

IMMUNOLOGICAL RESPONSES TO PROTEIN, CARBOHYDRATE AND LIPID FRACTIONS OF *NOCARDIA ASTEROIDES* IN MICE

RITA GUPTA, VIJAYKUMAR PANCHOLI, V. K. VINAYAK* AND G. K. KHULLER†

*Departments of Biochemistry and *Experimental Medicine, Postgraduate Institute of Medical Education and Research, Chandigarh-160012, India*

SUMMARY. The protein, polysaccharide and phospholipid constituents of *Nocardia asteroides* have been partially purified and their immunogenicity studied in mice. Humoral and cellular immune responses were demonstrated against a crude cytoplasmic protein fraction (CPF). Two fractions of CPF were prepared on a Sephadex G-200 column; a high mol. wt fraction, fraction-1(F₁) was capable of eliciting both types of immune responses, whereas fraction-2(F₂) behaved more like a haptén. Phosphatides elicited only humoral responses whereas polysaccharides were non-immunogenic.

INTRODUCTION

Nocardiosis, a systemic bacterial disease caused mainly by *Nocardia asteroides*, is no longer rare and its pathogenesis is incompletely understood. The relative importance of cellular and humoral immunity in nocardiosis is not clearly defined (Beaman and Maslan, 1977; Curry, 1980; Beaman *et al.*, 1982), although it has been suggested that cell-mediated immunity is more important than humoral. These studies appear to have been restricted to the response to whole nocardial cells which were either killed (Deem *et al.*, 1982) or viable (Krick and Remington, 1975; Hiramane *et al.*, 1981). Little attention has been given to the immune reactions induced by the proteins, polysaccharides and lipids of the nocardial cells. Because lymphocytes react with these components individually, it might be expected that the immune reactions that play a critical role in the pathogenesis of nocardiosis may be restricted to certain components of the nocardial cell rather than the whole cell. Hence, the present study was undertaken to investigate the immune responses elicited by constituents of *N. asteroides* in relation to the pathogenesis of the disease in mice.

MATERIALS AND METHODS

Bacterial strain. *N. asteroides* NCTC 8595 (equivalent to ATCC 14759) was obtained from the National Collection of Type Cultures, Collindale, U.K. and grown in Brain Heart Infusion Broth (BHI; HI-Media, Bombay, India) 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 and fed on a standard pellet diet (Hindustan Lever Ltd, Bombay, India) with water *ad libitum*.

Received 22 Nov. 1984; accepted 28 Jan. 1985.

† Requests for offprints should be sent to Dr G. K. Khuller.

Preparation of protein components. A 7-day growth of *N. asteroides* in BHI broth was harvested, suspended in acetone and left overnight. The cells were filtered, freed from acetone by evaporation at room temperature and dried. The dried cells were suspended in normal saline in a 10% (w/v) suspension. The cells were then sonicated at 60W (20 KC/s) intermittently in a "Sonifier" cell disrupter (Branson Sonic Power Co., Danbury, CT, USA) for 3 h in the cold. The unbroken cells were removed by centrifugation at 2800 *g* for 30 min and the supernate again centrifuged at 25 000 *g* for 50 min in a refrigerated centrifuge (Sorvall RC-2B, Newton, CT, USA) to remove cell walls. The clear supernate was subjected to ammonium sulphate precipitation at 70% saturation. The precipitates were kept at 4°C overnight, dissolved in the minimum amount of PBS, pH 7.4, and then dialysed against the same buffer for 3 days at 4°C to ensure complete removal of the ammonium sulphate as checked by the disappearance of white precipitates after addition of barium chloride to samples of the dialysate. This was denoted as crude protein fraction (CPF).

Four ml of the CPF (32 mg) was applied to a Sephadex G-200 (Pharmacia Fine Chemical, Uppsala, Sweden) column (2 × 80 cm) which had been packed under a pressure of 16 cm of Hg. The flow rate was adjusted to 20 ml/h and the proteins eluted with 0.01 M phosphate buffer, pH 7.4. The eluants were collected in 4.2-ml fractions with an automatic fraction collector (LKB, Bromma, Sweden) and the absorbance read at 280 nm in a Beckman (Model-25) Spectrophotometer (USA). The elution volume was plotted against optical density and two peaks were obtained. The fractions under the two peaks were pooled and concentrated by dialysis against solid PEG (Polyethylene glycol, mol. wt 20 000; Sigma Chemicals, St Louis, MS, USA). These fractions were designated F₁ and F₂ respectively.

The fractions CPF, F₁ and F₂ were subjected to polyacrylamide gel electrophoresis (Davis, 1964) to check the complexity of their protein constituents at 5 mA/gel for 2½ h in the presence of Tris-glycine buffer, pH 8.3. Protein estimation at each stage was done according to the method of Lowry *et al.* (1951).

Isolation and purification of polysaccharide fraction. *N. asteroides* was grown in BHI at 37°C for 7 days (stationary phase) and killed by autoclaving at 121°C for 20 min. The cells were then harvested and suspended in a mixture of chloroform and methanol (2:1, v/v) with constant stirring for 3 h to extract the lipids. The procedure was repeated twice and the filtrates pooled and used for the isolation of a lipid fraction. The defatted cells were used for the isolation of polysaccharide as described by Zamora *et al.* (1963). Briefly, the cells (10 g) were ground with 20 g of pyrex glass powder followed by treatment with 150 ml of 3 M potassium chloride (KCl). The cell-free supernate obtained after centrifugation (3500 *g* for 2 h) of the suspension was added to 10 volumes of methanol and kept at 4°C for 24 h. The precipitates were centrifuged (1000 *g* for 10 min.) and then suspended in 0.02 M PBS, pH 7.2. They were dialyzed against running tap water for 72 h and reprecipitated with 10 volumes of methanol. The precipitates were dried on acetone. The white precipitates were dissolved in 0.02 M carbonate buffer, pH 10.00. The soluble fraction was removed and designated as PS/I. The insoluble fraction was dissolved in 0.02 M citrate buffer, pH 5.0, and designated as PS/II. Polysaccharides in each fraction were estimated by the method of Dubois *et al.* (1956) with glucose as standard.

Purification of lipid fraction. Total phospholipids were used as an antigen. They were extracted according to the method of Folch *et al.* (1957). Autoclaved cells were extracted with chloroform:methanol (2:1, v:v) twice or thrice and all the extracts were pooled. To this, one-fifth volume of 0.7% potassium chloride was added, mixed and kept overnight. The aqueous layer was removed and the remaining organic layer transferred to a round-bottomed flask and dried under vacuum. After two washings with chloroform-methanol-water (16:8:1, v:v:v), the lipid was dried, dissolved in a known volume of chloroform and the phosphorus estimated (Bartlett, 1959). Neutral lipids were removed by repeated washings with acetone and the total phospholipids used for further work.

Identification and separation of individual phospholipids. Individual phospholipids were identified and separated by preparative thin-layer chromatography on silica gel-H (BDH, Glaxo Laboratories [India] Ltd, Bombay) plates by the method of Khuller (1976).

Immunisation schedule. Immunisation of mice was performed in a group of 20–30 animals with CPF, F₁, F₂, PS/I, PS/II and phospholipids as described below. They were designated as group I to group VI respectively.

Group Ia received three intraperitoneal injections each of CPF 50 µg in 0.1 ml, emulsified in an equal volume of Freund's incomplete adjuvant (FIA) at 5-day intervals. Group Ib was immunised with a single intraperitoneal injection of CPF 150 µg in 0.1 ml emulsified with FIA. Groups II and III received 150 µg of F₁ and F₂ respectively, emulsified in FIA, in three equally divided doses, on days 0, 5 and 10.

Groups IV and V received PS I and PS II, respectively, by three different schedules: (i) a single intraperitoneal injection of 1 mg of polysaccharide (PS I or PS II) emulsified in FIA; (ii) three subcutaneous injections (0.2 ml), each of 100 µg, of each polysaccharide fraction with an equal amount of FIA at 5-day intervals; (iii) 50 µg of polysaccharide (0.1 ml) by intravenous injection in the tail vein on days 0 and 3 followed by subcutaneous injection of 50 µg of polysaccharide and 50 µg of methylated bovine serum albumin (MBSA) emulsified with FIA on day 6 and 50 µg of polysaccharides intravenously on day 9.

Group VI was immunised with three subcutaneous injections each of phospholipid (25 µg lipid phosphorus) complexed with MBSA (Khuller and Subrahmanyam, 1971) and emulsified in FIA, at weekly intervals.

With each group a control group of animals (10 mice) was given in parallel, normal saline emulsified with or without FIA depending on the type of experiment in a corresponding schedule.

Measures of humoral immune response. The animals were bled before immunisation and samples of serum were stored at -20°C for further use. In groups of 5 or 6, the mice were bled to death and their spleens removed aseptically on days 7, 14, 21 and 28 after completion of immunisation. The sera were separated and stored at -20°C. The presence of specific or cross reacting antibodies or both were first detected by gel diffusion, immunoelectrophoresis and counter-current immunoelectrophoresis (CIE). A serum antibody titre for the animals immunised with the protein fractions was determined by indirect haemagglutination (IHA) as described by Herbert (1978), by use of 1.25% tanned sheep RBC coated with 88.5 µg of protein/ml. Anti-phospholipid antibody titres were determined by kaolin agglutination (Takahashi, 1962) with 0.1% w/v standard kaolin suspension sensitised with 60 µg/ml of total phospholipids.

Measures of cell-mediated immune response (CMI). Aseptically-removed spleens were used to study cellular immune response by leukocyte migration inhibition (LMI) (Falk and Zabriskie, 1971). Briefly, the capillary tubes (length 75 mm, internal diameter, 1.1 mm) were filled with spleen-cell suspension (10⁷ viable leukocytes/ml), centrifuged (200 g for 10 min) and cut at the cell-fluid interphase. The capillaries were placed in the leukocyte migration chambers (Laxbro, Pune, India) sealed with glass coverslips in the presence and absence of specific antigen (protein fractions F₁ 80 µg/ml, F₂ 50 µg/ml) polysaccharide (PS I 35 µg/ml; PS II 60 µg/ml) or total phospholipids (100 µg/ml) depending on the experiment. These antigen concentrations were the highest that gave no inhibition of migration with leukocytes from unimmunised animals. The percentage migration inhibition was calculated by the formula $(C - T/C) \times 100$ where C is the migration in the absence of antigen and T is migration in the presence of the antigen.

RESULTS

The elution profile obtained showed two distinct peaks which were designated F₁ and F₂ (fig. 1). The percentages of protein distributed in and recovered from the two fractions are shown in the table; 87.75% of the protein applied was recovered in fractions 1 (F₁) and 2 (F₂)—32.6% in F₁ and 55.1% in F₂.

After polyacrylamide-gel electrophoresis, the CPF gave eight major bands (fig. 2a) whereas six and two bands were seen in F₁ and F₂ respectively (figs 2 b and c). F₁ was more complex than F₂. Concentrated F₁ was subfractionated into four fractions by preparative PAGE (fig. 2b).

Immune responses after immunisation with protein fractions. The sera obtained from the animals immunised with multiple injections of CPF formed multiple precipitation

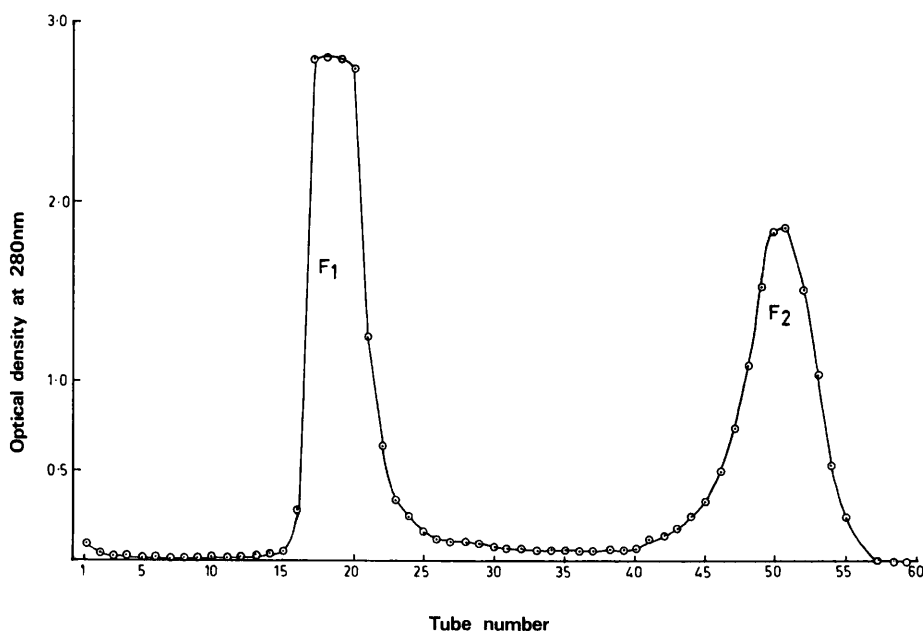


FIG. 1.—Elution profile of crude cytoplasmic protein fraction (CPF) on Sephadex G-200 column; F₁ and F₂ represent two distinct protein fractions.

TABLE
Elution profile of crude protein fraction on
Sephadex G-200*

Fraction number	Total volume (ml)	Protein (μg/ml)	Recovery of protein	
			Amount (mg)	Percentage of total CPF
1	36	290	10.44	32.625
2	49	360	17.64	55.125
Total recovery of protein			28.08	87.75

* Total of 32 mg protein (CPF) in 4.0 ml was fractionated.

lines with the homologous antigen in gel diffusion, immunoelectrophoresis and CIE (figs. 3 a,b). These antisera also formed multiple precipitation arcs with F₁ but only two with F₂. Agglutinating antibodies determined by the IHA test showed an increase in geometric mean titre from 844 (range 320–1280) in the first week after immunisation to a maximum of 5120 by 3 weeks after immunisation. The geometric mean titre obtained in the third week after immunisation was significantly higher ($p < 0.02$) than that obtained in the first week (fig. 4). In contrast to the results after immunisation by multiple injections, a single-dose immunisation procedure induced a relatively weak immune response. A significant agglutinating antibody response could not be detected in animals up to 2 weeks after immunisation.

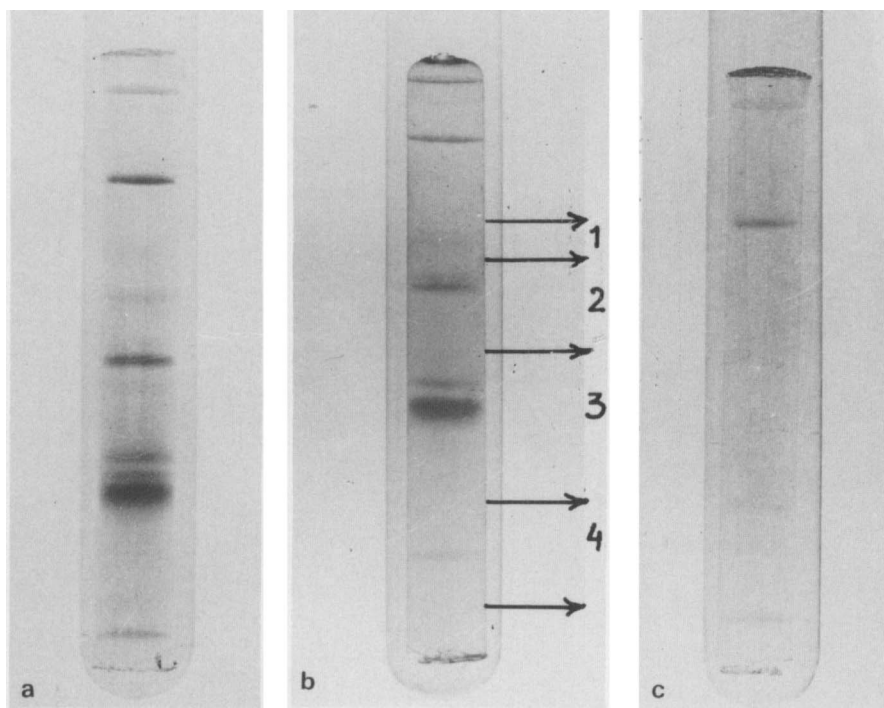


FIG. 2.—Polyacrylamide gel electrophoresis patterns of (a) CPF, (b) F_1 and (c) F_2 . The arrows indicate the points at which the gels were sliced to obtain different fractions of F_1 by preparative PAGE.

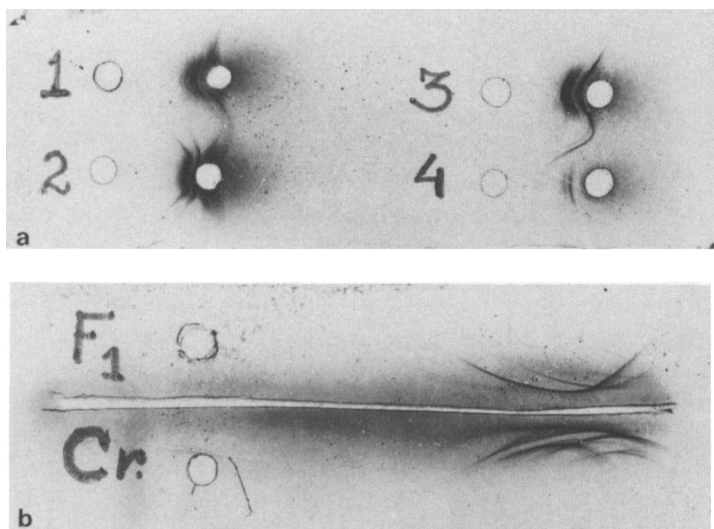


FIG. 3.—(a) Cross reactivity of the antisera raised against CPF demonstrated by CIE with different protein fractions obtained after gel filtration: (1) ammonium sulphate-precipitated CPF; (2) cytoplasmic extract; (3) F_1 , (4) F_2 .
(b) Immunoelectrophoretic patterns of F_1 and CPF (Cr) with antisera raised against CPF.

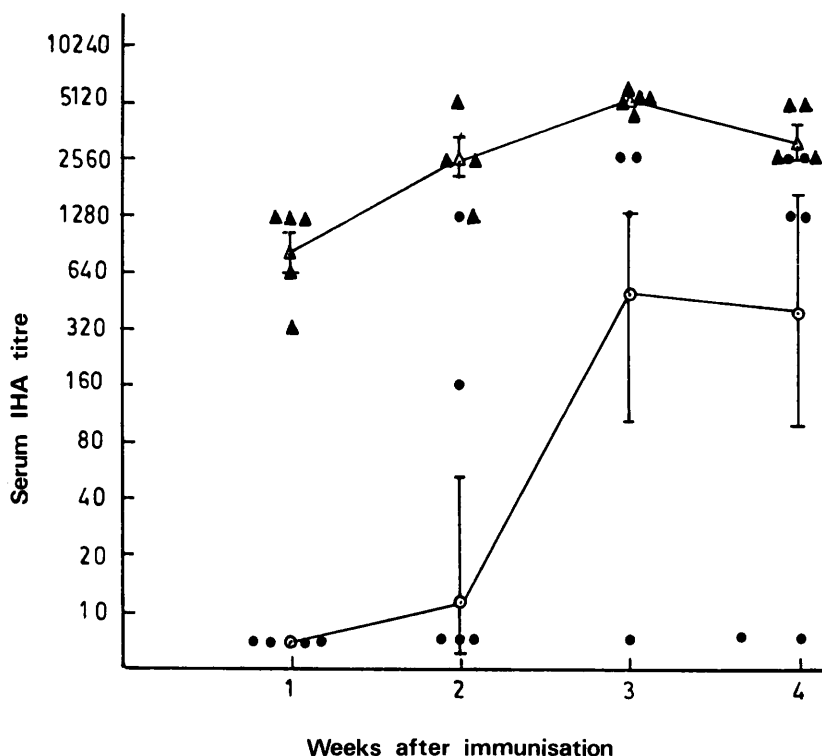


FIG. 4.—Comparison of indirect haemagglutinating titres in mice immunised with single (●—●) and multiple (▲—▲) doses of CPF. Vertical bars represent the standard error of the geometric mean.

The cellular immune response evaluated by LMI showed significant cellular sensitisation (fig. 5). CPF contains F_1 and F_2 and the LMI response was seen in the presence of F_1 and F_2 separately. A mean migration inhibition of $50.11 \pm 10.17\%$ and $44.68 \pm 4.62\%$ in the presence of F_1 and F_2 respectively was obtained in the first week after immunisation. The maximum cellular sensitisation with F_1 was seen 2 weeks after immunisation ($65.09 \pm 4.5\%$). A similar type of LMI was obtained with F_2 but the extent of migration inhibition was less than with F_1 . With the single-dose immunisation procedure, no significant migration inhibition ($p < 0.2$) was seen in the first week after immunisation but after 2 weeks significant migration inhibition ($p < 0.001$) was seen in the presence of F_1 or F_2 (fig. 5).

Immunisation with F_1 . Antisera obtained from mice immunised with F_1 formed multiple precipitation lines with the homologous antigen. The sera obtained from F_1 -immunised animals cross-reacted with CPF but did not cross-react with F_2 . Of the four subfractions of F_1 , three (nos. 2, 3 and 4) cross-reacted with CPF antisera. Maximum agglutinating antibody titre (geometric mean titre 5793) was detected 3 weeks after immunisation (fig. 6) and remained unchanged by 4 weeks.

The maximum LMI of $58.32 \pm 7.16\%$ was observed 4 weeks after immunisation. The migration pattern seen 1 week after immunisation and that seen after 4 weeks were essentially the same ($p < 0.1$) (fig. 7).

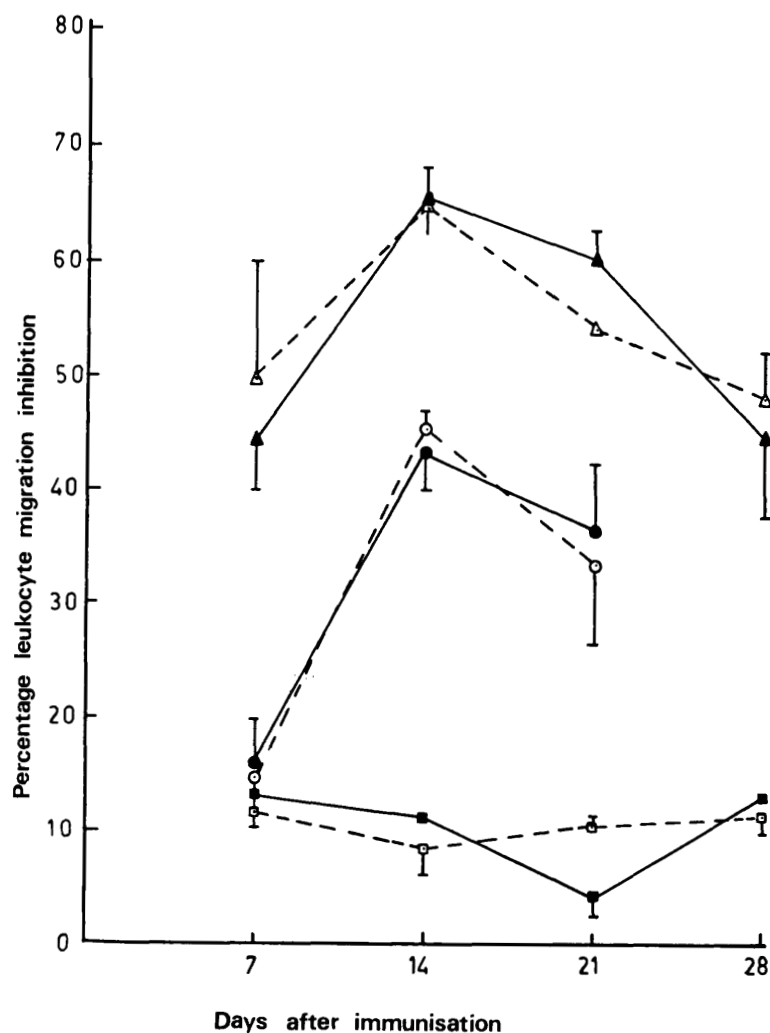


FIG. 5.—Migration inhibition of leukocytes obtained from the mice immunised with single and multiple doses of crude cytoplasmic fraction: ▲—▲, △---△ represent the multiple-dose immunization schedule and leukocyte migration inhibition (LMI) in the presence of F_1 and F_2 respectively; ●—●, ○---○ represent single-dose immunisation schedule and LMI in the presence of F_1 and F_2 respectively; ■—■, □---□ represent LMI in control group in the presence of F_1 and F_2 respectively. Vertical bars represent the standard errors of the means of the duplicate readings from at least three mice.

Immunisation with F_2 . Induction of immune responses after immunisation with F_2 was tested up to 4 weeks, but neither precipitating nor agglutinating antibodies were detected. The percentage LMI values were $<20\%$ in the presence of the homologous antigen. Thus, immunisation with F_2 protein did not induce either humoral or cellular immune responses.

Immunisation with PS I and PS II. The immunogenicity of PS I and PS II was studied with three different immunisation schedules. Precipitating or agglutinating antibodies were not detected up to 4 weeks after immunisation with any of the three

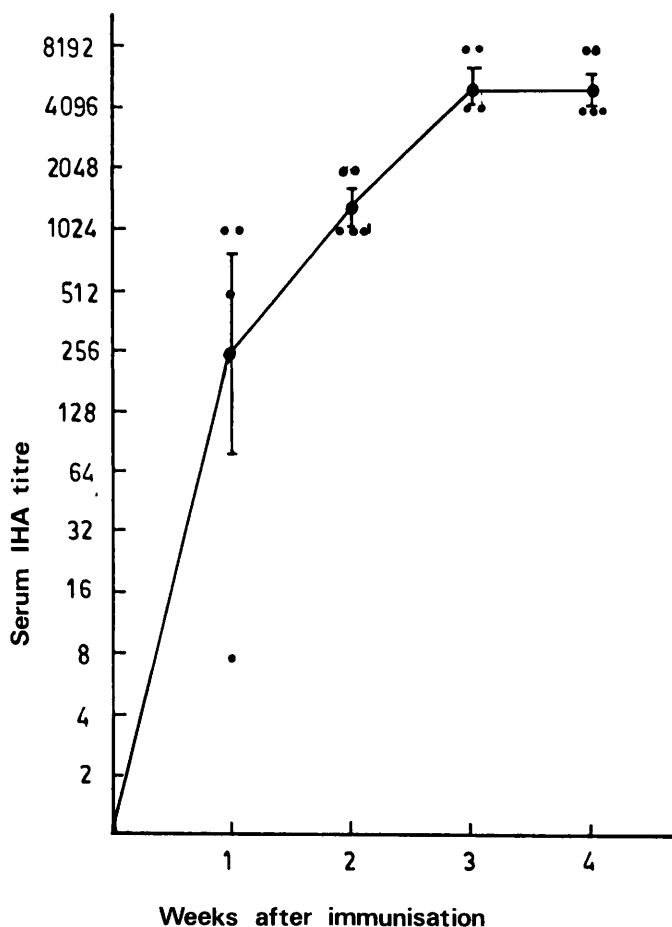


FIG. 6.—Indirect serum haemagglutinating antibody titre in mice immunised with F₁. Vertical bars represent standard errors of the geometric means obtained from 5 or 6 sera.

schedules. Moreover, there was no significant leukocyte migration inhibition ($\leq 20\%$) in immunised mice in the presence of homologous antigens.

Immunisation with total phospholipids. Precipitating antibodies against the homologous phospholipid antigen were detected from 1 week after immunisation with total phospholipids. However, these antibodies did not form precipitation lines with purified phosphatidylethanolamine (PE) or cardiolipin (CL) and were specific for the phosphatidylinositol-mannosides (PIM) fraction. The titres or agglutinating antibodies determined by the Kaolin agglutination test are shown in fig. 8. The agglutinating titres obtained up to the fourth week after immunisation varied from 320 to 1280.

Immunisation with phospholipids failed to elicit a significant CMI response even after 4 weeks.

DISCUSSION

Reports on the immunological activities of the various components of *N. asteroides* and their role in the pathogenesis of the disease are lacking, although various protein

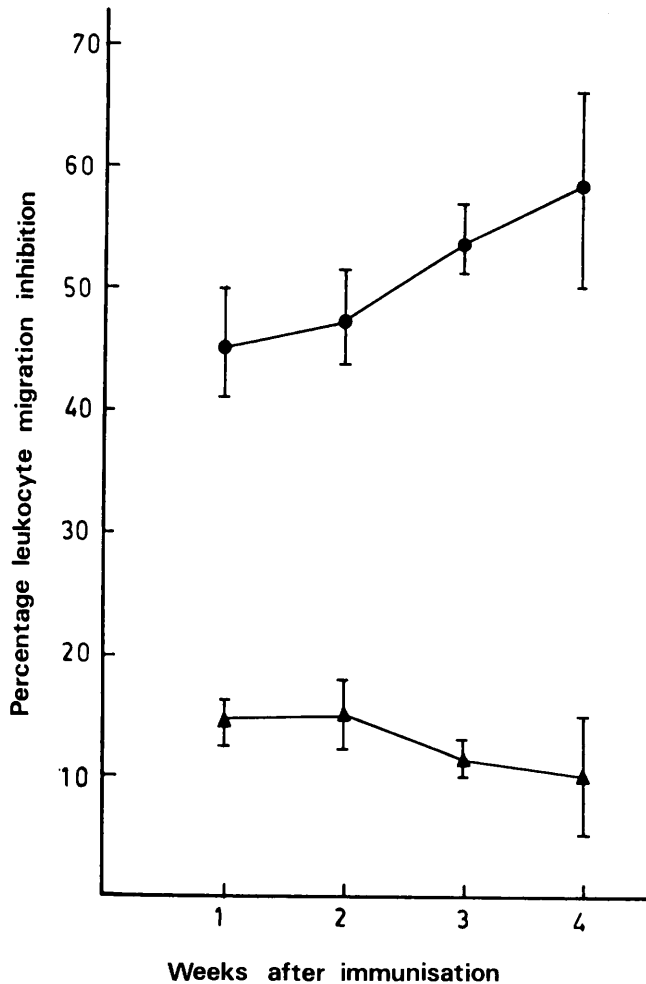


FIG. 7.—Migration inhibition of leukocytes obtained from mice immunised with F₁: ▲—▲ represents the control group; ●—● represents the immunised group. Vertical bars represent the standard errors of the means of duplicate readings from at least three mice.

(Palmer *et al.*, 1974; Shainhouse *et al.*, 1978; Blumer and Kaufman, 1979) and polysaccharide (Ortiz-Ortiz *et al.*, 1972) fractions from cultural filtrates and whole-cell extracts have been tested for use in diagnostic serology. In the present investigation, the proteins of *N. asteroides* elicited humoral and cellular responses. Lipids elicited only a humoral response whereas polysaccharides were not immunogenic.

Because the aim of the present study was to select immunologically active components with potential for use as immunoprophylactic agents, harsh physicochemical treatment of the bacteria, which may denature many of the proteins, was avoided. Acetone-killed cells were preferred for the isolation of protein antigens because this treatment carries relatively little risk of protein denaturation (Daniel and Janicki, 1978). Polyacrylamide-gel electrophoresis showed that the cytoplasmic protein fraction contained at least eight major protein bands. Although the exact mol.

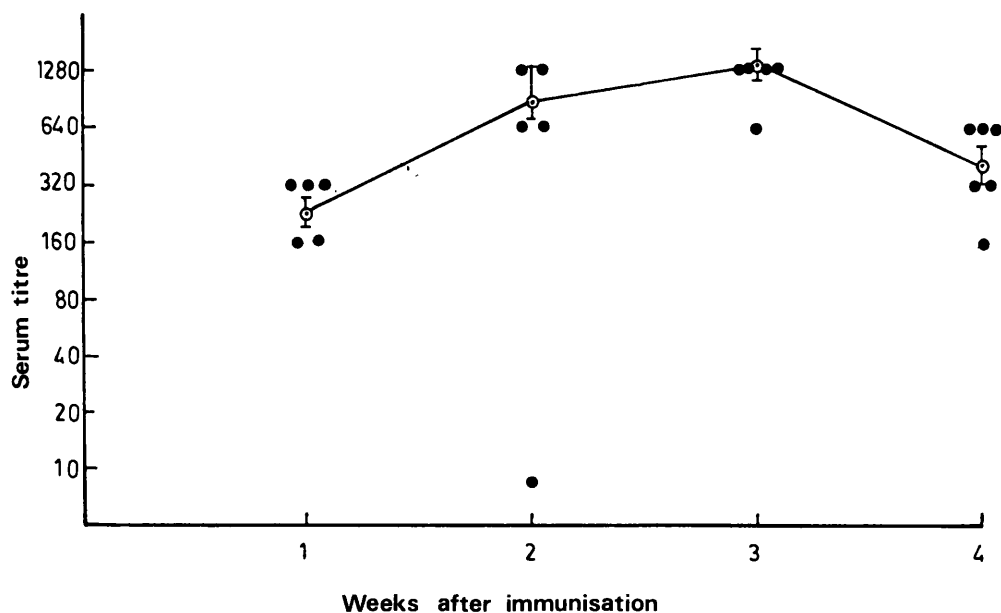


FIG. 8.—Kaolin agglutinating antibody titre of sera from mice immunised with total phospholipid. Vertical bars represent the standard errors of the geometric means of 5 or 6 sera.

wt of the F_1 and F_2 fractions were not determined, their elution volumes (V_e) indicated that the mol. wt of F_1 was *c.* 50 000 (V_e close to V_o) whereas F_2 was a low-mol. wt fraction with a V_e close to the bed volume (V_i). Furthermore, the electrophoretic patterns indicated that F_1 was more complex than F_2 . Preparative PAGE fraction 1 of F_1 was nonantigenic; it did not form precipitin line with antisera raised against F_1 . Although F_2 formed two precipitin lines with antisera raised against CPF, when used alone it did not induce an immunological response. This suggests that F_2 might act as a hapten which serves as an antigen when combined with a carrier protein such as F_1 . The failure of F_2 to induce an immunological response may be attributed to either its low mol. wt or its structure. In contrast to F_2 , F_1 induced a humoral as well as a cellular response as early as one week after immunisation. Use of an RNA-protein complex has been shown to induce cell-mediated immunity up to 70 days after immunisation in guinea pigs (Sundararaj and Agarwal, 1977), although it failed to elicit a humoral immune response.

Neither polysaccharide fraction elicited an immune response, even after selecting three different schedules of immunisation. Nocardial polysaccharide antigens have been investigated by Bojalil and Zamora (1963) for the diagnosis of mycetoma. The spleen cells from animals immunised with whole cells showed the production of MIF in the presence of homologous antigen (Ortiz-Ortiz *et al.*, 1972). The failure of purified polysaccharide fractions to elicit an immune response and the observation that precipitins could be detected against the same PS fractions in animals immunised with formalin-killed *Nocardia* (data not given) strongly suggested that these polysaccharides might be acting as haptens. An identical observation has been made with the closely related *Mycobacterium tuberculosis* (Boyden, 1956). The tuberculo-polysac-

charides are not themselves immunogenic although antigenicity is restored when they are conjugated with proteins or peptides. Our polysaccharide fractions were not completely free of contaminating proteins or peptides, but the treatment of the cells before extraction seem to have at least denatured them.

We have shown that the phospholipids of *N. asteroides* are antigenically similar to the mycobacterial phospholipids which are reported to elicit precipitating, agglutinating and complement-fixing antibodies (Khuller and Subrahmanyam, 1971). Furthermore, the antibodies raised against the total phospholipids did not cross-react with the PE and CL fractions but reacted only with PIM. Thus the PIM of *N. asteroides* is the only immunoreactive phospholipid as has also been found in mycobacteria (Khuller and Subrahmanyam, 1971; Banerjee and Subrahmanyam, 1978).

In contrast to the humoral response, CMI against lipid antigen was not detected. Although lipid-A of LPS, its synthetic analogs and related compounds have been shown recently to exhibit various immunological activities, including a cell-mediating immune response (Kotani *et al.*, 1983), the immunological role of phospholipids has not been investigated extensively. In view of the reports that the phospholipids of mycobacteria and *Nocardia* cross-react (Khuller and Chopra, 1983) and phospholipids are protective in nature (Khuller *et al.*, 1983), further studies on the immunological basis of the protective mechanism mediated by phospholipids are indicated.

From the present findings, it may be concluded that the protein antigens, especially the F₁ fraction, of *N. asteroides* show promise as active immunogens for prophylaxis and for the specific diagnosis of systemic or pulmonary nocardiosis.

This study was financed by the Indian Council of Medical Research, New Delhi, India.

REFERENCES

- Banerjee B, Subrahmanyam D 1978 Antigenicity of the phosphatidyl inositomannosides of Mycobacteria. *Immunochemistry* **15**:359-363.
- Bartlett G R 1959 Phosphorus assay in column chromatography. *Journal of Biological Chemistry* **234**:466-468.
- Beaman B L, Gershwin M E, Ahmed A, Scates S M, Deem R 1982 Response of CBA/N xDBA2/F₁ mice to *Nocardia asteroides*. *Infection and Immunity* **35**:111-116.
- Beaman B L, Maslan S 1977 Effect of cyclophosphamide on experimental *Nocardia asteroides* infection in mice. *Infection and Immunity* **16**:995-1004.
- Blumer S O, Kaufman L 1979 Microimmunodiffusion test for nocardiosis. *Journal of Clinical Microbiology* **10**:308-312.
- Bojalil L F, Zamora A 1963 Precipitin and skin tests in the diagnosis of mycetoma due to *Nocardia brasiliensis*. *Proceedings of the Society for Experimental Biology and Medicine* **113**:40-43.
- Boyden S V 1956 Antigens of *Mycobacterium tuberculosis*. *Advances in Tuberculosis Research* **7**:17-51.
- Curry W A 1980 Human nocardiosis: A clinical review with selected case reports. *Archives of Internal Medicine* **140**:818-826.
- Daniel T M, Janicki B W 1978 Mycobacterial antigens: a review of their isolation, chemistry and immunological properties. *Microbiological Reviews* **42**:84-113.
- Davis B J 1964 Disc electrophoresis II. Method and application to human serum proteins. *Annals of the New York Academy of Sciences* **121**:404-427.
- Deem R L, Beaman B L, Gershwin M E 1982 Adoptive transfer of immunity to *Nocardia asteroides* in nude mice. *Infection and Immunity* **38**:914-920.

- Dubois M, Gilles K A, Hemilton J K, Rebers P A, Smith F 1956 Colorimetric method for determination of sugars and related substances. *Analytical Chemistry* **28**:350-356.
- Falk R E, Zabriskie J B 1971 The capillary technique for measurement of inhibition of leucocyte migration. An assessment of cell-mediated immunity. In: Bloom B R, Glade P R (eds) *In vitro methods in cell-mediated immunity*, Academic Press, New York, pp 301-306.
- Folch J, Lees M, Sloane-Stanley G H 1957 A simple method for the isolation and purification of total lipids from animal tissues. *Journal of Biological Chemistry* **226**:497-509.
- Herbert W J 1978 Passive haemagglutination with special reference to the tanned cell technique. In: Weir D M (ed) *Handbook of experimental immunology*, 3rd edn, chapter 20, Blackwell, Oxford.
- Hiramine C, Hojo K, Yano I 1981 Development of cell-mediated hypersensitivity in guinea pigs following infection with *Nocardia asteroides*. *International Archives of Allergy and Applied Immunology* **65**:220-234.
- Khuller G K 1976 The mannophosphoinositides of *Nocardia asteroides*. *Experientia* **32**:1371-1372.
- Khuller G K, Chopra A 1983 Mannophosphoinositides of actinomycetales. *Actinomycetes* **17**:22-49.
- Khuller G K, Penumarti N, Subrahmanyam D 1983 Induction of resistance to tuberculosis in guinea pigs with mannophosphoinositides of mycobacteria. *IRCS Medical Science* **11**:288-289.
- Khuller G K, Subrahmanyam D 1971 Antigenicity of phosphatidyl inositomannosides of *Mycobacterium tuberculosis*. *Immunochemistry* **8**:251-256.
- Kotani S *et al* 1983 Immunobiological activities of synthetic lipid A analogs and related compounds as compared with those of bacterial lipopolysaccharide, re-glycolipid, lipid A and muramyl dipeptide. *Infection and Immunity* **41**:758-773.
- Krick J A, Remington J S 1975 Resistance to infection with *Nocardia asteroides*. *Journal of Infectious Diseases* **131**:665-672.
- Lowry O H, Rosebrough N J, Farr A L, Randall R J 1951 Protein measurement with Folin phenol reagent. *Journal of Biological Chemistry* **193**:265-275.
- Ortiz-Ortiz L, Bojalil L F, Contreras M F 1972 Delayed hypersensitivity to polysaccharides from *Nocardia*. *Journal of Immunology* **108**:1409-1413.
- Palmer D L, Harvey R L, Wheeler J K 1974 Diagnosis and therapeutic considerations in *Nocardia asteroides* infection. *Medicine (Baltimore)* **53**:391-401.
- Shainhouse J Z, Pier A C, Stevens D A 1978 Complement fixation antibody test for human nocardiosis. *Journal of Clinical Microbiology* **8**:516-519.
- Sundararaj T, Agarwal S C 1977 Cell-mediated immunity to *Nocardia asteroides* induced by its ribonucleic acid protein fraction. *Infection and Immunity* **18**:253-256.
- Takahashi Y 1962 Specific serum agglutination of Kaolin particles sensitized with tubercle phosphatide and its clinical evaluation as a serodiagnostic test for tuberculosis. *American Review of Respiratory Diseases* **85**:708-719.
- Zamora A, Bojalil L F, Bastarrachea F 1963 Immunologically active polysaccharides from *Nocardia asteroides* and *Nocardia brasiliensis*. *Journal of Bacteriology* **85**:549-555.