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Cutaneous Infection in Normal and Immunocompromised Mice

WILLIAM G. KRAFT, PRISCILLA T. JOHNSON, BRIEN C. DAVID, AND DONNA R. MORGAN*
Anti-Infective Research, Norwich Eaton Pharmaceuticals, Inc., Norwich, New York 13815

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Since a model of staphylococcal skin infection adequately reflecting human disease was unavailable, a self-limiting animal infection model specific for virulent *Staphylococcus* species was developed. A virulent strain of *S. aureus*, NCTC 9789 (ATCC 27700), was used to develop an infection model in adult, male CF-1 mice treated with 0 to 150 mg of cyclophosphamide (CY) per kg 4 days before challenge. Bacteria were inoculated onto the dorsal side of shaved mice at 0 to 10^6 CFU per mouse. Simultaneously, the skin was gently scraped to remove the superficial layers without drawing blood. The wound was occluded with impermeable film secured with surgical tape. At a CY dose of 50 mg/kg and an inoculum of 10^5 CFU, 89% of the mice (96 of 108) developed large abscesses (~15-mm diameter). Mice which were not immunocompromised developed fewer abscesses (20 of 68). Generally, no abscesses formed when the mice were not wounded (1 of 62), occluded (0 of 89), or inoculated (11 of 50). The abscesses developed 24 to 48 h after challenge and persisted for 2 to 3 weeks. The challenge organism was isolated from the abscesses. The rates of abscess formation of three additional *S. aureus* strains varied widely in normal and CY-treated mice. Three strains of *S. epidermidis*, one of *Micrococcus varians*, and one of *S. saprophyticus* failed to cause abscesses. Bacterial proliferation studies demonstrated that a strain of *S. aureus* and a strain of *S. epidermidis* proliferated to the same levels 48 h after challenge. Immunosuppression and wounding had little effect on the levels of proliferation of *S. aureus* ($P > 0.2$). Without occlusion, however, *S. aureus* proliferated to significantly lower levels ($P < 0.005$). This model may be be useful for screening topical anti-infective agents or studying the mechanisms of bacterial pathogenesis and host response.

Cutaneous infections are a serious problem in otherwise healthy and immunocompromised patients. Incidence data, however, are difficult to obtain. It is clear that *Staphylococcus aureus* is the major pathogen causing superficial pustular pyodermas (2). *Streptococcus pyogenes* is a second significant pathogen causing ulcerative pyodermas (2). *Pseudomonas aeruginosa*, a third pathogen, rarely causes pyoderma in healthy people; it is isolated most commonly from patients with malignancies, burn injuries, and other immunocompromising conditions (12).

Assessing the efficacy of topical antimicrobial agents is a difficult task. Clinical trial data are difficult to collect and interpret owing to the large difference in wound or lesion sites, inoculum size, severity of infection, and environmental factors. Several human inoculation models are available (10–13) but are costly and not without risk. Because of these limitations, an animal model is desirable for preclinical assessment of compounds with proven in vitro activity.

Capabilities for the in vivo assessment of anti-infective agents in animals were limited to evaluations with the mouse surgical wound and rat burn models (14, 15, 17). Both are models of severe trauma. These models are adequate for assessing the efficacy of anti-infective agents in the treatment of skin which has been grossly damaged. It is not clear that infection occurs in these models. The animals may simply function as incubators for the challenge organism. Proliferation of bacteria, followed by colonization of the trauma site, probably occurs in the absence of infectious lesions or abscesses. A review of the literature showed that the capability to evaluate treatments of true superficial infections was lacking.

This report describes a simple, reliable animal model for the study of superficial cutaneous bacterial infections resulting after a minor wounding process. The model can serve as a preclinical screening tool for anti-infective agents. Additionally, the model may be useful in studying the mechanisms of bacterial pathogenesis and host response in infections caused by *Staphylococcus* species.

MATERIALS AND METHODS

Animals. CF-1 male mice (24 to 28 g), obtained from Charles River Breeding Laboratories, Inc. (Wilmington, Mass.), were housed in groups of 5 to 10 in stainless-steel wire-bottom cages before use. The mice were given Purina Lab Chow (antibiotic-free) and water ad libitum throughout the experiments. Room temperature (21 to 24°C), relative humidity (25 to 50%), and lighting (12 h/day) were carefully controlled.

Bacterial strains. Nine strains of members of the family *Micrococcaceae* were studied extensively (Table 1). The cultures initially were grown on 5% sheep blood agar and harvested in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) with 15% glycerol. Portions (1 ml) of this suspension were frozen and maintained at −50°C until used.

Preparations of bacterial inoculum. Forty-eight hours before animal inoculation, a sample of the frozen bacterial suspension was transferred to a 5% sheep blood agar slant. After overnight incubation at 35°C, the bacteria were transferred to 50 ml of nutrient broth (BBL) supplemented with 0.2% NaCl and 1.0% yeast extract (BBL) in a 300-ml Erlenmeyer flask. The flask was placed in a shaking water bath set at 150 rpm and 37°C. After 6.5 h of incubation, 0.5 ml of the culture was transferred to 50 ml of fresh broth medium in a second 300-ml flask. The flask was placed in the

* Corresponding author.

shaker bath and incubated for 16 h. A 10-ml portion of the culture from the second flask was removed and placed into 90 ml of broth medium in a third 300-ml flask for incubation in the shaker bath for 1.25 h. The optical density was measured at 540 nm (an optical density of 1.4 to 1.6 was normal). A sample (45 ml) of the broth culture was harvested by centrifugation (12,000 × *g* for 10 min) at 4°C and suspended in 10 ml of 0.1% peptone water. The suspended cells were centrifuged again, and the pellet was resuspended in 10 to 12 ml of 0.1% peptone water. Serial 10-fold dilutions were performed as needed for animal inoculations. Plate counts were performed, in duplicate, to confirm the purity and accuracy of the inoculum.

Subcutaneous infection. A standard assay to determine virulence potential was performed as described previously (1, 6, 16). The dorsal area of the mouse was shaved with electric small animal clippers. Nair, a depilatory agent (Carter-Wallace, Inc., New York, N.Y.), was applied to the shaved area according to the manufacturer's instructions to remove remaining hair. The following day 0.1 ml of the bacterial suspension was injected subcutaneously into the shaved area. No occlusive dressing was applied. Mice were observed 48 h later for the presence of large necropurulent abscesses.

Immunosuppression. Four days before challenge with *S. aureus* mice were injected intraperitoneally with cyclophosphamide (CY; Sigma Chemical Co., St. Louis, Mo.). CY, administered as a single dose ranging from 0 to 150 mg/kg was reconstituted and diluted with pyrogen-free saline and glassware. Immunosuppression was used only in the wounded occluded mouse model (see below); CY was not used in the subcutaneous challenge model. Induction of neutropenia was verified from complete blood counts and differential blood smears. Complete blood count results were obtained with a model FN Coulter Counter. Differential leukocyte analyses were determined from May-Grünwald Giemsa-stained preparations.

Superficial wounding technique. One day before infection, mice were anesthetized with 75 mg of pentobarbital (Abbott Laboratories, North Chicago, Ill.) per kg. Mice were shaved and treated with a depilatory as described above. The following day mice were again anesthetized with 75 mg of pentobarbital per kg before superficial wounding. One drop (0.02 ml) of the prepared culture suspension was applied to the skin, which was scraped with a no. 24 scalpel blade until a reddened area appeared (just short of drawing blood). The wound site (approximately 10 mm^2) was occluded with a 15-mm^2 piece of sterile plastic film (Saranwrap; Dow Chemical Co., Indianapolis, Ind.) secured with a 25-mm-wide girdle of surgical tape (Micropore; Medical Products Division of 3M, St. Paul, Minn.). Mice were housed individually, in wire-bottom cages, to limit removal of the occlusive dressing. At specified intervals after challenge, occlusive dressings were removed, and wound sites were examined for white-gray abscesses. Only those mice with intact dressings at the time of examination were included in the analysis.

Semiquantitative bacterial analysis of wound site. A modification of the technique of Leyden et al. (10) was used to determine the total bacterial count at the wound surface. The occlusive dressings were removed, and the wound site was sampled with a sterile cotton swab premoistened in 0.1% peptone water–0.1% Tween 80. The swab was rinsed in 1 ml of the peptone water-Tween 80 solution. The site was sampled with the same swab and rinsed in the same tube a second time. Appropriate dilutions were made in 0.1% peptone water and plated in duplicate on brain heart infusion agar (BBL). Plates were incubated overnight at 37°C. Bacterial counts were expressed as CFU per site.

Histological processing of tissue samples. A 20-cm section of dorsal tissue was carefully excised from mice after sacrifice under CO_2. The section was fixed in 10% buffered Formalin and embedded in paraffin. Paraffin was then removed from cross sections (4 to 6 μm), and they were stained with hematoxylin and eosin. Stained preparations were examined for the presence of inflammation and invading bacteria.

Statistical analysis. Student's *t* test and analysis of variance were performed with the Minitab Release 82.1 program. Chi square and Fisher exact tests were performed on the chi square program. All data were entered into and analyzed by an HP3000 system.

RESULTS

Subcutaneous abscess development. Nine strains of members of the *Micrococcaceae* were selected for mouse skin virulence assays. In preliminary experiments, the subcutaneous route of inoculation was chosen because it was a fast, reliable method to obtain distinct skin abscesses. This assay was used to screen for virulence potential. No further characterization of the virulence properties was undertaken. Mice were examined for large, white-gray abscesses; examinations were made 48 h after challenge (Table 1). The number of CFU required to induce abscesses in 50% of the mice was determined (ID_{50}).

All four *S. aureus* strains tested had ID_{50} values below 10^8 CFU. The other strains tested had higher ID_{50} values; no abscesses developed in any of the mice at the levels reported. These results suggest that all four *S. aureus* strains were potentially virulent, while the remaining microorganisms were avirulent.

TABLE 1. Characterization of members of the family *Micrococcaceae*

Culture and strain no.	Source	ID_{50}[a]
Staphylococcus aureus		
270	SI97 from J. Leyden	6.0×10^7
271	NCTC 9789 (PS80 from W. Noble)	1.3×10^6
272	ATCC 10832 (FDA Wood strain)	5.6×10^7
274	Clinical isolate from a wound	1.5×10^7
Staphylococcus epidermidis		
249	Skin isolate from S. Haborman	—[b]
254	ATCC 17917 (Lever Brothers strain JS)	—
275	ATCC 12228 (FDA strain PCI1200)	—
Staphylococcus saprophyticus 276	Urinary tract infection isolate from T. J. Marrie	—
Micrococcus luteus 264	ATCC 9341 (FDA strain PCI1001)	—

[a] CFU required to cause subcutaneous abscess in 50% of mice within 48 h of challenge (0.1 ml administered subcutaneously).

[b] —, No abscesses formed in the mice challenged with these strains at $\geq 10^8$ CFU.

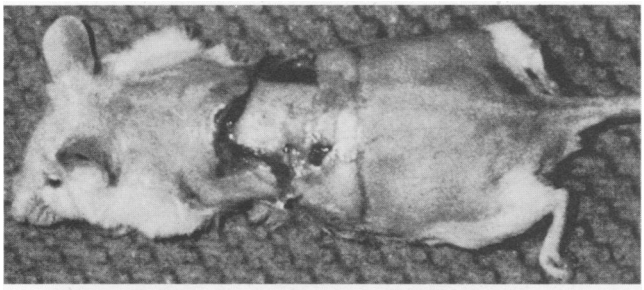

FIG. 1. Photograph of a mouse 6 days after challenge with Mi271, with a large abscess.

TABLE 2. Effect of CY on peripheral blood cell counts

Days posttreatment with CY[a]	CBC[b] after CY treatment of:							
	0 mg/kg				50 mg/kg			
	Total RBC	Total WBC	% PMN	% Lympho	Total RBC	Total WBC	% PMN	% Lympho
1	9.9	6.7	32	64	9.9	6.7	25	73
2	9.9	6.7	27	73	9.9	6.6	24	74
3	9.8	6.7	22	73	9.8	6.4	21	78
4	9.9	6.7	21	77	9.9	6.5	10*	89*
5	NT	6.8	22	77	NT	6.8	6*	92*
6	NT	6.8	25	76	NT	6.8	18	80
7	9.9	6.7	24	74	9.9	6.7	27	72

[a] CY administered intraperitoneally; animals were not challenged or wounded.

[b] Total erythrocyte (RBC) and leukocyte (WBC) counts are reported as the mean $\log_{10}$ where $n = 4$ to 16. Differential leukocyte count is reported as percentage of polymorphonuclear cells (PMN) and lymphocytes (Lympho). Monocytes and eosinophils were each ≤1%. Standard error of total counts was ≤0.01; standard error of differentials was 1 to 4%. * indicates that $P < 0.005$ when normal and CY-treated mice are compared; NT, not tested.

Definition of wound model. Abscess formation (Fig. 1) was studied with *S. aureus* Mi271 (also denoted NCTC 9789) over a range of CY doses followed by challenge with several bacterial inoculum levels (Fig. 2). In general, high doses of CY and high levels of bacterial inoculum yielded a higher percentage of and larger abscesses. Abscesses were obtained in 89% (96 of 108) of mice with CY levels as low as 50 mg/kg and an inoculum level of 10^5 CFU per animal. This combination of CY dose and bacterial inoculum was chosen for further studies. The challenge strain was isolated from the abscess site and determined by a battery of tests including biochemical properties, antimicrobial agent susceptibility patterns, and phage type. Neutropenia was most apparent in CY-treated mice (50 mg/kg) on the day of challenge (Table 2). Within 3 days of challenge, the neutrophils returned to normal levels. Fatality rates were determined 6 days after wounding. The fatality rate of normal (not immunocompromised) mice was 11% (10 of 90) when challenged with Mi271 or when wounded without challenge. Fatality rates of immunocompromised mice (50 mg of CY per kg) were identical; 11% of animals challenged with Mi271 (10 of 91) or wounded without challenge (9 of 83) did not survive. Histological samples revealed severe dermatitis, cellulitis, and ulceration, associated with bacteria, penetrating the subcutis (Fig. 3). Few abscesses developed in uninfected mice with CY doses of ≤50 mg/kg. At higher CY doses, there was a high rate of abscess formation resulting from environmental bacteria.

Development of abscesses. Mice receiving 0 to 150 mg of CY per kg and an inoculum of Mi271 (10^5 CFU per mouse) were observed for 24 to 72 h after challenge to determine when abscesses developed (Fig. 4). Mice receiving 150 mg of CY per kg developed abscesses within 24 h. Mice receiving 50 to 100 mg of CY per kg began to develop abscesses after 24 h; abscesses continued to develop up to 72 h after challenge. Only a few nonimmunocompromised mice developed abscesses within 72 h.

Persistence of abscesses. Three groups of mice (treated 4 days before with 50 mg of CY per kg) were observed at four time intervals for 20 days after challenge (Fig. 5). Mice challenged with *S. aureus* Mi271 developed abscesses which persisted for at least 20 days. After this time, the fur began to grow back, which allowed the wound site to dehydrate

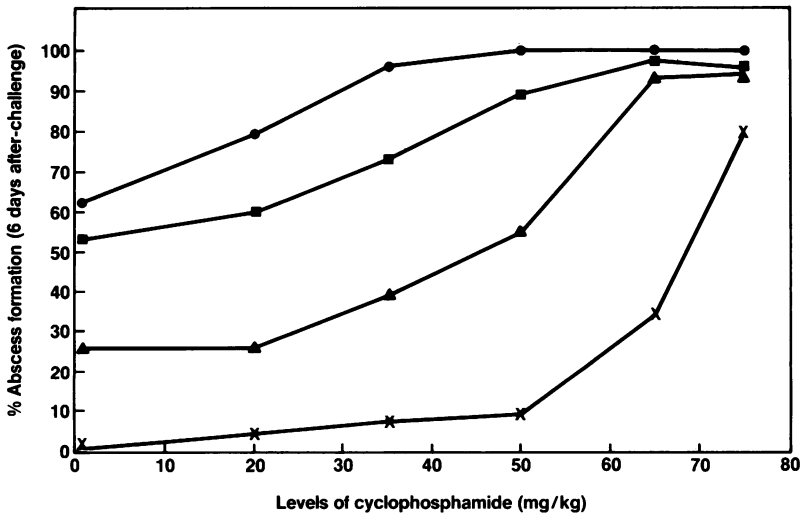

FIG. 2. Abscess formation in uninfected mice (×) and mice challenged with 10^4 (▲), 10^5 (■), and 10^6 (●) CFU of virulent *S. aureus*, Mi271. Mice were treated with 0, 20, 35, 50, 65, or 75 mg of CY per kg 4 days before bacterial challenge, wounding, and occlusion. Animals were observed 6 days after challenge.

FIG. 3. Hematoxylin-and-eosin-stained sections of infected mouse epidermis demonstrating dermatitis, ulceration, and cellulitis associated with invading bacteria (×50).

and facilitated healing of the abscess. Uninfected mice and mice challenged with *Staphylococcus epidermidis* Mi275 did not develop a significant number of abscesses over this time. Bacteremia occurred in up to 32% of mice (n = 20) challenged with Mi271. Fatality rates were determined 21 days after wounding. The fatality rate of infected mice (14 of 58) was equal to that of uninfected mice (13 of 53). This higher fatality rate compared with the rates at 6 days may be due to

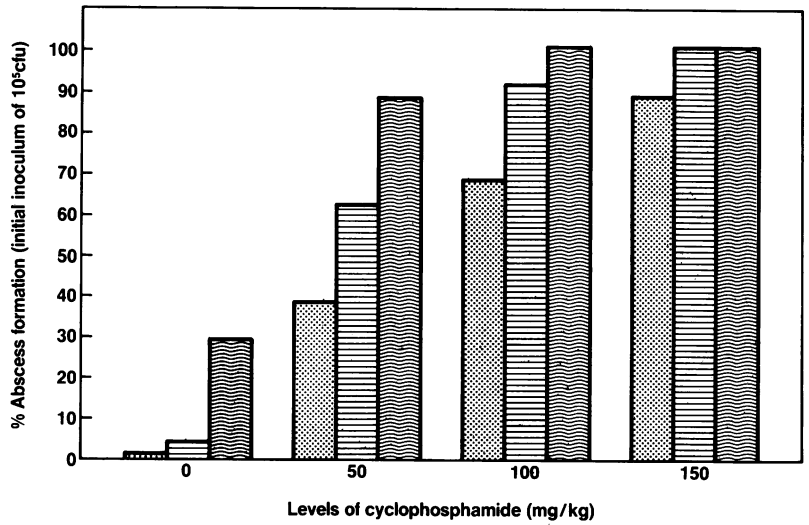

FIG. 4. Development of abscesses in mice inoculated with 10^5 CFU of virulent *S. aureus* Mi271. Mice were treated with 0, 50, 100, or 150 mg of CY per kg 4 days before bacterial challenge, wounding, and occlusion. Animals were observed 24 (dotted bars), 48 (bars with straight lines), and 72 (bars with curved lines) h after challenge.

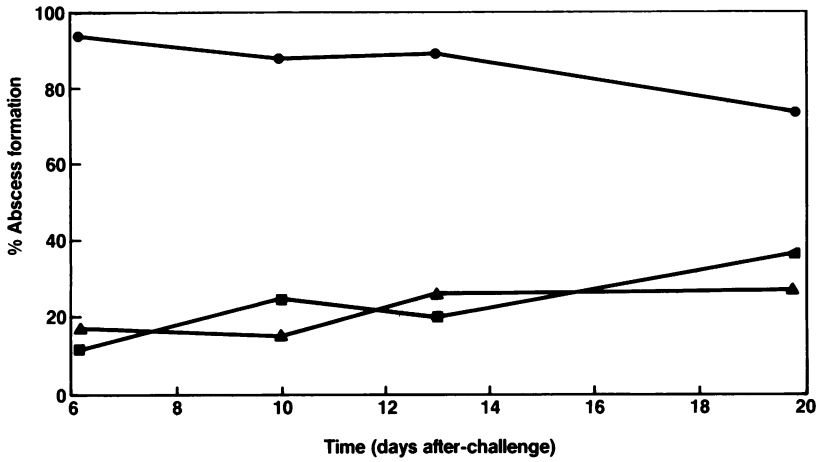

FIG. 5. Persistence of abscesses in uninoculated mice (■) and mice inoculated with 10^5 CFU of virulent *S. aureus* Mi271 (●) or 10^5 CFU of *S. epidermidis* Mi275 (▲). Mice were treated with 50 mg of CY per kg 4 days before bacterial challenge, wounding, and occlusion. Animals were observed 6, 10, 13, and 20 days after challenge.

the extended period under occlusive dressing. These results indicate that the infection established in animals challenged with virulent *Staphylococcus* species was self-limiting.

Specificity of wound model. Nine strains of members of the *Micrococcaceae* were studied for the ability to cause abscesses in normal and immunocompromised (50 mg of CY per kg) mice challenged with 10^5 CFU (Table 3). Only *S. aureus* induced abscesses. Two strains of *S. aureus* (Mi270 and Mi272) failed to cause abscesses in immunocompromised mice. Only Mi271 caused abscesses in normal (nonimmunocompromised) mice. Avirulent members of the *Micrococcaceae* did not cause abscesses in normal or immunocompromised mice. Such findings suggest that the model is specific for virulent *Staphylococcus* species.

Proliferation in the wound model. Two strains were studied for the ability to proliferate on the skin surface of the mouse. At nine time intervals (0 to 48 h postinoculation) mice were sacrificed by cervical dislocation, and the wound area was sampled for total bacterial counts. *S. aureus* Mi271 proliferated rapidly between 4 and 8 h postinoculation and reached a constant density by 24 to 48 h postchallenge (Fig. 6). *S. epidermidis* Mi275 proliferated to the same levels ($P > 0.05$) but after a longer lag phase (Fig. 7). In paired experiments bacteria proliferated in the absence of wounding and occlusion, but abscesses failed to develop (Table 4). Occlusive dressing did enhance bacterial proliferation ($P < 0.005$). Immunosuppression had no effect on the levels of proliferation ($P > 0.2$) but did affect abscess formation in mice challenged with Mi271.

TABLE 3. Abscess formation by members of the *Micrococcaceae*

Culture[a] and strain no.	Normal (%)[b]	Immunocompromised (%)[c]
S. aureus		
270	0/22 (0)	2/24 (8)
271	26/69 (38)	96/108 (89)
272	3/23 (13)	9/37 (24)
274	3/34 (9)	33/35 (94)
S. epidermidis		
249	2/22 (9)	0/25 (0)
254	0/23 (0)	1/27 (4)
275	2/36 (6)	9/62 (14)
M. luteus 264	1/19 (5)	5/21 (24)
S. saprophyticus 276	0/24 (0)	0/29 (0)
None	1/30 (3)	7/75 (9)

[a] Mice were challenged with 10^5 CFU during a wounding process; all mice were wrapped with an occlusive dressing and later observed for the presence of abscesses.

[b] Number of mice with abscesses/total number challenged; no prior treatment of mice with CY.

[c] Number of mice with abscesses/total number challenged; mice were treated with 50 mg of CY per kg 4 days before challenge.

DISCUSSION

Several skin-wounding and inoculation techniques have been described previously (3, 4, 7, 9–15, 17). In general, the models derived from these techniques use proliferation of the challenge organism as an indicator of the infectious process. Some models, including the mouse surgical wound and rat burn models (14, 15, 17), involve severe trauma to the skin. Our objective was to develop a suitable animal model that by visual inspection indicates an infection and requires only slight disruption of the skin.

Since the skin possesses major defenses against infection, including inherent dryness, the presence of fatty acids, and the immune system, we speculated that one or more of these parameters would have to be altered to establish an infection. Our approach was to alter the immune status of the host before infection.

CY induces leukopenia in mice (5, 8). The level of circulating leukocytes is depressed within 4 days after treatment. The most significant effect is the reduction of circulating neutrophils; the animals suffer total neutropenia. After day 5, the leukocyte count and profile gradually return to normal (5, 8). The data provided here support the concept that the immune system plays a significant role in preventing skin infections. We demonstrated that immunosuppression by CY is necessary to cause skin abscesses consistently. Neutropenia during the challenge phase presumably allows the virulent *Staphylococcus* strain to colonize the epidermal layers without clearance by phagocytes. Once established, the animal must be unable to clear the infection rapidly despite the return of circulating neutrophils. CY alone is not toxic to the animals, nor does it result in spontaneous

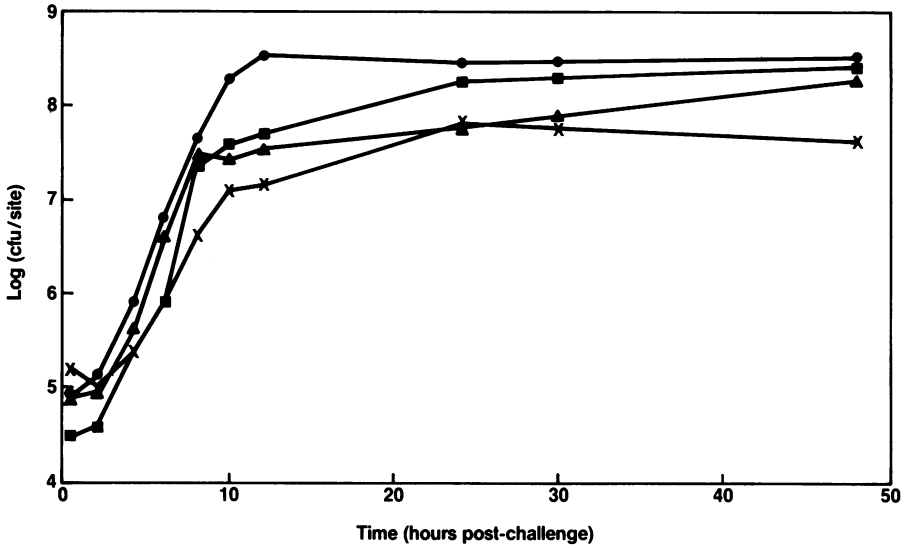

FIG. 6. Proliferation of *S. aureus* Mi271 observed 0, 2, 4, 6, 8, 10, 12, 24, and 48 h after challenge. Four groups were studied: (i) nonimmunocompromised mice which were wounded and occluded (▲); (ii) immunocompromised mice (50 mg of CY per kg) which were wounded and occluded (●); (iii) immunocompromised mice which were occluded without wounding (■); and (iv) immunocompromised mice which were wounded without occluding (×).

granuloma formation during the rebound phase. Immunosuppression does not affect abscess formation by avirulent members of the *Micrococcaceae*; therefore, we conclude that the model is specific for virulent *Staphylococcus* species.

Three additional factors are essential to the induction of abscesses. In the absence of wounding, no abscesses develop. It is also necessary to occlude the wound site; when allowed to dry the wound heals rapidly without abscess formation. The final element is inoculation with virulent *Staphylococcus* species. Uninfected mice or mice challenged with nonpathogenic members of the *Micrococcaceae* fail to develop abscesses. Avirulent *Micrococcaceae* proliferate readily on the surface of the mouse. Therefore, proliferation alone is not an adequate marker of infection.

In summary, mice which are immunocompromised with a single low dose of CY consistently develop cutaneous abscesses upon challenge with virulent *S. aureus* in the mouse scarification model. True infection (proliferation of bacteria and tissue invasion) occurs. This model may be useful as a preclinical screening tool. In addition, the model may be useful in the study of host-parasite relationships.

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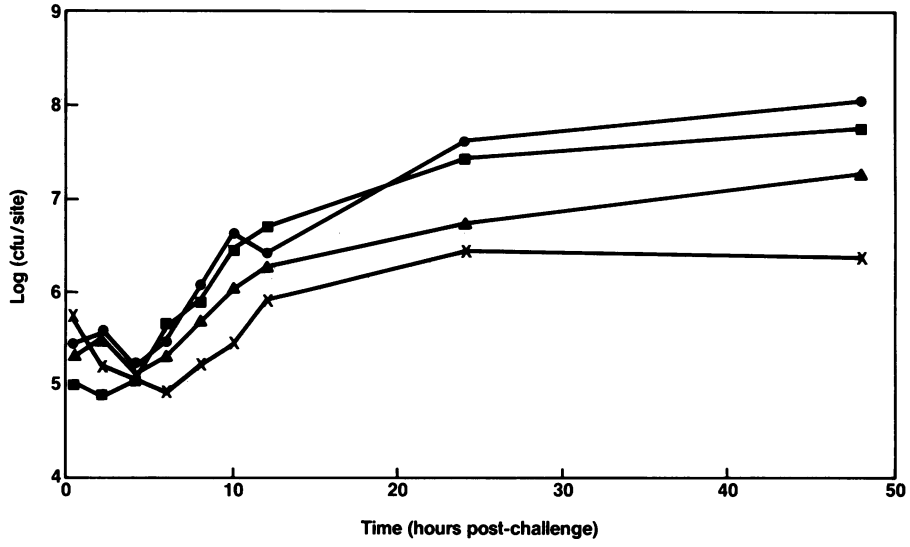

FIG. 7. Proliferation of *S. epidermidis* Mi275 observed 0, 2, 4, 6, 8, 10, 12, 24, and 48 h after challenge. Four groups were studied: (i) nonimmunocompromised mice which were wounded and occluded (▲); (ii) immunocompromised mice (50 mg of CY per kg) which were wounded and occluded (●); (iii) immunocompromised mice which were occluded without wounding (■); and (iv) immunocompromised mice which were wounded without occluding (×).

TABLE 4. Effect of wounding and wound occlusion on abscess development

Inoculum[a] and CY dose (mg/kg)[b]	Wounded[c]	Occluded[d]	Abscess (%)[e]
S. aureus			
None	+	+	20/68 (29)
50	+	+	94/105 (90)
50	+	−	0/89 (0)
50	−	+	1/62 (2)
S. epidermidis			
None	+	+	1/28 (4)
50	+	+	10/53 (19)
50	+	−	0/29 (0)
50	−	+	0/38 (0)

[a] Mice were inoculated with 10^5 CFU of *S. aureus* Mi271 or *S. epidermidis* Mi275.
[b] CY was administered 4 days before challenge with Mi271 or Mi275.
[c] Mice were wounded during inoculation (+) or inoculated without wounding (−).
[d] Mice were occluded after inoculation (+) or left without an occlusive dressing (−).
[e] Number of mice with abscesses/total number challenged; observed 6 days after challenge.

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