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Effect of Route of Inoculation on Host Resistance to *Nocardia*

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Virulent strains of *Nocardia asteroides* and *Nocardia caviae* were injected into mice by five different routes. When these organisms were grown to the same stage of growth in the same medium and otherwise prepared identically, it was found that they differed significantly in their ability to infect and kill the host, depending entirely upon the route of inoculation. Thus, *N. caviae* 112 was 30 times more virulent than *N. asteroides* GUH-2 when administered intranasally, whereas *N. asteroides* was at least 10 times more pathogenic than *N. caviae* when injected intravenously. They had similar degrees of virulence when given intraperitoneally. *N. asteroides* GUH-2 induced a more persistent and progressive infection than *N. caviae* 112 when injected into the footpads of mice; however, the latter strain was more lethal for the animals when given by this route. Different routes of infecting mice indicate a compartmentalization of the host response to different strains of nocardia. Therefore, the use of different strains of nocardia under carefully controlled and defined conditions should make it possible to dissect the nocardia-host interactions at the cellular levels.

The route of exposure of the host to a potentially pathogenic microorganism may affect significantly the ability of the organism to evade host defenses and cause disease (14). Thus, the pulmonary response to bacteremic challenge for a given pathogen such as *Staphylococcus aureus* differs from the pulmonary response to an aerosol exposure of the same pathogen (14).

There have been several studies concerning the relative pathogenicities of *Nocardia* for laboratory animals (1, 2, 8, 10-13, 16-24). Many of these reports have been inconclusive or contradictory. Many of the problems resulted from the fact that previous investigators did not standardize their techniques of growing, preparing, and inoculating the organisms into the host. Therefore, it is generally not possible to compare the results of one study with those of another. It has been demonstrated that the relative age of the bacterial culture (thus the phase of bacterial growth) greatly affects the pathogenic potential of *Nocardia asteroides* (6). To understand the mechanisms of nocardial pathogenesis one must establish further the conditions under which it is most or least capable of causing disease.

The effect of the route of inoculation of *Nocardia* into mice has never been reported. Therefore, the data presented here compare the effect of the route of inoculation on mouse susceptibility to virulent *N. asteroides* and *Nocardia caviae*. All experimental variables such as bacterial culture age, growth, and populations of mice were the same throughout this study. Thus, the only variable was the route of administration.

MATERIALS AND METHODS

Organisms. *N. asteroides* GUH-2 was isolated from a fatal human infection and maintained as described previously (5). *N. caviae* CDC 112, isolated from a human infection, was kindly supplied by W. Causey (Center for Disease Control, Atlanta, Ga.). It was grown and maintained as described for *N. asteroides* GUH-2 (6). The growth characteristics of *N. asteroides* GUH-2 have been published (6). *N. caviae* 112 has a similar rate of growth so that a 48-h broth culture appears to be equivalent to a 48-h culture of *N. asteroides* GUH-2 (this corresponds to the beginning of the stationary phase for both organisms). Therefore, the bacteria were grown to the early stationary phase; single-cell suspensions were prepared by differential centrifugation (3, 5, 6), suspended in 0.85% sterile saline, and diluted to the appropriate optical densities (580 nm with a Beckman Spectronic 20). Plate counts were made of the dilutions to quantitate the viable colony-forming units (CFU).

Animals. Groups of random-bred Swiss Webster mice (obtained from Simonsen's, Gilroy, Calif., and maintained as described previously [3, 4, 5, 6]) were given dilutions of the organism by different inoculation routes. All mice in these experiments were the same age (3 to 4 weeks), the same size (18 to 20 g), the same sex (female), and from the same source. Therefore, the animals represented controlled groups.

Infection schedules. Mice were infected by the following routes: intranasally, intravenously, subcutaneously in the tail or in the footpad, and intraperitoneally. They were monitored daily for 3 months. At the end of 3 months 50% lethal dose (LD_{50}) values were determined (9), and surviving mice were necropsied and evaluated for gross macroscopic lesions. Cultures were taken to establish the presence of the appropriate organism. The numbers of bacteria taken into the lungs during intranasal administration was

quantitated at 30 min after infection as previously described (4). In addition, organ clearance studies were done as previously described (4, 5). Quantitative recovery of the organisms from the organs of mice at 4 h and 1 week after intravenous injection was determined by asceptically removing the kidneys, lungs, spleen, liver, brain, adrenals, and blood. The blood (0.1 ml) was plated directly on brain heart infusion 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 Co.). Dilutions of the tissue homogenates were plated on brain heart infusion agar, incubated for 5 days, and quantitated for the numbers of nocardia. Each determination was done on five separate mice for each organ. All experiments were repeated at least once, with similar results.

RESULTS

Table 1 presents the calculated LD_{50} values for *N. asteroides* GUH-2 and *N. caviae* 112 when inoculated into mice by five different routes. When injected intraperitoneally both organisms gave similar LD_{50} values, and the infections inducted by this route were characterized by slowly progressive multiple lesions involving the spleen, mesenteric lymph nodes, liver, diaphragm, kidneys, lungs, and urogenital tract (Fig. 1). With the exception of very high inoculation doses (i.e., $>10^9$), mice inoculated intraperitoneally did not die in less than 2 weeks, and generally the infections progressed for 2 to 3 months before death ensued. It is important to point out that although the LD_{50} for *N. asteroides* GUH-2 given intraperitoneally was about 10^8 CFU/mouse (Table 1), the minimal infectious dose was about 50 times less than this amount. Figure 1 shows the peritoneal cavity of a mouse infected with 10^7 CFU at 3 months before necropsy. All mice (10 of 10) infected with this dose survived for 3 months and appeared at necropsy to resemble the mouse shown in Fig. 1. In subsequent experiments, mice infected with approximately 10^7 CFU of *N. asteroides* survived for more than 6 months even though there were large numbers of abscessed and granulomatous lesions present throughout the peritoneal cavity (viable nocardia were readily isolated from these lesions even at 6 months after infection). These observations were in sharp contrast to those obtained when the mice were inoculated intranasally (Table 1). By this route, the cells of *N. caviae* 112 were approximately 32 times more lethal than cells of *N. asteroides* GUH-2. Furthermore, the infectous process induced by both organisms resulted in an acute pneumonic re-

TABLE 1. *LD_{50} values for N. asteroides GUH-2 (early stationary phase) and N. caviae 112 (early stationary phase) inoculated by different routes into mice*[a]

Organism	Route of inoculation	No. of mice used	Calculated LD_{50} value (CFU/mouse)[b]
N. asteroides GUH-2	Intranasal	45	1.9×10^7
	Intravenous	30	2.5×10^6
	Intraperitoneal	30	1.5×10^8
	Footpad	29	$>5.3 \times 10^8$
	Tail (subcutaneous)	23	7.0×10^8
N. caviae 112	Intranasal	45	6.0×10^5
	Intravenous	23	2.7×10^7
	Intraperitoneal	30	2.5×10^8
	Footpad	30	10^7
	Tail (subcutaneous)	18	5.5×10^7

[a] LD_{50} values were determined at 3 months after infection.
[b] Calculated by the Reed-Muench method (9).

FIG. 1. *Photograph of a mouse sacrificed at 3 months after intraperitoneal injection of 10^7 CFU of N. asteroides GUH-2. None of the mice of this group (10 of 10) died; however, they were all infected as shown in this figure. Therefore, the infectious dose required to cause extensive, chronic infection was much smaller than the dose required to kill the mice within a 3-month period. Note the large, multiple abscesses throughout the peritoneal cavity. At 3 months after intraperitoneal injection of approximately 10^7 CFU of N. caviae 112, the mice did not have similar multiple abscesses as seen here; however, at 1 year after infection multiple abscesses were present.*

sponse in which the mice died within 10 days after infection. All mice that survived for 2 weeks appeared to recover completely from their pulmonary infection. Therefore, *N. caviae* 112 appeared to be most virulent when given intranasally, whereas *N. asteroides* GUH-2 and *N. caviae* had similar pathogenic capabilities when administered intraperitoneally (Table 1).

When *N. asteroides* GUH-2 or *N. caviae* 112 was injected intravenously there was an initial acute phase of illness, and some of the animals died during the first 2 weeks after infection. Those mice which survived this acute response then developed a chronic but progressive disease that ultimately resulted in death. To calculate LD_{50} values, we arbitrarily selected 3 months as the endpoint for these experiments. However, it should be noted that LD_{50} values calculated at 1 year after infection were considerably lower than those presented for the intravenous route shown in Table 1. For the intravenous and intraperitoneal routes of inoculation the minimal infectious doses were considerably less than the minimal lethal doses (Fig. 1).

It was shown previously that different strains of *N. asteroides* exhibited specific organ tropisms when injected intravenously into mice (5). Therefore, we followed the organ distribution of *N. caviae* 112 and *N. asteroides* GUH-2 after intravenous inoculation. We quantitated total recovery at 4 h after infection to determine body clearance (killing) and organ distribution of the organism. These data are presented in Tables 2 and 3. It is clear that the organisms were effectively cleared from the blood stream (>99.9%) in 4 h. Most of the organisms were removed by the liver. Approximately 100% of the inoculum of *N. asteroides* GUH-2 was recovered at 4 h (indicating no killing by the host), whereas only 51% of the *N. caviae* cells were recovered. These

TABLE 2. *Distribution of nocardial cells at 4 h after intravenous injection into mice*[a]

Organ	Distribution[b] of	
	N. asteroides GUH-2[c] (CFU/mouse)	*N. caviae* 112[d] (CFU/mouse)
Blood	7.5×10^2 ($\pm 1.0 \times 10^2$)[e]	<0.01% of total CFU
Brain	6.5×10^2 ($\pm 0.8 \times 10^2$)	2.0×10^2 ($\pm 0.2 \times 10^2$)
Kidneys and adrenals	9.3×10^3 ($\pm 2.0 \times 10^3$)	1.6×10^3 ($\pm 0.1 \times 10^3$)
Liver	4.0×10^6 ($\pm 0.7 \times 10^6$)	1.8×10^6 ($\pm 0.2 \times 10^6$)
Lung	8.5×10^4 ($\pm 0.5 \times 10^4$)	2.6×10^4 ($\pm 0.4 \times 10^4$)
Spleen	2.1×10^5 ($\pm 0.3 \times 10^5$)	7.1×10^4 ($\pm 0.7 \times 10^4$)
Total recovered	4.3×10^6 (100.0)[f]	1.9×10^6 (51.3)[f]

[a] Five mice were used for each determination.
[b] Numbers are based on plate count determinations of 0.1 ml of saline suspension used as the inoculum.
[c] Amount of inoculum, 4.3×10^6 CFU/mouse.
[d] Amount of inoculum, 3.7×10^6 CFU/mouse.
[e] Numbers in parentheses represent the standard error of the mean. Counts for blood are in CFU per milliliter.
[f] Percent of original inoculum.

TABLE 3. *Distribution of nocardial cells at 1 week after intravenous injection into mice*[a]

Organ	Distribution of	
	N. asteroides GUH-2[b] (CFU/mouse)	*N. caviae* 112[c] (CFU/mouse)
Blood	2.1×10^2 ($\pm 1.3 \times 10^2$)[d]	ND[e]
Brain	4.9×10^4 ($\pm 2.4 \times 10^4$)	4.8×10^3 ($\pm 4.3 \times 10^3$)
Kidneys and adrenals	5.2×10^6 ($\pm 3.0 \times 10^6$)	6.9×10^4 ($\pm 2.6 \times 10^4$)
Liver	5.4×10^5 ($\pm 3.7 \times 10^5$)	3.5×10^3 ($\pm 0.4 \times 10^3$)
Lung	4.3×10^3 ($\pm 2.9 \times 10^3$)	ND
Spleen	1.9×10^4 ($\pm 0.6 \times 10^4$)	10 (± 10)
Total recovered	5.8×10^6 (135.1%)[f]	7.7×10^4 (2.1%)[f]

[a] Five mice were used for each determination.
[b] Amount of inoculum, 4.3×10^6 CFU/mouse.
[c] Amount of inoculum, 3.7×10^6 CFU/mouse.
[d] Numbers in parentheses represent the standard error of the mean. Counts for blood are in CFU per milliliter.
[e] ND = None detected at a 10^{-1} dilution in any of five mice.
[f] Percent of original inoculum.

data suggested that some killing of the *N. caviae* 112 by the host occurred within the 4-h period. Furthermore, the 4-h incubation in the mice permitted an adequate assessment of the distribution of the organisms and host clearance because this amount of time was not long enough for nocardial multiplication to occur as the lag phase is about 6 to 8 h for both of these organisms (6). At 1 week after inoculation there were dramatic differences between *N. caviae* 112 and *N. asteroides* GUH-2 regarding clearance of the organisms and bacterial growth within the host (Table 3). Both organisms demonstrated a tropism for the brain, kidneys, and adrenals; however, *N. asteroides* GUH-2 increased within the brain 75-fold and in the kidneys and adrenals 560-fold, whereas *N. caviae* 112 demonstrated only a 24-fold increase in the brain and a 43-fold increase in the kidneys and adrenals. *N. caviae* was effectively eliminated from all other organs, whereas *N. asteroides* tended to persist in high numbers in the liver, lung, and spleen (Tables 2 and 3).

The footpad response of the mice to *N. caviae* differed from that to *N. asteroides* GUH-2 (compare Table 1 with Fig. 2). *N. caviae* induced a more acute illness with a rapid increase of inflammation which peaked at 14 days and then subsided. *N. asteroides* induced a similar footpad response that persisted for more than 28 days. The most striking difference was demonstrated by animal death at 7 to 14 days after injection with 2×10^7 *N. caviae* and not with *N. asteroides* GUH-2 (Table 1). Thus, by LD_{50} determinations, *N. caviae* appeared to be more than 50 times more virulent than *N. asteroides* GUH-2 when injected into the footpad; however, *N. asteroides* GUH-2 infections appeared to persist longer within the footpad than those caused by *N. caviae* 112. Similar, but not as striking, results were observed when the organisms were injected subcutaneously into the tail (Table 1).

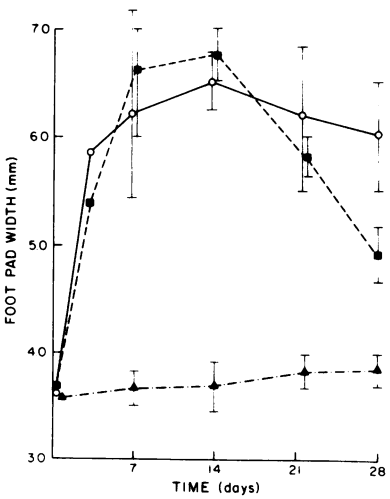

FIG. 2. *Footpad swelling associated with subcutaneous infection after inoculation of N. asteroides* (○) *and N. caviae* (■) *into the footpad. Most of the enlargement is due to extensive abscess formation involving the entire foot. Control mice had sterile saline injected into the footpads* (▲). *The bars represent the standard deviation.*

DISCUSSION

The data presented above show that the host responses to infection with *N. asteroides* GUH-2 and *N. caviae* 112 are quite different. Furthermore, these differences depend upon the route of exposure of the pathogen to the host defense systems. It has already been demonstrated that the method of exposing the lung to infection has a significant effect on how efficiently pulmonary defense mechanisms inhibit *N. asteroides* GUH-2 (4). Thus, *N. asteroides* was shown to be more capable of causing progressive pulmonary disease when the bacterial cells were aspirated into the lung than when aerosols of these organisms were inhaled (4). Similar observations have been reported with *Klebsiella pneumoniae* (7). Furthermore, it has been shown that the clearance of inhaled cells of *S. aureus* from the lung is different from the clearance of these bacteria when distributed in the lung after intravenous inoculation (14). Similar results were obtained for *Nocardia*.

The observations presented here demonstrate that some compartmentalization of host-parasite interactions occur, and this permits some evaluation of the specific mechanisms of host defense against nocardia. For example, the major cellular defense against bacteria introduced into the lungs is the alveolar macrophage, whereas organisms injected into the blood stream have early contact with circulating polymorphonuclear neutrophils as well as with mononuclear phagocytes present within the liver (14). In contrast, organisms injected into the peritoneal cavity initially interact with peritoneal macrophages which have been shown to be morphologically, ultrastructurally, and metabolically different from alveolar macrophages that reside in the alveoli of the lungs (15). Furthermore, nocardial cells introduced subcutaneously in either the footpad or the tail elicit a mixed, localized inflammatory process in which polymorphonuclear phagocytes predominate early, followed by an influx of macrophages and lymphocytes that persists for the duration of the response.

Many investigators have suggested that *No-*

cardia brasiliensis is more pathogenic than *N. asteroides*, whereas others claim that *N. caviae* is most virulent. Some investigators claim that *N. asteroides* is most virulent, whereas still others have indicated that *N. asteroides* is not pathogenic for mice (1, 2, 8, 10–13, 16–24). Unfortunately, all of these studies were done with completely different experimental procedures. That is, the age of the bacterial culture and the methods of preparing the inocula were not standardized and controlled. It has been established now that these variables greatly affect the relative virulences of *Nocardia* (6). In addition, most of the investigators used different routes of inoculation as well as completely different strains of *Nocardia* for their studies. Therefore, it is not possible to compare a study with intraperitoneal infection of *N. asteroides* suspended in hog gastric mucin with a study in which a different strain of *N. asteroides* was injected directly into the footpad or given intravenously (8, 10–13, 16–24).

All of these observations underscore the necessity of defining the route of infection and the methods of preparing the inoculum when analyzing host-parasite interactions.

Clearly, the host-parasite response to *Nocardia* is complex. However, the use of different routes of infection with different strains of *Nocardia* under carefully controlled conditions of growth should make it possible to systematically dissect host-parasite interactions at the cellular levels. These studies can be done with specifically manipulated murine models in combination with the use of genetically controlled immunodeficient animals such as hereditarily asplenic and athymic mice (3, 4, 5).

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