

## Characterization and Quantitation of Experimental Surgical-Wound Infections Used to Evaluate Topical Antibacterial Agents

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Reproducible experimental surgical-wound infections in mice for use in the evaluation of topical antibacterial agents are described. The experimental wound was created on the backs of mice by means of a midline incision and was infected by means of cotton sutures monocontaminated with *Staphylococcus aureus* or *Pseudomonas aeruginosa*. The course of these wound infections was followed by quantitation of surface bacteria through use of a surface rinse technique. Surface wound counts of the infecting organisms thus obtained appeared to reflect the dynamics of the total wound count, as determined by homogenization of biopsied tissue. Treatment of infected wounds with a placebo cream had only a slight effect on surface wound counts and on mortality in the case of the *S. aureus* infection but enhanced markedly the lethality of the *P. aeruginosa* infection.

The search for new drugs effective as topical antibacterial agents requires the careful and systematic evaluation of drugs not only in vitro but also in vivo, i.e., in the control and management of experimental infections. Several model infections for evaluating topical antibacterial agents have been developed in laboratory animals (5-7, 9, 18, 19, 21) and in humans (3, 14, 15, 20). Although many of the models have proved to be effective in assessing topical agents, most of them do not lend themselves easily to a mass screening of potential agents. This paper describes and characterizes a relatively simple surgical-wound model in mice in which potential topical antibacterial drugs can be evaluated easily and expeditiously against *Staphylococcus aureus* and *Pseudomonas aeruginosa* wound infections.

### MATERIALS AND METHODS

**Animals.** Female CF-1 mice, 18 to 20 g, purchased from a commercial source (Carworth Farms, New City, N. Y.) were used in all experiments. Mice were maintained, 10 to a cage, on wire, in temperature- and humidity-controlled quarters and were allowed food and water ad libitum.

**Test organisms.** *S. aureus* SC 2406 (phage type 53/77/84/85) and *P. aeruginosa* SC 8822, clinical isolates from the departmental culture collection, were used as test organisms. The two organisms were maintained on brain heart infusion (BHI) agar (BBL) slants stored at 4 C, with transfers to fresh slants made monthly. For each experiment, a fresh slant was inoculated from the appropriate stock culture and incubated at 37 C for 18 h. After incuba-

tion, the growth was removed from the slant by washing with 0.1 M phosphate buffer, pH 7.2, and the resulting suspension was adjusted to a concentration of  $2.0 \times 10^8$  cells/ml by use of a Klett-Summerson colorimeter with a 540-nm filter and a previously prepared standard curve. The suspension was then appropriately diluted and used for the contamination of sutures or the direct seeding of the experimental wounds.

**Preparation of contaminated sutures.** Commercial cotton thread (Coats and Clark's, mercerized, size 8) monocontaminated with *S. aureus* or *P. aeruginosa* was used as the suture material to initiate and potentiate the experimental infection. The thread was cut into 5-cm segments and placed in boiling water for 3 min. After removal from the water, the segments were allowed to cool and the excess water was removed by blotting with sterile paper towels. The segments were then dropped into a test tube containing 8 ml of the appropriate dilution of the *S. aureus* or *P. aeruginosa* suspension, mixed on a Vortex mixer for 10 s, and allowed to soak in the suspension for 30 min. During this time period, approximately  $10^3$  cells were adsorbed on each segment of thread. Since the adsorption of the cells was directly related to the concentration of organisms in the suspension, segments containing different numbers of cells could be prepared merely by varying the concentration of the test organism in the soak suspension. After the soak period, the segments were removed from the tube and blotted dry. The number of organisms adsorbed on each suture was ascertained by shaking a suture in a test tube containing 2 ml of sterile distilled water on a twist-action shaker (model N8, New Brunswick Scientific Co.) for 45 min at the lowest setting. Previous studies had shown that this procedure eluted more than

90% of the adsorbed organisms. After the bacteria had been eluted from the suture, the resulting suspension was diluted and plated on the appropriate agar medium.

**Experimental wound and infection.** The procedures used to produce the experimental wound and infections have been described previously (18). One day prior to infection, mice were anesthetized by an intraperitoneal injection of sodium pentobarbital (30 mg/kg). The backs of the mice were closely shaved with a fine-tooth electric clipper. On the day of infection superficial surgical wounds were produced on the backs of reanesthetized mice by making a longitudinal midline incision,  $2.3 \pm 0.2$  cm in length and extending down to the panniculus carnosus. The skin on either side of the incision was retracted, and the wound was infected either by direct seeding or by the insertion of a contaminated segment of thread (suture) through the skin with a suturing needle in such a way that the suture lay diagonally across the panniculus, with the ends extending slightly from the skin. The ends of the suture were secured with rubber cement. The wound eventually covered approximately 6% of the total body surface of a mouse (Fig. 1).

**Treatment of wounds.** For some experiments, the open wounds were treated topically with an emulsion-type cream base placebo that contained promulgen, protopet, sorbitol solution, silicone antifoam, distilled water, and methyl and ethyl parabens as preservatives. Approximately 400 mg of this placebo cream was applied with a tongue depressor over the entire wound and suture twice daily on the day of infection, 0.25 and 5.5 h after infection, and twice daily thereafter for a predetermined number of days.

**Quantitation of bacteria in wounds.** Several methods were used to follow the course of an infection, as reflected in the dynamics of wound counts of the test organism.

Surface bacterial counts were made by a surface rinse technique. At predetermined times after infection, mice were sacrificed with CO<sub>2</sub>; the suture, if present, was removed from the wounds. Each wound was cultured by placing a sterile test tube (22 mm in diameter) containing 5 ml of distilled water in an inverted position on the back of a mouse in such a manner that more than 90% of the wound was tightly enclosed by the mouth of the tube. The mouse and the test tube, held together with the tube upright, were shaken vigorously 25 times to remove the infecting organisms from the surface of the

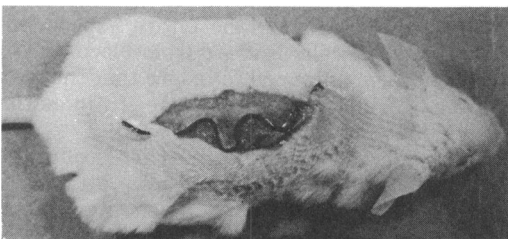


FIG. 1. Standard surgical-wound infection.

wound. Washings were appropriately diluted and then, because of the possibility of contamination, were plated on selective media, staphylococcus agar no. 110 (BBL) for *S. aureus* and MacConkey agar (BBL) for *P. aeruginosa*. In some cases washings were also plated on BHI agar (Difco). Plates were incubated for 24 to 48 h at 37 C, and then colony counts were performed.

Tissue bacterial counts were obtained by homogenizing weighed biopsy samples from infected wounds. Samples were homogenized for 3 min in a microblender (Eberbach); the homogenates were then diluted and plated on the appropriate selective agar medium, as described above.

The number of viable test organisms on sutures removed from infected wounds was determined as described earlier.

**Evaluation of systemic involvement.** Because the experimental wound infections often led to generalized infections, various organs were cultured over a period of time to characterize the magnitude and extent of the systemic involvement. At predetermined intervals after infection, mice were bled from the retro-orbital sinus or by cardiac puncture and sacrificed by CO<sub>2</sub>; liver, spleen, and kidney were removed aseptically. The organs were rinsed with sterile saline, blotted dry, and then minced. Small samples of the macerated tissue were incubated in BHI broth or were used to make impression smears on BHI agar to ascertain the absence or presence of the challenge organism or of any other bacteria. Blood was cultured directly in BHI pour plates or was streaked on BHI agar. In the case of the *S. aureus* infection, isolated organisms resembling staphylococci microscopically and culturally were phage typed to insure that the strain of *S. aureus* used for the infection was the same as that recovered.

## RESULTS

Grossly, the uninfected experimental surgical wound in the mouse presented the usual clinical picture associated with an uninfected open-type wound. Immediately after the incision had been made, there was only minimal bleeding, and it ceased spontaneously. Slight edema and erythema were evident during the early stages of wound healing, which progressed rapidly to almost complete repair in approximately 2 to 3 weeks. Microscopically, the uninfected wound initially demonstrated the classical signs of inflammation: edema, extravasated erythrocytes and leukocytes, dilated capillaries, etc. No deaths occurred as a result of the surgical procedure. When the wound had been infected with *S. aureus* or *P. aeruginosa*, clinical signs of infection were present but were not striking. Slight edema and erythema were observed around the margins of the wound, and suppuration was noted occasionally beneath the retracted skin. Wound healing generally required 3 weeks from the time of infection. No

deaths occurred in mice infected with *S. aureus*; only a few deaths were recorded in mice infected with *P. aeruginosa*.

When *S. aureus* was used as the test organism, two methods were employed to infect the experimental wound, direct seeding and the insertion of contaminated sutures. As can be seen in Table 1, during a period of 4 days there was no significant difference in bacterial counts in mice infected in these two ways with the same size inoculum. The peak count of *S. aureus* in the wounds appeared to occur earlier in mice infected by direct seeding. More important, however, was the fact that slightly less variation between individual counts of mice in the same group occurred when contaminated sutures were used to initiate the infection. Because of this finding, contaminated sutures were used to produce wound infections in all subsequent experiments.

To determine the smallest inoculum of *S. aureus* that could initiate the experimental infection, sutures were soaked in predetermined concentrations of the test organism that would yield  $10^1$ ,  $10^3$ , and  $10^5$  cells/suture after adsorption and were then evaluated in the experimental wound. The results (Table 2) indicate that  $10^1$  *S. aureus* cells/suture were insufficient to induce an infection, whereas an inoculum of  $10^3$  or  $10^5$  cells could elicit the infection. The use of an inoculum of  $10^5$  cells, as could be expected, initially produced slightly higher wound counts than did the use of  $10^3$  cells, but by 3 days after infection the counts were essentially equivalent. Since the inoculum of  $10^3$  cells/suture was sufficient to produce proliferation of the test organism in wounds, this inoculum was selected for use in the model infection.

As previously noted, mice infected with *P. aeruginosa* occasionally died, apparently as a result of the infection; this finding suggested systemic involvement leading to a generalized infection. The possibility of systemic involvement after the start of wound infections with

TABLE 2. Effect of inoculum size on the production of an experimental staphylococcal wound infection as reflected by surface wound counts

Days after infection	Mean surface count ( $\times 10^6$ ) <sup>a</sup>		
	10 cells/suture	$10^3$ cells/suture	$10^5$ cells/suture
1	NI <sup>b</sup>	$3.4 \pm 0.4$	$8.4 \pm 1.4$
2	NI	$6.8 \pm 1.2$	$22.5 \pm 1.5$
3	NI	$15.9 \pm 2.1$	$18.2 \pm 2.1$
6	NI	$4.5 \pm 0.8$	$3.2 \pm 1.0$
7	NI	$1.1 \pm 0.7$	$2.6 \pm 1.4$

<sup>a</sup> Mean  $\pm$  standard error of surface counts from five mice.

<sup>b</sup> NI, No infection; surface counts  $< 1.0 \times 10^6$  organisms.

either *P. aeruginosa* or *S. aureus* was investigated. At various intervals after infection, selected organs and blood were cultured to determine the absence or presence of the test organism. The results (Table 3) were somewhat surprising in that both infections exhibited some degree of systemic involvement very soon after suture insertion. In the *P. aeruginosa* infection, at 3 days after infection almost 75% of the mice demonstrated the presence of the organism in liver, kidney, and spleen although blood cultures were, for the most part, negative throughout the course of the experiment. *P. aeruginosa* persisted in the organs for up to 7 days but thereafter was rarely isolated. A few deaths were recorded during the first 2 weeks after infection, but none occurred after this period. *P. aeruginosa* was isolated from all internal organs cultured of mice that died, indicating generalized infection.

In the case of the *S. aureus* infection, the test organism was found in the liver, spleen, and kidney of most mice within 24 h after infection and persisted therein for 1 to 2 weeks. However, when the organs were cultured 2 weeks after infection, *S. aureus* was isolated only occasionally. No staphylococci were isolated when organs were cultured at the 3- and 4-week postinfection intervals. A few positive blood cultures were obtained only during the first week of infection, with the largest number found on postinfection day 1. No mortalities were recorded during the entire experiment. All *S. aureus* cells isolated from blood and organs were phage typed to insure that the organisms isolated were of the same strain as that used for the infection.

It should be noted that earlier studies, in which blood from the retro-orbital plexus was cultured, had indicated a much higher incidence of positive blood cultures than was demonstrated with cardiac puncture. However,

TABLE 1. Comparison of seeding versus insertion of contaminated sutures for producing the *S. aureus* wound infection

Days after infection	Mean surface count ( $\times 10^6$ ) <sup>a</sup>	
	Infected with sutures <sup>b</sup>	Infected by seeding <sup>b</sup>
1	$2.2 \pm 0.5$	$5.2 \pm 1.2$
2	$8.7 \pm 1.2$	$15.9 \pm 4.2$
3	$12.2 \pm 2.1$	$11.7 \pm 3.0$
4	$8.3 \pm 0.9$	$7.2 \pm 2.0$

<sup>a</sup> Mean  $\pm$  standard error of surface counts from five mice.

<sup>b</sup> Infection initiated with  $1.0 \times 10^3$  *S. aureus* cells.

these earlier data were not valid because, as was found later, the blood from the retro-orbital plexus was contaminated with pseudomonads from the hair surrounding the eye and from the conjunctivitis that is especially common in *P. aeruginosa*-infected animals. Thus, only the data obtained from the cardiac puncture blood cultures are presented in Table 3.

Because the signs of infection were not striking with either test organism, the course of the wound infections was followed through changes in the population dynamics of the infecting organisms. Three different quantitation tech-

niques were used: (i) the classical biopsy homogenization method, (ii) a surface rinse technique, and (iii) a suture culture technique. A comparison of the wound counts obtained via the three techniques is shown in Fig. 2. As would be expected, the homogenization technique yielded the largest wound counts, since both surface and tissue-associated organisms were quantitated, in contrast to only the surface organisms that were assessed by the surface rinse and suture culture techniques. In the *P. aeruginosa* infection, changes in the total wound population of the pseudomonads, as

TABLE 3. Localization of infecting organisms after the initiation of superficial wound infections

Infecting organism	Days after infection	No. of positive cultures/total cultures made				Cumulative mortality <sup>a</sup>
		Liver	Spleen	Kidney	Blood	
<i>P. aeruginosa</i>	1	4/15	2/15	4/15	0/15	0/40
	2	6/15	2/15	7/15	0/15	0/40
	3	13/15	10/15	10/15	3/15	2/40
	4	10/15	7/15	7/15	1/15	3/40
	7	6/15	7/15	7/15	0/15	5/40
	14	1/15	2/15	1/15	0/15	7/40
	21	0/15	0/15	0/15	0/15	7/40
	28	0/15	0/15	0/15	0/15	7/40
<i>S. aureus</i>	1	15/15	15/15	14/15	6/15	0/36
	2	14/15	15/15	14/15	1/15	0/36
	3	13/15	12/15	14/15	3/15	0/36
	4	15/15	14/15	15/15	3/15	0/36
	7	10/15	10/15	11/15	0/15	0/36
	14	1/15	1/15	0/15	0/15	0/36
	21	0/15	0/15	0/15	0/15	0/36
	28	0/15	0/15	0/15	0/15	0/36

<sup>a</sup> Number of mice dead/total infected.

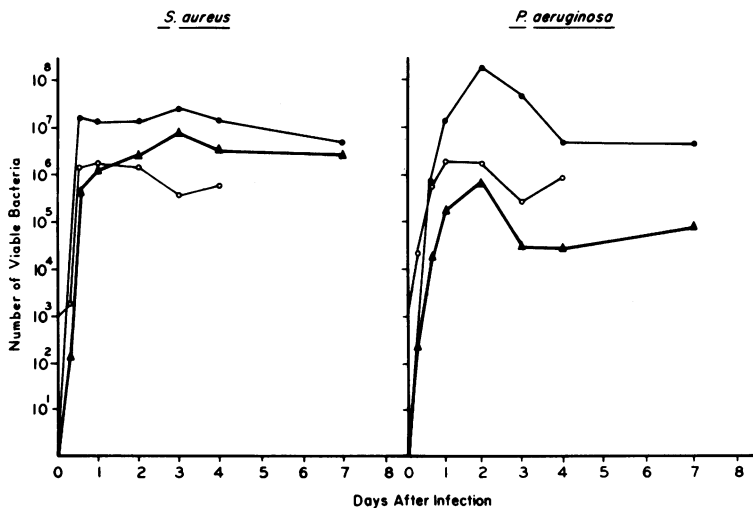


FIG. 2. Quantitation of test organisms in experimental wound infections by three different methods. Symbols: ●, mean number of bacteria per gram of tissue (homogenization procedure); ▲, mean number of bacteria per wound surface (surface rinse technique); ○, mean number of bacteria per suture (suture culture method). Each point represents results from a minimum of 10 mice.

demonstrated by the tissue (and surface) counts obtained by the homogenization technique, were quite accurately reflected by the results of the surface rinse technique. Wound counts obtained from the quantitation of pseudomonads eluted from wound-associated sutures to some extent also reflected the dynamics of the infection; however, sutures could not be retained in the wounds in adequate numbers for testing longer than 4 days. The same relationship between the different methods of quantitation was also observed in the *S. aureus* infection. Tissue and surface bacterial counts beyond the day 7 postinfection interval were generally too diverse to provide meaningful results and, therefore, are not presented.

The effect of a cream base vehicle on the two wound infections was investigated since test compounds must be formulated in some type of topical vehicle to be evaluated in the model. The cream base vehicle employed was selected because studies in vitro had indicated that it did not support the growth of either test organism when incubated at 37 C for several days in the presence of inocula equivalent to those used in the model infections. In addition, it was apparently compatible with various types of antibacterial agents, as indicated by the results of drug-release studies (unpublished data). The effect of treatment with the vehicle, utilized as a placebo cream, on the wound infections is shown in Fig. 3. The cream base treatment produced a marked effect on the *P. aeruginosa* infection, elevating the wound counts by more than 2 log<sub>10</sub> during the first 3 days after infection. By day 4, most of the mice had died as a result of the enhanced infection. In the *S. aureus* infection, the application of the placebo cream produced only a moderate increase in wound counts. These results were also demonstrated with other topical creams and ointments (unpublished data).

As is shown in Fig. 4, treatment with the placebo cream produced only a slight increase in the mortality resulting from the *S. aureus* infection but enhanced mortality markedly in the case of the *P. aeruginosa* infection. All treated mice had died by 2 weeks after infection, with a majority of the deaths occurring during the first 5 days after infection.

## DISCUSSION

In an effort to identify and evaluate substances that may be effective as topical antibacterial agents in humans, many experimental infections in laboratory animals have been developed (5, 7, 9, 18, 21). However, if a model

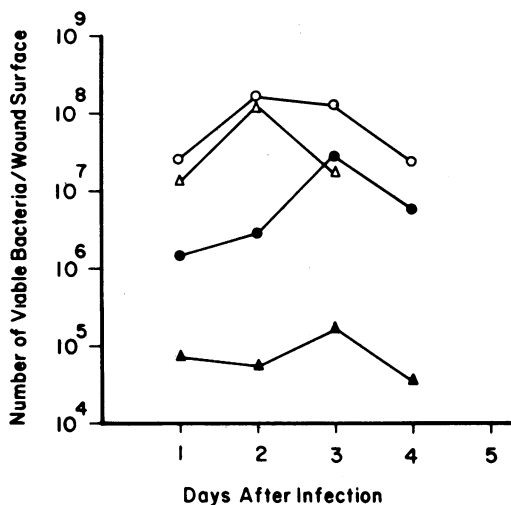


FIG. 3. Effect of topical treatment with placebo cream on wound counts of *P. aeruginosa* and *S. aureus* in experimental wound infections. *P. aeruginosa*: Δ, placebo treated; ▲, untreated. *S. aureus*: ○, placebo treated; ●, untreated. Each point represents the mean count from 10 mice.

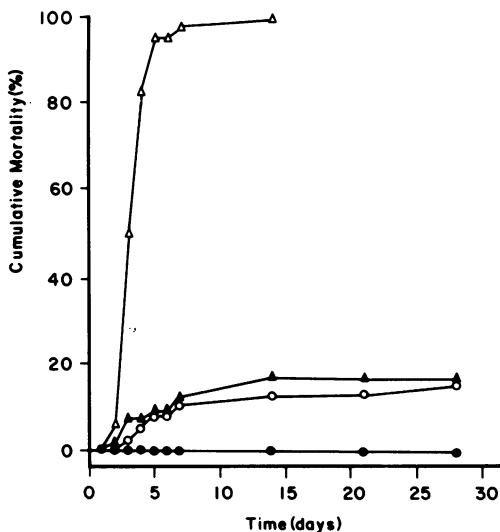


FIG. 4. Effect of topical placebo cream treatment on mortality in *P. aeruginosa* and *S. aureus* wound infections. *P. aeruginosa* infection: Δ, placebo treated; ▲, untreated. *S. aureus*: ○, placebo treated; ●, untreated. A minimum of 36 mice was used for each test group.

infection is to provide an evaluation of efficacy to which some degree of confidence can be attached, then it must satisfy several conditions: (i) because the model infection should simulate or approximate naturally occurring infections

in humans, the pathogenesis of infection must be known; (ii) the infection should be reproducible, readily controlled, and provide some measurable end point; (iii) it should be sensitive enough to permit detection of bacteriostatic, as well as of bactericidal, substances; (iv) clinically active antibacterial agents should demonstrate efficacy in the model; and (v) results from the model infection should provide a high degree of predictability of efficacy in humans. Moreover, if the model is to be used for screening purposes, it must meet additional requirements: (i) the model infection should be relatively simple to produce; (ii) the infection must be easily observed and quantitated; and (iii) it should be easy to evaluate several compounds at one time in an adequate number of animals. Many experimental models fail to meet the requirements that are necessary for a model to be truly relevant. The results described in this study and those presented in the following paper (13) indicate that most of the criteria are met in the surgical-wound model. It should be noted, however, that the extensive systemic involvement resulting from the experimental wound infections is rarely observed in clinical cases.

Since both gram-positive and gram-negative organisms are encountered in various skin and wound infections, *S. aureus* and *P. aeruginosa* were selected as representative test organisms for the wound model. The selection of these test organisms is even more appropriate since they are often the causative agents of open wound infections in humans. The rationale for using contaminated sutures to initiate the experimental infections was based on reported observations that: (i) contaminated sutures produce infections in laboratory animals; (ii) a relatively small number of *S. aureus* cells ( $10^8$ ) adsorbed on certain types of sutures can initiate an experimental infection in a closed wound; and (iii) various suture materials induce a foreign body response (1, 10). However, the results of our study indicated no difference between the course of the experimental infections elicited by placement of contaminated sutures and that initiated by direct seeding of wounds. This finding can probably be explained by the fact that most of the effects of suture-initiated infections were demonstrated in subcutaneous infections and not the open wound infection that is utilized in our model. Nonetheless, we decided to utilize the suture method of eliciting infection because a slight problem was encountered in the direct seeding of wounds. No matter how meticulously the 0.02-ml inoculum was applied, there was occasionally some runoff. The greater variation in wound counts in mice in-

fectured by direct seeding probably occurred as a consequence of runoff of inoculum.

In wound infections, either naturally occurring or experimental, some means must be employed to follow the course of the infection and evaluate the effectiveness of therapy. One procedure that has been utilized, both in animals and humans, is the quantitation of bacteria in infected tissues. Various methods and techniques have been devised for this quantitation, ranging from the classical tissue homogenization method (11) to various types of surface-sampling techniques using moist swabs (4), gauze pads (2), impression pads (8), suture counts (18), and other more elaborate procedures (17, 19). To utilize the experimental surgical-wound infections for screening purposes, it was necessary to have a method of quantitation that would be facile to perform and yet would yield accurate results. The surface rinse technique that was devised seemed to meet these requirements. Large numbers of wounds could be cultured in a relatively short period, and the resulting counts compared quite favorably with those obtained after homogenization of biopsied tissues.

The surface rinse technique collects only the superficially localized bacteria in the wound, whereas the homogenization method assesses both surface and tissue-localized organisms. Consequently, higher wound counts, indicative of the invasiveness of the infecting organism, are obtained via the homogenization method. However, wound counts obtained by the surface rinse technique, although lower, appear to reflect accurately the dynamics of the total wound count, surface- and tissue-localized bacteria, in the course of an infection. The homogenization method is not really suitable for use in screening for topical antibacterials because of the considerable amount of time required to evaluate a large number of tissue samples. Because the surface wound counts correlate well with the total wound counts, we decided to employ the surface rinse technique in evaluating potential topical agents. It should be mentioned that counts from wound-associated sutures to some extent also follow the dynamics of the wound bacterial population, but it was almost impossible to retain the sutures for more than 3 to 4 days in most mice.

One problem occasionally encountered in the model infection was contamination. As would be expected, the open wounds sometimes became contaminated with gram-positive and gram-negative organisms from skin, hair, and feces. However, these contaminating organisms, mainly gram-positive cocci, attained a population peak of only  $5.0 \times 10^4$  in uninfected

wounds, considerably less than counts obtained by either of the test organisms, and were rarely isolated from internal organs. To circumvent potential problems in colony counting that might be caused by contaminants, selective media were employed.

Since many topical antibacterial agents are generally formulated in cream or ointment vehicles for application to wounds or other skin infections, the effect of a commonly employed cream vehicle on the experimental infections was investigated. Although the placebo cream exhibited a moderate effect on the *S. aureus* infection, it affected the *P. aeruginosa* infection markedly, producing significant increases in wound counts and mortality. These results were not surprising, since it is known that many gram-negative organisms flourish in moist areas on the surface of a human body (16). The placebo cream, containing a high concentration of water, either increased the moisture content of the wound or prevented water loss from the wound per se, thereby creating a better environment for the growth of *P. aeruginosa*. The enhanced growth of the organism resulted in elevated wound counts, augmented invasiveness, and, consequently, increased mortality. This effect of the placebo cream can be duplicated with other cream and ointment bases or other procedures that keep the infected wound hydrated. The fact that the cream base vehicle used in this study does not support the growth of either test organism indicates that the higher bacterial counts found in placebo-treated mice represent microbial growth in the wound and not in the vehicle per se.

The application of the model infection to the evaluation of topical antibacterial agents is described in the following paper (13).

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