

## Cell-Mediated Immunity in Experimental *Nocardia asteroides* Infection

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Experimental mycetoma-like lesions developed in guinea pigs after subcutaneous injection of *Nocardia asteroides*. Although delayed hypersensitivity appeared earlier, increased macrophage migration inhibition and microbicidal activity appeared after 7 weeks. When the lesions healed, high cell-mediated immunity was present. Cell-mediated immunity was transferred to normal recipient guinea pigs from healed donor guinea pigs by spleen cell transfer. Recipient guinea pigs showed marked protection against challenge with *N. asteroides*.

Mycetomas are common in southern India. The majority of them are actinomycetomas caused mainly by *Nocardia asteroides* (2). *N. asteroides* also produces systemic infections either primarily or associated with diseases showing depressed cell-mediated immunity (CMI), such as Hodgkin's disease or leukemia (11). It has been observed that corticosteroids enhance the susceptibility of mice to *N. asteroides* infection (9). There is no report of a direct study correlating the development of CMI with localized or generalized *N. asteroides* infection. We have studied CMI during the progress of experimental mycetoma-like lesions in guinea pigs and its protective effect after spleen cell transfer from immunized animals.

### MATERIALS AND METHODS

**Animals.** Healthy outbred guinea pigs of both sexes, weighing 350 to 450 g, bred in our central animal house were used. They were fed on grams and fresh green vegetables.

**Bacteria.** The *N. asteroides* strain was obtained from L. N. Mohapatra (Department of Microbiology, All India Institute of Medical Sciences, New Delhi, India). Growth of *N. asteroides* on Sabouraud dextrose agar was passaged six times in guinea pigs and maintained as a stock culture. To obtain the bacterial inoculum, a 6-day growth of *N. asteroides* at 37°C on four to five Sabouraud dextrose agar slants was scraped and suspended in sterile 0.85% NaCl. Coarse particles were removed by filtration through loose, sterile cotton wool. The filtrate was centrifuged at 2,500 rpm for 20 min. The pellet was washed twice and resuspended in saline to give an optical density of 0.5 at 530 nm in a spectrophotometer. The viable count showed that this bacterial suspension contained  $2.6 \times 10^9$  viable organisms/ml.

**Listeria monocytogenes.** *L. monocytogenes* was kindly supplied by J. W. Osebold (Department of

Veterinary Microbiology, University of California, Davis). The culture was passaged three times in guinea pigs, and the organism was recovered from the spleens of infected animals. It was grown in brain heart infusion (BHI) broth at 37°C for 18 h and centrifuged at 2,500 rpm for 15 min. The pellet was washed and resuspended in BHI broth to the original quantity. The culture was distributed in 1-ml quantities and kept frozen at -70°C (stock culture).

**Media.** Hanks balanced salt solution (BSS) and Eagle minimum essential medium (MEM) with Earle BSS were made as described by Lennette and Schmidt (5). Enriched MEM was made by adding 10% heat-inactivated bovine serum to MEM.

***N. asteroides* antigens.** (i) Purified protein derivative. Purified protein derivative (N-PPD) was prepared by the method described by Magnusson (8). *N. asteroides* was grown in Sauton medium (6) for 4 to 5 weeks at 37°C. Cultures were heat killed at 100°C for 1 h. The organisms were removed by centrifuging at 2,000 rpm for 15 min and filtering through a sintered-glass filter. The protein fraction was precipitated by adding trichloroacetic acid to the filtrate to a final concentration of 4%. It was purified further by repeated isoelectric precipitation at pH 4.8 (14).

(ii) N-PP. A polypeptide skin test antigen from *N. asteroides* (N-PP) was prepared by the procedure described by Kingsbury and Slack (4). Briefly, *N. asteroides* was grown in modified Long and Seibert medium (12) for 10 days. The culture was autoclaved at 120°C for 15 min, and the growth was collected after centrifuging at 1,500 rpm for 15 min. The cells were defatted successively with acetone and ethyl alcohol-ether (1:1) and chloroform-methanol (3:1) mixtures. Finally, they were washed with acetone and dried. A 20-g amount of dried, defatted *N. asteroides* cells was extracted with 0.1 N HCl for 3 days. The supernatant was filtered and the pH was adjusted to 7.0. The precipitate was discarded. An equal quantity of saturated solution of picric acid was added to the filtrate with stirring. The yellow precipitate formed was collected and redissolved in

distilled water at pH 7. Ten volumes of acetone was added to it. The precipitate formed was washed with acidified acetone (1 ml of 1 N HCl to 100 ml of acetone) and ether. It was dissolved in 0.01 N HCl and dialyzed against 0.01 N HCl. The dialysate was collected and precipitated with picric acid. The precipitate was washed with acetone as described above and dried.

**Animal inoculation experiments.** Water-in-oil emulsion was prepared by mixing a 1-ml suspension of *N. asteroides* and 9 ml of incomplete Freund adjuvant (liquid paraffin plus lanolin [9:1]). From this, 0.5 ml was inoculated subcutaneously into guinea pigs. The inoculum contained  $1.3 \times 10^8$  viable nocardia. Animals were observed for the development of local lesions, i.e., granuloma and sinus formations at the site of inoculation. Groups of 4 to 6 guinea pigs were sacrificed at different time intervals and examined for lesions in internal organs. The development of CMI in these animals was also studied. For systemic challenge experiments, a 0.1-ml suspension of *N. asteroides* in saline containing  $2 \times 10^8$  organisms was inoculated intracardially into guinea pigs.

**Measurement of CMI. (i) Detection of DH.** Delayed skin hypersensitivity (DH) reactions were measured in guinea pigs that were injected subcutaneously with live *N. asteroides* at different time intervals during the development and progression of lesions. An area of skin in guinea pig flanks (8 by 6 cm) was shaved and the hair was removed. For eliciting DH, N-PPD and N-PP antigens (10  $\mu$ g in 0.1 ml of 0.85% NaCl) were injected intradermally. The diameter of the area of erythema and induration was measured 24 h after injection.

**(ii) MMI.** The macrophage migration inhibition (MMI) test was done by the method of David and co-workers (3) and as described by Agarwal and Sundararaj (1), except that different nocardia antigens, N-PPD and N-PP, were added to plastic chambers containing sensitized guinea pig peritoneal macrophages.

**(iii) MIC-A.** Macrophage microbicidal activity (MIC-A) was assayed by the technique described by Simon and Sheagren (13). Peritoneal exudate cells were collected from guinea pigs, washed in Hanks BSS, and adjusted to  $2 \times 10^6$  cells/ml of enriched MEM. A 2-ml amount of this suspension, with or without added N-PPD and N-PP, was placed in 50-mm glass petri dishes and incubated at 37°C in 5% CO<sub>2</sub> for 18 h. The petri dishes were then washed three times with MEM without antibiotics to remove nonadherent cells. Random petri dishes were evaluated by Gram staining, and the viability of cells was checked by vital dye exclusion. From stock culture, *L. monocytogenes* was grown in BHI broth at 37°C for 18 h. The culture was centrifuged at 1,500 rpm for 15 min. The pellet was washed and suspended in sterile 0.85% NaCl. The bacterial suspension was adjusted to an optical density of 0.5 at 530 nm, and the viable count was done on BHI agar plates. A 2-ml amount of enriched MEM without antibiotics but containing approximately  $5 \times 10^7$  *L. monocytogenes* organisms was added to each culture dish. The petri dishes were incubated at 37°C for 30

min in 5% CO<sub>2</sub> for phagocytosis to occur. They were washed three times with MEM to remove extracellular *L. monocytogenes*. The macrophages were lysed by exposure to distilled water for 15 min and scraping with a rubber policeman. Serial dilutions were made and viable *L. monocytogenes* were quantitated by the pour plate method. The colonies on the BHI agar plates were counted after incubation at 37°C for 24 to 48 h. The other petri dishes were incubated after adding enriched MEM without antibiotics. They were processed in the same manner after 1.5 and 4 h of incubation, and the number of viable *L. monocytogenes* was counted.

**(iv) MA.** The macrophage aggregation (MA) test was done by the method described by Lolekha and co-workers (7). A 2-ml amount of enriched MEM containing  $2 \times 10^6$  peritoneal exudate cells was cultured in silicon-treated test tubes (15 by 150 mm) in the presence of N-PPD (25  $\mu$ g/ml) and N-PP (25  $\mu$ g/ml). Control tubes did not contain antigen. All of the tubes were incubated at 37°C in 5% CO<sub>2</sub> for 24 h. After incubation, cells were resuspended by gentle mixing. One drop was placed in a slide and observed under low-power magnification ( $\times 100$ ) for clumping. The test was done in duplicate, and the results were graded from + to ++++ according to Lolekha et al. (7).

**Spleen cell transfer.** Guinea pigs that had been inoculated with one dose of live *N. asteroides* ( $1 \times 10^6$  in 0.5 ml) were sacrificed after different time intervals. CMI was measured in these guinea pigs. Their spleens were removed, rinsed three times with MEM to remove any adhering erythrocytes, and later snipped into small pieces measuring 4 to 6 mm. These pieces were forced through a 40-mesh stainless-steel sieve, and the spleen cells were suspended in MEM. The viability of spleen cells was checked by the trypan blue dye exclusion method. The cells were counted in a hemocytometer. The spleen cells were injected intravenously into normal guinea pigs in doses ranging from  $1 \times 10^6$  to  $3 \times 10^6$  cells.

**Challenge with *N. asteroides* and viable count in tissues.** Recipient guinea pigs that had been earlier injected with spleen cells from donor guinea pigs of known CMI were challenged either subcutaneously or intravenously with a standard dose of live *N. asteroides* ( $2 \times 10^6$  organisms/0.1 ml) within 48 h of spleen cell transfer. They were observed for survival or death for up to 3 months. Animals were sacrificed at definite time intervals, and viable counts were done in different tissues. Lungs, heart, liver, and kidneys were removed and homogenized. Serial dilutions of homogenates were made in 0.85% NaCl. A 0.1-ml amount from different dilutions was inoculated onto blood agar plates. The plates were incubated at 37°C for 3 to 5 days, and the number of colonies was counted.

## RESULTS

**Development of lesions.** At 7 days after subcutaneous injection of live *N. asteroides* into normal guinea pigs, no gross lesions were seen either locally or in internal organs, viz., lungs, heart, liver, and kidneys, nor was there any

swelling or sinus formation at the injection site, except a localized collection of cheesy material which showed a pure growth of *N. asteroides* upon culture. In 3 weeks a hard swelling appeared, which became progressively larger and softer. Subsequently, it led to the formation of a sinus from which a considerable amount of pus was discharged. In 4 to 5 weeks sinus formation and the amount of discharged pus progressively increased and localized in specific parts of subcutaneous tissues. It spread in different directions and more sinuses appeared. In 6 to 7 weeks the subcutaneous lesions started regressing and some of the old sinuses healed. In 8 to 10 weeks the regression was pronounced and many sinuses healed. From 12 weeks onward a complete healing of the lesions was observed. A total of 28 guinea pigs were inoculated with live *N. asteroides*. They were divided into six groups, each comprised of 4 to 6 animals. They were sacrificed at different time intervals. Before sacrifice, DH was tested with N-PPD and N-PP antigens. During the first 3 weeks, none of the animals showed any DH. DH appeared in 4 to 5 weeks in four out of six guinea pigs. As shown in Table 1, varying degrees of DH were observed in subsequent weeks. DH persisted up to 17 to 21 weeks. Some degree of DH was observed with N-PPD and N-PP antigens.

**Development of MMI, MA and MIC-A.** During the first 4 to 5 weeks, although a variable degree of MMI and MA appeared with N-PPD or N-PP antigens, it was not very marked (Table 1). In 6 to 7 weeks a considerable degree of MMI appeared. It was fairly marked after 7 weeks and persisted for 17 to 21 weeks. There was little or no microbicidal activity during the first 4 to 5 weeks. It appeared from week 6 onward, and in 8 to 10 weeks there was good microbicidal activity that persisted up to 21 weeks (Table 2).

**Transfer of CMI to *N. asteroides* in guinea pigs. (i) DH-positive and low CMI groups.** Donor guinea pigs were subcutaneously injected with live *N. asteroides*. After 4 to 5 weeks they were sacrificed and their spleen cell suspensions were injected intracardially into recipient guinea pigs. Donor guinea pigs had developed DH, but their CMI was low. Hence, upon transfer of spleen cells, DH was transferred and CMI remained low. When these animals were challenged intravenously with a standard dose of *N. asteroides*, their survival was not prolonged. Similarly, upon subcutaneous challenge, mycetoma-like lesions developed as in normal animals.

**(ii) Group with high CMI and loss of DH.** In another group of guinea pigs, when a second

transfer of spleen cells from immunized donor guinea pigs was done, there was a loss of DH although MMI, MA, and MIC-A remained the same. By a second transfer of spleen cells from guinea pigs with healed lesions to normal recipients, it was possible to get recipients with a high degree of CMI and no DH. When these animals were intravenously challenged with a standard dose of live *N. asteroides*, there was a considerable degree of protection, as shown by prolonged survival, even up to 80 days. There was also a marked reduction in the number of *N. asteroides* in various tissues (Table 3). After subcutaneous inoculation, there was a marked delay in the appearance of ulcers and sinuses followed by rapid healing.

TABLE 1. Development of CMI in guinea pigs subcutaneously inoculated with live *N. asteroides*

Guinea pig no.	Time (wks)	Mean erythema diam (mm)		MMI (%)		MA	
		N-PPD	N-PP	N-PPD	N-PP	N-PPD	N-PP
101	1-3	- <sup>a</sup>	-	17	24	-	-
102		-	-	25	-4 <sup>b</sup>	-	-
105		-	-	39	62	+	+
106		-	-	-20 <sup>b</sup>	1	-	-
7	4-5	10.5	12	43	56	++	++
107		-	-	20	21	+	+
108		14	13.5	-50 <sup>b</sup>	16	-	+
111		-	-	-65 <sup>b</sup>	37	-	+
112		12	13	-48 <sup>b</sup>	7	-	-
114		8.5	17	-14 <sup>b</sup>	-1 <sup>b</sup>	-	-
5	6-7	-	-	62	68	+++++	++++
9		10	14	87	78	+++++	++++
113		-	-	17	20	+	+
115		15	15.5	46	48	+++++	++++
6		8-10	10	12	36	40	++
8	-		-	52	49	++	++
19	14		13	63	60	+++++	++++
50	-		-	35	24	+	+
116	6		-	62	21	++	++
21	12-14		8	9	19	20	+
54		8.5	8	60	54	+++++	++++
55		8	8.5	75	39	+++++	++++
117		9	9	42	32	++	++
118		-	-	57	45	+++++	++++
11		17-21	13	14.5	87	84	+++++
51	8.5		11	48	52	+++++	++++
52	13		7.5	58	49	++	++
53	9		8.5	57	60	+++++	++++

<sup>a</sup> -, Negative reaction; +, ++, +++, extent of macrophage aggregation.

<sup>b</sup> Enhancement.

TABLE 2. Intracellular viable count of *L. monocytogenes* in peritoneal macrophages obtained from guinea pigs inoculated subcutaneously with live *N. asteroides* at different time intervals

Guinea pig no.	Time (wks)	Intracellular viable count ( $\times 10^3$ ) with:					
		No antigen added		N-PPD antigen added		N-PP antigen added	
		1.5 h	4 h	1.5 h	4 h	1.5 h	4 h
101	1-3	13,000	230,000	1,500	70	5,400	270
102		1,000	600	1,100	140	490	2,900
105		1,600	2,000	1,000	2,000	1,000	150
106		150	2,100	190	1,000	70	1,500
7	4-5	ND <sup>a</sup>	ND	ND	ND	ND	ND
107		370	2,500	140	30	90	65
108		53,000	60,000	11,000	10,000	8,600	8,500
111		5,800	23,000	3,900	490	1,000	360
112		7,000	45,000	2,100	5,000	9,100	6,000
114		750	45,000	3,600	20,000	12,000	10,000
5	6-7	ND	ND	ND	ND	ND	ND
9		ND	ND	ND	ND	ND	ND
113		10,000	37,000	8,700	5,500	7,000	6,000
115		30,000	9,750	6,250	4,500	7,000	750
6	8-10	ND	ND	ND	ND	ND	ND
8		ND	ND	ND	ND	ND	ND
19		ND	ND	ND	ND	ND	ND
50		1,000	1,200	280	9	ND	ND
116		8,200	240,000	1,900	450	1,900	450
21		12-14	2,000	11,000	400	30	ND
54	3,500		25,000	130	50	ND	ND
55	470		880	420	1	ND	ND
117	2,750		19,500	360	32	50	24
118	3,350		7,200	370	60	270	115
11	17-21	ND	ND	ND	ND	ND	ND
51		200	3,000	650	9	ND	ND
52		2,400	5,000	400	100	ND	ND
53		750	1,500	750	120	ND	ND

<sup>a</sup> ND, Not done.

## DISCUSSION

The results presented here indicate that guinea pigs developed CMI during the course of experimental nocardia infection. This appeared in 6 to 7 weeks. In the early period, although DH was present, as indicated by a positive skin test, there was no evidence of increased CMI as shown by increased MIC-A or MMI. Such a dissociation between DH and CMI has also been observed in other infections (10). In fact, these workers suggested that DH and CMI are mediated by a different population of T cells. We also noticed that not all of the guinea pigs developed dermal reactivity after an injection of live *N. asteroides*. When the immune spleen cells were transferred from DH-positive guinea pigs into normal guinea pigs, the DH disappeared after two passages, whereas MMI and

MIC-A persisted. This also confirms our view that the factor responsible for DH is different from the MMI factor, even though it may be difficult to distinguish between them. We felt that a better index for CMI in our animals was the extent of MMI, MA, and MIC-A. Based on these parameters, it was found that there was no significant CMI during the period when an active infection was present (4 to 5 weeks). From 6 to 7 weeks there was a gradual increase in MMI and MIC-A. This increase generally coincided with the onset of healing in ulcers. The CMI was seen to persist for at least 21 weeks. We tried to find the effect of DH alone in some of these animals, and the results of spleen cell transfer from donors with positive DH and little or no CMI (4 to 5 weeks) did not result in affording protection to the animals. These guinea pigs did not survive longer than the

TABLE 3. *MMI and MIC-A in peritoneal macrophages of donor and recipient guinea pigs before and after immune spleen cell transfer and the effect of intravenous challenge with N. asteroides on tissue counts in recipient and control guinea pigs*

Donor guinea pig no.	CMI in donor guinea pigs at time of transfer				Recip-ient guinea pig no.	Day of sac-rifice	Gross lesions in internal organs <sup>a</sup>	Total no. of viable <i>N. asteroides</i> in:			CMI in recipient guinea pigs at time of sacrifice after challenge							
	MMI (%)	MIC-A (no. of intracellular listeria [ $\times 10^6$ ])		N-PPD				Lungs	Heart	Kidneys	Liver	MMI (%)	MIC-A (no. of viable listeria [ $\times 10^6$ ])		N-PPD	With N-PPD		
		N-PPD	No antigen										4 h	1.5 h			4 h	1.5 h
	50	62	31,000	59,500				2,550	70	88	5	L <sup>+</sup>	2,800	NG <sup>b</sup>	1,700	5,000	48	49
86	50	62	31,000	59,500	2,550	70	89	10	NG	NG	NG	5,000	51	56	200	3,000	650	10
68	48	41	670	1,000	250	4	71	24	NG	NG	NG	NG	68	69	410	2,950	25	0.4
87	57	53	18,500	36,500	120	60	90	24	NG	NG	NG	NG	37	44	550	7,500	1,100	730
87	57	53	18,500	36,500	120	60	91	30	NG	NG	NG	NG	35	40	1,050	17,000	1,350	55
68	48	41	670	1,000	250	4	72	60	NG	NG	NG	NG	32	36	540	100	240	10
69	65	47	6,000	2,450	1,000	6	73	80	NG	NG	NG	NG	39	40	235	2,200	260	225
69	65	47	6,000	2,450	1,000	6	74	87	NG	NG	NG	NG	42	45	245	1,250	315	75
Control guinea pigs challenged intravenously with <i>N. asteroides</i> <sup>c</sup>																		
92							6	L <sup>+</sup> , H <sup>+</sup> , K <sup>+</sup>	15,000	280,000	52,000	3,000	16	10	150	10	185	6
93							6	L <sup>+</sup> , H <sup>+</sup> , K <sup>+</sup>	10,000	240,000	22,000	2,000	25	12	220	305	430	8
24							7	L <sup>+</sup> , K <sup>+</sup>	ND	ND	ND	ND	8 <sup>d</sup>	-31 <sup>d</sup>	ND	ND	ND	ND
48							5	L <sup>+</sup> , K <sup>+</sup>	ND	ND	ND	ND	10 <sup>d</sup>	-4 <sup>d</sup>	ND	ND	ND	ND
94							10	L <sup>+</sup> , H <sup>+</sup>	460,000	175,000	21,000	NG	28	31	1,900	1,800	400	3,500
95							10	L <sup>+</sup> , H <sup>+</sup>	330,000	28,000	510,000	NG	35	17	2,500	1,000	2,350	150
96							15	L <sup>+</sup> , H <sup>+</sup>	1,800,000	1,500	8,000	NG	16	14	17,050	1,250	700	1,800

<sup>a</sup> L, Lungs; H, heart; K, kidneys; +, gross lesions present; -, no gross lesions.

<sup>b</sup> NG, No growth; ND, not done.

<sup>c</sup> Normal guinea pigs challenged with *N. asteroides* became sick after 5 days. They were sacrificed in this state.

<sup>d</sup> Enhancement.

control animals, nor did they show any change in the duration and appearance of lesions. On the other hand, when CMI was transferred from guinea pigs possessing a high CMI, as shown by increased MMI and MIC-A, to normal guinea pigs, the recipients survived much longer than control animals after an intravenous challenge. Their tissues also did not show any growth of *N. asteroides*. In fact, they were sacrificed to obtain viable counts in different tissues. Such recipient guinea pigs with a high CMI also did not develop ulcers and sinuses to the same extent as control guinea pigs after subcutaneous infection with *N. asteroides*, and whenever some appeared they healed rapidly. We, therefore, feel that CMI plays an important role in protection against *N. asteroides* infection.

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