Effect of Cyclophosphamide on Experimental Nocardia asteroides Infection in Mice

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It was found that cells of Nocardia asteroides GUH-2 (virulent) were approximately 10 times more virulent than cells of N. asteroides 14759 (intermediate) and greater than 500 times more virulent than N. asteroides 10905 (avirulent) cells when early-stationary-phase cultures suspended in saline were injected intravenously into "normal" mice. There appeared to be a specific organ tropism for each strain. Thus, N. asteroides GUH-2 infected primarily the kidneys, N. asteroides 14759 infected the lungs and heart, and N. asteroides 10905 (in large doses) infected the lungs. Cyclophosphamide treatment of the mice 72 h prior to infection dramatically increased host susceptibility to nocardial infection, especially to N. asteroides 14759. However, cyclophosphamide treatment did not significantly alter the organ specificity for each strain. Cyclophosphamide greatly enhanced the ability of the nocardial strain to grow within its target organ and significantly altered normal host clearance from these organs.

In humans it is well established that Nocardia asteroides has the lung as its primary target organ, with the brain and central nervous system being frequent secondary sites (5, 28). However, it has also been reported that the heart, kidneys, eyes, and other organs, occasionally serve as primary sites of infection, especially in compromised hosts (5, 28). During the investigation of experimental infections in mice, we noted that there were marked differences in the ability of different strains of N. asteroides to cause either acute or chronic progressive infections (2, 4). We observed what appeared to be specific organ tropisms depending upon the relative virulence and strain of organism being studied (B. L. Beaman, unpublished data). Therefore, we designed experiments to establish clearly the relative virulence of several strains of N. asteroides for mice. Three strains that varied from low, intermediate, and high virulence were then selected and studied further.

The specific mechanisms of host immunity against nocardial infections has not been clearly established (4, 28). It has been shown that certain strains of N. asteroides can grow as facultative intracellular parasites (6). Furthermore, Ortiz-Ortiz and others have shown that specific components extracted from Nocardia can inhibit macrophage migration in vitro and elicit a delayed hypersensitivity response in animals (19, 20, 28). Therefore, it seems likely that Nocardia gives rise to a cell-mediated form of immunity similar to what has been observed with Mycobacterium, Brucella, Listeria, and Salmonella (24, 26, 28).

In the present study, we selected a polyfunctional cytotoxic agent (cyclophosphamide) as a model immunosuppressive agent to investigate further the host interaction with N. asteroides of low, intermediate, and high virulence. Cyclophosphamide was selected for this study for the following reasons: (i) a number of patients receiving cyclophosphamide therapy also develop nocardial infections (5); (ii) the effects of cyclophosphamide on host immunity and the host cell response have been studied (11, 13, 15, 21, 25); (iii) cyclophosphamide appears to affect specifically B-lymphocyte cell function the most (25); (iv) its effects on cell-mediated immunity to bacterial, fungal, and viral infections have been studied (1, 7, 12, 23, 26); and (v) trial experiments by us demonstrated that cyclophosphamide altered the host response to nocardial infection more profoundly than did comparable doses of cortisone (17), prednisolone, or azathioprine (Beaman, unpublished data).

MATERIALS AND METHODS

Microorganisms. N. asteroides 10905 was supplied by J. Rozanis, University of Western Ontario, London, Canada. N. asteroides 14759 was obtained from the American Type Culture Collection, Rockville, Md. N. asteroides GUH-2 was isolated from a fatal human infection at Georgetown University Hospital, Washington, D.C. The patient had a renal transplant, and the organism was isolated from the patient's kidney.

Each strain of Nocardia produces at least two
colony types upon initial isolation. On brain heart
infusion agar, these are recognized usually as either
chalk white colonies with extensive aerial fila-
mentation or as gray colonies without the extensive
aerial growth. In addition, there may be pigmented
variations of these colonial types. Thus, N. aster-
oides 10905 forms at least three colony types: white,
beige, and orange. Each colony type remains rela-
tively stable upon transfer and reverts to one of the
other colony types very infrequently. Therefore,
during this investigation only the beige clone of N.
asteroides 10905, the gray clone of N. asteroides
14759, and the gray clone of N. asteroides GUH-2
were used.

Preparation of inoculum. The nocardiae used
for the inoculum were isolated directly from animal
lesions and incubated in brain heart infusion broth
as described previously (3). Under the conditions
of growth used, it was found that 72-h cultures of the
three strains were in the early stationary phase of
growth and consisted primarily of a uniform sus-
pension of gram-positive, non-acid-fast short rods and
cocci (3). Twenty-milliliter samples of the bacterial
suspension were centrifuged at approximately 100 ×
g for 5 min to remove clumps of organisms. The
supernatant was collected and centrifuged at 500 ×
g for 15 min. The pellet was suspended in 10 ml of
sterile saline (0.85%, wt/vol), and the optical density
was determined at 580 nm with a Spectronic 20
spectrophotometer (Beckman). The optical density
was compared to standard curves for each organism,
determining colony-forming units (CFU) versus
optical density. Phase-contrast microscopy of wet
mounts of the cell suspension showed that few or no
clumps of bacteria were present. The samples of the
bacterial suspensions were then diluted to give the
approximate desired number of organisms per milli-
liter of saline. In addition, each sample was quanti-
tated by direct viability counts on brain heart infu-
sion agar as previously described (3). Therefore,
known and reproducible amounts of organisms (all
in approximately the same stage of growth) could be
given to the animals.

Animals. Female Swiss Webster mice, 4 weeks of
age and averaging 18 to 20 g in weight, were
obtained from Simonsen's, Gilroy, Calif., and used
throughout this study.

Determination of LD₅₀ in saline-versus cyclo-
phosphamide-treated mice. The saline suspensions
of bacteria were adjusted so that 1.0 ml contained
approximately 10⁶ CFU, and dilutions were pre-
pared to give 10⁶, 10⁵, and 10⁴ CFU/0.1 ml. (Mice
given N. asteroides 10905 also got 0.1 ml of approxi-
mately 10⁵ CFU/ml.) Ten mice in each group re-
ceived 0.1-ml intravenous (i.v.) (tail vein) injec-
tions of the appropriate dilutions. The mice were
pretreated by intraperitoneal injection of either
0.1 ml of sterile saline or 0.1 ml of cyclophosphamide
(2 mg/20-g mouse) in saline 72 h prior to i.v. injec-
tion with the bacterial suspension. Plate counts of
the individual dilutions indicated the actual number
of CFU of organisms received (Table 1). In addi-
tion, control mice were given saline plus cyclo-
phosphamide alone and cyclophosphamide plus 10⁷
to 10⁹ heat-killed nocardial cells. The 50% lethal
dose (LD₅₀) determinations were calculated by stan-
dard methods (8). Each experiment utilizing ap-
proximately 100 mice) was repeated at least three
times with essentially the same results (Table 1).

Bacterial quantitation in heart, kidneys, lungs,
and spleens. Initially, necropsies were conducted on
several mice from each group to determine the
course of infection and to establish which organs
appeared to be grossly infected at the time of animal
death. At the same time, mice were sacrificed and
the organs were removed, fixed, and embedded in
paraffin as described below.

For quantitation of organisms within each of the
four organs, the mice were given slightly fewer CFU
than the calculated LD₅₀ for saline-treated animals.
In all instances, this dosage represented a 100% lethal
dose for cyclophosphamide-treated mice. At
given time periods (3, 12, 24, 48, and 72 h), the mice
were killed by cervical dislocation. The heart, kid-
eys, lungs, and spleen were removed aseptically
to preweighed Virtis micro-homogenizer flasks. The
weight of each organ was determined, and 2 ml of
sterile saline was added. The tissues were homo-
genized in a Virtis 45 high-speed blender (Ivan Sor-
vall, Inc., Norwalk, Conn.) at moderate speed for 1
min. Appropriate serial dilutions of each homoge-
nate were plated on brain heart infusion agar and
incubated at 34°C for 3 days prior to counting. Each
sample was done in duplicate on two animals per
time period, and each experiment was repeated at
least twice with the same results. The data were
plotted as mean viable counts, with the range of
individual values shown by vertical lines (see Fig. 4
through 7).

Drug treatment. Cyclophosphamide (Cytoxin,
Mead Johnson and Co., Evansville, Ind.) was
injected to give a final concentration of 2.0 mg/0.1 ml
of sterile saline. A single dose of 0.1 ml was given
intraperitoneally into 20-g mice.

Light microscopy. The mice were sacrificed by
cervical dislocation, and the peritoneal and thoracic
cavities were opened. The internal organs of the
mice were flooded with a 10% buffered Formalin
solution (4). The lungs, heart, spleen, and kidneys
were removed from the animal and partially per-
fused with additional fixative. The samples were
placed in 3 to 5 ml of fixative and stored at 4°C for at
least 24 h. The fixed samples were washed with
buffer, dehydrated through a series of ethanol,
cleared, and then embedded in paraffin as described
earlier (4). Thin sections were cut with a Spencer
microtome (AO Co.), affixed to glass slides, and
stained by the Brown and Brenn modification of the
Gram stain (4) and by the Kinyoun acid-fast stain,
using 1% HCl in 70% ethanol as previously described
(4).

RESULTS

It was observed that under identical experi-
mental conditions three strains of N. asteroides
differed significantly in their relative virulence
when injected i.v. into mice (Table 1). The least
virulent strain was found to be N. asteroides
10905, with an LD₅₀ greater than 4.6 × 10⁸ CFU/mouse; *N. asteroides* 14759 was of intermediate virulence (LD₅₀ of 8.5 × 10⁷ CFU/mouse); the most virulent organism was *N. asteroides* GUH-2, with an LD₅₀ of 8.7 × 10⁶ CFU/mouse (Table 1). Therefore, saline suspensions of *N. asteroides* GUH-2 given i.v. were at least 530 times more virulent for mice than saline suspensions of comparably grown cells of *N. asteroides* 10905. Furthermore, cells of *N. asteroides* GUH-2 were approximately 10 times more virulent than comparable cells of *N. asteroides* 14759.

Groups of mice were injected (i.v.) with an approximate LD₅₀ dose of each of the nocardial strains. At 1 week the survivors were sacrificed and autopsied. All visible lesions were scored, and the distribution was noted (Table 2). In addition to the four organs listed in Table 2, it was found that occasionally there were lesions involving various lymph nodes, particularly within the mesentery. No other major organs were found to be involved. Organisms could not be recovered routinely from the blood, liver, or tail.

*N. asteroides* GUH-2, when injected i.v., always induced multiple, progressive abscesses throughout the kidney (Fig. 1), with the infectious foci initially being located within the glomeruli. Visible lesions within the heart were not observed; however, an occasional mouse developed multiple abscesses within the spleen, and the lung occasionally showed discolorations. In contrast, *N. asteroides* 14759 when injected i.v. always induced multiple, progressive abscesses throughout the lung (Fig. 2) and heart muscle (Fig. 3). Some mice also developed lesions within the spleen and occasionally within the kidney. The kidney involvement was not usually bilateral and was never as pronounced as with *N. asteroides* GUH-2. The less virulent strain, *N. asteroides* 10905, when injected i.v. in large doses (>10⁸ CFU/mouse) usually caused multiple abscesses throughout the lung only. The heart and kidney were rarely infected, but occasionally in a few of the mice the spleen became heavily infected.

Intraperitoneal injection of a single dose of cyclophosphamide (2 mg/mouse) 72 h prior to i.v. injection of nocardia had a profound effect on the susceptibility of the animal to infection (Table 1). The LD₅₀ of the less virulent *N. asteroides* 10905 was approximately 1.2 × 10⁷ CFU/mouse, whereas the LD₅₀ values for *N. asteroides* 14759 and *N. asteroides* GUH-2 were between 6 × 10⁶ and 9.1 × 10⁶ CFU/mouse, respectively. Therefore, cyclophosphamide rendered the mice 40 times more susceptible to infection with *N. asteroides* 10905 and about 100 times more susceptible to infection with the intermediate strain, *N. asteroides* 14759. Interestingly, the cyclophosphamide-treated mice became only 10 times more susceptible to the most virulent strain, *N. asteroides* GUH-2.

To determine the effect of cyclophosphamide on bacterial clearance from the organs, mice were injected with less than an LD₅₀ dose for the saline-treated mice (this dosage was, however, 100% lethal for the cyclophosphamide-treated mice). At given time intervals, the lungs, heart, spleen, and kidneys were removed aseptically and homogenized in saline, and viable colony counts were determined (see Fig. 4 through 7).

At the doses given, the organisms were gradually cleared from the lungs of the saline-treated mice (Fig. 4). Approximately 99% of the *N. asteroides* 10905 cells were destroyed within 24 h, and the same percent clearance was reached for *N. asteroides* GUH-2 in 48 h. However, it required 72 h for 99% clearance of the

### Table 1. Effect of cyclophosphamide on mouse susceptibility to *N. asteroides*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Total no. of mice used</th>
<th>LD₅₀ (CFU/mouse)</th>
<th>Cyclophosphamide</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>N. asteroides</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10905 (least virulent)</td>
<td>240</td>
<td>&gt;4.6 × 10⁸</td>
<td>1.2 × 10⁷</td>
</tr>
<tr>
<td>14759 (intermediate virulence)</td>
<td>300</td>
<td>8.5 × 10⁸</td>
<td>6 × 10⁶</td>
</tr>
<tr>
<td>GUH-2 (most virulent)</td>
<td>360</td>
<td>8.7 × 10⁶</td>
<td>9.1 × 10⁴</td>
</tr>
</tbody>
</table>

### Table 2. Mouse organs presenting visible lesions at 1 week postinfection with *N. asteroides*

<table>
<thead>
<tr>
<th>Strain</th>
<th>No. of mice studied</th>
<th>No. lesions observed</th>
<th>Distribution of lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>N. asteroides</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10905 (ca. 10⁸ CFU/mouse; saline; i.v.)</td>
<td>10</td>
<td>2/10</td>
<td>0/10</td>
</tr>
<tr>
<td>14759 (ca. 10⁸ CFU/mouse; saline, i.v.)</td>
<td>10</td>
<td>0/10</td>
<td>10/10</td>
</tr>
<tr>
<td>GUH-2 (ca. 10⁸ CFU/mouse; saline, i.v.)</td>
<td>10</td>
<td>0/10</td>
<td>10/10</td>
</tr>
</tbody>
</table>

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Fig. 1. (Top) Light micrograph of a section of mouse kidney 72 h postinfection with ca. 10^7 CFU of N. asteroides GUH-2 given i.v. The section was stained by the Kinyoun acid-fast method. Arrows indicate microcolonies of acid-fast nocardia growing throughout the kidney. This type of infection was never observed in kidneys of mice infected with either N. asteroides 14759 or N. asteroides 10905. (Bottom) High-magnification micrograph (of area "a" of top micrograph) showing individual acid-fast filaments growing within the kidney.

cells of N. asteroides 14759 from the lungs of the saline-treated mice. (It is important to remember that the dosage in each case was well below the LD₅₀ for normal mice.) During the first 12 h postinfection, the clearance of Nocardia from the lungs of cyclophosphamide-treated mice was about the same as that for the saline-treated animals (Fig. 4). However, N. asteroides 14759 and GUH-2 rapidly increased within the lungs of the compromised animals after 24 h. At 72 h postinfection, the viable cell number of N. asteroides 14759 was still increas-
Fig. 2. (Top) Light micrograph of a section of mouse lung 72 h postinfection with ca. $5 \times 10^7$ of *N. asteroides* 14759 given i.v. The section was stained by the Kinyoun acid-fast method. Arrows indicate microcolonies of acid-fast nocardia growing in abscesses formed throughout the lung. Massive, multiple abscesses such as shown here were never observed in the lungs of mice infected with *N. asteroides* GUH-2. (Bottom) High-magnification micrograph (of area "a" of top micrograph) showing individual acid-fast filaments growing within a lesion.

ing, as was that of *N. asteroides* 10905. On the other hand, the growth of *N. asteroides* GUH-2 within the lung appeared to peak at about 48 h and may have been decreased 72 h postinfection (Fig. 4). Fewer organisms were deposited within the
The nocardial cells observed commonly increased in the lungs, kidneys, or spleen. Stained by Kinyoun acid-fast method, unusual acid-fast organisms were frequently seen within the heart, lungs, or spleen. They were rarely seen within the heart (Fig. 2). The organism within the heart was observed to grow well in the hearts of cyclophosphamide-treated mice, but not in normal and saline-treated animals, although it was observed by E. coli. They were not cleared either.

![Diagram](Image)
asteroides GUH-2 appeared to induce a self-limited infection within the heart (Fig. 5).

Only *N. asteroides* GUH-2 increased significantly in the spleens of cyclophosphamide-treated mice (Fig. 6). Both *N. asteroides* 14759 and *N. asteroides* 10905 persisted at about the same level for 72 h in the spleens of both saline- and cyclophosphamide-treated mice (Fig. 6).

The viable cell numbers of *N. asteroides* GUH-2 increased dramatically in the kidneys of cyclophosphamide-treated mice. In addition, cells of this strain were not effectively cleared from the kidneys of the control animals. In contrast, both *N. asteroides* 10905 and *N. asteroides* 14759 were eliminated from the kidneys of normal and cyclophosphamide-treated mice (Fig. 7).

![Graph](image_url)

**Fig. 6.** Relative clearance of *N. asteroides* from the spleen of normal and cyclophosphamide-treated mice after i.v. injection. Each point represents the average of duplicate determinations in two mice per time period, and each bar represents the variability observed within a total of three separate experimental determinations. Symbols: (▲) 2.5 x 10^7 CFU of *N. asteroides* 10905 given i.v. in saline-treated mice; (△) 2.5 x 10^7 CFU of *N. asteroides* 10905 given i.v. in cyclophosphamide-treated mice; (●) 5.0 x 10^6 CFU of *N. asteroides* 14759 given i.v. in saline-treated mice; (○) 5.0 x 10^6 CFU of *N. asteroides* 14759 given i.v. in cyclophosphamide-treated mice; (■) 3 x 10^6 CFU of *N. asteroides* GUH-2 given i.v. in saline-treated mice; (□) 3 x 10^6 CFU of *N. asteroides* GUH-2 given i.v. in cyclophosphamide-treated mice. (At the dose levels used for each organism, the cyclophosphamide-treated mice generally did not survive beyond 72 h.)

**DISCUSSION**

Reports with mice as an animal model for studying nocardial pathogenicity have frequently had contradictory interpretations (2, 4, 9, 10, 14, 16, 18, 22, 24, 27). Most of these studies used bacterial cell pellets or crude suspensions of organisms grown from 1 to several weeks (9, 10, 14, 16, 18, 27). Smith and Hayward (24) studied the relative pathogenicities of a few strains of *N. caviae* and *N. asteroides* by injecting ox serum broth suspensions of 72-h cultures i.v. They felt that this type of suspension was required because they noted a 20-fold decrease in the viable CFU after 1 h of storage of the nocardia in saline (24). Their observations are in marked contrast to our data: we found less
than a twofold decrease in CFU after 5 h of storage in saline at ambient temperature (Beaman, unpublished data). In most other investigations involving mice, the bacterial cells were given either intraperitoneally or in the footpads (9, 10, 14, 16, 18, 27). Frequently, the suspension was given with some form of "adjuvant" (i.e., hog gastric mucin). Little attention was given to culture age and its effect on virulence, the effect of culture medium on virulence, or the homogeneity (i.e., single-cell suspensions) of the inoculum. We found that the most uniform and reproducible results were obtained by preparing homogeneous suspensions by differential centrifugation. Furthermore, i.v. inoculation gave much better results than intraperitoneal inoculation (Beaman, unpublished data).

By these techniques, we were able to determine reproducibly the relative virulences of several strains of *N. asteroides* for mice. Thus, we have a system whereby we can study in more detail specific factors (i.e., culture age) on nocardial virulence and the possible mechanisms of host resistance and immunity.

The mechanisms of organ specificity for the strains of *N. asteroides* used in this study remain a mystery but may reflect certain biological and biochemical properties of the organisms being studied. Smith and Hayward also found some degree of organ specificity with certain strains of nocardia given i.v. (24). However, the significance and interpretation of organ tropisms after i.v. injection with *N. asteroides* must be analyzed in light of the route of injection. Presumably, the usual route of infection in humans is by way of the lungs. Therefore, it is likely that the primary focus for the disease is set up in this organ. Spread to secondary foci is probably by direct extension into surrounding tissues or by hematogenous routes to other organs. Preliminary data show that host susceptibility to nocardia and the nature of the disease depend upon the route of inoculation (Beaman, unpublished data). Therefore, one must consider the route of infection when discussing organ tropisms and the specific host-parasite interaction.

Although circulating antibodies and delayed hypersensitivity to nocardial antigens have been demonstrated in humans and animals, their roles in the normal defense of the host against nocardial infections have not been established (4, 28). This study has clearly shown that cyclophosphamide increased the susceptibility of mice to infection with *N. asteroides*. These observations suggest that a B-cell response and humoral immunity are important in host defense against infection by *N. asteroides*. It has been shown that cyclophosphamide can inhibit the humoral immune response of an animal towards most antigenic insults (7, 11, 21, 25). Furthermore, B-cell functions were shown to be markedly affected, whereas cyclophosphamide-treated animals were still able to mount a cell-mediated immune response (11, 13, 21, 25). It has been shown that T-cell function appeared not to be affected as much as B-cell function, and the functioning of other cells of the reticuloendothelial system was not significantly impaired at the dose levels used during this investigation (11, 13, 15). Therefore, cyclophosphamide results in a marked increase of host susceptibility to infectious agents in which a humoral response is required for protection from infection or in recovery from an infection (1, 7, 11, 12, 23, 26).

The data presented above do not imply that cell-mediated immunity is not important in host defense against nocardial infections. However, based on the observations of others regarding the effects of cyclophosphamide (1, 7, 11-13, 15, 21, 23, 25, 26), it appears that the humoral response is important especially with *N. asteroides* 10905 and *N. asteroides* 14759, where cyclophosphamide enhances mouse susceptibility 40 and 100 times, respectively. Only a 10-fold enhancement to susceptibility towards the most virulent strain (*N. asteroides* GUH-2) may suggest that the humoral response is less important for resistance of the animal to infection with this organism.

Interestingly, the cyclophosphamide treatment did not appear to alter significantly the organ tropism for each organism. However, it did greatly affect the ability of the animal to clear the organisms from these regions. Thus, fewer organisms of *N. asteroides* GUH-2 grew much more rapidly in the kidneys of cyclophosphamide-treated mice than in the "normal" mice, whereas fewer organisms of *N. asteroides* 14759 grew more profusely in the lungs and hearts of cyclophosphamide-treated mice than in the "normal" mice.

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LITERATURE CITED


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