable optical densities at 450 nM (OD_{450}). Unmodified OMP preparations likewise gave OD_{450}'s too low for confident determinations of end-point titres (Figure 3). Unmodified OMP's gave a maximum OD_{450} of less than 0.2 at Mab reciprocal titres between 1.0 and 1.0 X 10^3. Below 10^3 the OD_{450} gradually declined to 0.014 at reciprocal titres over 10^6. Heating the OMP preparation at 100°C for 5 minutes prior to coating the ELISA plates increased the maximum OD_{450} to 0.475 and extended the maximal plateau to a reciprocal titre of 10^4 (Fig. 3). Heating at 100°C for 2 minutes also increased the maximal OD_{450} to over 0.400 though not to the same levels as heating for the longer period. Both heated preparations showed a gradual decline in OD_{450} to a final minimum value of approximately 0.100 at reciprocal titres above 10^6. Identical results were obtained for Mab 48H.5 (data not shown). Purification of Mab 48H.3 decreased the end-point dilution titre by 2-fold.

Similar results were obtained when whole cells were used as the coating antigen. Heat-killing the cells at 100°C for 5 minutes enchanced the maximal OD_{450} to between

0.400 and 0.550 for all organisms. Cells pre-incubated at 37°C for 5 minutes or killed in 0.3% formalin for 24 h. showed a maximal OD_{450} of approximately 0.100 (data not shown). Our 5 test strains, PADG1, PAO1, PA0503, Ps 338, and M-2 all gave similar OD_{450} profiles and end-point titres to Mab 48H.3 within one two-fold dilution (Table 1).

B. Specificity of Mab 48H.1

Mab 48H.1 was also reported as panreactive by Mackie et al. (97). Figure 4 shows a Coomassie-blue stained SDS-PAGE of heated OMP preparations of Homma serotype stain M (PAM) and PADG1 (lanes 2 and 3 respectively) with accompanying immunoblots demonstrating the specificity of Mab 48H.3 for protein F of both strains (lanes 4 and 5) and the specificity of Mab 48H.1 for a protein band of M_r 18 kd in both strains. No heat or 2-Me-modification of this 18 kd band was demonstrable with 11% page. ELISA's using heated and unheated OMP preparations and whole cells revealed no change in OD_{450} for Mab 48H.1 (data not shown).

Because some duplicate experiments showed reaction of Mab 48H.1 to lower M_r diffusely migrating material, thought to represent lipopolysaccharide due to its non-visualization on Coomassie-stained gels, it was felt that specificity for lipid A should be ruled out. An ELISA

using purified Pseudomonal lipid A and rabbit heterologous anti-lipid A antibodies as the positive control showed no recognition of lipid A by Mab 48H.1.

The M_r of the protein band recognized by Mab 48H.1 is consistent with protein-H complex, which was not separable into proteins H-1 and H-2 under the electrophoresis conditions employed.

C. Specificity of Mab's 48H.4, 48H.6, 48H.9 and 48H.10

Several monoclonal antibodies (48H.4, 48H.6, 48H.9, and 48H.10) identified as Homma serotype strain M (PAM) specific by Mackie et al. (97) were tested for antigenic specificity. Immunoblotting against PAM revealed that all had a specific affinity for a protein band of M_r approximately 30 kd, best detected in overloaded gels. The band is not heat-nor 2-Me-modifiable (Figure 5). There is no apparent cross-reactivity with LPS moieties on immunoblot.

D. Specificity of Mab 43_b

In an ELISA system Mab 43_b recognized only LPS of Homma serotpye B. The end-point dilution titre against purified LPS of PAO503 (a B serotype organism) was 1:10000 (L.E. Bryan and L. Lee, personal communication).

E. Isotyping of Monoclonal Antibodies

Four Mab's were isotyped using our ELISA system. Mab's 48H.1, 48H.3 and 48H.5 were all isotype IgG_{2a}. Mab 48H.9 was isotype IgG_{2b}. Minor cross-reactivity was observed with Mab 48H.9 to IgG3.

F. Passive Immunotherapy in Mice

1. Monoclonal Antibody Kinetics

After a single intravenous injection of a 1:10 dilution of Mab 48H.3 the mean peak ELISA titre obtained in serum sampled at 15 min. was 1:17,067 (Fig. 6). Levels declined rapidly to a mean titre of 1:7467 at 4 h. and declined gradually thereafter to 1:2400 at 96 h. No difference in the persistence of Mab 48H.3 could be detected in mice subjected to thermal injury 10 h. after immunization, but mice subjected to thermal injury and infection with PAO1 at 10 h. showed a subsequent rapid decline in titres to 1:1600 at 48 h.

Mortality in the thermally injured and infected group prevented extension of the data beyond 72 h. These "premature" fatalities were presumably due to infection, and death was usually preceeded by 12 h to 24 h of obvious illness characterized by ruffled fur, tremulousness, lethargy, rapid and deep respiration and somnolence. Mice that appeared ill at the time of sacrifice had lower

titres than healthy-appearing mice. The latter group comprised a greater proportion of those mice surviving beyond 48 h., which may account for the apparent increase in Mab levels at 60 and 72 h.

No signs of systemic toxicity such as sudden death, prostration or ill appearance as above were noted in response to Mab 48H.3 injection in any of the groups of mice.

2. Passive Immunotherapy with Mab 48H.3 in the Mouse Burn-Wound Model

Results of passive protection experiments in the mouse burn-wound model are summarized in Table 2. Control LD_{50} values for PADG1 were determined using placebo injections of either sterile PBS or Mab-ascites against Neisseria meningitidis outer membrane antigens which did not cross-react with Pseudomonas. No difference in LD_{50} was observed between these two groups.

Passive immunization with a single dose of Mab 48H.3 increased the LD_{50} by 100- to 300-fold for mice infected with PADG1 (Homma serotype G). The LD_{50} values for PAO1 and PAO503 (both Homma serotype B) were increased by 150- and 60-fold respectively. Mab 48H.3 was therefore shown to provide protection against strains of Homma serotypes different from the Homma serotype strain M against which this antibody was originally generated. At infecting inocula below 10^4 cfu/mL most mortality occurred between

36 and 48 h after infection. When inocula greater than 10^6 were administered, 40% to 100% of fatalities occurred 8 to 24 h after infection.

Mab 48H.3 failed to protect mice infected with Ps388 (Homma serotype F) or M-2 (Homma serotype B) (Table 2). Infection of control mice with Homma serotype strains A, B, C, D, F, and G resulted in no mortality with doses up to 10^6 cfu/mL (data not shown). No further studies were attempted with these strains.

In some experiments, TSB-DC or BHI were selected for pre-growth because of poor growth of some strains in S-medium. Strain M-2 shows a 54-fold difference in LD_{50} when grown in BHI versus TSB-DC, but no difference in protection afforded by Mab 48H.3.

Protection was found to be dose-dependent (Table 3). Eighty percent of control mice died when challenged with twice the LD_{50} dose of PADG1 (8.5×10^3 cfu/mL) while only 5% of mice pre-treated with a 1:10 dilution of Mab 48H.3 died when similarly infected. Pre-treatment with a 1:100 dilution gave intermediate mortality of 25%. Mean time until death was 39 h for both the control and the 1:100 pre-treated groups, versus 48 h for the 1:10 pre-treated group.

3. Passive Immunotherapy with Mab 48H.3 in the Cyclophosphamide-Treated Mouse Model

Cyclophosphamide-treated mice were reliably rendered leukopenic by day 4 after the initial dose of cyclophosphamide (Figure 7). Slide examination of selected blood smears confirmed that remaining white blood cells (WBC's) were predominantly mononuclear. Leukopenia, as defined by a WBC count of less than 1.0×10^9/L, persisted until day 8 after which counts gradually returned to normal by day 14 (data not shown). Mean weight of mice decreased from 21.8 g to a plateau of 17 g by day 9, then began to increase again by day 13.

The LD_{50} values for cyclophosphamide-treated control mice were 2.8×10^2 cfu/mL for PADG1 and 2.0×10^1 cfu/mL for PAO 503. Pre-treatment with a 1:10 dilution of Mab 48H.3 failed to change the LD_{50} values, but did appear to delay time to death in the pre-treated animals. We therefore attempted to demonstrate protection by life-table analysis (Fig. 8). All animals in both the control and immunized groups eventually died, however Mab 48H.3 significantly prolonged survival ($p<0.05$) by life-table analysis. Similar significant prolongation of survival by Mab 48H.3 was demonstrated for PAO503 (data not shown).

4. Passive Immunotherapy with Mab 48H.1 in the Mouse Burn-Wound Model

Pre-treatment of mice with either a 1:10 dilution or undiluted Mab 48H.1 failed to increase the LD_{50} value for PADG1. No prolongation of time to death was observed in immunized animals (data not shown). Kinetic experiments revealed an antibody titre almost identical to that shown for Mab 48H.3.

5. Passive Immunotherapy with Mab 43_b in the Mouse-Burn Wound Model

A single dose 1:10 dilution Mab 43_b increased the LD_{50} value for PAO503 by a factor of more than 10^5 (Table 4). Serotype specificity was confirmed by failure of Mab 43_b administration to change the LD_{50} for PADG1.

Passive protection by Mab 43_b was found to be dose-dependent (Table 5). Complete protection was achieved with a single dose of a 1:10 or a 1:100 dilution of Mab 43_b. Though statistical significance ($p < 0.05$) was not achieved, with a 1:1000 dilution 38% of infected mice survived as opposed to 8% of control mice. No mice receiving a 1:10,000 dilution of Mab 43_b survived.

6. Combination Therapy with Mab 48H.3 and Mab 43_b in the Mouse Burn-Wound Model

In view of the superior, but serotype-specific protection afforded by Mab 43_b, we investigated the possibility that Mab 48H.3 might offer additive or

synergistic effects when combined with Mab 43_b. The experiment was designed for maximal sensitivity and Table 6 shows the best results. Mab 48H.3 was used at a 1:100 dilution since previous data had shown partial protective effect at this concentration. Mab 43_b was used at 1:10,000 dilution after preliminary experiments showed 75% protection for a 1:5000 dilution.

The range of infecting doses used is approximately 100 times the previously established LD_{50} for PAO503 in this model. Lower inocula used in preliminary experiments yielded insufficient control mortality (less than 50%). As the infecting dose is increased, percent survival in control mice decreased or remained stable (Table 6).

Mab 48H.3 alone had no significant effect on percent survival. Parallel experiments done simultaneously using a 1:10 dilution of Mab 48H.3 showed no significant reduction in mortality at the infecting doses used. Mab 43_b alone provided partial protection when compared to the control groups, but this protection achieved statistical significance only in the fourth experiment ($p < 0.05$).

Combination of the two Mab's provided statistically significant protection (73% survival versus 20% control survival, $p < 0.05$) only in experiment 2, (Table 6). The difference in percent survival between the combined immunotherapy group and Mab 43_b alone in this experiment, as in all others, was not significant.

Other attempts using doses of Mab 43_b from 1:5000 to 1:20,000 alone and in combination with Mab 48H.3 in doses from 1:10 to 1:200 with various infecting doses likewise failed to show any benefit to adding Mab 48H.3 to Mab 43_b.

G. Radioiodination of Cell Surface Proteins

To evaluate the surface accessibility of outer membrane proteins, three strains of Pseudomonas aeruginosa were grown in three different media and surface-labelled with ^{125}Iodine. A protein of M_r 36-38 x 10^3 daltons, compatible with porin protein F was the major surface-labelled protein moiety in all three strains. In S-medium, however, the relative amount of protein F is decreased while the amount of higher M_r protein labelled is increased. This is much more evident for PADG1 and PAM than for PAO1.

Labelling of a second protein of M_r 17-18 x 10^3 daltons, compatible with protein H complex, is appreciable only in PAM grown in TSB or TSB-DC. Indistinct labelling is seen in S-medium and in PADG1 and PAO1 in TSB and TSB-DC.

H. Electron Micrographic Detection of Mab Binding with Colloidal Gold

Figure 10 shows the lack of specific binding of colloidal gold-labelled Mab 48H.3 to unheated cells of

PADG1. The field shown is typical of the entire preparation except for the small area of non-specific adherence of colloidal gold to amorphous material or a part of a cell. Two different cell types and intermediate forms are apparent, the majority of the forms being darker in appearance with a minority of lighter, mottled cells.

Binding of Mab 48H.3 to heat-killed PADG1 is shown in Figure 11. Again two different cell morphologies are present. Lighter appearing cells bind larger numbers of Mab-colloidal gold particles (mean 251 particles/cell; range 217 to 314 for 4 cells) than the darker appearing cells (mean 25 particles/cell; range 3 to 47 for 4 cells). Again, a few intermediate forms are present which bind an intermediate number of particles. Background particle counts were acceptably low (mean 4.5 particles/cell field; range 3 to 9). In estimating the background counts, an "average" size cell was selected for each micrograph. The dimensions of this cell were used as the cell field size and 10 cell fields were randomly selected and averaged.

Binding of Mab 48H.3 to heat-killed PAO503 is shown in Figure 12. No data were collected during scanning, but a typical field was selected for reproduction. Again, a lighter cell is present which binds Mab 48H.3 while darker cells do not. Background is negligible. Unheated PAO503 did not bind Mab 48H.3 in this experiment (data not shown).

Binding of Mab 48H.1 to live PADG1 is shown in Figure 13. The lighter cells bound an average of 160 particles/cell (range 143 to 181) while darker cells bound no more than background, which was less than 2 particles/cell field. Mab 48H.1 was not tested against heat-killed PADG1.

Mab 43_b binds to PAO503 but not to PADG1 (personal communication, A.J. Godfrey). No distinction is made between different cell morphologies in binding of Mab 43_b.

IV. DISCUSSION

The beneficial effects of immunotherapy for P. aeruginosa infection have been recognized for two decades (4,6,39,107) yet no single approach has gained widespread acceptance. Previous efforts have concentrated on sero- or immuno-type specific antigens. The data presented here examine some of the practical aspects regarding the theoretical advantages of using a widely conserved epitope, specifically porin protein F for passive immunotherapy.

The theoretical advantages of monoclonal antibodies rather than polyclonal sera for passive immunotherapy have been dealt with in the introductory chapters. Monoclonal antibodies can also be used to provide unique information regarding the nature and specificity of protective antigen-antibody interactions in studies of pathogenesis. The specificity of Mab 48H.3 for protein F has been established in this study by specific Western blotting techniques. Thus any protective effect from administration of this antibody can be attributed in some way to its binding to this protein.

The complex nature of polyclonal sera can obscure important interactions making precise identification of protective epitopes difficult. Protective capability initially claimed for anti-porin antibodies directed

against Salmonella typhimurium (84,98) was subsequently found to be due to previously undetected IgM antibodies directed against O-antigens present in the heterologous sera (155). Protection against Pseudomonas sepsis in mice by polyclonal rabbit sera raised against partially purified protein F has also been claimed (47). In that study, no attempt was made to rule out the presence of O-specific antibodies and protective capability was assessed only against the strain used to generate the immunizing antigen. Active immunization of mice with the same protein F preparation gave protection against lethal infection by the homologous strain (serotype O-5) as well as another strain of a different serotype (O-10), but the protein F preparation was found to be significantly contaminated with LPS. Equal protection could be achieved against the homologous strain by immunization with an amount of purified LPS equal to that present in the contaminated protein F. Protection in the heterologous strain however, was attributed to antibody response to protein F. No attempt was made to quantitate cross-reaction by anti-LPS antibodies nor antibodies to lipid A, which may have accounted for cross-protection.

Sawada et al. (154) achieved passive protection in a mouse burn-wound model using monoclonal antibodies to unspecified components in OMP preparations of P. aeruginosa. They demonstrated protection against lethal

infection by the homologous strain only, though immunologic recognition of OMP preparations from all Homma serotype strains was demonstrated by ELISA. Hancock et al. (62) have reported a 4- to 8-fold increase in LD_{50} value with the passive administration of Mab's to protein F in two mouse models of infection. This low level of protection was observed for both the homologous serotype and one strain of a different but unspecified serotype. Pennington et al. (130) found that their anti-protein F Mab failed to protect guinea pigs from lethal PA pneumonia.

We have shown that Mab 48H.3 is protective for 3 strains of PA of serotypes other than that from which this Mab was derived. The degree of protection varied from 60- to 340- fold over the control LD_{50} values in the mouse burn-wound model. Protection in this model was dose-dependent. In the cyclophosphamide-treated mouse model a lesser degree of protection was afforded. Protection was demonstrable only by prolongation of survival of neutropenic mice infected with strains of 2 serotypes different from the homologous strain. The difference in protection seen in the two models probably relates to differences in the type and severity of immune defects elicited.

Infliction of a burn-wound over a significant percentage of body surface area not only provides a portal

of entry and a suitable environment for local proliferation of bacteria, but also has deleterious effects on immune function at all levels. Pruitt (141) and Munster (112) have recently reviewed burn-associated immune defects and consequent infectious complications. The frequent occurrence of viral and fungal infections in addition to bacterial processes results from defects in cellular immunity (20) as well as humoral-phagocytic function.

Serum IgG levels are decreased in the first 48 h after burn injury due to mechanical leakage and catabolism (111). The decrease in IgG is unrelated to the burn wound size. In their study, sepsis reduced IgG levels only slightly more than thermal injury alone. Jones et al. (77) have shown that some burn patients consume exogenously administered PA hyperimmune globulin. Data presented above reveal a less rapid decline in levels of exogenously administered Mab 48H.3 in burned versus burned and infected mice but fails to show a difference between levels in unburned and burned mice. This discrepancy may be explained by an idiosyncracy of catabolism of this Mab as compared to IgG in general. Wide variation in the half-life of exogenously administered antibodies depending on the specific antigens to which the antibodies were directed was observed but not explained by Schiff (156). Total immunoglobulin levels were not measured in our mice.

Despite an early post-burn leukocytosis (95) neutrophil function is compromised after burn injury. Neutrophil chemotaxis is depressed in proportion to the area burned (178). Phagocytic activity, oxidative activity as measured by chemiluminescence, and bactericidal activity are also decreased (3). Whether these defects are intrinsic to the neutrophils or due to one or more circulating mediators or both is a matter of debate (95). A serum inhibitory factor is also implicated in post-burn reduction in alternate complement pathway mediated C3 conversion (13).

Cyclophosphamide affects immune response differently depending upon the dosage regimen. Lower doses lead to an increase in B-cell number and spontaneous immunoglobulin secretion, probably due to disruption of $CD4^+$ T-cell-mediated control of activated B-cells (102) and augmented delayed hypersensitivity due to a selective effect on specific T-suppressor cell subsets (23). Higher doses lead to a profound decrease in all T-cell subsets, B-cells and granulocytes, with less effect on erythrocyte and thrombocyte numbers (184). In addition, the lethal effect of cyclophosphamide on the proliferating cells of the gastrointestinal tract and the integument allows easier access by pathogens to the internal milieu.

Although antibody-mediated opsonophagocytosis has long been thought to be the major immune protective

mechanism against PA infection (186), it is clear that the humoral-phagocytic axis is not the sole consideration. Pier and Markham (132) have shown that passive transfer of splenic T-cells from mice immunized with purified PA polysaccharide in the absence of any antibody response can confer protective immunity in recipient mice. Vinblastine enhances this protective effect by selectively depleting a suppressor cell subset whereas cyclophosphamide does not (140). The effect of burn-wound injury on this interaction is unknown.

These murine data correlate with human results from the studies of Ziegler et al. (94,189) with passive immunotherapy using J5 antiserum. They were able to show statistically significant protection for all but neutropenic patients in whom a non-significant trend toward protection was observed.

Antibodies directed against LPS of PA have consistently demonstrated protective capabilities superior to those of antibodies directed against other antigens. Cryz et al. (30) were able to show protection in neutropenic mice with anti-LPS IgG but not with anti-elastase nor anti-exotoxin A IgA. Anti-LPS IgG protection was significantly enhanced by co-administration of gentamicin in a dose which produced no protection when given alone, with anti-elastase, nor with anti-exotoxin A antibodies. Sawada et al. (154) found that on a ug-per-

mouse basis, 4- to 2000- fold less anti-LPS Mab than anti-OMP Mab was required to protect mice from lethal intraperitoneal infection by PA. Several different Mab's of each type were tested and differences in the Ig subtype and epitope specificity may account for the variability in protection. Pennington et al. (130), using a guinea pig model of fatal PA pneumonia, showed significant protection with passive administration of murine Mab against LPS or human polyclonal IgG enriched for antibody to LPS of PA, but not with Mab against porin protein F.

In comparing Mab 48H.3 to the anti-LPS Mab 43_b I obtained similar results. A 1:10 dilution of Mab 48H.3 gave a 60- fold increase in the LD_{50} value for thermally injured mice challenged with PA0503 whereas the same dilution of Mab 43_b gave over 10^5-fold increase in the LD_{50} value. There was a 100-fold difference in the highest doubling dilution of Mab providing no significant protection against PA0503 for Mab 48H.3 (1:100 dilution) versus Mab 43_b (1:10,000 dilution). The end-point dilution titre of Mab 48H.3 in our OMP ELISA system was $1:10^6$ whereas the titre for 43_b was $1:10^3$ by ELISA using purified homologous LPS as the solid phase. Although the two Mab's were not compared on a ug to ug of antibody protein basis, it is clear that between 100- and 10,000-fold less Mab 43_b than Mab 43H.3 is required to achieve protection against PA0503. The efficiacy of anti-LPS

antibodies may be due to their dual modes of action as opsonic antibodies aiding in the destruction of intact bacterial cells and as detoxifying agents, binding circulating LPS to facilitate its clearance from circulation.

Given the clear protective superiority of anti-LPS antibodies, two possible roles for Mab 48H.3 were investigated. The first was to determine whether co-administration of 48H.3 could add to the protection afforded by 43_b. Combining various concentrations of 43_b and 48H.3, neither additive nor synergistic protective capability could be demonstrated against PA0503 in the mouse burn-wound model.

The second possible function of 48H.3 relates to the theoretical advantages of having a single antibody that recognizes a protective epitope common to all strains of PA. By providing protection against PA strains which are not recognized by a therapeutic mixture of anti-LPS antibodies due either to a deficiency of LPS O-side chains (61) or exclusion from the mixture of statistically uncommon serotypes of PA, Mab 48H.3 might significantly broaden the spectrum of coverage for LPS-based passive immunity. It was found, however, that protection by 48H.3 did not extend to all strains of PA tested despite panreactivity by ELISA and Western blot. Mice challenged

with two of the five strains tested experienced no benefit from receiving 48H.3.

The reasons for variability in protection remain unknown. The degree of protection did not correlate with the serotype of the infecting PA. Three different strains of B-serotype PA were tested and degrees of protection varying from none to 160-fold were observed. Degree of protection did not correlate with virulence. Although decreasing virulence as measured by LD_{50} value correlated with increasing protection among the three strains for which protection could be demonstrated, this relationship did not hold for the other two strains. Despite higher LD_{50} values in unprotected mice infected with these two strains, no protection was demonstrable with administration of Mab 48H.3. Increasing the virulence of PA strain M-2 by pre-growing it in different media failed to change its susceptibility to Mab 48H.3. Protection did not correlate with the medium used for pregrowth of infecting organisms.

Different media were used for some strains due to their nutritional requirements. Protection by 48H.3 was obtained against organisms grown in both S-medium and TSB-DC, but some organisms grown in TSB-DC were apparently not susceptible to 48H.3. This finding was unlikely to be due to differences in surface accessibility of protein F in organisms grown in different media since surface

radioiodination of three different strains of PA grown in TSB-DC, TSB, and S-medium confirmed that although the relative amount differed slightly, protein F was present and remained the major surface-accessible OMP in all cases. The problem of differential expression of protein F in in vivo growth conditions was not addressed.

Next, an attempt was made to directly visualize binding of Mab 48H.3 to bacterial cells in order to correlate the degree of protection with the amount of binding. It was found that live PADG1 grown in S-medium and PA0503 grown in TSB-DC did not bind colloidal-gold labelled 48H.3. The same cells, when heat-killed, showed avid binding of 48H.3 but only to a certain sub-population of cells distinguishable in electron photomicrographs by their lighter, often mottled appearance. Heat-killed PA0503 did bind less antibody than PADG1, consistent with the lesser degree of protection seen with PA0503. By contrast, Mab 48H.1. which provided no protection against PA infection actively bound to PADG1, either live or heat-killed. Mutharia and Hancock (114) were able to show by indirect immunofluorescent techniques that their anti-protein F Mab's bound to epitopes located on the surface of intact PA. Their Mab directed against protein H2 did not bind to the cell surface.

The lack of correlation between binding characteristics of Mab 48H.3 and protection observed

raises several questions regarding the mechanism of action of this antibody. Preferential or exclusive binding to heat-treated cells or OMP preparations sets Mab 48H.3 apart from previously reported anti-protein F Mab's, the affinity of which is either unaffected or decreased by heating or treatment with 2-Me (115). It would seem reasonable that Mab's which recognize intact cells should provide better passive immunity than a Mab with preferential affinity for an artificially altered protein. This may be the case despite the apparently greater changes in LD_{50} value associated with therapy with Mab 48H.3 in direct comparison with the results of Hancock et al. (62). One of the two strains for which this group reported an 8-fold increase in LD_{50} with passive immunotherapy is M-2, a strain against which Mab 48H.3 offers no protection. This degree of protection, however, would be difficult to accurately detect in our LD_{50} assay system since ten-fold dilutions of organisms are used. Given biologic variation and frequent problems with reproducibility with the mouse burn-wound model in our hands, less than a 10-fold increase in LD_{50} value is probably not significant. Direct comparison with other groups' Mab's are not appropriate at this time given the variability in protection with different strains of PA in different animal models of infection.

The explanation of why Mab 48H.3 works at all may depend on assessment of the OMP's of PA *in vivo* rather than *in vitro*. Surface accessibility of specific epitopes of protein F *in vivo* may correlate with changes produced by heat-treatment and/or 2-Me *in vitro* in one of the following ways:

Heating or 2-Me may cause a conformational change in protein F which exposes an epitope ordinarily hidden or partially hidden. such a structural change *in vivo* might be associated with opening and closing of pores with Mab 48H.3 recognizing the epitope exposed in the more rare open form (118). One would have to postulate host nutritional or defense factors causing an increase in the percentage of open pores *in vivo* to account for protection in the face of poor or non-existent *in vitro* binding. To study this possibility, PA could be isolated in amounts large enough for Mab-labelling for electron microscopy and SDS-PAGE from animal models of infection (50) or from clinical samples (16). Peptide mapping of *in vivo* and *in vitro* isolates to identify the epitope to which Mab 48H.3 binds and to infer its location relative to the orientation of the outer membrane would be valuable (115).

Exposure of the crucial epitope might also result from removal by 2-Me, heating or deficiency due to growth *in vivo* of molecules normally associated with protein F *in vitro* which hinder the binding of 48H.3. Other outer

membrane proteins or LPS moieties may function in this way. LPS- and OMP-deficient mutants grown *in vivo* and *in vitro* could be assessed for binding by 48H.3 to test this hypothesis.

The finding that only a distinct sub-population of cells binds 48H.3, even when heated, may be solely an *in vitro* phenomenon. In any case, the availability of protein F on the cell surface may vary according to the stage of the cell cycle, increasing at times when cells are more actively gathering nutrients. There may also be phase variation in either surface exposure or conformation. To investigate these possibilities, cell cultures could be synchronized and sequential samples taken and labelled with colloidal gold-tagged 48H.3 for electron photomicrography.

It seems unlikely that more rigorous treatment of cells may lead to exposure of the crucial epitope by cell killing and lysis with release of protein F. The lighter, better binding cells might then represent PA 'ghosts' surrounded by extruded protein F. This would not explain the enhanced affinity of 48H.3 for heated OMP preparations or the failure of killing by formalin to enhance binding by Mab 48H.3.

Finally, binding of colloidal gold to 48H.3 might hamper its subsequent binding to cells under certain conditions *in vitro* so that the electron micrographs are

not reflective of the situation *in vivo*. Mutharia and Hancock (114) had success with an indirect immunofluorescent assay using fluorescein isothiocyanate-conjugated antibody in demonstrating binding of their Mab's to surface-located protein F.

The mechanism of action of anti-protein F antibodies is presumed to relate to binding to protein F in intact cells and consequent opsonic enhancement of phagocytosis or interference with porin function. Hancock *et al*. (62) correlated a 2- to 7-fold increase in phagocytic uptake *in vitro* by human neutrophils with a 4- to 8-fold increase in LD_{50} value due to their anti-protein F Mab. Despite repeated attempts, we were unable to demonstrate any increase in phagocytic uptake for Mab 48H.3-treated PA. In these experiments 48H.3 also failed to kill PA cells *in vitro* (data not shown). This is consistent with the results of the other *in vitro* assays but again is at odds with the protective efficacy demonstrated by 48H.3. Again this might be explained by cell surface difference *in vivo*.

An alternative mode of action for Mab 48H.3 might involve binding to free protein F *in vivo*, but in the absence of any demonstrated virulence associated with protein F this seems unlikely.

Little has been learned about the other available theoretically less attractive Mab's apart from preliminary

characterization and determination of their specificities. Mab 48H.5 was felt to be identical to Mab 48H.3 and no individual studies were attempted. Mab 48H.1 recognized an epitope of the H-protein complex. It appeared not to be protective, but unlike other Mab's specific for protein H-2 (114) it did bind to cell surface-exposed epitopes. Mab's 48H.4, 48H.6, 48H.9, and 48H.10 recognized a band at 30,000 Mr, between OMP's F and G. This may be an unconserved epitope on a minor OMP or a non-proteinaceous antigen. In either case, while it may prove to be protective, as other minor OMP antigens have (80), it is serotype-specific and probably of minor interest.

V. CONCLUSIONS AND SUMMARY

This thesis examines the protective capabilities and *in vitro* correlates of Mab 48H.3, a monoclonal antibody directed against a well-conserved epitope of the major OMP of PA, porin protein F. A comparison of passive immunotherapy using this panreactive Mab, serotype-specific antibodies, and a combination of panreactive and serotype-specific Mab's is made. The results presented here allow some specific conclusions to be drawn, and in confirming the results of other workers suggests some general conclusions.

1) Passive administration of Mab 48H.3 can protect mice against lethal infection by *Pseudomonas aeruginosa*.

2) This passive protection is dose-dependent.

3) Protection is more convincingly demonstrated in the mouse burn-wound model than in the cyclophosphamide-treated mouse model, probably due to the greater severity of the immune deficit in the latter model.

4) Protection does not extend to all strains of PA despite panreactivity of Mab 48H.3 *in vitro*.

5) A panreactive antibody directed against protein H complex, Mab 48H.1 does not demonstrate protective capability in our system.

6) Mab 43_b offers better dose-dependent passive protection than Mab 48H.3, confirming the superiority of

antibodies directed against serotype-specific LPS antigens.

7) Co-administration of Mab 48H.3 does not improve the serotype-specific protection provided by Mab 43_b.

8) The mechanism by which Mab 48H.3 provides protection is unclear and further investigation of OMP expression and interaction with Mab 48H.3 in vivo is required.

Table 1

Affinity of Mab 48H.3 for Heated Whole Cells of P. aeruginosa

Experimental Strains	Reciprocal of End-Point Dilutions of MicroELISA Titres
PADG1	6.4×10^5
PAO1	3.2×10^5
PAO503	3.2×10^5
Ps 388	3.2×10^5
M-2	3.2×10^5

Affinity of Mab 48H.3 for Heated Whole Cells of Pseudomonas aeruginosa: ELISA plates were coated with 10^9 cells/mL heat-killed whole cells of 5 P. aeruginosa test strains. Two-fold dilutions of Mab 48H.3 were applied followed by ELISA development as per protocol, and end-point dilution titres were obtained.

Table 2

Effect of Mab 48H.3 on LD_{50} for P. aeruginosa Strains

Organism	Homma Serotype	Medium	LD_{50} (cfu/mL) Control	MCA 48H.3
PADG1	G	S-medium	3.8×10^3	3.8×10^5
PADG1	G	S-medium	3.5×10^3	1.2×10^6
PAO1	B	S-medium	7.2×10^1	1.1×10^4
PAO503	B	TSB-DC	1.6×10^1	9.7×10^2
Ps 388	F	TSB-DC	1.8×10^5	1.4×10^5
M-2	B	TSB-DC	1.5×10^4	1.5×10^4
M-2	B	BHI	2.8×10^2	2.1×10^2

Effect of Mab 48H.3 on LD_{50} for P. aeruginosa Strains: Mice were pre-treated as indicated then subjected to thermal injury and infection according to protocol. In each control and Mab-treated experiment 5 mice received each 10-fold dilution (from 10^0 to 10^6 or 10^7). The LD_{50} value was calculated by the method of Reed and Muench (147).

Table 3

Effect of Dose of Mab 48H.3 on Survival

Dilution of Mab 48H.3	Survival No. Alive/No. Animals (%)	p value
Control	4/20 (20%)	-
1:10	19/20 (95%)	< 0.001
1:100	15/20 (75%)	< 0.01

Effect of Mab 48H.3 Dose on Survival: The mouse burn-wound model was used. Each mouse received two times the LD_{50} dose of PADG1.

Table 4

Effect of Mab 43_b on LD_{50} Dose for P. aeruginosa Strains

Organism	Homma Serotype	LD_{50} (cfu/mL) Control	Mab 43_b
PAO503	B	2×10^1	$> 10^6$
PADG1	G	6.2×10^3	5.4×10^3

Effect of Mab 43_b on LD_{50} Dose for P. aeruginosa Strains: Each group of mice received either a placebo injection of PBS or Mab 43_b at a 1:10 dilution. The mouse burn-wound model was used and LD_{50} values calculated at 48 h. Five mice received each ten-fold dilution of the infecting organisms.

Table 5

Effect of Mab 43_b Dose on Survival of Thermally Injured and Infected Mice.

Infecting Organism	Dilution of Mab 43_b	Survival No. Alive/No. Animals (%)	p value
PAO503	control	1/13 (8%)	< 0.001
	1:10	15/15 (100%)	< 0.001
	1:100	13/13 (100%)	< 0.001
	1:1000	5/13 (38%)	n.s.
	1:10000	0/14 (0%)	n.s.

Effect of Mab 43_b Dose on Survival of Thermally Injured and Infected Mice: The mouse burn-wound model was used. Ten-fold dilutions of Mab 43_b were administered 10 h prior to thermal injury and infection. Each mouse received 3.1×10^3 cfu/ml of PAO503. The difference in survival was calculated at 96 h.

Table 6

Immunotherapy with Monoclonal Antibodies 48H.3 and 43_b Alone and in Combination

Experiment	Infecting Dose of PAO503 (cfu/ml)	Survival: No. Alive/No. Animals (%)			
		Control	48H.3	43b	Combination
1	1.5×10^3	11/20(55%)	6/20(30%)	9/20(45%)	10/20(50%)
2	2.7×10^3	3/15(20%)	2/15(13%)	7/15(48%)	11/15(73%)
3	3.3×10^3	4/15(27%)	4/14(29%)	6/15(40%)	6/14(44%)
4	6.5×10^3	2/15(13%)	4/15(27%)	8/15(54%)	6/15(40%)
5	1.1×10^4	4/20(20%)	3/20(15%)	9/20(45%)	5/20(25%)

Table 6. Immunotherapy with Monoclonal Antibodies 48H.3 and 43_b Alone and in Combination: The mouse burn-wound model was used. Mice were given placebo injections of PBS or a 1:100 dilution of Mab 48H.3, a 1:10,000 dilution of Mab 43_b or both Mab's together. Mortality was complete at assessment at 72 h.

Figure 1

Panreactivity of Mab 48H.3 for all Homma Serotype Strains of Pseudomonas aeruginosa and Three Test Strains

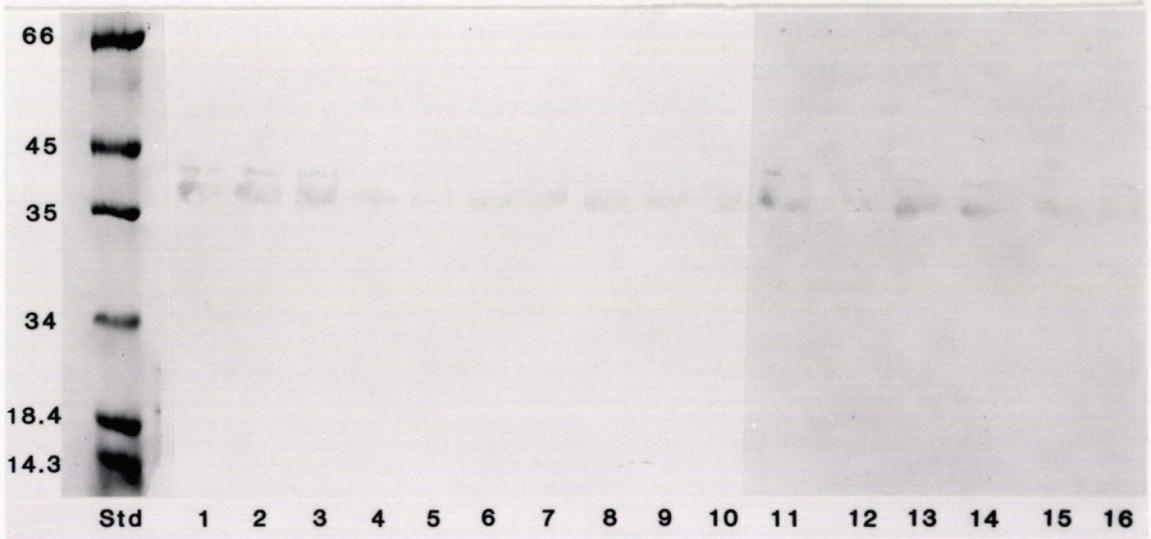

Figure 1. Panreactivity of Mab 48H.3 for all Homma Serotype Strains of Pseudomonas Aeruginosa and Three Test Strains: Isolated heat and 2-Me-treated OMP's from each of the 13 Homma serotype strains A to M (lanes 1-13) and PADG1, PAO1, and PAO503 (lanes 14-16) were applied to an 11% polyacrylamide gel (50 ug protein per lane) and electrophoresed. Proteins were then transferred to nitrocellulose paper and immunoperoxidase stained using Mab 48H.3 as the specific antibody. Protein molecular weight standards are shown at the left.

Figure 2

Effect of Heat and 2-Me-modification of Protein F on Affinity for Mab 48H.3

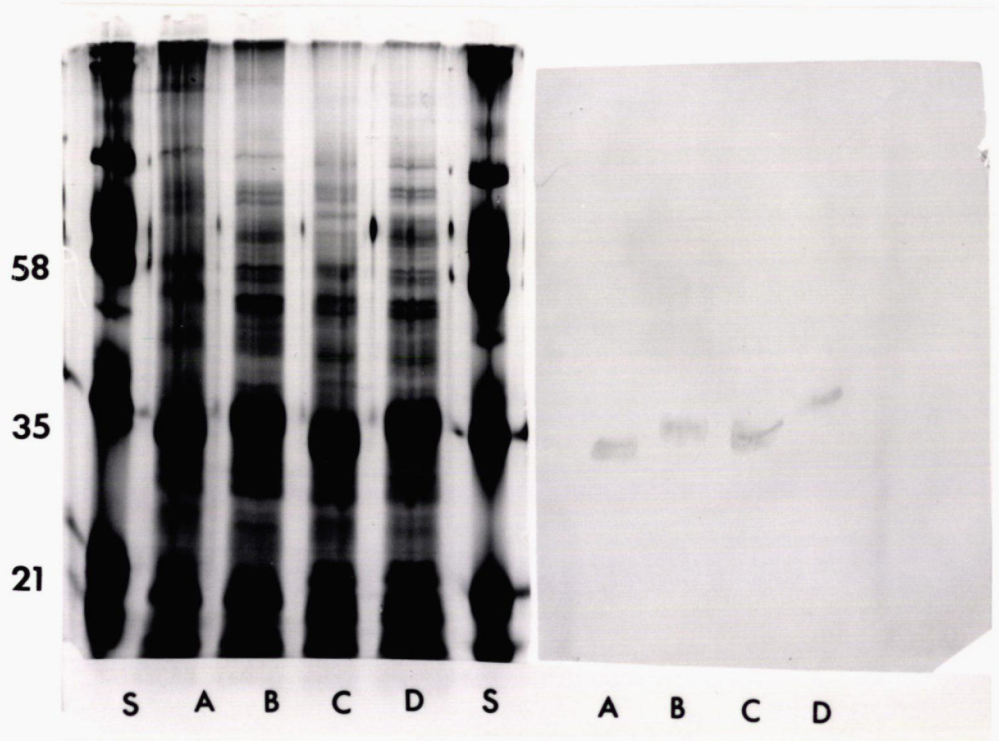

Figure 2. Effect of Heat and 2-Me-modification of Protein F on Affinity for Mab 48H.3: A silver-stained 11% PAGE of isolated OMP's (30 ug protein per well) of Homma serotype strain M and its corresponding immunoblot against Mab 48H.3 are shown. Molecular weight standards are shown in lanes S. Before PAGE, OMP's were incubated as follows: lane A- 37°C with no 2-Me; lane B- 37°C with 2-Me; lane C- 100°C with no 2-Me; lane D- 100°C with 2-Me.

Figure 3

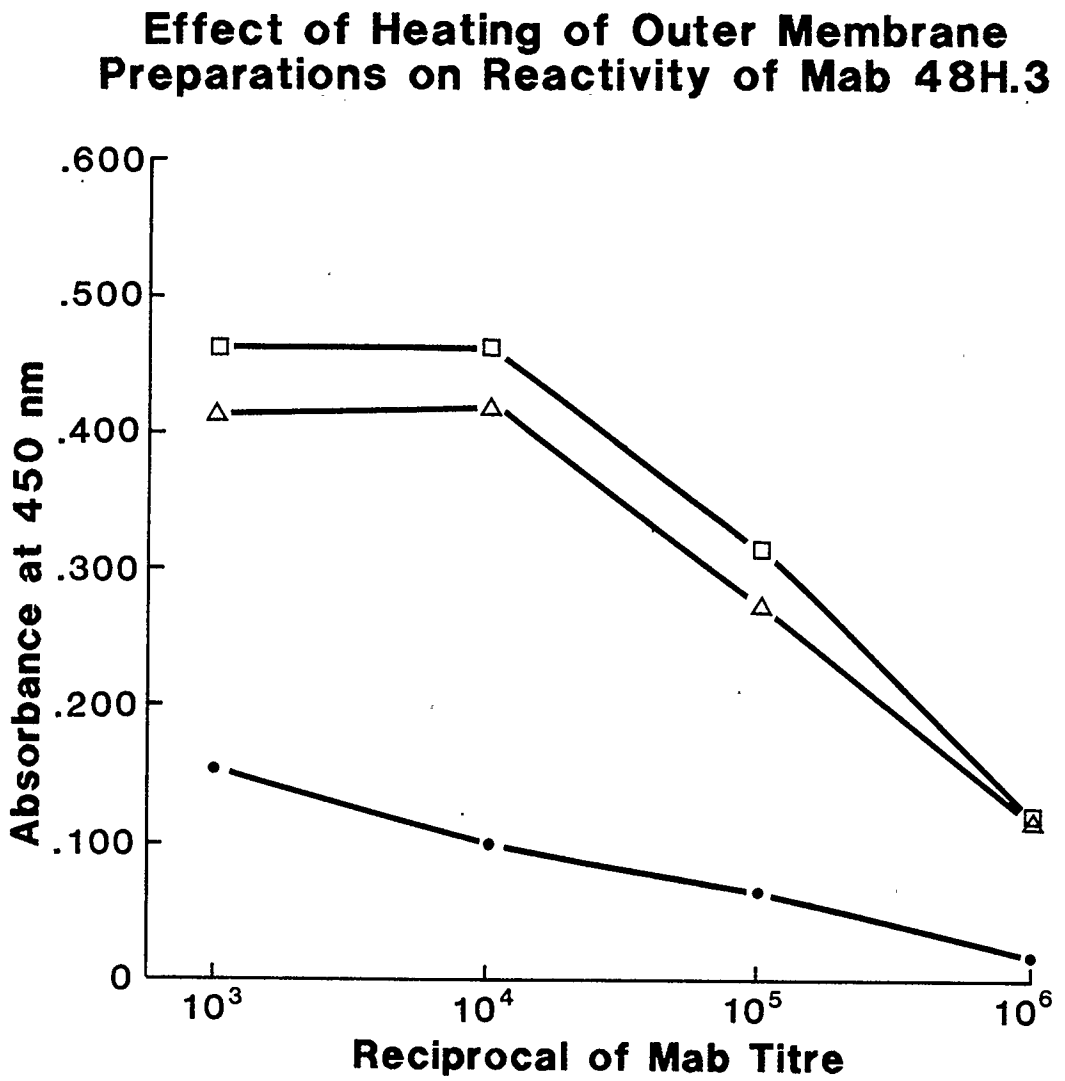
Effect of Heating of Outer Membrane Preparations on Reactivity of Mab 48H.3
Absorbance at 450 nm
.600
.500
.400
.300
.200
.100
0
10³
10⁴
10⁵
10⁶
Reciprocal of Mab Titre

Figure 3. Effect of Heating of Outer Membrane Preparations on Reactivity of Mab 48H.3: Ten-fold dilutions of Mab 48H.3 were applied to ELISA plates precoated with 200 uL of 5 ug/mL OMP protein from Homma serotype strain M which had been pre-incubated at 37°C for 10 minutes (●), 100°C for 2 minutes (Δ), or 100°C for 5 minutes (□).

Figure 4

Specificity of Mab 48H.3 and Mab 48H.1 in Two Strains of Pseudomonas aeruginosa

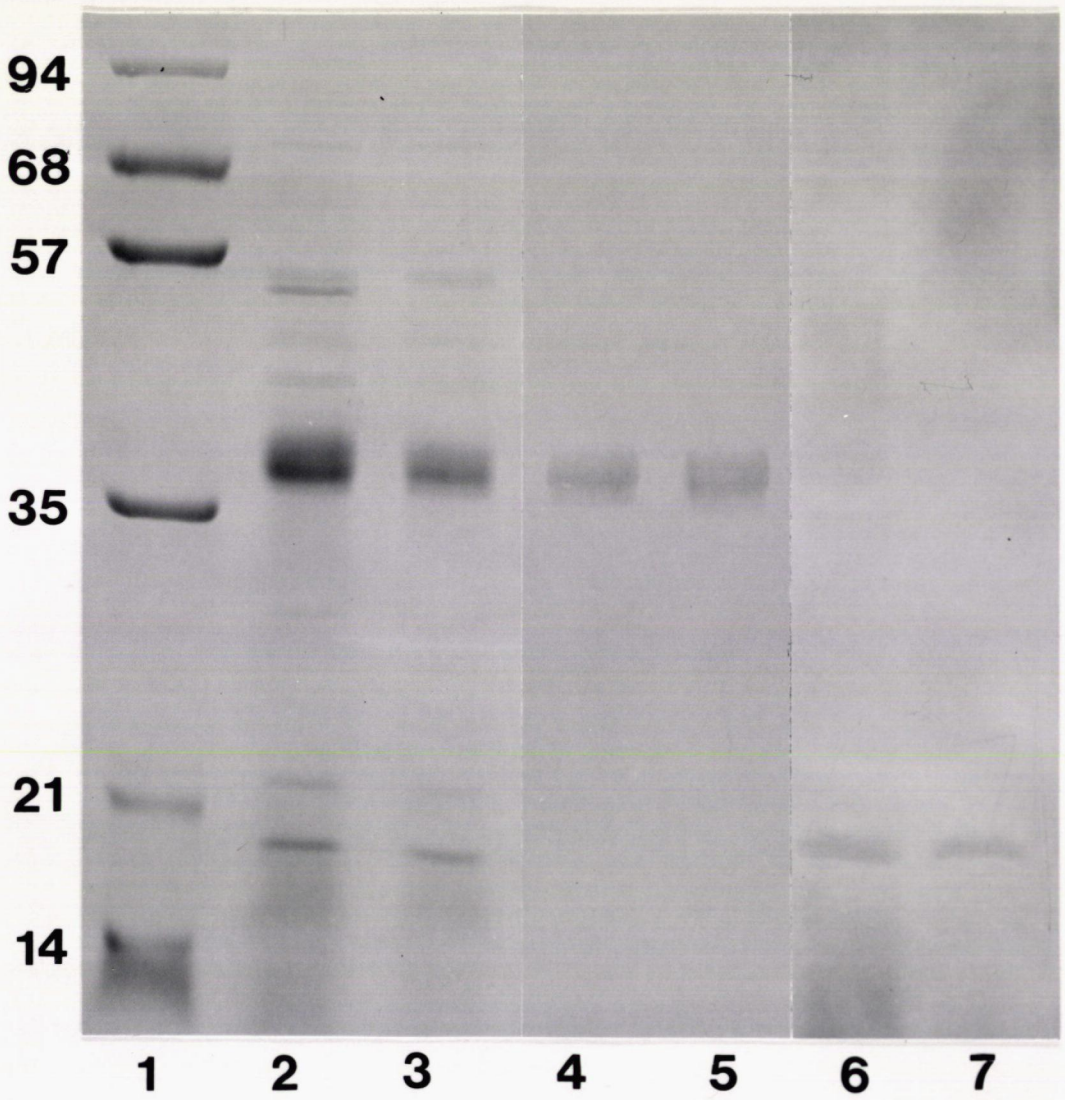

Figure 4. Specificity of Mab 48H.3 and Mab 48H.1 in Two Strains of Pseudomonas aeruginosa: Isolated OMP preparations from Homma serotype strain M and PADG1 were subjected to 11% PAGE. Strain M is shown in lanes 2, 4, and 6 and PADG1 is lanes 3, 5, and 7. Coommassie blue stained gel with molecular weight standards is shown in lanes 1, 2, and 3. Immunoblots using Mab 48H.3 (lanes 4 and 5) and Mab 48H.1 (lanes 6 and 7) are shown.

Figure 5

Specificity of Homma Serotype Strain M-Specific Monoclonal Antibodies

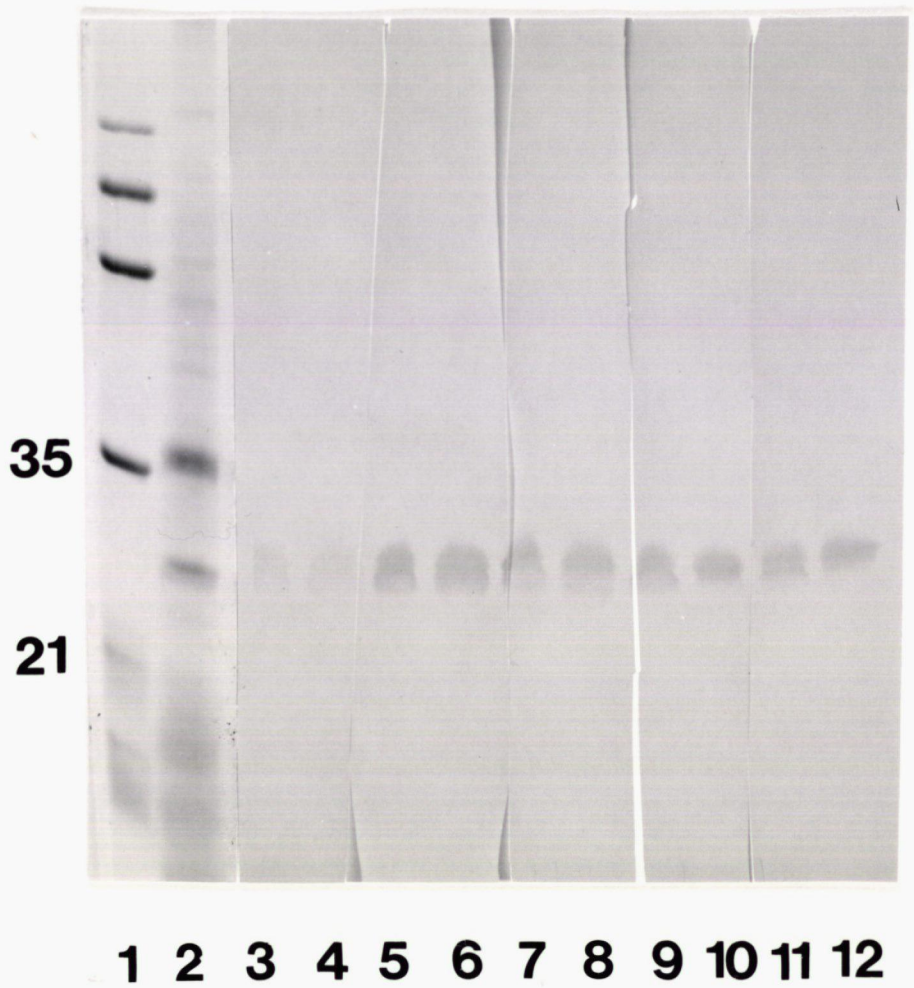

Figure 5. Specificity of Homma Serotype Strain M-Specific Monoclonal Antibodies: OMP preparations from Homma strain M were subjected to 11% PAGE then transferred to nitrocellulose for immunoblotting with Mab 48H.4 (lanes 3 and 4), Mab 48H.6 (lanes 5 and 6), Mab 48H.7 (lanes 7 and 8), Mab 48H.9 (lanes 9 and 10) and Mab 48H.10 (lanes 11 and 12). OMP's in lanes 3, 5, 7, 9, and 11 were pre-incubated at 37°C with no 2-Me. OMP's in lanes 2, 4, 6, 8, 10, and 12 were pre-incubated at 100°C with 2-Me. Coommassie blue stained gels of heated OMP's and molecular standards are in lanes 2 and 1 respectively.

Figure 6

Effect of Thermal Injury or Thermal Injury & Infection on Mab Titres in Mice

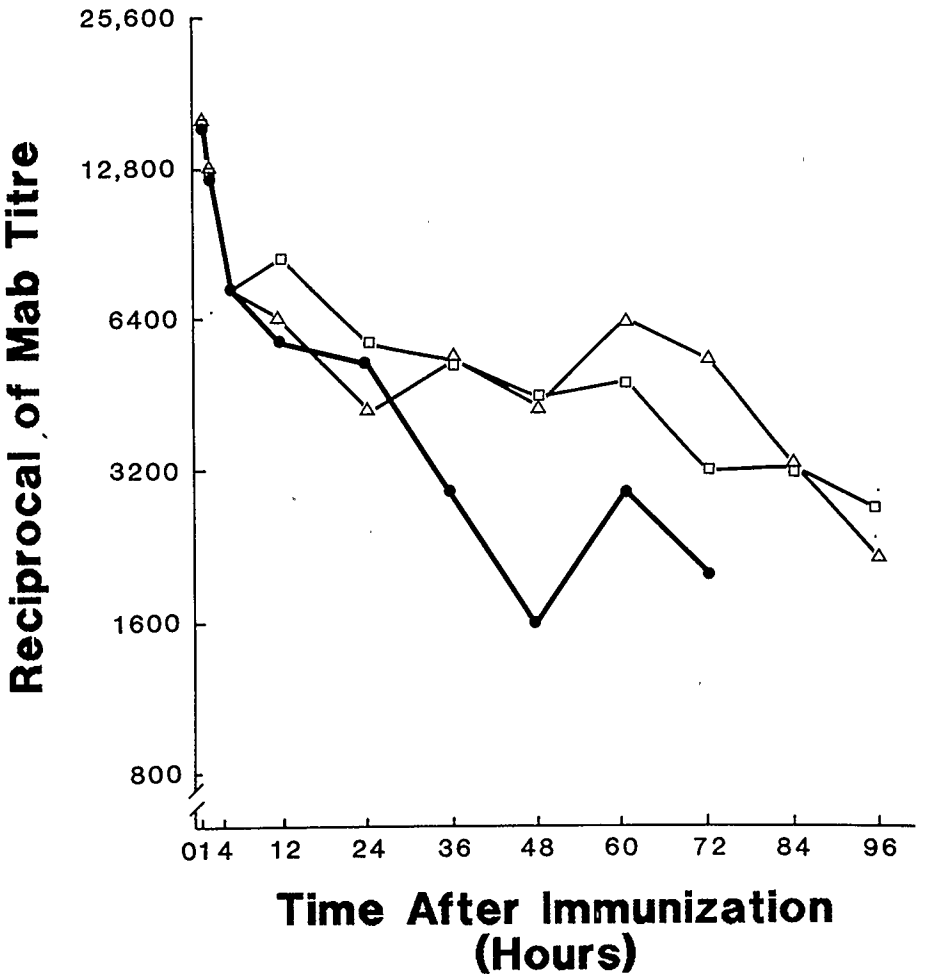

Figure 6. Effect of Thermal Injury or Thermal Injury and Infection on Mab Titers: All mice received 0.1 ml of a 1:10 dilution of Mab 48H.3 at time = 0 h. Control mice (□) received no further treatment. A second group of mice (Δ) was thermally injured and a third group thermally injured and infected (●) according to protocol at time = 10 h. Each point represents the mean titer of Mab 48H.3 from three mice. Mortality in the thermally injured and infected group prevented extension of data to 96 h. There was no mortality due to Mab injection or thermal injury in the untreated or the thermally injured groups.

Figure 7

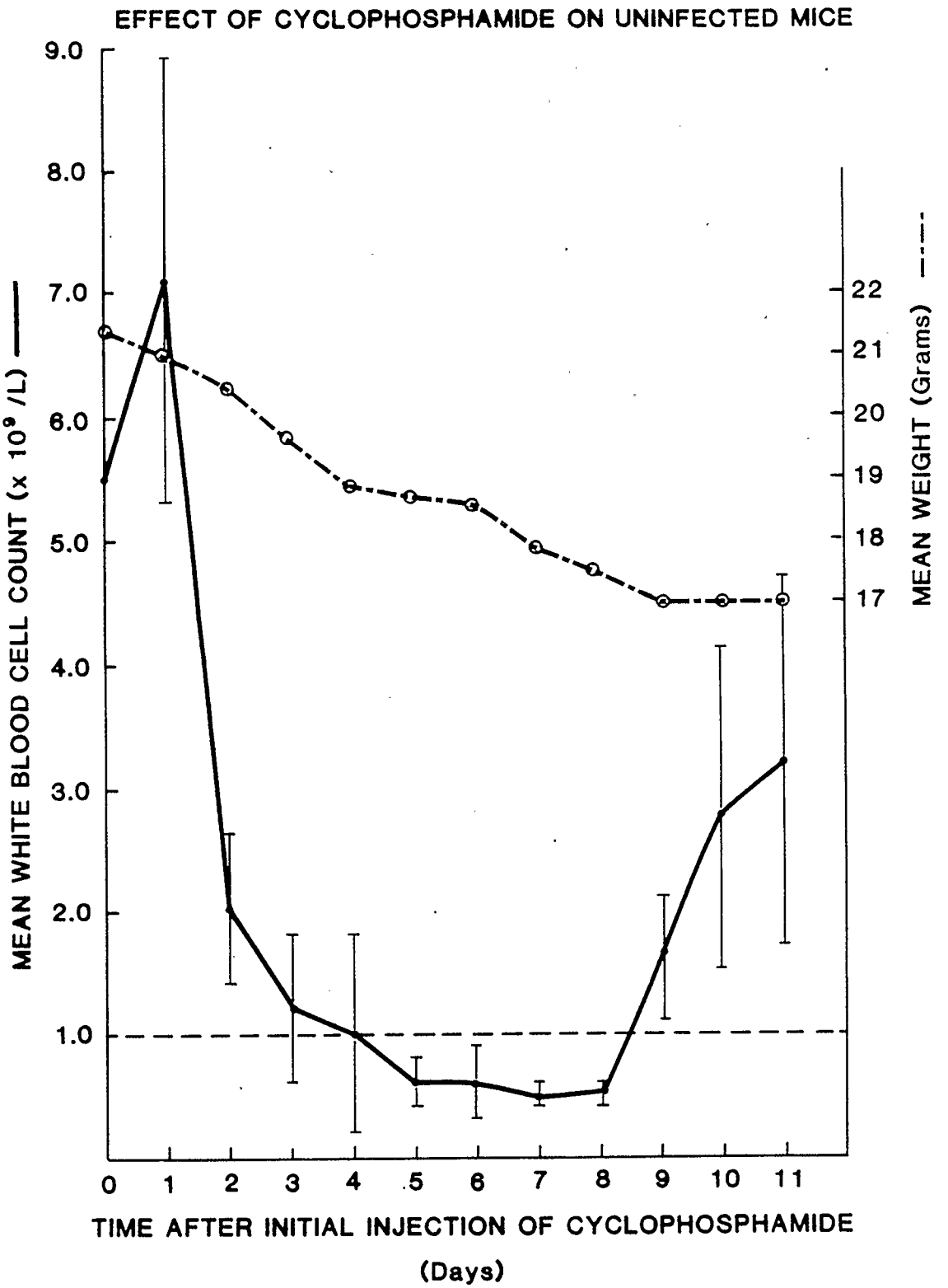
EFFECT OF CYCLOPHOSPHAMIDE ON UNINFECTED MICE
MEAN WHITE BLOOD CELL COUNT (x 10⁹ /L)
MEAN WEIGHT (Grams)
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
22
21
20
19
18
17
0 1 2 3 4 5 6 7 8 9 10 11
TIME AFTER INITIAL INJECTION OF CYCLOPHOSPHAMIDE
(Days)

Figure 7. Effect of Cyclophosphamide on Uninfected Mice: Cyclophosphamide was administered on days 0, 2, and 4. The dashed line shows the mean weight of all surviving mice for each day. After being weighed, 3 of the mice were chosen daily for sacrifice and white blood cell counts, expressed as the mean white blood cell count with standard deviation (solid line).

Figure 8

EFFECT OF PRE-TREATMENT WITH MAB 48H.3
ON SURVIVAL OF CYCLOPHOSPHAMIDE-TREATED MICE
INFECTED WITH PADG1

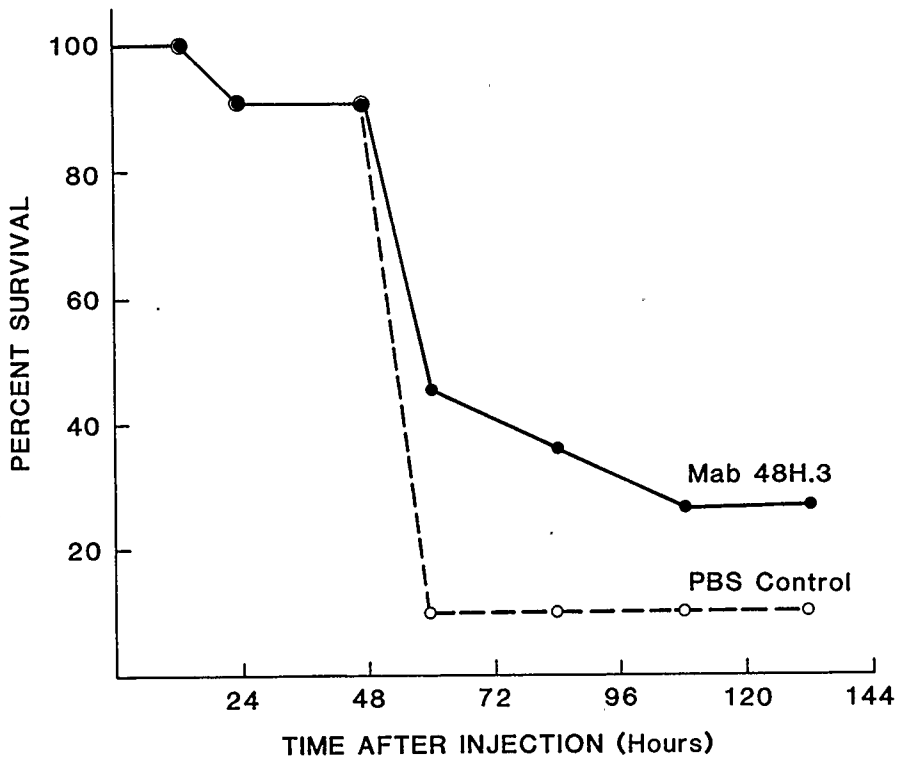

Figure 8. Effect of Pre-treatment with Mab 48H.3 on Survival of Cyclophosphamide-Treated Mice Infected with PADG1: Percent survival of PBS-injected mice (o) is compared to that of Mab 48H.3-injected mice (●). Each group began with 12 mice. Each mouse received 1 x 10^3 cfu/ml PADG1 and mortality was monitored for 30 days. The single mouse from each group which died at 28-30 days was omitted from statistical analysis. Mean time of death was 56.7 h in the PBS-injected group and 75.6 h in the Mab 48H.3 group. Survival was significantly different at 60 h ($p < 0.05$ by life table analysis)

Figure 9

Radioiodination of Cell Surface Proteins
of Pseudomonas Strains

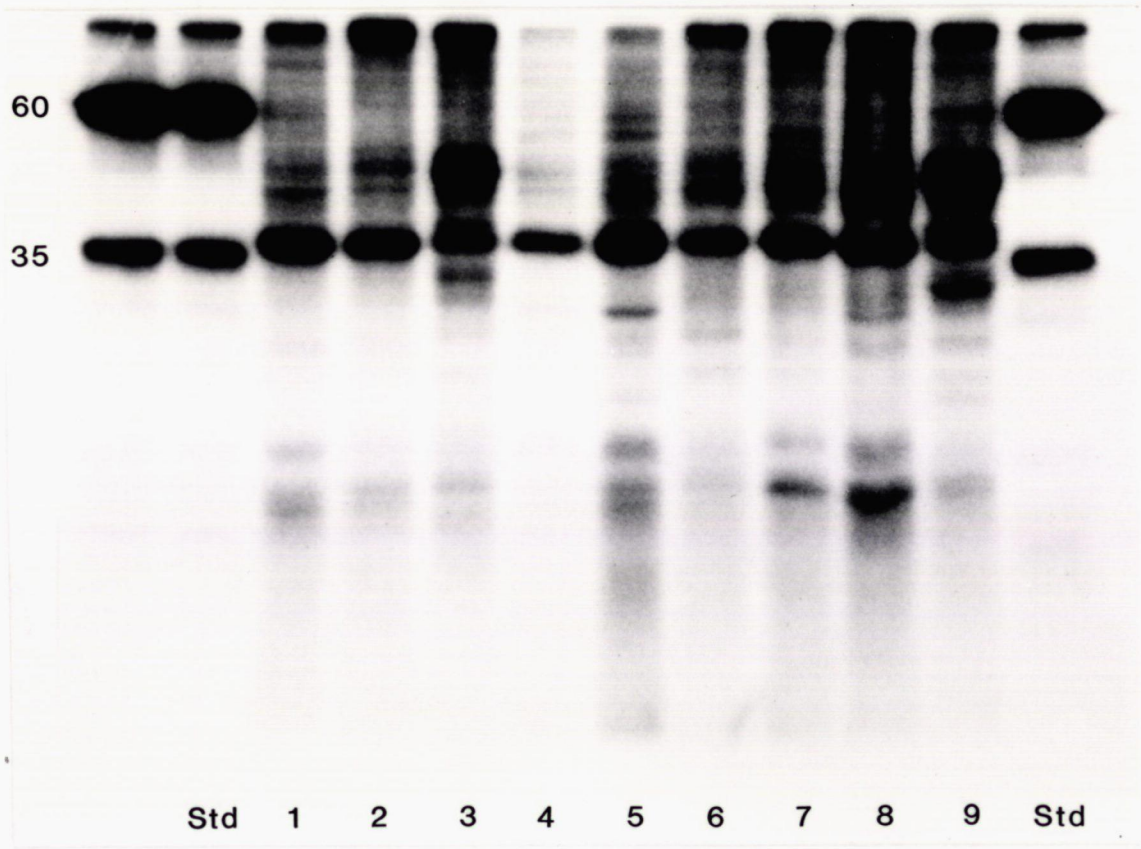

Figure 9. Radioiodination of Cell Surface Proteins of Pseudomonas Strains: Whole cells of Pseudomonas aeruginosa strains were radioiodinated, preincubated at 100°C in lysing buffer containing 2-Me and subjected to 14% SDS-PAGE followed by autoradiography. Shown are PADG1 grown in TSB (lane 1), TSB-DC (lane 2), and S-medium (lane 3), PAO1 grown in TSB (lane 4), TSB-DC (lane 5), and S-medium (lane 6) and PAM grown in TSB (lane 7), TSB-DC (lane 8), and S-medium (lane 9).

Figure 10

Colloidal Gold-Labelled Mab 48H.3 Binding
to Live PADG1 Cells

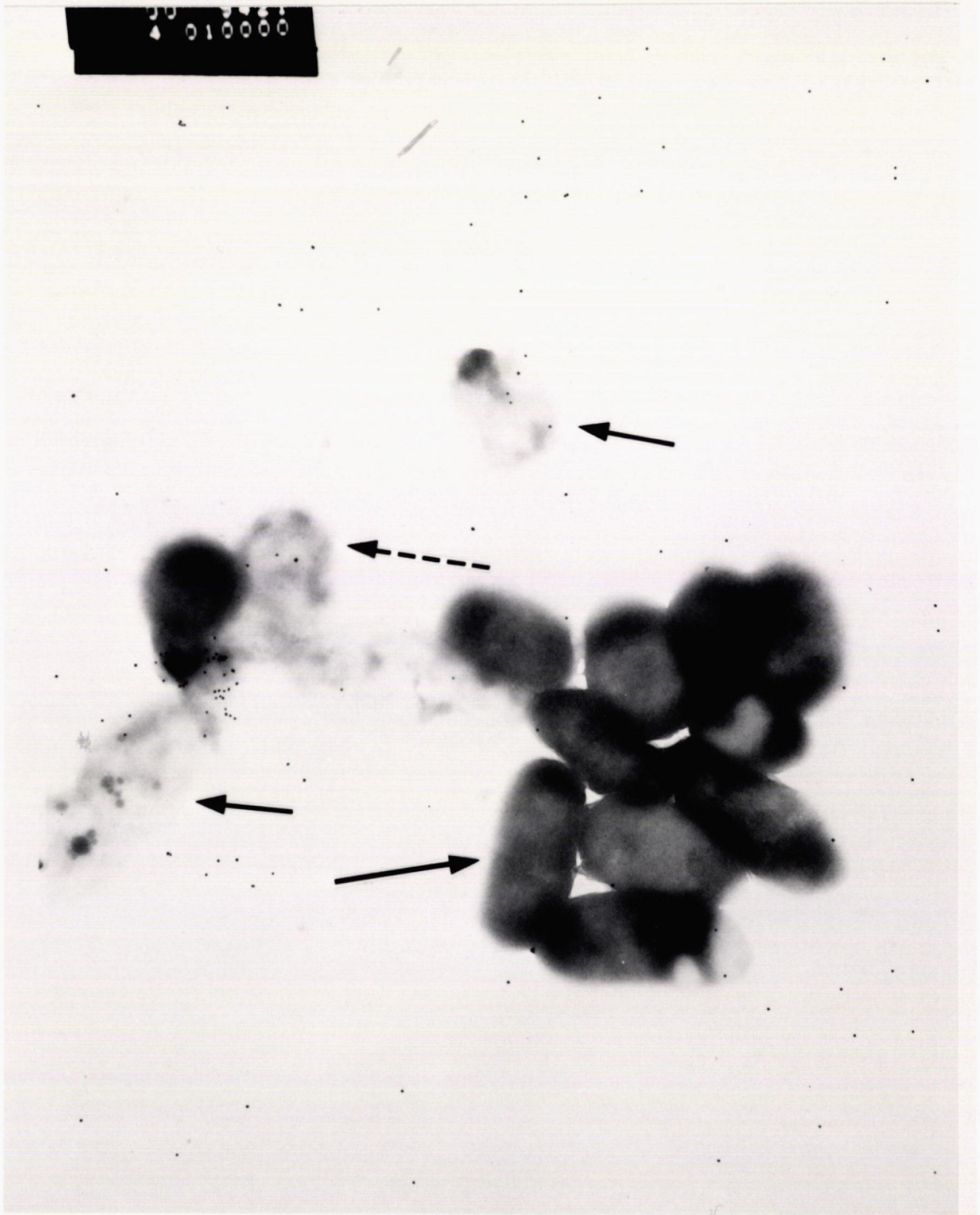

Figure 10. Colloidal Gold-Labelled Mab 48H.3 Binding to Live PADG1 Cells: Colloidal-gold-protein-A conjugate was used to detect binding of Mab 48H.3 to live, intact PADG1 cells. Gold particles were 15 nm in diameter. Two populations of cells are present in this electron micrograph. Small arrows mark the "lighter" cells, large arrows mark a representative "darker" cell, and a mottled "intermediate" cell is shown by a dashed arrow. None of the cells bind more than background amounts of Mab-colloidal gold particles.

Figure 11

Colloidal Gold-Labelled Mab 48H.3 Binding
to Heat-Killed PADG1 Cells

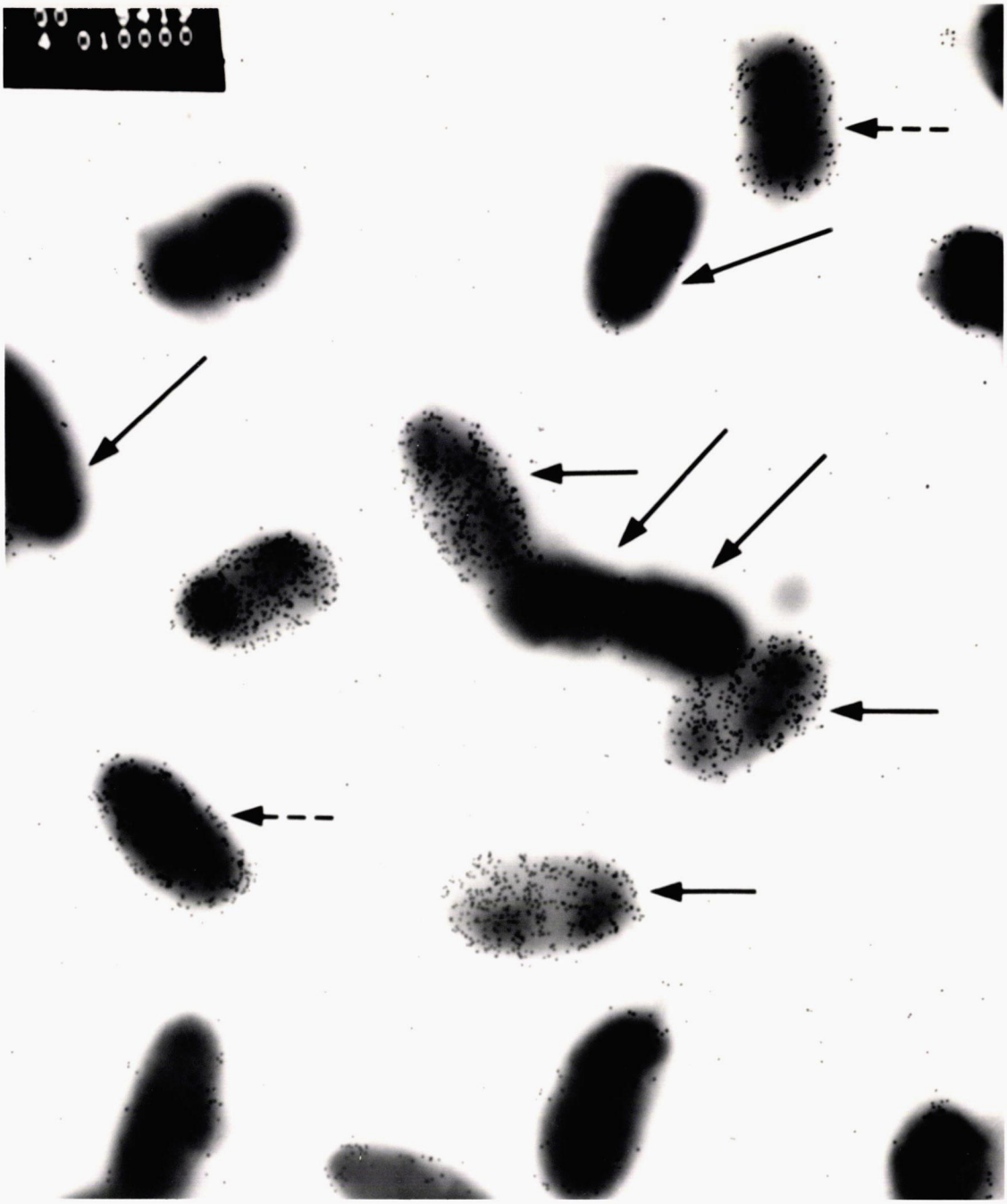

Figure 11. Colloidal Gold-Labelled Mab 48H.3 Binding to Heat-Killed PADG1 Cells: Colloidal-gold-protein-A conjuate was used to detect binding of Mab 48H.3 to heat-killed PADG1 cells. The large arrows mark 4 darker cells, the smaller arrows mark lighter cells, and dashed arrows mark cells of intermediate darkness. The colloidal gold particles are 15 nm in diameter.

Figure 12

Colloidal Gold-Labelled Mab 48H.3 Binding
to Heat-Killed PAO503 Cells

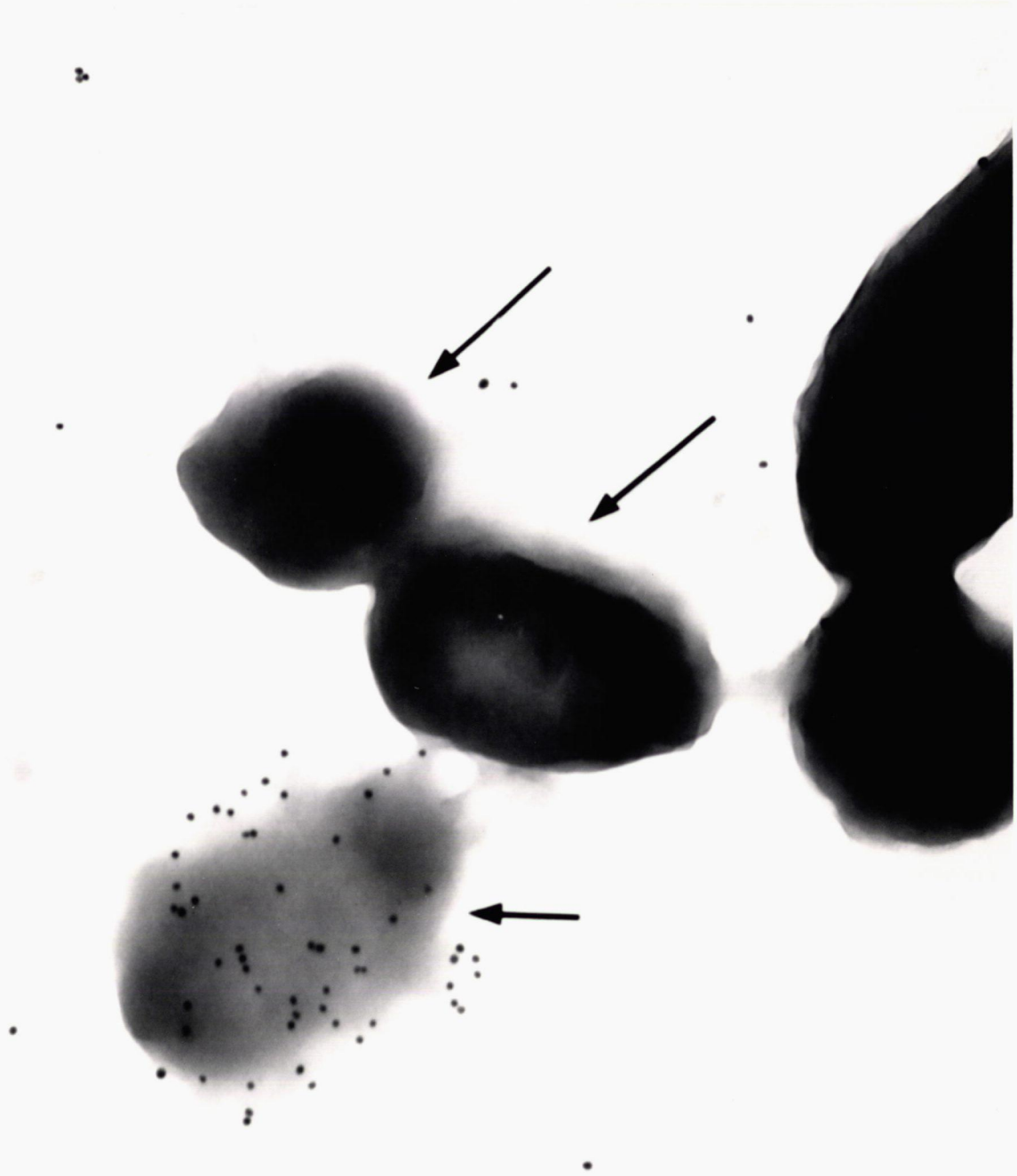

Figure 12. Colloidal Gold-Labelled Mab 48H.3 Binding to Heat-Killed PAO503 Cells: Heat-killed PAO503 cells were bound by Mab 48H.3 then by colloidal-gold-protein-A conjugate with a particle size of 15 nm. Small arrows mark a lighter cell and large arrows mark two representative darker cells.

Figure 13

Colloidal Gold-Labelled Mab 48H.1 Binding
to Live PADG1 Cells

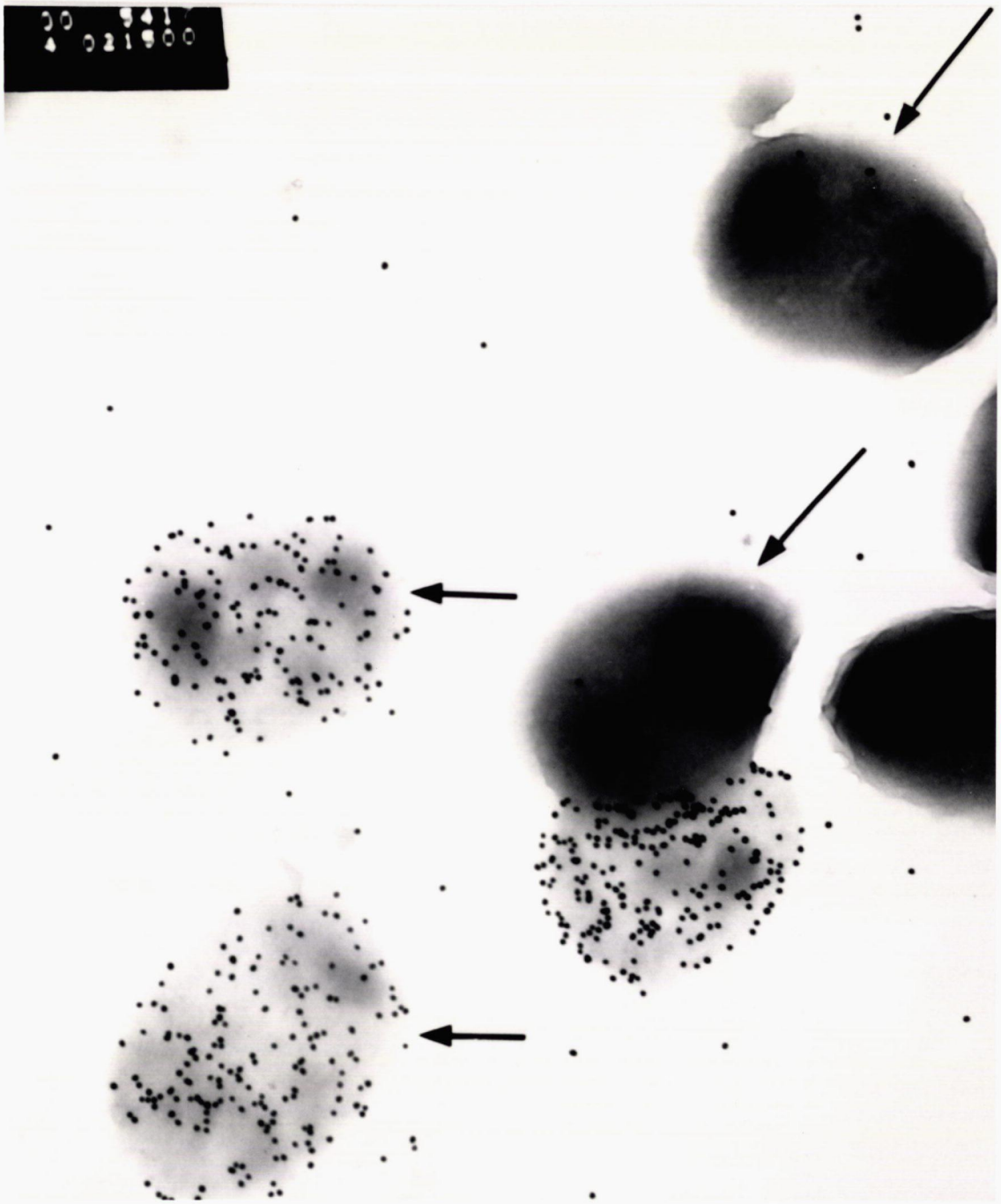

Figure 13. Colloidal Gold-Labelled Mab 48H.1 Binding to Live PADG1 Cells: The affinity of live PADG1 cells for Mab 48H.1 was assessed by subsequent binding of colloidal-gold-protein-A conjugate with a particle size of 15 nm. Lighter cells are marked by small arrows and darker cells by larger arrows.

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