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Response of CBA/N × DBA2/F_1 Mice to *Nocardia asteroides*

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Immunized and nonimmunized B-lymphocyte-deficient CBD2/F_1 (CBA/N × DBA/2) mice were infected with *Nocardia asteroides* GUH-2 by different routes of inoculation. The 50% lethal dose, organ clearance, footpad response, and antibody titers were measured. It was observed that B-cell-deficient male mice were not significantly more susceptible to infection than normal female controls even though the female CBD2/F_1 mice produced antinocardial antibodies while the deficient male animals did not. Preimmunized male and female mice were identical in their ability to clear *N. asteroides* from the adrenals, brain, kidneys, liver, lungs, and spleen. Both DBA/2 and CBD2/F_1 female mice were more susceptible than their male littermates to intravenous challenge with *N. asteroides* GUH-2. This enhanced susceptibility of the female mice as compared to the male littermates appeared to be due to a decreased resistance to nocardial infections in the brains of the female animals. These data indicate that antibody and certain B-lymphocyte subpopulations are not essential components in host resistance to *N. asteroides* GUH-2 in these mice.

The relative importance of humoral and cellular immunity to nocardiosis is not clearly defined. Previous studies have dealt with the in vitro interaction of *Nocardia asteroides* with macrophages (2, 14), polymorphonuclear neutrophils (13), monocytes (13), lymphocytes, and antibody (11). Furthermore, asplenic and athymic murine models were used to determine the roles of T-cell lymphocytes in host resistance (3, 5). The data from these investigations established that cells of *N. asteroides* are able to grow within macrophages. However, both specifically and nonspecifically activated macrophages appeared to be important in limiting nocardial growth (2, 4, 11, 14). In contrast, polymorphonuclear phagocytes and peripheral blood monocytes were not able to kill *Nocardia*, and their role in host resistance to *N. asteroides* is uncertain (13). T-cells were shown to be important to pulmonary clearance and prevention of dissemination of virulent strains of *N. asteroides* from the lungs (5), and T-cells were essential for an effective host response against systemic infection (3).

Although the data indicate that T-cells and cell-mediated immunity are important in host resistance to *Nocardia*, there is little information that defines the role of B-cells in the host response to *N. asteroides*. CBA/N mice are a distinct subline that have an X-linked deficiency in B-lymphocyte function. As a consequence, CBA/N mice have a poor antibody response to both T-dependent and T-independent antigens, and they possess abnormally low levels of immunoglobulin M (IgM) and IgG3 antibody (1, 12, 19). These mice appear to have a functional T-cell population. Furthermore, it has been shown that F_1 males derived from mating CBA/N females with immunologically normal males are deficient in B-cell function. These F_1 males are unable to respond to antigens, whereas the female littermates have an intact B-cell system and can respond to antigens to produce antibody (1, 9, 10, 12, 15, 16). These mice should provide an excellent model for studying the role of B-lymphocytes in host resistance to *N. asteroides*.

MATERIALS AND METHODS

Microorganisms. *N. asteroides* GUH-2 was isolated from a fatal human infection at Georgetown University Hospital, Washington, D.C. The organisms were grown in brain heart infusion (BHI) broth at 37°C as previously described (7). The pathogenicity of this strain of *Nocardia* for mice has been described (7, 8).

Mice. CBA/N (XID) B-cell-deficient female mice were mated with DBA/2 males to produce F_1 progeny; the CBD2/F_1 males are B-cell deficient, whereas CBD2/F_1 female littermates (controls) are immunologically intact. Both male and female mice were from matched groups, and all mice used in these experiments were of the same age and approximate size. Thus, nonimmunized mice were 6 weeks old and weighed approximately 20 g. Immunized mice were 12

weeks old and weighed approximately 25 g. Male and female DBA/2 mice were obtained from Charles River. All mice were 6 weeks old and weighed approximately 20 g. The mice were maintained at the Animal Resources Service of the University of California as previously described (8). All infected mice were maintained in a special animal room supplied with filtered air and were fed Purina laboratory chow ad libitum.

Preparation of inoculum. Fresh animal isolates of *N. asteroides* were grown in BHI broth to the early stationary phase of growth as previously described (7). 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 5 min to pellet the remaining bacteria. The organisms were suspended in sterile 0.85% saline. 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 desired number of colony-forming units (CFU) per milliliter.

Infection schedules. Intranasal infection of normal and immunized CBD2/F_1 male and female 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 quantitatively aspirated into the lungs as previously described (4). For 50% lethal dose (LD_{50}) determinations, six male and six female mice received between 1.3×10^8 and 1.3×10^6 CFU per mouse, and for lung clearance studies all mice received 1.7×10^6 CFU in the left lobe of the lung.

Intravenous (i.v.) infection and organ clearance studies of these mice were performed with serial quantitation of the organisms from the organs of five mice (3, 24, 72, 168, and 336 h after infection) as previously described (4). The blood (0.1 ml) was plated in duplicate onto BHI agar for quantitation. All other organs (adrenals, brain, kidneys, liver, lungs, and spleen) were homogenized with a Tekmar Tissumizer high-speed blender, and dilutions of the tissue homogenates were plated on BHI agar, incubated at 37°C, and counted (4). For the organ clearance determination, all nonimmunized mice received 3.3×10^6 CFU per mouse i.v., whereas all immunized mice received a dose of 1.3×10^7 CFU per mouse.

Immunization of mice. Male and female CBD2/F_1 mice were immunized subcutaneously (posterior to the head) with 18 mg of a 4% Formalin-killed suspension of *N. asteroides* GUH-2 in incomplete Freund adjuvant. Two booster immunizations of 5 mg of *N. asteroides* suspended in saline were given in the footpad at 2-week intervals. During the second booster, the footpad response was measured with adjustible calipers and compared with footpads of normal CBD2/F_1 mice that had not been immunized. The agglutinating antibody titer of the serum from infected, immunized and infected, nonimmunized mice was determined at the time of sacrifice.

Antibody determination (solid-phase RIA). Total antinocardial antibody titers of immunized mice were determined by solid-phase radioimmune assay (RIA) by the methods of Tsu and Herzenberg (21). 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 immunized and nonimmunized (control) CBD/N sera. 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.

Determination of animal susceptibility to *N. asteroides*. CBD2/F_1 male and female mice were infected with 10-fold dilutions of bacteria (six mice per group) by the following routes: intraperitoneal (9.1×10^8 to 9.1×10^6 CFU per mouse), intranasal (1.3×10^8 to 1.3×10^6 CFU per mouse), footpad (1.3×10^8 to 1.3×10^6 CFU per mouse), and i.v. (3.3×10^7 to 3.3×10^5 CFU per mouse). In addition, the susceptibilities of male and female DBA/2 mice were determined by using twofold serial dilutions (2.4×10^7 to 7.5×10^5 CFU per mouse). The mice were monitored daily, and at the end of 3 months LD_{50} values were determined by the Reed-Muench method (20). Surviving mice were necropsied and were evaluated for macroscopic and microscopic lesions and cultured for nocardial cells.

RESULTS

The LD_{50} values obtained for CBD2/F_1 mice after infection with *N. asteroides* GUH-2 are shown in Table 1. It was found that the B-cell-deficient male mice were eight times more resistant to i.v. challenge than were the female littermates. In contrast, when the organisms were administered intranasally, the male mice were two times more susceptible than the female controls, whereas no differences were observed in host susceptibility to either intraperitoneal or footpad challenge (Table 1). The differences between male and female mice after i.v. challenge appeared to be associated with sexual differences because similar results were obtained using DBA/2 male ($LD_{50} = 3.4 \times 10^6$ CFU per mouse) and female ($LD_{50} = 1.0 \times 10^6$ CFU per mouse) mice.

To assess the immunological responsiveness of CBD2/F_1 mice to *N. asteroides*, delayed-type

TABLE 1. LD_{50} of *N. asteroides* GUH-2 for CBD2/F_1 mice

Route	Sex	B-cell defect	LD_{50} (CFU per mouse)[a]
i.v.	M	+	8.3×10^6
i.v.	F	−	1.1×10^6
Intraperitoneal	M	+	1.6×10^8
Intraperitoneal	F	−	1.6×10^8
Intranasal	M	+	6.1×10^6
Intranasal	F	−	1.2×10^7
Subcutaneous (footpad)	M	+	$>1.0 \times 10^8$
Subcutaneous (footpad)	F	−	$>1.0 \times 10^8$

[a] Calculated by the Reed-Muench method (20) at 3 months after infection.

hypersensitivity, agglutinating antibody titers, and RIA titers were determined. The mice were immunized by repeated subcutaneous injections of killed cells of *N. asteroides* over a period of 6 weeks. After injection of 5 mg of dead cells into the posterior right footpads, it was shown that all mice exhibited delayed-type hypersensitivity as measured by increased footpad size (Fig. 1). The male mice did not differ from the female animals in the intensity of the response. Nonimmunized mice demonstrated no increase in footpad size after injection of dead *Nocardia* (Fig. 1). The agglutinating antibody titers for the immunocompetent female mice immunized as described above averaged 1:128 ± 75, and all of the mice had titers greater than 1:64 (Table 2). None of the B-cell-deficient male mice had agglutinating antibody titers at a 1:2 dilution (Table 2). These results indicate that both the B-cell-deficient and control mice were able to develop delayed-type hypersensitivity and presumably cell-mediated immunity, whereas the B-cell-deficient males were not able to mount an agglutinating antibody response (probably IgM) to *N. asteroides*. Furthermore, it was shown that only the female mice had detectable levels of antibody as determined by RIA (Table 2).

The pulmonary response of intranasally instilled cells of *N. asteroides* was assessed in both immunized and nonimmunized male and female CBD2/F1 mice (Fig. 2). The nonimmunized, B-cell-deficient males were not as capable of inhibiting the growth of *N. asteroides* in their lungs as were the female controls (Fig. 2). On the other hand, both the male and female immunized animals were equally capable of clearing *N. asteroides* GUH-2 from their lungs (Fig. 2).

TABLE 2. Antibody responses of CBD2/F1 mice immunized with killed cells of *N. asteroides* GUH-2[a]

Mouse no.	Sex	B-cell defect	Agglutinating titer[b]	RIA titer
1	F	–	1:128	1:100
2	F	–	1:64	NT[c]
3	F	–	1:64	1:750
4	F	–	1:128	1:10,000
5	F	–	1:256	1:500
6	M	+	0[d]	0
7	M	+	0[d]	0
8	M	+	0[d]	NT
9	M	+	0[d]	0
10	M	+	0[d]	0

[a] Antibody titer was determined 1 week after the final booster.
[b] Antibody titer determined 3 h after infection.
[c] NT, Not tested.
[d] Undiluted antibody.

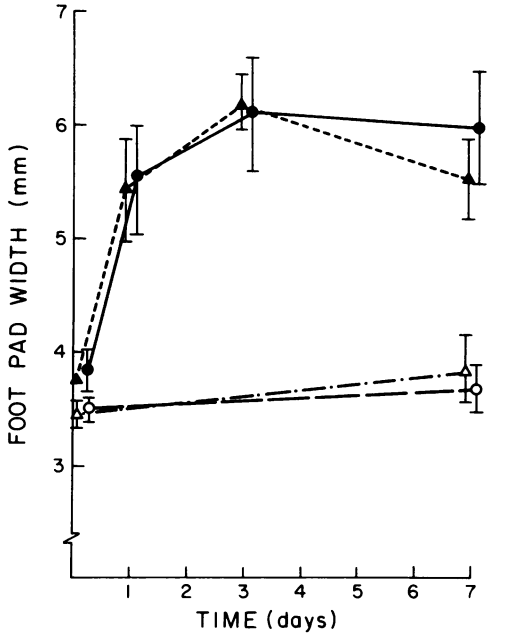

FIG. 1. Footpad response to injection of Formalin-killed cells (5 mg per mouse) of *N. asteroides* GUH-2 into immunized and nonimmunized CBD2/F1 mice. (○) Nonimmunized female control mice; (△) nonimmunized male B-cell-deficient mice; (●) immunized female mice; (▲) immunized male B-cell-deficient mice. (Bars represent standard deviation).

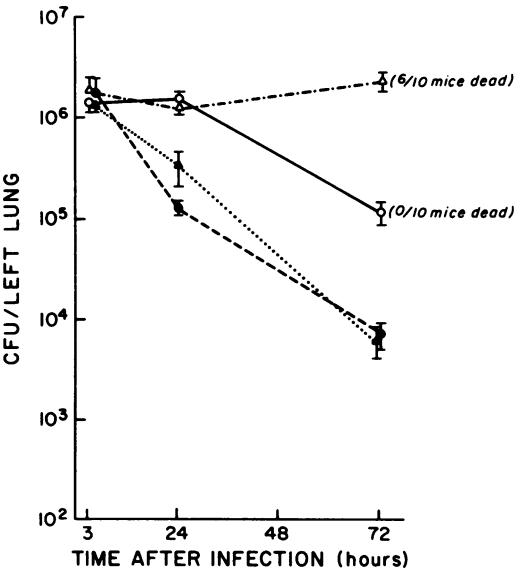

FIG. 2. Pulmonary clearance of *N. asteroides* GUH-2 after intranasal administration of 1.7×10^6 ($\pm 0.2 \times 10^6$) CFU into the left lobe of the lung of immunized and nonimmunized CBD2/F1 mice. (△) Nonimmunized male, B-cell-deficient mice; (○) nonimmunized female controls; (▲) immunized male, B-cell-deficient mice; (●) immunized female controls. (Bars represent standard error).

Because of the early increased susceptibility of the male mice to pulmonary infections (in less than 72 h), these results probably represent differences in pulmonary clearance of *N. asteroides* in nonimmune mice independent of B-cell functions. Furthermore, cell-mediated immunity probably played the major role in host resistance to pulmonary challenge with *N. asteroides* since the immunized B-cell-deficient males and female littermate controls behaved identically (Fig. 2).

In a separate series of experiments, male and female immunized and nonimmunized CBD2/F_1 mice were given i.v. injections of saline suspensions of *N. asteroides* GUH-2. The numbers of bacteria in the lungs, adrenals, brain, kidneys, liver, and spleen (Fig. 3A through F, respectively) were quantitated at 3 h and at 1, 3, 7, and 14 days after injection. No substantial differences were observed between the male and female immunized mice (Fig. 3). The cells of *N. asteroides* were uniformly inhibited or eliminated from all organs as compared to the nonimmunized controls (Fig. 3); greater than 99% of the bacteria were removed from the blood of all animals at 3 h, and the blood was sterile in 72 h. However, there was a greater initial growth of *N. asteroides* in the brains of the nonimmunized female controls than in those of the B-cell-deficient male mice (Fig. 3C). This rapid growth within the brains of nonimmunized mice proba-

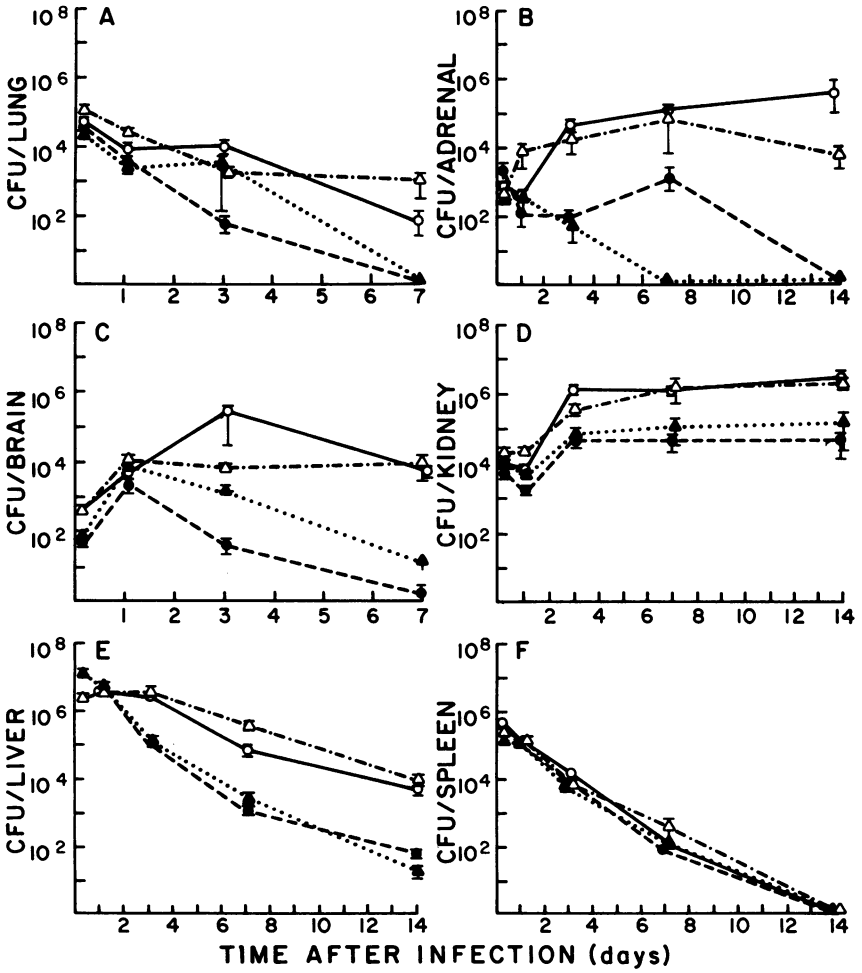

FIG. 3. Clearance and distribution of *N. asteroides* GUH-2 after i.v. injection into CBD2/F_1 mice. All nonimmunized male and female mice received 3.3×10^6 CFU per mouse, whereas, all immunized mice were given a normally lethal dose of 1.3×10^7 CFU per mouse. None of the immunized mice died; however, all of the nonimmunized mice given this same dose died within 72 h after injection. (○) Nonimmunized female control mice; (△) nonimmunized male B-cell-deficient mice; (●) immunized female mice; (▲) immunized male, B-cell-deficient mice. (Bars represent standard error).

bly accounted for the increased susceptibility of female mice over the males to acute death after i.v. inoculation (Table 1, Fig. 3C); this altered susceptibility may be related to hormonal (sexual) differences in the mice rather than the status of the B-cell population. Using normal DBA/2 male and female mice as controls, similar differences were found in the brain at 72 h after i.v. injection with 6×10^6 CFU per mouse; the DBA/2 males had only 3.8×10^3 ($\pm 2.3 \times 10^3$) CFU in their brains, whereas the brains of the females had 3.4×10^4 ($\pm 2.3 \times 10^4$) CFU.

DISCUSSION

Studies have shown that both CBA/N and CBD2/F_1 male mice do not lack functional B-lymphocytes completely (1, 12). However, it was demonstrated that there is a major defect within these mice that results from a subpopulation of B-cells that normally appears late in the development of the immature mouse (12). Furthermore, there is an inability of the macrophages within these mice to present antigens to Lyb 5-lymphocytes (A. Singer et al., Fed. Proc. 39:807, 1980). These defects within the subpopulation of B-cells result in reduced levels of IgM and IgG3 antibodies within the serum as well as the inability of these mice to respond to certain antigens (1, 9, 12, 18, 19).

Several investigators have utilized CBD2/F_1 B-cell-deficient mice to study the role of B-cells in host resistance and immunity to infectious agents (9, 10, 15, 16, 18, 19). O'Brien et al. (19) showed that B-cell-deficient male mice were 1,000 times more susceptible to infection with *Salmonella typhimurium* than were the normal CBD2/F_1 females. Furthermore, the deficient males produced significantly less IgM and IgG antibody against *S. typhimurium* than did the normal females. These investigators concluded that B-cells and humoral responsiveness play a significant role in host resistance to *S. typhimurium* and that CBD2/F_1 mice represent a good model for studying the roles of B-lymphocytes in immunity to *Salmonella* (18, 19).

The role of B-lymphocytes in host resistance and immunity to *N. asteroides* has not been determined adequately. Furthermore, the importance of antibody to protection of the host against *N. asteroides* is not clear. Even though circulating antibodies to nocardial antigens can be detected in individuals, passive transfer of these antibodies does not appear to enhance host resistance to *N. asteroides* (17). However, it was shown that treating mice with cyclophosphamide, which can inhibit the humoral response, significantly enhanced host susceptibility to *Nocardia* (6). As a consequence, it was suggested that a B-cell response and humoral immunity are important in host resistance to *N. asteroides* (6). Since cyclophosphamide is a polyfunctional, cytotoxic compound it is not possible to attribute its action in mice solely to B-cell function. Therefore, the utilization of genetically controlled B-cell-deficient mice that have defined B-cell deficiencies would permit a more precise evaluation of the role of B-cells in host immunity to *N. asteroides*.

The data presented herein demonstrated that B-cell-deficient CBD2/F_1 male mice were not significantly more susceptible to infection with *N. asteroides* than the CBD2/F_1 female mice that possessed a normal B-cell population. In fact, the deficient male mice were eight times more resistant than the normal females to i.v. challenge with a lethal dose of *N. asteroides*. There was a slightly greater, but possibly insignificant, susceptibility (twofold) of the deficient mice to intranasal infection, but since this enhanced susceptibility occurred during the acute phase of infection (less than 72 h) it is likely that these results were not due to a B-cell defect.

Both deficient and normal CBD2/F_1 mice that were immunized with killed cells of *N. asteroides* developed the same enhanced ability to resist either an intranasal or i.v. challenge with a lethal dose of viable cells of *N. asteroides*. Injection of the antigens of *N. asteroides* into the footpads of both B-cell-deficient and normal immunized mice demonstrated that these animals possessed identical abilities to mount a delayed-type hypersensitivity response as measured by footpad swelling. These observations suggested that the cell-mediated immune response was most important in host resistance to *N. asteroides* GUH-2.

The determination of both agglutinating antibody and RIA titers in the immunized mice demonstrated that all normal female CBD2/F_1 mice mounted a measurable humoral response to cells of *N. asteroides*, whereas, in contrast, none of the B-cell-deficient males was able to produce measurable levels of antibody against *Nocardia*. These findings are consistent with the results of other investigators, who found decreased levels of antibody in immunized CBD2/F_1 male mice (9, 10, 18, 19). It seems that the humoral immune response of CBD2/F_1 mice plays little role in increasing host resistance to infection with *N. asteroides* GUH-2 because both the B-cell-deficient male and the normal female mice were equally resistant to lethal infection in spite of the measurable differences in antibody responses.

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