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Virulence of Different *Pseudomonas* Species in a Burned Mouse Model: Tissue Colonization by *Pseudomonas cepacia*

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The virulence of *Pseudomonas aeruginosa* and other pseudomonads was examined in a burned mouse model. *P. aeruginosa* M-2 was highly virulent causing 100% mortality by 38 h with an injection of 10^2 CFU by either a subcutaneous or intraperitoneal route. Subcutaneous injection of 10^2 CFU revealed rapid multiplication of the bacteria at the burn wound with 10^8 CFU/g detectable in the burned skin by 28 h postinjection, 10^5 CFU/g of liver, and 10^3 CFU/ml of blood. Non-*P. aeruginosa* clinical isolates were markedly less virulent; an injection of $\geq 10^7$ CFU caused $\leq 60\%$ lethality. *P. cepacia* SMH colonized the burned skin of thermally injured mice, persisting at levels of 10^7 to 10^8 CFU/g of burned skin after an initial injection of 10^5 CFU. *P. cepacia* persisted in the burn wound for at least 3 weeks. No organ invasion was detectable throughout this period. Studies with an additional clinical isolate of *P. cepacia* yielded similar results. An injection of a 10^2 CFU dose revealed that the level of persistence is dose dependent. Results suggest that the tenacious persistence of *P. cepacia* in the burn wound may provide a model for the study of persistent colonization and infection in a compromised host.

It has recently been documented that *Pseudomonas* species other than *P. aeruginosa*, especially *P. cepacia*, are emerging as clinically important pathogens (6, 10, 12, 32). *Pseudomonas* species, other than *P. aeruginosa*, account for 7.2% of the isolates from burn wound infections (13). Only *Staphylococcus aureus* and *P. aeruginosa* are isolated more frequently from burn wound infections (13). The recovery of *P. cepacia* in cystic fibrosis patients is associated with a poor prognosis for long-term survival (L. L. MacKensie and P. H. Gilligan, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, C34, p. 317). *P. cepacia* is highly resistant to many antibiotics that are useful in treating other gram-negative bacilli (25, 28, 30). Of great concern in the hospital is the resistance of *P. cepacia* to aqueous quaternary ammonium disinfectants and its ability to grow in distilled water with no visible turbidity even at 10^7 cells per ml (2, 7, 25).

The burned mouse model (27) was developed to investigate burn wound sepsis due to *P. aeruginosa* (4, 8, 9, 15, 17, 21, 24). This model has been used to study differences in virulence of isogenic motile and nonmotile derivatives of *P. aeruginosa* (15) and *Salmonella typhimurium* (M. Carsiotis, A. D. O'Brien, and I. A. Holder, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, B83, p. 37) and also to test the protective effects of *P. aeruginosa* flagellar vaccines (9). Little is known concerning the pathogenesis of *Pseudomonas* species other than *P. aeruginosa* in the currently used animal models. Therefore, the studies reported herein were performed to assess the relative virulence of different *Pseudomonas* species in the burned mouse model and the effect of active immunization with flagellar antigen (FAg).

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MATERIALS AND METHODS

Bacteria. *P. aeruginosa* M-2 (originally isolated from the small intestine of a CF1 mouse) was obtained from I. A. Holder, Shriner's Burns Institute, Cincinnati, Ohio. *P. cepacia* SMH (a human clinical isolate) and *Escherichia coli* ATCC 25922 were obtained from M. Camblin, St. Mary's Medical Center, Knoxville, Tenn.; *P. cepacia* strains E4119 Ca, E8980(1) Col, E2973 Ma, E7427 PR, E7893 Ill, E7427 PR, and *P. maltophilia* B69 Fla (all human isolates), were obtained from the Centers for Disease Control, Atlanta, Ga.; *P. stutzeri* HEW (a human clinical isolate) was obtained from M. Moody, Baltimore Cancer Research Program, University of Maryland, Baltimore.

The original cultures received from various sources were suspended in a solution of 25% glycerol (vol/vol)

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in saline (0.85% [wt/vol] NaCl) and maintained frozen at −70°C. Stock cultures were maintained at 4°C as a dilute suspension in Luria Broth (1% NaCl, 1% tryptone, 0.5% yeast extract [pH 7.0]).

Quantitation of bacteria. Bacteria were grown in Luria Broth for 12 h at 37°C on a gyratory shaker at 175 rpm. Cells were harvested by centrifugation (4,000 × *g* for 15 min) and subseqently washed twice in a standard diluent (25 mM potassium phosphate [pH 7.0], 0.9% NaCl, 0.01% gelatin, 0.2 mM $MgSO_4 \cdot 7H_2O$). Cells were then diluted in the same standard diluent. The desired inocula were calculated upon reference to a predetermined standard curve (log_{10} CFU/ml versus optical density at 590 nm). Viable cell counts were determined by serial dilution plate counts done in duplicate on Pseudomonas Isolation Agar (American Scientific Products, Stone Mountain, Ga.), Desoxycholate agar (BBL Laboratories, Cockeysville, Md.), or Trypticase soy agar plates (BBL).

Laboratory animals. Virulence studies of the *Pseudomonas* species were performed with Ha/ICR mice (Cumberland View Farms, Clinton, Tenn.). Female mice weighing 22 to 24 g were used in all experiments. Before experimental use, the animals were maintained in cages at 10 mice per cage with food and water available ad libitum.

Preparation of FAg. FAg were prepared from *Pseudomonas* strains by a modification of the method of Montie et al. (14).

Enzyme assays. Protease activity was assayed with dialyzed brain heart infusion milk plates (26). Zones of hydrolysis around individual colonies were measured after 24 and 48 h of incubation at 37°C.

Experimental burn procedure. The burned mouse model of Stieritz and Holder (27) was used to study virulence of various *Pseudomonas* species and strains. The backs of mice were shaved one day before the burn procedure. The mice were then anaesthetized by placing them in a closed container containing methoxyflurane (Metofane; Pitman-Moore, Washington Crossing, N.J.) for ca. 90 s. An asbestos board with a window (35 by 25 mm) was pressed firmly against the shaved back of the mouse. Ethanol (0.5 ml, 95%) was applied to the back outlined by the window, ignited, and allowed to burn for 10 s and was immediately extinguished. This burn resulted in a second degree burn of ca. 30% of the total body surface (27). Immediately after the burn, all mice were injected intraperitoneally with 0.5 ml of sterile physiological saline for fluid replacement therapy to prevent overt shock.

Bacterial challenge of burned mice. Bacteria to be injected into mice were cultured and quantitated as described previously. For virulence studies, animals were challenged immediately postburn by subcutaneous or intraperitoneal injections of 0.1 ml of the bacterial suspension.

Two types of control were used. One consisted of an injection of organisms into nonburned skin 10 mm anterior to the burn site. Subcutaneous injection of organisms into nonburned mice served as an additional control.

Bacterial quantitation of animal tissues, organs, and blood. At various times postinoculation, animals were killed by cervical dislocation or decapitation. The liver and full thickness specimens of burned or nonburned skin or both were removed, weighed and diced, and placed in sterile 10-ml capacity Potter Elvehjem tissue grinders (Fisher Scientific, Pittsburgh, Pa.) containing 5 ml of the standard diluent. The sample was homogenized for 5 min. The bacterial content of the homogenates was determined by serial dilution plate counts done in duplicate on Pseudomonas Isolation Agar (American Scientific Products) or Desoxycholate agar plates (BBL). Colonies were counted after 24 to 48 h of incubation at 37°C. The bacterial count was expressed as CFU per gram weight of tissue or organ.

Numbers of bacteria in the blood were similarly determined from samples collected after decapitation. Blood, collected immediately after death, was diluted 1:10 in the standard diluent to prevent clotting, and the bacterial counts were expressed as CFU per milliliter. At the same time, swabs were also taken from the surface of the burned skin site and the surface of nonburned skin 10 mm anterior to the burn site. Pseudomonas Isolation Agar plates were streaked with the swabs and were incubated for 36 to 48 h at 37°C.

In our studies, we used the following definitions concerning pathogenicity. Colonization is defined as persistence of a microorganism without disease (e.g., without tissue damage, bacteremia, or organ invasion). Infection is defined as the production of disease (e.g., tissue destruction, bacteremia, or organ invasion).

Immunization of mice. Female Ha/ICR mice were injected intramuscularly in a hind limb with FAg (1 g/0.1 ml in 0.9% saline) (9). After 14 days, the immunized mice were burned and challenged.

RESULTS

High numbers of *P. aeruginosa* are required to cause death in normal mice; however, in thermally injured mice, *P. aeruginosa* is extremely virulent (27). The other *Pseudomonas* species were tested in the burned mouse model to determine their virulence relative to the highly lethal *P. aeruginosa* M-2. The comparison of relative virulence revealed significant differences between *P. aeruginosa* and other pseudomonads (Table 1). The injection of ca. 10^2 CFU of *P. aeruginosa* M-2 caused 100% mortality; however, injection of $\geq 10^7$ CFU of other pseudomonads caused ≤60% lethality (Table 1). *P. aeruginosa* is also highly lethal when applied topically to the burned skin of mice (9). In contrast, topical application of 5.2×10^7 CFU of *P. cepacia* SMH onto burned skin of CF1 mice resulted in no deaths.

Low virulence in the burned mouse model was not limited to *Pseudomonas* species other than *P. aeruginosa*. The injection of 3.3×10^7 CFU of *E. coli* ATCC 25922 resulted in only 20% lethality (Table 1). Similar results were obtained by Stieritz (27).

The measurement of proteolytic activity of the various *Pseudomonas* species and *E. coli* showed that only *P. aeruginosa* M-2 exhibited protease activity. *P. aeruginosa* M-2 is known to produce exotoxin A (24).

P. aeruginosa is a highly invasive organism in

TABLE 1. Comparison of the lethality of *Pseudomonas* species in the burned mouse model (Ha/ICR mice)

Organism	Dose[a]	% Lethality[b]	Dose[c]	% Lethality[b]
P. aeruginosa M-2	2.1×10^2	100	6.5×10^2	100
P. cepacia SMH	1.7×10^7	20	9.7×10^7	20
P. cepacia E4119 Ca	3.9×10^7	0	1.5×10^7	40
P. cepacia E8980(1) Col	3.2×10^7	20	3.3×10^7	20
P. cepacia E7893 Ill	2.7×10^7	0	1.4×10^7	0
P. cepacia E2973 Ma	2.9×10^7	0	1.6×10^7	20
P. cepacia E7427 PR	2.6×10^7	0	3.7×10^7	0
P. maltophilia B69 Fla	1.6×10^7	0	2.8×10^7	0
P. stutzeri HEW	1.7×10^8	0	3.6×10^8	60
E. coli	3.3×10^7	20	3.3×10^7	20

[a] Subcutaneous injection of bacteria (in 0.1 ml of buffer) into burn site.
[b] Percent lethality of a group of five mice.
[c] Intraperitoneal injection of bacteria (in 0.1 ml of buffer).

compromised hosts. Within 28 h of subcutaneous injection of ca. 10^2 CFU of *P. aeruginosa* M-2 into the burn site, the bacteria rapidly multiplied with 10^8 CFU/g detectable in the burned skin, 10^5 CFU/g of liver, and 10^3 CFU/ml of blood (data not shown). These data confirm previous studies (27). Although less virulent, *P. cepacia* SMH colonized the burned skin of thermally injured mice. When ca. 10^5 CFU of *P. cepacia* SMH was injected subcutaneously into the burn site, the levels of *P. cepacia* increased within 24 h to 10^7 to 10^8 CFU/g of burned skin and persisted at this level for 3 weeks before declining to undetectable levels by 4 weeks postinjection (Fig. 1). It was observed that by 3 weeks, the burned skin is rapidly healing. When a lower level (10^2 CFU) of *P. cepacia* was injected, a similar persistent pattern was observed, except that the highest level obtained was 10^4 CFU/g of burned skin (data not shown).

Another clinical strain of *P. cepacia* was compared with *P. cepacia* SMH for the ability to colonize burned skin. Approximately 10^5 CFU of *P. cepacia* strains SMH and E7427 PR were injected subcutaneously into the burn site. Both strains were able to colonize and persist in burned skin. *P. cepacia* E7427 PR was identical to strain SMH in that it rapidly multiplied to levels of 10^7 to 10^8 CFU/g of burned skin and persisted at that level.

Although *P. cepacia* SMH colonized burned skin, it did not invade deep body tissues since it was not detected in the liver, blood, or unburned skin. Unburned mice injected subcutaneously with approximately 10^5 CFU exhibited marked decreases in *P. cepacia* CFU/g of skin within 72 h; this was also observed in unburned skin of burned mice that had been similarly injected (Fig. 1). *P. cepacia* was not detected in burned skin of mice that had been injected subcutaneously with *P. cepacia* into unburned skin. Swabs were taken to determine the amount of *P. cepacia* present on the surface of the skin of mice injected subcutaneously with *P. cepacia*. Swabs of both burned and unburned skin revealed no *P. cepacia* present on the skin surface of the majority of mice; however, a few mice had ≤ 80 CFU on the skin surface at the first timepoints. The same lack of surface colonization occurred with mice injected with *P. aeruginosa*. This may represent slight leakage from the injection site.

Mice immunized with *P. cepacia* SMH FAg (type I) did not have significant reductions in the number of homologous *P. cepacia* SMH colonizing the burned skin after challenge (Fig. 2). Likewise, there was no difference in colonization by *P. cepacia* SMH in burned skin of mice that had previously been immunized with FAg of a heterologous *P. cepacia* E8980(1) Col (type II), *P. aeruginosa* M-2 (type b), or saline control (Fig. 2).

DISCUSSION

Preliminary 50% lethal dose experiments revealed the low virulence of non-*P. aeruginosa* species. *P. aeruginosa* M-2 caused 100% mortality with a dose as low as 10^2 CFU, regardless of the challenge route. This confirms recent reports documenting the virulence of this organism in the burned mouse model (9, 15). In sharp contrast were the results obtained from *P. maltophilia*, *P. stutzeri*, and six *P. cepacia* strains. Even at doses as high as 10^7 CFU, these other pseudomonads caused little or no mortality.

P. aeruginosa elaborates several extracellular toxins and enzymes that have been implicated as important determinants of virulence (11). Most notable of these are the proteases and exotoxin A (31). *P. aeruginosa* is also the only species of *Pseudomonas* known to produce exotoxin A (1, 18); strain M-2 has been reported to produce exotoxin A (24). Moreover, although *P. aeruginosa* M-2 was highly proteolytic, the other *Pseudomonas* spp. used in this study were nonproteolytic. Different investigators have shown that

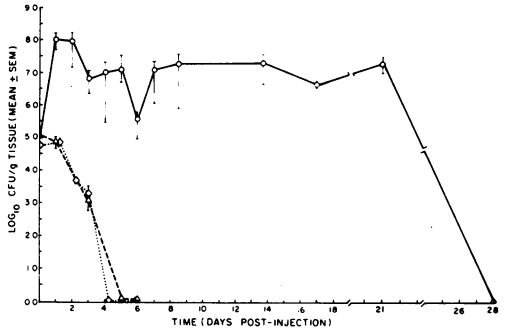

FIG. 1. Quantitative bacteriology of skin after subcutaneous injection of *P. cepacia* SMH. Injection of 5.7 (±1.1) × 10^4 CFU/0.1 ml into unburned mice (·····); injection of 1.1 (±0.1) × 10^5 CFU/0.1 ml into unburned skin of burned mice (-----); and injection of 1.0 (±0.1) × 10^5 CFU/0.1 ml into burned skin (———).

proteolytic activity is an important factor contributing to the expression of full virulence when *P. aeruginosa* is administered via a subcutaneous route in burned animals (9, 24). It is thought that proteases serve as "spreading factors," damaging and degrading tissues allowing for motile organisms to invade the host. Also, such enzyme activity may enhance the growth of bacteria at the burn site by releasing valuable nutrients from the injured tissues (3). Therefore, we suggest that the absence of proteolytic activity observed with all of our non-*P. aeruginosa* spp. may account for the lack of invasion of tissues and the subsequent low mortality. *P. aeruginosa* M-2 rapidly invaded the host by 28 h wtih 10^5 CFU/g of tissue detectable in the liver and 10^3 CFU/ml of blood. All of the mice injected with 10^2 CFU of *P. aeruginosa* M-2 died within 38 h. In contrast, the nonproteolytic *P. cepacia* SMH remained at levels of 10^7 to 10^8 CFU/g of burned skin for 3 weeks without any subsequent invasion or mortality. Another clinical strain of *P. cepacia* (E7427 PR) showed an identical pattern of colonization and persistence. The multiplication of these bacteria does not appear to delay healing. Healing of the skin after 3 weeks is correlated with no detection of the organisms in the skin. The ability of *P. cepacia* to colonize and persist in a burn wound without subsequent mortality is a striking difference with *P. aeruginosa*. PA103, a noninvasive strain of *P. aeruginosa*, can be lethal (21, 24). This strain has been shown by others to multiply to high levels in the burn wound without subsequent invasion. However, this strain does elaborate exotoxin which enters the bloodstream and can cause death (21, 24). Experiments are currently in progress to further examine the role of proteases and the lack of invasion by *P. cepacia* from colonized burn wounds.

The injury resulting from a 10-s alcohol burn is a partial thickness burn (27). Granulation tissue with a rich vasculature, existing beneath the burn wound, would allow host defenses (e.g., plasma proteins and phagocytic cells) into the burn site (16). This may account for the low level of intraeschar colonization observed with 10^2 CFU of *P. cepacia* was injected into the burn site. Perhaps with such a low dose exposure, the magnitude of the host response is sufficient to limit the rapid multiplication of *P. cepacia*; whereas a higher dose (10^5 CFU) of *P. cepacia* appears to overwhelm the host response, thereby allowing *P. cepacia* to proliferate to levels of 10^7 to 10^8 CFU/g of burned skin. Therefore, bacteria colonizing the burn wound appear to be in dynamic interaction with the host response with the level of multiplication occurring being dose dependent.

In contrast, injection of 10^2 CFU of *P. aeruginosa* M-2 results in rapid intraeschar bacterial growth and then invasion of adjacent viable tissues with subsequent destruction of the granulation bed and septicemia (16, 27, 29). *P. aeruginosa* has the ability to convert partial thickness burns to a full thickness injury as a result of tissue destruction and ischemia, perhaps owing to proteases (16, 20). The extracellular products of *P. aeruginosa* (e.g., slime, elastase, leucocidin, exotoxin A) may be important in the survival and proliferation of this organism in the burn wound (5, 11, 19, 22, 23).

This ability of *Pseudomonas* spp. to colonize and persist in a trauma wound may be signifi-

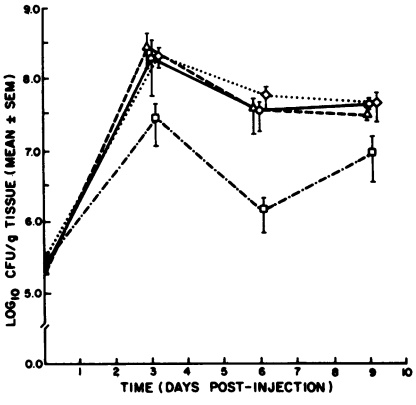

FIG. 2. Quantitative bacteriology of skin from immunized mice after subcutaneous injection of *P. cepacia* SMH (average injection of 2.7 × 10^5 CFU/0.1 ml). Mice were immunized as follows: *P. cepacia* E8980(1) Col FAg (-----); *P. aeruginosa* M-2 FAg (———); *P. cepacia* SMH FAg (····· and -----); and saline control (·····).

cant. It is known that pseudomonads and numerous other gram-negative bacilli are very opportunistic in their ability to infect compromised hosts (6, 20, 32). In a hospital environment, the extreme antibiotic resistance and nutritional versatility of some *Pseudomonas* spp. invariably results in selective colonization by these organisms. Opportunistic infections caused by *Pseudomonas* species other than *P. aeruginosa* are difficult to manage and may require frequent and sometimes toxic antibiotic treatment. Thus, the ability of these organisms to tenaciously persist in burn wounds may provide a model reflecting on persistent colonization and infection in compromised patients.

Holder and Montie recently demonstrated a significant level of protection against normally lethal *P. aeruginosa* burn infection by immunizing mice with a flagellar vaccine (9). Mice so protected had a ca. 50% reduction in the number of bacteria in the liver; however, bacterial counts at the burned skin were unchanged (9). In these studies, mice immunized and challenged with the homologous *P. cepacia* SMH (type I FAg) also did not have a significant decrease in numbers of bacteria persisting in the burn wound. No differences were seen with other immunogens and heterologous challenge. Thus, although immunization with flagella significantly protects against lethal infection by blocking the invasive capacity of *P. aeruginosa*, such treatment has no effect on the initial proliferation and persistence at the burn wound by either *P. aeruginosa* or *P. cepacia*. Burn wound sepsis consists of two major phases: proliferation and invasion (16, 27, 29). Studies examining the role of motility of *Pseudomonas* spp. in the transition between these two phases are currently in progress, along with competition experiments involving *P. aeruginosa* and other *Pseudomonas* spp. in the burned mouse model.

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