Resistance of Athymic Nude Mice to Experimental Cutaneous Bacillus anthracis Infection

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Background. Previous studies in a murine cutaneous anthrax model have demonstrated that hairless and haired HRS/J mice are extremely resistant to Bacillus anthracis. Because these mice are relatively thymus deficient, we used C57BL/6 athymic nude and euthymic mice to evaluate the relationship between T cell deficiency and this heightened resistance.

Methods. Animals were epicutaneously inoculated with $1 \times 10^7$ B. anthracis (Sterne) spores onto abraded skin or injected with the spores intradermally or subcutaneously. The mice were then either monitored for survival or killed for quantitative histological experiments.

Results. Athymic mice were found to be markedly resistant to all 3 inoculation routes, compared with euthymic C57BL/6 mice. Athymic mice rendered leukopenic with cyclophosphamide became susceptible. Histological examination demonstrated increased inflammation and absence of organisms in the skin of athymic mice, compared with euthymic ones. The numbers of organisms in the athymic animals increased markedly after cyclophosphamide treatment. Superficial exudate fluids of inoculated skin showed many more neutrophils and ingested bacilli in the athymic mice.

Conclusions. These experiments demonstrate that athymic nude C57BL/6 mice are markedly resistant to experimental cutaneous anthrax, apparently because of a superficial neutrophilic response that clears the inoculated organisms before they can invade the underlying skin.

Anthrax in humans is $\sim$95% cutaneous and $\sim$5% respiratory in form [1, 2]. Because animals rarely develop cutaneous anthrax, there have been few experimental studies of this form of the disease. Therefore, the pathogenesis of cutaneous anthrax and the local host defenses against it have received relatively little study. To learn more about this disease, we recently developed a murine model of cutaneous anthrax, using the Sterne strain of Bacillus anthracis [3]. This unencapsulated strain is essentially noninfectious for humans but retains significant pathogenicity for mice (which are more sensitive to anthrax than are humans). Many previous studies have used experimental infections with the Sterne strain in mice as a model for human anthrax, with the caveat that lack of a capsule could cause differences in the way the organism is handled by the host. Initial experiments in our cutaneous infection system demonstrated that hair follicles were a major site of organism proliferation in the inoculated skin.

Further experiments were done with hairless HRS/J mice to determine whether the lack of hair follicles with properly formed hair shafts would decrease susceptibility to B. anthracis after epidermal inoculation. Indeed, these animals were found to be extremely resistant to this type of infection. However, this resistance was found to be unrelated to the abnormal hair follicles of these animals by a number of lines of evidence, including (1) resistance to intradermal and subcutaneous spore injections (which bypass the hair follicles as portals of entry), (2) resistance of the haired heterozygotes of this strain, and (3) development of susceptibility in hairless HRS/J mice after they were rendered leukopenic [4]. Further examination of infections in HRS/J mice.
revealed that these animals were able to mount a superficial neutrophilic response in the cutaneous exudate fluids of the abraded inoculated skin and that this response appeared to clear the organisms before they could infect the underlying skin [5].

Previous studies have shown that various inbred mice strains vary markedly in their susceptibility to anthrax in a number of experimental infection models, including the cutaneous system described above [3, 4, 6]. Therefore, HRS/J mice may be more resistant because of some as yet undefined characteristic in their genetic background. On the other hand, these mice have some other traits in addition to their lack of hair. They do have a mutation in the hr gene of chromosome 14 that causes them to lose their hair and to have shallow, nonfunctioning hair follicles, but they have also been found to undergo late thymic cortical atrophy, resulting in significant T cell abnormalities that predispose them to Listeria infections [7–9]. Haired HRS/J mice that are heterozygous for the hr gene mutation have also been found to be susceptible to Listeria infections and to have an increased rate of lymphomagenesis late in life [7–10]. Therefore, it would appear that HRS/J mice (either hairless hr/hr mutants or haired hr/+ heterozygotes) have some relative defects in T cell function and cell-mediated immunity, although these are perhaps not as severe as those seen in athymic nude mice. The comparison is interesting, however, because athymic nude mice have been shown to be very resistant to certain infections, including those caused by Pseudomonas aeruginosa and Candida albicans [11–13], and T cell deficiency could be protective against specific infections. On the other hand, the background of HRS/J mice is complex and based on several strains. Their immunity to anthrax could be due in some way to their T cell deficiency or, perhaps, to some other aspect of their genetic background.

Because athymic nude mice of the C57BL/6 strain are available, the present study was undertaken to compare athymic with euthymic C57BL/6 mice to determine whether the former might be more resistant to experimental cutaneous anthrax. We wanted the experiments to answer 2 fundamental questions: (1) Do athymic nude mice have increased resistance to experimental cutaneous infections with B. anthracis? (2) If so, is this resistance due to an enhanced superficial neutrophilic exudation, as in the HRS/J animals?

**METHODS**

**Organisms.** We used the Sterne strain of B. anthracis for these experiments; it was obtained from the Colorado Serum Company and cultured on brain-heart infusion agar plates. B. anthracis Sterne is a toxigenic, unencapsulated strain that is relatively noninfectious in humans but retains significant pathogenicity for certain inbred strains of mice [3, 14]. Spores were prepared for inoculation as described elsewhere [5].

Staphylococcus aureus (strain 25923) was obtained from the American Type Culture Collection and cultured on tryptic soy agar overnight before use. The organisms were washed 3 times with saline, adjusted by optical density, and confirmed by colony counts. Cutaneous staphylococcal infections of euthymic C57BL/6 mice were used in this study to determine whether a previous infection with a different organism might increase the resistance of the euthymic animals to B. anthracis.

**Animals.** C57BL/6 and BALB/c mice were obtained from Charles Rivers Laboratories, and athymic nude C57BL/6 mice were obtained from Taconic Farms. All of the animals were described by the suppliers as being specific pathogen free; they included both male and female mice, 8–14 weeks of age. Some mice were treated with cyclophosphamide (150 mg/kg intraperitoneally 3 days before and 100 mg/kg 1 day before the inoculations) to render them leukopenic, as described elsewhere [15]. The mice for these experiments were housed in a separate biosafety level 2/3 section of the Veterinary Medical Unit at the Milwaukee VA Medical Center. Previous work has shown BALB/c mice to be highly resistant and C57BL/6 mice to be intermediate resistant to B. anthracis infection (compared with highly susceptible, complement-deficient strains, such as DBA/2) [14].

**Experimental inoculations.** One day before inoculation, the animals were carefully shaved over their flanks with an electric razor; the sites were examined for cutaneous defects the following day, and only animals with clear skin were used. The inoculation sites were prepared by scalp knife abrasion, and the organisms were applied under occlusive dressings, as described elsewhere [5]. The inocula consisted of 1 × 10⁷ B. anthracis spores or, in some cases, 1 × 10⁷ cfu of S. aureus, added to 4-mm filter paper disks (GB002; Schleicher & Schuell) placed onto an abraded area on the animal’s left flank, with a similar quantity of saline alone added to the disk on the opposite side. In other experiments, the inoculum of 1 × 10⁷ B. anthracis spores was injected intradermally or subcutaneously into the shaved flank skin of the mice. The animal studies were approved by the appropriate institutional review committees.

**Monitoring of infections.** After 6 or 24 h, the occlusive dressings were removed, and filter touch preparations were made by touching a glass slide with the skin side (underside) of the 4-mm filter disk. The slides were then stained by the LeukoStat method (Fisher). Some animals were killed after removal of the dressings, and skin samples were obtained for histological study, as described below. In other experiments, the inoculated mice, including those that had undergone epicutaneous, intradermal, or subcutaneous spore injections, were examined daily for 14 days for development of local lesions, cutaneous edema, or a moribund state, as described elsewhere [3, 4].

**Histology.** Skin from inoculated areas was obtained for histology, with paraffin sections prepared and stained with tissue Gram stains. Each of 10 interfollicular fields on each section was examined by light microscopy (magnification, ×400) in a blinded fashion for the presence and depth (below the skin sur-
face) of organisms. In addition, the outlet of each hair follicle infundibulum seen on the entire section was examined for the presence of vegetative bacilli, with the data expressed as the percentage of hair follicle outlets infected. Inflammatory cell infiltrates in the upper dermis were graded in comparison to a set of standard photomicrographs and were scored on an arbitrary scale.

**Examination of touch preparations.** After staining, the slides for the filter touch preparations were examined by light microscopy (magnification, ×400) using a 20 × 20 square ocular micrometer to enumerate vegetative bacilli, host inflammatory cells, and the percentage of host cell-associated bacilli; that percentage represents the number of bacilli in contact with host cells divided by the total number of bacilli counted. For this study, 10 random fields were examined for each slide. Each bacillus in a chain was counted as a single organism. The data were expressed as the total numbers of bacilli and host cells divided by the total number examined. Hair follicles were examined for infection, and the data were expressed as the number of follicles with bacilli present per the number examined. The number of bacilli or host inflammatory cells seen on filter touch preparations was expressed as the mean ± SE per square millimeter, and the number of cell-associated bacilli seen on filter touch preparations was expressed as a percentage. These values for athymic and euthymic animals were compared using the nonparametric Mann-Whitney U test or the Kruskal-Wallis test with Dunn’s multiple-comparison posttest (for 3 or more groups). The numbers of animals that died or developed edema after *B. anthracis* inoculation were compared using Fisher’s exact test. The GraphPad Prism statistical package (version 4.0c) was used to make these determinations. Differences of *P* < .05 were considered significant.

**RESULTS**

**Death and development of regional edema.** The primary intent of these experiments was to determine whether the resistance to anthrax previously seen in HRS/J mice would also be found in T cell–deficient mice from a defined genetic background—in particular, athymic nude C57BL/6 mice. As shown in table 1, significant mortality occurred in the euthymic C57BL/6 mice after inoculation by the epidermal, intradermal, or subcutaneous route; however, none of the athymic nude mice died after either epidermal or intradermal inoculation, and only 1 of 5 died after subcutaneous spore inoculation. Similarly, most of the euthymic C57BL/6 mice developed edema after epidermal or intradermal inoculation, whereas the athymic nude mice did not; on the other hand, these animals did develop edema after subcutaneous spore inoculation (even though most later recovered), indicating that resistance in the athymic animals was not complete.

Because enhancement of innate immunity by previous infections is one explanation for increased resistance to infection in athymic animals, we tested some of the euthymic nude mice with intradermal *B. anthracis* spore injections after they had recovered from a cutaneous *S. aureus* infection at the same site; as shown in table 1, 13 of 22 animals with previous cutaneous staphylococcal
infections died, compared with 24 of 25 animals without such infections (P < .005). Moreover, for the euthymic mice with previous S. aureus infections that did die or develop edema after intradermal B. anthracis inoculation, the time to death or to development of edema was significantly extended. Therefore, the euthymic C57BL/6 mice with previous experimental S. aureus infections were protected to some extent against intradermal B. anthracis spore inoculation, although the extent of this protection was less than that seen in the athymic nude mice.

Presence of organisms and inflammation in skin samples. Skin sections from animals that had undergone epicutaneous B. anthracis inoculation either 6 or 24 h previously were examined for the presence and location of organisms in the skin and the degree of dermal inflammation present. The results summarized in table 2 show a marked difference in the numbers of interfollicular fields or hair follicle outlets that were seen to contain bacilli. In fact, virtually no organisms at all were seen in sections from the athymic nude C57BL/6 mice, but organisms were frequently found in their euthymic counterparts and in the BALB/c mice, which are known to be resistant to B. anthracis. If the athymic animals were rendered leukopenic by cyclophosphamide treatment, then their skin and hair follicles were readily invaded by the bacilli, as is also shown in table 2. Inflammation in the upper dermis was increased in the inoculated skin from athymic mice at both 6 and 24 h but was not so in that from uninfected or cyclophosphamide-treated athymic mice (table 3). Figure 1A shows the presence of B. anthracis bacilli in the epidermal remnants and hair follicles of inoculated abraded skin from an athymic C57BL/6 mouse at 24 h. In comparison, figure 1B demonstrates the lack of organisms in a corresponding section from an athymic nude animal; this figure also shows a significant dermal inflammatory infiltrate, greater than that in the euthymic animal. After cyclophosphamide treatment, the athymic nude mice did become susceptible to the epicutaneous infections, with bacilli readily invading deep into the skin, as shown in figure 1C.

### Table 2. Bacillus anthracis bacilli and infected hair follicles in histological sections of skin biopsy specimens after epicutaneous inoculation of spores.

<table>
<thead>
<tr>
<th>Time point, mice</th>
<th>Fields with bacilli, proportion</th>
<th>Depth of bacilli, mean ± SE, μm</th>
<th>Hair follicles infected, proportion</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL/6</td>
<td>23/120</td>
<td>3.2 ± 1.2</td>
<td>102/256</td>
</tr>
<tr>
<td>C57BL/6 nude</td>
<td>0/100</td>
<td></td>
<td>5/416</td>
</tr>
<tr>
<td>24 h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL/6</td>
<td>59/130</td>
<td>14.1 ± 4.6</td>
<td>215/273</td>
</tr>
<tr>
<td>C57BL/6 nude</td>
<td>0/130</td>
<td></td>
<td>2/526</td>
</tr>
<tr>
<td>C57BL/6 nude, cyclophosphamide treated</td>
<td>95/117</td>
<td>107.3 ± 42.1</td>
<td>428/539</td>
</tr>
<tr>
<td>BALB/c</td>
<td>21/90</td>
<td>3.3 ± 1.9</td>
<td>106/270</td>
</tr>
</tbody>
</table>

**NOTE.** Data are from 5–7 mice tested in 5–7 experiments per point. Significantly fewer bacilli and infected hair follicles were found in athymic nude mice than in their euthymic counterparts (P < .001 for each comparison at 6 or 24 h, Fisher’s exact test).

### Table 3. Inflammation in histological sections of skin biopsy specimens after epicutaneous inoculation of Bacillus anthracis spores.

<table>
<thead>
<tr>
<th>Mice</th>
<th>Inflammatory cell infiltrates score, mean ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6 h</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>C57BL/6 nude</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>C57BL/6 nude, cyclophosphamide treated</td>
<td>NT</td>
</tr>
</tbody>
</table>

**NOTE.** Data are from 5–7 mice tested in 5–7 experiments per point. Inflammation was scored on arbitrary scale from 0 to 3. Inflammation scores were higher in the athymic animals but decreased after cyclophosphamide treatment (for nude vs. euthymic mice, P < .05 at 6 h and P < .01 at 24 h; for nude vs. cyclophosphamide-treated nude mice, P < .001 at 24 h; Kruskal-Wallis and Dunn’s multiple-comparison tests). NT, not tested.
imals demonstrated that 92.4% ± 1.7% of these cells were neutrophils.

**DISCUSSION**

The results of the present study demonstrate that athymic nude C57BL/6 mice are much more resistant to *B. anthracis* than their euthymic counterparts. Lack of susceptibility in the athymic animals extended also to inoculations given intradermally or subcutaneously; because these administration routes bypass hair follicles or other epidermal skin components, resistance of the athymic animals seems unrelated to abnormalities in their epidermal structures. Studies at 6 h after inoculation, when the

**Table 4. Accumulation of *Bacillus anthracis* bacilli and inflammatory cells in epicutaneous exudates after inoculation of spores onto abraded skin.**

<table>
<thead>
<tr>
<th>Mice</th>
<th>Total bacilli/mm²</th>
<th>Cells/mm²</th>
<th>Bacilli/cell</th>
<th>Free bacilli/mm²</th>
<th>Cell-associated bacilli, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td>512.5 ± 134.4</td>
<td>16.7 ± 5.4</td>
<td>34.8 ± 18.6</td>
<td>492.6 ± 131.1</td>
<td>4.5 ± 1.5</td>
</tr>
<tr>
<td>C57BL/6 nude</td>
<td>127.3 ± 24.4</td>
<td>151.6 ± 57.2</td>
<td>1.6 ± 0.4</td>
<td>71.0 ± 14.7</td>
<td>40.7 ± 8.6</td>
</tr>
</tbody>
</table>

**NOTE.** Data, given as means ± SEs, are from 6-h filter preparations obtained from 7 mice tested in 7 experiments per point. Total bacilli indicates all of the organisms seen, and free bacilli are those not associated with host cells. Athymic nude mice had more host cells and cell-associated bacilli and fewer free (i.e., not cell-associated) bacilli than did their euthymic counterparts (*P* = .002, *P* = .001, and *P* = .001, respectively, Mann-Whitney *U* test).
Infection is just beginning in this model system, demonstrated that the organisms never entered the skin of the athymic animals, as opposed to invading and then being cleared by an efficient phagocytic cell response. The athymic animals mounted a more efficient superficial inflammatory response to these infections than did the euthymic ones, and the cells involved were predominantly neutrophils. Rendering the athymic nude mice leukopenic with cyclophosphamide made them susceptible to the epicutaneous inoculations with *B. anthracis*. Taken together, these findings suggest that the athymic nude mice are particularly resistant to cutaneous or subcutaneous inoculations with *B. anthracis* because these mice are better able to mount an early superficial neutrophilic response that clears the inoculated organisms before they can invade the skin below. These results closely parallel those previously described in HRS/J mice [4, 5]; however, mouse strains differ markedly in their susceptibility to *B. anthracis*, and the relatively complicated background of the HRS/J strain may have been responsible for their resistance to anthrax. Because the comparisons in the present study involved inbred C57BL/6 counterparts of the athymic mice, the results support the possibility that the T cell deficiency in these animals is some way related to *B. anthracis* resistance.

Athymic nude mice are severely deficient in T cell–dependent cell-mediated immunity and are known to be particularly susceptible to many fungal and bacterial infections [16–21]. Therefore, their heightened resistance to certain pathogens such as *B. anthracis* seems counterintuitive. On the other hand, it has been suggested that the athymic animals may be more prone to development of nonlethal infections that enhance their innate immunity to certain other pathogens through increases in antibacterial activity of their phagocytic cells [13]. Because athymic nude mice are particularly susceptible to *S. aureus* skin infections, we tested in the present system the possibility that a preceding cutaneous staphylococcal infection might protect euthymic mice from later challenge with *B. anthracis*. Such a protective effect was found, but it provided only partial resistance to intradermal spore injections in the euthymic animals, compared with almost complete resistance in the athymic ones. However, it is difficult to know exactly what exposure the athymic animals in our study may have had to *S. aureus* or other microbes before *B. anthracis* inoculation in our experiments. Innate immunity may have been stimulated by normal flora or a variety of bacterial pathogens, even though the animals were described by the supplier as being specific pathogen free. In addition, the animals may have had multiple minor infections or other microbial exposures over time that had a cumulative effect on their innate immunity to *B. anthracis*.

Another possibility is that a lack of regulatory T cells in the athymic mice may have augmented the effect of prior microbial exposures in stimulating innate immunity. Regulatory T cells represent 5%–10% of peripheral CD4⁺ T cells in mice and humans and constitutively express CD25 (the interleukin-2 receptor α-chain); these cells help maintain immunologic self-tolerance, and their removal has been shown to enhance immune responses against a variety of infectious microbes [22]. Athymic nude mice lack CD4⁺CD25⁺ regulatory T cells, which can be repopulated by appropriate thymic tissue grafts [23]. Regulatory T cells can suppress innate immune pathology [24], but they may also interfere with pathogen resistance through this mechanism. Therefore, a lack of regulatory T cells in athymic nude mice may promote resistance to certain pathogens, because innate immune mechanisms may be more readily up-regulated owing to this lack of control. Athymic nude mice may have up-regulated innate immunity, not only because they are susceptible to recurrent nonlethal infections but also because they lack the suppressive control of regulatory T cells.
In summary, the present study indicates that athymic nude C57BL/6 mice are resistant to experimental cutaneous anthrax, apparently because of a superficial neutrophilic response that acts at an early point in the infectious process to clear the inoculated organisms before they can invade the underlying skin. The relationship of the T cell defect to this resistance is unclear at present, and it may well be indirect in nature.

References