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Experimental Studies on Mice Subcutaneously Challenged with Heat-Killed Cells of *Pseudomonas aeruginosa* (39269)

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Infections caused by *Pseudomonas aeruginosa* have increased considerably over the past few years, especially in patients already debilitated by other diseases, corticosteroids, antibiotics, and antineoplastic agents (1, 2). Cutaneous infections and lesions due to this organism are frequently observed as a manifestation of both systemic as well as localized wound infections; however, the significance of *P. aeruginosa* in dermatology has been largely ignored. Several separate pathological types of cutaneous lesions have been described and consist of gangrenous ulcers surrounded by erythema and induration, which has been termed "ecthyma gangrenosum," a striking erythema with sharply demarcated borders with eruptive petechia, and roseolalike maculopapular plaques (3, 4). It has been postulated that certain of these dermatologic conditions associated with *P. aeruginosa* infections may be related to a Shwartzman-like reaction rather than to toxic factors of the organism (5, 6). Recent studies in our laboratory indicate that both acute and chronic systemic infections in mice can be established by sc administration of *P. aeruginosa* without having to predispose the animals to infection through the use of burn wounds or antineoplastic chemotherapy (7). In addition, ecthyma gangrenosumlike lesions developed at the inoculation site. During this study, preliminary results indicated that the dermal response and susceptibility to lethality of the animals challenged with dead cells of *P. aeruginosa* was markedly different from that obtained with live cells (7). The use of dead cells of *P. aeruginosa* for experimental *in vivo* studies is of importance because of the potential role of *Pseudomonas* endotoxin as a virulence factor during the infectious process. At present the status of *Pseudomonas* endotoxin is subject to some controversy (8–12). Therefore, the present report is a continuation of these dermatological studies in an effort to develop and further characterize a convenient experimental model for studying the response of animals sc challenged with heat-inactivated cells of *P. aeruginosa*.

Materials and methods. Organism. A strain of *Pseudomonas 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. The organism was routinely cultured in 5% peptone (Difco) and 0.25% trypticase soy broth (BBL) for 24 hr at 35° with constant aeration.

Preparation of inoculum. The 24-hr cultures were centrifuged at 17,000 rpm for 15 min with a Sorvall RC2B centrifuge. The supernatant was discarded, and the soft pellet of cells was resuspended in 5 ml of sterile saline. The cell suspensions tended to undergo some autolysis over extended periods of time or upon additional centrifugation, so they were used immediately for the *in vivo* studies. Cell suspensions were first plated for the viable count determination and then autoclaved at 121° under 15 psi pressure for 15 min. The sterility of each suspension was then verified by plating on tryptose agar.

In vivo studies. The heat-killed cell suspensions (0.5 ml) were inoculated sc into white Swiss-Webster female mice weighing 18–22 g. Control animals received an equivalent amount of sterile saline. A minimum of six animals were employed for each dilution after pretitration studies. In an attempt to determine the sequence of possible pathological responses of the animals, a 21-day holding period was employed. Animals were autopsied after death, while those surviving the 21-day period were sacrificed by cervical detachment and also autopsied. The final mean lethal dose (LD_{50}) values were calculated by the method of Reed and Muench

(13) for a 72-hr holding period, since animals surviving the challenging dose of organisms never died after this time period.

Chemical agents. Several antineoplastic drugs were examined, each having a different biochemical mechanism of action in an attempt to predispose the mice to altered skin reactions and susceptibility to toxic shock (14, 15). The drugs were administered ip in the following concentrations; methotrexate (Lederle Labs, Pearl River, N.Y.) 160 mg/kg; vincristine sulfate (Oncovin, Eli Lilly and Co., Indianapolis, Ind.) 1.25 mg/kg; cytosine arabinoside (Cytosar, The Upjohn Co., Kalamazoo, Mich.) 500 mg/kg; and actinomycin D (Cosmogen, Merck, Sharpe and Dohme, W. Point, Pa.) 0.595 mg/kg. In addition, epinephrine (adrenalin chloride, Parke, Davis and Co., Detroit, Mich.) 4.64 mg/kg, cortisone acetate (Upjohn Co., Kalamazoo, Mich.) 625 mg/kg; and 5% hog gastric mucin (Wilson Labs, Chicago, Ill.) were used. All of the drugs and mucin were diluted to the desired concentration in nonpyrogenic sterile saline and were prepared immediately before each experiment. A volume of 0.5 ml was administered ip either 24 hr before, simultaneously, or 24 hr after the sc bacterial challenge. Control animals received one of each of the agents in the absence of the bacterial challenge. With the exception of Cytosar, the concentrations of the various antineoplastic agents was one-half the LD_{50} dose of the agent as determined by pretitration studies in order to reduce the possibility of death or altered histological responses due to the drug in the absence of the bacterial challenge. The LD_{50} of Cytosar is unknown and is reported by the manufacturer to be greater than 1000 mg/kg, and in our hands it was not lethal for the mice at this concentration.

Histopathological studies. After gross postmortem observations were recorded, the heart, lungs, liver, spleen, stomach, intestines, pancreas, kidneys, portions of blood vessels, and skin at the inoculation site 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.

Results. Previous studies indicated that mice sc challenged with *P. aeruginosa* were generally refractory to infection and that unusually large numbers of cells were needed to produce dermonecrosis and death, although the LD_{50} values were substantially reduced in the presence of immunosuppressive agents (7). Therefore, initial studies to be described herein were designed to determine whether dead bacterial cells were capable of eliciting similar adverse skin reactions and/or death. Titration studies were heat-inactivated cells administered sc yielded a 72-hr LD_{50} value of 3.4×10^{10} cells as compared to 4.8×10^{8} viable cells. Animals surviving after 72 hr always recovered from the bacterial challenge. Some of the animals receiving a sublethal dose of 1.0×10^{10} dead cells developed black crusty skin lesions within 3 to 5 days at the inoculation site, which were somewhat similar in appearance to clinically observed ecthyma gangrenosum (3, 4). Of particular interest was that the appearance of the lesion was sporadic and unpredictable. Thus, consecutive values for three experiments employing sets of six mice each yielded 33, 50, and 33%, respectively. The total average for the three sets of mice was 38.6%. Inoculation of doses higher than 10^{10} dead cells did not increase the incidence in the number of animals yielding a black lesion and only resulted in rapid death within 24 hr or less, which was probably due to toxic shock. Unlike the dermonecrotic lesions experimentally obtained with viable cells (7), the black lesions were dry, nonpurulent, and did not extend deep into the underlying musculature. Extensive hemorrhage was macroscopically visible and large numbers of erythrocytes were seen in the surface dermal layers. Also, the inflammatory response at the inoculation site seemed to be less severe than that seen with live cells (7), and was usually associated with surface blood vessels. The perivascular infiltrates were made up primarily of PMN's with some mononuclear leukocytes. Gross studies of internal organs of animals receiving 3.4×10^{10} dead cells indicated only a slightly enlarged spleen, while histologically the liver displayed several areas of hydropic degeneration of hepatocytes along with the accumulation of a few erythrocytes and PMN's in the periportal

regions. No unusual gross or histopathological lesions were noted in sacrificed animals receiving sublethal doses of dead cells other than the skin lesions.

Several attempts were made to increase the frequency of the black lesions. These included administration of 10^{10} dead cells or 10^6 live cells ip or sc either 1 day or 1 week prior to the sc challenge with 10^{10} dead cells. However, the incidence of the black lesions did not change. But when a longer time period was used between the initial inoculation and challenge, then an increase in the frequency occurred. Thus, fourteen animals receiving sc either 10^6 viable cells or 10^9 dead cells, and challenged 16 days later, yielded frequencies of 78.6 and 50%, respectively. These lesions appeared much more severe, covered a greater skin area with extensive hemorrhage, and showed a more intense black discoloration than obtained with animals receiving only the one sc inoculation of dead cells. Microscopically, an increased cellular response made up of erythrocytes, PMN's, and mononuclear leukocytes was evident in the animals receiving two injections 16 days apart. The internal organs appeared unaffected, except for a slightly enlarged spleen.

Only use of the sc route for the initial injection resulted in a significant increase in the lesions, because when animals were given 10^6 viable cells or 10^9 dead cells ip prior to a 16-day challenge, the incidence of the black lesions remained unchanged. Also, 10^{10} heat-killed or 10^7 viable cells were given sc and then followed 24 hr later with a challenge dose of 10^7 heat-killed cells or 10^5 viable cells given iv in an attempt to invoke a Shwartzman reaction. However, the iv challenge did not elicit any unusual reactions at the sc site of inoculation.

In an attempt to increase the susceptibility of mice to subcutaneous challenge, sets of six mice were treated with 0.5 ml of 5% hog gastric mucin given ip 24 hr prior, simultaneously, or 24 hr following challenge with dead cells However, no significant change in the LD_{50} or frequency of the skin lesions was observed. Mixtures of mucin and heat-killed cells administered sc also did not decrease the LD_{50} as had been noticed in the previous study employing live cells (7).

The increased lethality associated with bacterial endotoxins as potentiated by actinomycin D and other antitumor drugs has been well established, and this interaction has implications of clinical importance (14, 15). Therefore, several antineoplastic drugs were singly administered to the mice in an attempt to enhance the toxicity of the dead cells or their cellular components such as endotoxin. The drugs tested were methotrexate, vincristine sulfate, cytosine arabinoside, and actinomycin D. However, only methotrexate was found to significantly increase the lethality of heat-killed cells. These results are summarized in Table I. With regard to the skin lesions, it was found that of the four drugs only methotrexate and actinomycin D decreased the incidence of its appearance (to 17% of the animals).

The simultaneous treatment of mice with either epinephrine or cortisone acetate administered either ip or sc in the same area, but at a different site, did not appear to have any significant effect on the LD_{50} values (Table II). However, cortisone acetate completely suppressed the lesions when administered either ip or sc at the same time as the bacterial challenge, while epinephrine had no effect. The two hormones were not administered 24-hr pre- or postinfection due to their relatively short-lived pharmacological effects.

Discussion. The potential role of *P. aeruginosa* endotoxin in the infectious disease process has been subject to much controversy. Previous studies by Homma *et al.* (8) indicated that a potent endotoxin could be obtained from this organism; however, the bulk of investigations from several other laboratories indicate that neither dead cells nor endotoxin are particularly toxic to laboratory animals unless very high concentrations were employed (9–12). The present study tends to support these latter findings. With regard to the many types of skin reactions clinically observed, one mechanism by which they occur could be the result of the various proteases produced by this organism since hemorrhage and dermonecrosis can be easily produced when either elastase or collagenase from pseudomonas is administered sc (16, 17). In the present study, the black lesions that arose with sublethal injections

TABLE I. EFFECT OF ANTINEOPLASTIC DRUGS ON THE LETHALITY OF HEAT-KILLED *P. AERUGINOSA* CELLS SUBCUTANEOUSLY INJECTED INTO MICE.

Antineoplastic drug given	Time of drug administration relative to bacterial challenge	Subcutaneous LD_{50} of Strain L-1	Decrease in LD_{50}	
			Log	Fold
None	None	3.4×10^{10}	0	1.0
Methotrexate	24 hr before[a]	1.1×10^{10}	0.2	3.1
160 mg/kg (0.55 LD_{50})	Simultaneously[b]	1.8×10^{9}	1.2	18.9
	24 hr after[a]	4.0×10^{9}	0.9	8.5
Vincristine sulfate	24 hr before[a]	3.0×10^{10}	0.0	1.2
1.25 mg/kg (0.50 LD_{50})	Simultaneously[b]	1.5×10^{10}	0.2	2.3
	24 hr after[a]	2.0×10^{10}	0.1	1.7
Cytosine arabinoside	24 hr before[a]	3.0×10^{10}	0.0	1.2
500 mg/kg (LD_{50} not determined)	Simultaneously[a]	2.8×10^{10}	0.1	1.2
	24 hr after[a]	3.2×10^{10}	0.0	1.1
Actinomycin D	24 hr before[a]	1.8×10^{10}	0.2	1.9
0.59 mg/kg (0.51 LD_{50})	Simultaneously[a]	2.6×10^{10}	0.1	1.3
	24 hr after[a]	1.8×10^{10}	0.2	1.9

[a] Total of 6 mice per dilution.
[b] Total of 12 mice per dilution.

TABLE II. EFFECT OF SUPRARENAL HORMONES ON THE LETHALITY OF HEAT-KILLED *P. AERUGINOSA* CELLS SUBCUTANEOUSLY INJECTED INTO MICE.

Type of hormone treatment given simultaneously with bacterial challenge		Subcutaneous LD_{50} of strain L-1	Decrease in LD_{50}	
Hormone	Route		Log	Fold
None	None	3.4×10^{10}	0	1.0
Epinephrine	Intraperitoneal[a]	3.0×10^{10}	0	1.1
4.6 mg/kg (0.49 LD_{50})	Subcutaneous[a]	1.8×10^{10}	0.2	1.9
Cortisone acetate	Intraperitoneal[b]	3.2×10^{10}	0	1.1
62.5 mg/kg (LD_{50} not determined)	Subcutaneous[b]	1.8×10^{10}	0.2	1.9

[a] Total of 12 mice per dilution.
[b] Total of 6 mice per dilution.

of dead cells suggest that the whole cell or a fraction thereof may also play an important clinical role in certain dermatologic conditions such as pyoderma gangrenosum, purpura fulminans, and dermatitis nodularis necrotica. The Shwartzman reaction has been suggested by others as a possible mechanism for these lesions although no direct evidence has been obtained to support this supposition (5, 6, 18, 19). Our current results imply that an unmasking or activation of a reactive substance such as an endotoxinlike substance occurs during the heating of the cells since sublethal doses of viable cells are unable to elicit this same kind of lesion. Our results are also reminiscent of Hall *et al.* (3) who inadvertently produced prolonged, severe dermal lesions by the id inoculation of autogenous heat-killed cells to a patient who had a *P. aeruginosa* leg infection.

Since the incidence in the formation of our lesions was increased by the sc inoculation of either viable of heat-killed cells 16 days, but not 7 days before the sc dead-cell challenge, it would appear that these lesions in mice may be due to a classical immunological response, such as an Arthus reaction or delayed hypersensitivity, rather than a Shwartzmanlike phenomenon. In addition, attempts to produce a dermal Shwartzman reaction in mice is normally very difficult and our attempts to provoke this response were unsuccessful.

In light of the increasing reports of *pseudomonas* infections in patients with malignant diseases, especially in those who are undergoing antineoplastic or hormonal therapy, the capability of several of these agents to detect endotoxin and predispose mice to toxemia and death was tested. This is of potential clinical relevance since a con-

tinuous stream of endotoxin may be liberated during the lysis of the invading cells in both acute and chronic infections. Previous studies of experimentally induced chronic *pseudomonas* infections indicated that any of the four of the antineoplastic agents tested herein depressed the number of viable cells needed to kill the mice (7). However, with the current study only methotrexate was able to potentiate the toxicity of the heat-killed bacteria, although both methotrexate and actinomycin D depressed the incidence of the black lesions. Epinephrine, an adrenal medullary hormone, was also used in these studies because it has been shown to enhance the toxic action of endotoxin as well as the Shwartzman reaction (20). However, the drug did not significantly alter the LD_{50}, nor the incidence or the intensity of the lesions. On the other hand, cortisone acetate, an adrenal cortical hormone, which has previously been shown to negate the activity of endotoxin (21), did inhibit the formation of the black lesions, but not the toxicity of the dead cells. Consequently, these results give further support to the contention that the cells of *P. aeruginosa* and the endotoxin therein are relatively nontoxic and are probably of minor significance in its virulence, but may be of potential importance in some of the dermatological phenomena associated with *pseudomonas* infections.

Summary. Mice were found to be generally refractory to sc challenge with heat-killed cells of *Pseudomonas aeruginosa* and did not die unless unusually high concentrations were employed. Approximately 38.6% of the animals receiving a single, sublethal dose of 1×10^{10} dead cells developed black, crusty, necrotic skin lesions within 3 to 5 days. No major gross and histopathological changes were detected in internal organs. If the animals were sc administered sublethal doses of either live or dead cells of *P aeruginosa* 16 days prior to sc challenge, then the incidence of black lesions rose to 78.6 and 50% of the animals, respectively. Of several antineoplastic agents tested, only methotrexate significantly affected the 72-hr LD_{50} resulting in a drop to 1.8×10^9 from 3.4×10^{10} cells. However, both methotrexate and actinomycin D decreased the incidence of the black lesions.

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