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Immunobiology of Germfree Mice Infected with *Nocardia asteroides*

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Nocardia asteroides GUH-2 was administered either intranasally or by intravenous inoculation into (i) conventionally grown, (ii) germfree, and (iii) lipopolysaccharide-treated germfree NIH:S mice. The number of bacteria within the lungs, brain, kidneys, adrenals, liver, spleen, and blood was quantitated at 3, 24, 72, and 168 h after infection. Further, the histological changes that occurred in each of these organs after infection were studied. The data demonstrated that germfree mice were significantly more susceptible to the acute phase of infection caused by *N. asteroides* than the conventionally grown animals. The brains and lungs of these mice were affected most dramatically. Pretreatment of the germfree mice with lipopolysaccharide completely reversed this enhanced susceptibility and rendered the animals more resistant to infection than the conventionally grown mice. These observations establish further the role of macrophage activation and the development of cell-mediated immunity in host resistance to nocardia. In addition, the presence of a resident microflora within the host appears to be important in the development of resistance to systemic nocardial infections.

The specific mechanisms of host resistance to nocardial infections appear to be complex and not yet fully defined. By combining both in vivo and in vitro studies, it has been shown that *Nocardia asteroides* grow intracellularly in both alveolar and peritoneal macrophages (4, 5, 12, 13); T cell lymphocytes are important in effective host responsiveness (7, 8, 16); cell-mediated immunity may be important for host protection (3, 9, 25, 30); and in vitro, both specifically and nonspecifically activated macrophages can temporarily inhibit intracellular growth (5, 15). In contrast, peripheral blood monocytes and polymorphonuclear neutrophils from humans do not kill or inhibit these organisms (Filice, Beaman, Krick, and Remington, unpublished data), and most data suggest that humoral immunity and specific antibody with complement have little effect on host resistance to virulent strains of *N. asteroides* (5, 25). Further, the lung is the primary target organ for *N. asteroides*, whereas the brain and central nervous system represent important secondary sites (6).

We have developed murine models that permit the assessment of the viability of *N. asteroides* in one lung, while at the same time histological analysis of the interrelationships of the nocardia to macrophages and polymorphonuclear neutrophils can be determined in the other lung (8). Thus, using specifically manipulated animal model systems, we can further define the pathogenesis of *N. asteroides* introduced into the intact murine lung. Since dissemination of *N. asteroides* from the lung to other organs, such as the brain and kidney, plays an important role in nocardiosis, it is important to compare and contrast organ clearance and host responsiveness after intravenous challenge. It has been shown previously that the route of inoculation has a major effect on the interaction of nocardia with the host, and there appears to be a compartmentalization of the host response to *N. asteroides* (11).

The use of germfree animals potentially offers an important and unique tool in the study of host-nocardia relationships because the germfree environment isolates the host from both its own normal microflora and from the influence of exogenous microbial interactions. It also permits the study of definitive immunological reactions against single or specifically controlled external factors (19, 29). There have been no previous reports on nocardial interactions in germfree hosts.

MATERIALS AND METHODS

Microorganism. *N. asteroides* GUH-2 was isolated from a fatal human infection and maintained (9, 10). The pathogenicity of this strain of nocardia for mice has been described (10).

Mice. A colony of inbred germfree N:NIH(S) mice is maintained in the Maximum Isolation Building of the Animal Resources Branch at the University of California. This colony was originally derived from cesarian section of timed pregnant N:NIH(S) mothers

with subsequent foster-nursing by germfree CD-1 mothers (Charles River Laboratories, Cambridge, Mass.) and maintained in sterile solid plastic isolators (Germfree Laboratories, Miami, Fla.). The colony has been maintained under sterile conditions for more than 2 years with biweekly monitoring of potential aerobic, anaerobic, and fungal contaminants. Germfree mice of both sexes, 6 to 10 weeks of age, were used throughout and were randomized to each of the experimental groups.

Preparation of inoculum. Fresh animal isolates of *N. asteroides* were grown in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.) and grown to the early stationary phase of growth at 37°C with 150 rpm rotational agitation (2, 10). The culture was centrifuged at low speed (ca. 50 × *g*) for 5 min to sediment clumps of cells, and the supernatant suspension was centrifuged at about 1,000 × *g* for 15 min to pellet the bacterial cells. The organisms were suspended in sterile saline (0.85%). Phase-contrast microscopy revealed that the suspensions were composed of uniform coccobacillary cells with few or no bacterial clumps. These cell suspensions were diluted to give the appropriate number of colony-forming units (CFU) per milliliter to be injected intravenously or administered intranasally into appropriate populations of mice.

Infection schedules. Intranasal infection of conventionally grown, germfree, and lipopolysaccharide (LPS)-treated germfree mice was accomplished by lightly anesthetizing the animals by intraperitoneal injection of tribromo-ethanol (approximately 1.3 mg/10 g of animal weight). Saline suspensions of the organism (0.05 ml) were introduced into the nares, and the mice quantitatively aspirated the suspensions into the lungs as previously described (8).

Intravenous infection and organ clearance studies of the conventionally grown, germfree, and LPS-treated germfree mice were done as previously described (8, 11). Quantitation of the organisms from the organs of five mice from each group at 3, 24, 72, and 168 h after infection was determined by asceptically removing the kidneys, lungs, spleen, liver, brain, adrenals, and blood. The blood (0.1 ml) was plated directly onto BHI agar for quantitation. All other organs were placed in 3.0 ml of sterile saline and homogenized asceptically for 30 s with a Tissumizer high-speed blender (Tekmar). Dilutions of the tissue homogenates were plated on BHI agar, incubated for 5 days, and counted as previously described (8, 11).

LPS treatment. Because of observations suggesting that macrophages of conventionally housed mice are chronically activated compared to cells from germfree mice, groups of germfree animals were injected with 50 μg of LPS (L3129 *Escherichia coli*, Sigma Chemical Co., St. Louis, Mo.) three times each week for 2 weeks before infection with nocardia. This dose and schedule were chosen because observations from this laboratory and elsewhere indicate that such treatment significantly enhances macrophage activity (17, 18).

Response to sheep erythrocytes. To determine whether the altered response of germfree mice to nocardia was unique, and to assess the immunological response of these mice, groups of mice were immunized by intraperitoneal injection of 2×10^8 sheep erythrocytes. Five days later, spleens from the immunized animals were resected, and the number of splenic plaque-forming cells was quantitated (18).

Light microscopy. The mice were sacrificed by an overdose of ether. The peritoneal, thoracic, and brain cavities were opened. The internal organs were carefully removed and placed in 4% buffered Formalin. The lungs were perfused with Formalin before removal. The tissue samples were prepared for histology as previously described (1, 8, 9). The stained sections were observed with a Zeiss research microscope equipped with planachromatic objectives. Micrographs were taken with panatomic X film (Kodak) and green or blue filters at the light source.

RESULTS

Pulmonary response. Figure 1 illustrates the clearance data for mice infected intranasally with *N. asteroides* GUH-2. During the first 24 h there was an increase in nocardial numbers isolated from the left lungs of both LPS-treated and untreated germfree mice. The conventionally grown mice received a slightly lower mean

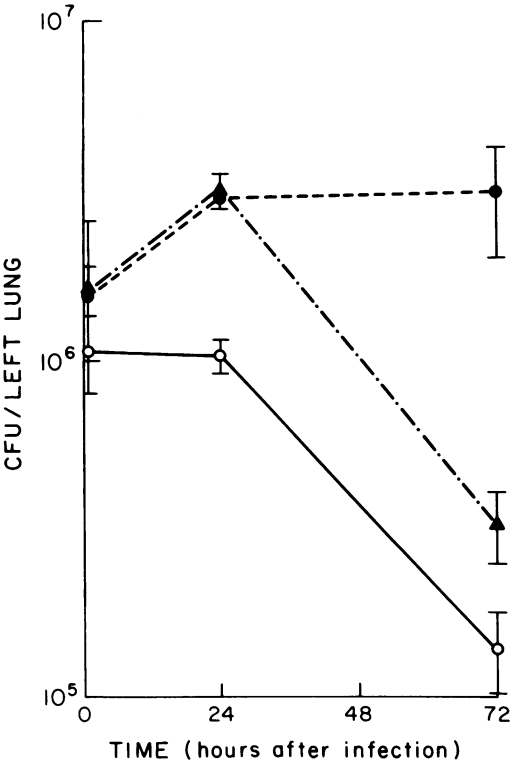

FIG. 1. *Pulmonary clearance of intranasally administered cultures of N. asteroides GUH-2. Symbols: ●, unmanipulated germfree N:NIH(S) mice; ▲, LPS-treated germfree N:NIH(S) mice; ○, conventionally grown N:NIH(S) mice. The bars represent the standard error of the mean.*

dose than the germfree groups, and the numbers of nocardia did not appear to increase during the first 24 h. However, as shown previously, these results are probably misleading since the nocardial cells grow as tangled masses of filaments and homogenates containing clumps of filaments would not represent the true numbers of cells present (8). Histological analysis of the lungs of these mice demonstrated that considerable nocardial growth occurred in the lungs since only coccoid cells were used to inoculate these mice, and these cells had now developed into long, branching filamentous organisms. It is important to emphasize that there were no filamentous cells visualized within the initial inoculum (Fig. 2A). These results are basically the same as those reported in a previous study using conventionally grown specific pathogen-free Swiss Webster mice (8). Therefore, no apparent differences were observed among the conventionally grown and the LPS-treated germfree mice by microscopic analysis of sections of the lungs at 24 h after infection (compare Fig. 2A with reference 8). In contrast, the sections of lungs of germfree mice at 24 h after intranasal infection demonstrated significant cellular elongation of the nocardia, but there was very little host cell response within these infected lungs. Instead, the bacteria were growing profusely within the alveolar spaces, and filaments of nocardia had invaded the alveolar interstitium and the epithelial cells lining the bronchi (Fig. 3A, B). At 72 h after infection, differences among the three populations of mice were clearly evident by both viable plate counts of lung homogenates (Fig. 1) and light microscopy (Fig. 2C, D and 3C, D). The lungs of germfree mice were heavily infected with long, branching filaments, and the alveolar spaces were now packed with macrophages and polymorphonuclear cells (cf. Fig. 3B and D). There was still extensive bacterial invasion of the bronchial epithelium and alveolar walls (Fig. 3C, D). The mice were acutely ill, and many of them died. The bacterial numbers had not decreased at 72 h after infection. This was in sharp contrast to the LPS-treated germfree and conventionally grown mice (Fig. 2C, D). Light microscopy demonstrated that nocardial growth was still evident, but there was a marked decrease in severity of infection (cf. Fig. 2B and D). Further, the overall numbers of nocardial filaments visualized within the lungs were decreased as compared to the germfree animals (Fig. 1). The lesions in the conventional mice were composed of large numbers of macrophages and lymphocytes (Fig. 2C, D) whereas, at 72 h after infection, lymphocytes were not prominent in the lungs of germfree animals. The numbers of bacteria recovered from the lung homogenates of LPS-treated germfree and conventionally grown mice were significantly less than those observed in the unmanipulated germfree animals (Fig. 1, 2C, 3C). At 1 week after intranasal infection, the number of bacteria isolated from the lungs of all surviving mice was decreased; however, there were still more organisms in the lungs of germfree mice than in the lungs of their LPS-treated counterparts. Light microscopy revealed that the infectious process within these mice was subsiding.

It has been shown that the route of inoculation can significantly affect the organism's ability to evade host defenses and cause disease. Thus, the pulmonary response of mice to bacteremic challenge with *N. asteroides* differs from the pulmonary response to an aerosal exposure of the same organism (8, 11). Therefore, the lungs of conventionally grown mice, germfree mice, and LPS-treated germfree mice were assessed after intravenous injection of *N. asteroides* GUH-2 and compared to groups that had received the organisms intranasally (Fig. 4). During the first 24 h after intravenous inoculation, the number of organisms deposited within the lungs decreased in all of the groups of mice (Fig. 4). Histological analysis demonstrated small, localized areas of inflammation within the lung associated with capillaries and blood vessels but not within the alveolar spaces. At 72 h after infection, the organisms had initiated an invasive process within the lungs of the germfree mice that resulted in alveolar consolidation. In contrast, the lungs of the conventionally grown and LPS-treated mice had small, well-defined, abscess-like lesions that appeared to remain localized. The bacteria within the lungs of all of the mice had initiated growth, as evidenced by the formation of branched, filamentous cells from the coccobacillary form that was initially injected into the bloodstream. The numbers of organisms recovered from homogenates of the lungs from germfree animals increased at 72 h, whereas the numbers recovered from conventional and LPS germfree mice continued to decrease (Fig. 4). The infections induced in the lungs of the germfree mice were self limited since the numbers of organisms recovered from the lungs of these mice at 1 week after infection were reduced as compared to the 3-h inoculum level (Table 1). However, the number of organisms remaining in the lungs of germfree mice 1 week after infection was still significantly greater than that in the LPS-treated germfree mice (Table 1). These data clearly establish that the pulmonary response of germfree animals to infection with *N. asteroides* is significantly altered when compared to that of the conventionally grown mice, and LPS treatment resulted in re-

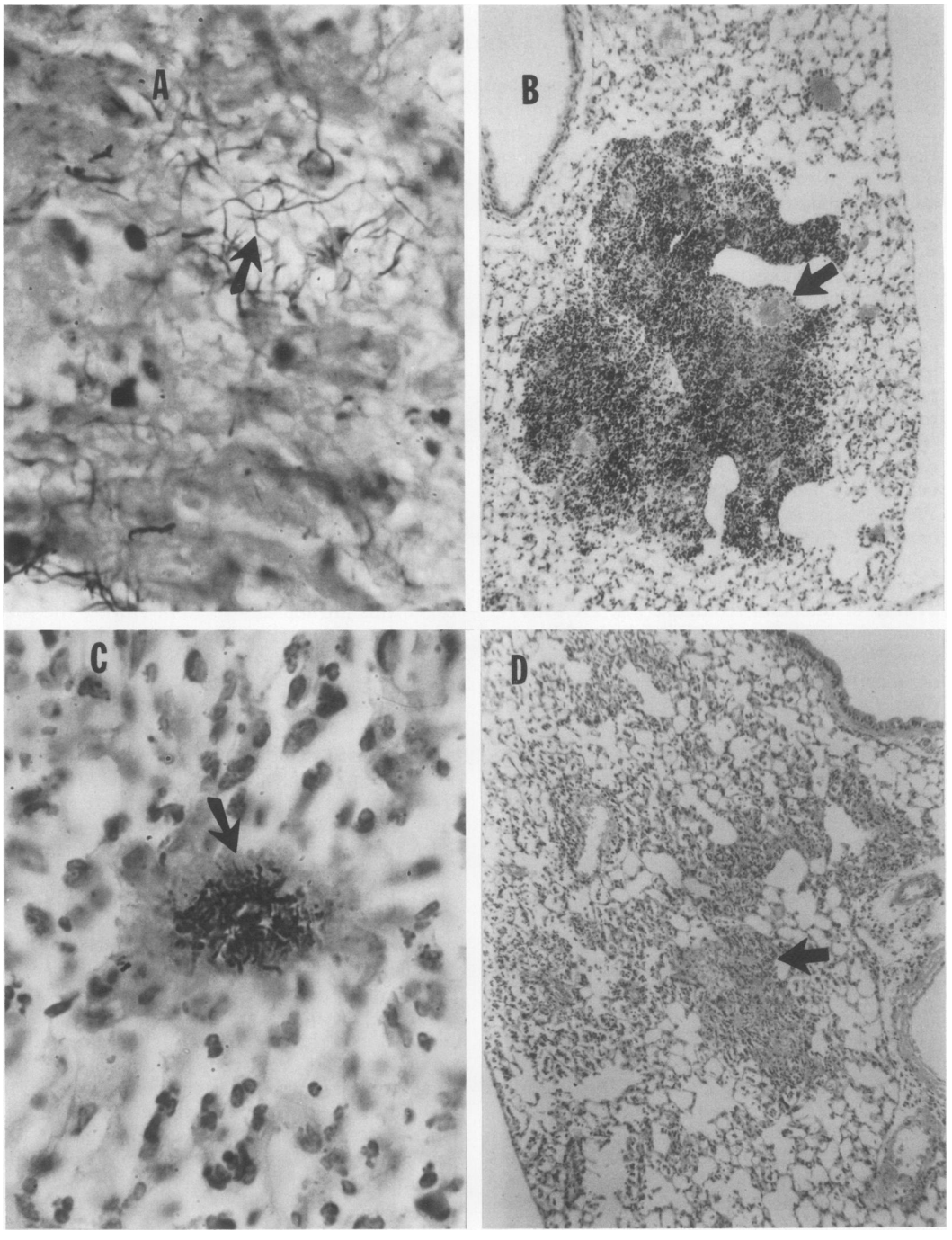

FIG. 2. *Representative sections of lungs of LPS-treated germfree N:NIH(S) mice at time intervals after intranasal administration of suspensions of N. asteroides GUH-2 (Contrast with Fig. 3). (A) A Brown and Brenn Gram stain of a section of lung 24 h after infection. Arrow indicates nocardial filaments growing throughout the pulmonary infiltrate. (B) The same lung as in A except stained by hematoxylin and eosin. Arrow indicates the region of the lung shown in A. This micrograph is a low-magnification view of a large portion of a representative area of the lung. Note the extensive, well-defined region of pulmonary inflammation and alveolar consolidation (Contrast with Fig. 3B). (C) A Brown and Brenn Gram stain of a section of lung 72 h after infection. Arrow indicates a clump of nocardial filaments surrounded by inflammatory cells. (D) The same lung as in C except stained by hematoxylin and eosin. The arrow indicates the region of the lung shown in C. This micrograph of a large portion of a representative area of the lungs shows a general decrease in severity of pulmonary inflammation (Contrast with Fig. 3D).*

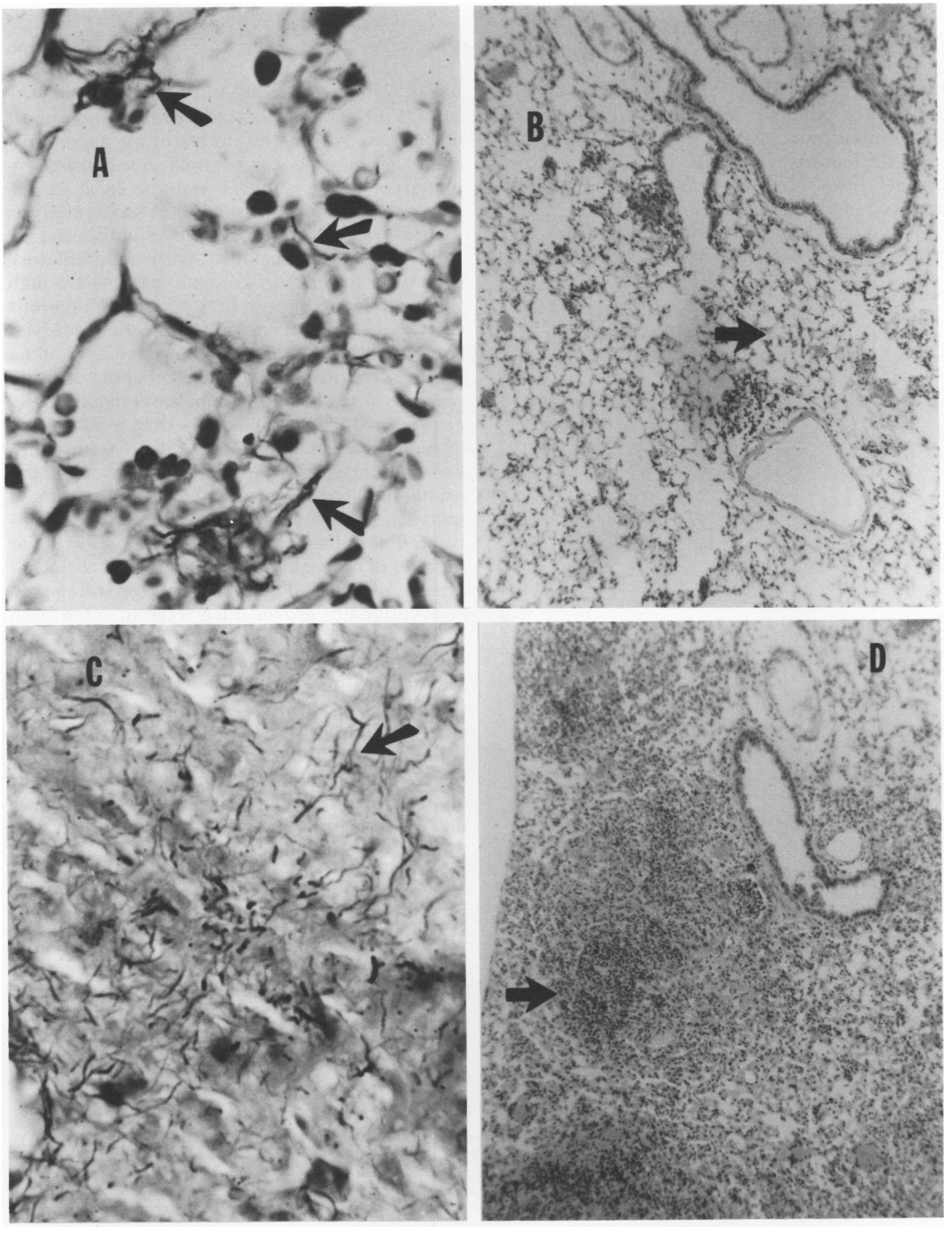

FIG. 3. *Representative sections of lungs of unmanipulated germfree N:NIH(S) mice at time intervals after intranasal administration of suspensions of N. asteroides GUH-2. (A) A Brown and Brenn Gram stain of a section of lung 24 h after infection. Arrows indicate nocardial filaments growing within the cells of the alveoli. Note the absence of an inflammatory response. (B) The same lung as in A except stained by hematoxylin and eosin. Arrow indicates the region of the lung shown in A. This micrograph is a low-magnification view of a large portion of a representative area of the lung. Note the overall absence of pulmonary inflammation and cellular infiltration within the lung. (C) A Brown and Brenn Gram stain of a section of lung 72 h after infection. Arrow indicates nocardial filaments growing profusely throughout the pulmonary infiltrate. (D) The same lung as in C except stained by hematoxylin and eosin. The arrow indicates the region of the lung shown in C. This micrograph of a large portion of a representative area of the lung shows that extensive pulmonary inflammation is present (Contrast with B).*

storing or enhancing the host's resistance to pulmonary infection after both intranasal and intravenous inoculation.

Response in the brain. The brain and central nervous system are the most frequent sites involved in disseminated nocardiosis (6). Therefore, the brains of conventionally grown, germfree, and LPS-treated germfree mice were studied after intravenous administration. During the first 24 h there was a rapid increase in numbers of bacteria within the brains of all mice; however, the increase was greatest in the unmanipulated germfree animals and the least in the LPS germfree mice (Fig. 5). Microscopic analysis demonstrated that the capillaries within the cerebrum became occluded with inflammatory cells with nocardial filaments growing through the wall into the cerebral tissues. Numerous scattered foci of infection were observed throughout the cerebrum; however, few or no lesions were observed in the cerebellum or other portions of the brain. At 72 h, the conventionally grown mice that received less than a 50% lethal dose of organisms had a slight decrease in numbers of organisms in the brain, whereas the unmanipulated germfree mice had a continued increase (Fig. 5). In sharp contrast, the LPS-treated germfree mice had a significantly lower number of organisms in the brain than the other groups of mice (Fig. 5). When germfree and conventionally grown mice were injected with twice as many organisms, the differences in response were even more pronounced during the first 24 h (Fig. 5). The nocardia grew in the brains of the germfree mice at a rate greater than they grew in BHI broth in vitro (10), whereas the numbers of organisms in the brains of the conventionally grown mice increased at about the same rate as observed with the lower dose. Between 24 and 72 h after infection, the numbers of organisms found in the brains of both groups had continued to increase at about the same rate (Fig. 5). The numbers of organisms isolated from the brains of all surviving mice were decreased at 1 week after infection (Table 1); however, the brains of the unmanipulated germfree mice still had significantly more organisms than those of the LPS-treated germfree and conventionally grown animals (Table 1).

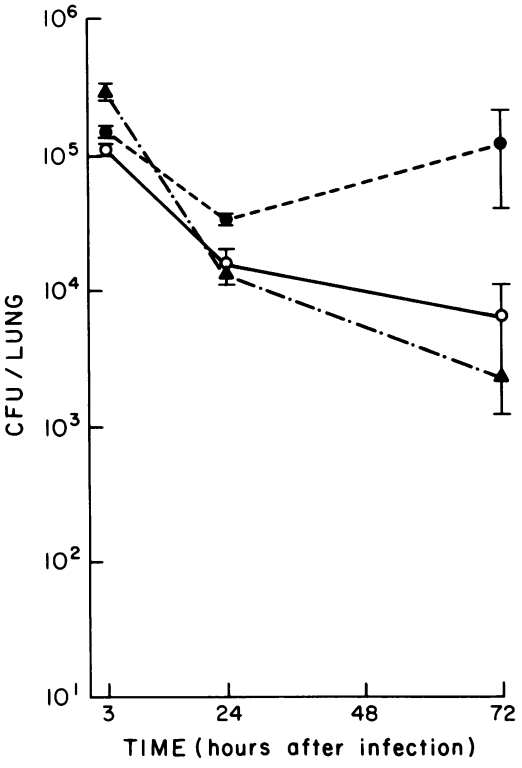

FIG. 4. *Pulmonary clearance of cells of N. asteroides GUH-2 after intravenous injection (2.8 × 10^6 CFU/mouse). Symbols: ●, unmanipulated germfree N:NIH(S) mice; ▲, LPS-treated germfree mice; ○, conventionally grown mice. The bars represent the standard error of the mean.*

Response in the kidneys and adrenals. Previous experiments have shown that *N. aster-*

TABLE 1. *Organ distribution of conventionally grown, axenically grown, and LPS-stimulated axenically grown N:NIH(S) mice*[a]

Organ	*N. asteroides* in mouse type:		
	Conventionally grown	Gnotobiotic	Gnotobiotic, LPS-treated
Adrenals	1.3×10^3 ($\pm 0.9 \times 10^3$)	1.3×10^5 ($\pm 0.3 \times 10^5$)	4.3×10^2 ($\pm 3.6 \times 10^2$)
Brain	2.3×10^2 ($\pm 0.7 \times 10^2$)	7.8×10^3 ($\pm 0.6 \times 10^3$)	5.5×10^1 ($\pm 2.3 \times 10^1$)
Liver	1.2×10^6 ($\pm 0.2 \times 10^6$)	1.0×10^6 ($\pm 0.1 \times 10^6$)	9.1×10^5 ($\pm 1.1 \times 10^5$)
Lung	2.0×10^2 ($\pm 0.6 \times 10^2$)	8.5×10^3 ($\pm 2.1 \times 10^3$)	1.1×10^2 ($\pm 0.4 \times 10^2$)
Kidney	5.5×10^4 ($\pm 1.3 \times 10^4$)	1.1×10^6 ($\pm 0.5 \times 10^6$)	5.8×10^3 ($\pm 2.6 \times 10^3$)
Spleen	2.1×10^4 ($\pm 0.8 \times 10^4$)	1.1×10^5 ($\pm 0.2 \times 10^5$)	1.1×10^5 ($\pm 0.4 \times 10^5$)
Total recovered	1.3×10^6	2.4×10^6	1.0×10^6

[a] At 1 week after intravenous injection of *N. asteroides* GUH-2 (2.8×10^6 CFU per mouse).

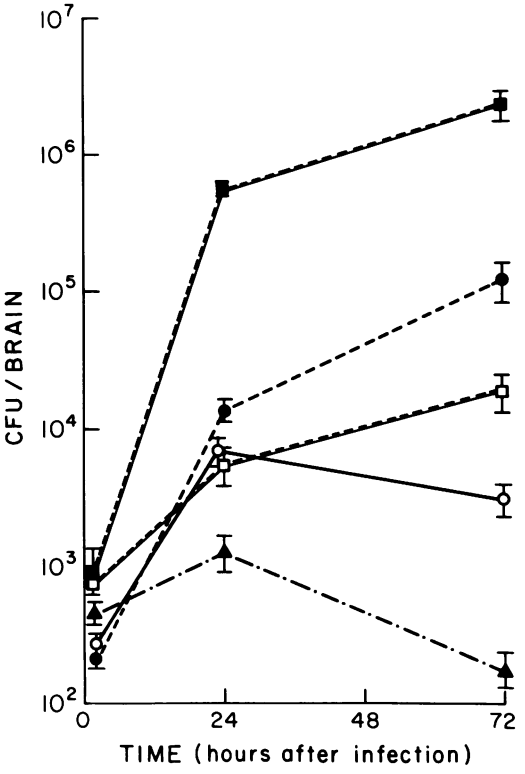

FIG. 5. *Growth of N. asteroides GUH-2 in the brains of N:NIH(S) mice after intravenous inoculation. Symbols: ■, unmanipulated germfree mice injected with 4.3 × 10⁶ CFU/mouse; □, conventionally grown mice injected with 4.3 × 10⁶ CFU/mouse; ●, unmanipulated germfree mice injected with 2.8 × 10⁶ CFU/mouse; ▲, LPS-treated germfree mice injected with 2.8 × 10⁶ CFU/mouse; ○, conventionally grown mice injected with 2.8 × 10⁶ CFU/mouse. The bars represent the standard error of the mean.*

oides GUH-2 uses the adrenals and kidneys as target organs when injected intravenously into mice (7, 9, 11). The data presented in Fig. 6 clearly demonstrate that unmanipulated germfree mice were less able to inhibit the growth of nocardia in the kidneys and adrenals than either the conventionally grown or LPS-treated animals. The LPS-treated germfree animals effectively inhibited the growth in the adrenals at 72 h after infection but only retarded the growth in the kidneys at this time period (Fig. 6). Conventionally grown mice were intermediate in their response to infection of both the adrenals and kidneys. At 1 week after infection the germfree mice still had significantly more organisms in their adrenals than either the LPS-treated germfree or the conventionally grown mice (Table 1). Similar results were observed in the kidneys of the germfree mice (Table 1). These data established that the host's ability to respond to infections of both the adrenals and kidneys was greatly impaired in germfree mice, but LPS treatment of these mice rendered them significantly more resistant than the conventionally grown animals.

Response in the liver, spleen, and blood. Intravenously injected cells of *N. asteroides* GUH-2 were rapidly and efficiently removed from the blood stream of all groups of mice. At 1 h after injection more than 99.9% of the organisms injected into the mice were removed from the blood stream. The numbers of bacteria remaining in the blood continued to decline until several mice had no detectable organisms. There appeared to be no differences between the conventionally grown and germfree mice.

The liver and spleen served as the primary filtering organs that removed the organisms

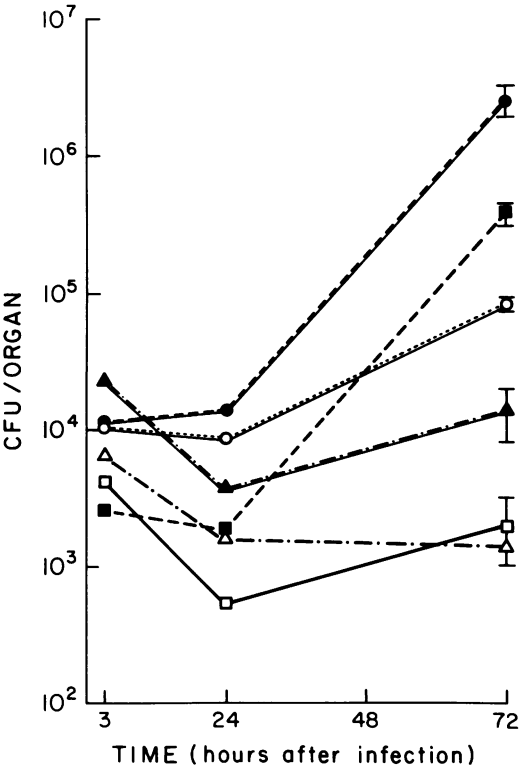

FIG. 6. *Growth of N. asteroides GUH-2 in the adrenals and kidneys of N:NIH(S) mice after intravenous inoculation (2.8 × 10⁶ CFU/mouse). Symbols: ■, adrenals of unmanipulated germfree mice; △, adrenals of LPS-treated germfree mice; □, adrenals of conventionally grown mice; ●, kidneys of unmanipulated germfree mice; ▲, kidneys of LPS-treated germfree mice; ○, kidneys of conventionally grown mice. The bars represent the standard error of the mean.*

from the blood stream. In all of the mice more than 89% of the inoculum was recovered from these two organs at 3 h after injection. The spleens of the LPS-primed germfree mice consistently contained more than the other mice, but the spleens of these animals were significantly enlarged; thus, on a gram-weight basis all spleens of all mice had similar numbers of organisms (Fig. 7).

At 24 h after infection, the number of organisms increased slightly in the livers of conventionally grown and germfree mice. The increase was less (probably not significant) in the LPS-treated animals. At 72 h they were beginning to decrease, and at 1 week there was a greater reduction in numbers as compared to the inoculum levels (Table 1). There appeared to be no difference in the response in the livers of conventionally grown, germfree, and LPS-treated germfree mice. Similar results were observed in the host's ability to eliminate the organisms from the spleens (Fig. 7). Therefore, LPS treatment of germfree mice did not have any significant effect on the clearance of *N. asteroides* GUH-2 from the blood, liver, and spleen (Fig. 7, Table 1).

Immunological assessment and responsiveness. There was significant individual variation in the numbers of splenic plaque-forming cells in germfree mice injected with nocardia (Table 2). The standard error of the mean varied from approximately 15% in mice injected intravenously with nocardia to 40% in LPS-treated mice injected intravenously with nocardia (Table 2). In contrast, germfree mice immunized with sheep erythrocytes but without nocardia had significantly less variation (approximately 10%). The mean number of plaques per spleen was similar in all groups. There was a slight, but statistically significant depression in the numbers of plaques per spleen of mice treated with LPS and having nocardia injected intravenously. The numbers of plaque-forming cells per 10^6 spleen cells appeared to be enhanced in germfree mice injected intravenously with *N. asteroides* and decreased in LPS-treated animals injected in the same manner (Table 2). Infecting the LPS-treated mice intranasally with *N. asteroides* GUH-2 appeared to have little or no effect on the numbers of plaque-forming cells within the spleens. No data are presented for nocardia administered intranasally in germfree mice without LPS treatment because of insufficient survivers at 7 days postinfection. Finally, the responses of control germfree (noninjected mice) and LPS-treated mice without nocardia were essentially the same as that for the conventionally grown N:NIH(S) mice (data not shown).

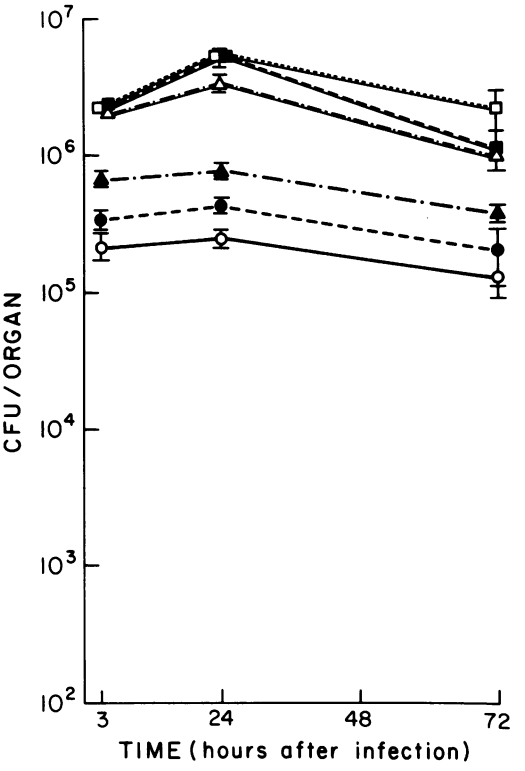

FIG. 7. *Clearance of N. asteroides GUH-2 in the livers and spleens of N:NIH(S) mice after intravenous inoculation (2.8 × 10^6 CFU/mouse). Symbols: ■, livers of unmanipulated germfree mice; △, livers of LPS-treated germfree mice; □, livers of conventionally grown mice; ●, spleens of unmanipulated germfree mice; ▲, spleens of LPS-treated germfree mice; ○, spleens of conventionally grown mice. The bars represent the standard error of the mean.*

TABLE 2. *Response of germfree mice to sheep erythrocytes*[a]

Group[b]	Plaques/spleen	Plaques/10^6 spleen cells
Nocardia IV	29,002 ± 4,573[c]	383 ± 105
LPS + IV nocardia	17,413 ± 7,361	155 ± 62
LPS + IN nocardia	27,606 ± 10,059	217 ± 65
Control	25,620 ± 1,633	261 ± 25

[a] Seven days post intravenous (IV) or intranasal (IN) nocardia infection.
[b] Four mice per group.
[c] Standard deviation.

DISCUSSION

The data presented herein demonstrate that germfree mice respond in a manner significantly different from that of conventionally grown mice after either intranasal or intravenous inoculation with *N. asteroides* GUH-2. Pretreatment of

germfree mice with LPS abrogates this enhanced susceptibility of the germfree mice to nocardia and increases host resistance to levels equal to or greater than the constitutive levels present in the conventionally grown animals that possess a normal resident microflora. Each organ system responds differently, and in an apparently independent manner, to *N. asteroides* GUH-2.

The extreme susceptibility of the brains of germfree mice to invasion by *N. asteroides* was an unexpected observation. The fact that LPS treatment of these mice before infection resulted in complete protection from cerebral invasion strongly implied that the status of macrophages and perhaps other components of the reticuloendothelial system present within the brains of germfree mice was quiescent or retarded in ability to respond to nocardia. In contrast, the host defense mechanisms present within the brain in the LPS-treated animals must have been effectively activated. These data suggest that the presence of a resident microflora within the conventionally grown animal plays a significant role in continuously, but probably non-specifically, activating the host defense capabilities against nocardial infections of the brain, lungs, kidneys, and adrenals. The fact that no differences were observed in the liver, spleen, and blood of conventionally grown, germfree, and LPS-treated germfree mice indicates that host defense mechanisms against *N. asteroides* in these organs are not significantly affected by the absence of a normal, resident microflora. These observations appear to be in sharp contrast to the results reported for *Mycobacterium bovis* Ravenel (33) and *Mycobacterium tuberculosis* (21, 22, 31).

The immunological system of gnotobiotic animals has received considerable attention. Earlier work demonstrated that the lymph nodes of germfree mice were smaller than those of conventionally housed animals (19, 24). This difference was most prominent within Peyer's patches, and it was felt to be attributed to the normally close proximity of Peyer's patches to the resident microbial flora. Histologically, the lymph nodes and spleen in germfree mice have a decreased number of plasma cells within germinal centers (19, 24).

Functional studies, however, reveal that such differences are due to the absence of specific antigenic stimuli because appropriate numbers of plasma cell germinal centers are present and antibody responsiveness can be elicited under proper challenge (19, 24, 29). Indeed, the data presented herein show that the response of germfree mice to the dose of sheep erythrocytes (a thymus-dependent antigen) used in this study is similar in both uninfected mice and animals infected with nocardia. It should be pointed out, however, that because a limited number of mice were available in the germfree isolators, it was not possible to do an extensive study on dose response to sheep erythrocytes. Therefore, a dose lower than the 2×10^8 sheep erythrocytes used in our study may have detected subtle changes. Nevertheless, the size and weight of lymphatic organs in germfree and conventional mice were similar. Further, the number and distribution of the thymus-dependent and thymus-independent areas of lymph nodes were not significantly different in these two groups of animals. The study of macrophages in the spleen and lymph nodes of germfree and conventional mice reveal a similar macrophage frequency, distribution and content of iron and lipochrome (19, 24, 29). Some studies have suggested that there were major quantitative defects in monocyte function in germfree mice (19, 24, 29); however, more recent studies failed to confirm this but suggest that the functional activities of monocytes from germfree animals are reduced or delayed. Even though germfree mice have normally functioning lymphocytes, they have reduced ability to express delayed-type hypersensitivity (19, 24, 32). The changes in the behavior of macrophages in germfree mice might contribute to this (23, 28, 29, 32). Morland et al. (28) studied the morphology, lysosomal enzyme activities, and phagocytic characteristics of peritoneal macrophages obtained from germfree and conventionally grown NMRI mice. They found that peritoneal macrophages from both germfree and conventional mice shared several properties. However, macrophages from germfree mice had increased β-glucuronidase activity but decreased ability to spread on glass, decreased chemotactic response, decreased in vitro induction of lysosomal enzymes and decreased ability to phagocytize particles by way of the C_3b receptors, even though Fc receptor-mediated phagocytic ability was not affected (28).

Most studies involving infectious agents in germfree animals have dealt with either oral or gastrointestinal models (14, 19, 20, 27, 29). Some studies have dealt with the interaction of either *Mycoplasma* or viruses in the respiratory tract (26, 29), but there have been relatively few investigations concerning facultatively intracellular pathogens after systemic or respiratory challenge in germfree mice (19, 21, 22, 31, 33). Ueda et al. (33) demonstrated that germfree mice were more susceptible to infection with the virulent *M. bovis* strain Ravenel than were conventionally grown mice. They found that the major differences were observable in the lungs, liver,

spleen, and heart of the mice and that the differences between the germfree and conventionally grown mice depended upon the size of inoculum used to infect the animals (33). Moreover, there were no differences in these animals until at least 2 weeks after infection. These data disagree with the findings of others who could not demonstrate differences between germfree and conventionally grown mice when infected with *M. bovis* BCG or *M. tuberculosis* $H_{37}Rv$ (21, 22, 31). Similarly, it was shown that the host-parasite interactions of *Salmonella typhi* and *Salmonella enteritidis* injected intravenously into germfree and conventionally grown mice did not differ. However, dramatic differences in these groups of animals were observed when the salmonellae were administered orally (14). In sharp contrast, *N. asteroides* GUH-2 injected intravenously or administered intranasally into germfree mice differed significantly when compared to other pathogens inoculated into germfree animals by the same routes (14, 19, 21, 22, 31, 33). These observations establish further the necessary role of the state of macrophage activation and the development of cell-mediated immunity in host resistance to infection by *N. asteroides*, and they point out the importance of a resident microflora in systemic host resistance to nocardial infections.

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