INFECTION AND IMMUNITY, Oct. 1977, p. 253-256

Vol. 18, No. 1
Printed in U.S.A.

NOTES

Cell-Mediated Immunity to *Nocardia asteroides* Induced by Its Ribonucleic Acid Protein Fraction

T. SUNDARARAJ AND S. C. AGARWAL*

Department of Microbiology, Jawaharlal Institute of Postgraduate Medical Education and Research, Pondicherry-605 006, India

Received for publication 23 February 1977

Guinea pigs immunized with ribonucleic acid protein fraction from *Nocardia asteroides* developed high cell-mediated immunity within 2 weeks, as shown by increased macrophage microbicidal activity and macrophage migration inhibition. This immunity protected against intravenous and subcutaneous challenge with *N. asteroides* and persisted for at least 70 days.

In an earlier communication, we reported the production of experimental mycetoma in guinea pigs and development of cell-mediated immunity (CMI) during experimental infection with *Nocardia asteroides* (4). For immunoprophylaxis against *Nocardia* infection, it is necessary to look for antigens that produce protective CMI. The protective effect of ribosomal vaccines has been demonstrated in some bacterial infections (2, 5, 6). There is no report on development of CMI and its protective effect after administration of ribonucleic acid protein fraction (P-RNA) of *N. asteroides*. We have therefore investigated it in guinea pigs.

Healthy, outbred guinea pigs of both sexes, weighing 350 to 450 g, bred locally and fed on grams and fresh green vegetables, were used. They were immunized with P-RNA obtained from *N. asteroides*. The protein content of the P-RNA was determined by the method of Lowry et al. (1), with the modifications that for removing traces of remaining phenol, ether was added, and nitrogen was bubbled through the phenol-extracted P-RNA solution to remove ether. This was repeated three to four times. The RNA content of P-RNA was estimated by pentose analysis as described by Schneider (3). P-RNA contained RNA (325 μg/ml) and protein (250 μg/ml); the RNA/protein ratio was 56:44. Besides P-RNA, purified protein derivative (PPD) and polypeptide (PP) antigens obtained from *N. asteroides* were used to stimulate immune peritoneal macrophages. These were prepared as described before (4).

Guinea pigs were immunized by intradermal injection of 0.2 ml of a mixture containing equal volumes of P-RNA solution and incomplete Freund adjuvant. It contained 6 μg of RNA and 5 μg of protein. A second injection was given after 1 week. Groups of three guinea pigs were sacrificed at different times, and the development of CMI post-immunization was observed. Spleen cell transfer, challenge with *N. asteroides*, viable counts in tissues, and measurement of CMI were done as described before (4). The parameters used for measuring CMI were dermal reactivity or skin hypersensitivity, macrophage migration inhibition (MMI), macrophage aggregation (MA), and microbicidal activity. An increase in MMI above 20% was interpreted as significant. For measurement of microbicidal activity, the 1.5- to 4-h viable counts of *Listeria monocytogenes* in macrophages not stimulated by antigen served as a control. These counts were compared with the microbicidal activity of immune macrophages stimulated by PPD, PP, or P-RNA. A decrease in the 1.5- to 4-h viable *Listeria* cell counts after stimulation of immune macrophages with antigen indicated microbicidal activity.

CMI to *N. asteroides* appeared as early as 14 days after immunization with P-RNA (Tables 1 and 2). These immunized guinea pigs, sacrificed 14 days after immunization, did not develop any dermal reactivity, MMI was about 25%, MA was absent, but microbicidal activity was present. Because of the presence of microbicidal activity, we feel that immunized animals developed CMI within 14 days. After 25 days, CMI was more marked. Although dermal reactivity was again absent, MMI increased above 20%. MA and microbicidal activity also increased. At 35 and 45 days post-immunization, CMI was high and a considerable increase in MMI and microbicidal activity was observed in the presence of PP, PPD, and P-RNA. At 70

TABLE 1. *Development of CMI in guinea pigs after intradermal inoculation of P-RNA from N. asteroides*[a]

Guinea pig no.	Time (days)	MMI (%)			MA[b]		
		PPD	PP	P-RNA	PPD	PP	P-RNA
119	14	24	6	25	+	+	+
120		31	−22[c]	6	−	−	−
169		26	23	19	+	+	+
121	25	54	20	70	+++	+	+++
122		69	12	67	+++	±	+++
168		41	25	47	+	+	+
170	35	36	37	44	++	++	++
171		52	23	33	+++	+	++
172		43	33	32	++	+	++
123	45	30	15	27	−	−	+
124		41	62	26	−	+	+
125		59	49	39	++	++	++
126	70	5	−21[c]	−12[c]	−	−	−
127		41	20	51	++	++	++
128		−5[c]	−48[c]	−18[c]	−	−	−

[a] No dermal reactivity was seen in any immunized guinea pigs.
[b] +++, ++, +, −, and ± denote degree of MA.
[c] Enhancement.

TABLE 2. *Microbicidal activity of peritoneal macrophages obtained from guinea pigs immunized intradermally with P-RNA from N. asteroides*

Guinea pig no.	Time (days)	Intracellular viable count (×10³) of *L. monocytogenes*											
		No antigen added		PPD antigen added			PP antigen added			P-RNA antigen added			
		1.5 h	4 h	1.5 h	4 h		1.5 h	4 h		1.5 h	4 h		
119	14	1,000	7,500	3,250	1,850	$P = 0.05$	950	160	$P = 0.01$	145	90	$P = 0.05$	
120		9,000	105,000	2,250	5,450		4,500	1,250		8,250	205		
169		36,500	140,000	13,500	520		25,500	5,550		1,050	650		
121	25	7,200	17,500	1,975	575	$P < 0.01$	6,500	550	$P < 0.01$	625	500	$P < 0.01$	
122		12,050	15,000	920	75		11,800	355		560	300		
168		10,000	85,000	39,500	4,500		22,000	3,950		12,000	7,850		
170	35	5,150	13,000	11,000	4,600	$P = 0.01$	50,000	10,650	$P < 0.01$	36,000	7,500	$P < 0.01$	
171		10,000	68,500	16,500	750		3,000	475		24,000	950		
172		3,000	3,800	5,000	295		10,000	200		1,500	250		
123	45	65	190	8	5	$P < 0.01$	25	1	$P < 0.01$	6	1	$P < 0.01$	
124		1,700	16,000	155	30		120	30		250	50		
125		30,500	46,000	1,850	85		1,300	45		2,650	60		
126	70	12,500	32,500	265	115	$P < 0.01$	675	165	$P < 0.01$	455	580[a]	$P < 0.01$	
127		305	2,500	48	48		435	125		53	210[a]		
128		3,000	35,000	20,000	450		500	1,500		3,750	20,000[a]		

[a] Increase in number of viable *L. monocytogenes* was not to same extent as in control, unstimulated macrophages.

days, CMI was still present as measured by an increase in microbicidal activity, although MMI was less than 20% and MA was negative in two guinea pigs (no. 126 and 128). In another animal (no. 130), increased MMI persisted after induction with PPD and P-RNA but not with PP. We have therefore observed that, unlike CMI in experimental nocardiosis produced by injecting small, sublethal doses of live *N. asteroides*, which appears in 6 to 7 weeks, the CMI after injection of P-RNA appeared earlier (within 2 weeks) and persisted for at least 10 weeks.

To investigate the protection afforded by P-RNA, several guinea pigs were actively immunized with it and challenged with *N. asteroides*. These animals were sacrificed at different days

post-immunization. Gross lesions in the liver, spleen, kidneys, heart, and lungs were investigated, and the tissue count for *N. asteroides* was done. The results were compared with those obtained from normal healthy guinea pigs challenged with *N. asteroides* (Table 3). Unimmunized guinea pigs (no. 165, 166, and 167) became sick within 5 days, lost weight rapidly, and died within 15 to 20 days. On postmortem, gross lesions, i.e., white nodules (micro-abscesses), were found in the heart, liver, spleen, kidneys, and lungs. The tissue counts were high. In immunized animals (no. 138 and 139) sacrificed 10 days after challenge, gross lesions were absent in most of the internal organs, and total viable numbers of *Nocardia* in tissues were much lower than in controls; some tissues did not show any organisms at all. In other guinea pigs (no. 142, 143, 144, and 145), 34 days after challenge with *Nocardia*, gross lesions were completely absent from the heart, liver, spleen, kidneys, and lungs, and the tissues were completely free from any organisms. This shows that CMI produced by administration of P-RNA is highly protective, as is also suggested by the increased survival of experimental animals up to at least 56 days and absence of sickness, gross lesions (micro-abscesses), or *Nocardia* in internal organs. The organisms seen early, within 10 days after challenge, were probably all killed by the increased specific microbicidal activity of immune macrophages. This has been corroborated by further work (unpublished data).

To eliminate the effect of antibodies in protection against *N. asteroides* challenge, CMI was transferred passively by immune spleen cells from actively immunized (P-RNA) donor guinea pigs into normal recipient guinea pigs. The recipient guinea pigs were challenged intravenously with *N. asteroides*. These animals were sacrificed at different days after challenge. Development of gross lesions in internal organs was investigated as before, and total tissue counts were done (Table 4). Gross lesions in the heart, liver, and kidneys and viable *Nocardia* were found in animals sacrificed on day 5 (no. 177 and 181). In guinea pigs sacrificed 10 days after challenge (no. 178 and 179), gross lesions were present only in the kidneys. Viable bacteria were grown from kidneys only. In guinea pigs sacrificed 30 days and later after challenge (no. 174 and 175), no gross lesions in any internal organs and no viable *Nocardia* were present. This shows that CMI induced by P-RNA has a predominant role in protection against *N. asteroides*.

The present work shows that guinea pigs immunized with P-RNA of *N. asteroides* develop CMI within 14 days. This immunity appeared earlier than that induced during the course of experimental *Nocardia* infection, where it appeared in 6 to 7 weeks. The CMI was protective, and the immunized animals survived much longer after intravenous challenge. It was also observed that the guinea pigs did not become sick for at least 56 days. This immunity was cell mediated and not dependent on circulating antibodies, as shown by protection in passively immunized animals. After administration of immunogenic P-RNA, the animals not only survived longer, but the course of disease was restricted, since the gross lesions on various organs (liver, spleen, kidneys, and lungs) became fewer until finally no gross nocardial lesions were seen. Similarly, the viable count of *Nocardia* in various tissues was much less than in controls after

TABLE 3. *Effect of intravenous challenge with N. asteroides in guinea pigs with CMI actively induced by P-RNA from N. asteroides*[a]

Guinea pig no.	Day of sacrifice (post-challenge)	Gross lesions	Total no. of viable *N. asteroides* in tissues				
			L	H	K	Sp	Li
138	10	H	NG	180,000	NG	NG	NG
139	10	—[b]	NG	150	1,000	1,350	150
140	13[c]	—	200	NG	50	NG	NG
141	16	H	400	65,000	350	450	2,550
143	34	—	NG	NG	NG	NG	NG
142	37	—	NG	NG	NG	NG	NG
144	56	—	NG	NG	NG	NG	NG
145	56	—	NG	NG	NG	NG	NG
167[d]	6[c]	L,K	1,500,000	40,000	475,000	15,000	75,000
165[d]	15	L,H,K	150,000	200,000	100,000	5,000	300
166[d]	15	L,H,K	1,000,000	200,000	500,000	15,000	3,000

[a] Abbreviations: L, Lungs; H, heart; K, kidneys; Sp, spleen; Li, liver; NG, no growth of *N. asteroides*.
[b] —, No gross lesions.
[c] Died.
[d] Control guinea pigs.

TABLE 4. *Effect of intravenous challenge with N. asteroides in guinea pigs with CMI passively transferred (spleen cell transfer) from guinea pigs actively immunized with P-RNA from N. asteroides*[a]

Recipient guinea pig no.	Day of sacrifice after challenge	Gross lesions	No. of viable *Nocardia* in different tissues				
			L	H	Li	Sp	K
177	5	L,H,K	30,000	17,000	16,000	15,000	15,500
181	5	L,H,K	35,000	85,000	40,000	15,000	13,500
178	10	K	NG	NG	NG	NG	4,000
179	10	K	NG	NG	NG	NG	100,000
174	30	—[b]	NG	NG	NG	NG	NG
175	30	—	NG	NG	NG	NG	NG
176	45	—	NG	NG	NG	NG	NG
180	45	—	NG	NG	NG	NG	NG

[a] Abbreviations are as in Table 3.
[b] —, No gross lesions.

challenge until finally there were no living organisms in the tissues. Similarly, on subcutaneous challenge of immunized animals with *N. asteroides*, the lesions (subcutaneous abscesses) appeared later and were fewer. Our findings, therefore, stress the immunogenic nature of P-RNA of *N. asteroides* and suggest its use in the prevention and restriction of *Nocardia* infection.

LITERATURE CITED

1. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
2. **Misfeldt, M. L., and W. Johnson.** 1976. Variability of protection in inbred mice induced by a ribosomal vaccine prepared from *Salmonella typhimurium*. Infect. Immun. **14**:652–659.
3. **Schneider, W. C.** 1957. Determination of nucleic acids in tissues by pentose analysis. Methods Enzymol. **3**:680–684.
4. **Sundararaj, T., and S. C. Agarwal.** 1977. Cell-mediated immunity in experimental *Nocardia asteroides* infection. Infect. Immun. **15**:370–375.
5. **Venneman, M. R., and L. J. Berry.** 1971. Cell-mediated resistance induced with immunogenic preparations of *Salmonella typhimurium*. Infect. Immun. **4**:381–387.
6. **Youmans, A. S., and G. P. Youmans.** 1965. Immunogenic activity of a ribosomal fraction obtained from *Mycobacterium tuberculosis*. J. Bacteriol. **89**:1291–1298.