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Subcutaneous Multiplication of Exfoliatin-Producing Staphylococci

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After subcutaneous inoculation of approximately 10^6 cocci, changes in staphylococcal populations were followed by the enumeration of organisms in excised tissues. In contrast to conventional *Staphylococcus aureus* strains, exfoliatin-producing strains were able to multiply in the subcutaneous tissues of neonatal and adult mice. Although strains capable of producing large quantities of exfoliatin were better able to proliferate than strains producing lesser amounts of toxin, it was not determined whether exfoliatin was directly responsible for the observed multiplication. Two variants exhibiting a partial loss in exfoliatin production showed minor changes in proliferative capability. A third strain, after being cured of its exfoliatin plasmid, manifested a marked reduction in exfoliatin production and was unable to multiply subcutaneously. With some strains multiplication proceeded for several hours but was then followed by a decline in the number of viable organisms. Histological sections of subcutaneous lesions revealed a rapid influx of neutrophils, but leukocytes accumulated in the region regardless of whether the organisms multiplied or were eliminated.

During our initial studies with *Staphylococcus aureus* exfoliatin, it was noticed that certain strains of staphylococci, when inoculated subcutaneously into neonatal mice, could cause exfoliation after about 24 h but when grown in culture did not elaborate detectable amounts of toxin into the supernatant fluid (4). One explanation that could be offered for this observation is that these strains simply produced very little exfoliatin but upon inoculation could multiply and eventually accumulate sufficient toxin to elicit a visible response. Since skin loosening could be demonstrated with as little as 0.15 μg of exfoliatin, it did not seem unreasonable for a proliferating population of staphylococci to elaborate this quantity of toxin during a 24-h period. What caused the explanation to be viewed with scepticism was that prior studies had shown that staphylococci, in the absence of a foreign body, did not multiply in the subcutaneous tissue of adult mice (1, 8). Therefore, for the explanation to be tenable it appeared necessary to demonstrate either that neonatal mice differed from adults or that exfoliatin-producing strains differed from conventional strains. Investigation of these questions indicated that neonates did not behave differently towards staphylocci than did adults, but that exfoliatin-producing strains seemed unique in being able to multiply subcutaneously.

MATERIALS AND METHODS

Staphylococcal strains. *S. aureus* strains EV, TG, JF, and JH were originally obtained from M. E. Melish and L. A. Glasgow (6). These strains were selected because they differed significantly in their capacity to produce exfoliatin (4). Recently some of these strains have been redesignated (7). *S. aureus* strains UT-0003 (formerly TA), UT-0003-4, and UT-0002-19 were furnished through the courtesy of M. Rogolsky. Strain UT-0003-4 is a substrain of UT-0003, and UT-0002-19 is a substrain of EV (M. Rogolsky, personal communication). Both substrains exhibited a partial loss in exfoliatin production when compared to the parent strains.

S. aureus strain TG-Sp^R, derived from strain TG, also exhibited reduced exfoliatin production. This strain was isolated from the parent as a spectinomycin-resistant mutant, but the association between antibiotic resistance and decreased exfoliatin production proved fortuitous, since other spectinomycin-resistant mutants were not deficient in toxin production.

S. aureus 18Z, an exfoliatin-negative strain, has been described previously (3, 8).

Inoculation and enumeration of organisms. Staphylococci were grown overnight in Trypticase soy broth with constant aeration. The cultures were centrifuged, and the sedimented cocci were washed and suspended in the same medium and stored at −70 C until needed. Aliquots of frozen suspensions were enumerated by plate counts prior to use.

Groups of neonatal Swiss mice, less than 72 h old, were inoculated subcutaneously with 0.02 ml of sus-

pension containing approximately 10^6 cocci. In some cases larger or smaller doses were used. The site of inoculation was standardized on the right flank, and the overlying skin was marked with dye. Any animals exhibiting leakage of fluid from the inoculation site were discarded. At zero time and various intervals thereafter, four to eight neonates were sacrificed with chloroform. The carcasses were immersed in ethanol and blotted dry with sterile gauze pads, and the entire right flank (skin, subcutaneous tissue, and muscle) was aseptically excised. The tissues were homogenized and the staphylococci were counted as previously described (8).

Neonatal mice inoculated with staphylococcal strains capable of causing exfoliation in the doses used were separated from their mothers to avoid molestation. By providing supplemental heat with a conventional light source, survival could be assured for at least 48 h unless terminated sooner by the elaboration of bacterial toxins.

In experiments studying the behavior of staphylococci in adult mice, the same procedure was followed except that animals between 20 to 30 g in weight were used.

Histological sections. Two small spots, approximately 4 mm apart, were marked on the flanks of neonatal mice. Organisms were inoculated subcutaneously in a 0.02-ml volume, taking care that the tip of the needle was located midway between the overlying spots. At zero time and various intervals thereafter, animals were sacrificed, the inoculation site was excised, and the tissues were placed in neutral buffered formalin. After fixation, and by using the spots as guides, tissues were trimmed in such a manner that subsequently cut sections would be directed through the center of the inoculation site. Several nonserial, 5-μm-thick sections, stained with hematoxylin-eosin, were prepared from each tissue sample.

RESULTS

Inoculation of *S. aureus* 18Z into neonatal mice revealed that this strain did not fare any better in neonates than it did in adults (Fig. 1). Histological sections obtained at zero time from similarly inoculated neonates readily permitted visualization of cocci. The organisms were rather uniformly distributed throughout the extracellular matrix of the region and did not appear aggregated. An influx of neutrophils was evident within 4 h and increased in intensity over the next 24 to 48 h. During this time cocci rapidly became more difficult to locate in the lesion, but it could not be determined whether this was the result of phagocytosis or simply crowding of the region with leukocytes. Undiminished accumulations of neutrophils were still present after 96 h, the longest interval examined. In some animals the size and intensity of the response was such that the lesions were readily visible through the intact skin.

Inoculation of neonates with *S. aureus* EV, a "good" exfoliatin producer (4), demonstrated that this strain could multiply subcutaneously after a short lag (Fig. 2). However, the period of observation was limited, since infection resulted in generalized exfoliation and death of the hosts.

Strain EV could also multiply in adult mice. Even with smaller doses (approximately 10^5 cocci) a period of multiplication was noted, but after several hours proliferation was checked (Fig. 3). Larger doses led to a more prolonged period of multiplication and in some animals, after a week, resulted in subcutaneous abscesses containing several milliliters of pus.

To avoid early death from the action of exfoliatin, *S. aureus* JF was selected for further study. This strain was characterized as a "moderate" exfoliatin producer (4) and, although capable of initiating generalized exfoliation and death, in smaller doses led to a more limited degree of exfoliation with survival of a significant percentage of infected neonates. Strain JF was able to multiply in the subcutaneous tissues of neonates, but after the initial period of proliferation the staphylococcal population declined (Fig. 4).

Infection with 10^6 cocci of strain JF did cause loosening of the epidermis overlying the inoculation site (manifested by a positive Nikolsky sign), but in most animals spontaneous tearing or removal of the loosened skin did not occur. The possibility was entertained that the ob-

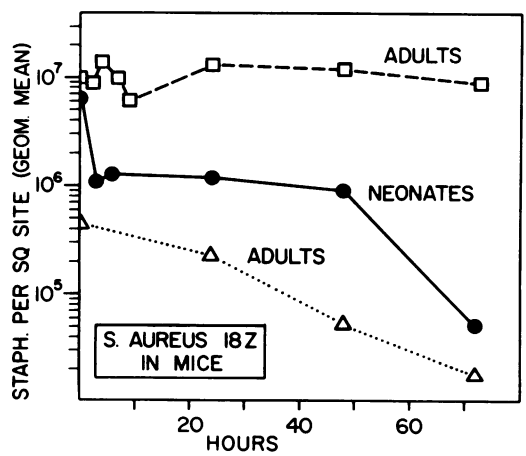

FIG. 1. *Survival of S. aureus 18Z in the subcutaneous tissues (SQ) of neonatal and adult mice. Each point represents the geometric mean derived from eight animals. Results of three experiments are shown: adults infected with either 5 × 10^5 cocci or 10^7 cocci and neonates infected with 10^7 cocci. There is no evidence for multiplication of the organism.*

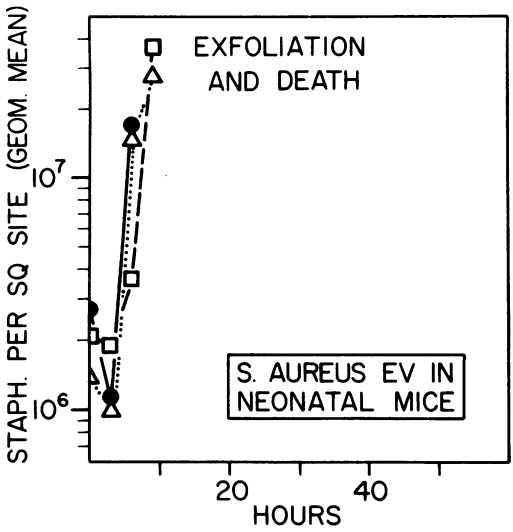

FIG. 2. *Multiplication of S. aureus EV in the subcutaneous tissues (SQ) of neonatal mice. Each point represents the geometric mean derived from eight animals. The figure depicts data from three experiments. Due to death of neonates from exfoliation, observations could not be extended beyond 9 h. The maximum staphylococcal population is significantly greater than the zero-time population in all three experiments.* □, △: $P = <0.001$; ○: $P = 0.008$.

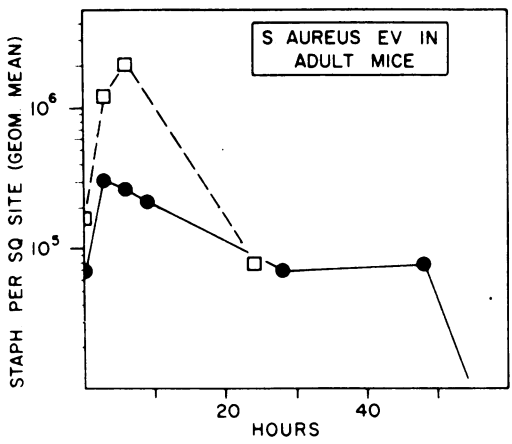

FIG. 3. *Multiplication of S. aureus EV in the subcutaneous tissues (SQ) of adult mice. Each point represents the geometric mean derived from four animals. Two experiments are depicted. The maximum staphylococcal population is significantly greater than the zero-time population in both experiments; P = 0.05 in both cases.*

served increase in the staphylococcal population might be the consequence of multiplication in fluid accumulating within the cleavage plane rather than multiplication in the subcutaneous tissue. This possibility was evaluated

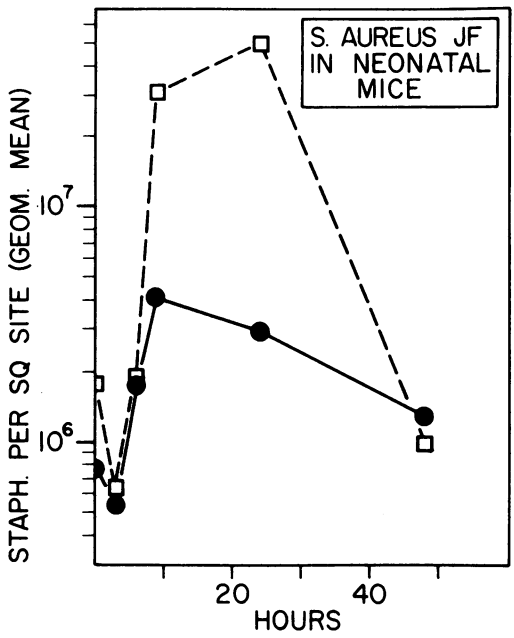

FIG. 4. *Multiplication of S. aureus JF in subcutaneous tissues (SQ) of neonatal mice. Each point represents the geometric mean derived from eight animals. The results of two experiments are depicted. The maximum staphylococcal population is significantly greater than the zero-time population in both experiments.* □: $P = <0.001$; ●: $P = 0.05$.

by culturing separately fluid recovered from the developing bullae. A small quantity of sterile saline (approximately 0.05 ml) was repeatedly injected into, and aspirated from, the cleavage plane and, after final withdrawal, plated to enumerate any organisms present. Invariably this material was sterile.

When they develop their hair coat, usually after 5 to 6 days of age, neonatal mice become markedly resistant to the skin-loosening effects of exfoliatin (4, 6). In spite of an inability to cause intraepidermal cleavage, strain JF did multiply in the subcutaneous tissues of 7- to 9-day-old mice during the first 24 h after infection (Fig. 5). As with neonates, the initial period of multiplication was followed by a decline in the bacterial population.

S. aureus strain JH was selected to represent a "poor" exfoliatin producer (4). This strain, in doses of approximately 10^6 cocci, did not proliferate subcutaneously but persisted for varying periods before being eliminated (Fig. 6). However, larger doses (10^7 cocci) did exhibit significant multiplication in neonates (Fig. 7).

Because our data suggested an association between exfoliatin production and ability to multiply subcutaneously, consideration was

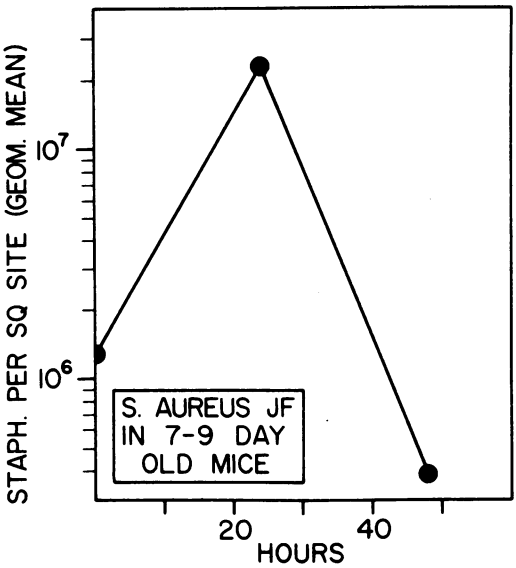

FIG. 5. *Multiplication of S. aureus strain JF in the subcutaneous tissues (SQ) of 7- to 9-day-old mice. Each point represents the geometric mean derived from four animals. The maximum staphylococcal population is significantly greater than the zero-time population; P = <0.005.*

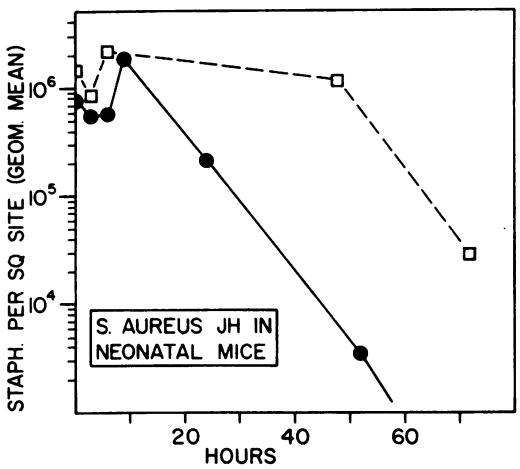

FIG. 6. *Persistence of S. aureus strain JH in the subcutaneous tissues (SQ) of neonatal mice. Each point represents the geometric mean derived from eight animals. Results of two experiments are shown. There is no significant increase in the staphylococcal population in either experiment.*

given to the possibility of comparing the in vivo behavior of exfoliatin-negative mutants with that of parent strains. Unfortunately, such mutant pairs were not available and an effective screening method for the isolation of nontoxigenic variants did not seem practical. In lieu of these options it was decided to examine some plasmid-cured strains, which exhibited a partial loss in exfoliatin production as recently reported by Rogolsky et al. (7).

Strain UT-0002-19, a plasmid-cured substrain of EV, produced approximately 1/10 as much exfoliatin as the parent strain; yet, when inoculated subcutaneously into neonates, UT-0002-19 multiplied as well as EV. The only noticeable difference among these organisms was that about one-half the animals inoculated with the plasmid-cured strain survived for 24 h. At this time the staphylococcal population in subcutaneous sites was 2×10^8 to 3×10^8 cocci.

S. aureus strain TG, like EV, was classified as a "good" exfoliatin producer (4) and, like EV, multiplied in the subcutaneous tissues of neonates (Fig. 8). The TG-Sp^R mutant, producing about 1/10 as much exfoliatin as TG, also multiplied but only after a 3-h lag period.

S. aureus strain UT-0003, a "moderate" exfoliatin producer, multiplied in neonates after a short lag (Fig. 9). The plasmid-cured substrain, UT-0003-4, produced no detectable exfoliatin and did not multiply subcutaneously, but was rapidly eliminated from the tissues.

Histological sections prepared from foci initiated with exfoliatin-producing strains exhibited features similar to those seen with strain 18Z, with the exception that a cleavage plane through the granular layer or frank exfoliation was frequently evident in advanced lesions. Inflammatory cells rapidly accumulated after infection, but the number and persistence of neutrophils in these sites had no

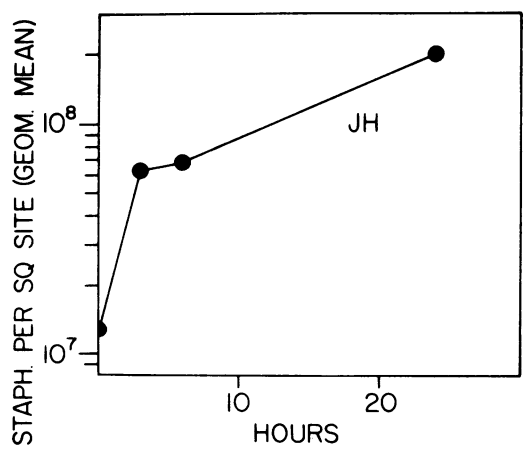

FIG. 7. *Multiplication of S. aureus JH in the subcutaneous tissues (SQ) of neonatal mice infected with 10^7 cocci. Each point represents the geometric mean derived from four animals. The maximum staphylococcal population is significantly greater than the zero-time population; P = <0.001.*

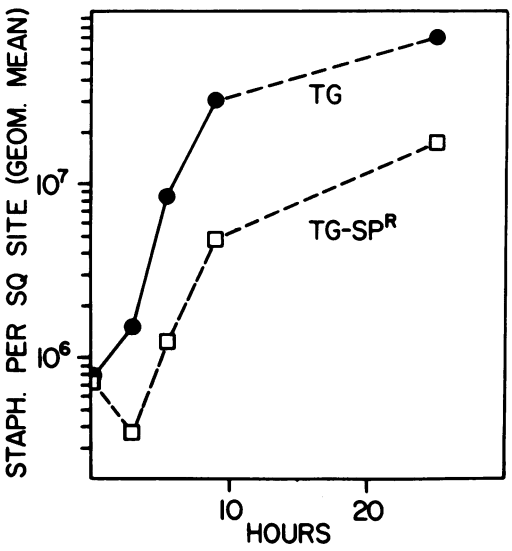

FIG. 8. *Multiplication of S. aureus strains TG and TG-Sp^R in the subcutaneous tissues (SQ) of neonatal mice. Each point represents the geometric mean derived from eight animals except for the 24-h sample with TG, which is based upon four survivors. The maximum staphylococcal population is significantly greater than the zero-time population with both strains. TG: P = <0.001; TG-Sp^R: P = <0.001.*

obvious relationship to the number of viable staphylococci present in the tissues.

DISCUSSION

One of the more difficult challenges confronting anyone attempting to discuss staphylococcal host-parasite interactions is to explain how the organism manages to initiate a lesion in the host. Man and other species appear rather resistant to *S. aureus*, and in experimental models large inocula are frequently used to establish a focus of infection (2). Since it seems unreasonable to expect that this will occur during the course of spontaneous infections, it appears necessary to explain how a relatively small number of cocci can become established, proliferate, and counteract the defenses of the host.

Certainly the most common human staphylococcal infection is the furuncle; yet the early events leading to this type of infection are poorly understood, primarily because a suitable experimental model does not exist. Ordinarily, strains isolated from such lesions are reluctant to multiply when inoculated into the same tissues of a fresh host; therefore, the isolation of strains capable of multiplying subcutaneously would be useful for experimental purposes.

Our studies demonstrated that certain exfoliatin-producing staphylococci differed significantly from other strains previously examined (1, 8) in being able to multiply in the subcutaneous tissue of mice in the absence of foreign materials. Although the ability to multiply subcutaneously was associated with exfoliatin production, it cannot be ascertained from the available data whether toxin production is directly responsible for this behavior. If exfoliatin is what imparts this capability upon a strain, it would suggest a possible alternate function for this toxin, other than its commonly recognized skin-loosening property, since multiplication was observed in older animals, which are quite resistant to exfoliation. Furthermore, if exfoliatin is actually responsible for multiplication, our data suggest that maximal toxin production is not a necessity for proliferation since strains JF and JH (in high doses), as well as substrains UT-0002-19 and TG-Sp^R, did multiply, if only to a limited degree or after a lag. Only upon comparing the UT-0003 strain with its plasmid-cured substrain UT-0003-4, which exhibited a marked reduction in exfoliatin production, was a major difference in survival evident. Our data do imply that strains producing large amounts of exfoliatin are capable of a more prolonged period of multiplication than are strains elaborating lesser quantities of toxin, but further work is required before this can be determined with certainty, since multi-

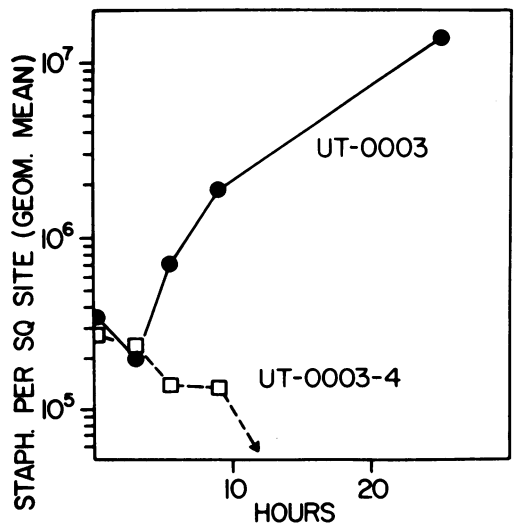

FIG. 9. *Staphylococcal populations in the subcutaneous tissues (SQ) of neonatal mice infected with S. aureus strain UT-0003 or UT-0003-4. Each point represents the geometric mean derived from eight animals. The maximum staphylococcal population is significantly greater than the zero-time population in the case of strain UT-0003; P = <0.001.*

plication also appears influenced by the dose inoculated. The effect of inoculum size on the persistence of conventional strains in subcutaneous tissues has been noticed previously (8).

Rogolsky and co-workers (7, 9) have obtained evidence indicating that in certain strains the genetic determinant for exfoliatin production is plasmid associated. These strains, when cured, manifest significant decreases in exfoliatin production, but some residual toxin production can usually be demonstrated. Keyhani et al. (5) have suggested that toxin production may be under both chromosomal and extrachromosomal control. The greatest relative reduction in exfoliatin production after plasmid elimination seems to occur with parent strains that are not "good" toxin producers (M. Rogolsky, personal communication), as exemplified by UT-0003 and UT-0003-4. This pair also manifested the greatest difference in behavior within the subcutaneous tissue from among the strains examined.

Histological sections were not a reliable means of judging multiplication in the subcutaneous tissues. Regardless of the behavior of the inoculum, neutrophils quickly entered the region and remained in the vicinity for extended periods. These observations are consistent with the report by Agarwal (1), who found that leukocytes accumulated around nonproliferating staphylococci located in the subcutaneous tissues of mice, but are in disagreement with the findings of Melish and Glasgow (6), who did not observe an inflammatory response in their infected neonates. Aside from disparities possibily attributable to use of different mouse strains, we have no explanation for the discrepancy.

In several experiments, after an initial period of multiplication, the staphylococcal population was seen to decline (Fig. 3-5). Whether the reduction in viable organisms resulted from phagocytosis or was mediated by another mechanism cannot be determined at this time.

Also unresolved at present are the questions whether strains capable of multiplying subcutaneously can also multiply in other tissues, and whether these strains can proliferate in species other than mice.

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