IMMUNE RESPONSES TO THE PROTEIN, CARBOHYDRATE AND LIPID ANTIGENS OF *NOCARDIA ASTEROIDES* IN EXPERIMENTAL NOCARDIOSIS IN MICE

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SUMMARY. The intravenous injection of *Nocardia asteroides* into mice produced systemic nocardiosis involving all the vital organs. Infection of the kidneys and adrenals was more persistent and progressive than in other organs as evidenced by increased bacterial counts and histopathological findings. During the course of the experimental infection, no humoral immune response was detected against various protein antigens up to 4 weeks after challenge, but significant cell-mediated immunity (CMI) was found. Phospholipid antigens elicited only a humoral immune response. The increased CMI responses with protein antigens correlated well with the decreasing bacterial load, which suggested that CMI against proteins was important in the pathogenesis of this disease.

INTRODUCTION

Infections of man with *Nocardia asteroides* have received little attention (Curry, 1980) although it is now recognised that the disease has a wide spectrum of clinical manifestations and is cosmopolitan in its geographic distribution (Randhawa and Khan, 1977). Studies of experimental infection in mice have shown marked differences in the ability of different strains of *N. asteroides* to cause either acute or chronic progressive infections with specific tissue tropism (Beaman and Maslan, 1977). These studies reveal that immunological responses to specific strains play an important role in the pathogenesis and final outcome of the diseases.

Although many studies have shown that the cell-mediated immune (CMI) response produced against the whole cells (Krick and Remington, 1975; Deem *et al.*, 1982) or ribonucleo-protein fraction of *N. asteroides* is protective (Sundararaj and Agarwal, 1977), the relative immunological role played by individual components of *N. asteroides* during infection have not been studied. The present study was performed to explain the clinical and pathological significance of various components of *N. asteroides* in experimental nocardiosis.

MATERIALS AND METHODS

Bacterial strains. *Nocardia asteroides* NCTC 8595 (equivalent to ATCC 14759) was obtained

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from the National Collection of Type Cultures, Colindale, U.K. It was maintained on Brain Heart Infusion Agar (BHI agar; Hi-media, Bombay, India). The bacteria were grown in BHI broth at 37°C for 7 days (stationary phase).

Animals. Swiss albino mice (outbred) of either sex weighing 18–20 g (4–5 weeks old) were used throughout the study. They were fed on standard pellet diet (Hindustan Lever Ltd, Bombay, India) and provided with water *ad libitum*.

Preparation of inoculum. To obtain a uniform suspension, *N. asteroides* was grown in BHI broth containing Tween-80 0.5% v/v and incubated in a shaker at 37°C. The cells were harvested after 72 h in early stationary phase from the supernate of the culture, avoiding clumps settled at the bottom. The supernate containing a relatively uniform suspension of the bacteria was centrifuged at 1000 *g* for 15 min. The pellet was resuspended and washed twice with sterile normal saline to remove unwanted cellular debris and then adjusted to a maximum concentration of 10⁹ cfu/ml. Ten-fold serial dilutions of the suspension were made in sterile normal saline. For each concentration, each of six mice was given 0.1 ml of bacterial suspension by injection into the tail vein. Mortality and morbidity were studied for 3 weeks and the LD50 dose calculated according to the method of Karber (1931). A total of 24 mice was used for each experiment and each experiment was repeated with three different cell populations of *N. asteroides* to establish the reproducibility of the method.

Organ distribution and clearance of *N. asteroides*. Organ distribution and clearance of bacteria after intravenous injection of an LD50 dose were determined after different intervals. Animals in groups of 4–6 were killed by exsanguination on days 1, 3, 7, 14 and 21 after infection. Lungs, liver, spleen, kidneys-adrenals and brain were removed aseptically and homogenised in sterile saline (3 ml) in glass homogenisers. Serial dilutions of each homogenate (10 µl) were inoculated on to BHI agar and viable counts were performed after incubation for 3 days at 37°C. Average viable counts on different days after infection for all the organs were determined from 4–6 animals, each with triplicate observations.

Histopathology. Representative infected tissues were fixed in 10% formol saline, embedded in wax, cut into 5-µm sections and stained by haematoxylin and eosin.

Preparation of antigens. Protein antigens were prepared from acetone-killed, sonicated cells from which the proteins were extracted and fractionated as described by Gupta *et al.* (1985). Total phospholipid antigen was prepared from total lipids by acetone precipitation as described by Gupta *et al.* (1985); the phosphorus content of the phospholipids was determined by the method of Bartlett (1959).

Immune responses during experimental infections. Each group of 40 mice was given an LD50-dose of *N. asteroides* by intravenous injection. On days 7, 14 and 21 after infection, the mice, in groups of six, were killed by exsanguination. Sera were separated for studying the humoral immune response. The spleens were removed aseptically and spleen cell suspensions used to study the CMI. The techniques used were those described by Gupta *et al.* (1985). The relative concentrations of T and B lymphocytes in the spleen-cell suspensions were investigated with the methods of Mysliwski *et al.* (1977) and Gravely *et al.* (1976) respectively.

Statistical analysis. Statistical analysis was undertaken with Student's *t* test.

RESULTS

The mean LD50 value obtained at the end of 3 weeks for three different groups was 2.89×10^7 cfu/mouse (table I).

Groups of 40–50 mice were challenged intravenously with an LD50-dose of *N. asteroides* and the surviving mice in groups of 4 or 5 were killed on days 1, 3, 7, 14 and 21 after challenge. The numbers of cfu in the aseptically removed lungs, liver, brain, spleen and kidneys-adrenals were investigated. The distribution and subsequent clearance pattern of *N. asteroides* from the various organs are shown in fig. 1. The total number of cfu recovered from all the organs of the infected mice 24 h after infection accounted for > 90% of the initial inoculum, indicating that there was no significant killing of the bacteria by the host during first 24 h. Maximum numbers of bacteria

TABLE I
LD50 of *N. asteroides* for mice infected intravenously

Experiment no.	Number of organisms injected	Number of mice challenged	Mortality after 3 weeks	Percentage mortality	Calculated* LD50 (cfu/mouse)
1	3.1×10^8	6	6	100	3.1×10^7
	3.1×10^7	6	3	50	
	3.1×10^6	6	0	0	
	3.1×10^5	6	0	0	
2	5.7×10^8	6	6	100	2.6×10^7
	5.7×10^7	6	4	71.42	
	5.7×10^6	6	1	12.50	
	5.7×10^5	6	0	0	
3	4.7×10^8	6	6	100	2.98×10^7
	4.7×10^7	6	3	51.14	
	4.7×10^6	6	1	12.50	
	4.7×10^5	6	0	0	

$$\text{*Log LD50 value} = 0.5 + \log \text{ of highest bacterial dilution} - \frac{\text{Sum of percentages of dead animals}}{100}$$

FIG. 1.—Distribution and clearance of *N. asteroides* in organs of mice infected intravenously with an LD50 dose: ○—○ lung, ●—● liver, △—△ brain, ▲—▲ spleen, ■—■ kidneys-adrenals. (each point represents mean $\pm$ SEM of results for 4–6 animals).

($1.37 \pm 0.35 \times 10^7$ cfu) were recovered from the liver 24 h after challenge. No viable bacteria could be isolated from the lungs on day 7 and all other organs except kidneys-adrenals were free from infection by 21 days after challenge. In contrast with the other organs, the kidneys-adrenals of the infected mice showed a gradual increase in the numbers of cfu to about a 200-fold increase in cfu on day 21 after challenge. This experiment was repeated once to check the reproducibility of the results.

Histopathology

Histopathological studies were undertaken for all the organs removed between 9 and 15 days after infection. The lungs showed marked infiltration of leukocytes (mainly lymphocytes) in the interstitial area of the lung parenchyma without the formation of granulomata. The liver showed sinusoidal congestion as a result of septicaemia with a few microabscesses producing a focal spotty necrosis. No inflammatory changes or formation of abscesses were seen in the spleen or brain. In contrast with all the other organs, the kidneys showed a marked infiltration of neutrophils with multiple microabscesses throughout the parenchyma and especially in the cortex area.

Immune response during experimental nocardiosis

(1) Humoral immune response

Sera from the infected animals did not contain antibody to any of the protein fractions (CPF, F₁ and F₂) up to 3 weeks after infection by either gel diffusion or CIE. No antibodies could be detected by IHA. In contrast, precipitins were detected against phospholipid antigen from one week up to 3 weeks after infection, by gel diffusion and by CIE.

(2) Cellular immune response

Leukocyte migration inhibition (LMI). The percentage LMI values of infected animals tested with F₁ and F₂ are shown in fig. 2. The mean LMI values (%) of 53.03 ± 3.46 and 55.23 ± 2.68 observed with F₁ at weeks 1 and 2 after infection, respectively, were significantly more ($p < 0.01$) than that obtained at week 3 (36.56 ± 2.60). A similar pattern of LMI was observed with the F₂ fraction but the LMI was significantly lower ($p < 0.01$) than that in the presence of F₁ at any time after challenge.

In contrast with this, there was no significant LMI ($< 20\%$) in the presence of the phospholipid antigen.

T and B cell counts. Mean T and B lymphocyte differential counts on day 0 (before infection) were $33.23 \pm 1.18\%$ and $33.13 \pm 0.68\%$ of spleen-cell population respectively. Seven days after infection, the percentage of T cells in the spleens of infected animals had increased markedly and a mean value of $41.32 \pm 0.69\%$ was obtained. This was statistically more ($p < 0.01$) than the T-cell count on day 0. This trend was maintained up to the second week after infection ($43.85 \pm 1.05\%$) and thereafter the T-cell counts started decreasing to the normal values ($31.99 \pm 1.03\%$) by day 21. Splenic B-lymphocyte populations, however, remained unchanged throughout the course of the experiment (table II).

FIG. 2.—Migration inhibition of leukocytes obtained from mice infected with *N. asteroides*: ■—■ represents the migration in the presence of protein fraction F₁; □—□ represents the migration in the presence of protein fraction F₂.

TABLE II
Effect of intravenous infection with N. asteroides on T and B lymphocyte populations of mice

Lymphocyte	Percentage (mean ± SEM) of spleen-cell population represented by given type of lymphocyte on days after infection			
	0	7	14	21
T	33.23 ± 1.18	41.33 ± 0.69*	43.85 ± 1.05*	31.99 ± 1.03
B	33.13 ± 0.68	32.10 ± 0.91	33.82 ± 0.42	34.65 ± 0.79

* $P < 0.001$ (Student's *t* test) in comparison with the count on day 0.

DISCUSSION

Systemic nocardiosis has been induced by intravenous injection in Swiss Webster mice (Beaman and Maslan, 1977). They have further shown in this model a specific organ tropism for different reference strains of *N. asteroides*, including *N. asteroides* ATCC 14759 which showed a predilection to infect primarily the lungs and heart. In contrast to this finding, the present study demonstrated that *N. asteroides* NCTC 8595 (equivalent to ATCC 14759) has a predilection for the kidney instead of the lung and heart; 7 days after infection there was complete clearance of bacteria from the lung

whereas in the kidneys the bacteria persisted and showed a gradual increase up to 4 weeks after infection. The histopathological study supported the bacteriological findings. The persistence of bacteria in the kidneys might reflect the lack of a large number of resident macrophages in the kidneys (Beaman *et al.*, 1982). The liver and spleen are the two largest organs of the reticuloendothelial system and the fixed macrophages might be responsible for the microbial elimination from these organs.

Studies on the immunological responses against purified chemical constituents of *N. asteroides* during nocardiosis are rare. In the present study, cellular and humoral responses against the purified protein and lipid antigens were studied. Polysaccharides were not included because they were found to be non-immunogenic (Gupta *et al.*, 1985). During the course of infection, significant CMI against F₁ and F₂ was detected by LMI. The increase in the level of CMI response with a concomitant decrease in the bacterial count suggests that the CMI response plays an important role in the resolution of the disease. The decline in CMI after 3 weeks is probably due to the sequestration of specific antigen by activated macrophages leading to a gradual dilution of the antigen in circulation. The role of CMI in bacterial clearance during the infection is also supported by an increase in T-cell numbers (table II). Lack of antibody response up to 3 weeks further supports the finding that the CMI response is more important than the humoral response in the elimination of the bacteria and limiting the progress of the diseases. The lack of humoral response is in agreement with the findings of Krick and Remington (1975) and Conde *et al.* (1983). A few studies have, however, detected a very low titre of antibody in nocardiosis in man (Stevens *et al.*, 1981) and in cattle (Pier *et al.*, 1968), and in experimentally induced local infection in guinea pigs (Hiramine *et al.*, 1981) against a whole-cell extract of *N. asteroides*. The absence of circulating antibodies against the cytoplasmic protein fraction during infection, despite the fact that these fractions elicit antibody responses when used for immunisation (Gupta *et al.*, 1985), is noteworthy. A possible reason for this might be an overload of antigen due to the overwhelming multiplication (100–200-fold increase) of the bacteria during the first 2 weeks after the challenge, which may lead to the state of B-cell unresponsiveness, due probably to the activation of T-suppressor cells as has been seen in tuberculosis (Collins and Watson, 1979) and leprosy (Watson and Collins, 1979). As the infection recedes, and the bacterial load decreases, the antibody might appear at a later stage of the infection, as has been seen in infection with *N. brasiliensis* (Conde *et al.*, 1983). The possibility suggested for *N. brasiliensis* that the antibody produced during the initial stages of the infection remained adsorbed in the infected tissue as an immune complex, and the detectable level of antibody appeared only during the chronic stage of the disease after the infected tissue has been saturated, might also be true in *N. asteroides* infections. However, it needs further experimental investigation.

CMI to phospholipid antigen was not detected during infection, although an antibody response could be seen from the first week. The appearance of antiphospholipid antibodies during the infection can be explained by the fact that the phospholipid content of the bacteria is too low to cause immune tolerance against this particular antigen. Instead, the increasing numbers of bacteria help in raising the antigen concentration to that necessary for an antibody response. Antiphospholipid antibodies have also been demonstrated in sera from patients with tuberculosis (Khuller

and Subrahmanyam, 1972). It is expected that these antibodies might prove to be of significance in the serodiagnosis of systemic and local nocardial infections.

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