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Experimental Studies on Mice Challenged Subcutaneously with *Pseudomonas aeruginosa* (38925)

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Pseudomonas aeruginosa has gained increased medical importance as a human pathogen within the last few years. Of particular significance is its increasing incidence of nosocomial infections of patients already debilitated by other diseases, as well as in those receiving extensive radiation therapy, prolonged treatment with corticosteroids, antineoplastic drugs, or antibiotics (1, 2). The significance of *P. aeruginosa* in dermatology has been largely ignored despite the fact that cutaneous lesions are frequently observed as a manifestation of both systemic as well as localized wound infections. Thus, there have been sporadic reports of nonburn wound infections by this organism as well as infections of fingernails (green nail syndrome), toewebs, and external otitis (3, 4). In addition, *P. aeruginosa* plays an important role in burn wound infections. Some cutaneous lesions due to systemic infections result in gangrenous ulcers which are termed "ecthyma gangrenosum" (4). Another type of skin lesion more characteristic of an allergic reaction has also been described (4).

At present, little is currently known as to the susceptibility of healthy mice to subcutaneous challenge with live cells of *P. aeruginosa*. Previous studies with subcutaneously infected mice indicated that they were resistant to infection unless treated with 6-mercaptopurine for several days prior to infection (5). In this experimental system the mice developed acute infections of 3 days duration or less. However, preliminary studies in our laboratory indicate that chronic infections leading to lethality could be established in normal mice, thus allowing us to follow the sequence of tissue damage over prolonged intervals. Therefore, the present study was undertaken to develop and characterize a convenient experimental model for studying the susceptibility and response of normal mice challenged with *P. aeruginosa* via a dermatological route.

Materials and Methods. Organism. A proteolytic strain of *P. aeruginosa* (L-1) isolated from a patient with emphysema suffering from *Pseudomonas* pneumonia at St. Johns Hospital, Harper Woods, Michigan, was employed in this study.

Cultivation. The organisms were cultivated in a medium composed of 5% peptone (Difco) and 0.25% trypticase soy broth for 24 hr at 35° with constant aeration. The cultures were then centrifuged at 15,000 rpm for 15 min with a Sorvall RC2B centrifuge and resuspended in sterile saline. Viable cell counts were determined on tryptose agar (Difco) in order to establish the number of viable bacteria in cell suspensions injected into the mice. The cells tended to undergo some autolysis over extended periods of time or upon additional centrifugation so they were used immediately for *in vivo* studies.

In vivo *studies.* Live cell suspensions were inoculated into white Swiss–Webster, female mice weighing 18–22 g. A minimum of 6–18 animals were employed for each dilution after pretitration studies. Each experiment was usually performed in duplicate or triplicate. In an attempt to determine the sequence of the pathological response of the animals the LD_{50} values for chronically infected animals were determined after a 21-day holding period. Animals surviving the bacterial challenge were sacrificed by cervical detachment and autopsied. The final mean dose (LD_{50}) values were calculated by the method of Reed and Muench (6). Although most studies employed the subcutaneous route, the intradermal, intraperitoneal, and intravenous routes were also employed in some cases for comparative purposes. With the exception of the intradermal route (0.1

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ml) a volume of 0.5 ml of cell suspensions was administered in each case. Control animals received an equivalent amount of sterile saline.

Immunodepressant agents. Several antineoplastic agents (each having a different biochemical mechanism of action) were used in this study in an attempt to predispose mice to subcutaneously induced infections. The drugs were administered intraperitoneally in the following concentrations: Methotrexate (Lederle Labs., Pearl River, NY), 160 mg/kg; vincristine sulfate (Oncovin, Eli Lilly & Co., Indianapolis, Ind.), 1.25 mg/kg; cytosine arabinoside (Cytosar, The Upjohn Co., Kalamazoo, Mich.), 500 mg/kg; and actinomycin D (Cosmogen, Merck, Sharpe & Dohme, West Point, Pa.), 595 mg/kg. With the exception of Cytosar, the drug concentrations tested were one-half of the predetermined LD_{50} dose of the drug alone. The LD_{50} of Cytosar is unknown and is reported by the Upjohn Co. to be greater than 1000 mg/kg, and in our hands it was not lethal for the mice at this concentration. Use of one-half of the LD_{50} drug concentrations was effective in avoiding lethality and maintained any detectable histological changes in uninfected control animals to a minimum. Other agents tested included the following: epinephrine (Adrenalin chloride, Parke, Davis & Co., Detroit, Mich.) 4.64 mg/kg; and cortisone acetate (The Upjohn Co., Kalamazoo, Mich.), 625 mg/kg; and 5% hog gastric mucin (Wilson Labs, Chicago, Ill.). All drugs and chemical agents were made up fresh in sterile saline before each experiment and 0.5 ml was administered intraperitoneally either simultaneously, 24 hr before or 24 hr after subcutaneous bacterial challenge. For comparative purposes, the agents were also administered subcutaneously in some cases.

Histopathological studies. After gross postmortem observations were recorded, the heart, lungs, liver, spleen, stomach, intestines, skin, pancreas, kidneys, and portions of skin and blood vessels were removed and fixed in a phosphate-buffered Formalin, pH 7.2. Sections were cut on a rotary microtome and stained with hematoxylin and eosin. Verhoeff's (7) and the Brown–Brenn (8) staining procedures were used on select sections to determine the presence or absence of elastin and bacteria in the tissues, respectively.

Results. Initial studies attempted to determine whether subcutaneous injections of viable cells of *P. aerubinosa* could establish a chronic, systemic infection in healthy mice which were not debilitated by burn wounds or antineoplastic chemotherapy. Using a 21-day holding period, an LD_{50} value of 4.6×10^8 colony forming units (CFU) for 18 animals was established with strain L-1. However, a suspension of cells in 5% mucin yielded an LD_{50} value of 3.2×10^4 CFU. The use of mucin did not affect the type or degree of pathology obtained in the chronically infected mice as compared to infected mice which had not received mucin.

Comparative studies on the susceptibility of mice to infection by other parenteral routes was also performed. The LD_{50} values for the intraperitoneal and intravenous routes were 1.4×10^7 and 1.0×10^8 CFU, respectively (Table I). These results indicate that of the three routes tested, the subcutaneous route required the largest inoculum of CFU to cause death.

In an attempt to determine the course of infection during chronic infections the animals were infected with 1.1×10^8 CFU which is a concentration slightly below the LD_{50} of 4.6×10^8. Thus, gross and histopathological studies of mice sucutaneously infected yielded the following results: Within 24–48 hr animals developed large hemorrhagic, necrotic lesion surrounded by edema, redness, and induration only at the site of inoculation (Fig. 1). After 3–4 days ecthyma gangrenosum-like lesions would often ulcerate giving off a greenish purulence and formed a hard crusty core. Viable *P. aeruginosa* could be

TABLE I. SUMMARY OF 21-DAY LD_{50} VALUES FOR MICE INFECTED WITH *P. aeruginosa* BY VARIOUS ROUTES

Route of injection	LD_{50} values CFU
1. Subcutaneous[a]	4.6×10^8
2. Intradermal[b]	3.2×10^8
3. Intraperitoneal[b]	1.4×10^7
4. Intravenous[b]	1.0×10^8

[a] Total of 18 mice/dilution.
[b] Total of 12 mice/dilution.

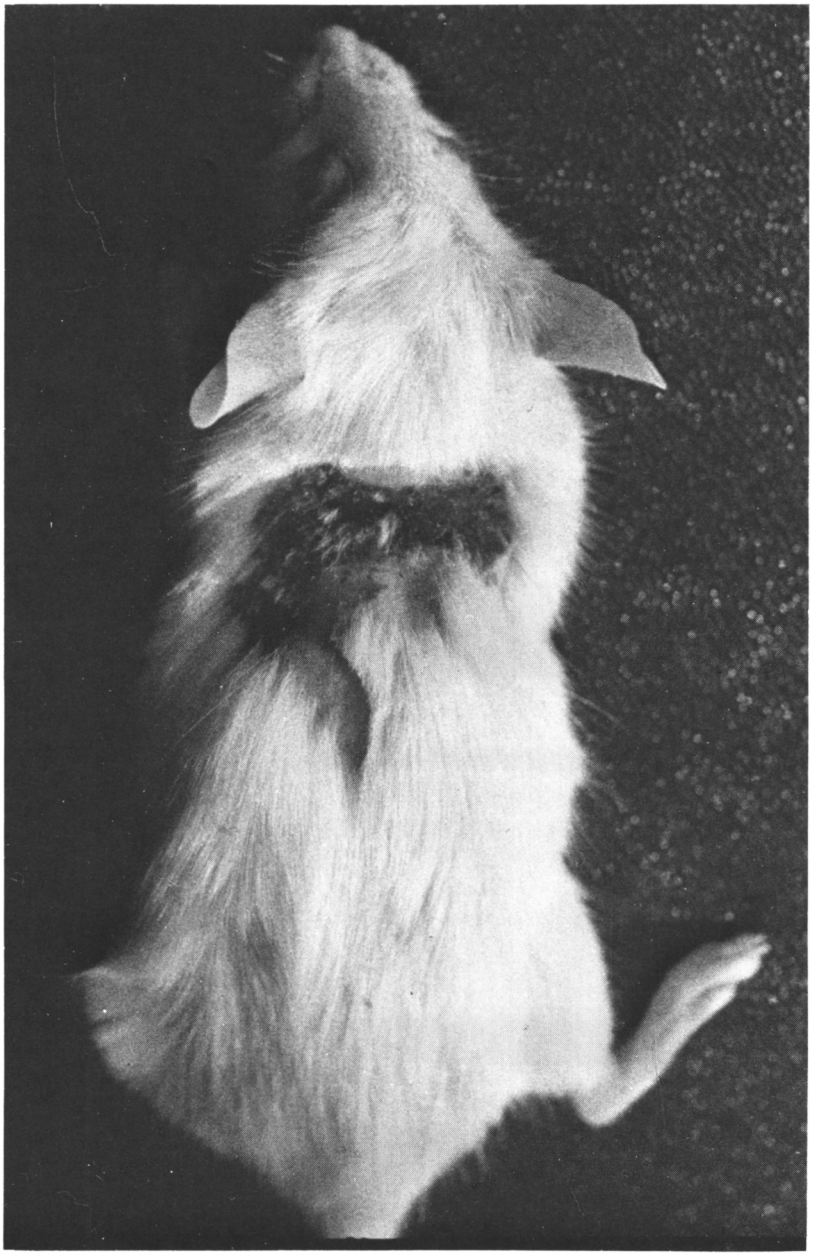

FIG. 1. Photograph taken of mice showing black lesions 4 days after they were infected with 1.1×10^8 CFU of *P. aeruginosa*.

cultured from the purulent exudate or from crushed necrotic dermal tissue but not from internal organs until after 96 hr postinfection.

Microscopically, the skin lesions obtained with live cells were typical of an acute bacterial inflammation, with a mixed cellular infiltrate consisting of predominantly polymorphonuclear leukocytes (PMNs) and some mononuclear cells. After 7 days the normal histological appearance of the skin was extensively obliterated with lesions extending deep into the connective tissues and muscle layers. At no time was a necrotizing vasculitis detectable in any of the skin lesions,

and no destruction of elastin in the dermal blood vessels was observed. After 12 days, the lesions showed some healing with fibrous scarring. No evidence of new skin reactions such as ecthyma gangrenosum appeared in any of the chronically ill animals at sites away from the inoculation site.

Within 4–8 days postinfection, animals exhibited a hematogenous spread of *P. aeruginosa* and a few of the animals began to die from systemic infection. Upon autopsy, both the livers and lungs of these animals showed a great deal of hemorrhage and frequently contained large necrotic foci. By 5 days, foci of frank interstitial pneumonia were apparent and large walled off abscesses were detected by the ninth or tenth day The kidneys showed petechial hemorrhage and occasional renal abscesses by the fifth day. Interstitial nephritis was observed by then and cortical nephritis was detected by the eighth day. Five days postinfection hepatomegaly was detectable and by the tenth day, large hepatolobular abscesses with central necrosis were evident with widespread degeneration. Viable organisms were cultured from the liver, lungs, peritoneal sacs, skin, and occasionally the kidneys. In addition, splenomegaly was always present in these animals. Microscopic examination of the spleens showed the splenic architecture essentially intact with an increase of megakaryocytes.

Animals infected subcutaneously with doses higher than the LD_{50} (i.e., 10^9 CFU) died within the first 24–48 hr and showed little gross pathology outside of very hemorrhagic lungs and spotty hemorrhagic livers. In addition, no dermonecrosis or draining skin lesions were detected. Microscopically, the livers were congested and hepatocytes showed widespread degeneration. A great deal of interstitial hemorrhage was found in the lungs suggesting that these animals may have died from toxic shock. On the other hand, animals receiving sublethal doses of 5×10^7 CFU or less, survived the challenge and showed little or no striking pathological response when sacrificed at 21 days.

Since *P. aeruginosa* is normally of low virulence and mice are generally refractory to infection by this organism, mouse susceptibility to infection was tested by administration of various antineoplastic drugs. Each drug produced a transient leukopenia in the circulation within 24–48 hr but the white cell counts returned to normal within 72–96 hr. The average drop in circulating leukocytes at 48 hr ranged from 28.5 to 47.7% depending on the drug. Consequently, a single injection of one of the four of the antineoplastic agents used in this study markedly reduced the normal resistance of mice to subcutaneous challenge with *P. aeruginosa*. In mice injected intraperitoneally either 24 hr preinfection, simultaneously, or 24 hr postinfection with 160 mg/kg methotrexate, 1.25 mg/kg vincristine sulfate, 500 mg/kg of cytosine arabinoside, or 0.59 mg/kg actinomycin D, the bacterial LD_{50} was decreased by at least one log or more (Table II). Methotrexate and actinomycin D potentiated the greatest reduction in the LD_{50} value and both were the most effective when administered 24 hr after the bacterial challenge. In mice receiving methotrexate postinfection the LD_{50} value was altered from 4.6×10^8 to 2.0×10^6 or a 230-fold decrease while actinomycin D also yielded over a two log reduction to 3.9×10^6 from 4.6×10^8 CFU. For vincristine sulfate and cytosine arabinoside, the simul-

TABLE II. EFFECT OF VARIOUS ANTINEOPLASTIC AGENTS ON THE LD_{50} VALUES OF INFECTED MICE

Drug (intraperitoneal)	Time of drug administration relative to bacterial challenge	LD_{50}
—	—	4.6×10^8
Methotrexate	24 hr before infection	1.0×10^7
"	simultaneous[a]	7.2×10^6
"	24 hr postinfection	2.0×10^6
Vincristine sulfate	24 hr before infection	3.9×10^7
"	simultaneous[a]	1.9×10^7
"	24 hr postinfection	3.2×10^7
Cytosine arabinoside	24 hr before infection	3.9×10^7
"	simultaneous	1.8×10^7
"	24 hr postinfection	3.2×10^7
Actinomycin D	24 hr before infection	2.5×10^7
"	simultaneous	2.5×10^7
"	24 hr postinfection	3.9×10^6

[a] Total of 6 mice/dilution. The remainder of the experiments employed 12 mice/dilution.

taneous administration of the drug and cells was the most effective regimen and resulted in a LD_{50} decrease to 1.9×10^7 and 1.8×10^7 CFU, respectively.

Histological studies of mice which had received antineoplastic drug therapy exhibited some differences in tissue response as compared to infected mice which had not received one of the drugs. For example, in mice which had received vincristine sulfate or actinomycin D, the cellular response at the site of infection was greatly reduced, and the slight cellular infiltrates which were present consisted of predominantly mononuclear leukocytes. Otherwise, skin lesions appeared similar to those obtained in infected animals which had not received one of the antineoplastic agents. The lungs of the infected-drug treated animals usually appeared congested with a great deal of hemorrhage by 3 days suggesting the development of a more rapid systemic infection than in untreated, infected animals. Discrete pulmonary abscess formation was never noted in these animals, nor was it detected in the liver and kidney. Uninfected, control animals receiving only the drug did not exhibit this pulmonary hemorrhage. Histological examination of the major internal organs indicated that all four drugs exhibited a marked suppression of the inflammatory response. However, suppression of the leukocytic response was quantitatively not as great in animals receiving methotrexate, despite the fact that it exhibited the greatest potentiation of infection.

The use of two suprarenal hormones also significantly increased the lethality of L-1 as can be seen in Table III. A simultaneous injection of 4.6 mg/kg epinephrine given subcutaneously in the same general area as the infection site, reduced the LD_{50} value 2.9 logs from 4.6×10^8 to 5.6×10^5 CFU. Intraperitoneal adrenalin injections were slightly less effective, resulting in a 2 log decrease to 4.3×10^6. Although an LD_{50} value for cortisone acetate in uninfected control mice could not be determined, simultaneous injection of 625 mg/kg either intraperitoneally or subcutaneously did depress the LD_{50} 14.4- and 82.1-fold, respectively. The two hormones were not administered 24 hr pre- or postinfection due to their short-lived pharmacological effects.

TABLE III. EFFECT OF SUPRARENAL HORMONES ON THE LD_{50} VALUES OF INFECTED MICE

Hormone[a]	Route	LD_{50}
—	—	4.6×10^8
Epinephrine	Intraperitoneal[b]	4.3×10^6
Epinephrine	Subcutaneously[c]	5.6×10^5
Cortisone acetate	Intraperitoneal[c]	3.2×10^7
Cortisone acetate	Subcutaneous[c]	5.6×10^6

[a] The hormones were administered simultaneously with the bacterial challenge.
[b] Total of 12 mice/dilution.
[c] Total of 6 mice/dilution.

Discussion. One of the clinically significant portals of entry of *P. aeruginosa* bacteremias is infected wounds. This is particularly true in burn wound infections where the significant feature of *Pseudomonas* infection is extensive local invasion that rapidly leads to fatal bacteremia (9). Seeding of the bloodstream from cutaneous lesions is probably common and the reverse process may also occur and might be one factor that is responsible for ecthyma gangrenosum (4, 10). The results described herein were somewhat unexpected since Mull and Callahan (5) previously reported that healthy mice were resistant to subcutaneous infection by *P. aeruginosa* unless predisposed to infection by 6-mercaptopurine. However, our results indicated that mice which were not debilitated by burns or antineoplastic chemotherapy were susceptible to systemic infection leading to death when infected subcutaneously. The results described herein reproduce many features of burn wound sepsis in that once the wound is colonized by *P. aeruginosa*, the organism subsequently proliferates and thereby spreads hematogenously to produce pneumonia and systemic sepsis (11).

One of the results of the present study is that no external skin lesions developed during chronic infection other than at the original inoculation site. The reason for this is not as yet understood, especially since ecthyma gangrenosum is pathognomonic of systemic infections by *P. aeruginosa* in moribund, leukemic, or severely burned patients (4, 12). However, it appears that most, if not all experimental animal models do not exhibit this

lesion other than at the site of infection and therefore, it seems to be associated only with human infections. Furthermore, the appearance of multiple skin lesions of this type does not always occur even in patients with systemic *P. aeruginosa* infections. However, when ecthyma gangrenosum-like lesions occur at the site of infection in mice the mechanism is most likely due to the elaboration of extracellular proteases and possibly other dermonecrotic enzymes. This is supported by previous studies in which purified preparations of either elastase or collagenase from *P. aeruginosa* were dermonecrotic when administered subcutaneously to mice (13, 14).

The present study indicates that the test animals were least susceptible to infection and death when the subcutaneous route was employed as opposed to the intraperitoneal and intravenous routes. *Pseudomonas aeruginosa* appears to be an organism of very low virulence in healthy individuals as well as in mice as witnessed by the large number of cells necessary to produce a fatal infection. However, a variety of experimental techniques can be employed to potentiate the disease processes. The increased lethality associated with bacterial endotoxins as potentiated by actinomycin D and other antitumor drugs has been well established for some time (15, 16). However, there have been few experimental studies of this kind wherein infectious agents have been studied in place of endotoxin (17). In light of the increasing reports of *Pseudomonas* infections in patients with malignant disease, especially in those who are undergoing antineoplastic therapy or corticosteroids, the capability of several of these agents to predispose mice to infection was tested. The antitumor drugs used in this study were selected because of their current clinical importance in cancer therapy as well as their potential immunosuppressive action. Also, each represented a major class of antineoplastic agents exerting different mechanisms of biochemical action at the cellular level. For example, methotrexate is a folic acid antagonist (16), vincristine sulfate arrests mitotic division at the stage of metaphase by some undetermined mechanism (18), cytosine arabinoside acts as a deoxycytidine antagonist and inhibits nucleic acid synthesis (19), and actinomycin D intercalates with guanine residues in DNA thereby interfering with DNA dependent synthesis of mRNA (20). Comparison of the lethality between the untreated infected mice and mice treated with one of these oncolytic agents showed that all four drugs significantly lowered the number of colony forming units needed to produce a systemic infection and death. These data suggest that patients already immunologically debilitated from neoplastic diseases and antineoplastic treatment, are very susceptible to serious iatrogenically associated systemic infections. The use of two suprarenal hormones was also examined since patients receiving corticosteroids or other hormone therapy, especially on a prolonged basis, may also be inadvertently predisposed to infection as was noted in these experiments. These results are consistent with the reports that corticosteroids and immunosuppressive agents are associated with a poor prognosis for survival of infected patients and experimental animals. It appears that granulocytopenic patients with leukemia and cancer are particularly predisposed to infection and frequently die from *P. aerubinosa* sepsis (21, 22). Thus, even the transitory leukopenia induced by a single dose of antineoplastic drug as described herein was enough so that the other natural defense mechanisms of the animals was overwhelmed by a 10- to 100-fold decrease in the number of organisms needed to initiate a terminal infection.

Summary. By use of the subcutaneous route, chronic *Pseudomonas aeruginosa* infections were established in normal mice undebilitated by burn wounds or leukopenic agents. Using a 21-day holding period, an LD_{50} value of 4.6×10^8 colony forming units was obtained. After subcutaneous infection, the dermis was completely necrosed with the lesions reaching deep into the subcutaneous tissue and musculature within 3–4 days. Ecthyma gangrenosum-like skin lesions at the site of infection appeared during this time period. By 7–15 days all mice exhibited a systemic infection. Both the livers and lungs showed a great deal of hemorrhage and frequently contained large necrotic foci, while the kidneys showed petechial hemorrhage and occasional renal abscesses. The suscepti-

bility to infection was markedly increased by use of various antineoplastic agents and suprarenal hormones. However, the type of tissue damage or severity was not significantly altered as compared to infected mice which had not received any of the chemical agents.

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