To the Graduate Council:

I am submitting herewith a dissertation written by David Ray Drake entitled "Motility and Virulence of Pseudomonas aeruginosa; Passive Protection with Flagellar Antisera." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Microbiology.

Thomas C. Montie

Thomas C. Montie, Major Professor

We have read this dissertation
and recommend its acceptance:

Alfred M. Legendre

Robert N Moore

Barry Tkacz

David A Bemis

Accepted for the Council:

Lawrence

Vice Provost
and Dean of The Graduate School

MOTILITY AND VIRULENCE OF *PSEUDOMONAS AERUGINOSA*; PASSIVE PROTECTION WITH FLAGELLAR ANTISERA

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

David Ray Drake

March 1986

DEDICATION

Over the last five years, a lot of hard work and sacrifice have taken place in completing the research and the writing of this dissertation. Although I have invested many hours of work, the sacrifice was suffered by my family. My wife, Barbara, had to endure many hours of loneliness as I was always working in the laboratory. Although we were apart for so many evenings, she was always understanding and patient. Her sacrifice at times seemed unending; yet, she always offered her love and support. She was my strength through the many disappointments and setbacks. She was my biggest fan, and I love her dearly.

At times, it was difficult trying to explain to my daughter, Theresa, why her father had to be away so much. While it was hard for me to always be gone, I realize her sacrifice was so much greater. I know there isn't any way for me ever to repay my family for being a part-time husband and father over the last five years. Therefore, even though the gesture is wholly inadequate, I dedicate this dissertation to my wonderful wife Barbara, and my children, Theresa Lynn and Alexander Ryan. This accomplishment is as much theirs as it is mine. Barbara, Theresa, Alex, . . . this is for you.

ACKNOWLEDGEMENTS

Several people have helped me in one way or another on the long road in completing this dissertation and deserve mention.

I wish to thank my committee members, Drs. Montie, Moore, Bemis, Rouse, and Legendre for their guidance, helpful criticisms, and support throughout this project. A special thanks goes to Dr. Legendre for joining my committee at such a late date.

I convey sincere gratitude towards my major professor, Thomas C. Montie, for his encouragement, support, and especially his friendship. I wish him much success in his research and will always remember my time working with him.

Becoming almost a fixture in the laboratory, I have seen many people come and go. I thank Becky, Janice, Joyce, Melissa, and especially Mary Anne and Greg for their friendship. I wish to express special thanks to Byron Stover who became a wonderful friend. I thank David Horohov who was a companion both at Purdue and here at UT. I also thank Burke Martin for his friendship and wonderful introduction to quality bird calls.

The current generation of people in Dr. Montie's lab have been wonderful even while suffering through my moodiness in preparing this dissertation. Dr. Helen Sellin has become an instant friend; I thank her for her wonderful optimism and cheery support. I wish to thank Otto Slater also for his friendship and excellent sports analyses and discussions. I convey thanks to Sarah Edmonds for her friendship as well; she deserves special thanks for sharing her Western blot

technique and other technical assistance. Jane Raulston has become a very special friend to me. I thank her for sharing her hopes, dreams, aspirations, and ups and downs with me. She will always be a special friend. I wish to thank Fathers Chuck and Pete and all my friends at John XXIII Catholic Church for their continued support. All of these people are very dear to me; I will miss them all and will always cherish the memories.

I wish to thank Janet Lathey for sharing her ELISA technique with me and Sandi Woolf for her excellent blood work.

Finally, sincere gratitude is extended to my parents and in-laws for their love and wonderful financial support. I would have never been able to complete my studies without their help.

ABSTRACT

The role of motility as a virulence factor in Pseudomonas aeruginosa burn wound sepsis was examined using mutants deficient in the Fla or Mot phenotype. Differences in physiological profiles, antibiograms, O antigen types, growth rates, and proteolytic activity were not observed in the Fla^- and Mot^- mutants, providing evidence for the similarity of these strains. Animal susceptibility studies showed that Fla^- and Mot^- mutants are significantly less virulent than the wildtype, parent strains by subcutaneous, topical, and intraperitoneal routes of challenge. Tissue colonization experiments revealed a characteristic, rapidly systemic infection in burned mice challenged with wildtype organisms. Nonmotile cells similarly proliferated in the burn wound, but the characteristic bacteremia and systemic invasion was markedly absent. The infection remained localized in the skin wound and the mice survived. Non-motile bacteria appeared in the liver following i.p. challenge at much higher levels than motile cells, suggesting a difference in the rate of clearance from the peritoneal cavity.

Significant protection against burn wound sepsis was achieved with flagellar antisera. Cross-protection experiments revealed that protection was not only H antigen dependent but specific for the flagella antigen type. Antiserum raised against a-type flagellin would only protect against homologous bacterial challenge and not against b-type flagellated strains, and vice versa. The specificity of flagellar antisera was also demonstrated in cross-agglutination assays,

motility inhibition, Western blot analyses, and an ELISA. Reactivity was not detected against somatic antigens.

Motile bacteria colonized the burn wounds of passively-protected mice to similar levels as seen in non-protected animals, but remained localized and did not cause systemic infection, a pattern remarkably similar to infections with mutants deficient in motility. Animals rendered neutropenic were not protected with flagellar antisera, indicating an important role for phagocytic cells in protection. Microscopic studies revealed that motility is directly inhibited by a specific action of IgG antibody with the flagellum. Antiflagellar antibody is hypothesized as exerting its protective capacity by (1) inhibiting the motility of invading bacteria by a cross-linking, agglutination reaction and (2) acting as an opsonin, targeting immobilized cells for phagocytosis.

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INTRODUCTION

General Introduction

Pseudomonas aeruginosa, a gram-negative bacillus, was isolated by Gessard in 1882 and described as a microbial pathogen by Charrin in 1890 (Stanley, 1947; Bodey *et al.*, 1983). This organism has since been characterized as an aerobic, gram-negative short rod that is physiologically versatile (Doggett, 1979). It grows on a number of carbon sources and can be found in many different surface waters and soils; as such, *P. aeruginosa* is described as being ubiquitous in nature (Doggett, 1979; Cross, 1979).

Within the last 20 or so years, *P. aeruginosa* has become established as a highly successful opportunistic pathogen. It is recognized as one of the most serious nosocomial and iatrogenic pathogens (Doggett, 1979; Bennett, 1974). In the hospital surveillance program of the Center for Disease Control, 10% of urinary tract infections, 9% of surgical wound infections, 17% of lower respiratory tract infections, and 11% of bacteremias were caused by *P. aeruginosa* (Bennett, 1974). Moreover, it is the most common pathogen recovered from patients who have been hospitalized for more than a week (Bodey *et al.*, 1983).

Normal, healthy individuals are not at risk of infection by *P. aeruginosa*. However, patients who are rendered immunocompromised due to burn injury, surgical procedures, chemotherapy for cancer, diabetes, genitourinary manipulation, chronic lung and heart diseases are all

susceptible to P. aeruginosa infection. A common factor among many of these patients is an interruption of the natural barriers that protect against invasion. Subsequent invasion of tissues can result in devastating infection. This general pathogenic process is particularly observed in cancer and burn patients.

Patients are frequently neutropenic with acute leukemia from the radiation and chemotherapy effects of cancer treatment (Schimpff, 1970; Schimpff et al., 1974; Tapper and Armstrong, 1974; Froland, 1981). Neutropenic patients are exceedingly susceptible to bacterial infections. In studies of nosocomial infections, patients with leukemia are approximately 15 times more susceptible to infection than the average hospital population (Schimpff, 1970; Schimpff et al., 1974; Tapper and Armstrong, 1981). There are positive correlations between low polymorphonuclear leukocyte counts, and high infection rates. From these studies, it has been surmised that one of the most serious characteristics of P. aeruginosa infection in cancer and burn patients is the overwhelming propensity for the organism to become bacteremic; in fact, P. aeruginosa has been consistently associated with the highest case fatality rate for any of the bacteremic gram-negative rod infections which occur in compromised patients (Doggett, 1979; Cross, 1979). An examination of why this organism becomes rapidly bacteremic, particularly in burn patients, involves identification and study of a number of cellular and extracellular products that have been associated with P. aeruginosa virulence. A review of these is appropriate in attempting to elucidate the reasons why this organism is highly invasive, a hallmark of P. aeruginosa infection.

Virulence Components of Pseudomonas aeruginosa

Cell Wall Antigens. Pseudomonas aeruginosa, like most other gram-negative bacteria, has a cell envelope structure consisting of an outer membrane, a peptidoglycan layer, and an inner cytoplasmic membrane. Some strains also produce a substantial capsule or glycocalyx, defined as the specific polysaccharides located outside the oligosaccharide portion of the cell wall lipopolysaccharide (Cross, 1979; Doggett, 1979). The cell envelope is apparently of importance in the persistence and growth of this organism at sites of infection (Bodey et al., 1983; Cross, 1979; Doggett, 1979). Some components of the cell envelope control adhesion and microcolony formation, while others seem to play a role in the control of the passage of nutrients and, importantly, the exclusion of humoral and synthetic antibacterial agents from the bacterial cell.

The slime of P. aeruginosa has been described as a macromolecular complex that covers the entire surface of the organism (Liu, 1979; Sensakovic et al., 1974). It is not synonymous with a capsule as it is not rigid or well defined, does not stain negatively with capsule strains, and readily disperses from the bacteria in liquid batch culture (Liu, 1979; Dimitracopoulos et al., 1974). The role of slime in the pathogenesis of P. aeruginosa was described by Sensakovic and Bartell (1974). It was shown to exhibit considerable toxicity upon injection into mice. Additional studies by Dimitracopoulos et al. (1974) and Schwarzmann and Boring (1971) revealed that slime is produced in vivo and has significant antiphagocytic properties on rabbit neutrophils. Moreover, immunization of animals with purified

slime appears to provide a type-specific immunity against viable bacterial challenge (Liu et al., 1961; Almt and Bass, 1967a, b; Bartell et al., 1970).

Slime has also been associated with mucoidy, a characteristic associated with P. aeruginosa strains isolated from cystic fibrosis (CF) patients. It is now widely recognized that the majority of CF patients harbor strains exhibiting this phenotype (Pollack, 1984). The slime polysaccharide layer is very heterogeneous; however, a major component has been identified as alginate. The role of this extracellular polysaccharide in chronic CF infections is somewhat controversial. However, it is likely that it contributes to the virulence of P. aeruginosa in this clinical setting in some manner. It is not known if this is the same material identified as slime in earlier studies.

The role of slime in virulence of P. aeruginosa in non-cystic fibrosis infections is still somewhat obscure. The purified material is toxic in itself and appears to protect the organism against phagocytosis. Active immunization with a glycolipoprotein component of slime protects against lethal challenge of some P. aeruginosa strains. Also, protection has been achieved following administration of rabbit anti-glycolipoprotein serum (Sensakovic et al., 1977; Dimitracopoulous et al., 1980). However, production of large amounts of this material does not directly correlate with increasing virulence and some high slime producing strains have been characterized as having low virulence (Liu, 1979).

Since the isolation and early description of P. aeruginosa in 1882, a vast number of reviews have dealt with the lipopolysaccharide (LPS) or endotoxin moiety, which was originally thought to be responsible for the pathogenesis of this organism. Early studies by Charrin (1890) demonstrated the toxicity of cell-free filtrates of this organism (Liu, 1979). Ensuing studies over the next three decades seemed to indicate that the pathogenesis of P. aeruginosa was mostly due to heat-labile extracellular toxins (Liu, 1966b, 1979). However, the importance of these results declined and much emphasis was placed upon the role of LPS as the major virulence component. The toxicity of P. aeruginosa endotoxin appears to be considerably less than classical E. coli endotoxin; however, significant differences observed between preparations described in the literature apparently were due to differences in the techniques used for extraction. In fact, studies done by Dyke and Berk revealed that five commonly employed methods used resulted in five endotoxin preparations with widely varying toxicities (Liu, 1979). Concluding from these studies, it appeared that P. aeruginosa endotoxin is weak at best when compared to other gram negatives and plays a less important role in the pathogenesis of this organism than other components.

However, recent studies have indicated that the role of lipopolysaccharide as a virulence component may not have been properly addressed (Cryz et al., 1983a, 1984).

Several lines of evidence suggest that lipopolysaccharide actually contributes substantially to P. aeruginosa virulence including the fact that LPS may confer serum resistance (Young, 1972) and that antibody to

LPS has been shown to be highly protective in experimental models (Cryz et al., 1983a, 1984). An elegant study by Cryz et al. (1984) revealed that an extensively characterized LPS mutant, derived from a smooth strain, was comparatively nonvirulent with a 50% lethal dose greater than 1,000 fold higher than that of its parent. Moreover, the LPS mutant was unable to establish an infection and was serum sensitive. These data suggest that an intact, smooth LPS is required for P. aeruginosa to express full virulence.

Another cellular component that has been implicated as a virulence factor is a leukocidin as described by Scharmann (1976a, b). This cell-bound substance was shown to be toxic to leukocytes only and caused considerable morphological destruction; hence, it was designated as a leukocidin by analogy with the well characterized Panton-Valentine leukocidin of Staphylococcus aureus. Leukocidin is cell-bound in the form of a precursor toxin with very low toxicity. It is converted into a highly toxic form by the action of endogenous proteases. Isolation and purification of leukocidin is accomplished by autolysis of batch culture cells, ammonium sulfate precipitation, and gel filtration (Scharmann, 1976). Purified, leukocidin damaged all tested leukocytes and some tissue culture cells in vitro, but was ineffective against erythrocytes and thrombocytes (Scharmann, 1976a, b). A lethal dose for mice by intravenous injection was determined to be 1 ug (Scharmann, 1976a,b).

Additional characterization of P. aeruginosa leukocidin revealed that as little as 13-20 nanograms of crystallized toxin was cytotoxic for polymorphonuclear leukocytes (Hirayama et al., 1983). Time course

experiments examining the degradation of labeled, membrane phospholipids in leukocytes exposed to leukocidin were performed to gain further understanding of the mode of action of this substance (Hirayama and Kato, 1983). The initial action of leukocidin was a stimulation of phosphatidic acid production and concomitant degradation of polyphosphoinositides. The authors hypothesized that this activity might be related to a calcium movement from extracellular and intracellular spaces, resulting in the activation of calcium-dependent enzymes in the leukocidic process.

The role of leukocidin in the pathogenesis of P. aeruginosa is not clear. By selectively killing polymorphonuclear leukocytes, this substance may enhance the evasion of the phagocytic line of defense of the host and allow for the colonization and spread of this organism. Its general importance is questionable however, in that only 4 out of 110 strains tested have leukocidin activity (Scharmann, 1976a, b).

Exotoxins. As previously discussed, P. aeruginosa elaborates various toxic fractions that are demonstrable in vitro and also in experimental animals (Liu, 1966a,b, 1973, 1974; Pavloskis 1974; Woods and Iglewski, 1983; Armstrong et al., 1971; Young, 1980; Homma, 1980). Special attention has focused on exotoxin A, a heat-labile extracellular protein first described by Liu et al. (1961, 1973). Exotoxin A has been shown to be the most toxic product of P. aeruginosa with a mean lethal dose so in mice of 0.2 ug (Liu, 1974; Pavlovskis et al., 1974). Its toxicity is due to its ability to inhibit protein synthesis in susceptible cells by catalyzing the ADP-ribosylation of

elongation factor 2 (EF-2) (Iglewski and Kadat, 1975; Nicas and Iglewski, 1985; Homma, 1980). Enzymatic activity of toxin A is absent from native toxin, but can be initiated by a variety of treatments including proteolytic nicking and partial denaturation and reduction (Woods and Iglewski, 1983).

Because of the lethal properties of exotoxin A, a considerable amount of effort has been directed towards examining the role of this protein toxin in the virulence of P. aeruginosa in animal models. To detect whether or not exotoxin A is produced and elaborated in vivo, investigators utilized the inhibition of EF-2 in serum and tissue homogenates as an assay for toxin. Exotoxin A is produced by P. aeruginosa multiplying in the burn wound of a mouse (Saelinger et al., 1977). The toxin was detected in extracts of infected burn skin only and not burn controls. Moreover, exotoxin A activity was found in the serum, liver and spleen of infected mice. These results suggested that bacteria elaborate the toxin upon rapid multiplication in the burn site, that it appears in the bloodstream in increasing amounts in correlation with the increasing bacterial load in the skin, and that protein synthesis of the liver and spleen are significantly depressed (Saelinger et al., 1977; Holder, 1985). Because of the involvement of this extracellular product in the pathogenesis of P. aeruginosa, it was suggested that active or passive immunization may serve as a useful therapeutic regimen (Saelinger et al., 1977; Holder, 1985; Cross et al., 1980).

Excitement generated by the possible use of exotoxin A as a wholly protective immunogen was soon diminished. Two independent studies

revealed that neither active or passive immunization delivered long term protection (Snell et al., 1978; Pavlovskis et al., 1981). Active immunization with toxoid preparations did increase the mean time to death, but no long duration survival was accomplished (Snell et al., 1978; Pavlaskis et al., 1981). Moreover, long term survival of mice receiving antitoxin antibody could be obtained only if the infected mice received simultaneous antibiotic therapy (Cryz et al., 1983a; Pavlovskis et al., 1977). Antitoxin therapy did apparently neutralize toxin that was produced by the organisms colonizing the burn wound, since protein synthesis in the major organs was not inhibited in treated animals. This suggests, therefore, that toxin A, although an important virulence component, is not the sole virulence determinant involved in fatal P. aeruginosa infections in burned mice (Holder, 1985).

Another toxin moiety, designated exoenzyme S, was initially described by Bjorn et al. (1979). This extracellular protein differs from toxin A and diphtheria toxin in that it does not adenosine diphosphate (ADP)-ribosylate elongation factor 2, but rather catalyzes the transfer of the ADP-ribose moiety of nicotinamide adenine dinucleotide to a number of different proteins extracted from eucaryotic cells (Iglewski et al., 1978; Sokol et al., 1981). It also differs from toxin A in its heat stability and its inactivation, rather than potentiation, by pretreatment with urea and dithiothreitol (Iglewski et al., 1978; Sokol et al., 1981). Exoenzyme S has been shown to be elaborated in vivo in a burned mouse model (Bjorn et al., 1979). Analysis of 124 clinical isolates of P. aeruginosa revealed

that whereas 80% of the strains tested produced toxin A, only 38% produced toxin S (Sokol et al., 1981).

An elegant study utilizing a transposon (Tn1) induced mutant strain deficient in only exoenzyme S production has provided evidence for the role of this product in virulence (Nicas and Iglewski, 1985). This mutant and others were tested in different accepted animal models and it was discovered that the loss of exoenzyme S resulted in significant decrease in virulence. This was particularly apparent in burn wound infections where a 2,000-fold difference was observed between the parent and the mutant. These genetic studies therefore have identified exoenzyme S as an important virulence factor of P. aeruginosa.

Proteases. A well established characteristic of most strains of P. aeruginosa is that they are proteolytic (Morihara, 1964; Liu, 1974, 1979; Bodey et al., 1983; Holder and Haidaris, 1979; Janda et al., 1980). Liquefaction of gelatin, clearing of skim milk, digestion of hemoglobin, and dissolution of fibrin, elastin, and collagens have all been used as assays for P. aeruginosa proteases (Liu, 1979). The first study examining the general role of proteases as virulence components demonstrated an association between hemorrhagic and necrotic skin lesions and protease activity (Liu, 1966a). By using strain PA103, a relatively nonvirulent strain ($LD_{50} > 10^5$ cfu) in the burned mouse model that produces toxin A, but only small amounts of alkaline protease and elastase, Holder and Haidaris (1979) were able to show that supplementation of a nonlethal challenge dose of PA103 with

alkaline protease or elastase caused significant increases in mortality. Furthermore, a significant reduction in protein synthesis of the liver of the PA103 protease supplement group was observed compared to the PA103 or protease alone groups (Snell et al., 1978). These data collectively indicate that proteases are also important virulence determinants of P. aeruginosa.

Another approach in defining the role of proteases in the pathogenesis of this organism utilized specific protease inhibitors (Holder and Haidaris, 1979; Holder, 1983). These studies revealed that localized treatment (at the burn site) of P. aeruginosa infected mice with the serum protease inhibitor, alpha macroglobulin, which inhibits both P. aeruginosa alkaline protease and elastase, provided a significant enhancement of survival compared to controls. Whereas 100% of non-treated or albumin treated mice succumbed to infection, only 21% of the protease inhibitor-treated mice failed to survive (Holder, 1983).

Still another approach in examining the role of proteases as virulence components of P. aeruginosa has made use of protease-deficient mutant strains (Pavlovskis and Wretlind, 1979). These studies showed that the LD_{50} of these mutants is approximately one log higher than the wild type parent when tested in mice burned according to the method of Stieritz and Holder (1975). Moreover, consistently fewer viable bacteria were observed in the blood of mice infected with protease-deficient strains than those infected with the parent organisms. Collectively, then, these data provide

additional support for the role of proteases in the virulence of P. aeruginosa.

How exoproteases serve as virulence factors is still unclear. These exoenzymes were shown to cleave serum components and serum alpha 1-antitrypsin (Schultz and Miller, 1974; Morihara, et al., 1979). Also, it was reported that P. aeruginosa elastase can degrade human immunoglobulin G (IgG) in vitro (Holder, 1985). All of these components are important for normal host defenses; therefore, a role for the proteases can be envisioned as substances that specifically reduce host resistance capabilities.

Another possible role for proteases is that, as extracellular enzymes, they may provide nutrients for growth of P. aeruginosa. These enzymes could degrade large protein molecules into small peptides which could be easily transported into the cell. Proteases may also serve to provide nitrogen and carbon to the organism in cases where proteins are the sole source of these requirements. Alternative nitrogen sources have been shown to decrease protease production by P. aeruginosa (Liu, 1973). However, more recent studies have shown that sodium salts are at least as effective as ammonium salts in inhibiting protease production (Kessler and Safrin, 1983). It was concluded that the well documented inhibition by ammonium ions represents a non-specific effect, most likely related to the high ionic strength of the medium. Support for the hypothesis that extracellular proteases help provide nutrients was provided by a study conducted by Cicmanec and Holder (1979). A wild-type, protease (+) strain grew equally well in normal skin and burn skin extracts whereas a protease (-) P. aeruginosa strain

(PA103) exhibited a much reduced growth rate in burned skin. Supplementation of exogenous protease to PA103 and the burned skin resulted in a significantly more rapid growth rate, comparable to the parent strain. Moreover, the addition of ammonium sulfate, an inhibitor of protease activity, and/or production reduced the mean doubling time of the parent strain in burned skin extracts to that of the protease (-) strain. These results further indicate that proteases may serve as virulence factors of P. aeruginosa by modifying the available nutrients in burned skin and thus permitting an enhanced growth rate and a more rapid establishment of the infection in the host (Cicmanec and Holder, 1979).

Pseudomonas aeruginosa Infections in Burn Patients

Types of Infections. Every year, more than two million Americans are burned, 100,000 of them severely enough to require hospitalization (Gelfand, 1984). Despite many advances in medical research, burn injuries remain a serious cause of morbidity and mortality (Gelfand, 1984; FrØLand, 1981; Pruitt et al., 1984; Mayhall, 1981). Infection is a major complication of burn injury, being the major cause of death in hospitalized burn patients.

Burn injury obviously destroys the skin and reduces this organ from a primary mechanical barrier resisting bacterial colonization to a preferred growth site (Cicmanec and Holder, 1979). Although this is the most common infection, other types of infection also occur. In debilitated burn patients, P. aeruginosa is a frequent causative agent of either hematogenous or airborne pneumonia. At one point,

hematogenous pneumonia, usually arising from an infected burn wound, was most common; however, the use of topical burn wound antimicrobial agents has reduced its incidence and airborne pneumonia is now the most common form of pulmonary infection occurring in burn patients (Pruitt and Lindberg, 1979). Hematogenous pneumonia begins as an infection of pulmonary capillaries, initiated by bacterial emboli. Autopsies of patients that have died from hematogenous pneumonia revealed multiple, hemorrhagic nodules throughout the lungs (Pruitt and Lindberg, 1979). Interestingly, it has been shown that the organisms causing bronchopneumonia are commonly those colonizing the wound site of the burn patient (Pruitt and Lindberg, 1974).

Another type of infection in burn patients is suppurative thrombophlebitis, which can occur within any previously cannulated vein (Pruitt and Lindberg, 1979; Froland, 1981). The duration of the cannulation of a vein, the type of cannula used, and the location of the cannulation site with respect to the burn wound are all important in understanding the septic complications that can occur. Suppurative thrombophlebitis is of primary concern since it can serve as a source of hematogenously disseminated P. aeruginosa in patients with hemotogenous pneumonia.

Ocular infections, such as conjunctivitis and corneal ulceration, may occur throughout the postburn convalescence. These infections can be very serious and rapidly progressive with destructive dissolution of the eye within 24-48 hours (Ohman et al., 1980). The severity of corneal damage which occurs has been attributed to extensive

proteolysis by P. aeruginosa exoproteases (Ohman et al., 1980; Pruitt and Lindberg, 1979).

There are several additional infections observed in burn patients, including suppurative chondritis, pyelonephritis, and endocarditis. The incidence of these varies, but all can be life-threatening in the immunocompromised burn patient (Gelfand, 1984).

Microbiology of Burned Skin. Life-threatening bacteremic P. aeruginosa infections in burn infections begin as localized infections of the burn wound (Pruitt et al., 1979; Holder et al., 1973). The history of burn wound microflora is interesting and reflects the development of different antibiotics. Before the development of penicillin, B-hemolytic streptococci were the organisms most commonly associated with fatal infections in burn patients. Following the use of penicillin, staphylococci became a common cause of sepsis until the development of the semisynthetic penicillins (Pruitt et al., 1979). The clinical use of these antibiotics was followed by the emergence of the gram negative bacteria, particularly Pseudomonas aeruginosa, as the most problematic pathogens in burn patients.

Examination of burn wounds over time has shown an interesting pattern. Immediately post-burn and up to 24 hrs post-burn, the flora of the burn site is sparse and predominantly consists of gram positive bacteria. However, after 24 hours, the rate of colonization of P. aeruginosa increases rapidly (Pruitt et al., 1979). Studies at the Institute of Surgical Research (Fort Sam Houston, Texas) have shown that over 20% of patients admitted at 30 hours post-burn have their

wounds colonized by P. aeruginosa (Pruitt et al., 1979). Moreover, 48% of admitted patients have wound cultures positive for P. aeruginosa, and greater than 60% are colonized by P. aeruginosa by the fifth day post-burn.

A major controversy continues over whether colonizing P. aeruginosa is from endogenous or exogenous sources. A number of studies seem to indicate that the major source of these colonizing organisms in burn wounds is the flora of antecedent patients (Holder et al., 1973; Mayhall, 1981). One investigation revealed that contact spread was the major route of contamination in a burn ward (Lowbury, 1960). Bacterial analyses demonstrated that approximately half of the nurses working in their unit carried P. aeruginosa on their hands. Strict environmental control measures and reverse isolation techniques have been shown to significantly restrict the number and kinds of bacteria colonizing the burn wound in such cases (Holder et al., 1973).

Other studies, however, have provided evidence for colonization of bacteria in the burns wounds derived from endogenous sources (Pruitt et al., 1979). An investigation at the Shriner's Burns Institute (Cincinnati, Ohio) used reverse isolation techniques to prevent environmental contamination (Holder et al., 1973). It was shown that the transfer of P. aeruginosa did not occur from one patient to another in a closely-confined ward. However, these procedures did not control the rapid colonization of burn wounds from endogenous sources, as determined by phage typing and serotyping. Chitkara and Fierabend (1981) demonstrated that the rectum was the source of P. aeruginosa

which caused burn wound infections in a group of patients. Jarret et al. (1978) have shown that the administration of antibiotics to eliminate or reduce the levels of the indigenous GI tract flora of burn patients also delays colonization of the burn wound by these bacteria. It is also now recognized that many of the bacterial infections of cancer patients may originate from their own indigenous bowel flora (Bodey, 1981). Collectively, these studies indicate that P. aeruginosa strains that colonize a patient's burn wounds may be isolated from their own intestinal microflora.

Although there is evidence that the burn wound may be colonized by P. aeruginosa from the patients' G.I. tract, the frequency of colonization by this route is still uncertain. A key point also is that it is very difficult to determine whether these isolates are part of the normal fecal microflora or are acquired after a period of time in a burn ward (Mayhall, 1981). While it is clear that the gastrointestinal tract as a possible route for infection of burn wounds cannot be ignored, the bulk of evidence indicates that surface contamination by health care personnel and environmental reservoirs provides the most important modes of spread (Mayhall, 1981; Holder et al., 1973; Pruitt et al., 1979).

Experimental Animal Models

The sequence of events of invasive P. aeruginosa burn wound infection has been delinated in three distinct animal models (Teplitz et al., 1964a,b; Stieritz and Holder, 1975). The scalded rat model developed by Teplitz et al. (1964a,b), involves subjecting the shaved

dorsal surface of Sprague-Dawley rats to a 10 second exposure of boiling water; animals are subsequently challenged with 10^8 colony-forming units (cfus) of P. aeruginosa via a topical application. The scalded mouse model follows an essentially identical procedure (Stover et al., 1985). Development of these animal models have allowed for the study of the pathogenesis of burn wound sepsis, particularly the events at the junction of the burn wound and the underlying viable tissue. Initial seeding of the wound surface is followed by a period best defined as wound colonization, characterized by a rapid proliferation of the bacteria in the eschar. Localization of bacteria to hair follicles is considered an important initial stage of wound colonization. Intra-eschar spread and multiplication of the bacteria continues until the fifth day post-burn, at which point the first signs of invasion into the underlying granulation tissue is observed. It has been shown that granulation tissue provides protection of the wound, specifically against invasion of bacteria into deeper tissues and the bloodstream (Holder et al., 1973). In addition, the presence of healthy granulation tissue is important from the standpoint of graft take. Thus, the integrity of this tissue is vital; invasion by P. aeruginosa leads to its rapid destruction, allowing for penetration into deeper tissues and subsequent bacteremia.

There appears to be a critical bacterial load in the burn wound that leads to the spread from the initial colonization site. Early studies demonstrated that systemic spread from the burn wound does not occur until the wound level reaches 10^5 cfu/gram tissue. For clinical protection the proliferation of the bacteria in the burn wound

must be controlled or maintained below this level (Moncrief et al., 1966). This is usually accomplished by topical antimicrobial agents (Pruitt and McManus, 1984).

The burned mouse model, developed by Stieritz and Holder (1975), is different from the scalded rat model in that a 10 second alcohol flame burn is employed. This has been shown to cause a partial thickness burn of approximately 30% of the total body surface area. Mice burned in this manner are highly susceptible to fatal infections by subcutaneous challenge of P. aeruginosa. Of particular interest is that burned mice are susceptible mainly to P. aeruginosa infection, and not other gram negative or positive bacteria (Stieritz and Holder, 1975). Quantitative bacterial counts of the burn eschar, blood, and various internal organs have shown that bacterial invasion only occurs when skin counts exceed 10^5 cfu/gram, results comparable to data from the scalded rat model. Thus, both the burned mouse model and the scalded rat and mouse models appear to be clinically relevant and mimic the pathogenesis of P. aeruginosa in burn wound infections.

Burn Wound Induced Host Immunosuppression

Infection is the most frequent cause of morbidity and mortality in the severely burned patient. The pronounced susceptibility of burn patients reflects myriad changes in host physiology. The destruction of viable skin not only serves as a disruption of a normally impervious barrier, it creates a select, avascular site that allows for the rapid proliferation of colonizing microorganisms. This susceptibility to infection increases in a sigmoid dose response manner as the severity

of the burn wound increases (Pruitt et al., 1984). Systemic organ dysfunction also parallels the extent of the burn injury (McMenamy et al., 1981). The predisposition to infection by opportunistic pathogens also reflects in large part the overwhelming impairment of essentially every facet of the host immune system (Gelfand, 1984). Couple to this the effect of numerous exoproducts and virulence components elaborated by P. aeruginosa and one begins to understand why these infections are so frequently fatal. Since virtually all branches of the immune system are depressed, it is best to separate a review of these processes into humoral and cellular components.

Alterations in the Cellular Response. A number of investigations have determined that burn patients (and experimentally burned animals) suffer both qualitative and quantitative alterations of their granulocytes. Studies by McManus examining total white blood cell counts and absolute neutrophil counts in scalded rats revealed a temporal pattern of peripheral leukocyte numbers similar to that observed in humans (McManus, 1983). An initial rapid leukocytosis appeared immediately after animals were burned, which peaked at one day post-burn, and then rapidly declined by 2-3 days postburn. A short-term, moderate depression was seen by 2-3 days followed by a second elevation which persisted for the remainder of the experiment (10 days). Differential counts demonstrated that neutrophils were primarily responsible for these observed elevations. Lymphocyte counts showed a significant depression early in the experiment but returned within the preburn levels by day four. Interestingly, the absolute

neutrophil counts remained above normal levels throughout the experiment. However, burned animals were found to have decreased inflammatory responses to intraperitoneal injections of various killed bacterial species. It was concluded that this hyporesponsiveness is partially responsible for the susceptibility of burn patients to infection (McManus, 1983).

A series of studies examining the phagocytic, bactericidal, and chemotactic abilities of neutrophils in burn patients and experimental animals have identified a number of alterations. The phagocytic capability of both granulocytes and alveolar macrophages is reduced after burn injury (Rittenbury and Hanback, 1967; Schmidt et al., 1983). However, the reduction in uptake, particularly in neutrophils, appears to be minor compared to the severe reduction in bactericidal activity (Grogan, 1976a). Examination of phagocytosis and killing of neutrophils from patients with severe burns (greater than 30% surface area) demonstrated a much reduced killing of many different species of bacteria (Lennard et al., 1974; Alexander et al., 1968, 1978a,b). Depression of the bactericidal activity of burn neutrophils is apparently due to significantly lower intracellular contents of lactoferrin, myeloperoxidase, and chymotrypsin-like cationic proteins (Ransjo et al., 1977). Collectively, then these studies indicate that the ability of neutrophils to kill ingested bacteria in burn patients is deficient and this loss of integrity appears to be profoundly important in the development of invasive P. aeruginosa sepsis (Alexander and Wixson, 1970a).

Comparisons of the chemotactic response of peripheral blood neutrophils between burn patients and control individuals have revealed a significant decrease in those from burn patients (Grogan, 1976b; Meakins *et al.*, 1978; Alexander *et al.*, 1978a,b). This depression was detected after approximately five days post-burn and was observed in both patients with burn wound sepsis and those not infected, although it was significantly more pronounced in the former group. The ability of neutrophils to respond to chemotactic stimuli may be very important in burn patients. Considering the overwhelming capacity of *P. aeruginosa* to spread from the burn wound, it is conceivable that a reduced *in vivo* chemotactic response could play an important role in the development of invasive infection. In fact, it has been shown that burned, infected mice can be partially protected when normal peritoneal exudate cells are administered (Markley and Smallman, 1969).

In addition to multiple defects in neutrophil bactericidal activity and chemotaxis, burned rats were observed to exhibit an apparent aberration in granulocyte regulatory function (Eurenius and Brouse, 1973). Moreover, investigation of the regulation of granulopoiesis in human burn patients has indicated a possible correlation between serum colony-stimulating factor (CSF) levels and patient survival of burn wound sepsis (Peterson *et al.*, 1983). These authors have attempted to associate severity of invasive, life-threatening infection with delayed rise in serum CSF concentrations. They concluded that patients with sluggish CSF responses experienced aberrations in granulopoiesis sufficient to predispose them to fatal infections.

Immunological changes are not limited to nonspecific cellular responses as cell-mediated immunity is also altered (Munster et al., 1972; Alexander, 1979). Studies in burned animals have shown that skin allograft rejection is delayed in proportion to burn size and that these animals are essentially anergic to certain tests of delayed hypersensitivity (Munster et al., 1972; Gelfand, 1984; Meakins et al., 1977). Also, changes in lymphocyte response to mitogenic stimulation were depressed (GuXi-ming et al., 1983). General dysfunction of cell mediated responses and elevated levels of suppressor T-lymphocytes may be important in burn patients suffering from disseminated viral infections (Foley et al., 1970).

Alterations in the Humoral Response. Humoral immune systems are also disrupted by burn injury (Gelfand, 1984). Opsonizing antibodies have been shown to be vitally important in human immunity to P. aeruginosa infections (Young and Armstrong, 1972; Young, 1972). In fact, it has been demonstrated that specific antibody significantly increases the bactericidal function of neutrophils from burn patients (Alexander and Fisher, 1970b). The presence of high levels of circulating antibody apparently partially compensates for burn neutrophil abnormalities.

Levels of fibronectin are markedly depressed after burn injury of greater than 30 percent of the body surface area. Low fibronectin levels often precede sepsis and infusion of exogenous fibronectin may improve the clinical course of infections in burn patients (Robbins et al., 1981; Gelfand, 1984). Immunoglobulin levels are depressed early

following burn injury, with IgG levels being more significantly altered (Alexander et al., 1978a,b). A massive hypocomplementemia which occurs in burn patients may have multiple effects on the immune system, including resultant deficiencies of opsonization, chemotaxis, and bacteriolysis (Gelfand, 1984). Other alterations are not as clearly defined, such as alterations in the secondary humoral response and the primary response to new antigens.

Mechanisms of Burn-Induced Immunosuppression. The high incidence of fatal septicemia associated with severe thermal injury has been shown to result from a loss of immunocompetence (Alexander and Moncrief, 1967; Alexander, 1979). Initial studies implicated the role of suppressor T-lymphocytes in burn induced immunosuppression (Miller and Baker, 1979). Evidence was provided by Ninneman and Stein (1980) that prostaglandin E_2 released by macrophages after exposure to endotoxin may be the circulating factor which acts directly in lymphocyte suppression. Other studies have described the appearance of small molecular weight immunosuppressive factors (Hakin, 1977; Ninnemann et al., 1982) and reticuloendothelial depressing substances (Loegering, 1983). These factors are characterized as small peptides, and probably represent degradation products of the burn wound. Such factors are not found in normal serum and may exert their effects through the activity of a specific suppressor population of lymphocytes (Ninnemann et al., 1982).

Monocytes and macrophages may be important in post-burn immunosuppression as described by Hansbrough et al. (1982). Their

studies demonstrated that cell-mediated immunity (CMI), as assayed by a contact sensitivity assay, was severely depressed in burned mice over a two week period. However, immediate removal of the post-burn eschar resulted in avoidance of immunosuppression. In addition, transfer of burned tissue subcutaneously into unburned normal mice caused a severe immunosuppression. A normal CMI could be restored by intravenous infusion of peritoneal macrophages from normal mice, but not burned mice. The authors conclude that suppression of CMI appears to result specifically from impairment of monocyte function. Thus, burn induced immunosuppression seems to involve numerous factors and cell interactions. It may well be that monocyte-macrophage dysfunction plays a central role and is related to the presence of burn tissue components. Macrophage products such as prostaglandin E_2 suppresses T-helper activity and augments T-suppressor function and may account for many of the immunological defects observed in the burn patient.

Treatment and Prevention of P. aeruginosa Infections

Pseudomonas aeruginosa is resistant to many different antibiotics and seems to develop resistance against new generations of antimicrobials very rapidly (Cross, 1979). Complicating this problem is the frustration in treating susceptible patients who, for the most part, have severe underlying diseases such as severe burns or acute leukemia that can be fatal. It is not surprising, that in many cases, antimicrobial therapy for *P. aeruginosa* infections is regarded as a temporary measure initiated to limit the spread of infection while

attempts are made to ameliorate the underlying disease (Cross, 1979). Because of this approach and other factors complicating antibiotic treatment of P. aeruginosa infections including the need to initiate therapy very early in the course of infection, a more feasible approach has been the development of preventative vaccines (Cryz, 1984).

The first clinically-evaluated immunotherapy regimens employed whole cell vaccines (Cryz, 1984). A substantial level of protection was observed in patients actively immunized with monovalent, heat-killed cells or passively with human hyperimmune antiserum. Protection was also achieved with a multicomponent vaccine consisting of six formalin-killed P. aeruginosa strains. However, problems with small numbers of patients treated and concurrent advances in burn wound management do not allow for absolute evaluations of effectiveness (Cryz, 1984).

Another clinically-evaluated, multivalent vaccine consists of LPS antigens extracted from strains of defined serotypes. Chemical analyses have shown that the vaccine is composed primarily of LPS. The efficacy of this vaccine has been tested in several patient populations. A number of these clinical trials have shown great promise of protection in susceptible patient groups, but serious problems may exist. In many cases vaccine evaluations were done without adequate controls and limited protection was observed in some of the trials. The vaccine's greatest limitation appears to be the adverse reactions elicited upon immunization. The severity varied in patient groups with severe reactions occurring in a substantial proportion of cancer patients (Cryz, 1984).

A third vaccine that shows promise is the polyvalent extract vaccine (PEV-01), which consists of cell surface antigens extracted from 16 P. aeruginosa serotypes with EDTA and glycine (Cryz, 1984). Therapeutic evaluations in burn hospitals in England and India have demonstrated that protection against infection can be achieved; however, several aspects concerning these clinical trials and the nature of the vaccine itself need to be noted. In the Indian trials, no topical burn wound therapy was employed, a procedure used routinely in the West that is an integral part of supportive medicine preventing P. aeruginosa sepsis. Most importantly, the PEV-01 vaccine consists of a group of unknown antigens. The composition of each of the vaccine components vary and may consist of LPS and several outer membrane proteins. Thus, its future in commercial development seems severely limited at this point.

Many other candidate vaccines have appeared within the last decade and warrant review. Most of these have been evaluated in animal models only. A series of studies have described the isolation and purification of high molecular weight polysaccharide, an apparent O antigen derivative from culture supernatants of P. aeruginosa and the use of this antigen as a protective immunogen (Pier et al., 1978a,b, 1981, 1982a,b, 1983; Pollack et al., 1984). It was observed that high molecular weight polysaccharide induced antibodies that protect mice against live bacterial challenge. It appears to be nontoxic and elicits type-specific opsonic antibody in man.

Lieberman (1977, 1978) has described the development of a P. aeruginosa ribosomal vaccine that consists of RNA-protein complexes.

Active immunization of mice with these preparations provided significant serotype-specific protection against viable, intraperitoneal challenge (Lieberman, 1978). Moreover, protection was achieved against P. aeruginosa challenge in mice administered anti-ribosomal vaccine antibody (Lieberman et al., 1980). The mechanism of protection and the exact nature of the ribosomal vaccine is still somewhat obscure.

A quite different approach has involved the development of a vaccine that does not contain any P. aeruginosa antigens (Cryz, 1984). An LPS mutant strain of Escherichia coli JS lacks O-antigen specific side chains and apparently has an exposed core region. The rationale for use of isolated JS LPS as an immunogen is that antibody is elicited against this exposed core region which is shared by several gram-negative bacteria; therefore, cross-reactive protection should be achieved. There is some evidence for such protection, but a number of independent studies have revealed little, if any protection in JS-immunized anmals and if protection is elicited, it appears to be serotype-specific (Cryz, 1984).

Rationale: Statement of the Problem

It is well established that Pseudomonas aeruginosa is a serious opportunistic pathogen in immunocompromised patients. Traditional therapy to combat both acute and chronic infections of P. aeruginosa has consisted of overwhelming antibiotic treatments (which accentuates the resistance problem), immunization of susceptible patients with toxoids and other inactivated virulence factors, and immunization with

lipopolysaccharide vaccines. Indeed, the virulence of P. aeruginosa is multifactorial and many of the extracellular products have a documented role in pathogenesis. However, immunization of experimental animals with many of these virulence components delays the time to death only and does not elicit long-term protection.

Immunization with LPS preparations have led to multiple problems of pyrogenicity and varied responses. Also, there is a requirement for complex multifactorial vaccines that might contain as many as 17 distinct O antigen types.

Previous results indicated that a broader view of pathogenicity and prevention of infection by P. aeruginosa was needed (Holder et al., 1982; Montie et al., 1982a,b). A hallmark of P. aeruginosa infection is the overwhelming propensity of this bacterium to rapidly disseminate from a local infection. Once systemic invasion occurs in a burn patient, the prognosis for survival is poor. It is hypothesized that motility is an integral component that contributes to the invasive capacity of P. aeruginosa. A possibly more rational approach would be to treat the susceptible patient in such a way as to inhibit the initial spread from a localized infection. It is this concept that has led to the novel approach of developing an anti-flagellar vaccine that prevents invasive infection by arresting motile cells (Holder et al., 1982; Montie et al., 1982a). A vaccine or specific flagellar antisera that selectively inhibits motility also would allow for more extensive examination of the invasive potential of this organism and the role of motility in the pathogenesis of P. aeruginosa.

It seems probable that the relative contribution of the many described virulence factors may vary widely, depending on the disease (Nicas and Iglewski, 1985). To investigate the role of one factor, such as motility, bacterial strains are required that differ only in the presence or absence of that one factor. The research reported herein used a series of mutants that differed only in the state of flagellation of the bacterium and/or the ability of the cells to "swim."

The specific aims of this research were focused into two central areas:

(1) Is motility a virulence factor? If a loss of motility is associated with a subsequent decrease in virulence, would nonmotile mutants cause localized infections only and not exhibit the propensity to spread? The importance of motility may vary depending on the route of challenge. The actual mechanism as it relates to virulence may reflect on differences in challenge routes and also in susceptibility differences to phagocytosis and intracellular killing. What is the role of motility in invasive burn wound infection?

(2) Initial studies have demonstrated that active protection could be achieved with partially purified flagella antigen. Is the protection specific and can protection be obtained passively with hyperimmune antiflagellar antisera?

CHAPTER II

MATERIALS AND METHODS

Bacteria

Origin and Description of Cultures. Several Pseudomonas aeruginosa strains were used in this study. Strain M-2, originally an isolate from the small intestine of a CF-1 mouse, and WR5, GNB-1, SBI-H, SBI-S, SBI-I, GNB-1, and 1210, all clinical isolates, were kindly provided by Dr. Ian Alan Holder, Shriner's Burn Institute, Cincinnati, Ohio. The Fla^- mutant derived from M-2 was obtained through ethyl methane sulfonate mutagenesis and screening as previously described (Montie et al., 1982b). The wild type strain PaO-1 and its corresponding Fla^- mutants, AK1152 and AK1153, were graciously supplied by Dr. A. Kropinski, Queen's University, Kingston, Canada. Strains MT1200 (Fla^+ Mot^+) and MT1200-80 (Fla^+ Mot^-) were a gift of Drs. M. Tsuda and T. Iino, Hongo University of Tokyo, Japan, and have been previously described (Tsuda et al., 1981, 1983a,b). Strain MT1200 was derived from PaO-1, and has been extensively characterized, genetically. The flagellar genes of P. aeruginosa PaO-1 have been shown to be clustered in two distinct regions (I and II), which are located in the very late region of the linkage map of the chromosome (Tsuda and Iino, 1981, 1983a,b). One Mot and five Fla cistrons have been identified in region I, and 10 Fla and two Che cistrons have been demonstrated in region II. Moreover, complementation tests of over 200 strains using transducing phage (E79 tv-1) have revealed 6 complementation groups in region I and at least

11 in region II. Strain MT1200 has been used in these mapping studies studies and in determining the order of the flagellar genes and has the following anabolic, catabolic and antibiotic markers: methionine, isoleucine, catechol, carnosine, rifampin. Strain MT1200-80 (Fla^+ Mot^-) was derived initially from MT1200 through ethyl methane sulfonate (EMS) mutagenesis. It has also been extensively characterized as a region II mutant, with a defect in the Fla E cistron. It is nonmotile and possesses a long, straight flagellar filament. In addition, it demonstrates nonreciprocal complementation in transductions, behaving as if the mutation is dominant to the wild-type alleles.

Strains 5142 (a type) and 5933 (a type) were originally from Collection de l'Institut Pasteur, Paris, France (Ansorg, 1978). Strains 13030 (a type), 170001 (b type), and 170018 (a type) were obtained from B. Lanyi, National Institute of Hygiene, Budapest, Hungary (Lanyi, 1970). These strains represent different flagellar types and have been characterized according to molecular weight and immunological cross-reactivity very recently (Allison et al., 1985).

Maintenance of Stock Cultures. Upon receipt of the cultures, bacteria were grown in Brain Heart Infusion Broth (BHIB) in 30 ml volumes and incubated on a gyratory shaker at 180 rpm for 15 hours at 37°C. Cells were pelleted by centrifugation (6,000 x g) for 15 minutes and resuspended in an equivalent volume of sterile BHIB. One milliliter aliquots were dispensed into ultralow freezer vials

consisting of 0.5 ml washed cells and 0.5 ml 50% glycerol (in distilled water). Vials were stored frozen at -70°C.

Day to day stock cultures were maintained as dilute suspensions of bacteria in BHIB (8 mls per tube) at 4°C. Stocks were renewed every 2-3 months by inoculating 30 mls of Brain heart infusion broth with 0.1 ml of the appropriate stock culture and incubating on an orbital shaker at 180 rpm, for 15 hours at 37°C. New stocks were prepared by inoculating fresh tubes of Brain heart infusion broth with 0.5 ml of the overnight cultures.

Construction of Standard Curves. Working standard curves related the log of viable bacteria to absorbance at 590 nanometers. Such curves provided for estimates of the number of viable cells in prepared suspensions of bacteria. Thirty milliliters (flasks) of Brain heart infusion broth were inoculated with appropriate strains and incubated for 15 hours at 37°C, shaking at 180 rpm. Cells were harvested by centrifugation (6,000 x g) and subsequently washed twice in a standard diluent (25 mM potassium phosphate buffer, pH 7.0, 0.85% NaCl, 0.2 mM magnesium sulfate, and 0.01% gelatin) and finally resuspended in 30 mls of the same diluent. Four, serial, two-fold dilutions of the final washed cell suspension were initially made, and then additional 10-fold dilutions to obtain countable plates. The absorbance at 590 nm was recorded for each of the two-fold dilutions and appropriate further 10-fold dilutions were plated in duplicate onto trypticase soy agar plates, which were incubated overnight at 37°C. Plates were counted and calculations were done to determine the number of colony-forming

units (cfu) per milliliter of each of the two-fold dilutions. These data were collectively entered into a simple linear regression calculator program which provided the best fitting line. Representative curves for each of the Fla and Mot phenotypes were made.

Protease Assays. P. aeruginosa strains were initially cultured overnight on trypticase soy agar plates at 37°C or in Brain heart infusion broth. To determine general protease activity, dialyzed Brain Heart Infusion Agar plates were made. Brain Heart Infusion Broth was made up at 10x normal concentration by adding 18.5 grams to 50 mls distilled water. This solution was dialyzed against one liter of distilled water for 18 hours at 4°C. The contents of the dialysis bag were discarded and the dialysate was supplemented with 3% agar and autoclaved at 121°C for 15 minutes. Skim milk was prepared at a concentration 3% as well and autoclaved separately at 112°C for 15 minutes. After cooling to approximately 60°C, the two preparations were mixed in equal volumes and poured into plates at a volume of 15-20 mls per plate. Bacteria were inoculated onto the plates either by band inoculations (single streak down center of plate) using inocula from trypticase soy agar plate cultures or by spread plates with 0.1 ml inocula from diluted Brain heart infusion broth cultures and incubated for 48 hours at 37°C. Zones of clearance due to proteolytic activity could be readily discerned and were carefully measured in millimeters around bands of culture growth or colonies using a manometer.

Two assays were used to detect specific elastase activity. In the first, elastin agar plates were prepared. Nutrient broth was made up at a concentration of 1.6% and was supplemented with 3.2% agar. Elastin obtained from Bovine neck ligament (Sigma) was prepared at a concentration of 0.3%. Elastin is not soluble in water, so a suspension as fine as possible (no large aggregates or clumps) was prepared by rapid stirring for 30 minutes. These components were autoclaved in separate containers and mixed in equal volumes and stirred well to ensure adequate dispersion of the elastin suspension. Plates were poured at a volume of 15-20 mls per plate. Elastin-agar plates were band-inoculated with inocula obtained from trypticase soy agar plate cultures and were incubated at 37°C for 72 hours. Areas of clearance around the subsequent bands of growth could be determined by examining the plates under direct illumination, and were measured in millimeters.

A second elastase assay utilized an elastin-congo red conjugate as the substrate. Bacteria were cultured in 18 mls of 5% peptone and 0.25% trypticase soy broth in a 250 ml flask, shaking at 200 rpm at 37°C until an optical density at 590 nm of approximately 0.5 was obtained. Two mls of this culture were inoculated into a second 250 ml flask containing 18 mls of the same medium. This secondary culture was incubated in a gyratory water bath at 200 rpm for 16 hrs at 37°C. The cells were pelleted by centrifugation (6,000 x g) and the supernatant fluid was filter sterilized using a 0.45 uM Acrodisc filter (Gelman, Ann Arbor, MI). The assay mixture consisted of 2 mls of sterile culture supernatant fluid, four mls of reaction buffer (0.1 M

Tris-maleate, pH 7.0; 1.0 mM $CaCl_2$), 10 mg elastin-congo red (Sigma) and 0.025% SDS. The assay was carried out in a 25 ml flask, shaking on a gyratory shaker at 200 rpm at 37°C. After a 2-hour incubation, the reaction was terminated by the addition of two mls of 0.7 M sodium phosphate, pH 6.0. Controls were run simultaneously and consisted of the same reaction mixture except sterile medium was substituted for the culture supernatant. Samples were measured spectrophotometrically at 495 nm for the appearance of a light red color, indicative of elastase activity. One enzyme unit was defined as the amount of elastase that caused an increase in .01 optical density units at the measured wavelength.

Growth Curves. Primary cultures of P. aeruginosa were grown in 30 mls of Luria broth (1.0% trypticase peptone, 0.5% yeast extract, 1.0% NaCl) for 15 hours at 37°C, shaking at 180 rpm. Secondary cultures consisted of 30 mls of Luria broth in 250 ml side arm flasks and were inoculated with 0.5 ml of the respective primary culture. The secondary cultures were incubated at 37°C, shaking in a gyratory water bath at 180 rpm. Readings were taken at different hourly intervals on a Klett colorimeter at 660 nm. Klett units were converted to optical density values according to the relationship, 1 KU = .02 O.D. units, and plotted versus time in hours on semi-logarithmic paper.

Physiological Profiles and Antibiograms. Whole physiological profiles of each parent and respective mutant strains were determined using a computerized, micro-plate assay system (American Micro Scan), courtesy of Dr. Mark Camblin, Director of Clinical Microbiology, St.

Mary's Hospital, Knoxville, TN. A total of 27 biochemical tests were performed. The oxidation/fermentation of glucose, and the fermentation of the sugars sucrose, sorbitol, raffinose, rhamnose, arabinose, inositol, melabiose, and adonitol were assayed. Others tests included urea hydrolysis, oxidase, nitrate reduction, hydrogen sulfide production, utilization of lysine, arginine, ornithine, citrate, malonate and the presence of indole and tryptophan deaminase, among other standard biochemical tests.

Antibiograms of each strain were also compiled with several antibiotics examined including piperacillin, tobramycin, kanamycin, amikacin, gentamicin, carbenicillin, moxacillin, chloramphenicol, cefamandole, cefataximine, and a number of semi-synthetic penicillins and 3rd generation cephalosporins.

Serological Typing. The O antigen types of the P. aeruginosa strains studied were determined using the International Antigen Typing System (IATS) provided by Difco. This scheme consists of a set of 17 antisera raised against autoclaved cells of known O antigen type and control sample suspensions of each of these types of cells. Bacteria were cultured overnight at 37°C on trypticase soy agar plates. Moderately turbid suspensions of cells in 0.85% saline were prepared on microscope slides with care taken to ensure fine suspensions without clumps or aggregates. A 45 ul drop of one of the respective antisera (10 fold dilution) was added to the suspension, and gently mixed. Slides were rocked back and forth gently for a period of 2 minutes or until agglutination was observed. The procedure was repeated for each

of the 17 antisera. Controls consisting of homologous cell suspensions and antisera were run periodically to ensure potency of the antisera.

Motility Plate Assays. Motility of the P. aeruginosa strains was determined in motility medium consisting of 1% tryptone, 0.3% yeast extract, 0.5% NaCl, and 0.3% agar. Primary cultures of each of the strains were grown overnight on trypticase soy agar plates at 37°C. Motility plates were stab-inoculated carefully in the center of each plate with the respective primary culture. Each strain was assayed in triplicate. Following inoculation, the plates were incubated in an upright position at 37°C for 24 hours. The circular zone of bacterial growth emanating from the initial stab could be discerned by examining the plates under direct light; the diameter of the zones was measured in millimeters in each of the three plates and presented as an average value.

Electron and Phase Contrast Microscopy. For electron microscopy, bacteria were grown for 15-18 hours in 5 mls of Brain heart infusion broth at 37°C without shaking. In some instances, dilutions were made in distilled water. Cells were negatively stained with 0.5% phosphotungstic acid, pH 7.0. Specimens were examined using an H-600 Hitachi transmission electron microscope.

For phase contrast microscopy, bacteria were cultured overnight at 37°C on trypticase soy agar plates. Lightly turbid suspensions in 0.85% saline were made on microscope slides and mounted with

coverslips. Observations were made using a Bausch and Lomb phase contrast microscope (magnification: 400x).

Animal Models Employed

Mice Used. Female CF-1 mice (Charles River), were used in all animal burn experiments. Mice were kept at 8-10 animals per cage in a temperature and light cycle controlled room. Water and mouse chow (Purina) were provided ad libitum.

Burned Mouse Model. A modification of the burn mouse procedure of Stieritz and Holder (1975) was used. The dorsal surfaces of mice weighing 20-22 grams were shaved with a small animal clipper one day prior to or the morning of a burn experiment. Mice were anaesthetized by placing them in a closed, dry seal glass container containing cotton soaked with the inhalation anaesthetic methoxyflurane (Metofane, Pittman-Moore), which was separated from the mice by a perforated steel platform. The breathing rates of the animals were closely monitored and when breathing appeared slow and somewhat labored, the animals were heavily sedated and ready for the burn procedure. A teflon template with a precisely cut window (35 x 25 mm) was firmly placed over the shaved backs of the mice. The dimensions of the template allow for an approximate 30% surface area burn in 20-22 gram mice. Alcohol (95%, 0.5 ml) was pipetted onto the exposed skin outlined by the template and ignited. The burn was precisely timed for 13 seconds with a digital electronic clock and extinguished by a forceful breath of air. Mice were immediately injected intraperitoneally with 0.5 ml of sterile 0.85% saline for fluid replacement to prevent overt shock from the burn

injury. The bacterial challenge was administered topically (0.3 ml) or subcutaneously (0.1 ml) in the burn wound or intraperitoneally (0.1 ml), depending on the study. In some experiments, mice were administered 0.5 ml of 100-fold diluted (in 0.85% saline) flagellar antisera intraperitoneally.

Scalded Mouse Model. The scalded mouse model was done according to the procedure described by Stover et al. (1985). Female, CF-1 mice, weighing 26-27 grams, were used. The backs of the animals were shaved one day prior to burning. Mice were anaesthetized in the methoxyflurane container until heavily sedated. Each mouse was restrained in a holding device with the shaved back exposed through a template window of dimensions 55 x 25 millimeters. The outlined, exposed surfaces were immersed into 80°C water; the scald burn was precisely timed for 7 seconds with a digital electronic clock. This is an optimal time for burning, resulting in a 30% body surface area, 3rd degree burn (Stover et al., 1985). Immediately following the burn, mice are administered 0.5 ml 0.85% saline intraperitoneally for fluid replacement therapy. The appropriate bacterial challenge was delivered either topically (0.3 ml) or subcutaneously (0.1 ml).

Virulence Studies

For all experiments comparing the lethality of P. aeruginosa strains differing in the Fla or Mot phenotype, the following protocol was employed. Bacteria were cultured in Brain heart infusion broth (30 mls in 250 ml flasks) for 15 hours in a 37°C gyratory water bath, shaking at 180 rpm. The cells were washed two times (6,000 x g, 15

minutes) in standard diluent and finally resuspended in 30 mls of the same diluent. A dilution of the washed cell suspension was measured in a spectrophotometer (Spec 20) at 590 nm, and the approximate number of viable cells per ml was determined upon reference to an appropriate standard curve. Serial dilutions of the washed cell suspension were done and appropriate tubes were spread or pour-plated onto trypticase soy agar plates in duplicate to determine the actual number of viable cells per ml or 0.1 ml.

Mice were burned (flame or scald) as previously described. In topical challenge studies, 0.3 ml of a predetermined bacterial suspension was layered gently over the burn wound. To examine the subcutaneous route of challenge, an appropriate bacterial suspension was injected into the burn eschar, just underneath the surface layer, in a 0.1 ml volume. Mice challenged intraperitoneally received 0.1 ml of a bacterial suspension in the lower right flank of the abdomen. Each experimental group consisted of 5-10 mice, depending on the study. Mice were observed over a 7 day period postburn, and resultant mortalities recorded.

Bacterial Quantitation of Burned Skin, Liver, and Blood

Mice were burned and challenged subcutaneously, topically, or intraperitoneally with washed bacterial suspensions as previously described (Stieritz and Holder, 1975). At various times postburn and challenge, mice were sacrificed by decapitation. Full thickness samples of burned skin and liver were removed, weighed and diced, and added to sterile 10 ml capacity Potter Elvehjem tissue grinders (Fisher

Scientific, Pittsburgh, PA) containing 5 mls of sterile diluent. Each sample was homogenized for 5 minutes using an electrical-powered homogenizer (Bellco, Vineland, NJ). Enumeration of the bacterial content of the homogenates was done by duplicate serial dilution plating on Pseudomonas Isolation Agar (American Scientific Products, Stone Mountain, GA) or Cetrimide Agar (Difco). Colonies were counted after 24-48 hours of incubation at 37°C, and the bacterial content was expressed as colony forming units per gram of skin or liver, as determined by the following formula:

$$\frac{[\text{colony forming units (cfu) per ml}] \times (5 \text{ mls})}{\text{gram weight of tissue specimen}} = \text{CFU/gram weight tissue}$$

Final results are expressed as the average $\pm$ standard error of the mean from three mice per time point.

Blood samples were collected immediately following decapitation in small beakers containing approximately 0.5 ml of 10% sodium citrate (in 0.85% saline). Serial dilutions and plating were done on cetrimide agar. Numbers of bacteria in the blood were expressed as colony forming units per milliliter of blood.

Evaluation of Antiflagellar Serum

Production in Rabbits. Flagella antigen (FAg) from Pseudomonas aeruginosa is routinely prepared and stored in a lyophilized state as previously described (Montie et al., 1982a). For immunization of rabbits, 250-350 ug of lyophilized flagella antigen was reconstituted in one ml of distilled water. An equal volume of Complete Freund's Adjuvant (CFA) was added, and the two were thoroughly mixed by repeatedly pushing the mixture through a syringe until a fine emulsion

was made. The back of a rabbit was shaved, and 0.3-0.5 ml of the emulsion was injected into several sites of the shaved area subcutaneously. Each rabbit was bled before injections with FAg to collect preimmune sera. A booster injection was given two weeks following the first; rabbits were bled one week after the booster was administered. Approximately 25 mls of blood were collected, allowed to clot, and incubated at 4°C overnight. Sera were collected from the retracted clot, and clarified by centrifugation (6,000 x g). One ml aliquots of sera were stored frozen at -70°C.

Adsorption Experiments. Hyperimmune, antiflagellar sera was subjected to the following procedure to remove any nonspecific, background antibody. P. aeruginosa strains M-2 and M-2 Fla$^-$ were cultured for 15 hours in Brain heart infusion broth at 37°C, shaking at 180 rpm. The cells were washed two times as previously described and resuspended in 5 mls of 0.85% saline. A 0.5 ml aliquot of M-2 Fla$^-$ suspension was added to the immune sera, gently mixed, and incubated (stationary) for 30 minutes at 37°C. The sample was centrifuged at 6,000 x g for 15 minutes, and the serum fraction removed. This adsorption procedure was repeated several times, and the final adsorbed serum sample was filter-sterilized and stored frozen in one ml vials at -70°C. Similar adsorption assays were performed with M-2 suspensions that had been heated in 100°C water for one hour and formalin (0.3%) killed M-2 Fla$^-$ for comparison.

Cross-agglutination Assays for Flagella Antigen. Bacteria were cultured overnight on trypticase soy agar plates at 37°C.

Moderately-turbid suspensions of cells in 0.85% saline were made on microscope slides. A 45 ul drop of a-type or b-type flagellar antiserum (10x diluted) was added to the suspension and gently mixed. The slides were rocked back and forth and observed over a period of two minutes. Agglutination was recorded according to the following characterization: ++--strong, rapid agglutination, +--moderate, much slower agglutination, (-)--no agglutination. Control slides with normal, preimmune sera were always done simultaneously for comparison.

Determination of Antibody Type, Specificity, and Titer by an ELISA Technique. P. aeruginosa FAg was reconstituted in carbonate buffer (pH 9.6) at a concentration of 0.2 ug/ml. Fifty ul of this antigen preparation was added to appropriate polyvinyl chloride, microtiter plate wells (Fisher Scientific, Pittsburgh, PA) giving a protein concentration of 10 ug per well. Plates were covered to prevent evaporation and incubated overnight at 4°C. Each plate was washed at least three times with Phosphate-buffered saline + Tween (0.8% NaCl, .02% KH_2PO_4, 0.12% Na_2HPO_4, .02% KCl, .05% Tween 20), with caution taken not to allow the plates to dry between steps as this increases nonspecificity. After washing, 50 ul of whole antiflagellar serum or appropriate dilutions were added to each well. Dilutions of the flagellar antiserum were made in PBS-Tween containing 1% gelatin. Plates were covered and incubated for 60 minutes at 37°C. Following this incubation, plates were washed three times with PBS-Tween. Fifty ul of goat anti-rabbit IgG conjugated with peroxidase or goat

anti-rabbit IgM-peroxidase diluted 1:10,000 in PBS-Tween + 1% gelatin were added to respective wells. The plates were again incubated for 60 minutes at 37°C, and subsequently washed three times with PBS-Tween. Fifty ul of substrate solution (0.1 M citric acid anhydrous, 0.2 M disodium phosphate anhydrous, 1% orthophenylenediamine (Sigma), .03% H_2O_2, pH 6.0) were added to each well, and the plates were covered (substrate is light sensitive) and incubated at room temperature for 15-30 minutes. The reaction was terminated by the addition of 25 ul of 2.5 M sulfuric acid to each well. Plates were read on an ELISA plate reader at 488 nm. Wells were considered positive if they developed to a level twice as dark as the control wells, which consisted of identical reagents except preimmune sera or buffer were substituted for the antiflagellar sera.

The level of nonspecific antibody in adsorbed flagellar antisera was determined as follows. FAg was coated onto wells of polyvinylchloride microtiter plates as previously described. Suspensions of autoclaved cells (O antigen type 5 and 10) provided in the International Antigen Typing Scheme (Difco, Detroit, Michigan) were diluted 1000-fold in PBS-Tween + 1% gelatin and added to respective wells. Dilutions of antiflagellar sera and IATS antisera number 5 were used as the primary antibody. The remaining assay steps were performed as described above.

Polyacrylamide Gel Electrophoresis and Western Blot Techniques. The methods used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) have been described previously (Montie *et*

al., 1982a; Allison et al., 1985). P. aeruginosa flagellin was transferred to nitrocellulose paper (Schleicher and Schuell, Inc.) and an immunoautoradiography procedure was done according to the procedure originally described by Towbin et al. (1979). Transfer was accomplished at 100 V for 2.5 hours in buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol (transfer buffer). The nitrocellulose paper was immersed in 3% bovine serum albumin (Sigma) containing 0.9% saline and 10 mM Tris-hydrochloride (pH 7.4) for one hour before the immunoadsorption. The paper was then rinsed with distilled water. Hyperimmune, adsorbed M-2 flagellar antiserum was used at a dilution of 1:50 in buffer containing 150 mM NaCl, 5 mM EDTA, 50 mM Tris-hydrochloride (pH 7.4), 0.25% gelatin, and 0.05% Nonidet P-40. The nitrocellulose paper was incubated for 2 hours at room temperature. The antiserum was then removed, and the blots were washed with 10, 20 ml volumes of wash buffer [150 mM NaCl, 5 mM EDTA, 50 mM Tris-hydrochloride (pH 7.4), 0.25% gelatin, 0.1% SDS, 0.5% Triton X-100]. Following the washes, the nitrocellulose paper was reacted with ^{125}I-protein A for 2 hours with constant shaking. The washing steps were repeated and the blots received a final distilled water rinse. The blots were then allowed to air dry and were exposed on Kodak X-Omat AR film.

Growth Experiments in Preimmune and Flagellar Antisera. Primary cultures of strains M-2 and M-2 Fla$^-$ were grown in Brain heart infusion broth for 15 hours at 37°C, shaking at 180 rpm. Whole sera and 10-fold dilutions (in 0.85% saline) were inoculated with 0.1 ml of

the respective primary culture and incubated shaking at 180 rpm at 37°C and in small test tubes stationary at 37°C overnight. Observations on the turbidity of the secondary cultures were made periodically during an 8 hour period and at 24 hours post-inoculation.

Tube Immobilization Assay. To determine the titer of antiflagellar antibody that inhibits the motility of P. aeruginosa, the following assay was designed. Small test tubes containing 2.7 mls of soft agar (0.8% nutrient broth, 0.3% agar) were made, autoclaved at standard temperature and pressure, and incubated in a 48°C water bath. Whole and serial two-fold dilutions of flagellar antisera (in 0.85% saline) were added to the soft agar tubes, 0.3 ml per tube. Control tubes consisted of whole preimmune sera in soft agar. Each tube was gently mixed and allowed to solidify at room temperature. Immediately thereafter, tubes were stab inoculated with bacteria previously cultured on trypticase soy agar plates, and incubated at 37°C for 18 hours. The spreading of bacteria from the initial stab line was easily detected with the immobilization titer defined as the highest dilution of flagellar antisera which restricted bacterial growth to the initial stab line.

Motility Inhibition: Phase Contrast and Electron Microscopy. Observations of motility inhibition by flagellar antisera were performed as follows. P. aeruginosa strains M-2 and PJ108 were cultured overnight on trypticase soy agar plates at 37°C. Lightly turbid suspensions were prepared in 0.85% saline on microscope slides. A 20 ul drop of 10-fold diluted M-2 flagellar antiserum (in distilled

water) was added to the suspensions, and gently mixed. Coverslips were added, and observations were made immediately under phase contrast microscopy at 400x.

For electron microscopy, a turbid suspension of M-2 or PJ108 was made in one ml of 10x diluted M-2 antiserum from the overnight plate cultures. Negative strains with 0.5% phosphotungstic acid were made immediately, and observations were made using an H-600 Hitachi transmission electron microscope.

Bloodstream Clearance Assays

Mice were subjected to the standard 13 second burn as previously described. After burning, the animals were allowed a one hour recovery period, which was followed by incubation for one hour under a heat lamp (250 W, reflecting bulb) for tail-vein dilation. Each mouse was placed in a restraining device allowing for easy access to the tail, and injected with 0.1 ml of a washed bacterial suspension intravenously. At 10 minutes and 30 minutes following injection, each mouse was bled through the retroorbital sinus with approximately 30 ul collected. Three mice were done per time point. Blood samples were spread plated on cetrimide agar, and results were expressed as colony forming units per milliliter of blood.

Cyclophosphamide Treatment of Mice

Cyclophosphamide (Sigma) was prepared in distilled water at a concentration of 200 mg/kg. Mice were injected with 0.1 ml intraperitoneally 3 days prior to burning and challenge. Differential, 100-blood cell counts (Wright Staining Method) were done to determine

the number of polymorphonuclear neutrophils following cyclophosphamide treatment.

CHAPTER III

RESULTS

Characterization of P. aeruginosa Strains

Growth Curves. Primary cultures of each strain were grown up in Luria broth for 15 hours at 37°C, shaking at 180 rpm. Secondary Luria broth cultures were initiated with standardized inocula, and incubated under identical conditions. Optical density measurements were taken at different increments of time up to 11 hours post-inoculation. Semi-log graphs of the data revealed little difference in the growth rates of wild type P. aeruginosa strains and their Fla^- or Mot^- mutant counterparts (Figure 1). No significant difference was observed in the lag (pre log), logarithmic, and exponential phases of growth between the strains.

Serotyping. P. aeruginosa strains were cultured overnight at 37°C on trypticase soy agar plates. Moderately turbid suspensions of cells were made in physiological saline (0.85%). A single 45 ul drop of an appropriate antiserum type of the IATS typing kit was added. Typing results demonstrated no difference between O antigen types of the parent strains and their respective mutants (Table 1).

Phase Contrast and Electron Microscopy. The phenotypes of M-2, M-2 Fla^-, PaO-1, AK1152, and AK1153 have been previously documented (Montie et al., 1982; A. Kropinski, personal communication). To examine strains MT1200 and 1200-80, static overnight (37°C) cultures of

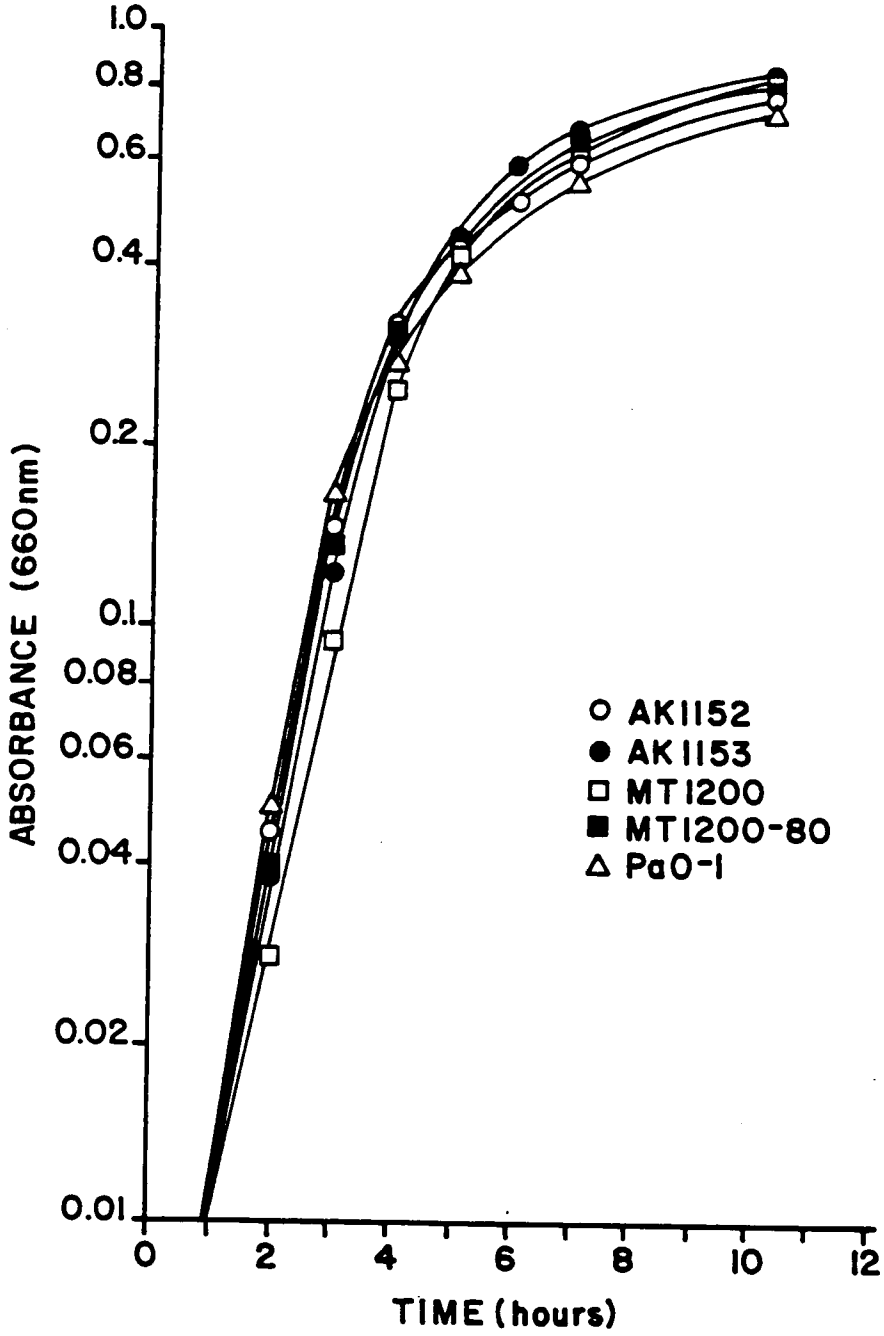

Figure 1. Comparison of growth rates of P. aeruginosa strains in Luria broth.

Table 1. Characteristics of parent and corresponding motility mutants

Strain	Phenotype	Motility (mm)[a]	O-antigen Type[b]
M-2	Fla^+ Mot^+	25.0	5
M-2 Fla^-	Fla^- Mot^-	3.6	5
PaO-1	Fla^+ Mot^+	25.0	5
AK1152	Fla^- Mot^-	6.3	5
AK1153	Fla^- Mot^-	6.2	5
MT1200	Fla^+ Mot^+	25.0	5
MT1200-80	Fla^+ Mot^-	3.2	5

[a]Zone of growth from the inoculation site measured in millimeters.

[b]Slide agglutination assay using Difco-IATS serotyping kit.

P. aeruginosa were prepared in small tubes of BHI broth. Negatively-stained preparations confirmed the designated phenotypes (Figures 2 and 3). It can be seen that the parental strain MT1200 (Fla^+ Mot^+) possesses a normal, wavy flagellum. In contrast, the nonmotile strain MT1200-80 exhibits a long, straight flagellum. Phase contrast microscope observations were made with lightly-turbid suspensions of all of the respective strains. All of the parental, wild-type strains were observed to be actively motile whereas none of the mutant strains exhibited motility.

Motility Plate Assays. P. aeruginosa strains were cultured overnight on trypticase soy agar plates at 37°C. Motility agar plates were stab-inoculated in triplicate with the respective cultures. Measurements of bacterial spreading at 24 hours coincided with the motility observations made under phase contrast microscopy (Table 1). The parental strains spread in concentric circles from the inoculation sites whereas no spreading was observed in the Fla^- and Mot^- plates.

Proteolytic Activity. P. aeruginosa strains again were cultured overnight on trypticase soy agar plates at 37°C. Proteolytic activity of the P. aeruginosa strains was initially assayed on skim milk agar plates. Each strain was band inoculated or spread plated. All of the strains exhibited large zones of clearance around bacterial colonies or streaks (Table 2). These results are indicative of general protease activity since casein is susceptible to hydrolysis by all of the elaborated P. aeruginosa proteases. To specifically determine whether

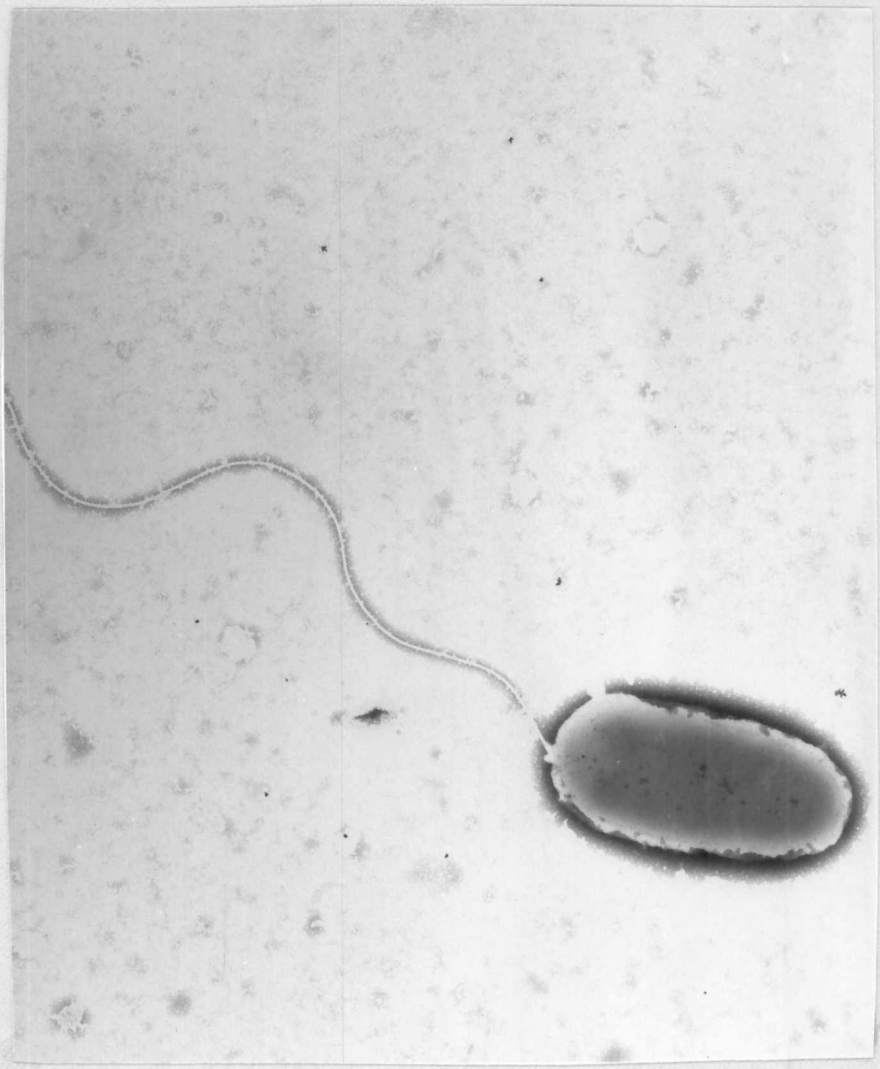

Figure 2. Electron micrograph of P. aeruginosa strain MT1200 stained with 0.5% phosphotungstic acid. Magnification: 10,000X.

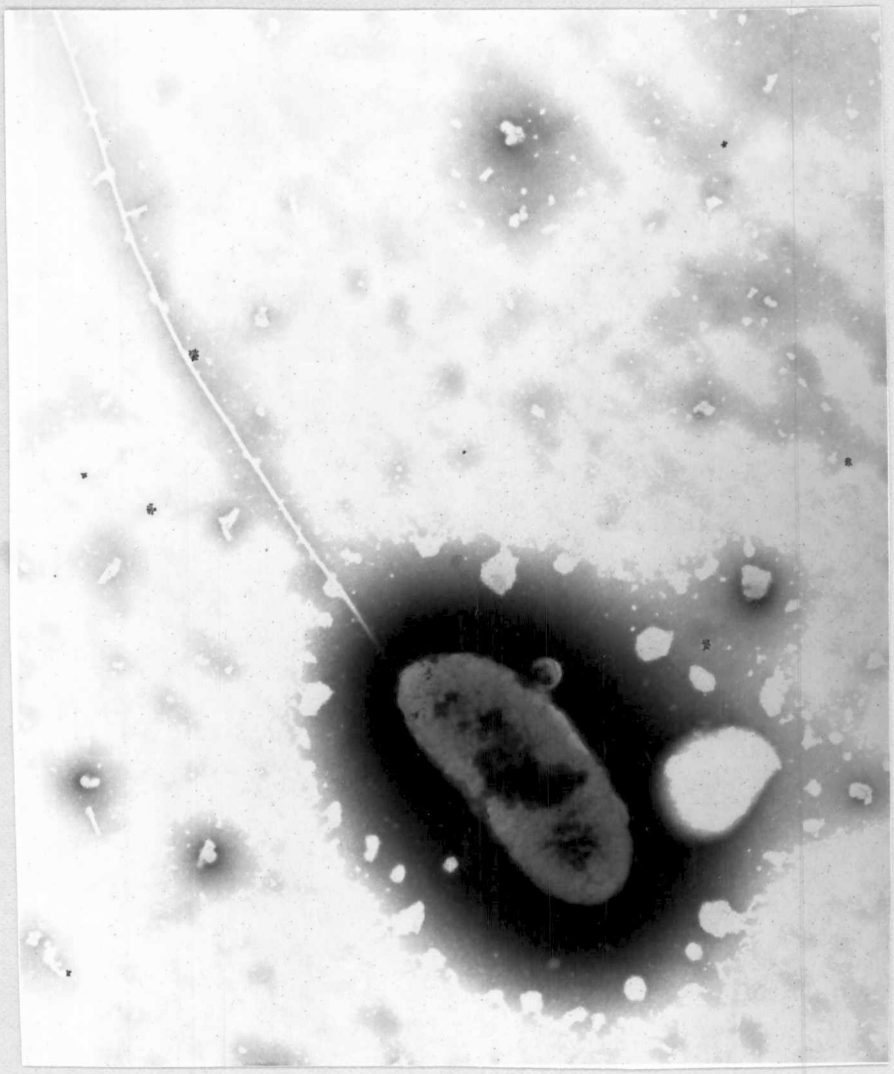

Figure 3. Electron micrograph of P. aeruginosa strain MT1200-80 stained with 0.5% phosphotungstic acid. Magnification: 10,000X.

or not elastase is produced, elastin-agar plates were band-inoculated with similar inocula; observations at 72 hours revealed large zones of clearing around the streaks of all of the P. aeruginosa strains (Table 2). No significant differences between the parent and mutant strains were observed. To determine if a quantitative difference in elastase activity existed between the strains, culture supernatants were assayed by the elastin-congo red method. P. aeruginosa strains M-2 and M-2 Fla$^-$ showed the highest activities, 51.3 enzyme units and 43.8 units respectively (Table 2). Values obtained for strains PaO-1, AK1152, AK1153, MT1200 and 1200-80 were significantly lower; however, the activities of the parental strains and their respective mutants were very similar.

Physiological Profiles and Antibiograms. A physiological profile consisting of 29 biochemical tests was performed on each of the P. aeruginosa parental strains and Fla$^-$ or Mot$^-$ mutants. No differences in profiles were observed in any of the strains tested. Moreover, they matched identically to standard, reference isolates of P. aeruginosa. Antibiograms consisting of 16 different antibiotics were obtained. Again, no differences were found between the parent and mutant strains, and very little difference was noted among any of the strains tested.

Susceptibility Studies in Burned Mice

Initial experiments examined the lethality of the parental strains and the respective mutants in the burned mouse model. Strain M-2 was found to be highly virulent when injected intradermally into the burn

Table 2. Characteristics of parent and corresponding motility mutants: proteolytic activity

Strain	Phenotype	Proteolytic activity[a]	Elastase (qualitative)[b]	Elastase (quantitative)[c]
M-2	Fla^+ Mot^+	13.7	+	51.3
M-2 Fla^-	Fla^- Mot^-	13.1	+	43.8
PaO-1	Fla^+ Mot^+	8.6	+	18.9
AK1152	Fla^- Mot^-	7.7	+	18.8
AK1153	Fla^- Mot^-	7.9	+	18.6
MT1200	Fla^+ Mot^+	8.4	+	17.6
MT1200-80	Fla^+ Mot^-	11.0	+	19.1

[a]Zone of hydrolysis measured in millimeters of colonies on dialyzed brain heat infusion broth--skim milk agar plates.

[b]Zone of hydrolysis (+ or -) around streaks on elastin-nutrient agar plates.

[c]Enzyme units of elastase as determined by elastin-congo red assay. 1 enzyme unit = increase in .01 O.D. units at 495 nanometers.

wound (Table 3). At a dose of only 480 cfu, all of the challenged mice succumbed to the infection by the third day postburn. Analysis of the time to death kinetics revealed an inverse relationship between the death rate and the challenge dose. The LD_{50} was only 5.3 cfu. Control mice survived throughout the seven day postburn period.

Strikingly different results were observed when burned mice were challenged intradermally with the isogenic counterpart M-2 Fla^- strain (Table 3). A significant loss of virulence was demonstrated as doses four and five logs higher were needed to achieve one hundred percent mortality. The LD_{50} for M-2 Fla^- was determined to be approximately 5×10^4 cfu, a value 10,000 times higher than that of the wildtype strain M-2. These results confirm previous, initial findings (Montie et al., 1983b) and demonstrate the extent of the loss of virulence associated with a Fla^- phenotype.

To extend these initial observations and further document the association between loss of virulence and a Fla^- phenotype, similar studies were conducted with P. aeruginosa strains PaO-1 (Fla^+ Mot^+), AK1152 (Fla^-), and AK1153 (Fla^-). As shown in Table 4, the parent strain was highly virulent with a LD_{50} comparable to that of M-2. A sizable difference was observed with the Fla^- mutants, however. Only one mortality occurred in mice challenged with doses of 10^6-10^7 cfu of strains AK1152 and AK1153. Respective LD_{50} values were approximated at greater than 10^7 cfu, a value five logs higher than that of PAO-1 (Fla^+ Mot^+). These data, therefore, further document the severe loss of virulence observed in isogenic P. aeruginosa strains of the Fla^- phenotype.

Table 3. Virulence of two *Pseudomonas aeruginosa* strains differing in the Fla phenotype challenged subcutaneously

Strain	Challenge Inocula (CFU)	Cumulative Mortalities (Died/Total Inoc.) Days Postburn 1	2	3	4	5	6	7
M-2 (Fla^+- WT parent)	4.8×10^3	0/10	10/10	-	-	-	-	-
	4.8×10^2	0/10	6/10	10/10	-	-	-	-
	4.8×10^1	0/10	1/10	4/10	7/10	9/10	9/10	9/10
	4.8×10^0	0/10	1/10	1/10	2/10	3/10	3/10	3/10
	control	0/10	0/10	0/10	0/10	0/10	0/10	0/10
M-2 (Fla^- EMS mutant)	1.44×10^8	2/5	5/5	-	-	-	-	-
	1.44×10^7	0/5	5/5	-	-	-	-	-
	1.44×10^6	0/5	2/5	4/5	4/5	4/5	4/5	4/5
	1.44×10^5	0/5	0/5	2/5	2/5	3/5	3/5	3/5
	1.44×10^4	0/5	0/5	1/5	1/5	1/5	1/5	1/5
	1.44×10^3	0/5	0/5	0/5	0/5	0/5	0/5	0/5
	controls	0/5	0/5	0/5	0/5	0/5	0/5	0/5

Table 4. Virulence of three Pseudomonas aeruginosa strains differing in the Fla phenotype challenged subcutaneously

Strain	Challenge Inocula (CFU)	Cumulative Mortalities (Died/Total Inoc.) Days Postburn 1	2	3	4	5	6	7
PaO-1 (Fla^+ WT-parent)	5.3×10^3	1/5	5/5	-	-	-	-	-
	5.3×10^2	0/5	4/5	4/5	4/5	4/5	4/5	4/5
	5.3×10^1	0/5	1/5	2/5	2/5	2/5	2/5	2/5
AK1152 (Fla^-, EMS-mutant)	8.3×10^6	0/5	0/5	0/5	1/5	1/5	1/5	1/5
	8.3×10^5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
	8.3×10^4	0/5	0/5	0/5	0/5	0/5	0/5	0/5
	8.3×10^3	0/5	0/5	0/5	0/5	0/5	0/5	0/5
AK1153 (Fla^-, EMS-mutant)	1.1×10^7	0/5	1/5	1/5	1/5	1/5	1/5	1/5
	1.1×10^6	0/5	0/5	0/5	0/5	0/5	0/5	0/5
	1.1×10^5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Controls	0.85% Saline	0/15	0/15	0/15	0/15	0/15	0/15	0/15

Strains MT1200 (Fla^+ Mot^+) and MT1200-80 (Fla^+ Mot^-) presented a unique opportunity to address the question whether or not the loss of virulence results simply from the loss of a flagellum or the loss of the capability of the bacteria to swim. The wildtype parent (MT1200) was lethal in burned mice challenged subcutaneously, although this strain did not exhibit the same level of virulence as the other parental strains, M-2 and PaO-1 (Table 5). However, the nonmotile mutant strain (MT1200-80) was essentially avirulent as challenge doses of 9×10^4 cfu resulted in no mortalities (Table 5). This is in striking contrast to the parent strain where 80 percent mortality was achieved at a marginally lower inoculum of 6.2×10^4 cfu. These results are in agreement with the hypothesis that active motility is an important virulence factor of P. aeruginosa in burn infections.

The route of bacterial challenge and colonization may be important in regards to the role of motility in invasive burn wound infection. To gain understanding of where in the infectious sequence of events motility may be the most advantageous to an infecting organism, different routes of challenge were examined.

Mice receiving the standard 13 second flame burn were challenged with very high doses of bacteria topically by layering 0.3 ml of washed cells onto the surface of the burn wound. As shown in Table 6, the wildtype strain M-2 (Fla^+ Mot^+) was highly virulent by this route. Inoculation with 2.75×10^7 cfu resulted in 60% mortality of a group of 10 mice; 10 and 100-fold higher doses caused 80 and 100 percent mortality respectively. However, challenge of burned mice with topical

Table 5. Virulence of two Pseudomonas aeruginosa strains differing in the Mot phenotype challenged subcutaneously

Strain	Challenge Inocula (CFU)	Cumulative Mortalities (Died/Total Inoc.) Days Postburn 1	2	3	4	5	6	7
MT1200 (Fla$^+$ Mot$^+$)	6.2×10^5	0/10	6/10	10/10	-	-	-	-
	6.2×10^4	0/10	4/10	7/10	7/10	8/10	8/10	8/10
	6.2×10^3	0/10	1/10	3/10	4/10	4/10	5/10	5/10
	6.2×10^2	0/10	0/10	0/10	1/10	1/10	1/10	1/10
MT1200-80 (Fla+ Mot-)	9.4×10^4	0/10	0/10	0/10	0/10	0/10	0/10	0/10
	9.4×10^3	0/9	0/9	0/9	0/9	0/9	0/9	0/9
	9.4×10^2	0/10	0/10	0/10	0/10	0/10	0/10	0/10

Table 6. Virulence of two Pseudomonas aeruginosa strains differing in the Fla phenotype challenged topically

Strain	Challenge Inocula (CFU)	Cumulative Mortalities (Died/Total Inoc.) Days Postburn 1	2	3	4	5	6	7
M-2 (Fla^+-WT parent)	2.75×10^9	0/10	0/10	8/10	10/10	-	-	-
	2.75×10^8	0/10	1/10	7/10	7/10	8/10	8/10	8/10
	2.75×10^7	0/10	1/10	3/10	5/10	5/10	5/10	6/10
	2.75×10^6	0/10	1/10	2/10	2/10	3/10	4/10	4/10
M-2 (Fla^-, EMS mutant)	2.13×10^9	0/10	0/10	0/10	3/10	4/10	4/10	4/10
	2.13×10^8	0/10	0/10	0/10	1/10	1/10	3/10	4/10
	2.13×10^7	0/9	0/9	0/9	0/9	0/9	0/9	0/9

applications of M-2 Fla$^-$ (Fla$^-$ Mot$^-$) again demonstrated a reduction in virulence (Table 6). A challenge dose of 2.13 x 10^9 cfu reulted in only 40% mortality, whereas a similar dose of the wildtype parent caused 100% mortality; moreover, 60% mortality was achieved with a 10^7 cfu challenge dose of M-2 (Fla$^+$ Mot$^+$) with no mortalities occurring from an equivalent dose of the nonmotile mutant M-2 Fla$^-$ (Fla$^-$ Mot$^-$). These data, then, support the hypothesis that motility contributes to the virulence of P. aeruginosa in burn wound infections regardless of the surface mode of inoculation. Similar experiments were conducted with MT1200 (Fla$^+$ Mot$^+$) and MT1200-80 (Fla$^+$ Mot$^-$). However, the wildtype (MT1200) was not as virulent by the topical route of challenge and therefore adequate comparisons with the mutant phenotype were not possible.

The virulence of P. aeruginosa strain M-2 (Fla$^+$ Mot$^+$) and M-2 Fla$^-$ (Fla$^-$ Mot$^-$) was examined in burned mice challenged intraperitoneally. There is some evidence for gastrointestinal translocation in burned animals (Maejima et al.); experiments focusing on intraperitoneal challenge addresses the issue of whether or not the Fla$^+$ Mot$^+$ phenotype is more virulent than the Fla$^-$ Mot$^-$ phenotype by this route. As seen in Table 7, 100 percent mortality was achieved in burned mice challenged intraperitoneally with 2.8 x 10^5 cfu of M-2 (Fla$^+$ Mot$^+$) and 70 percent with a 10-fold less dose of 2.8 x 10^4 cfu. In contrast, a slightly higher dose of strain M-2 Fla$^-$ (Fla$^-$ Mot$^-$), 3.3 x 10^4 cfu, caused only 30 percent mortality. Additionally, a 10-fold higher dose of 3.3 x 10^5 cfu resulted in only 60% mortality. These data show that a difference in

Table 7. Virulence of two Pseudomonas aeruginosa strains differing in the Fla phenotype challenged intraperitoneally

Strain	Challenge Inocula (CFU)	Cumulative Mortalities (Died/Total Inoc.) Days Postburn 1	2	3	4	5	6	7
M-2 Fla^+-	2.8×10^7	10/10	-	-	-	-	-	-
WT parent)	2.8×10^6	1/10	10/10	-	-	-	-	-
	2.8×10^5	0/10	8/10	10/10	-	-	-	-
	2.8×10^4	0/10	6/10	7/10	7/10	7/10	7/10	7/10
	2.8×10^3	0/10	1/10	1/10	1/10	1/10	1/10	1/10
	2.8×10^2	0/10	0/10	0/10	0/10	0/10	0/10	0/10
M-2 (Fla^--	3.3×10^7	10/10	-	-	-	-	-	-
EMS mutant)	3.3×10^6	2/10	9/10	10/10	-	-	-	-
	3.3×10^5	0/10	5/10	6/10	6/10	6/10	6/10	6/10
	3.3×10^4	0/10	1/10	3/10	3/10	3/10	3/10	3/10
	3.3×10^3	0/10	0/10	0/10	1/10	1/10	1/10	1/10
	3.3×10^2	0/10	0/10	0/10	0/10	0/10	0/10	0/10

virulence of approximately one log exists between a wildtype and Fla^- phenotype when injected via the intraperitoneal route.

Similar studies were conducted with strains PaO-1 (Fla^+ Mot^+) and AK1152 (Fla^- Mot^-) (Table 8). Challenge of burned mice intraperitoneally with as little as 6.29×10^3 cfu of strain PaO-1 caused 70 percent mortality by the seventh day postburn. However, a dose effect was not observed with this strain as a 100-fold higher challenge inoculum, 6.29×10^5 cfu, also resulted in 70 percent mortality. A key point to be noted is that differences in time to death were observed; at the 6.29×10^5 cfu dose, six out of 10 mice succumbed to the infection by the second day postburn and challenge whereas a significantly delayed occurrence of mortalities was observed with the 6.29×10^3 cfu inoculum. This may be important in colonization of the burn wound and tissue invasion. Similar results to those obtained with M-2 Fla^- were observed after intraperitoneal challenge of burned mice with AK1152 (Fla^- Mot^-) (Table 8). Inoculation with 1.03×10^5 cfu resulted in only 30% mortality, compared to the 70 percent mortality achieved with 100-fold less wildtype (Fla^+ Mot^+) bacteria. These data, therefore, are further indicative of differences in virulence between Fla^- Mot^- and Fla^+ Mot^+ phenotypes by an intraperitoneal route of challenge in burned mice.

To again determine if the Fla^+ Mot^- phenotype exhibited an analogous loss of virulence, burned mice were challenged intraperitoneally with either the parental strain MT1200 (Fla^+ Mot^+) or the nonmotile mutant 1200-80 as shown in Table 9. An

Table 8. Virulence of two Pseudomonas aeruginosa strains (PAO-1 and AK1152) differing in the Fla phenotype challenged intraperitoneally

Strain	Challenge Inocula (CFU)	Cumulative Mortalities (Died/Survived) Days Postburn 1	2	3	4	5	6	7
PaO-1 (Fla^+ WT-parent)	6.29×10^6	2/10	10/10	-	-	-	-	-
	6.29×10^5	0/10	6/10	7/10	7/10	7/10	7/10	7/10
	6.29×10^4	0/10	2/10	6/10	6/10	6/10	6/10	6/10
	6.29×10^3	0/10	1/10	4/10	4/10	5/10	6/10	7/10
AK1152 (Fla^-- EMS mutant)	1.03×10^7	7/10	10/10	-	-	-	-	-
	1.03×10^6	3/10	6/10	7/10	8/10	9/10	9/10	10/10
	1.03×10^5	0/10	2/10	2/10	2/10	3/10	3/10	3/10

Table 9. Virulence of two *Pseudomonas aeruginosa* strains differing in the Mot phenotype challenged intraperitoneally

Strain	Challenge Inocula (CFU)	Cumulative Mortalities (Died/Total Inoc.) Days Postburn 1	2	3	4	5	6	7
MT1200	3.05×10^7	6/10	10/10	-	-	-	-	-
(Fla^+ Mot^+)	3.05×10^6	0/10	8/10	8/10	9/10	9/10	9/10	9/10
	3.05×10^5	0/10	2/10	2/10	2/10	2/10	2/10	2/10
	3.05×10^4	0/10	0/10	2/10	2/10	2/10	2/10	2/10
MT1200-80	5.5×10^7	2/10	10/10	-	-	-	-	-
(Fla^+ Mot^-)	5.5×10^6	0/10	0/10	0/10	0/10	0/10	0/10	0/10

inoculum of 3.05 x 10^6 cfu of MT1200 caused 90% mortality of the test animals by the fourth day postburn. In contrast, a higher dose of the Fla^+ Mot^- mutant, 5.5 x 10^6 cfu, resulted in no mortalities within the seven days postburn period. A one log difference in virulence is apparent between the two strains, which is very similar to previous results. Collectively, then, these studies document the importance of motility as a virulence component in P. aeruginosa burn wound infections.

Quantitative Bacteriology of Tissues in Burned Mice

To increase our understanding of the pathogenesis of P. aeruginosa burn wound infections and the role of motility, experiments were designed to follow the progress of bacterial colonization and spread into different tissues. Mice were subjected to the standard 13 second burn and immediately challenged with the respective inoculum. The results of the first comparative study are illustrated in Figure 4. The wildtype parent strain (Fla^+ Mot^+) was inoculated directly into the burn site, immediately postburn, at a concentration of 10^2 cfu. A rapid proliferation in the burn wound ensued, with counts reaching above 10^7 cfu per gram of skin by 30 hours post-challenge. Enumeration of liver bacterial counts revealed an interesting parallel increase. Bacteria were not detected in liver homogenates at 8 hours postburn; however, as the bacterial load in skin increased, colonization of the liver concomitantly increased. By 30 hours post-infection, liver homogenates were found to contain approximately 10^5 cfu/g tissue. Essentially identical results were observed from

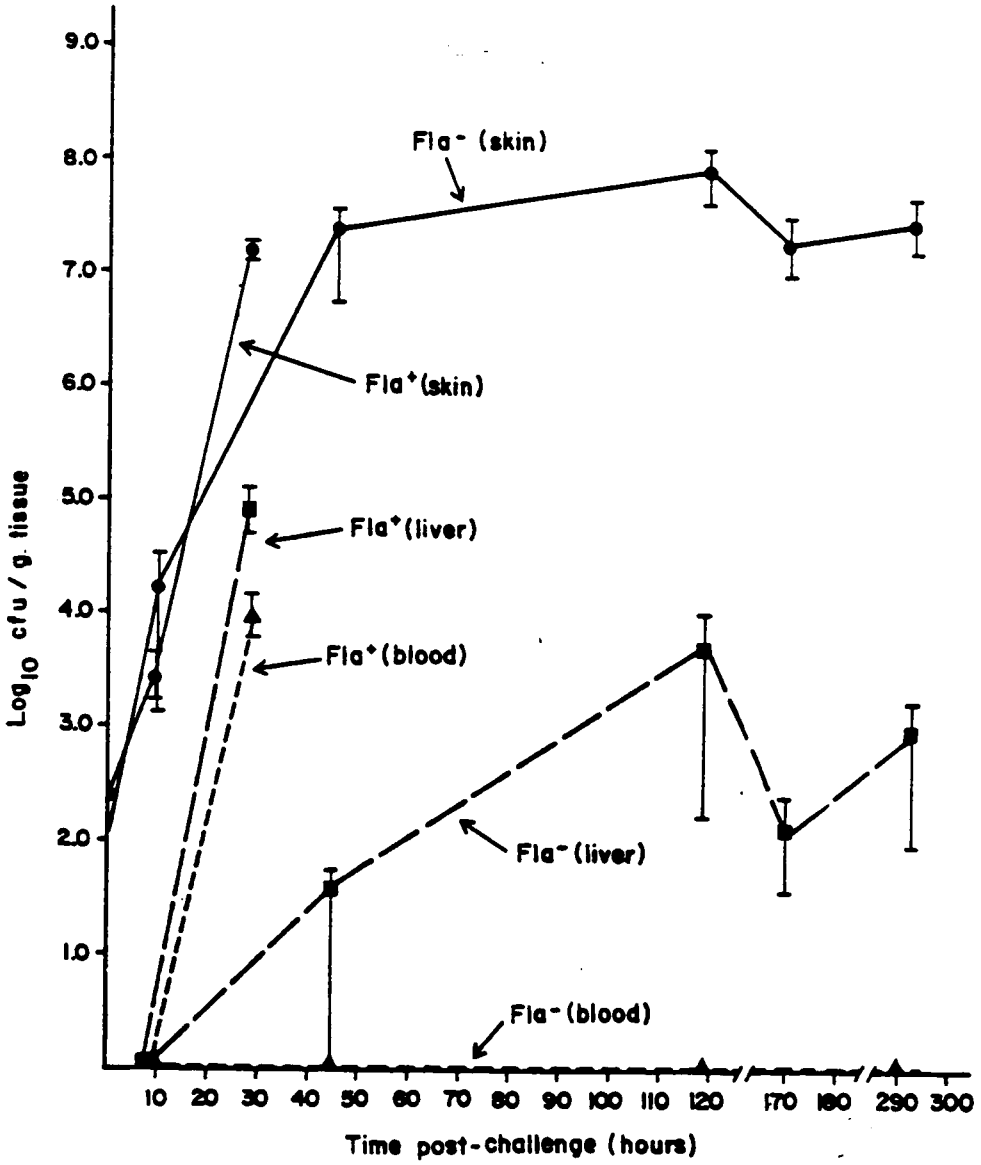

Figure 4. Quantitative bacteriology of skin, liver, and blood after subcutaneous challenge with M-2 or M-2 Fla$^-$. Data are expressed as means of three mice $\pm$ standard error of the mean. Earlier skin counts (M-2) Fla$^+$ from a similar experiment are: 8 hours--3 x 10^3 cfu, 16 hours--5 x 10^6 cfu, 22 hours--10^7 cfu.

enumeration of bacteria in the bloodstream of these mice. Bacteria were not detected in the blood at 8 hours post-challenge; however, by 30 hours post-infection, approximately 10^4 cfu of bacteria were found per milliliter of blood. Mice were very ill by 30 hours post-challenge and succumbed to infection within the next 5-10 hours.

A strikingly different pattern of events was observed when burned mice were challenged with 10^2 cfu of the mutant strain M-2 Fla^- (Fla^- Mot^-) (Figure 4). An important observation is that the mutant strain multiplied within the burn wound to similar levels as the parent, reaching 10^7 cfu per gram of burned skin. Significant differences were observed in liver and blood counts, however. Counts in liver homogenates revealed an almost transient colonization of the liver, with less than 10^2 cfu per gram tissue by 45 hours post-challenge. The latter is in sharp contrast to the 10^5 cfu per gram liver by 30 hours post-burn observed with the wild-type challenge. The liver counts never approached those obtained with the parent strain; by 290 hours postburn, only 10^3 cfu per gram liver were detected. A more sharp contrast can be made with the blood counts obtained between the two strains. Strain M-2 Fla^- (Fla^- Mot^-) was never detected in the bloodstream, even by 290 hours post-infection, whereas 10^4 cfu per milliliter of blood were observed by only 30 hours post-challenge with the parent strain M-2 (Fla^+ Mot^+).

To determine if a similar progression of infection occurs following a route of bacterial challenge other than a subcutaneous one, the following experiments were done. Challenge of burned mice by a

topical application of strain M-2 provided results similar to those previously described, as illustrated in Figure 5. At 24 hours, enumeration of the bacterial load of the burn wound revealed counts of approximately 3×10^7 cfu per gram of skin. However, the bacteria rapidly proliferated in the burn wound, with bacterial counts reaching 5×10^8 cfu per gram of skin by 48 hours post-challenge. Bacteria were detected in liver and blood at 24 hours postburn with levels of 6×10^2 cfu and 9×10^1 cfu, respectively. Paralleling the increase in the bacterial concentration of the burn skin were rapidly increasing numbers of bacteria in liver and blood preparations. By 48 hours postburn and challenge, liver counts had increased to approximately 5×10^6 cfu per gram and blood to 7×10^5 cfu per milliliter.

To examine the sequence of events of burn infection in mice challenged intraperitoneally, the following experiments were performed. Figure 6 illustrates tissue colonization and invasion following i.p. challenge with 4.85×10^4 cfu of strain M-2 (Fla^+ Mot^+). At one hour postburn and challenge, an average of 5×10^2 cfu were detected in liver homogenates; bacteria were not found in burned skin and blood samples, however. By 8 hours post-challenge, a slight decrease in liver counts occurred. Also, approximately 10^2 cfu per milliliter of blood were detected. Skin counts at 8 hours postburn remained at zero. A large increase in skin counts was observed at the 24 hour time point, however, with levels of bacteria reaching 3×10^6 cfu per gram of skin. An increase in liver counts to 4×10^2 cfu occurred whereas no bacteria were detected in the blood. It is important to note that

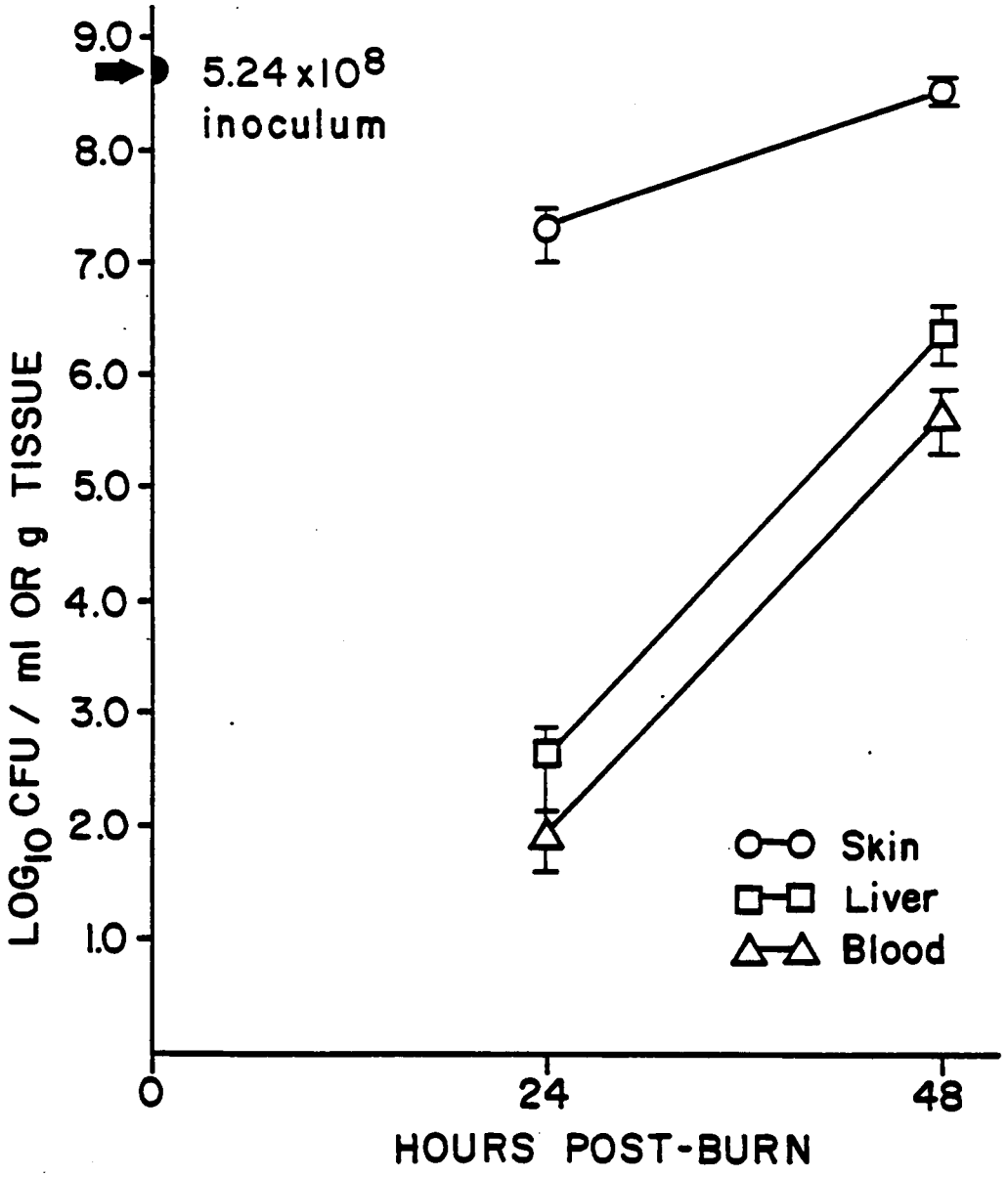

Figure 5. Quantitative bacteriology of skin, liver, and blood after topical challenge with M-2. Data are expressed as means of three mice ± standard error of the mean.

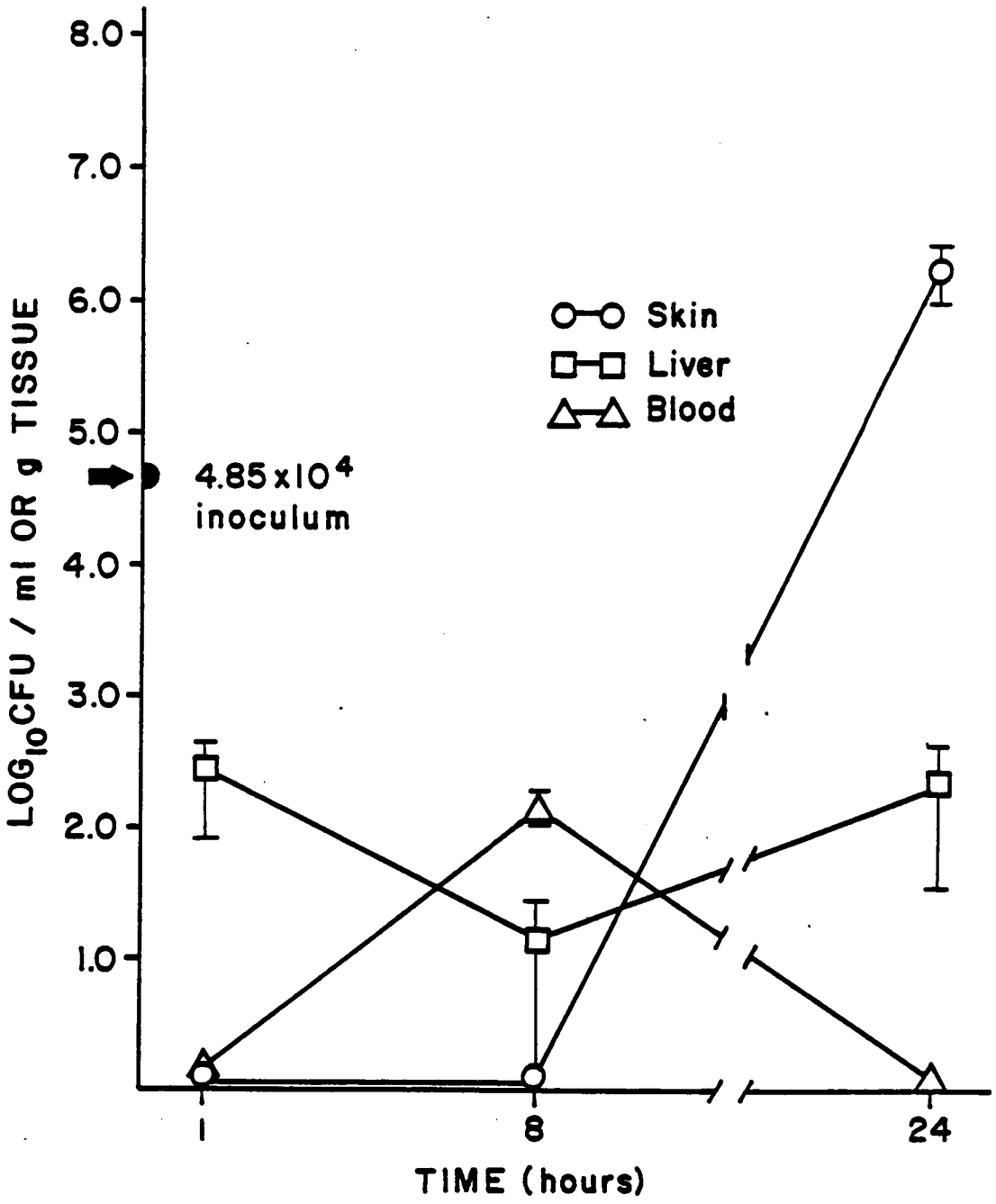

Figure 6. Quantitative bacteriology of skin, liver, and blood after intraperitoneal challenge with M-2. Data are expressed as means of 3 mice ± standard error of the mean.

remaining experimental mice all succumbed to fatal P. aeruginosa infection by 48 hours postburn and challenge.

A similar pattern was observed in mice challenged intraperitoneally with 1.2 x 10^6 cfu of another wildtype strain, MT1200 (Fla^+ Mot^+) as shown in Figure 7. Enumeration of bacteria in liver homogenates revealed a level of colonization of 4 x 10^3 cfu by only one hour postburn and challenge. A different finding, from the M-2 pattern, can be seen in bacterial counts in the blood at one hour; whereas no counts were detected in the previously shown M-2 challenge, 10^2 cfu per milliliter were found in the blood samples of MT1200-challenged mice. This may reflect the 100-fold higher dose of MT1200, but other explanations are possible. Bacteria again were not detected in burned skin homogenates at the one hour time point. By seven hours postburn and challenge, a level of 10^2 cfu per gram of burned skin was seen, a finding which also may reflect the higher inoculum. Coinciding with the increase in skin counts was a sharp decline in blood counts and essentially no change in numbers of bacteria of the liver. Results of the 26 hour time point were almost identical to the previously discussed M-2 experiment. The bacterial load of the liver hovered around 5 x 10^2 cfu, counts were not detected in the bloodstream, and a rapid proliferation of bacteria had occurred in the skin. Remaining mice all succumbed to a P. aeruginosa infection, results identical to the previous experiment. A very different picture was observed in tissue colonization of burned mice challenged intraperitoneally with the mutant MT1200-80 (Fla^+ Mot^+) (Figure 8). At one hour postburn and inoculation, 5 cfu were detected

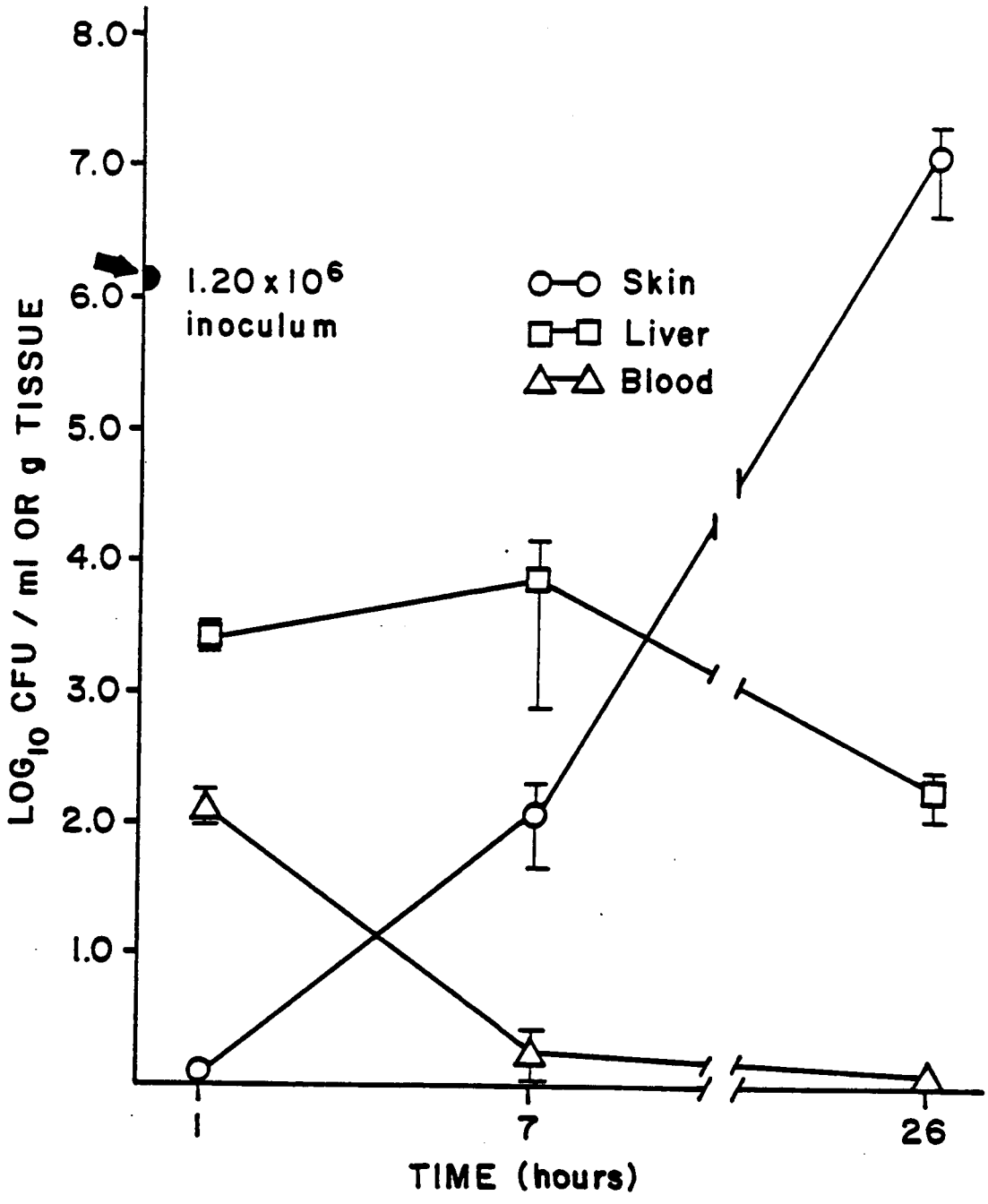

Figure 7. Quantitative bacteriology of skin, liver, and blood after intraperitoneal challenge with MT1200. Data are expressed as means of 3 mice ± standard error of the mean.

in skin homogenates. Although this is a very low level of colonization, this was the first time bacteria were detected at such an early period after the burn. Bacterial counts were not observed in the blood at one hour postburn in contrast to the 100 cfu/ml observed in mice challenged with MT1200. This may be due to levels of bacteria being below the limits of detection of the plate assay employed. A striking difference was seen in liver colonization at one hour postburn. Plate counts of liver homogenates revealed a level of 5 x 10^5 cfu per gram tissue, 100-fold higher than respective counts observed in MT1200-challenged mice (Figure 8). By seven hours postburn, a rapid decrease of the bacteria in the liver had taken place. The bacteria colonizing the burn wound proliferated at a rate comparable to previous observations, reaching a level of 5 x 10^2 cfu by 7 hours. At the 26 hour time point, the bacterial counts in the liver had continued to rapidly decrease with barely detectable levels of only 10 cfu. Possibly of importance, bacteria continued to multiply in the burn wound, but only to an approximately 100-fold lower level than what was observed in mice challenged i.p. with the wildtype phenotype. Another key observation is that bacteria were never detected in the bloodstream of MT1200-80 challenged mice (Figure 8). This may be a result of the very rapid clearance of these cells by the reticuloendothelial system, in comparison to the clearance of the motile strain, MT1200. Remaining mice all survived for a two week period following the 26 hour time point, radically different results than what was observed in mice challenged with wildtype, parental strains (Fla^+ Mot^+).

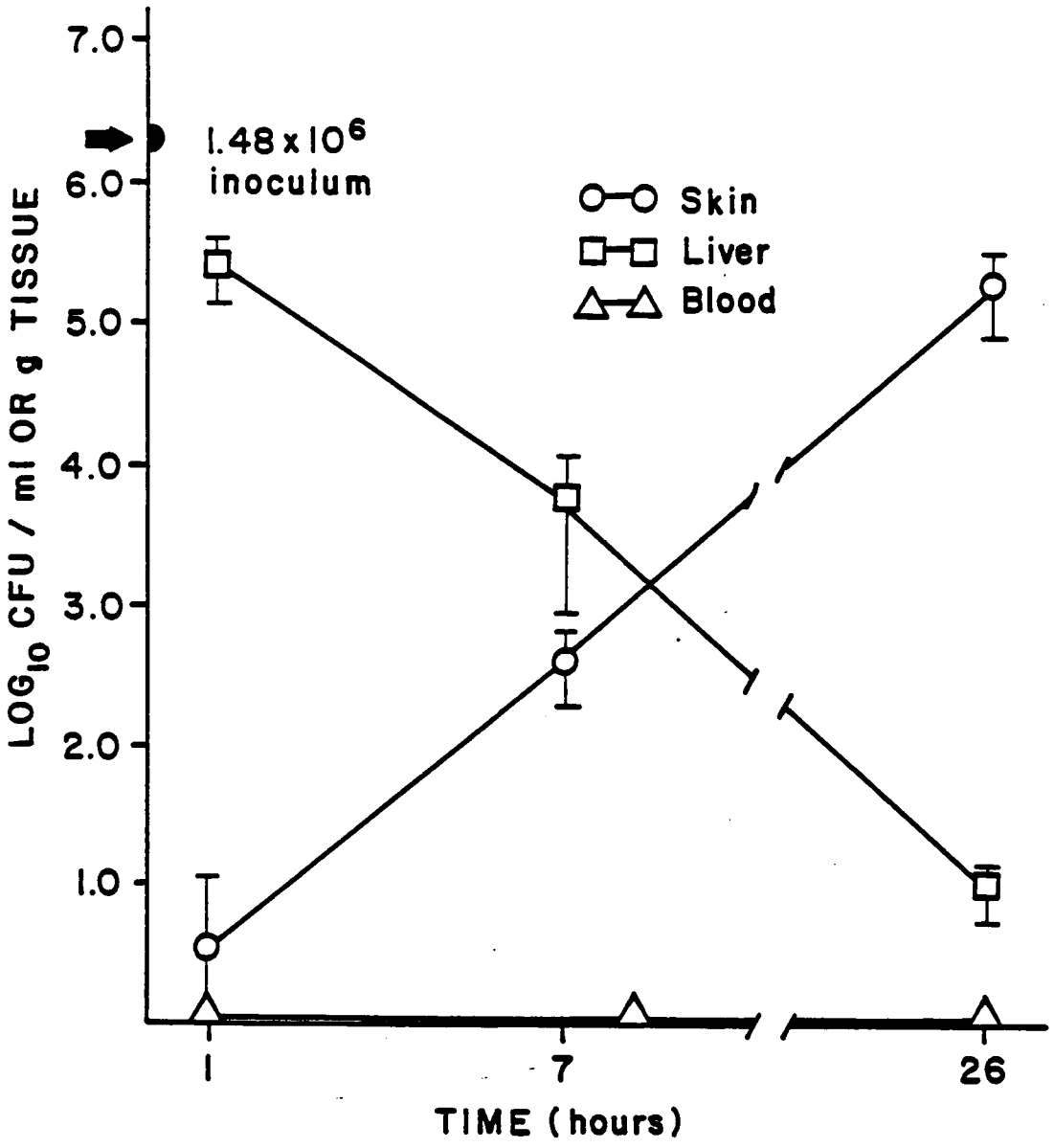

Figure 8. Quantitative bacteriology of skin, liver, and blood after intraperitoneal challenge with MT1200-80. Data are expressed as means of 3 mice ± standard error of the mean.

To determine if differences existed in the ability of strains MT1200 (Fla^+ Mot^+) and MT1200-80 (Fla^+ Mot^-) to colonize and proliferate within the burn wound, low numbers of washed cells of each strain were administered into the burn wound (Table 10). Essentially no difference was observed in quantitative bacteriological counts of burned skin of the two strains. Numbers of MT1200 and 1200-80 in burned skin homogenates at 26 hours were almost identical.

Another well-characterized animal model developed for the study of burn wound infections is the scalded mouse model of Stover et al. (1985), as previously discussed. It was of interest to determine if a similar difference in virulence due to the absence of motility would exist in this scald model as compared to the burned mouse model. Initial experiments examined the virulence of M-2 (Fla^+ Mot^+) and M-2 Fla^- (Fla^- Mot^-) by both subcutaneous and topical routes of challenge as shown in Table 11. Surprisingly, little difference was observed in the lethality of the wildtype-parent and Fla^- mutant following a subcutaneous challenge. At a dose of 1.2×10^2 cfu M-2, only 50% mortality was achieved by day 7; challenge with 1.4×10^2 cfu of M-2 Fla^- resulted in 3 out of 8 mice succumbing to infection. This apparently is the first report of a subcutaneous challenge in the scald mouse model as the developers of the model have exclusively followed a topical route of infection. There may be problems of toxicity in this type of burn that would not allow for the colonization of the burn wound by small numbers of bacteria. A different picture developed when scalded mice were challenged topically. A dose of 3.1×10^7 cfu of M-2 proved to be highly fatal as 100% of the test animals

Table 10. Quantitative bacteriology of burned skin in mice challenged with two Pseudomonas aeruginosa strains differing in the Mot phenotype[a]

Time Postburn (hrs)	MT1200 (Fla^+ Mot^+)	MT1200-80 (Fla^+ Mot^-)
0 (Inocula)	7.1×10^2	9.2×10^2
7	2.8×10^4	1.8×10^4
26	5.4×10^6	5.5×10^6

[a]Colony-forming units per gram of homogenized burned skin.

Table 11. Virulence of two Pseudomonas aeruginosa strains differing in the Fla phenotype challenged topically and subcutaneously in the scalded mouse model

Strain	Route	Challenge Inocula (CFU)	Cumulative Mortalities (Died/Total Inoc.) Days Postburn 1	2	3	4	5	6	7
M-2 (Fla^+ Mot^+)	s.c.	1.2×10^2	0/8	2/8	4/8	4/8	4/8	4/8	4/8
	topical	3.1×10^7	2/8	6/8	8/8	-	-	-	-
M-2 Fla^-	s.c.	1.4×10^2	0/8	2/8	3/8	3/8	3/8	3/8	3/8
	topical	3.6×10^7	0/8	2/8	3/8	6/8	6/8	6/8	6/8
Controls	topical	-	0/5	0/5	0/5	0/5	0/5	0/5	0/5

died by the 3rd day post-challenge. Somewhat different results were seen with the group of mice challenged with M-2 Fla^-. By day 3, only 3 out of 8 mice had succumbed to infection and 6 out of 8 by the end of the experiment. Although M-2 Fla^- was able to kill a substantial number of the animals, a significant delay in time to death occurred compared to that observed in mice in the wildtype infection. This again probably reflects the ability of the motile strain to spread rapidly from the burn wound, invade and colonize viable tissues, causing organ failure and death.

To further address the role of motility in infection of scalded mice, animals were challenged topically with either strain MT1200 (Fla^+ Mot^+) or MT1200-80 (Fla^+ Mot^-). Results of this experiment are shown in Table 12. A challenge dose of the more virulent strain M-2 (Fla^+ Mot^+) served as a positive infection control. Topical application with 2.6×10^9 cfu of the parental strain MT1200 resulted in 5 out of 8 mice succumbing to infection by day 4 postburn. It is not surprising that 100 percent mortality was not achieved since it was previously shown that strain MT1200, although a wildtype, is not as virulent as M-2. Exceedingly different results were seen in the MT1200-80 challenged mice (Table 12). A challenge inoculum of 1.5×10^9 cfu did not cause any mortalities in the test animals. Therefore, these data from the scald mouse model studies add further support to the previous documentation that motility enhances the virulence of infecting P. aeruginosa by allowing for the spread from the burn wound, resulting in multiple organ invasion.

Table 12. Virulence of two Pseudomonas aeruginosa strains differing in the Mot phenotype challenged topically in the scalded mouse model

Strain	Challenge Inocula (CFU)	Cumulative Mortalities (Died/Total Inoc.) Days Postburn 1	2	3	4	5	6	7
M-2 (Fla^+- WT parent)	4×10^7	1/8	6/8	8/8	-	-	-	-
MT1200 (Fla^+ Mot^+)	2.6×10^8	1/6	1/6	1/6	1/6	1/6	1/6	1/6
	2.6×10^9	1/8	1/8	4/8	5/8	5/8	5/8	5/8
MT1200-80 (Fla+ Mot-)	1.5×10^9	0/8	0/8	0/8	0/8	0/8	0/8	0/8
Controls	-	0/6	0/6	0/6	0/6	0/6	0/6	0/6

Preparation and Characterization of Anti-Flagellar Antiserum

Adsorption of Background Antibody. The hyperimmune anti-flagellar antiserum was raised in rabbits against partially purified flagella preparations. Previous studies by Montie et al. (1982a) have demonstrated the lack of major contaminants in these preparations by SDS-gel electrophoresis. However, small significant amounts of lipopolysaccharide (LPS) have been detected. To remove any nonspecific, "background" antibody, the immune sera were subjected to several rounds of adsorption with either heated M-2 cell suspensions, which destroys the labile flagellin, but leaves the membrane components intact, or M-2 Fla$^-$, the flagella-less isogenic mutant. Slide and tube agglutination assays were carried out with the adsorbed sera against M-2 and M-2 Fla$^-$ viable cells. Rapid agglutination was observed when dilutions of antisera were mixed with M-2, as expected. No agglutination, however, occurred against M-2 Fla$^-$ or heated M-2 cells at all concentrations of antiserum tested. These data indicate that any nonspecific antibody initially present in the antisera was removed by repeated adsorption.

Specificity of Adsorbed Anti-Flagellar Antiserum. To examine the specificity of the adsorbed antisera, a series of cross-agglutinating, slide assays were performed. The flagella antigen types of P. aeruginosa have been described elsewhere (Allison et al., 1985). Briefly, P. aeruginosa flagella can be separated into two basic types, "a" and "b", by molecular weight, indirect immunofluorescence, and slide coagglutination techniques (Ansorg et al., 1984). The a-types

can be further divided into several subgroups whereas the b-types comprise a homologous group. The slide agglutination assays, as shown in Table 13, demonstrate specificity as b-type antisera reacted against only b-type strains and a-type antisera agglutinated homologous cells as well. The a_0-type strains seem to be an anomaly in that reaction with b-type and not a-type antisera occurred. However, these strains were most likely incorrectly typed as pointed out in a recent paper from our laboratory.

The type of reactive antibody observed in these assays was determined by an enzyme-linked-immunosorbant-assay (ELISA). Flagella antigen was applied to ELISA plates at a concentration of 0.01 ug per well. The primary antibody was from either an a or b-type antisera, depending on the antigen used. The horseradish peroxidase linked secondary antibody was either an anti-IgG or anti-IgM. The results of these experiments are shown in Table 14. Adsorbed M-2 antiserum was shown to consist predominantly of IgG antibody with only a very low concentration of IgM. Similar results were determined for adsorbed 1210 antiserum (a-type).

To further document the specificity of the H-antisera for flagella antigens and demonstrate the lack of reactivity against somatic antigens, the following experiments were conducted. ELISA plate wells were coated with M-2 (b-type) flagella antigen and Difco antigen no. 5, a suspension of autoclaved P. aeruginosa cells used in the Difco International O antigen typing scheme (IATS). These cells are used in the typing kit as a positive control for serotype number 5, i.e. the respective antiserum number 5 also supplied in the IATS kit will

Table 13. Cross-agglutination reactions with FAg antisera[a]

H antisera[b]	Agglutination[c] of indicated P. aeruginosa strain										
	M-2 (b)	SBI-H (b)	SBI-I (b)	SBI-S (b)	170001 (b)	13030 (a_0)	5142 (a_0)	GNB-1 (a type)	WR5 (a type)	5933 ($a_0a_1a_2$)	170018 ($a_0a_3a_4$)
M-2 (b)	++	++	+	+	-	+	++	-	-	-	-
170001 (b)	++	++	++	++	++	ND[d]	ND	-	-	-	-
5933 ($a_0a_1a_2$)	-	-	-	-	-	-	-	-	-	++	-
170018 ($a_0a_3a_4$)	-	-	-	-	-	-	-	-	-	-	++

[a]Taken from Infect. Immun. 1985. 49:770-774 of which D. Drake is a co-author.

[b]FAg components are in parentheses. All sera except 170001 sera were adsorbed for O antibodies.,

[c]Agglutinations were graded as follows: ++, strong; +, moderate to weak; -, none.

[d]ND, Not done.

Table 14. Type of anti-flagellar antibody as determined in an ELISA[a]

Antigen Type[c]	Secondary Antibody[d]	Antiserum Titer[b]
M-2 (b)	anti-IgG	512,000
	anti-IgM	64
1210 (a)	anti-IgG	256,000
	anti-IgM	64

[a]Procedure described in Methods section.

[b]Reciprocal of the highest dilution of antiserum which has a value 2x that of control wells without antigen.

[c]Antigen was coated onto the wells at a concentration of .01 ug per well.

[d]Horseradish peroxidase conjugated antibody.

rapidly agglutinate these cells. This O-antigen type was chosen because strain M-2, of which the b-type flagella were used to raise specific anti-H antigen antibodies, possesses an O antigen serotyped as number 5. Thus, if contaminating LPS in the flagella antigen preparations elicited antibody, a positive reaction in the O antigen coated wells would be expected from the M-2 H-antigen adsorbed antiserum. The results from this study are illustrated in Table 15. As previously shown, M-2 anti-flagellar antisera reacted strongly against the homologous H-antigen with a titer of 512,000. Difco antiserum number 5 reacted against O antigen number 5 with a titer of 3200, a level expected from such a commercially supplied typing serum. It should be noted that the Difco antiserum also recognized contaminating, homologous O antigen type LPS in the M-2 flagella antigen preparation. Also, Difco antiserum number 5 did not react at all with a different O antigen type, demonstrating the specificity of the O antiserum in this assay. Of particular importance is the observation that adsorbed M-2 H antigen-antisera did not react against the Difco antigen 5 coated wells. These data show that the adsorbed anti-flagellar antiserum does not contain any background antibody against somatic antigen.

Another approach for determining the specificity of anti-flagellar antiserum utilized a Western Blot technique. M-2 (b-type) flagella antigen was subjected to electrophoresis in an SDS gel and then blotted onto nitrocellulose paper as described in the Methods and Materials section. Adsorbed b-type antiserum was used as the coating antibody. ^{125}I-labeled Staphylococcus aureus protein a, which specifically

Table 15. Specificity of anti-flagellar antiserum and lack of somatic antigen reactivity as determined in an ELISA[a]

Antigen Type[c]	Primary Antisera	Antiserum Titer[b]
M-2 (b)	M-2 (b)	512,000
Difco #5[d]	Difco #5[e]	3,200
Difco #10	Difco #5	0
M-2 (b)	Difco #5	8,000
Difco #5	M-2 (b)	0

[a]Procedure described in Methods section.

[b]Reciprocal of the highest dilution which has a value 2x that of control wells without antigen.

[c]Flagella antigen was coated onto the wells at a concentration of .01 ug per well.

[d]Autoclaved cells of O antigen type 5 from the Difco IATS typing kit, diluted 1000-fold.

[e]Antiserum raised against autoclaved cells of O antigen type 5, from the Difco IATS typing kit.

binds to antigen-antibody complexes, was added. The results are shown in Figure 9. It can be seen that the anti-flagellar antibody singly reacted strongly against the b-type flagellin band, a further strong indication of the specificity of the anti-flagellar antisera.

Motility Inhibition In Vitro by Anti-Flagellar Antiserum. Upon demonstrating the specificity of the flagellar antisera against flagellin, it was of interest to examine if the anti-flagellar antibody would inhibit motility of viable cells and to obtain some understanding of the mechanism of this inhibition. Growth experiments were performed initially to determine if the antisera were bactericidal. It was discovered that strain M-2 grew quite well in whole and diluted anti-flagella serum, indicating an absence of bactericidal activity.

Immobilization of viable cells was assayed in motility tubes containing low agar concentrations (0.3%), nutrient broth, and dilutions of the appropriate adsorbed antiserum. An immobilization titer was determined for the flagellar antisera and defined as the highest dilution of antiserum that restricted bacterial growth to an initial stab line, as shown in Table 16. Strain M-2 (b-type H antigen) exhibited a significant depression in the ability to spread in the motility agar supplemented with anti-flagellar (b) serum. It should be noted that no inhibition of motility was observed in tubes containing normal preimmune serum. Another b flagella-type, P. aeruginosa strain (SBII) showed an inhibition of motility in the M-2 antiserum tubes of an identical magnitude, illustrating the cross-reactivity among the homologous b-flagellin types. A key point in this regard is that

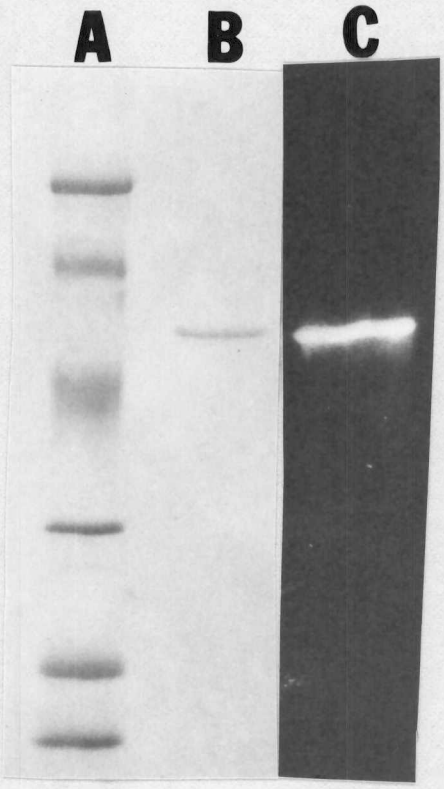

Figure 9. SDS-PAGE and Western blot of M-2 flagellin. (Lane A) From top, Phosphorylase B (92,500); Bovine serum albumin (BSA) (66,200); Ovalbumin (45,000); Carbonic anhydrase (31,000); Soybean trypsin inhibitor (21,500); Lysozyme (14,400). (Lane B) M-2 flagellin. (Lane C) M-2 flagellin reacted with M-2 adsorbed flagellar antiserum and ^{125}I-Staph Protein A.

Table 16. Immobilization of bacteria in motility tubes incorporated with flagellar antisera[a]

Organism	Antisera[b]	H Ag Type	O Ag Type[c]	Titer of Immobilization[d]
M-2	b	B	2,5	2,560
SBI-I	b	B	10	2,560
1210	b	$a_0a_1a_2$	6	0
1210	a	$a_0a_2a_2$	6	1,280
M-2	a	B	2,5	40
SBI-I	a	B	10	40

[a]Motility tubes were stab inoculated with the appropriate organism from 18 hr plate cultures. The tubes were incubated for 18 hrs at 37°C.

[b]Raised against a-type or b-type flagellin.

[c]O Ag types were determined using the IATS set (Difco).

[d]The immobilization titer is defined as the highest dilution of antiserum that restricts bacterial spreading to the stab line. No motility inhibition was observed in saline or normal preimmune serum tubes.

although strain SBI-I possesses the same b-type flagellin, it has a different O antigen type. This serves as additional proof of the specificity of the antiserum. Furthermore, P. aeruginosa strain 1210, possessing a-type flagella, did not show an inhibition of motility in tubes incorporated with b-type antiserum, additionally indicating the lack of cross-reactivity between heterologous flagella antigen-antiserum mixtures.

Phase Contrast Microscopy: Observations of Motility Inhibition by Flagellar Antiserum. An interesting question concerning the mechanism of inhibition of motility by the specific flagella antiserum arises. Does the antibody inhibit the motility of a single bacterium or does inhibition of motility occur due to an agglutination of cells? To address this question, lightly turbid suspensions of bacteria were made in dilutions of adsorbed antisera on slides and observed under phase contrast microscopy. Small clumps of cells were observed, sometimes 2, 3, or more, in what best would be described as a "pinwheel arrangement." The bacteria appeared to be bound only end-to-end and never "cemented" together, membrane to membrane. This arrangement was also observed under electron microscopy as seen in Figure 10. The bound cells appeared not to be in direct membrane contact at the tips. Such bound bacteria spun around very rapidly in futile attempts to escape the entanglement. Single cells were observed swimming freely and only stopped upon apparent contact with the flagellum of another cell. From these observations, it is concluded that the specific flagella antibody inhibits motility by causing a cross-linking

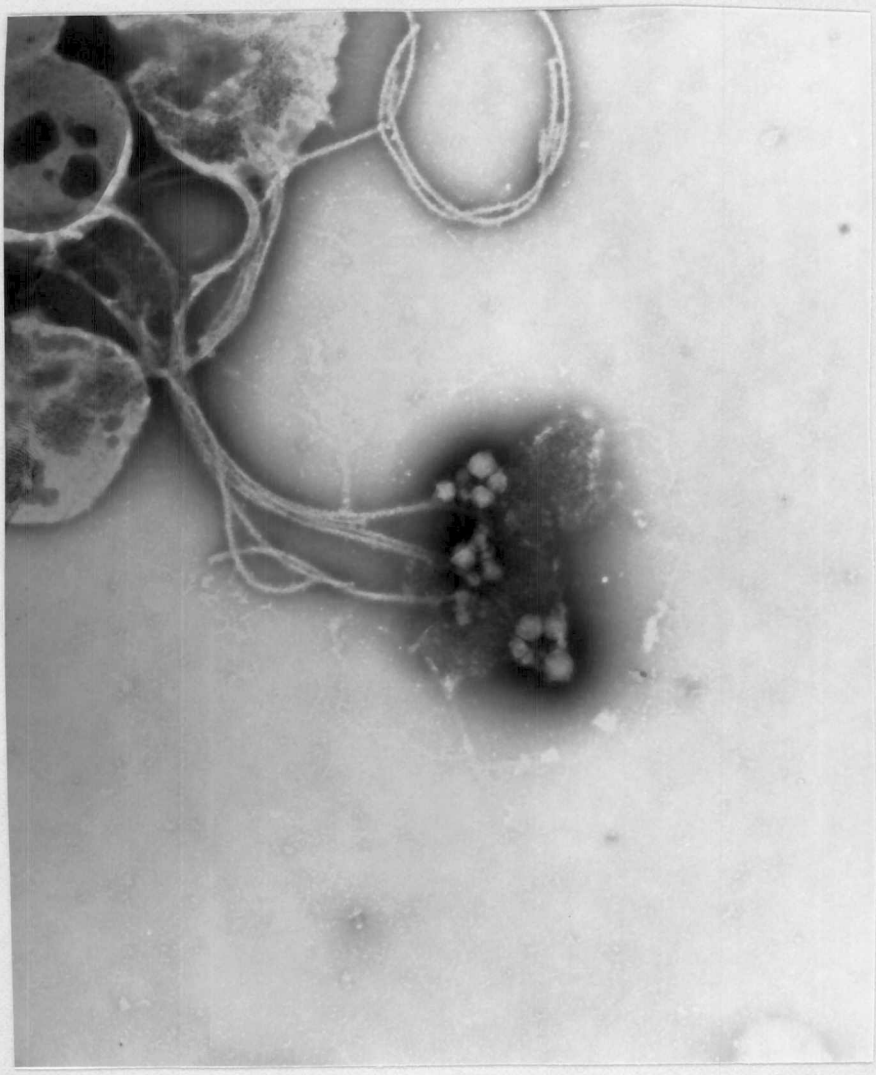

Figure 10. Electron micrograph of P. aeruginosa (b-type) suspended in 100-fold diluted homologous flagellar antiserum and stained with 0.5% phosphotungstic acid. Magnification: 20,000X.

phenomenon between the flagella of bacteria that come in contact with each other. Such a microagglutination reaction rapidly builds until large aggregates of cells are bound together.

Protection Against Pseudomonas aeruginosa Infection: Passive Immunotherapy with Flagellar Antiserum

Studies with P. aeruginosa strains deficient in the Fla or Mot phenotype demonstrated the corresponding loss of virulence, particularly the invasive potential, associated with such a defect. Additional investigations showed specificity of anti-flagellar antiserum and the ability to inhibit the motility of cells in vitro. Of particular interest at this point was the question, will such an antiserum protect against normally invasive P. aeruginosa burn wound infection?

The results of an initial study conducted to address this question are shown in Table 17. Non-immunized mice rapidly succumbed to a fatal P. aeruginosa infection with 100 percent mortalities recorded by the 4th day postburn and challenge. A much different picture developed in mice receiving 100-fold diluted flagellar antiserum, however. It can be seen that passively immunized mice which received an identical challenge dose all survived. Moreover, no mortalities were observed at a 10-fold higher challenge dose and only one death occurred at a concentration equivalent to 1700 LD_{50} doses. These data indicate that protection can be achieved with anti-flagellar serum against normally lethal challenge doses.

Table 17. Homologous challenge using B type antisera and challenging with a flagellar B type organism (M-2) subcutaneously[a]

Mice	Challenge (CFU)	Cumulative Mortalities (Died/Total Inoc.) Days Postburn 1	2	3	4	5	6	7
Non-injected	1.4×10^2	0/8	1/8	5/8	8/8	-	-	-
Antiserum administered	1.4×10^2	0/8	0/8	0/8	0/8	0/8	0/8	0/8
Antiserum administered	1.4×10^3	0/8	0/8	0/8	0/8	0/8	0/8	0/8
Antiserum administered	1.4×10^4	0/8	1/8	1/8	1/8	1/8	1/8	1/8

[a]Antisera (100-fold diluted--0.5 ml) injected i.p. immediately after burning and one day postburn.

The specificity of flagellar antiserum for homologous antigen types was demonstrated by slide agglutination and motility inhibition assays *in vitro*. It was important to determine if such specificity would also exist in *in vivo* protection studies. The results of the first experiment addressing this issue are shown in Table 18. Non-immunized mice were highly susceptible to strains M-2 and SBI-I (b-types). Normal, preimmune serum did not afford any protection to infected mice. A key result is that complete protection against both b-type *P. aeruginosa* strains was achieved by administration of flagellar b-type antiserum. The specificity of the protection should be emphasized in that strain SBI-I, although of the same flagellar type as M-2 FAg (which was used to raise the antiserum), has a different, non-cross reactive O antigen type.

Flagellar antiserum raised against b-type FAg did not protect against challenge with a-type FAg *P. aeruginosa* strains (Table 19). As can be seen, all test groups rapidly succumbed to a fatal infection with no apparent differences between the control and passively immunized groups of mice. Similar studies were done with a-type flagellar antiserum as shown in Table 20. Protection was observed only in mice receiving a homologous type challenge strain and not in mice challenged with heterologous strains. These data, then, correlate well with the *in vitro* immobilization assays demonstrating that protection achieved by administering flagellar antiserum is FAg-type specific.

In the protection studies to this point, flagellar antiserum was administered immediately following the burn or one day prior to inflicting the burn injury. A question to be asked at this point is

Table 18. Homologous protection using B type H specific antisera to challenge with flagellar B type organisms[a]

Mice	Challenge (CFU)[b]	Cumulative Mortalities (Died/Total Inoc.) Days Postburn 1	2	3	4	5	6	7
Non-injected	M-2 (1.43×10^2)	0/8	5/8	8/8	-	-	-	-
Non-injected	SBI-I (4.0×10^4)	0/8	6/8	7/8	7/8	7/8	7/8	7/8
NRS administered[c]	M-2 (1.4×10^2)	0/8	6/8	8/8	-	-	-	-
NRS administered	SBI-I (4.0×10^4)	0/8	7/8	7/8	7/8	7/8	7/8	8/8
Injected with M-2 H antisera	M-2 (1.43×10^2)	0/8[d]	0/8	0/8	0/8	0/8	0/8	0/8
Injected with M-2 H antisera	SBI-I (4.0×10^4)	0/8[d]	0/8	0/8	0/8	0/8	0/8	0/8

[a]Antisera (100-fold diluted--0.5 ml, i.p.) injected one day prior to burning.

[b]Subcutaneous.

[c]NRS = Normal rabbit serum.

[d]Day 15 = 0/8.

Table 19. Cross-challenge using M-2 B type H specific antisera and challenging with flagellar A type organisms[a]

Mice	Challenge (CFU)[b]	Cumulative Mortalities (Died/Total Inoc.) Days Postburn 1	2	3	4	5	6	7
Non-injected	1210 (7.9×10^3)	2/8	8/8	-	-	-	-	-
Non-injected	GNB-1 (4.0×10^4)	0/8	6/8	8/8	-	-	-	-
NRS administered[c]	1210 (7.9×10^3)	0/8	8/8	-	-	-	-	-
NRS administered	GNB-1 (4.0×10^4)	1/8	6/8	8/8	-	-	-	-
Injected with M-2 H antisera	1210 (7.9×10^3)	0/8	7/8	8/8	-	-	-	-
Injected with M-2 H antisera	GNB-1 (4.0×10^4)	0/8	5/8	7/8	8/8	-	-	-

[a]Antisera (100 fold diluted--0.5 ml, i.p.) injected one day prior to burning.

[b]Subcutaneous.

[c]NRS = Normal rabbit serum.

Table 20. Cross-challenge using 1210 A-type specific antisera and challenging with flagellar B-type organisms[a]

Mice	Challenge (CFU)[b]	Cumulative Mortalities (Died/Total Inoc.) Days Postburn 1	2	3	4	5	6	7
Non-injected	M-2 (1.54×10^2)	0/8	4/8	7/8	7/8	7/8	7/8	7/8
NRS administered[c]	M-2 (1.54×10^2)	0/8	2/8	4/8	6/8	7/8	7/8	7/8
NRS administered	1210 (1.09×10^2)	0/8	5/8	8/8	-	-	-	-
Injected with 1210 antisera	M-2 (1.54×10^2)	1/8	5/8	7/8	7/8	7/8	7/8	7/8
Injected with 1210 antisera	1210 (1.09×10^2)	0/8	0/8	1/8	1/8	1/8	1/8	1/8

[a]Antisera (100 fold diluted--0.5 ml, i.p.) injected immediately postburn.

[b]Subcutaneous.

[c]NRS = Normal rabbit serum.

whether or not protection can be achieved if administration of the flagellar antiserum is delayed. The results of these studies are illustrated in Table 21. Mice passively immunized immediately postburn and challenge were completely protected, as previously shown. Delaying the administration of flagellar antiserum by one day postburn and challenge resulted in an almost total abrogation of protection as 7 out of 8 mice died by day 3. Incorporation of a second, booster injection of antiserum on day 2 following the day 1 injection also did not alter the lethality of infection. Delay in administering flagellar antiserum by 11 hours caused a significant loss in protection although a delay in time to death was noted. These data indicate that complete protection is achieved only upon immediate administration of flagellar antiserum which may reflect the need for protective antibody very early in burn wound infection to inhibit the spread from the burn site.

It was reported previously that the topical route of challenge has merit in considering surface contamination of burn wounds; in fact, investigators using scald animal models exclusively use this route of challenge (Pruitt and McManus, 1984). To determine if flagellar antiserum would afford protection in mice challenged topically, the following study was done (Table 22). Non-protected mice were all moribund by the fifth day postburn following challenge with 2.8×10^9 cfu strain M-2. Similar results were obtained when mice were given preimmune serum before bacterial challenge. Mice that received anti-flagellar serum exhibited a significant delay in time to death and partial protection. It is important to point out that 100 fold lower doses of strain M-2 also cause 100 percent mortality. It may well be

Table 21. Delay in administration of flagellar antiserum in subcutaneously challenged mice[a]

Mice	Dose[b]	Cumulative Mortalities (Died/Total Inoc.) Days Postburn 1	2	3	4	5	6	7
Non-injected	9.7×10^1	0/8	4/8	8/8	-	-	-	-
Injected on day 0	9.7×10^1	0/8	0/8	0/8	0/8	0/8	0/8	0/8
Injected 11 hours postburn	1.5×10^2	0/8	1/8	3/8	7/8	7/8	7/8	7/8
Injected on day 1 postburn	9.7×10^1	0/8	5/8	7/8	7/8	7/8	7/8	7/8
Injected on days 1,2 postburn	9.7×10^1	0/8	2/8	7/8	7/8	8/8	8/8	-

[a]Antiserum (100 fold diluted--0.5 ml) injected intraperitoneally.

[b]*P. aeruginosa* strain M-2.

Table 22. Homologous challenge using B type H antisera and challenging topically with flagellar B type organisms[a]

Mice	Challenge (CFU)	Cumulative Mortalities (Died/Total Inoc.) Days Postburn 1	2	3	4	5	6	7
Non-injected	M-2 (1.11×10^3 s.c.)	0/8	6/8	8/8	-	-	-	-
Non-injected	M-2 (2.78×10^9)[b]	0/8	0/8	6/8	6/8	8/8	-	-
Injected with NRS	M-2 (2.78×10^9)[b]	0/8	0/8	5/8	8/8	-	-	-
Injected with M-2 H antisera	M-2 (2.78×10^9)[b]	0/8	0/8	3/8	3/8	3/8	3/8	5/8

[a]Antisera (100 fold diluted--0.5 ml) injected i.p. one day prior to burning and one day postburn.

[b]Topical applications.

that a greater level of protection would be observed at lower challenge doses.

Quantitative Bacteriology of Tissues in Passively Immunized, Burned Mice

At this point, it has been documented that protection against P. aeruginosa infection can be achieved in burned animals upon administration of flagellar antiserum. These experiments were performed to follow the progression of infection following subcutaneous bacterial challenge in such protected mice. Data from the first study are illustrated in Figure 11. Values for skin, liver and blood counts from normal, non-immunized mice are shown for comparison. Control mice usually succumbed to infection within 24-36 hours postburn; therefore, only the time points immediately prior to death are shown for purposes of comparison. Passively-immunized mice survived through the eighth day postburn. At 24 hrs postburn and challenge, bacteriological counts of burned skin in mice administered anti-flagellar serum had reached a level of only 10^5 cfu per gram of skin, a concentration 2 logs less than what was observed in nonimmunized, burned mice. Also, no bacteria were found in either liver or blood samples (at 1 day) of passively-immunized mice in complete contrast to numbers occurring in control mice. In agreement with the lethality studies, mice administered anti-flagellar serum all survived to the end of the experiment (8 days) whereas non-protected mice succumbed to infection by days 1-3. Bacteriological counts in tissues over the 8 day period postburn illustrate this difference. Bacteria never exceeded 10^3 cfu

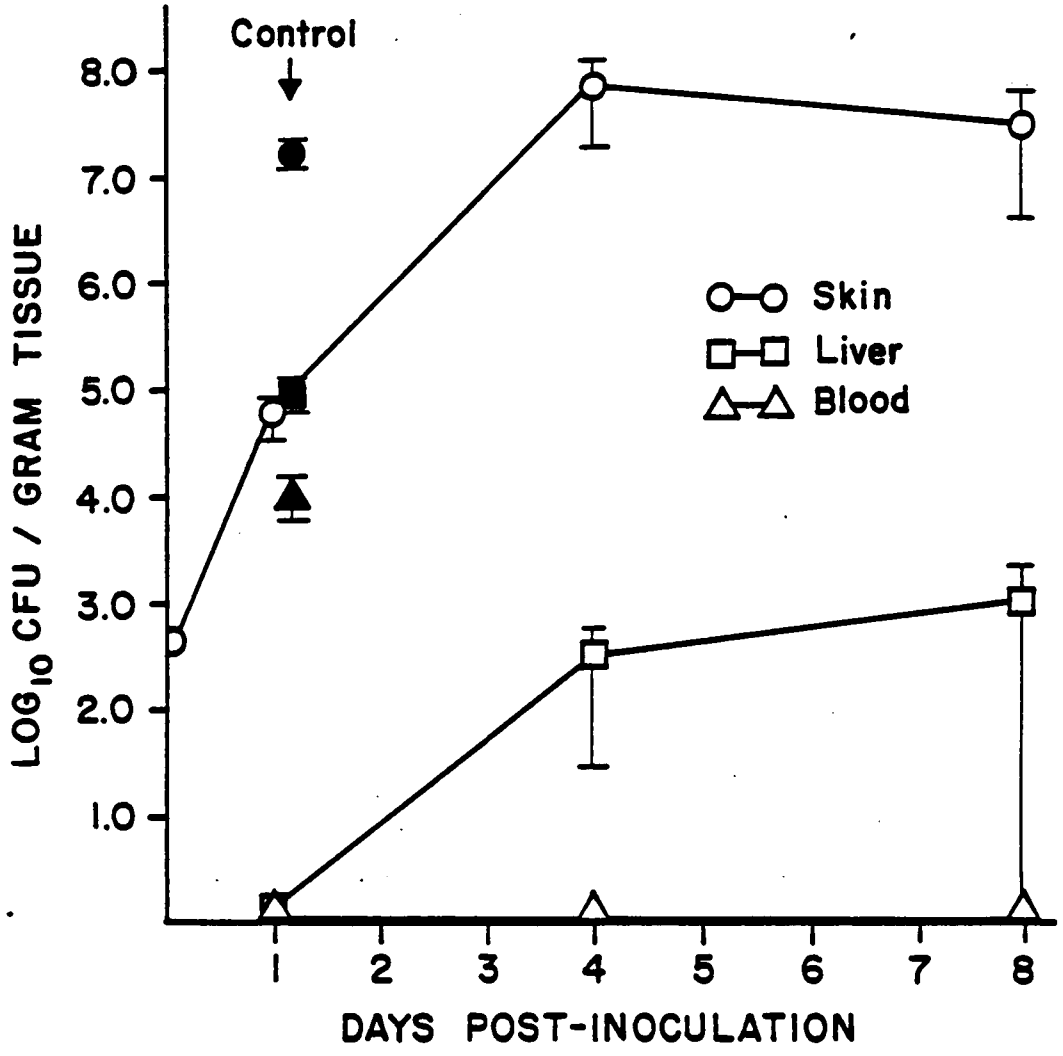

Figure 11. Quantitative bacteriology of skin, liver, and blood of mice after administration of b-type anti-flagellar serum at day 0 and challenged at day 0 with a homologous P. aeruginosa (M-2) subcutaneously.

in the liver and were never detected in the bloodstream. It is pertinent to point out that these tissue data are very similar to what is observed in burned mice challenged with P. aeruginosa strains deficient in the Fla or Mot phenotype.

In some of the lethality experiments, mice received more than one injection of flagellar antiserum. To see what effect this might have on tissue colonization and invasion, a study was conducted as shown in Figure 12. Mice were administered anti-flagellar serum one day prior to burning and on the 2nd day postburn. Quantitative bacteriology of tissues revealed a very similar pattern to that observed in mice receiving a single administration of antiserum. Skin counts were almost identical and bacteria again were not detected in the blood throughout the experiment. A notable difference, however, was seen in bacteriological counts of the liver. In doubly-immunized mice, bacteria were at barely detectable levels, below 10 cfu per gram of liver. As in the first study, mice passively-immunized with anti-flagellar serum all survived until the termination of the experiment whereas control mice rapidly succumbed to a lethal P. aeruginosa infection by days 1-3.

Clearance of Bacteria from the Bloodstream and Passive Immunization of Neutropenic Mice

Lethality experiments have demonstrated the level of protection achievable in burned mice upon administration of the specific flagellar antiserum. Tissue colonization experiments demonstrated the lack of invasion of P. aeruginosa from the burn wound in passively immunized

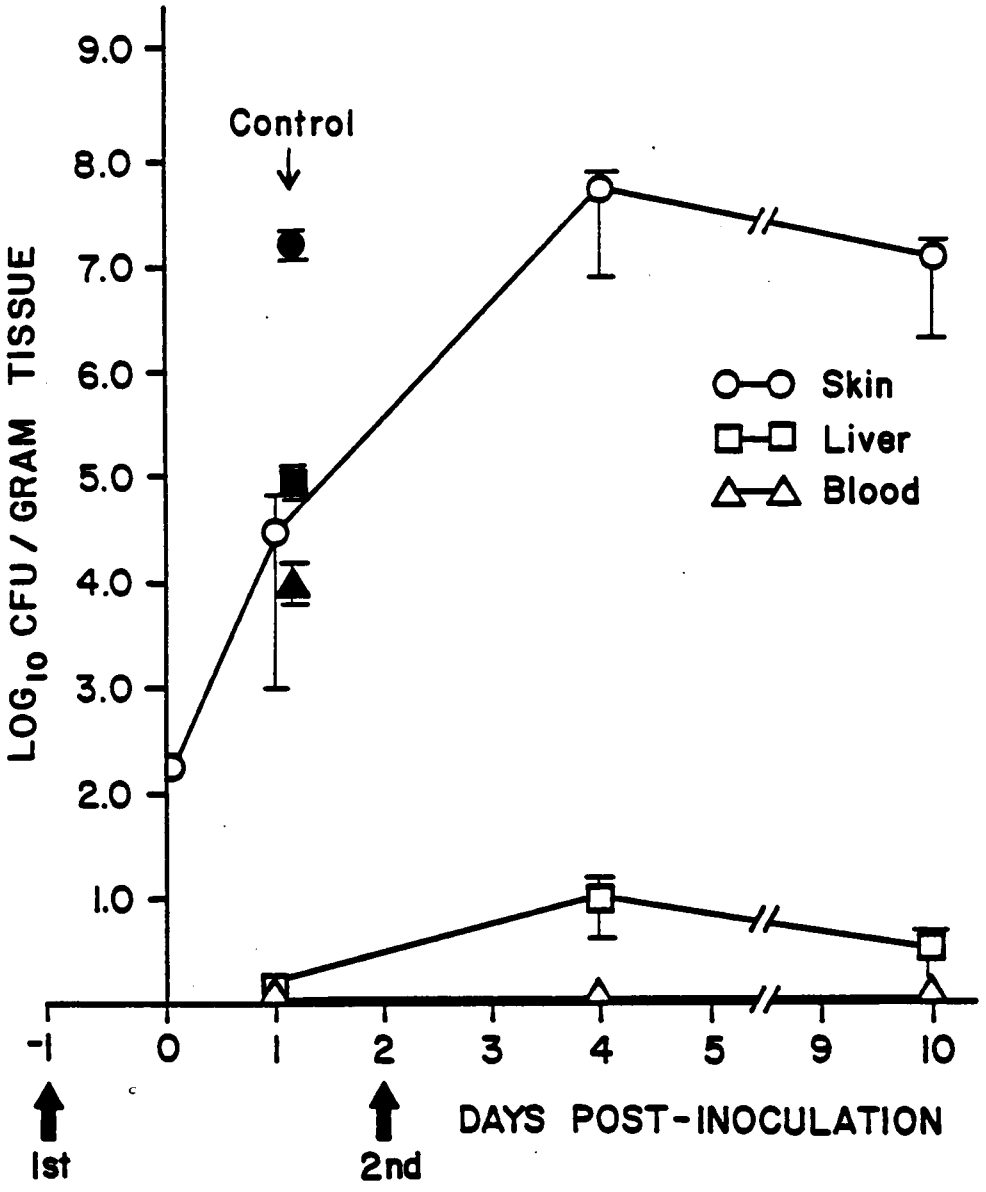

Figure 12. Quantitative bacteriology of skin, liver, and blood of mice passively immunized twice with b-type antiflagellar serum at days -1 and 2 and challenged at day 0 with a homologous P. aeruginosa (M-2) subcutaneously.

mice. A question arises at this point concerning the nature of this protection. How does anti-flagellar antibody protect burned, normally highly susceptible mice against lethal infection? With the data presented, one hypothesis envisions an agglutination reaction occurring in vivo, thus inhibiting motility and blunting the invasive capacity of P. aeruginosa. Another equally important role may involve a cellular line of defense, the phagocytes. In an attempt to address the latter, the following experiments were conducted.

It is known that burned animals undergo an overwhelming immunosuppression due to their injury (see Introduction). To determine if administration of anti-flagellar serum enhances the function of the reticuloendothelial system (RES), clearance of bacteria from the blood in immunized and non-immunized mice was measured as shown in Table 23. A number of P. aeruginosa strains differing in the Fla phenotype were injected intravenously in several experiments. Interestingly, the clearance of MT1200 from non-burned mice seemed slightly less than that observed in burned animals, although the significance of this difference is marginal at best. There was no apparent difference in clearance rates of any of the other test groups, including the passively immunized mice.

Burned animals are known to suffer a multifactorial dysfunction of their neutrophils which can be partially overcome upon transfer of normal granulocytes. To further address the role of phagocytes in protection in flagellar antiserum-treated animals, mice were rendered neutropenic by a single injection of cyclophosphamide (Table 24). A challenge of only 18 cfu resulted in 4 out of 6 nonimmunized control

Table 23. Clearance of bacteria from the bloodstream in burned and burned-passively immunized mice[a]

Mice	Challenge[c]	Time Post-Intravenous Challenge (Minutes)[b] 10	30
Normal	MT 1200 (2×10^7)	4.2×10^3 (15 min.)	9.4×10^2 (40 min.)
Burned	MT1200 (1.6×10^7)	4.8×10^2	9.4×10^1
Burned	M-2 (1.9×10^7)	1.1×10^3	1.5×10^2
Burned	M-2 (2.3×10^7)[d]	6.7×10^2	1.8×10^2
Burned	AK1152 (2.6×10^7)	3.1×10^3	7.3×10^2
Burned, Passively Immunized[e]	M-2 (1.4×10^7)	8.7×10^2	8.9×10^1

[a]Mice were burned for 13 seconds, allowed to recover for 1 hour, placed under a heat lamp for an additional hour for tail vein dilution, and injected intravenously.

[b]Numbers of bacteria expressed are averages of 3 mice bled 3x at 10 and 30 minutes.

[c]Bacteria were washed and suspended in bacterial diluent; volume injected intravenously was 0.1 ml.

[d]Intravenous challenge was delayed 11 hours after burning.

[e]Mice were administered 0.5 ml (i.p.) 100x diluted M-2 antisera immediately postburn.

Table 24. Effect of cyclophosphamide treatment on mice prior to burn and challenge[a,b]

Mice	Total WBC/mm^3	Number of polymorphonuclear leukocytes/mm^3	Number of Lymphocytes/mm^3
Non-treated	1.0×10^3	2.7×10^2	7.3×10^2
Injected with CY	0.4×10^3	1.2×10^1	3.8×10^2

[a]Mice were injected with cyclophosphamide (CY) at a concentration of 200 mg/kg 3 days prior to burn and challenge.

[b]Total and differential white blood cell (WBC) counts were determined by the Wright Staining Method.

deaths by the 3rd day postburn (Table 25). Complete protection, again, was achieved by administration of anti-flagellar serum whereas normal serum treated mice did not survive. Treatment of mice with cyclophosphamide significantly reduced their neutrophil counts as shown in Table 24. Neutropenic mice were highly susceptible to P. aeruginosa infection, even after administration of flagellar antiserum (Table 25). A slight delay in time to death was observed in passively-immunized mice, but 100 percent mortality had occurred by the 3rd day postburn and challenge.

Table 25. Effect of cyclophosphamide treatment on protection in burned mice administered anti-flagellar serum[a]

Mice	Challenge (CFU)[b]	Cumulative Mortalities (Died/Total Inoc.) Days Postburn 1	2	3	4	5	6	7
Non-injected	1.84×10^1	0/6	3/6	4/6	4/6	4/6	4/6	4/6
Injected with NRS	1.84×10^1	0/6	2/6	6/6	-	-	-	-
Injected with M-2 antisera	1.84×10^1	0/6	0/6	0/6	0/6	0/6	0/6	0/6
CY treated, injected with NRS[c]	1.84×10^1	0/6	6/6	-	-	-	-	-
CY treated, injected with M-2 antisera[c]	1.84×10^1	0/6	3/6	6/6	-	-	-	-

[a]Mice were injected i.p. with cyclophosphamide (CY), 4 mg per mouse, 3 days prior to burning.

[b]Subcutaneous.

[c]Antisera or normal sera (0.5 ml, 100x diluted, i.p.) injected immediately postburn.

CHAPTER IV

DISCUSSION

Similarity of Fla and Mot Mutants

The virulence of *Pseudomonas aeruginosa* appears to be multifactorial in that several isolated components have been implicated in the pathogenesis of this organism. The relative contribution of many of these virulence factors may vary widely, however, depending on the disease or animal model studied. Moreover, not all clinical isolates seem to produce the entire repertoire of described virulence components. Therefore, a more realistic approach in adequately studying the role of one factor in the pathogenesis of *P. aeruginosa* would involve using mutants derived from well-characterized, wild-type strains that differ from the parents in the absence of that one factor only. This was the approach taken in this study on the role of motility in the pathogenesis of *P. aeruginosa* in burn wound infections.

An exhaustive number of tests were employed to demonstrate the similarity between the wild-type parent strains and their respective derived Fla or Mot mutants. Although it is apparent that some feature could be overlooked, the overall consensus from this battery of tests is that no difference in the overall physiology, serum reactivity, growth, or elaborated products could be detected. These data lend support for the apparent isogenicity of the motility mutants and allow for interpretation of differences observed in virulence and tissue

infection studies on the basis of the presence or absence of motility alone.

Virulence Studies

Initial experiments were performed to examine the virulence of strains M-2 and M-2 Fla$^-$ via the standard subcutaneous challenge of burned mice. As indicated in the results section, a significant loss of virulence was observed with M-2 Fla$^-$ challenge, with an LD_{50} value approximately 10,000 times higher. These data confirm and elaborate on previous findings that the loss of flagella and subsequent loss of motility correspond well with loss of virulence (Montie et al., 1982b). To extend these initial findings, experiments were conducted with two additional sets of mutants. Again, strains possessing a Fla$^-$ or Mot$^-$ phenotype were significantly less virulent than their respective parents. The parental strain MT1200 was less virulent than the other two wild types, PaO-1 and M-2. This may reflect the presence of the anabolic and catabolic markers on the mapped flagellar genes of MT1200 or may be indicative of slight strain differences not measured in this study. However, as with the Fla$^-$ mutants, the Mot$^-$ mutant MT1200-80 was essentially avirulent. It is important to stress that the characteristic loss of virulence is observed not only with Fla$^-$ mutants, but also with strain MT1200-80, which is flagellated but nonmotile. These data indicate, then, that decreases in virulence do not seem to result from the physical absence of flagella, but more from the loss of the function of motility.

The colonization of burn wounds of thermally-injured patients has been shown to occur from both endogenous and exogenous pathways. Therefore, it was considered important to examine multiple routes of challenge to properly address where the ability to swim would be most advantageous to an infecting organism.

Studies by McManus et al. (1984) have shown that very high inocula are needed to initiate a lethal P. aeruginosa infection in scalded rats by a topical route. This has been a point of issue within the burn research field with the developers of the scalded mouse model claiming that a topical, surface infection is the most clinically relevant. An opposing view is that burn wounds in clinical settings seldom, if ever, are contaminated with such a high dose; therefore, the developers of the burned mouse model have always followed a regimen where very low numbers of bacteria are injected directly into the burn eschar (subcutaneous route). Both arguments have scientific merit, and it is doubtful that one animal model is better or more clinically relevant than the other. Studies of topical wound infection with M-2 and M-2 Fla^- in both animal models revealed that a significantly lower number of mortalities occurred in mice challenged with the Fla^- strain, demonstrating that a decrease in virulence due to loss of flagella and subsequent motility is not limited to a subcutaneous challenge. Such comparisons were not able to be made with the other two groups of strains as it was discovered that the parent MT1200 and PAO-1 exhibited very low virulence via the topical route of challenge.

An intraperitoneal route of challenge revealed similar differences in virulence as seen in the subcutaneous experiments between parent and

respective mutants but at a lower magnitude. In all cases, estimated LD_{50} doses of the mutant strains were one log higher than each wildtype parent. An explanation for these differences may be derived from analysis of tissue colonization data, which will be discussed later.

The initial lethality experiments, therefore, have documented that motility is an integral virulence component which enhances the capability of a colonizing P. aeruginosa strain to cause lethal burn wound infection. A much lower level of killing resulted from infection with strains of a Fla^- or Mot^- phenotype in all of the routes of challenge examined. Motility may not be as important in localized infections, such as keratitis. Extensive corneal damage in P. aeruginosa eye infections apparently results from the action of elaborated proteases and possibly other exoenzymes and exotoxins. Whether or not a strain is flagellated or motile seems to be irrelevant in this type of infection as Fla^- or Mot^- mutants are as virulent as wildtype strains (B. Iglewski, personal communication).

Motility may also play a minor role in such chronic infections as cystic fibrosis. In fact, strains isolated from CF patients in poor clinical condition usually exhibit a non-flagellated phenotype. It has been hypothesized that selection pressure against an invasive, wildtype phenotype exists in the lungs of cystic fibrosis patients (Luzar and Montie, 1985; Luzar et al., 1985). Nonmotile bacteria persisting in mucoid microcolonies seem to be more characteristic of chronic, cystic fibrosis infections. Therefore, motility seems to be more important in

acute, systemic infections which are characterized by a rapid invasion from an initial focus of infection.

To gain further understanding of the progression of P. aeruginosa burn wound infection, and to determine why motility is important at different levels of magnitude depending on the route of challenge, experiments were designed to monitor the colonization and spread of bacteria in thermally-injured mice.

Progress of Infection in Burned and Burned, Passively Immunized Mice

Tissue colonization experiments revealed interesting differences between infecting strains differing in the Fla or Mot phenotype. In non-protected, burned mice challenged with wildtype strain M-2 subcutaneously, a very rapid, invasive infection was observed. Bacteria multiplied from initially very low numbers to greater than 10^7 cfu within 24 hours. Coinciding with this proliferation in the burn wound was evidence of rapid tissue invasion characterized by bacterial counts in the liver and bloodstream increasing at similar, rapid rates. It was observed that systemic organ invasion did not occur until bacteria multiplying in the burn wound approached levels of 10^5-10^6 cfu per gram. This pattern of burn wound sepsis is very similar to what is observed in human patients and underscores the tremendous capacity of P. aeruginosa to become bacteremic from a local wound. These tissue experiments support the initial virulence studies and demonstrate why the wildtype strains are so lethal in burned mice.

A much different pattern of infection occurred in mice challenged with M-2 Fla$^-$ subcutaneously. Although a similar proliferation of

bacteria in the burn wound was observed the characteristic, rapid systemic invasion was strikingly absent. The nonmotile bacteria did not suffer from an apparent inability to colonize the burn wound, but did not seem to be able to spread from this initial focus of infection. Low counts in the liver were observed which may simply represent a passive, "spillover" from the expanding burn skin population into the bloodstream. The key point, here, is that without motility, P. aeruginosa is limited to a localized infection and subsequently is not nearly as lethal. These studies further indicate then that motility is an important component of the virulence complex of P. aeruginosa and that abrogation of motility significantly decreases the invasiveness of this organism such that systemic infection is not observed. These data and the previously discussed virulence studies are in agreement with an investigation done by McManus et al. (1980). Nitrosoguanidine induced Fla^- mutants were shown to be much less virulent than motile, parent strains when inoculated topically onto scalded rats. The authors concluded that the progressively invasive nature of P. aeruginosa indicates a vital role for motility in infection and that alterations of motility significantly reduce virulence.

Additional evidence suggesting a role for motility as a virulence factor was provided by studies with Vibrio cholerae (Yancey et al., 1978, 1979). These experiments demonstrated that motile and chemotactic cells were significantly more invasive than nonmotile cells, and able to colonize the intestinal tracts of animals to a much higher degree.

Another approach has been to use isolated flagella as an immunogen. Previous studies documented the high levels of protection achieved in mice after immunization with flagella antigen preparations (Holder et al., 1982). It was hypothesized that specific anti-flagellar antibody protected the animals by arresting the motility of the infecting bacteria, thus blunting the characteristic invasiveness (Holder et al., 1982). In this study, administration of specific, adsorbed flagellar antiserum to burned mice also resulted in protection against normally lethal challenge doses. To begin to understand the mechanism of this protection, tissue colonization experiments were conducted in mice passively protected with one or two doses of diluted flagellar antiserum. The progress of infection observed in these mice challenged with low doses of the wildtype parent M-2 subcutaneously was remarkably similar to that seen following M-2 Fla$^-$ infection. The normally, highly invasive bacteria multiplied to high levels within the local burn wound but did not exhibit the characteristic rapid spread of bacteria into the bloodstream and liver. Rather, very low levels of cells were found in the liver, and bacteria were never detected in the blood. The overall description of infection observed in mice protected with flagellar antiserum is one essentially indistinguishable from the pattern observed with a Fla$^-$ strain. It is conceivable then, to postulate that the protection observed following administration of anti-flagellar serum is due in part to the action of antibody specific for the flagellum, arresting the motility of the infecting cells, thus preventing lethal invasion. This would account for the similarities seen between passively protected, wildtype

challenged mice and nonimmunized mice challenged with bacteria deficient in motility. In essence, the motile-parent cells have also lost the function of motility due to immobiizing antibody and are restricted to the burn site as are the nonmotile mutants. Data from these tissue colonization experiments in passively protected mice also add further support for the importance of motility. By blunting motility, the virulence of infecting bacteria is greatly reduced.

Characterization, Protection and Specificity of Adsorbed Flagellar Antiserum

There are several lines of evidence for the specificity of the antibody for the flagellum. Hyperimmune antisera were raised in rabbits against flagella antigen preparations that were shown to be free of major contaminants (Montie *et al.*, 1982a). Antisera were extensively adsorbed with M-2 Fla$^-$ or heated M-2 cells to remove any background antibodies against somatic antigens. This was important as it has been shown that protection against *P. aeruginosa* infection can be achieved by administering anti-LPS antiserum (Cryz *et al.*, 1983a). The lack of LPS antibody in the flagellar antisera was demonstrated in an ELISA; no detectable antibody was observed against cells of O antigen type 5, a homologous O antigen type to the cells from which the FAg was used to raise the antiserum. Anti-flagellar serum would not agglutinate cells of a different H antigen type even though the O antigen type was identical. Tube immobilization assays gave further indication for this specificity as flagellar antisera would inhibit the motility of homologous H antigen cells only, regardless of the O

antigen type. Western blot assays revealed a strong reactivity of the anti-flagellar serum for the major flagellin band only, with no other binding observed.

The phase contrast microscopy observations of cells suspended in dilutions of flagellar antiserum provided further evidence for the mode of action of the specific antibody. The swimming of individual bacteria did not seem to be inhibited; however, small aggregates of two and three cells were observed to occur upon apparent contact of their flagella. Cells did not seem to be bound to each other via a membrane interaction but rather by an entanglement of their flagella. Each individual cell in these aggregates violently attempted to render itself free, causing a spinning pinwheel effect as the cells spun around each other. These small aggregates quickly grew into large aggregates, leading to the visible, flocculent agglutination seen in the slide agglutination assays. Collectively, then, these in vitro assays point out the specificity of the flagellar antisera and the corresponding lack of O antigen reactivity.

Additional support for the monospecificity of the anti-flagellar sera is provided by the cross-protection experiments. Mice administered a b-type antiserum were protected only against homologous challenges and not against infection with an a-type strain. Similar and consistent results were obtained with the reverse experiment: mice passively immunized with a-type antiserum were protected against a-type challenge but were highly susceptible to heterologous challenges. A key point again is that the O antigen type of the immunizing (for Hag antiserum) or infecting organism is irrelevant. Thus, the in vivo data

correlate well with the results demonstrating specificity of the flagellar antisera *in vitro* for the flagellum.

It is known that neutrophils and the nonspecific cellular defense of the host is impaired following thermal injury, enhancing the susceptibility of a host to lethal burn wound infection. Moreover, it has been shown that opsonizing antibodies are also important in protection against these infections and that administration of such antibodies partially compensates for the loss of immunocompetence. To examine the contribution of phagocytic cells in the protection achieved by passive immunization with flagellar antiserum, experiments were designed to investigate the status of the reticuloendothelial system in burned mice by comparing blood clearance rates in nonprotected and protected animals. An interesting finding was that clearance of bacteria from the bloodstream in burned mice was actually heightened rather than depressed as compared to normal animals. Also, no difference in clearance was observed in burned mice administered flagellar antisera. This is consistent with results from studies performed by McManus *et al*. (1984) and Alexander and Wixson (1970a). It was shown that although rats and mice are rendered immunosuppressed by thermal injury, an actual increase in the numbers of circulating neutrophils occurs by the first day postburn. This might explain the increased clearance of bacteria from the bloodstream as it has also been shown that the phagocytosis of neutrophils is not so severely depressed as other immunological phenomena.

To further examine the role of phagocytes in passively-protected mice, animals were rendered neutropenic with cyclophosphamide before

burning and challenge. Neutropenic mice have been shown to be very susceptible to subcutaneous infection with P. aeruginosa strain M-2 with as few as 8 cfu causing 83% mortality (Cryz et al., 1983b). The results obtained revealed that neutropenic, burned mice were highly susceptible to burn wound infection as expected. Mice administered anti-flagellar serum also succumbed to a fatal infection although a slight delay in time to death was observed. Thus, the protection gained by administration of flagellar antiserum was greatly diminished upon losing the nonspecific cellular defense, emphasizing an important role of phagocytes. It is important to point out that cyclophosphamide can cause multiple defects in the immune response, and is not limited to effects on neutrophils. Depending on the dosage, lymphocytopenia can also occur. However, in these studies a single injection of cyclophosphamide resulted in only a two-fold decrease in peripheral lymphocytes but a 22.5-fold decrease in neutrophils. Therefore, it is probable that the increased susceptibility to P. aeruginosa is associated with the state of neutropenia in these animals. It is possible that specific anti-flagellar antibody serves as an effective opsonin, targeting invading bacteria for phagocytosis by neutrophils and macrophages. Cryz et al. (1983a) demonstrated a high level of protection against P. aeruginosa burn wound sepsis after administration of anti-LPS antibody. They found a similar lack of invasion from the burn wound in such protected mice as shown in the studies reported here. They concluded that since similar numbers of bacteria were observed in the burn wound in all experimental groups at the time bacteremia was first noted, the anti-LPS antibody acted as an opsonin

to promote the rapid clearance of the bacteria. This is observed in human infections also as anti-LPS, opsonizing antibody is important in protection and plays a significant role in recovery from P. aeruginosa infections (Young, 1972a,b).

In a related study protection against lethal infection by the spore-forming bacterium, Clostridium chauvoei, was achieved by administration of anti-flagellar serum (Tamura and Tanaka, 1984; Tamara et al., 1984). This bacterium causes blackleg, a disease with economic impact in cattle and sheep, and has been studied in a number of mouse models. The authors demonstrated that mice passively immunized with specific, antiflagellar antibody and challenged intramuscularly with C. chauvoei spores all survived, with no bacterial spread to the liver as observed in controls. Importantly, they also showed that abrogation of polymorphonuclear neutrophils by cyclophosphamide treatment significantly reduced the level of protection afforded by flagellar antiserum. It was concluded that anti-flagellar antibody exerts its effect by opsonic function and that neutrophils are vitally important in this protection.

In light of these findings, the sequence of events following passive immunization is hypothesized to occur as follows. Wildtype, P. aeruginosa rapidly proliferates in the burn wound after inoculation of low numbers due to: (1) the avasculature of the site, which prevents influx of soluble and cellular defense components and (2) the array of elaborated proteases which function to degrade burn tissue, supply nutrients for the rapidly multiplying bacteria and promote spread of the bacteria by intensifying the burn damage. In nonprotected mice,

motile cells quickly bridge the viable-nonviable tissue interface and hematogenously spread, resulting in systemic, multiple organ invasion. Nonmotile cells lack the capacity to rapidly invade viable tissues from the burn site, and remain in the burn wound as a localized infection. A few cells spill over into the bloodstream due to the expanding bacterial population. In protected mice, invading bacteria are quickly immobilized at the viable tissue interface as a result of the agglutinating anti-flagellar antibody. It would be interesting to examine the tissue pathology during this phase of infection.

The antibody may exert a dual role as an opsonin; thus, bacteria are rapidly cleared by neutrophils and blood monocytes, resulting in substantially decreased colonization of the liver. Opsonophagocytosis studies with preimmune and flagellar antiserum would help clarify whether or not anti-flagellar antibody serves as an opsonin in addition to an agglutinating-immobilizing function.

Interpretation of the results obtained in tissue colonization experiments following an intraperitoneal challenge with the motility mutants is somewhat more difficult. Interesting differences at one hour post-challenge with 10^6 cfu of strains MT1200 (Fla^+ Mot^+) and MT1200-80 (Fla^+ Mot^-) were observed. An approximate 100-fold higher number of bacteria was detected in livers of mice injected with 1200-80. Also, no counts were detected in the bloodstream compared to 100 cfu per ml of blood in the 1200-challenged mice. This may reflect differences in clearance rates from the peritoneal cavity. It has been shown that bacteria and inert particles injected into the peritoneal cavity are cleared within minutes through phagocytosis by local

phagocytes and translymphatic adsorption (Dunn et al., 1985). Bacteria are rapidly removed into regional lymph nodes and eventually through the lymphatic duct into the bloodstream, where they are cleared by the liver and spleen. It is possible that nonmotile cells are subject to a much more rapid removal. This would lead to the higher liver counts observed in the 1200-80 challenged mice and the absence of bacteria in the blood. The less rapidly cleared motile strain MT1200 may be more able to evade sessile and circulating phagocytic cells, and thus persist in the peritoneal cavity and bloodstream for longer periods of time. Recent studies by Carsiotis et al. (1984) and Weinstein et al. (1984) demonstrated that differences were not observed in the phagocytosis of Fla^+ and Fla^- Salmonella typhimurium, but that flagellated cells survived intracellularly in macrophages for much longer periods of time than nonflagellated cells. However, differences in killing were not observed between phagocytized motile and nonmotile, flagellated cells, indicating that the presence of the flagellum, not motility, somehow prolonged intracellular survival.

An analogous situation may exist with the P. aeruginosa strains. The straight flagellum of MT1200-80 may not protect the bacterium from intracellular bactericidal factors as a normal, wavy flagellum would. Perhaps the flagellum wraps around the cell, providing some sort of a "cloaking" effect, shielding the bacterium from the effects of the antibacterial components of the phagolysosome. The straight, rigid flagellum may be unable to protect the cell in this manner. Therefore, strain 1200-80 may be significantly more susceptible to killing by

phagocytic cells, and thus not able to persist in the peritoneal cavity and liver, compared to the wildtype strain.

Another possibility is that strain 1200-80 may be more readily phagocytized in addition to being more susceptible to killing. Nonmotile cells may not be able to evade phagocytic cells and may be more susceptible to a "walling-off" phenomenon, enhancing their removal.

Colonization of the burn wound to similar levels had occurred by 2 hours post-challenge with either 1200 (Fla^+ Mot^+) or 1200-80 (Fla^+ Mot^-). Thus, motility did not appear to be important in the ability of the bacteria to initially reach and colonize the burn site. It is possible that bacteria were carried to the burn wound by circulating phagocytes. Both strains rapidly proliferated in the burn wound following the initial seeding, but a notable 100-fold difference in skin counts was seen by 26 hours postburn and challenge. Strains 1200 and 1200-80 were injected subcutaneously into burned mice in very low numbers to determine if this difference was due to the inability of the nonmotile strain 1200-80 to colonize and multiply. Results from this experiment showed that strain 1200-80 was able to colonize and rapidly proliferate within the burn eschar to levels identical to those obtained by the wildtype parent, MT1200. To explain the differences observed in skin counts in intraperitoneally challenged mice, the following is hypothesized. No difference was observed between the motile and nonmotile cells in reaching the burn site. However, motility may impart a significant advantage to cells in the ability to bridge the viable-nonviable tissue interface and spread up into the

avasculature burn wound. Nonmotile cells are able to colonize the burn wound but may be restricted to the immediate area where they are initially deposited, and are thus subjected to infiltrating phagocytes at the interface. Therefore, motility may not be important in the ability of cells to reach the burn site following intraperitoneal injection, but may play a vital role in bacteria actively moving into the burn wound.

A very large difference was seen in the survival of remaining experimental mice challenged with either 1200 or 1200-80. By 48 hours postburn and challenge, 100% mortality of mice injected i.p. with the wildtype strain 1200 had occurred whereas no mortalities were observed in 1200-80 infected mice. These results are consistent with and confirm earlier virulence studies. This may be due in part to the invasive capacity of the motile cells spreading from the burn wound once high counts are achieved whereas the nonmotile cells remain in the burn site as a localized infection. One might consider that systemic bacterial invasion in mice initially challenged intraperitoneally does not occur until bacteria have multiplied to a high level in the burn wound, similar to the pattern seen in subcutaneously challenged mice. It may well be that an increase in the concentration of elaborated proteases and toxin parallels the rise in the bacterial load, enhancing the exodus from the burn site.

However, the role of exotoxin A in invasive infection is still somewhat obscure. One possible role is that toxin may promote dissemination of motile cells from the burn wound by significantly reducing the nonspecific cellular response, as it has been shown to be

highly toxic for peripheral blood monocytes (Pollack and Anderson, 1978). Another study has demonstrated that toxin suppresses granulocyte-macrophage progenitor cells in bone marrow (Stuart and Pollack, 1982). However, its importance in promotion of systemic infection may be slight as it has been shown that administration of antitoxin antiserum significantly reduces levels of circulating toxin in burned mice, but fails to provide any protection against systemic infection of motile cells (Cryz, 1983a). Toxin may play a more important role by serving as the primary cause of death in systemically infected mice due to a "focused toxemia." In other words, heavy bacterial colonization of the liver may result in total liver dysfunction due to locally high levels of toxin. Nonmotile cells, on the other hand, also colonize the burn wound and produce toxin, but mice survive this type of infection. A key feature is that they do not exhibit a rapid and sustained invasion from this site due to the inability to swim and thus do not cause a lethal infection.

Summary and Conclusions

From these studies, the following conclusions are made:

(1) Motility is an important virulence factor of *Pseudomonas aeruginosa* and contributes substantially to the invasive capacity of this organism in lethal burn wound infections. Nonmotile mutants were significantly less virulent than motile, wildtype strains in burned mice.

(2) Nonmotile strains colonize and multiply within burn wounds similarly to motile cells. Also, differences in *in vitro* growth

rates, O antigen reactivity, physiological profiles, antibiograms and assays of elaborated proteases were not observed, indicating the apparent isogenicity of the mutants differing in the Fla or Mot phenotypes.

(3) Although nonmotile strains proliferate and reach similar levels in the burn site, the characteristic, rapid systemic invasion of wildtype strains is not seen. The bacteria remain localized, and mice survive the infection.

(4) Adsorbed flagellar antisera inhibits the motility of bacteria in vitro, and is specific for flagella antigen as demonstrated by cross-agglutination assays, motility inhibition, western blot analyses, and an ELISA. Phase contrast microscopy revealed an apparent cross-linking phenomenon of flagella by anti-flagellar antibody, inhibiting motility by an agglutination reaction.

(5) Protection against P. aeruginosa burn wound infection not only is achieved upon administration of adsorbed flagellar antisera but also antisera is specific for FAg as determined by cross-protection experiments. These data correlate well with the in vitro motility inhibition data.

(6) Motile bacteria colonize the burn wounds of passively protected mice, but are not able to invade. This pattern of wound infection is remarkably similar to that occurring in infection with bacteria deficient in motility.

(7) Neutropenic, burned mice were not protected by flagellar antiserum, indicating a vital role for phagocytic cells in protection. It is hypothesized that anti-flagellar antibody serves a dual role in

protection. The motility of invading bacteria apparently is blunted by a cross-linking, agglutination reaction. Anti-flagellar antibody may act as an opsonin, targeting immobilized cells for phagocytosis by neutrophils and macrophages.

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APPENDIX

Table A.1. Summary of virulence differences of *Pseudomonas aeruginosa* strains differing in the Fla or Mot phenotype[a]

Strain	Phenotype	Lethal Dose 50[b]	Comparative decrease (log) in virulence[c]
M-2	Fla^+ Mot^+	5	
M-2 Fla^-	Fla^- Mot^-	5×10^4	4
PaO-1	Fla^+ Mot^+	10^2	
AK1152	Fla^- Mot^-	$>10^7$	5
AK1153	Fla^- Mot^-	$>10^7$	5
MT1200	Fla^+ Mot^+	6×10^3	
MT1200-80	Fla^+ Mot^-	$>>10^5$	>>2[d]

[a]Subcutaneous route of challenge.

[b]Challenge dose resulting in 50% mortality of experimental animals.

[c]Approximate decrease in virulence measured as increase (log) in LD_{50} values.

[d]Value may be significantly higher as no mortalities occurred from the highest bacterial challenge tested.

Table A.2. Adsorption of flagellar antibody and subsequent loss of protection in burned mice[a]

Mice	Challenge Inocula (CFU)[b]	Cumulative Mortalities (Died/Total Inoc.) Days Postburn 1	2	3	4	5	6	7
Non-injected	1.1×10^2	0/6	4/6	5/6	6/6	-	-	-
Injected with mock-adsorbed flagellar (b-type) antiserum[c]	1.1×10^2	0/6	0/6	0/6	0/6	0/6	0/6	0/6
Injected with AK1152 (Fla^-) adsorbed flagellar (b-type) antiserum	1.1×10^2	0/6	0/6	0/6	0/6	0/6	0/6	0/6
Injected with M-2 (Fla^+) adsorbed flagellar (b-type) antiserum	1.1×10^2	0/6	3/6	6/6	-	-	-	-

[a]Suspensions of formalin (0.3%)-killed cells were added to 100-fold dilutions of flagellar antiserum and incubated at 37°C for 30 minutes. Bacteria were removed by centrifugation (6,000 x g) and the procedure was repeated 7 times. Final antisera preparations were filter sterilized before injection.

[b]*P. aeruginosa* strain M-2 subcutaneously

[c]Antisera adsorbed 7 times with sterile 0.85% saline.

[d]Strains AK1152 and M-2 have O antigen type S.

Table A.3. Level of protection achieved by active immunization of mice with flagella antigen[a]

Strain	Challenge Inocula (CFU)[b]	Cumulative Mortalities (Died/Total Inoc.) Days Postburn 1	2	3	4	5	6	7
M-2	4.6×10^{5}[c]	0/5	0/5	0/5	0/5	0/5	0/5	0/5
M-2	4.6×10^{4}	0/5	0/5	0/5	0/5	0/5	0/5	0/5
M-2	4.6×10^{3}	0/5	0/5	0/5	0/5	0/5	0/5	0/5

[a]Mice were injected with 1 ug FAg b-type (in 0.85% saline) intraperitoneally (0.1 ml) one week prior to burn and challenge with a homologous strain (M-2).

[b]Subcutaneous.

[c]Equivalent to 86,000 LD_{50} doses in non-immunized mice.

VITA

David Ray Drake was born to Mary Ann and Ronald Drake on June 25, 1955. He attended public schools in Evansville, Indiana, and graduated from William Henry Harrison High School in June, 1973. The following September, he entered Purdue University in West Lafayette, Indiana, where he majored in Entomology. After receiving the Bachelor of Science degree from Purdue in 1977, he entered graduate school in the Department of Entomology at Purdue University and began research in the area of Invertebrate Microbiology and Biochemistry. He completed his research and accepted a teaching assistantship in the Department of Microbiology at The University of Tennessee, Knoxville, in September, 1980, and began study toward a Ph.D. After completing his written thesis, he went back to Purdue and was awarded the Master of Science degree in May, 1982. He completed his doctoral work in Pathogenic Microbiology and received the Doctor of Philosophy degree in March, 1986.