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Infectious Agents in Immunodeficient Murine Models: Pathogenicity of *Nocardia asteroides* in Congenitally Athymic (Nude) and Hereditarily Asplenic (Dh/+) Mice

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Congenitally athymic (Nu/Nu), hereditarily asplenic (Dh/+), and littermate control mice were given intravenous injections of homogeneous cell suspensions of the virulent *Nocardia asteroides* GUH-2. Kill curve, 50% lethal dose, and kidney clearance data were obtained over a period of 3 months postinfection. *N. asteroides* initiated both an acute infectious process and a chronic, progressive disease in these animals when given intravenously. The heterozygous (Nu/+) mice appeared to be slightly more susceptible to the acute phase of infection than their nude littermates. In contrast, nude mice were at least 50 times more susceptible to chronic nocardial infection than were the heterozygous (Nu/+) controls. Swiss Webster specific pathogen-free mice were similar to heterozygous (Nu/+) mice in their susceptibility to *N. asteroides*. The hereditarily asplenic (Dh/+) mice were not as susceptible to lethal infection as were nude mice. However, asplenic mice demonstrated an inability to eliminate nocardia from infected kidneys, whereas their littermate control (+/+) mice were able to mount an effective response and destroy most of the organisms within the kidneys. Similar observations were noted when nude and heterozygous (Nu/+) littermate mice were infected in the footpad. The nude mice developed a systemic infection and died within 4 weeks with little inflammation of the footpad and no macroscopic lesions. In contrast, heterozygous (Nu/+) mice developed extensive local abscesses in the foot that persisted for at least 4 weeks. There was no animal death and no evidence of dissemination. The data presented herein indicate that T cells are essential for adequate host response against infection with a virulent strain of *N. asteroides*.

Substantial information has accumulated on the myriad of immunological factors responsible for retardation of disease, modification of host susceptibility, and influence of anamnestic responses on infectious agents (1, 18, 23). A significant source of this information has been derived from observations of patients with either congenital or acquired defects in their immune system; although such observations have added to our clinical insight, they have practical limitations. Indeed, in studying specific mechanisms of host immunity against a number of chronic infections of humans, appropriate animal model systems may be more useful. Many of the studies on such relationships between host immunity and infectious agents have used experimental animals manipulated either by thymectomy, splenectomy, corticosteroid, and/or cytotoxic drug therapy (9, 24, 28). All of these methods, even when performed in the neonatal period, do not entirely eliminate the role of the thymus or spleen on ontogenic influences exerted during gestation. Even the use of thymectomized, irradiated, bone marrow-restored mice (with and without anti-theta treatment) can only approximate a T-less state. Over the past several years, however, a number of unique spontaneous animal models have been discovered that lend themselves to versatile application in the study of host immunity to infectious agents. These include the congenitally athymic (nude) and hereditarily asplenic (Dh/+) mice (8, 12).

The immunobiology of nude mice is well known and has been recently reviewed (15, 19). Nude mice have a major deficiency of thymic-dependent function as determined by a large number of histological and functional criteria (19). Although they are not totally devoid of lymphocytes with T-cell characteristics, they are devoid essentially of all T-cell function (14, 15, 19). In contrast, heterozygous littermates (Nu/+) appear to be immunologically intact and

have been demonstrated to be similar to conventional (+/+) control mice (15). Comparisons of the immunopathology of nude and Nu/+ mice infected with a variety of organisms are beginning to be explored in depth (16, 20, 21, 22, 25, 29, 30, 31).

Another animal model that has direct relevance to these kinds of studies is the hereditarily asplenic mouse. In 1959 the hereditary absence of the spleen was discovered as a mutant in a mouse colony in England (8). Asplenia is inherited as an autosomal dominant disorder with 100% penetrance. Heterozygotes, besides lacking spleens, have preaxial triphalangy and/or poly-, syn-, or oligodoctyly of the hind feet; affected mice are thus referred to as carrying the Dh gene for dominant hemimelia (i.e., Dh/+). Because of the effect of the spleen on the maturation of T-cell function, Dh/+ mice, in contrast to splenic littermate controls (+/+), manifest a major age-dependent qualitative deficiency of T-cell function as well as a quantitative reduction in serum immunoglobulins (8).

There are only a limited number of investigations that suggest that cell-mediated immunity is important in host resistance to nocardial infections. A very recent study suggests, based entirely on mortality data, that nude mice are more susceptible than Nu/+ littermates to *Nocardia* (13) and that Nu/+ littermates are intermediate in behavior to conventional strains of mice (13). Using more quantitative parameters, we have expanded and clarified the use of these animal models in studying host resistance to infection by a virulent strain of *Nocardia asteroides*. We have established that T cells are required for host clearance of *N. asteroides* by using two different host models.

MATERIALS AND METHODS

Microorganism. *N. asteroides* GUH-2 was isolated from a fatal human infection at Georgetown University Hospital, Washington, D.C. The grey clone of this organism was used throughout this investigation (7). This organism is maintained in animals and transferred on brain heart infusion agar as previously described (7).

Animals. Congenitally athymic mice on an N:NIH(S) background and hereditarily asplenic mice on a B6·CBA background were maintained at the Animal Resources Service, University of California, Davis, as previously described (8, 14, 15). Nude mice were raised by mating Nu/+ females to Nu/Nu males. Hereditarily asplenic mice were raised by mating +/+ females to Dh/+ males. All infected mice were maintained in a special animal room supplied with filtered air and fed Purina laboratory chow ad libitum and acid water (pH 2.5 to 2.8).

Preparation of inoculum. *N. asteroides* GUH-2 was isolated from the kidneys of "normal" Swiss Webster mice 1 week postinfection (by intravenous [i.v.] injection) as previously described (7). Erlenmeyer flasks (250 ml) containing 50 ml of brain heart infusion broth (Difco Laboratories) were inoculated with 0.05 ml of the starter culture and incubated for 72 h at 34°C with 150 rpm rotational agitation. The culture was centrifuged at low speed (ca. $50 \times g$) for 5 min to sediment the clumps of cells, and the supernatant suspension was centrifuged at $3,000 \times g$ for 15 min to pellet the bacterial cells. Phase-contrast microscopy revealed uniform cell suspensions with few or no bacterial clumps (3, 7). The organisms were resuspended in sterile saline (0.85%) and adjusted so that 1.0 ml contained ca. 10^7 organisms; 10-fold dilutions were made.

Five to 10 mice for each datum point were injected i.v. with less than 50% lethal dose (LD_{50}) dose of organisms (determined previously for the "normal" Nu/+ littermate control mice).

Determination of LD_{50} in nude, heterozygous control (Nu/+), Swiss Webster (specific pathogen-free [SPF]), asplenic Dh/+, and control +/+ B6·CBA mice. The saline suspensions of bacteria were adjusted so that 1.0 ml contained ca. 10^8 colony-forming units (CFU), and dilutions were prepared to give 10^7, 10^6, 10^5, and 10^4 CFU per 0.1 ml. Between 10 and 14 mice in each group received 0.1-ml i.v. (tail vein) injections of the appropriate dilutions. After 3 months, the LD_{50} was determined by the method of Reed-Muench (10). In addition, kill curve data were plotted.

Bacterial quantitation in kidneys. Initially, necropsies were conducted on several mice from each group to determine the course of the infection and to establish which organs were involved at the time of animal death. As previously shown, *N. asteroides* GUH-2 when injected i.v. into mice causes progressive fatal destruction of the kidneys. All other organs appeared to be normal. This was also found to be the case with Nu/Nu, Nu/+, Dh/+, and +/+ mice. Therefore, only the kidneys were used for bacterial quantitation (7). Five mice from each group were sacrificed at 3 h and at weekly intervals up to 12 weeks postinfection. At the dose levels of nocardia used, the nude mice began to die between 6 and 7 weeks postinfection. The kidneys were removed aseptically, placed in microhomogenization flasks (Virtis) containing 3.0 ml of sterile saline (0.85%), and homogenized at medium speed for 1 min using a Virtis "45" homogenizer. Serial dilutions were plated from the homogenate onto brain heart infusion agar for direct colony counts. The organisms were quantitated and plotted as CFU per kidney.

Although about 10% of the Dh/+ mice also have hydronephrosis (8), it was found that this kidney defect did not alter the total numbers of nocardia present within the kidneys during the course of the experiment.

Footpad inoculation. We have found that with *N. asteroides* there is a significant difference in host response, depending upon the route of inoculation (B. L. Beaman, unpublished data). Therefore, saline suspensions of the organism (as described above) were prepared, and 0.05 ml containing 10^7 organisms were injected into the hind, left footpads of 8 to 10 nude

and heterozygous (Nu/+) mice of each group. The mean width of the footpads was measured with calibrated calipers before infection and at weekly intervals.

RESULTS

Survival data and LD_{50} values were obtained by using several normal and immunodeficient mice infected i.v. with suspensions of *N. asteroides* GUH-2 (Table 1, Fig. 1a and b). The LD_{50} for "normal" Swiss Webster (SPF) mice during these studies averaged 3.0×10^6 CFU per mouse. Interestingly, the heterozygous (Nu/+) N:NIH-S mice were more resistant to i.v. challenge than were the other mice studied. The control B6·CBA mice were of intermediate susceptibility, while the asplenic B6·CBA and nude (Nu/Nu) N:NIH-6 mice were significantly more susceptible to infection by *N. asteroides* GUH-2 (Table 1). Thus, we infected nude and heterozygous (Nu/+) mice with less than a standard LD_{50} dose (2.6×10^6 and 2.6×10^5 CFU per mouse; Fig. 1a and b). During the acute phase of nocardial infection, the Nu/+ mice appeared to be slightly more susceptible to *N. asteroides* than were nude mice (14/14 Nu/+ mice appeared acutely ill with weight loss while only 4/14 nude mice demonstrated overt illness). In contrast, nude mice were significantly more susceptible than were the heterozygous (Nu/+) littermate control mice to chronic, progressive infection by *N. asteroides* (Fig. 1a and b). Over a period of 18 weeks postinfection, the LD_{50} for the heterozygous (Nu/+) mice was ca. 5.9×10^6 CFU per mouse while the LD_{50} for the athymic mice was 1.1×10^5 CFU per mouse (Table 1). Thus, nude mice were about 50 times more susceptible than the heterozygous controls. It was shown that when *N. asteroides* GUH-2 is injected i.v., the chronically infected mice develop progressive lesions almost exclusively within the kidneys (7). Thus, all of the mice were given i.v. injections of ca. 10^6 CFU of *N. asteroides* GUH-2. The kidneys were removed,

TABLE 1. *LD_{50} values for N. asteroides GUH-2 (3 days) inoculated (i.v.) into different murine hosts*[a]

Mouse strain	No. of mice used	Calculated LD_{50}[b] (CFU/mouse)
Swiss Webster (SPF)	30	3.0×10^6
Nude (Nu/Nu) NIH-S	28	1.1×10^5
Heterozygous (Nu/+) NIH-S	28	5.9×10^6
B6·CBA (normal)	30	8.0×10^5
B6·CBA (asplenic)	27	4.8×10^5

[a] LD_{50} values determined 3 months after i.v. inoculation.

[b] Calculated by the Reed-Muench method (10).

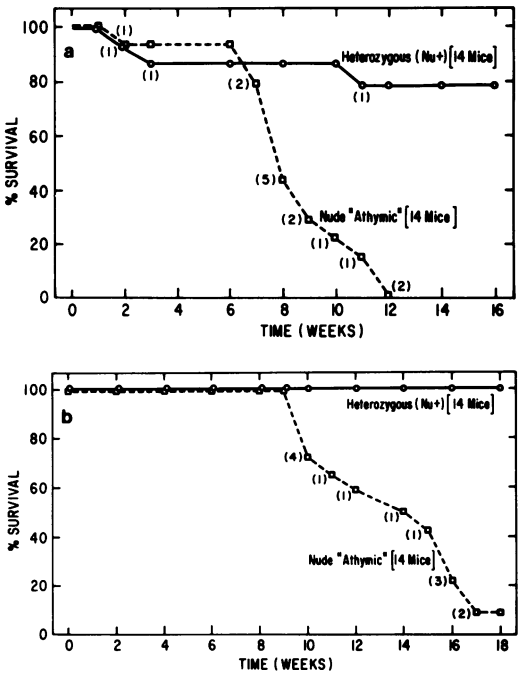

FIG. 1. *Kill-survival plots of mice infected with N. asteroides GUH-2. (a) Plots of mice infected i.v. with 2.6×10^6 cells of N. asteroides per mouse. (b) Plots of mice infected i.v. with 2.6×10^5 cells of N. asteroides per mouse.*

and the number of nocardiae per kidney was determined. The growth of *N. asteroides* within the kidneys of nude, Nu/+, and Swiss Webster mice is shown in Fig. 2 and 3. At 3 h postinfection ca. 10^3 organisms are localized within the kidneys. It has been shown previously that most of the organisms given i.v. became localized within the lung, liver, and spleen (7). However, *N. asteroides* GUH-2 does not grow in these organs, but grows only within the kidney (7). At 1 and 2 weeks after i.v. inoculation there was a proliferation of nocardiae within the kidneys of both animal systems (Fig. 2 and 3). In fact, the growth within the kidney and the severity of kidney lesions appeared to be slightly enhanced in the "normal" heterozygous (Nu/+) littermate controls over those in the T-cell deficient, athymic mice (Fig. 3; $P > 0.05$). Further, some Swiss Webster and heterozygous mice died during this period while only one nude mouse succumbed during the 2 weeks after infection. The most dramatic differences were observed 4 weeks after i.v. injection (Fig. 2 and 3). At 4 weeks postinfection large numbers of organisms were isolated from the kidneys of the athymic (nude) mice. Further, massive kidney destruction was clearly evident. In contrast, few organisms could be isolated from the kidneys of either heterozygous

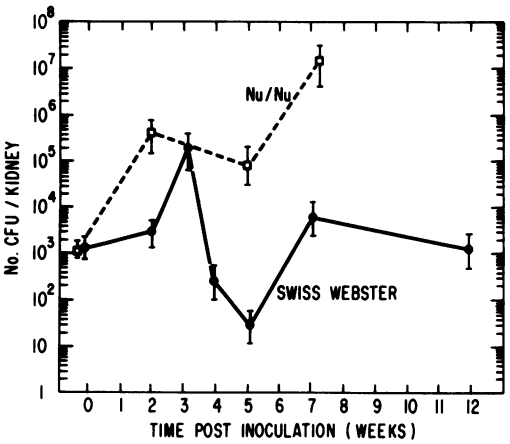

FIG. 2. *Kidney clearance of N. asteroides GUH-2 injected i.v. into nude and Swiss Webster (SPF) mice. The bars indicate standard error of the mean. Each point represents a mean of five mice.*

control or Swiss Webster control mice, and the kidneys showed little macroscopic evidence of infection (Fig. 2 and 3). The infected nude mice began to die at 6 weeks postinfection while the heterozygous mice remained relatively healthy (Fig. 1). Only small numbers of *N. asteroides* GUH-2 cells were able to persist within the kidneys of the heterozygous (Nu/+) control and "normal" Swiss Webster mice for at least 18 weeks. However, the number of organisms within the kidneys of "normal" mice never exceeded 10^4 CFU/kidney even at 18 weeks postinfection. The kidneys of the nude mice always had more than 10^6 CFU/kidney until animal death (Fig. 2 and 3), and at death the kidneys usually had about 10^7 CFU/kidney (Fig. 2).

Injection of 10^7 CFU of *N. asteroides* GUH-2 into the footpads of nude and heterozygous mice gave a very different response than i.v. injection (Table 2). The nude mice infected by the footpad route demonstrated very little inflammation in their feet. In contrast, the littermate controls (Nu/+) developed a dramatic and acute inflammatory response in their footpads. The nude mice became systemically infected after footpad inoculation, and they began to die within 1 week postinfection. Lesions were present in the kidneys, lungs, and spleen. At 2 weeks postinfection, about 40% of the nude mice had died but none of the littermate controls were dead. However, 100% of the heterozygous control mice had developed massive footpad infections with multiple abscesses (Table 2). Four weeks after injecting nocardiae into the footpads, 100% of the nude mice had died of systemic nocardial infection and the footpads showed no macroscopic evidence of infection. In contrast, 100% of the heterozygous, littermate control mice were alive,

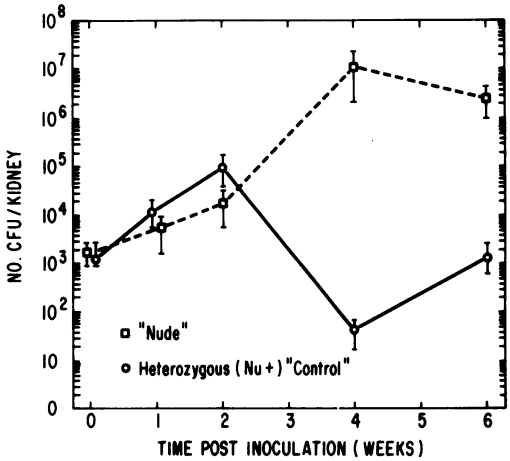

FIG. 3. *Kidney clearance of N. asteroides GUH-2 injected i.v. into nude (Nu/Nu) and heterozygous (Nu/+) littermate control mice. The bars indicate standard error of the mean. Each point represents a mean of five mice.*

TABLE 2. *Footpad response to infection with N. asteroides GUH-2 (10^7 CFU/footpad)*

Mouse type	No. of mice	Time after infection (week)	Mean footpad width (mm)[a]	Animal deaths	Systemic infection at autopsy
Nude (Nu/Nu) NIH-S	8	0	3.2 ± 0.08	0/8	0/8
		1	4.5 ± 0.2	0/8	0/8
		2	4.4 ± 0.2	3/8	3/8
		4	—	5/8	5/8
Heterozygous (Nu/+) NIH-S	10	0	3.5 ± 0.07	0/10	0/10
		1	6.4 ± 0.2	0/10	0/10
		2	6.9 ± 0.2	0/10	0/10
		4	Not measured (about the same as at 2 weeks)	0/10	Sacrificed (10); none had disseminated lesions

[a] ± Standard deviation.

and there was no evidence of disseminated or systemic disease (Table 2). The control mice were sacrificed, and the only lesions we observed were present within the footpads. No organisms were isolated from the kidneys of the heterozygous control mice; however, large numbers of organisms could be isolated from the infected footpads of these control mice. The opposite was true for the infected nude mice.

The LD_{50} data on hereditarily asplenic (Dh/+) mice and their littermate controls being injected i.v. with *N. asteroides* GUH-2 reveal that both are more susceptible to infection than are the Swiss Webster or heterozygous (Nu/+) mice (Table 1). However, kidney clearance studies show a significant difference between the asplenic (Dh/+) and the littermate control (+/+) mice in eliminating *N. asteroides* GUH-2 from the kidneys over a period of 12 weeks (Fig. 4). The mechanisms for this increased susceptibility to *N. asteroides* in asplenic (Dh/+) mice is unclear; however, the young mice (when they received the injection) had a depressed T-cell function which becomes normal as the mice grow older (8). The older mice (i.e., 12 weeks postinfection) have essentially a normal T-cell response, but have major quantitative defects in B-cell function (8). These timed responses may explain, in part, why the asplenic mice are only slightly (twofold) more susceptible to lethal infection to *N. asteroides* while they are greatly altered in clearance of the organism from infected tissues (Table 1, Fig. 4).

DISCUSSION

It appears from the observations presented herein that the heterozygous (Nu/+) control mice are more susceptible during the acute phase of nocardial disease than are the nude mice. These data are similar to previous studies of *Listeria monocytogenes* in nude mice (11), although the acute-phase susceptibility of heterozygous mice to *N. asteroides* is not as great as observed with *Listeria*. The precise mechanism of this apparent enhanced susceptibility during the acute phase of infection remains to be elucidated. It has been attributed to the chronic activated nature of macrophages from nude mice and/or an elevated number of clonogenic PFU-C and CFU-C bone marrow and spleen cell populations in nude mice, but not in the heterozygous (Nu/+) littermate controls (33). However, it becomes very clear that the Nu/+ mouse can mount an effective host response to 10^6 CFU of *N. asteroides* GUH-2 so that the survivors of the acute illness overcome the nocardia and the infection becomes self-limited and usually resolves. Nevertheless, small numbers of nocardial cells may persist within the previously infected tissues indefinitely. In contrast, the nude mouse cannot mount a response sufficiently effective to eliminate the nocardia. Thus, the nocardial cells ultimately replicate, and it appears that the T cell is an essential component of the host defense against *N. asteroides* GUH-2.

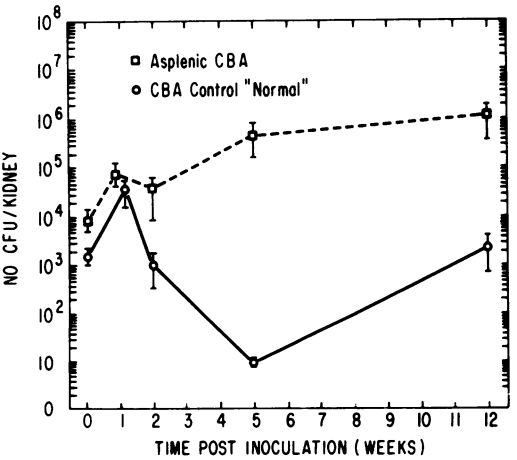

FIG. 4. *Kidney clearance of N. asteroides GUH-2 injected i.v. into asplenic B6·-CBA (Dh/+) and littermate B6·CBA control mice. The bars indicate error of the mean. Each point represents a mean of five mice.*

There have been some studies of the response of nude mice to listerial and mycobacterial infections. It was shown that nude mice differ significantly from their heterozygous (Nu/+) littermates in response to *Mycobacterium bovis* Ravenel, *M. bovis* BCG, *M. avium, M. leprae,* and *M. kansasii* (26, 29). For example, bacterial growth in nude mice after inoculation of these mice with *M. bovis* Ravenel and *M. bovis* BCG was significantly greater than when inoculated into heterozygous controls (29). In contrast, the response of nude and heterozygous (Nu/+) mice after injection with *M. avium* Flamingo was essentially the same, and no statistically significant differences were noted in mycobacterial growth rates in both groups of mice (29). However, the histology of the mycobacterial-infected nude mice was different from that of the littermate controls when all strains of *Mycobacterium* were used (26). Granuloma formation is readily apparent in the heterozygous (Nu/+) mice, whereas granulomas are not readily induced in nude mice (26). Instead, the lesions observed in the nude mice were characterized primarily by either necrotic exudative changes or proliferation of bacilli-loaded cells. During the acute phase of listeriosis, nude mice were more resistant (LD_{50}, 1.2×10^6 CFU) than were the immunologically intact heterozygous controls (LD_{50},

6×10^4 CFU) (11). Similar, but not as pronounced, processes occur during the acute phase of nocardial infection. Although nude mice are 20 times more resistant to acute infection by *Listeria* than the heterozygous control mice (11), the data presented herein demonstrate that nude mice are only slightly more resistant (about twofold) than were the heterozygous controls toward acute infections with *N. asteroides* GUH-2. Further, the nude mice appeared to be more susceptible than the heterozygous (Nu/+) controls to chronic nocardial infections than *Listeria monocytogenes* (11). These results are different from those observed with other microorganisms such as *Candida albicans* and *Staphylococcus aureus* (25). For example, it was shown that nude mice are more resistant to i.v. challenge with *C. albicans* than littermate controls (25), and thymus cell transplants make nude mice susceptible to challenge with *Candida* (25).

The mechanisms of nocardial pathogenesis and specific mechanisms of host immunity against nocardial infections have not been established clearly (4, 5, 6, 32). However, it seems likely that cell-mediated immunity plays a significant role in certain types of nocardial infections (5, 6, 17, 27). Sundararaj and Agarwal (27) presented some data that suggest cell-mediated immunity could be induced in guinea pigs by infecting them with *N. asteroides* emulsified in incomplete Freund adjuvant. It is difficult to assess the effect the oil emulsion has on a normal host response to nocardial infection. The authors claim to have transferred adoptively cell-mediated immunity to *N. asteroides* by spleen cells (27). However, because they used outbred gunea pigs, without regard to histocompatibility type, and because of all the attendant hazards of allogeneic interference, it is difficult to interpret their data (27). In contrast, Krick and Remington reported that resistance to nocardial challenge in "normal" mice could not be transferred by either sera of spleen cells from immune donor mice (17). They found that peritoneal macrophages from mice given *N. asteroides* were "activated" as determined by in vitro L-cell inhibition assay systems. Mice infected with *Nocardia* were resistant to challenge by *L. monocytogenes*. Finally, mice infected with *Listeria* appeared to be more resistant to infection with *Nocardia* (17). Such observations may be critical to understanding the "acute macrophage-dependent period" of the infection. Unfortunately, these investigators used *Nocardia* in hog gastric mucin to enhance nocardial virulence. It is not known what effect the mucin has on the host response to nocardial infection. We have demonstrated that virulent forms of *N. asteroides* that infect mice without using either oil or mucin behave in the animal differently from those strains that are not virulent unless mucin or oil is used (2). However, the data presented by Krick and Remington suggest that "activated" macrophages are important in preventing nocardial infection in mice (17). Similarly, Folb et al. (13) used intraperitoneal injections of wet weight preparations of *Nocardia* to assess immunity. They felt that heterozygous (Nu/+) littermate mice were not good controls for their experiments involving intraperitoneal challenge with crude suspensions of *Nocardia* (i.e., 10 mg [wet wt] of cells); however, they made no attempt to quantitate their results other than by animal death to a single dose of organism. The use of intraperitoneal challenge is fraught with problems in nude mice because of the significant difference of peritoneal exudate cells from nude and Nu/+ mice.

There have been no studies reported that define or identify the specific role of T cells in host resistance to nocardial infection. Our data presented herein, on both nude and asplenic mice, strongly suggest that T cells are generated, which are very important in host resistance against *N. asteroides* GUH-2. Nevertheless, these data do not imply that other factors such as the humoral immune response, polymorphonuclear cell function, and macrophage function are not also important factors necessary for host resistance to infection by *Nocardia*.

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