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ACTIVITY OF FLAGELLAR ANTIBODY
IN THE PHAGOCYTOSIS OF
PSEUDOMONAS AERUGINOSA BY
POLYMORPHONUCLEAR LEUKOCYTES

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ABSTRACT

The H (flagellar) antigen of *Pseudomonas aeruginosa* isolates is expressed as either a homologous (b) or heterologous ($a_0a_1a_2a_3a_4$) type. *P. aeruginosa* rabbit anti-flagellar IgG was shown to enhance phagocytosis of *P. aeruginosa* by mouse peritoneal polymorphonuclear leukocytes (PMNs) in two different phagocytosis assays. An indirect assay was initially used in which the decrease in viable bacterial counts from the supernatant of a mixture consisting of bacteria and PMNs was measured. The results were confirmed and extended using an assay in which bacteria were labeled by growth in [^3H] thymidine, and radioactive counts of internalized bacteria were recorded. With this method, intracellular bacteria were quantitated directly. The opsonic activity of the flagellar antibody was specific for the flagella type (a or b). It was shown that *P. aeruginosa* strain M-2 (b-type) antiserum increased opsonophagocytosis of strain PAO1 (b-type) by seven-fold over normal rabbit serum (NRS), whereas a-type antiserum was essentially unreactive. To ascertain the degree of opsonic cross-reactivity between heterologous a-types, four different strains were used: 1210 ($a_0a_1a_2$), 170018 ($a_0a_3a_4$), 5940 (a_0a_2), and SBI-N (a_x). Using 1210 antiserum, it was shown that all four a-types were phagocytized specifically at a level four to six fold over NRS. In contrast, M-2 antiserum was not opsonic. The opsonic property of flagellar antiserum and the

demonstration of cross-reactivity among a-types further supports previous evidence favoring the use of only two H antigen types (a and b) in a potential vaccine.

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Chapter I

Introduction

Pseudomonas aeruginosa is well-documented as an opportunistic pathogen in a wide range of immunocompromised patients (14). This organism, while seldom causing infection in normal healthy individuals and exhibiting low carriage rates, is most frequently acquired nosocomially. Patients with a suppressed immune or host-defense system are often infected with this organism, in particular, those suffering from serious burn wounds, cystic fibrosis, and malignancies. *P. aeruginosa* is the organism most frequently isolated from cystic fibrosis patients, and once colonization by mucoid strains occurs, it is virtually impossible to eradicate (14). It is the leading causative agent of mortality in burn wound patients with *P. aeruginosa* bacteremia in burn patients resulting in 60% to 75% mortality (14). Other *P. aeruginosa* septicemias, including neonatal sepsis, result in high mortality rates in neutropenic patients and account for 50% of bacteremias (14).

The nature and characteristics of this organism dictate the need for a successful treatment program prior to or as soon as possible following infection. Not only is this organism well characterized as resistant to a wide range of antibiotics (64), but it is also very

ubiquitous and able to survive in moist environments with few nutritional requirements. It frequently is isolated from a wide variety of surfaces in hospitals including distilled water containers (14). The ability of this organism to survive, and multiply in diverse conditions and its resistance to many disinfectants make it difficult to eradicate, and therefore protection must begin at the patient level.

A wide range of extracellular and somatic factors also contribute to the virulence of this organism in immunocompromised patients. Among the extracellular enzymes indicted in pathogenicity are toxin A, elastase, hemolysins, and alkaline protease. In addition, somatic antigens such as flagella, pili, lipopolysaccharide, and alginate contribute to invasiveness and persistence of the organism (14).

Cell surface components have frequently been utilized as agents for immunotherapy which includes passively administering antibodies directed against the component or actively administering the antigen itself. For a cell surface component vaccine to be successful, it should be immunogenic and induce a memory (IgG) response. The antigen should be non-toxic and well conserved among the species in which it is found. Surface exposure and production in the disease state are also necessary requirements (22). Most importantly, it must result in protection following vaccination of the target subject. Flagella of *P.*

aeruginosa potentially fulfill the requirements of a successful vaccine candidate. Immunization of rabbits with flagellar antigens elicits a specific immunoglobulin, primarily IgG, response as detected by enzyme-linked immunosorbent assay (ELISA) (2, 41).

Flagella of *P. aeruginosa* are classified as two types, designated a and b. This typing scheme is based on slide agglutination assays and the molecular weight (M_r) of the flagella. Ansorg (5, 6) and Lanyi (34) used immunofluorescence assays to discern the two types. The heterologous a-types can be divided into several subgroups consisting of molecular weights of 45,000 to 52,000 (2). The a-type flagella exhibit one to three of the sub-antigen types, a_1 , a_2 , a_3 , and a_4 , and appear to have a common sub-antigen designated a_0 . The b-types comprise a homologous group with a molecular weight of 53,000 (2). Approximately 60% of clinical isolates exhibit b-type flagella. Recently, flagella types have been distinguished by use of an enzyme-linked immunosorbent assay (ELISA) which is to be more sensitive than slide agglutination techniques (39). This detection method is applicable to both flagellar filaments and whole bacterial cells.

Protection against *P. aeruginosa* by passive immunotherapy with flagellar antiserum has been demonstrated (18, 19). It is

thought that flagella serve as a virulence factor in the initial colonization of burn wounds and may aid in further spread of the organism from the original site resulting in bacteremic conditions (18). Passive protection is specific for the flagella type as demonstrated on a burned-mouse model (18). Protection against challenge by b-type strains was observed with passive transfer of b-type antiserum. Among the heterologous a-types, cross-protection has been demonstrated supporting the evidence for a common a_o sub-antigen in this type of flagella. Although the burn wounds of protected mice were colonized to the same extent as those of non-protected mice, systemic infection did not occur in immunized mice. These results indicate that passive protection occurred by binding of the antibody to flagella resulting in antibody-mediated cytotoxicity, either by initiating phagocytosis, activating the complement cascade, or by immobilizing the cells themselves. In related work, it has been shown that passive treatment of mice with *Clostridium chauvoei* flagellar antiserum results in protection against intramuscular challenge with the bacteria (65, 66, 67).

Flagella are a major virulence factor in several organisms. With *P. aeruginosa* which exhibits a single polar flagellum, non-flagellated (Fla^-) non-motile (Mot^-) mutants are significantly less virulent than the flagellated (Fla^+) motile (Mot^+) parental strains in a burned-mouse model (41). Motile organisms rapidly

disseminated from the original burn wound resulting in bacteremia, while non-motile mutants, although able to multiply in the wound site, did not demonstrate invasiveness. When flagellated *Salmonella typhimurium* Mot⁺ and Mot⁻ strains were tested orally and intraperitoneally in mice, there was no difference in the virulence of the Mot⁺ and Mot⁻ organisms indicating that flagella, not motility or chemotaxis, are necessary for virulence (13, 71). Additionally, no differences were seen in the survival of motile and non-motile cells within macrophages supporting the role of flagella in prolonging the survival of the bacterium.

Investigators using isogenic pairs of *S. typhimurium* showed that Fla⁺ strains were considerably more virulent than the Fla⁻ phenotype (13, 71). It has been demonstrated that flagella of *S. typhimurium* serve as virulence factors by prolonging survival of the bacteria within macrophages (71). While no differences were observed in the phagocytosis of Fla⁺ and Fla⁻ cells, flagellated *S. typhimurium* survived intracellularly longer than non-flagellated organisms. Recently, however, researchers presented results questioning the specificity of the Fla⁻ mutation. In studies with five pairs of isogenic mutants, one of each pair being Fla⁻ due to a mutation in one of the flaFI through flaFV cistrons, approximately 100% mortality was observed in animal models with all constructs. From these results, the researchers concluded that a

virulence gene exists near the *flaF* which was presumably deleted in the original *Fla⁻* derivative (Carsiotis, M., B. Stocker, I. A. Holder, D. Weinstein, and A. D. O'Brein, 1987 Ab. Ann. Meet. Am. Soc. Microbiol. p. 53).

One of the primary defense mechanisms of all members of the animal kingdom against invading microorganisms and particles is the cellular immune system. Most bacterial species to which the host animal is exposed are not pathogenic due to phagocytosis and subsequent killing of the organisms as soon as they cross the protective barriers of the host. The majority of pathogenic bacteria have developed complex mechanisms which allow them to evade ingestion or digestion by phagocytes. The ability of bacteria to avoid host defense systems resides mainly in surface components such as capsules, proteins, pili, and O-antigens - all of which serve to increase the virulence of the organism (45).

There are two major types of phagocytes. Polymorphonuclear leukocytes (PMNs) or granulocytes circulate throughout the host. Neutrophils comprise approximately 98% of the PMN population and are the primary defense against invading particles and organisms (28, 69). Circulating and fixed macrophages mature from monocytes and comprise the second category of phagocytic cells.

Recognition and attachment of invading organisms by phagocytes is greatly facilitated by the presence of specific immunoglobulin G antibodies (IgG1 and IgG3). PMNs, as well as

macrophages, have a receptor for the exposed Fc fragment of the antibody molecule. Complement component C3b also acts as an opsonin and serves as a "bridge" to bring the organisms in close contact with the leukocyte membrane (69). These particle-associated opsonins (IgG and C3b) enhance the adhesive phase by binding in a specific receptor-ligand manner with complementary receptors on the phagocytes' surface. While phagocytosis can occur in the absence of opsonins due to non-specific recognition of the particle, the phagocyte-particle interaction is greatly enhanced in the presence of the Fc receptor-ligand interaction (1).

Once a foreign particle has been recognized and a chemotactic signal has been received by the phagocyte bringing it into physical contact with the particle, ingestion can ensue. The first critical step involves the adhesion of the particle to receptors of the phagocyte. The mechanism of this adhesive phase may be either a van der Waals, a hydrophobic, or an electrostatic attraction, and/or a receptor-mediated bond (1). Once adhesion has occurred, the particle is engulfed by endocytosis; a phagocytic vesicle is formed, and then fused with a lysosome containing hydrolytic enzymes which destroy most bacteria (1).

In burned mice rendered neutropenic by cyclophosphamide treatment, protection afforded by *P. aeruginosa* flagellar antiserum is eliminated (18). Treatment with cyclophosphamide also results in loss of protection by flagellar antibody against

intramuscular challenge of *C. chauvoei* (66). This has led to the hypothesis that the phagocytic system, especially PMNs, is an important part of the mechanism of protection by flagellar antiserum. Protection against *P. aeruginosa* is afforded mainly by PMNs. The method of protection of flagellar antiserum may be due, in part, to its opsonizing capabilities. Opsonin-enhanced phagocytosis of strains of *P. aeruginosa* has been demonstrated by several investigators (10, 48, 72, 74).

Complement appears to be a more important factor in the opsonization of rough Gram-negative isolates than in smooth clinical isolates (10, 19, 54, 55, 71). Antibodies produced against rough mutants of *E. coli* and *Salmonella* strains promoted phagocytosis only when complement was present (70). These findings led researchers to propose that recognition of the *Enterobacteriaceae* by PMNs requires one of three different opsonizing mechanisms: (1) antibody alone, (2) antibody or complement, and (3) complement alone (70). Most smooth strains are in category two, while most rough strains have an absolute requirement for complement. Mucoid strains of *P. aeruginosa* require the presence of complement in addition to IgG in order to be phagocytized, whereas a non-mucoid revertant strain required a heat-stable opsonin (11).

Antibodies to several surface antigenic components of *P. aeruginosa* may serve as opsonins, and, in this capacity, augment

the ingestion of bacteria by phagocytic cells. Cell surface antigens important in this interaction include mucoid exopolysaccharide (3), lipopolysaccharide (73), high molecular-weight polysaccharide (49, 50), and protein F (7, 22, 37). In serum resistant strains of *P. aeruginosa*, opsonization may be accomplished by monoclonal antibodies to protein F (porin) in the absence of complement (7). Although phagocytosis by PMNs was increased two- to seven-fold by the addition of specific antibody to protein F, killing did not occur (7). When antibody to *P. aeruginosa* mucoid exopolysaccharide was used, mucoid cystic fibrosis strains of *P. aeruginosa* exhibiting rough lipopolysaccharide (LPS) were killed effectively by blood leukocytes only when both complement and specific antibody were present (3). *P. aeruginosa* LPS elicits type-specific opsonizing antibodies (73). Antibodies, especially IgG, to high molecular-weight polysaccharide also serve as effective opsonins (49, 50). In general, IgG antibodies are more effective in promoting opsonophagocytosis than other immunoglobulins (10, 36). These results demonstrate the requirement of specific antibody to cell surface components in order for effective phagocytosis to occur.

Antibodies to surface components appear to be more effective in promoting phagocytosis than antibody to extracellular products. In experiments with a *P. aeruginosa* exoenzyme S-producing parent

strain and an exoenzyme S-deficient mutant, both the parent and mutant were killed by PMNs when incubated with fresh serum (44). The addition of specific antibody to exoenzyme S resulted in the inability of the parental strain to disseminate in a burned-mouse model. The researchers concluded that exoenzyme S, while not important in colonization, aids in dissemination of the organism by adversely affecting host defenses (44). This data requires confirmation since the purity of antigen and resulting antibody may be questioned (personal communication, Dr. T. C. Montie with Dr. T. Nicas).

Non-opsonic phagocytosis of non-mucoid strains of *P. aeruginosa* from cystic fibrosis patients occurs (61, 62, 63). In the absence of serum opsonins, phagocytosis has been shown to be correlated with piliation and hydrophobicity of the bacterium. Those strains which are subject to non-opsonic phagocytosis were more heavily pilated and more hydrophobic than strains which were not phagocytized in the absence of opsonins. This mechanism of host defense was suggested to occur in areas where serum opsonins are not available, particularly in the respiratory tract.

Flagella may serve as effective surface antigens against which opsonizing antibodies may be formed. Flagellar antiserum promotes phagocytosis of *C. chauvoei* in addition to serving as protective antibodies (65, 66, 67). The protective effect against *C. chauvoei* appears to be mediated only by PMNs and not by

macrophages. As previously stated, flagella and motility are well-documented factors in the virulence of *P. aeruginosa*.

The purpose of this study was to determine the role of flagellar antiserum in the phagocytosis of *P. aeruginosa*. Two types of *in vitro* phagocytic assays were used to investigate whether: (a) flagellar immune serum enhances the phagocytic response of PMNs, (b) this response is specific for the flagellar antibody (a or b), and (c) cross-reactivity is demonstrable among the heterologous a-types.

Chapter II

Materials and Methods

Bacterial Strains

The following strains of *Pseudomonas aeruginosa* were used in this study: strains M-2, 1210, SBI-N, CF-LL, WR-5, and GNB-1 from Dr. I. A. Holder, Shriners Burn Institute, Cincinnati, Ohio; strains 86f, 414ii, and 572b from Dr. M. J. Thomassen, Case Western Reserve University School of Medicine, Cleveland, Ohio; strains 5933, 5939, 5940, and 7191 from Dr. R. Ansorg, Collection d'Institut Pasteur, Paris, France; strains 170002, 170012, and 170018 were from Dr. B. Lanyi, National Institute of Hygiene, Budapest, Hungary; strain 19660 from Dr. R. S. Berk, Wayne State University Medical School, Detroit, Michigan; strains 3592, 3598, and 3614 from the Tennessee Department of Health and Environment, Nashville, Tennessee; and the wild-type strain PAO1 and its ethyl methane sulfonate non-flagellar (Fla⁻) mutants AK1152 and AK1153 from Dr. A. Kropinski, Queen's University, Kingston, Canada.

Maintenance of Stock Cultures

Upon receipt of the cultures, bacteria were grown overnight at 37°C in 25 mls Brain Heart Infusion Broth (BHIB) in a 175 rpm

rotary shaker. After harvesting and washing twice with 0.85% saline, cells were resuspended in 10 mls of sterile BHIB. One ml aliquots consisting of 0.5 ml washed cells and 0.5 ml 50% glycerol (in 0.85% saline) were stored frozen at -70°C . Day to day stock cultures were maintained as dilute suspensions in BHIB at 4°C .

Quantitation of Bacteria

Bacteria were grown in 25 mls BHIB for 15 hours at 37°C on a 175 rpm gyratory shaker. Cells were harvested by centrifugation ($4000 \times g$ for 15 min) and washed twice in a standard diluent (25 mM KH_2PO_4 , pH 7.0, 0.9% NaCl, 0.01% gelatin, 0.2 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$). Cells were resuspended in an equivalent volume of standard diluent, and four two-fold dilutions were made of this suspension. The absorbance at 590 nm was recorded for each dilution. Five further 10-fold dilutions were made and plated in duplicate on BHI plates which were incubated overnight at 37°C . A standard curve was constructed ($\log_{10}$ colony forming units/ml vs. optical density, 590 nm) and used as a reference to calculate desired inocula.

Preparation of Formalin-Killed Whole Cells

An overnight culture (25 mls in 250-ml flasks) was grown in mineral salts medium supplemented with succinate at 37°C on

rotary shaker at 175 rpm. Two-liter flasks containing 500 mls of the mineral salts medium were inoculated with the culture and incubated with slow shaking at 30°C overnight to promote flagella production. Bacteria were harvested by centrifugation at 4200 x g for 15 min at 4°C, resuspended in 0.85% saline containing 0.6% formalin, and incubated statically at room temperature for 48 hr. Cells were harvested by centrifugation, washed twice with 0.85% saline, dialyzed, and stored in a lyophilized state for use in the ELISA.

Laboratory Animals

Peritoneal leukocytes were harvested from 18-21 g female CF-1 mice (Harlan Sprague Dawley, Indianapolis, IN). Prior to experimental use, animals were maintained in cages of 10 mice per cage with food and water available *ad libitum*.

Female New Zealand white rabbits (Myrtle's Rabbitry, Thompson Station, TN) weighing three kg were used for the production of antiserum. Prior to and during immunization, the animals were maintained one to a cage with food and water available *ad libitum*.

Preparation of Polymorphonuclear Leukocytes

Inflammatory peritoneal phagocytes were obtained from CF-1 mice injected intraperitoneally with two mls of 10% proteose peptone. After four hours, mice were sacrificed by carbon dioxide

asphyxiation, and cells were harvested by peritoneal lavage with 10 mls Hanks' Balanced Salt Solution (HBSS) (Gibco Laboratories, Grand Island, NY) (16). For most isolates, cells were pelleted by centrifugation at $400 \times g$ for 10 min and resuspended in RPMI(Gibco) + 5% fetal calf serum (FCS) (Hyclone Laboratories, Logan, UT) at a concentration of 1×10^7 cells/ml. For samples in which further purification was attempted, the pellet was resuspended in eight mls phosphate-buffered saline (PBS). The cell suspensions were placed in 15 ml plastic tubes, underlaid with 3.0 ml Ficoll-Paque (Pharmacia, Piscataway, NJ) and centrifuged at $400 \times g$ for 30 min at 20°C . The granulocyte-rich pellet was washed three times with HBSS and resuspended in RPMI + 5% FCS at a cell concentration of 1×10^7 per ml. Cell counts were performed by hemocytometer. Cytocentrifuge (Shandon) smears (5 min at 700 rpm) were prepared and stained with Diff-Quik (American Scientific Products, Stone Mountain, GA). Viability was assessed by trypan blue exclusion.

Production and Adsorption of Flagellar Antiserum

P. aeruginosa flagellar antigen was routinely prepared as previously described (2, 41) and stored in a lyophilized state. An emulsion consisting of equal volumes of Complete Freund's adjuvant and 250-350 ug of lyophilized flagellar antigen reconstituted in one ml distilled water was made. Several

subcutaneous injections consisting of 0.3-0.5 ml of the emulsion were made into a shaved area on the backs of female New Zealand white rabbits. Prior to the injection series, rabbits were bled to obtain preimmune sera. Two weeks later a booster injection was given, and rabbits were bled one week later. After collecting the blood, it was allowed to clot and incubated overnight at 4°C. Sera were collected and clarified by centrifugation at 6000 x g. One ml aliquots were stored frozen at -70°C.

Antiflagellar sera was adsorbed to remove any non-specific background antibody. Undiluted immune serum was added to a 0.5 ml sample of AK1152, M2 Fla⁻ formalin-killed cells, or homologous boiled bacteria. After gently vortexing, the mixture was incubated statically for 30 min at 37°C. The sample was centrifuged at 6000 x g for 15 min and the serum fraction was removed. This procedure was repeated several times. After filter-sterilization, one ml aliquots were stored frozen at -70°C.

Enzyme-linked Immunosorbent Assay

The indirect method of enzyme-linked immunosorbent assay (ELISA) was used to determine the flagella type of *Pseudomonas aeruginosa* flagellar antigen preparations or formalin-killed whole cells (39). Fifty ul of the antigen was adsorbed to a polyvinyl-chloride microtiter plate (Fisher Scientific, Pittsburg, PA) by overnight incubation at 4°C. The antigen was diluted in

carbonate buffer to a concentration of 10 ng/well for flagellar antigens or 1 ug/well for whole-cell preparations. The plate was washed three times with phosphate-buffered saline (PBS) + Tween 20 (0.8% NaCl, 0.8% KH_2PO_4 , 0.12% Na_2HPO_4 , 0.02% KCl, 0.05% Tween 20), and 100 ul of coating buffer consisting of PBS + 3% gelatin was added to each well. After a 45 min incubation at 37°C , the plate was washed three times with PBS-Tween. Fifty ul of the appropriate rabbit polyclonal antiserum diluted 1 in 1000 in PBS-Tween + 1% gelatin was added, and 1:1 doubling dilutions were made to a final concentration of 1 in 512,000. After a 60 min incubation at 37°C , the plate was washed, and 50 ul of goat anti-rabbit IgG conjugated to peroxidase diluted 1 in 10,000 in PBS-Tween + 1% gelatin was added to each well. The plate was incubated at 37°C for 60 min and washed three times. Fifty ul of the substrate (0.1 M citric acid anhydrous, 0.2 M disodium phosphate anhydrous, 1% ortho-phenylenediamine (Sigma Chemical Corporation, St. Louis, MO), and 0.03% H_2O_2 , pH 6.0) was added to each well and allowed to develop at room temperature for 3-5 min. The reaction was stopped by the addition of 2.5 M sulfuric acid. The amount of substrate hydrolyzed was proportional to the amount of antibody present. The net absorbance at 490 nm was read on an ELISA plate reader (Bio-Tek Instruments), and the titer was determined to be the reciprocal of the last dilution in which

the absorbance value was twice that of the normal rabbit serum (NRS) control.

Opsonization

Bacteria suspended in HBSS + 1% gelatin at 1×10^8 cells/ml were opsonized by the addition of 0.1% serum and incubation for 30 min at 37°C with shaking.

Phagocytosis Assay

Equal volumes of opsonized bacteria in HBSS with 0.1% gelatin and PMNs in RPMI medium with 5% FCS were mixed at time zero to give a ratio of 10 bacteria to one PMN. Prior to mixing, a sample of bacteria was taken, serially diluted, and plated to determine the number of bacteria at time zero. Reaction mixtures were incubated at 37°C with shaking. At designated times, portions were mixed with an equal volume of ice-cold HBSS and centrifuged at $130 \times g$ for seven min at 25°C. Supernatant was diluted appropriately in 0.85% saline and plated on Brain Heart Infusion agar plates for bacterial enumeration. Plates were incubated overnight at 37°C, and bacterial colonies were counted.

Electron Microscopy

PMNs in RPMI + 5% FCS were mixed 1:1 with PAO1 in HBSS + 1% gelatin (GHBSS) to achieve a ratio of 10 bacteria to one PMN. PAO1

was opsonized in anti-M-2, anti-1210, and NRS. After 10 min, phagocytosis was stopped by the addition of ice-cold HBSS. PMNs were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7) for one hour at room temperature and were post-fixed in osmium tetroxide in phosphate buffer. Cells were embedded in epoxy resin, cured, and sectioned. After staining with uranyl acetate and lead citrate, cells were examined with a Hitachi H-600 transmission electron microscope.

Radiolabeled Phagocytosis Assay

A phagocytosis assay using radiolabeled bacteria was performed as modified after the method of Johnson *et al.* (31). Bacteria were labeled by growth for 15 hr at 37°C in a gyratory shaker (175 rpm) in mineral salts medium (MSM) (KH_2PO_4 0.04 M, K_2HPO_4 0.02 M, $(\text{NH}_4)_2\text{SO}_4$ 7.6×10^{-3} M, $\text{MgSO}_4 \times 7\text{H}_2\text{O}$ 2.0×10^{-4} M, $\text{FeCl}_3 \times 6 \text{H}_2\text{O}$ 9.2×10^{-6} M) supplemented with 0.4% sodium succinate to which 20 μCi of [^3H] thymidine/ml (specific activity 85 Ci/mmol; E. I. Dupont, Boston, MA) was added. Bacteria were washed three times with 0.85% saline and resuspended in HBSS + 1% gelatin at a final concentration of 1×10^8 cfu/ml as determined spectrophotometrically. Equal volumes of opsonized, radiolabeled bacteria and PMNs were mixed to achieve a ratio of 10:1. The mixture was incubated at 37°C in a shaking water bath

for 10 min. A control tube consisting of bacteria and RPMI + 5% FCS was also incubated as a determination of total bacteria available for phagocytosis. Phagocytosis was stopped by the addition of one ml of ice-cold HBSS. The reaction mixture was centrifuged at 130 x g for seven min to separate PMNs with cell-associated bacteria from non-cell-associated bacteria and washed three times with 0.85% saline. The control tube was centrifuged at 4000 x g for 15 min to determine total bacteria available. Pellets were resuspended in Bray's Solution (Research Products International Corporation, Elk Grove Village, IL) and counted in a Beckman LS 7000 Liquid Scintillation System. Counts were adjusted for background activity by subtracting counts obtained from the control tube containing bacteria only. The fold-increase of opsonophagocytosis above NRS was determined by the following: $\text{cpm (opsonin)/cpm (NRS)} \times 100$.

Intracellular Killing Assay

The assay for the antimicrobial action of PMNs was performed by the method of Stubner *et al.* (64). Equal volumes of opsonized bacteria in PBS and PMNs suspended in RPMI + 5% FCS were mixed to achieve a ratio of 10 bacteria to one PMN. The mixture was incubated at 37°C with shaking for 10 min. Bacteria were washed twice by centrifugation at 130 x g to remove non-engulfed bacteria. The granulocyte pellet was resuspended in PBS and

incubated at 37°C with shaking. Immediately after resuspension and 15, 30, 60, and 90 min later, aliquots were taken. PMNs were lysed in an equal volume of ice-cold distilled water. Dilutions were made in 0.85% saline and plated on BHI agar to determine total viable counts of intracellular bacteria.

Chapter III

Results

H Antigen Determination

A summary of strains used in this study and their H antigen type as determined by slide agglutination is shown in Table 1 (5, 6).

As an extension of previous work using the isolated flagella antigens as standard antigens, formalized whole bacterial cells were used to determine the H antigen type of *P. aeruginosa* strains by the indirect ELISA method (Table 2). The ELISA was shown to be more sensitive than the slide agglutination method. It was possible to determine (by ELISA) the flagella type of unknown strains which were not reactive in the slide agglutination method. Cross-reactivity with three well-characterized sera was based on ELISA titers. All b-type strains had a titer greater than 256,000 with PJ108 (b) antiserum and titers of 16,000 or less with the a-type antisera, 1210 and 170018. Similarly, most a-type strains had high titers with anti-1210 and anti-170018 and low titers with anti-PJ108. In general, most strains were less reactive with 1210 antiserum than with 170018 antiserum. The cross-reactivity demonstrated by the a-type strains also supports

Table 1. Antigenic Components of *Pseudomonas aeruginosa* Flagella.

Strain designation	Antigenic components(s)	Flagellin Mr
M2	b	53,000
PJ108	b	53,000
PAO1	b	53,000
19660	b	53,000
5939	a ₀ a ₃	52,000
5933	a ₀ a ₁ a ₂	51,000
1210	a ₀ a ₁ a ₂	51,000
7191	a ₀ a ₁ a ₂	51,000
5940	a ₀ a ₂	47,000
170018	a ₀ a ₃ a ₄	45,000
GNB-1	a _x	49,000
3598 (F)	a _x	48,000
3614 (F)	a _x	48,000
86-f (CF)	a _x	48,000
572b (CF)	a _x	48,000
SBI-N	a _x	---

GNB - General clinical non-burn isolate.

F - Folliculitis isolate.

CF - Cystic fibrosis isolate.

SBI - Shriners Burn Institute isolate.

a_x - Undetermined a-sub-antigen type.

Table 2. Determination of *Pseudomonas aeruginosa* Flagella Type by ELISA.

Strain designation	Determination of flagella type		Cross-reactivity		
	Slide agglutination	ELISA	PJ108	1210	170018
			(b)	(a0a1a2)	(a0a3a4)
PJ108	b	b	+++	+	+
5939	NR	a	+	++	+++
19660	a/b	b	+++	+	+
5933	a	a	+	+	+++
1210	a	a	+	++	++
7191	a/b	a	+	++	++
5940	a	a	+	++	+++
170018	a	a	-	++	+++
GNB-1	a	a	+	+	++
3598	NR	a	+	++	++
3614	a	a	+	++	+++
86-f	a	a	+	++	++
572b	NR	a	-	+	++

Cross-reactivity is based on ELISA titers:

<1000 -; 1,000-16,000 +; 32,000-128,000 ++;
256,000-512,000 +++.

NR - no reaction.

data indicating a common a_o sub-antigen in a-type flagella.

Polymorphonuclear Leukocyte Characteristics

Leukocytes harvested from the peritoneal cavities of CF-1 mice were shown to be 95% viable by trypan blue exclusion. Cytocentrifuge smears stained with Diff-Quik showed that preparations consisted of approximately 73% PMNs and 27% macrophages. Of the PMN population, approximately 5% were immature band cells. Repeated attempts to further purify the leukocyte preparation using Ficoll-Pacque were unsuccessful.

Sera Characteristics

Hyperimmune sera were prepared from female New Zealand white rabbits as previously described (41). Sera were shown in an ELISA to contain predominately IgG antibodies and were free of somatic antibodies after adsorption with Fla⁻ whole bacterial cells (41). Titers, expressed as the reciprocal of the last dilution in which the absorbance value was twice that of the NRS control, were typically greater than 128,000 against homologous flagellar filaments.

Indirect Determination of Opsonophagocytosis

The opsonizing capacity of flagellar antibody was tested by an indirect phagocytosis assay was used in which the reduction of

viable bacterial counts was measured. Bacteria were opsonized in 0.1% sera without causing aggregation of the bacteria. With this method, it was shown that homologous flagellar antiserum resulted in an increase in phagocytosis of *P. aeruginosa* as compared to NRS as shown in Figure 1. Data shown are the means of three replicate experiments. Maximum uptake occurred by approximately 20 minutes. Hyperimmune flagellar antiserum resulted in 75% uptake at 10 minutes compared with 45% uptake of bacteria treated with NRS. The addition of FCS in the reaction mixture essentially did not affect the results, and further experiments were performed without FCS in the opsonizing mixture.

The ability of flagellar antiserum to enhance phagocytosis was determined using homologous and heterologous flagellar antisera. Strain PAO1 (b) was opsonized with M-2 (b) and 170018 ($a_0a_3a_4$) antiserum. Figure 2 shows that homologous antiserum resulted in 89% uptake of PAO1, while only 25% of bacteria were engulfed by 10 minutes when opsonized by the heterologous antiserum. This represented a greater than three-fold increase in opsonophagocytosis of homologous serum over heterologous serum. Figure 3 illustrates the analogous results obtained when strain SBI-N (a_x) was opsonized in M-2 and 170018 antiserum and non-cell-associated bacteria were assayed after 15 minutes.

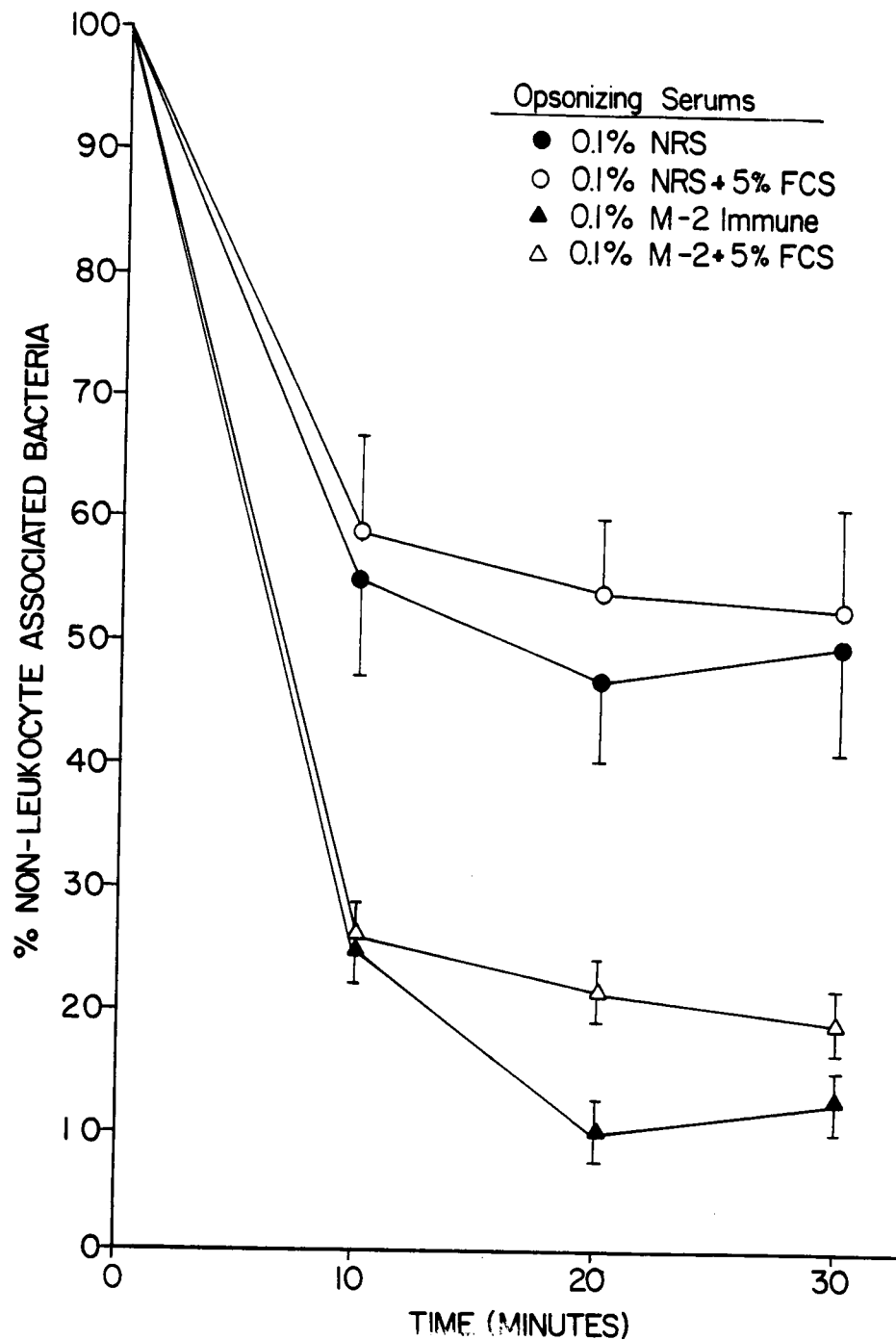


Figure 1. Opsonizing Effect of Homologous Immune Serum. Strain PAO1(b) was opsonized with 0.1% normal rabbit serum (NRS), 0.1% NRS + 5% fetal calf serum (FCS), 0.1% M-2 (b) antiserum, and 0.1% M-2 antiserum + 5% FCS.

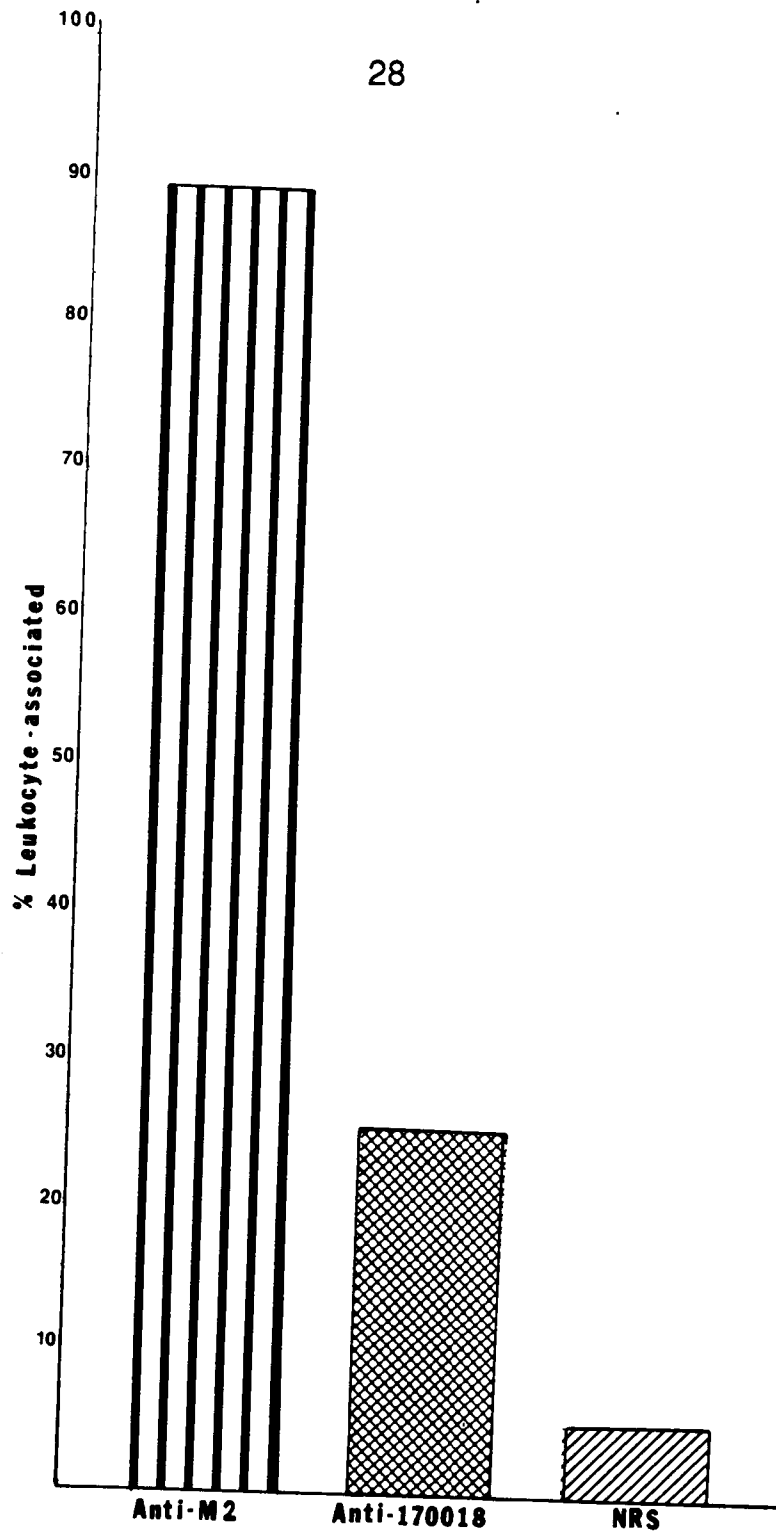


Figure 2. Specificity of Anti-flagellar Serum in Opsonization of PAO1(b). Bacteria were opsonized with anti-M-2 (b), anti-170018 ($a_0a_3a_4$), and NRS. Analyses by Student *t* test of the mean value differences between M-2 and 170018 gave significant *t* values beyond the 0.001 level.

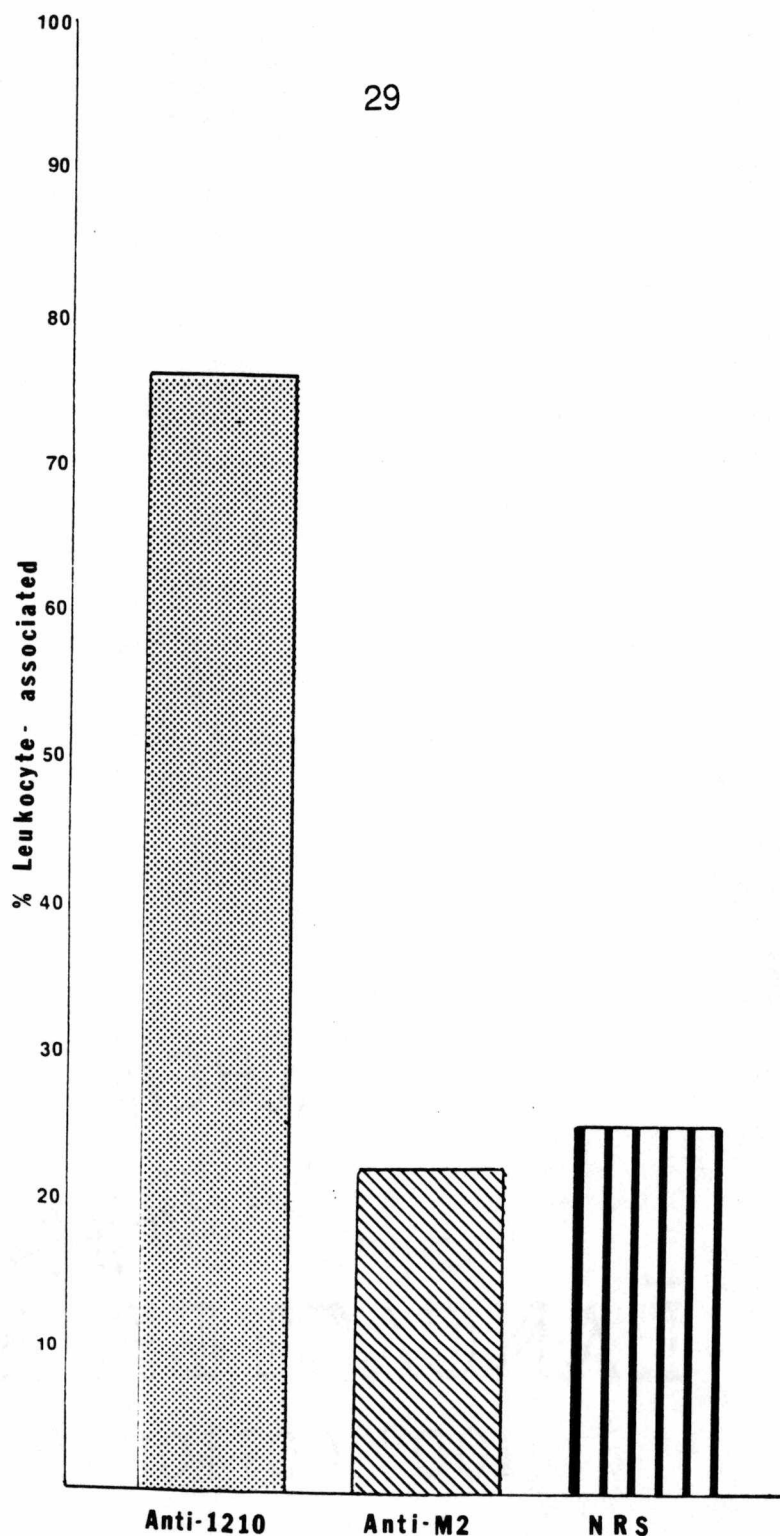


Figure 3. Specificity of Anti-flagellar Serum in Opsonization of SBI-N (a). Bacteria were opsonized with anti-1210 ($a_0a_1a_2$), anti-M-2, and NRS. Analyses by Student t test of the mean value differences between 1210 and M-2 gave significant t values beyond the 0.001 level.

Again, there was greater than three-fold increase in phagocytosis of strains opsonized with homologous serum compared with heterologous serum. These results represent the mean of at least three experiments, with a significant *t* value beyond the 0.001 level. Flagellar antiserum was shown to be opsonophagocytic specifically for the flagellar type.

Microscopy to Verify Attachment and Uptake by PMNs.

Cytocentrifuge smears of PAO1 opsonized in M-2 antiserum or NRS after a 60 minute incubation with PMNs were examined under 1000x oil-immersion, and cell-associated bacteria were counted directly. Results consistent with previous data indicating that flagellar antibody is opsonic are shown in Figure 4. Approximately twice as many bacteria opsonized in homologous serum are associated with the PMNs than bacteria opsonized in NRS.

Transmission electron microscopy verified the uptake of bacteria into the PMNs. Figure 5 shows the internalization of PAO1 opsonized in M-2 antiserum as indicated by arrows. This method was not able to be used quantitatively as no internalized bacteria opsonized in heterologous or NRS were seen in PMNs.

Confirmation by a Direct Radiolabeled Method of the Opsonophagocytosis Specificity of Flagellar Antibody

The opsonic property of flagellar antiserum and the specificity

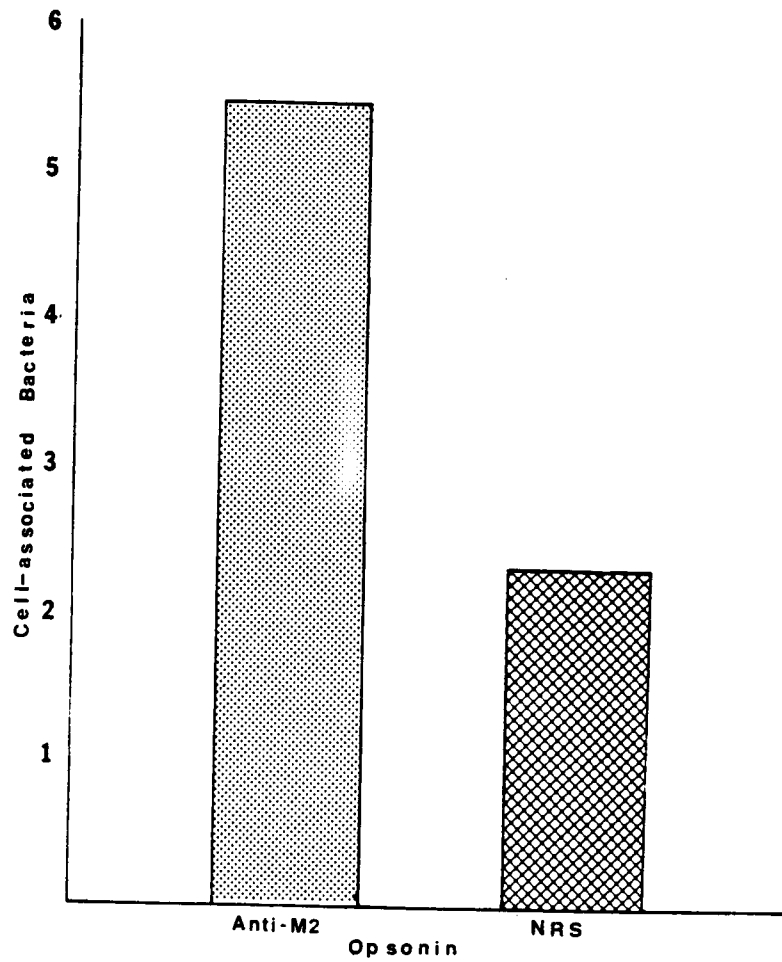


Figure 4. Cell-associated Bacteria Determined Microscopically. PAO-1 (b) was opsonized with M-2(b) antiserum and NRS.



Figure 5. Transmission Electron Micrograph of Polymorphonuclear Leukocyte with Ingested PAO1. Strain PAO1 was opsonized with M-2 (b) antiserum.(x 15,000 magnification).

of the opsonin for the flagella type was verified using a radiolabeled assay in which cell-associated labeled bacteria could be quantitated directly. In this approach, the PMNs were washed three times to assure removal of cell-associated bacteria; therefore, results reflect the amount of internalized bacteria. A representative experiment is shown in Figure 6. PAO1 which had been [^3H] labeled was opsonized with M-2, 1210, and NRS. Cell-associated and non-cell associated bacterial counts were recorded. Increased numbers of PMN-associated bacteria were seen when PAO1 (b) was opsonized with homologous b-type antiserum than when opsonized with the a-type serum or NRS. Correlating with the latter observation was the appearance of corresponding fewer counts of non-PMN-associated bacteria when PAO1 was opsonized with M2 antiserum.

In Table 3, the counts per minute (cpm) were used to obtain values for the percentage of leukocyte-associated bacteria. The specificity of the flagellar antibody in opsonophagocytosis was demonstrated again.

The presence of a common cross-reactive α_0 antigen among the heterologous a-type flagella recently has been shown in an ELISA (38) and in animal protection models (18). The cross-reactivity among a-type strains is also demonstrated in the phagocytosis of opsonized *P. aeruginosa* by PMNs. Table 4 shows not only the specificity of the antiserum, but also the capacity of 1210

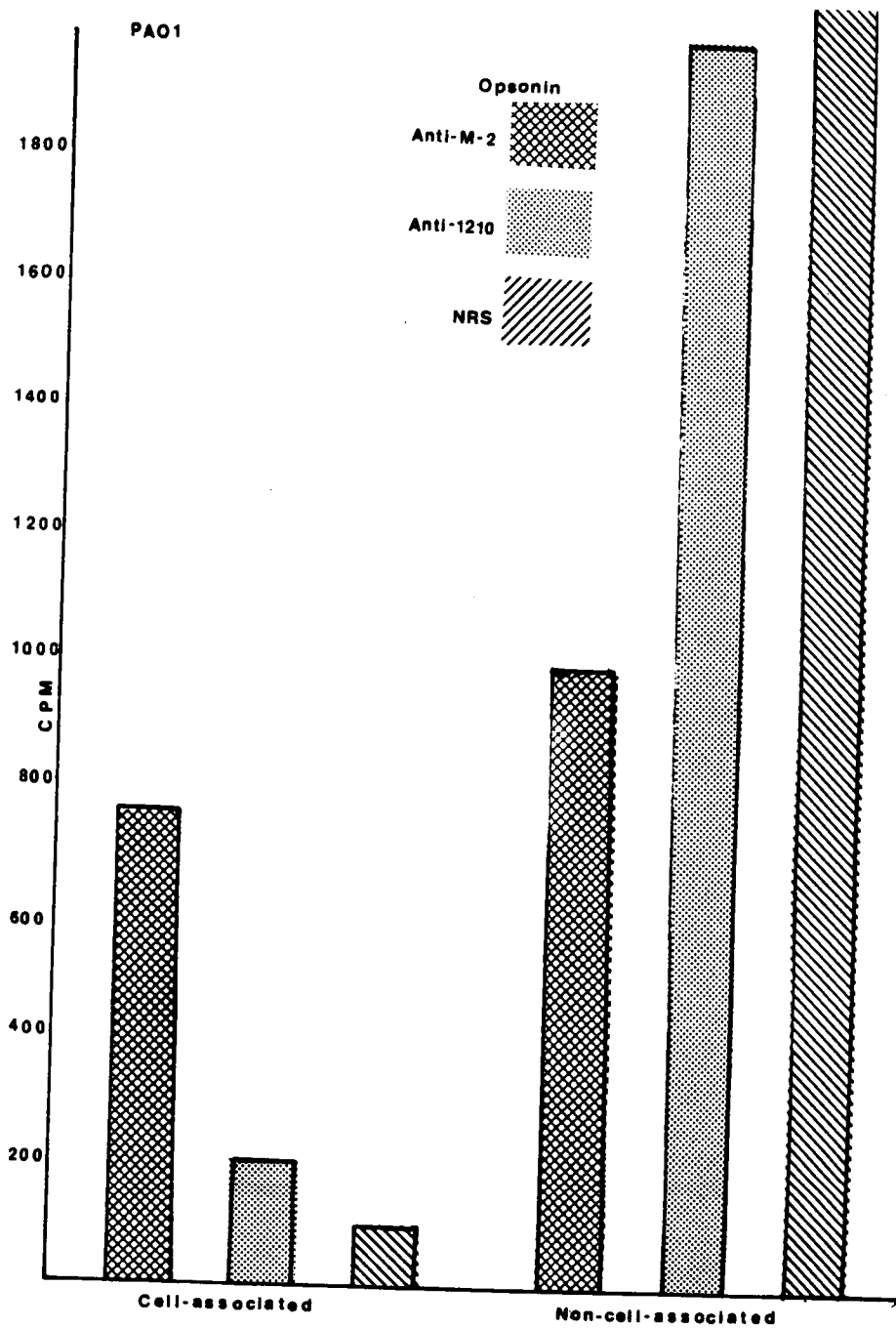


Figure 6. Cell-associated and Non-cell-associated PAO1 as Determined by Radioactive Method. Strain PAO1 (b) was opsonized in anti-M-2 (b), anti-1210 ($a_0a_1a_2$), and NRS. CPM-counts per minute.

Table 3. Specificity of Anti-flagellar Serum in the Opsonization of *Pseudomonas aeruginosa* Determined by Direct Radioactive Method.

Strain	Opsonin	Percent leukocyte associated bacteria
PAO1 (b)	M2 (b)	76
PAO1	1210 (a ₀ a ₁ a ₂)	34
PAO1	NRS	35
SBI-N (a _x)	1210	67
SBI-N	M-2	28
SBI-N	NRS	26

Percent leukocyte-associated bacteria was determined by the following: (cpm cell-associated bacteria/ cpm total bacteria available for phagocytosis) x 100. All cpm were adjusted for background.

Analyses by Student *t* test gave significant *t* values beyond the 0.01 level.

Table 4. Opsonic Cross-reactivity Among a-type Strains of *Pseudomonas aeruginosa*.

Strain	Opsonin	Fold increase in phagocytosis
PAO1(b)	M-2(b)	6.7
PAO1	1210(a ₀ a ₁ a ₂)	1.9
1210	1210	5.6
1210	M-2	1.5
170018(a ₀ a ₃ a ₄)	1210	3.5
170018	M-2	1.1
5940(a ₀ a ₂)	1210	4.1
5940	M-2	1.2
SBI-N(a _x)	1210	4.2
SBI-N	M-2	0.6

Fold increase in phagocytosis was determined by the following: cpm immune serum treated cell-associated bacteria/ cpm NRS treated cell-associated bacteria. Cpm are adjusted for background.

Analyses by Student *t* test gave *t* values significant beyond the 0.01 level.

($a_0a_1a_2$) antiserum to enhance the phagocytosis of bacterial strains 1210, 170018 ($a_0a_3a_4$), 5940 (a_0a_2), and SBI-N (a_x) strongly supporting previous evidence for a common cross-reactive a_0 antigen.

A level of non-opsonic phagocytosis has been demonstrated using non-mucoid strains of *P. aeruginosa* isolated from cystic fibrosis patients (61, 62, 63). Results of phagocytosis of PAO1 opsonized with M2 antiserum, normal rabbit serum, and untreated are shown in Figure 7. Phagocytosis of cells not treated with serum occurred to approximately the same extent as cells treated with normal rabbit serum which may be due to the non-specific opsonization stimulated by FCS in the PMN suspension due to complement in FCS or nonspecific binding to PMN membranes (hydrophobic or other interactions).

Previous research has demonstrated the requirements of flagella and motility for invasiveness and virulence of *P. aeruginosa* in burn wounds (19, 40). Preliminary experiments were performed to determine the opsonophagocytosis of a strain which produces flagella monomeric units (Mon) but does not assemble them in a flagellum ($\text{Mon}^+ \text{Fla}^-$) and a non-flagellated clinical isolate ($\text{Mon}^- \text{Fla}^-$). Strain AK1153 ($\text{Mon}^+ \text{Fla}^-$), a derivative of PAO1 (b), was opsonized with M-2 (b) antiserum and NRS. The fold-increase in phagocytosis of anti-M2 treated above NRS

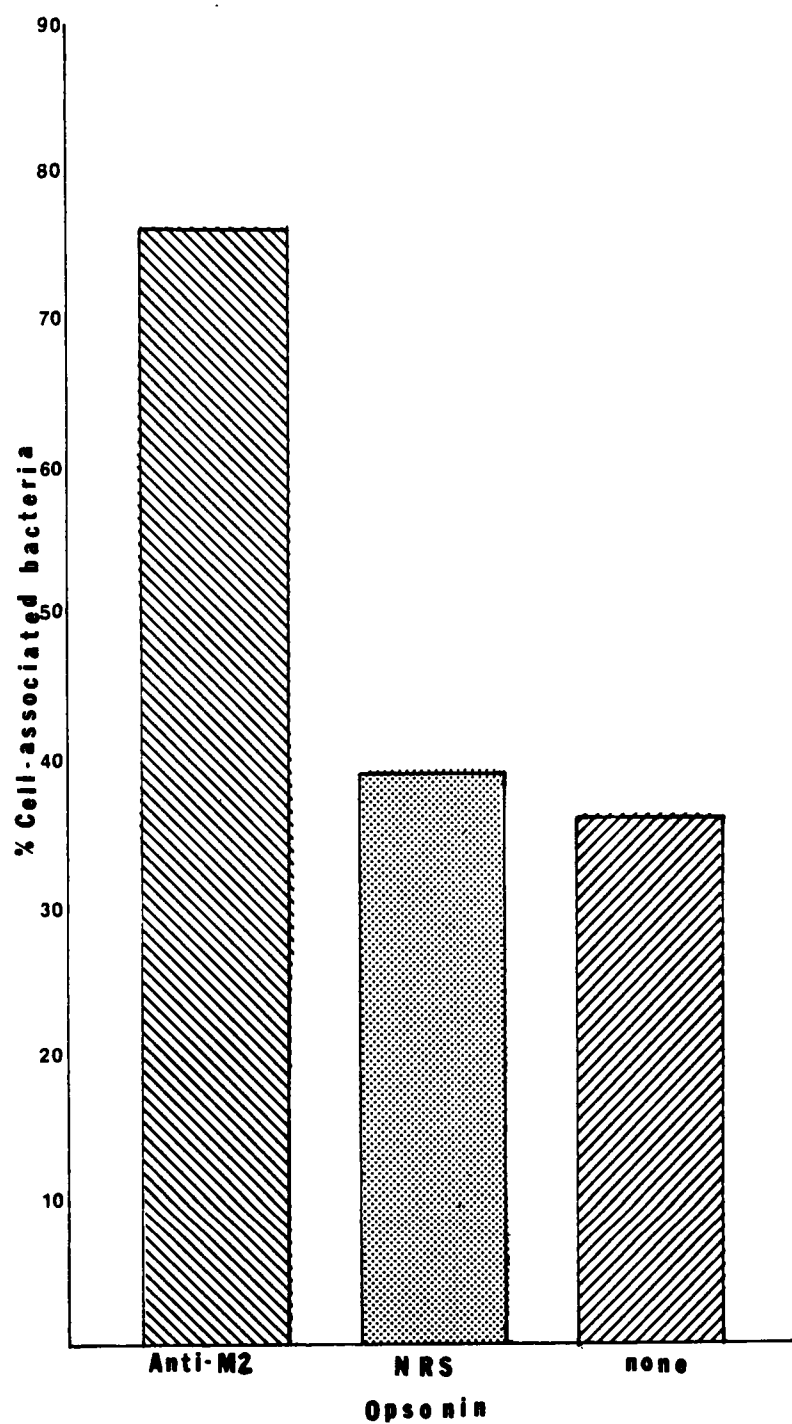


Figure 7. A Comparison of Opsonic and Non-opsonic Phagocytosis. PAO1 (b) was treated with M-2 (b) antiserum, NRS, and untreated.

treated bacteria was approximately 0.9. Strain 414ii (Mon⁻ Fla⁻) also had a fold-increase value of 0.9 when comparing anti-M-2 treated with NRS treated. Strain PAO1 had a fold-increase of 1.6 when opsonized with M-2 antiserum as compared to NRS.

Intracellular Killing

To determine the killing capacity of the PMNs, strain PAO1 was opsonized with anti-M-2, anti-1210, and NRS. In preliminary experiments, bacteria were shown to be internalized to a greater extent when opsonized with homologous serum. Approximately two to three more viable PAO1 treated with anti-M-2 were observed within PMNs at time zero than the numbers of internalized bacteria treated with heterologous or NRS. However, no decreases were observed in bacterial numbers within PMNs within 90 min indicating that killing of the bacteria did not occur. This could possibly be due to the lack of fresh serum as a complement source. This is consistent with preliminary data which showed that heat-treated serum (HTS) (30 min, 56°C) with inactive complement resulted in approximately the same rate of phagocytosis as untreated serum. By 10 min, 81% of PAO1 opsonized in M-2 antiserum were phagocytized compared to 86% of PAO1 internalized when opsonized in HTS. Comparable results

were obtained when the bacteria were treated with NRS and HT NRS, in that approximately 20% uptake of bacteria occurred with both treatments.

Chapter 4

Discussion

Phagocytosis and intracellular killing by circulating polymorphonuclear leukocytes serves as one of the major defense mechanisms by which an animal delays the spread of organisms throughout the body. The ability of a phagocytic cell to engulf and subsequently kill organisms is greatly enhanced by the presence of opsonins - both heat-stable immunoglobulin and heat-labile complement components.

Optimal phagocytic function is essential in the case of *P. aeruginosa* infections since most strains are resistant to the bactericidal effects of serum (72, 74). The importance of the bactericidal effects of serum is inferred by the observation that Gram-negative isolates from localized infections are usually serum sensitive, whereas bacteremic isolates are serum resistant (73). With the exception of rough, mucoid isolates from chronic cystic fibrosis patients, the majority of *P. aeruginosa* isolates are serum resistant. It has been proposed that smooth strains which exhibit long lipopolysaccharide O side chains are able to resist killing by their ability to prevent the stable insertion of the complement complex C5b-9 into the outer membrane (32, 56).

This mechanism has been proposed for other Gram-negative bacteria.

With *P. aeruginosa*, one of the major functions of IgG appeared to be as an opsonin for phagocytosis, since most strains are resistant to phagocytosis without antibodies (9, 10, 72, 74). Antibodies enhance the uptake of microorganisms, but a requirement for complement is necessary for the stimulation of intracellular killing. This has been demonstrated for *Proteus mirabilis* (24). Fresh serum containing both IgG and C3b is necessary for optimal killing of *Staphylococcus aureus* and *Escherichia coli* (35). In addition, maximal killing by PMNs of *Candidia albicans* occurred only with the addition of fresh serum as a complement source (47). Previous researchers proposed that the interaction of phagocytic (PMN) cell surface receptors with antibody and complement component C3b occurs so that particle-bound C3b is required for adherence of the microorganisms to the phagocytic cell. The Fc region of bound IgG appeared to serve as a trigger for internalization of the organism (35, 56). The addition of Fc fragments or Fab fragments inhibited internalization without affecting binding of the organism to the phagocytic cell. Intracellular killing, however, was not determined by these researchers.

An organism's susceptibility to phagocytosis can be divided into three categories based on the requirement of opsonins: (1) no

opsonins, (2) fresh normal serum or heat-inactivated immune serum, or (3) specific antibody and heat-labile serum factor (25). *P. aeruginosa* reportedly requires both specific antibody and a heat-labile serum factor for optimal phagocytosis and killing by PMNs (74). While antibody and complement-dependent phagocytosis and killing of *P. aeruginosa* are the most influential factors preventing colonization and dissemination of the organism, the role of extracellular and somatic factors in infection cannot be excluded, and the combined effects of virulence factors serve to increase the pathogenicity of *P. aeruginosa* immunocompromised patients. The importance of phagocytic cells in circumventing infection is emphasized by the finding that leukopenic patients have an increase susceptibility to *P. aeruginosa* infections (53).

Specific antibody to a variety of cell surface components can passively protect animals against challenge by *P. aeruginosa*. Flagellar antibodies (IgG) are protective in burned mice against challenge by virulent strains (18, 41). This protection is specific for the flagellar type and was eliminated in leukopenic mice which led to the hypothesis that clearance of the organism from wound sites depended on opsonization of the bacteria by antibody and subsequent recognition by PMNs.

In this study, the capability of flagellar antiserum to serve as an opsonin and the specificity of opsonization were determined using two different phagocytosis assays. Rabbit anti-flagella IgG

was shown to be opsonic and to increase the phagocytosis of *P. aeruginosa* by mouse PMNs when compared to NRS. Maximum uptake occurred by 20 minutes. The ability of specific flagella antibody to enhance phagocytosis correlated with previous results demonstrating the protective efficacy of antiserum against serum-resistant, virulent challenge strains (18, 41). Opsonization was specific for the flagellar type (a or b) in that addition of homologous antiserum increased phagocytosis four to seven fold whereas heterologous antiserum had a marked decrease in opsonic ability when compared to the homologous serum (Figures 2 and 3). These results were observed in an indirect phagocytosis assay in which the viable bacterial counts decreased in the supernatant of a mixture consisting of opsonized bacteria and PMNs was observed.

Initial attempts to initiate phagocytosis consisted of using 1-5% immune serum in the assays. These concentrations resulted in aggregation of the bacteria and made interpretation of the results difficult. By reducing the serum concentration to 0.1%, aggregation did not occur, but phagocytosis was still enhanced. It has been reported that serum concentrations as low as 0.1% resulted in ingestion of *E. coli* and *S. aureus*; however, a minimum concentration of 2.5% was required for killing of *S. aureus* and 0.5% for *E. coli* (35).

The phagocytic cells used in these assays consisted of approximately 73% PMNs and 27% macrophages. It was not

possible to purify the mixture using a density centrifugation method. Although it is not possible to rule out the contribution of macrophages to these results, protection against *P. aeruginosa* appears to be derived mainly from PMNs (18, 53) and would support the assumption that these cells are the major line of phagocytic defense against the organism.

Transmission electron microscopy was used to verify uptake of bacteria within PMNs. Thin sections of PMNs showed internalization of bacteria opsonized with homologous flagella antiserum. Bacteria opsonized with heterologous flagella immune serum or NRS were not observed in PMNs, however, these results should not be attributed solely to the absence of uptake since it is possible that the number of bacteria internalized was small enough that greater numbers of PMNs would have needed to be examined in order to demonstrate uptake. These observations did, however, serve to confirm that bacteria were indeed phagocytized and not simply attached to the surface of the PMNs.

For further confirmation and definition of the specificity of the opsonic property of flagellar antiserum, a direct phagocytosis assay was used in which bacteria were labeled with [^3H] thymidine. The cpm of internalized bacteria was used to calculate the percentage of leukocyte associated bacteria and the fold increase in phagocytosis of immune serum-treated over NRS-treated bacteria. These results confirmed the opsonic capability

of flagella antiserum and its specificity for the flagella type in enhancing phagocytosis. In addition, the ability of an a-type antiserum to opsonize several a-type bacteria with varying sub-antigen types demonstrated the presence of a common cross-reactive a_0 sub-antigen.

A common cross-reactive a_0 sub-antigen has also been demonstrated in passive protection studies (18). The presence of the cross-reactivity would allow incorporation of only two antigens in a potential flagella vaccine - one a- and one b-type. The cross-reactivity of a-type flagella has also been determined in an ELISA using formalin-killed whole bacterial cells to detect flagella types. This assay is an adaptation of the method used to detect isolated flagella antigen and could be applicable to the detection of *P. aeruginosa* in patients' sera. In order for an antigen to be used for detection, it must be produced in the disease state and must be available for antibody production. The ELISA technique has been adapted to detect the production of several *P. aeruginosa* products including protease and exotoxin A (30) and alginate (12); however, these products are not always produced in the disease state and may be inappropriate as detectible antigens. The O antigen of the lipopolysaccharide has also been used to detect *P. aeruginosa* in an ELISA (60), although the presence of 17 different O immunotypes limits the use of this antigen for

detection. Outer membrane protein (OMP) detection has more potential (23, 38); however, the feasibility of using this antigen clinically for detection may be limited since some of the organisms used in these studies may overproduce the OMP and may not be representative of clinical isolates. Flagella of *P. aeruginosa* are produced by 95% of clinical isolates (2, 5, 34, 46), induce protective IgG antibody in rabbits (41), and could be potential antigens for use in a detection method for *P. aeruginosa*. Likewise, the detection of *Salmonella* flagella by ELISA has been shown to be both sensitive and specific for the flagella (8).

The inability of PMNs to kill ingested bacteria could be attributed to the absence of fresh serum as a complement source. The absence of complement in the assay system is also supported by preliminary experiments which show that heat-inactivated serum enhances phagocytosis to approximately the same extent as untreated serum. This would appear to indicate that specific antibody is mainly required for uptake of bacteria. The effects of complement component C3b need to be further studied in order to determine its role in phagocytic uptake and killing. In this case fresh unabsorbed serum should be used in studies.

Flagella and motility are required for invasive virulence of *P. aeruginosa* (19), and in addition to aiding in dissemination of the bacteria from a wound, flagella may also exert an inhibiting effect against phagocytosis by PMNs. The lack of killing could also be

partially related to a protective mechanism of the flagella itself. Intracellular killing assays could be done to determine the protective capacity of flagella by comparing Fla⁻ mutants with the parental strain.

Preliminary experiments were performed to test whether mutants lacking a filament, but showing a positive colony blot (Mon⁺) would react in the opsonophagocytosis assay. The uptake of a Mon⁺ Fla⁻ strain opsonized with homologous serum was approximately the same as NRS treated bacteria. While homologous immune serum would have been expected to increase phagocytosis to some extent, it is possible that Mon⁺ Fla⁻ strains do not have an adequate amount of reactive antigen which is exposed to an extent which it can be recognized by antibody and subsequently by the Fc receptor of the phagocytic cells. The excretion of the monomer into the medium so that only small amounts are present on the cell surface to react with antibody could also explain the results obtained. It is also possible that the polyclonal antibody favors reaction with the polymer filament rather than the monomeric form of the flagella. Further studies to elucidate the nature of the phagocytic interaction with the Mon⁺ Fla⁻ strains would need to be performed.

Non-opsonic phagocytosis of *P. aeruginosa* mucoid strains is attributed to changes in surface characteristics (i. e. the presence of pili and hydrophobicity) of the strain (61, 62, 63). Macrophage

cationic proteins have also been shown to alter the cell surface LPS and morphology of the outer membrane of *P. aeruginosa*. These alterations resulted in increased phagocytosis when compared with untreated bacteria (54). Comparison of the differences in phagocytosis of Mon⁺ and Mon⁻ strains in relation to cell surface changes could also be studied in addition to the effects of opsonins on these types of organisms.

In summary, *P. aeruginosa* flagella antiserum was shown to enhance phagocytosis of the organism in a manner specific for the flagella type. Cross-reactivity among a-type flagella correlated with previous data indicating a common a₀ sub-antigen and would support the necessity of only two antigens in a potential vaccine - one a- and one b-type. The ability of flagella antiserum to opsonize *P. aeruginosa* and subsequently enhance phagocytosis would serve as an important mechanism in host defense using passively transmitted antibodies.

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