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Adoptive Transfer of Immunity to *Nocardia asteroides* in Nude Mice

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Nude mice on a BALB/c background were adoptively transferred with unprimed spleen cells, *Nocardia*-primed spleen cells, or *Nocardia*-primed splenic T lymphocytes from syngeneic, heterozygous (nu/+) littermates. Two days later, these recipient mice and unmanipulated (control) nude mice were infected intravenously with a 50% lethal dose of *Nocardia asteroides* GUH-2 from an early stationary-phase culture. Antibody titers, spleen weights, percent mortality, and organ clearance of the microorganisms were measured at 3 h to 28 days after infection. Adoptively transferred nude mice had larger spleens and greater titers of anti-nocardial antibody 7 to 28 days after infection as compared with control nude mice. Adoptive transfer with either primed spleen cells or primed splenic T lymphocytes enhanced both the survival of recipient nude mice and their ability to eliminate *N. asteroides* from the liver and spleen. These data indicate that adoptive immunity to infection with *N. asteroides* can be transferred with either specifically primed spleen cells or splenic T lymphocytes. Thus, it appears that cell-mediated immunity and T lymphocytes are of uppermost importance in host resistance to nocardial infection.

Host resistance to *Nocardia asteroides* involves several aspects of innate and acquired immunity. Specific observations indicate that humoral immunity is not an important mechanism of resistance to nocardial infection. It has been demonstrated that antibody is produced irregularly or in low titers during both human infections (21) and experimental murine infections (B. L. Beaman, unpublished data). In addition, attempts to transfer immunity to *N. asteroides* with serum from immunized mice have been unsuccessful (16). Further, antibody-deficient CBD2/F1 male mice are no more susceptible to infection with *N. asteroides* than are normal female littermates (2). Similarly, innate mechanisms of resistance to *Nocardia* have been shown to be ineffective in assays of nocardicidal activity. Human polymorphonuclear neutrophils do not kill *N. asteroides* in vitro (11) but do inhibit their growth (G. Filice, personal communication). Likewise, normal rabbit alveolar macrophages (1) and mouse peritoneal macrophages (12) cannot kill virulent strains of *N. asteroides* in vitro.

In contrast to these observations, cell-mediated immunity appears to play an important role in host resistance to nocardial infection. Non-specifically activated mouse peritoneal macrophages (12) and alveolar macrophages from immunized rabbits in the presence of primed lymphocytes (9) can kill *Nocardia*. T cells have been shown to play a role in host defense against infection with other facultatively intracellular pathogens, such as *Listeria monocytogenes* (10), *Brucella abortus* (8), *Histoplasma capsulatum* (25), and *Mycobacterium* (20, 24). Likewise, T cells seem to be important in pulmonary clearance of *N. asteroides*, since congenitally athymic (nude) mice cannot eliminate the intranasally administered bacteria (5). In addition, nude mice are 50-fold more susceptible than heterozygous (nu/+) littermates to intravenous challenge with *N. asteroides* (3). Because these data suggest that T cells are critical during murine host resistance to nocardial infection, this study attempts to determine whether resistance to *N. asteroides* can be adoptively transferred to nude mice with T cells from unmanipulated versus *Nocardia*-immunized heterozygous (nu/+) littermates.

MATERIALS AND METHODS

Microorganisms. *N. asteroides* GUH-2 was originally isolated from a fatal human infection at Georgetown University Hospital, Washington, D.C. The organisms were grown in brain heart infusion broth at 37°C and maintained as described previously (7). The pathogenicity of this strain of *Nocardia* for mice has been described previously (6).

Mice. Heterozygous (nu/+) mice on a BALB/c background were used as donors of primed and unprimed

lymphocytes. Congenitally athymic (nude) littermate mice (nu/nu) were used as recipients in adoptive transfer experiments. The nude and heterozygous littermates were maintained at the Animal Resource Service of the University of California as described previously (3). The genetic background of these mice has been described previously (13). All mice were maintained in sterile isolaters supplied with filtered air and fed rodent chow and acid water (pH 2.8) ad libitum.

Immunization of mice. nu/+ mice were immunized with 4% Formalin-killed, early stationary-phase cells of *N. asteroides* GUH-2 by injecting subcutaneously 0.2 ml of cells (18 mg) in incomplete Freund adjuvant. Then, at 2-week intervals, these mice received two footpad boosters of 0.05 ml of nocardial cells (5 mg) in sterile saline.

Preparation of lymphocyte suspensions. Two weeks after the last booster, immunized and unmanipulated nu/+ mice were sacrificed by an overdose of diethyl ether, and their spleens were removed and placed in 35-mm petri dishes with 3 ml of Hanks balanced salt solution (HBSS). A single cell suspension was made by injecting HBSS through a 25-gauge needle into each spleen. The cell suspension was incubated in 0.017 M Tris-buffered (pH 7.2) ammonium chloride (0.16 M) for 5 min at 37°C to lyse erythrocytes and then centrifuged at 200 × *g* for 5 min and washed three times with HBSS. The lymphocytes were filtered through a loosely packed glass wool column to remove clumps and dead cells and adjusted to 10^7 cells per ml in HBSS with 5% fetal bovine serum. A T cell-enriched population of lymphocytes was obtained by the panning technique (26). Affinity chromatography-purified rabbit anti-mouse kappa light-chain antibody was diluted to 10 μg/ml in 0.05 M Tris (pH 9.5). Five milliliters of the antibody was added to 100-mm polystyrene petri dishes which were incubated for 60 min at room temperature and then decanted and washed four times with HBSS and once with HBSS with 5% fetal bovine serum. Five milliliters of the lymphocyte suspension (5×10^7 cells) was added to each plate, incubated for 40 min at 4°C, and then swirled and incubated for an additional 30 min at 4°C. The plates were swirled gently, and the nonadherent cells were removed. These cells were greater than 90% T cells, with approximately 1% contaminating B cells, as determined by immunofluorescence microscopy. All lymphocyte populations were washed and resuspended to 5×10^7 cells per 0.1 ml in HBSS.

Adoptive transfer of lymphocytes. Nude mice were irradiated with 150 rad of X-rays (30 rad/min for 5 min) before the adoptive transfer of lymphocytes. This procedure was found to be necessary to ensure successful adoptive transfer of lymphocytes into nude mouse recipients. Each mouse was injected intravenously with either HBSS, 5×10^7 spleen cells from immunized or unmanipulated nu/+ mice, or 2.5×10^7 splenic T cells from immunized nu/+ mice. Two weeks before the adoptive transfer, immunized and unmanipulated nu/+ donor mice were injected intraperitoneally with 10^8 sheep erythrocytes in sterile saline. Two days after adoptive transfers, all nude mice were injected intraperitoneally with 10^8 sheep erythrocytes to assess the success of the T cell reconstitution. All nude mice were bled 5 days later, and hemagglutinating titers were determined. Nearly all (97%) of the adoptively transferred nude mice developed significant hemagglutinating titers (1:16 or greater), whereas all control nude mice had no or very low titers (1:2 or less). Only these adoptively transferred nude mice which had developed significant agglutinating titers to sheep erythrocytes were used at subsequent time points.

Preparation of inoculum. A 48-h (early stationary-phase) culture of *N. asteroides* GUH-2 was used for all experiments. The culture was centrifuged at 50 × *g* for 5 min to remove clumps of cells, and the supernatant was centrifuged at 500 × *g* for 5 min to pellet the bacterial cells. The pellet was suspended in sterile saline, and the suspension was adjusted to an optical density at 580 nm of 0.20 (approximately 5×10^7 colony-forming units [CFU] per ml) with a Spectronic 20 spectrophotometer. The actual number of CFU per mouse was determined by a plate count on tryptic soy agar plates. Two days after the adoptive transfers, all nude mice were injected intravenously with approximately 5×10^6 cells of *N. asteroides* GUH-2 (a 50% lethal dose for nude mice at 2 weeks after infection).

Quantitation of organisms. The number of *Nocardia* in the organs of five mice from each group was determined at 3 h and 7, 14, and 28 days after challenge. The mice were sacrificed by an overdose of diethyl ether, and the blood was collected for an analysis of serum antibody titers. The livers, spleens, kidneys, and brains were removed asceptically, placed in 3.0 ml of sterile saline, and homogenized for 12 s with a Tekmar Tissumizer high-speed blender as described previously (7). The homogenized tissues were diluted appropriately in sterile saline and plated on duplicate tryptic soy agar plates. The plates were incubated for 48 h at 37°C and then counted to determine the number of CFU per organ.

Antibody determination. Anti-nocardial antibody titers of challenged mice at each time point were determined by solid-phase radioimmune assay, using the methods of Tsu and Herzenberg (23). The antigens for the assay were from the cytoplasmic extract (adjusted to 1 mg of protein per ml) from a 48-h culture of *N. asteroides* GUH-2. The test antibody consisted of dilutions of serum from challenged mice and uninfected control mice. The second antibody was a ^{125}I-labeled rabbit anti-mouse antibody prepared against the whole mouse immunoglobulin fraction. A threefold or greater difference between control and tested sera was considered significant in the determination of antibody titers.

RESULTS

Antibody titers and spleen weights were measured to demonstrate the successful adoptive transfer of *Nocardia*-primed nu/+ lymphocytes into nude mouse recipients. A solid-phase radioimmune assay was used to detect antibodies against nocardial cytoplasmic antigens produced in adoptively transferred and control nude mice. No control nude mice developed any detectable antibody to the nocardial cytoplasmic antigens at any time during the course of infection (Table 1). Nude mice adoptively transferred with unprimed nu/+ spleen cells produced low but significant ($P < 0.05$) levels of anti-nocardial antibodies 14 days after nocardial infection (Ta-

TABLE 1. Demonstration of successful transfer of *Nocardia*-primed lymphocytes into nude mice

Donor cells	Recipient	Anti-nocardial antibody titer[a]		Spleen wt (g)
		RIA	Agglutinating	
None	nu/nu	0 ± 0	1:8 ± 3	0.0701 ± 0.0114
Unmanipulated nu/+ spleen	nu/nu	1:29 ± 12	1:9 ± 4	0.1729 ± 0.0255
Nocardia-primed nu/+ spleen	nu/nu	1:8,110 ± 2,430	1:33 ± 25	0.2573 ± 0.0680
Nocardia-primed nu/+ splenic T	nu/nu	1:192 ± 47	1:12 ± 2	0.2043 ± 0.0578
None	Immunized nu/+	1:16,000 ± 1,570	1:230 ± 26	0.3315 ± 0.0250
None	Unmanipulated nu/+	0 ± 0	0 ± 2	0.1335 ± 0.0054

[a] Numbers represent the mean ± standard error at 14 days after intravenous challenge with 5×10^6 CFU of *N. asteroides* GUH-2 (nu/nu recipients).

ble 1), indicating that the nocardial antigens used in this assay are T dependent. Nude mice which received *Nocardia*-primed spleen cells developed high, early titers characteristic of an anamnestic response (Table 1). For example, at 14 days after nocardial infection, the mean antibody titer of nude mice adoptively transferred with *Nocardia*-primed spleen cells was 280-fold greater than that of nude mice adoptively transferred with unprimed spleen cells ($P < 0.001$). Those nude mice which were adoptively transferred with *Nocardia*-primed splenic T cells developed a low antibody titer 7 days after infection (approximately equal to the titer of nude mice adoptively transferred with unprimed spleen cells), which increased logarithmically through 28 days. Thus, at 14 days, this titer was 42-fold less and at 28 days only 4-fold less than that of nude mice adoptively transferred with *Nocardia*-primed spleen cells.

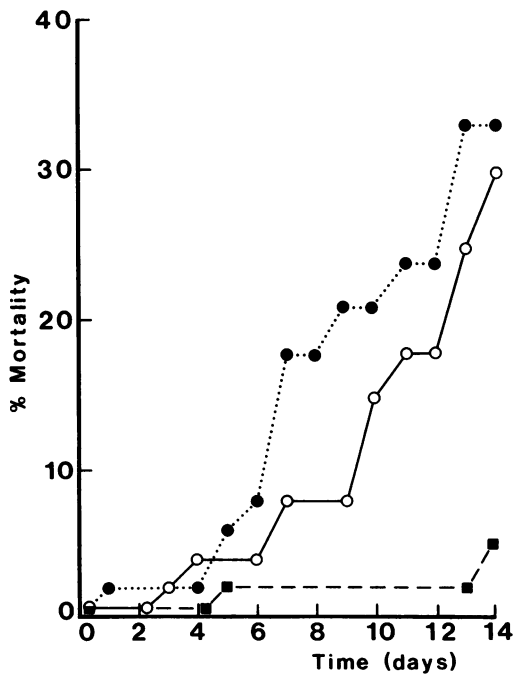

FIG. 1. Percent mortality of nude mice after intravenous infection with 5.3×10^6 CFU of *N. asteroides* GUH-2. Symbols: ○, control nude mice; ●, nude mice which received 5×10^7 unprimed nu/+ spleen cells; ■, nude mice which received 5×10^7 *Nocardia*-primed nu/+ spleen cells. Each group represents a total of 71 to 73 mice combined from three separate experimental determinations.

Mice adoptively transferred with *Nocardia*-primed spleen cells or *Nocardia*-primed splenic T cells developed enlarged spleens compared with control nude mice ($P < 0.025$ to 0.05, respectively) 14 days after intravenous infection with *N. asteroides* (Table 1). Nude mice which received unprimed spleen cells also developed enlarged spleens as compared with those of control nude mice (Table 1). It was also observed that the spleen size of nu/+ mice increased significantly after immunization with *N. asteroides* (Table 1).

Adoptive transfer of *Nocardia*-primed spleen cells, but not unprimed spleen cells, protected nude mice from a 50% lethal dose challenge with *N. asteroides* (Fig. 1). No unchallenged nude mice died during the course of this study. Both control nude mice and nude mice adoptively transferred with unprimed spleen cells had considerably higher mortality rates (29 and 32%, respectively) at 14 days after infection with *N. asteroides* than nude mice adoptively transferred with *Nocardia*-primed spleen cells (5%). The total number of CFU of *Nocardia* recovered per mouse was determined by the summation of total bacterial numbers in the liver, spleen, kidneys, and brain. This number accounted for greater than 99% of the nocardiae found during the course of infection after intravenous inoculation. The total number of CFU of *Nocardia* was reduced significantly in nude mice adoptively transferred with *Nocardia*-primed spleen cells as compared with control nude mice at 7 ($P <$

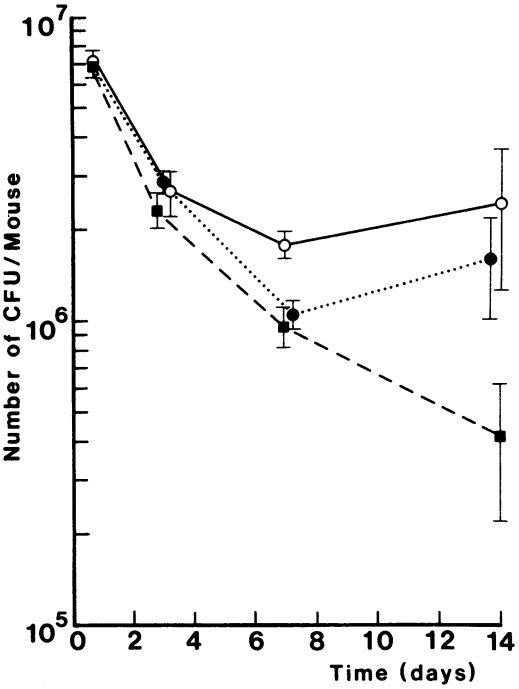

FIG. 2. Total body elimination of *N. asteroides* GUH-2 after intravenous infection with 5.3×10^6 CFU per mouse. Symbols: ○, control nude mice; ●, nude mice which received 5×10^7 unprimed nu/+ spleen cells; ■, nude mice which received 5×10^7 *Nocardia*-primed nu/+ spleen cells. Each point represents the mean ± standard error of between 12 and 22 mice obtained from four separate experimental determinations.

0.005) and 14 ($P < 0.05$) days after challenge with *N. asteroides* (Fig. 2). Thus, at 14 days after infection, nude mice which received *Nocardia*-primed spleen cells were infected with sixfold fewer *Nocardia* than control nude mice, whereas nude mice which received unprimed spleen cells were infected with less than twofold fewer *Nocardia* than control nude mice.

The elimination of *Nocardia* from the liver was greatly enhanced in nude mice adoptively transferred with *Nocardia*-primed spleen cells or *Nocardia*-primed splenic T cells (Fig. 3). Both groups of mice had a greater than 60-fold reduction of *Nocardia* in their livers as compared with control nude mice at 28 days after nocardial infection. Mice reconstituted with unprimed spleen cells did not show the same enhanced clearance as those which received *Nocardia*-primed spleen cells. Thus, at 14 days after challenge, mice which received unprimed spleen cells had only a fourfold increase in nocardial elimination from the liver, whereas mice adoptively transferred with *Nocardia*-primed spleen cells had a 42-fold decrease in bacterial numbers in the liver as compared with control nude mice. In all experiments, the nude mice adoptively transferred with unprimed or *Nocardia*-primed spleen cells appeared to retain larger numbers of *Nocardia* in their spleens initially than control nude mice (Fig. 4). The elimination of *Nocardia* from the spleens was enhanced in nude mice adoptively transferred with primed spleen cells or primed T cells (Fig. 4) and, to a lesser extent, in nude mice which received unprimed spleen cells. Thus, at 14 days after nocardial infection, nude mice adoptively transferred with *Nocardia*-primed spleen cells had a 10-fold decrease of bacterial numbers in the spleen, whereas nude mice adoptively transferred with unprimed spleen cells had only a 2-fold decrease of bacterial numbers as compared with control nude mice. These data suggest that T cells play a role in splenic clearance of bacteria from the bloodstream. The number of *Nocardia* recovered from the brain and kidneys was generally decreased in nude mice adoptively transferred with *Nocardia*-primed spleen cells or *Nocardia*-primed splenic T cells (Table 2); however, the number of organisms recovered from these organs was more variable than that observed in either the spleen or liver.

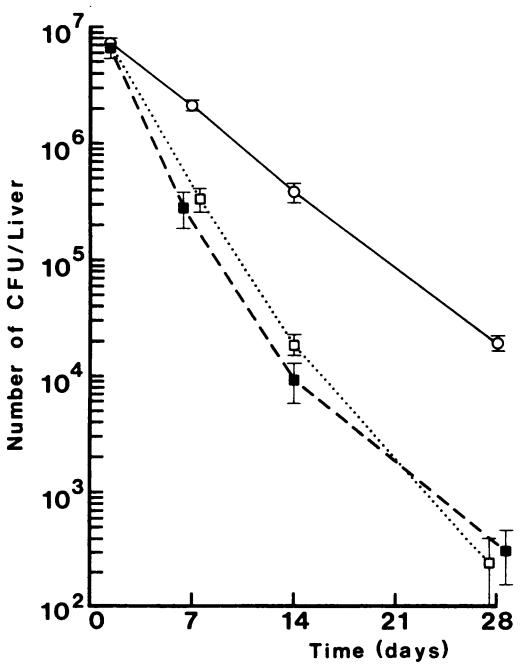

FIG. 3. Elimination of *N. asteroides* GUH-2 from the liver after intravenous infection with 5.6×10^6 CFU per mouse. Symbols: ○, control nude mice; ■, nude mice which received 5×10^7 *Nocardia*-primed nu/+ spleen cells; □, nude mice which received 2.5×10^7 *Nocardia*-primed nu/+ splenic T cells. The bars represent the standard error of the mean.

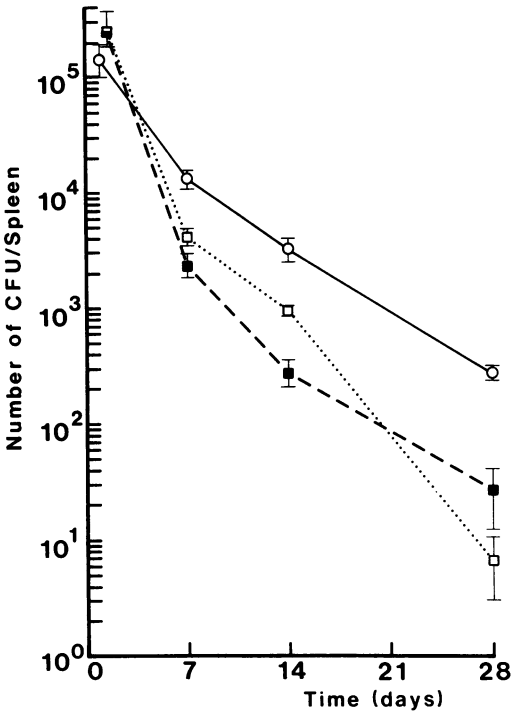

FIG. 4. Elimination of *N. asteroides* GUH-2 from the spleen after intravenous infection with 5.6×10^6 CFU per mouse. Symbols: ○, control nude mice; ■, nude mice which received 5×10^7 *Nocardia*-primed nu/+ spleen cells; □, nude mice which received 2.5×10^7 *Nocardia*-primed nu/+ splenic T cells. The bars represent the standard error of the mean.

DISCUSSION

The adoptive transfer of *Nocardia*-primed spleen cells or *Nocardia*-primed splenic T cells enhanced the elimination of *N. asteroides* GUH-2 most dramatically from the spleen and liver of infected nude mice. The liver and spleen are the two largest organs of the reticuloendothelial system, and it is probable that the fixed macrophages of the liver and spleen are responsible for microbial elimination from these organs. Splenic lymphocytes from heterozygous (nu/+) mice have been shown to home primarily to the spleen and liver when transferred into nude mice (14). In addition, it was shown that *Nocardia*-primed lymphocytes enhanced the ability of rabbit alveolar macrophages to kill *N. asteroides* in vitro (9). Thus, primed T cells appear to activate macrophages of the spleen and liver to enhance elimination of *Nocardia* from these organs.

It was noted that adoptive transfer of *Nocardia*-primed spleen cells did not greatly affect the elimination of *N. asteroides* GUH-2 from the brains or kidneys of nude mice (Table 2). Growth of *Nocardia* in the brains of intravenously inoculated normal mice occurs rapidly during the first 72 h after infection (2). Surviving mice are then able to eliminate *N. asteroides* from the brain after this time (2). None of the nude mice used in these experiments developed significantly increased numbers of *Nocardia* in the brain (less than a fivefold increase above inoculum levels) during the course of infection. In contrast, conventionally housed N:NIH(S) mice typically have a 50- to 100-fold increase of *Nocardia* in the brain at 72 h after challenge (4). The ability of *N. asteroides* to grow in the brain may be a function of the level of macrophage activation, since germfree N:NIH(S) mice had 100 times more *Nocardia* in their brains at 72 h after intravenous inoculation than the same conventionally housed N:NIH(S) mice (4). This increased susceptibility of the brain to nocardial infection could be abrogated by injecting lipopolysaccharide, a nonspecific activator of macrophages, into the germfree mice before challenge (4). It appears that nude mice have enhanced defense mechanisms within the brain,

TABLE 2. Determination of viable *Nocardia* in the kidneys and brains of adoptively transferred nude mice infected intravenously with 5×10^6 CFU of *N. asteroides* GUH-2

Donor cells	Mean ± SE CFU in:	
	Kidneys[a]	Brain[b]
None	$1.59 \times 10^6 \pm 0.86 \times 10^6$	$1.18 \times 10^3 \pm 0.63 \times 10^3$
Unmanipulated nu/+ spleen	$1.37 \times 10^6 \pm 0.56 \times 10^6$	$1.80 \times 10^2 \pm 0.59 \times 10^2$
Nocardia-primed nu/+ spleen	$5.41 \times 10^5 \pm 2.35 \times 10^5$	$2.66 \times 10^2 \pm 0.99 \times 10^2$
Nocardia-primed nu/+ splenic T	$1.00 \times 10^6 \pm 0.71 \times 10^6$	$1.59 \times 10^2 \pm 0.73 \times 10^2$

[a] Results are for 12 to 19 mice obtained from four separate experimental determinations at 14 days after challenge.

[b] Results are for 20 to 25 mice obtained from four separate experimental determinations at 7 days after challenge.

which may be due to the presence of nonspecifically activated macrophages (8). Thus, even specifically primed T cells could not significantly ($P < 0.15$) enhance elimination of *Nocardia* from the brains of nude mice.

Growth of *N. asteroides* GUH-2 in the kidneys follows a more chronic course. The number of *Nocardia* increases in the kidneys after 72 h and continues at high levels for several weeks in both normal (2) and nude (3) mice. Immunization of mice before challenge with *N. asteroides* lowers the number of *Nocardia* in the kidneys only 10-fold as compared with nonimmunized mice (2), and the *Nocardia* can still persist in high numbers within the kidneys of immunized mice (2). Since the kidneys do not have a large population of resident macrophages, initial host defense against infection of the kidneys probably involves circulating blood monocytes and neutrophils. Other studies have shown that these cells are not effective at killing *N. asteroides* (11).

Although antibody was produced in adoptively transferred nude mice, it appears unlikely that it was protective. Very low agglutinating antibody titers to *Nocardia*, but high anti-nocardial radioimmune assay antibody titers were found in adoptively transferred nude mice. Other studies have shown that high radioimmune assay antibody titers (2) and agglutinating antibody titers (2, 16) have little effect on the course of murine infection with *N. asteroides* GUH-2.

Before this study, there have been no reports in the literature regarding the role of T lymphocytes in adoptive transfer of immunity to *Nocardia asteroides*. Sundararaj and Agarwal (22) reported on the adoptive transfer of cell-mediated immunity to *N. asteroides* in guinea pigs. Unfortunately, these workers used outbred guinea pigs and whole-spleen cell transfers by intracardial injection without appropriate antigen controls to measure the success of their adoptive transfers. Because of allogeneic interference and subsequent nonspecific activation of macrophages in this system, it is not possible to interpret their results. Further, no attempt was made to define the role of T cells in their adoptive transfer experiments, and the adoptive transfer of spleen cells from unmanipulated donors was not included as a control in their system.

Although nude mice have some lymphocytes that express a low density of Thy-1 antigen on their surfaces (17, 18), they have little or no T cell function (19). The lack of helper T cell function was also demonstrated in this study by the inability of these mice to produce antibody against T-dependent antigens (sheep erythrocytes and nocardial cytoplasmic antigens). The adoptive transfer of *Nocardia*-primed nu/+ spleen cells to nude mice enhanced the survival of these mice to intravenous inoculation with *N. asteroides* GUH-2. It is probable that the enhanced survival of these mice is due to a population of specifically primed T cells. These T cells can activate macrophages in vitro to enhance their ability to kill *N. asteroides* (9). In addition, preliminary evidence in our laboratory indicates that a subpopulation of specifically primed T cells can kill *N. asteroides* directly in vitro. The increased resistance of nude mice reconstituted with specifically primed T cells establishes the importance of these cells in host defense against infection with *N. asteroides*.

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