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Cytoplasmic Antigens from *Nocardia* Eliciting a Specific Delayed Hypersensitivity

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Purified cytoplasmic extracts from *Nocardia asteroides* and *N. brasiliensis* elicit delayed hypersensitivity in *Nocardia*-sensitized guinea pigs. The differences in skin reactivity clearly show that it is possible to distinguish between different *Nocardia* species. When peritoneal exudate cells from the latter animals were obtained and treated with the purified cytoplasmic extracts, their migration was inhibited to a significant degree only by means of the homologous antigen. A mild delayed reactivity was observed when the cytoplasmic antigens were used as skin test materials on animals sensitized with BCG. On the other hand, no inhibition of migration was observed when peritoneal exudate cells from BCG-sensitized guinea pigs were exposed to the cytoplasmic antigens.

Cytoplasmic components from acid-fast bacilli have proved to be useful in obtaining materials with a highly specific skin reactivity over conventional culture filtrate preparations (1, 7, 8). With this in mind, efforts to isolate from the cytoplasm of nocardiae substances with a capacity to elicit delayed hypersensitivity in *Nocardia*-sensitized animals were realized. The data indicate that purified cytoplasmic extracts (PCE) elicit delayed hypersensitivity in *Nocardia*-sensitized animals and permit the differentiation among animals sensitized with different *Nocardia* species.

MATERIALS AND METHODS

Animals. Random-bred guinea pigs weighing 400 to 500 g were used throughout this study.

Antigens. PCE were obtained from *N. asteroides* IP-766 (PCENa) and *Nocardia brasiliensis* UPHG-24 (PCENb) strains. These microorganisms were grown in a modified Proskauer and Beck medium (9). They were harvested 21 days after incubation at 37 C, washed with warm distilled water, defatted with ethyl alcohol-diethyl ether (1:1) solution, and vacuum dried. The dried bacteria were suspended in a tris(hydroxymethyl)aminomethane (Tris) buffer (0.01 M, *p*H 7.4) containing 0.01 M magnesium acetate (approximately 50 g of bacteria in 200 ml of Tris). Deoxyribonuclease was added to a level of 2 μg/ml. The suspension was broken in a SorvallRibi cell fractionator (model RF-1) at pressures ranging from 15,000 to 30,000 psi. To permit extraction of protoplasmic components, the disrupted cell mass was placed at 4 C overnight. The ruptured cellular mass was centrifuged first at 3,020 $\times$ *g* for 15 min to separate the unbroken whole cells, cell walls, and cellular debris. The supernatant fluid was again centrifuged at 12,000 $\times$ *g* for 30 min and then at 48,000 $\times$ *g* for 15 min. The supernatant fluid was decanted and further centrifuged at 144,000 $\times$ *g* for 3 hr. The above centrifugation steps eliminated most of the ropy highly polymerized deoxyribonucleic acid and ribosomes. The supernatant fraction was then dialyzed against water, lyophilized, and stored at -20 C until used.

Chemical and physical studies. Protein was determined by the biuret method (5) using bovine serum albumin (Pentex) as a standard. Determination of the total hexose concentration in the cytoplasmic preparations was carried out by the anthrone method (3). Glucose was used as the standard. Nucleic acids were determined by the formula of Warburg and Christian (4). A spectral absorption scan was obtained by use of a Zeiss spectrophotometer and a 1-cm cuvette over the wavelengths 220 to 300 nm.

Sensitization. Guinea pigs were injected subcutaneously each week for 3 weeks in the footpads with 1 mg of living *N. asteroides* or *N. brasiliensis* emulsified in Freund's incomplete adjuvant. The animals were skin-tested 21 days later with the cytoplasmic antigens.

Skin testing. Animals were skin-tested on the flank by intradermal injection of different amounts of the test substance in 0.1 ml of saline. Two diameters of induration at right angles to one another were measured (in millimeters) 24 hr later. The reactions were reported as the product of these diameters. Readings of less than 25 were arbitrarily considered negative.

Specificity of PCE antigens. The difference in specificity (SPD) of PCENa and PCENb was characterized by the difference between the homologous and heterologous reactions. The difference was calculated as described by Magnusson (6), namely, SPD = $(A_a + B_b) - (A_b + B_a)$, where A_a is the homologous reaction obtained with PCENa in animals sensitized to *N. asteroides*. A_b is the heterologous reaction obtained

TABLE 1. *Chemical analysis of purified cytoplasmic extracts (PCE) from Nocardia*

PCE	Contents (%)[a]		
	Proteins	Carbohydrates	Nucleic acids
Nocardia asteroides.....	53.2	31.2	9.0
N. brasiliensis.....	56.3	26.9	7.5

[a] Calculated in basis of dry weight of antigen.

with PCENa in animals sensitized to *N. brasiliensis*; B_b and B_a denote homologous and heterologous reactions with PCENb. When SPD values are 0, it means that the heterologous reaction was as large as the homologous. On the other hand, larger SPD values reflect that the homologous reaction was much larger than the heterologous.

In vitro macrophage migration test. The in vitro macrophage migration test was carried out by the method of Bloom and Bennett (2). In brief, peritoneal exudate cells (PE) were induced in sensitized and nonsensitized guinea pigs by intraperitoneal injection of 30 ml of mineral oil (Nujol, Plough de Mexico, S.A. de C.V.) 3 days prior to harvesting the cells. After exsanguination, the abdomen was shaved, wiped with ethanol, and a mid-line incision was made. Peritoneal washings were done by pouring 100 ml of chilled heparinized (0.5 unit/ml) Hanks balanced salt solution (HBSS) into the peritoneal cavity. HBSS was withdrawn by using a pipette inserted into a polypropylene tube perforated at its lower end. The cells were harvested at 250 × *g*, washed three times in HBSS, resuspended in Eagle minimal essential medium (MEM) containing penicillin and streptomycin plus glutamine and 15% normal guinea pig serum and adjusted to 2×10^7 viable cells. Suspensions of PE were drawn up in capillary tubes, sealed at the lower end with paraffin, and plugged with rubber Crito-caps (Clay-Adams, New York, Cat. no. A-2940). After centrifugation of the capillary tubes at 120 × *g* for 2 min at 4 C, they were cut at the cell-fluid interface and the part containing the packed cells was held in place on the lower cover slip of a 1.0-ml Bloom-type chamber (Berton Plastics, S. Hackensack, N.J. 07606). The cut portions of the capillary tubes were mounted on the lower coverslip with Dow silicone grease. The chamber was then covered with a cover slip and sealed with paraffin around the edges. It was then filled with MEM containing 100 μg of protein/ml of cytoplasmic antigen or with MEM without antigen as control. After 24 hr of incubation at 37 C, the area of cell migration from each tube was recorded by microphotography. The negative film was projected, and the image of the migrated cells was traced on paper. The tracings were cut out, weighed, and the mean for each set of tubes was calculated. The per cent inhibition of migration was determined by using the formula: per cent inhibition of migration = 1 − (mean area of migration with antigen/mean area of migration without antigen) × 100.

RESULTS

Purified cytoplasmic extracts from *N. asteroides* (PCENa) and *N. brasiliensis* (PCENb) contained protein, carbohydrate and nucleic acid material (Table 1). They showed an absorption peak at

TABLE 2. *Mean reactions to purified cytoplasmic extracts (PCE) after 24 hr in 10 sensitized guinea pigs*

Skin test		Induration area (mm^2) of sensitizing strain		
Antigen	Dose (μg)	*Nocardia asteroides*	*Nocardia brasiliensis*	BCG
PCE from *Nocardia asteroides*	10.0	225	100	49
	2.0	196	81	25
	1.0	100	49	16
	0.2	64	36	4
PCE from *N. brasiliensis*	10.0	169	225	64
	2.0	100	169	36
	1.0	81	144	25
	0.2	49	81	16
PPD[a]	1.0	64	64	121

[a] Purified protein derivative.

TABLE 3. *Inhibition of migration of sensitized guinea pig peritoneal exudate cells in the presence of purified cytoplasmic extracts from either Nocardia asteroides (PCENa), Nocardia brasiliensis (PCENb) or PPD[a]*

Antigen added to cells[b]	Mean inhibition of migration of cells from animals sensitized with:		
	N. asteroides	*N. brasiliensis*	BCG
PCENa.....	64 ± 12.9 ($P < 0.001$)[c]	7 ± 0.3 ($P > 0.2$)	11 ± 0.6 ($P > 0.5$)
PCENb.....	6 ± 0.7 ($P > 0.7$)	61 ± 17.2 ($P < 0.001$)	14 ± 1.7 ($P > 0.1$)
PPD[a].....	24 ± 1.0 ($P < 0.05$)	21 ± 0.7 ($P < 0.05$)	46 ± 1.5 ($P < 0.001$)

[a] Purified protein derivative.
[b] Antigen concentration was 100 μg/ml in each case.
[c] Mean inhibition of migration ± standard error (%) of four experiments. *P* values were determined by Student's *t* test by comparison to cells without antigen.

FIG. 1. *Inhibition of macrophage migration. Top row: cells from guinea pigs sensitized with Nocardia asteroides, N. brasiliensis or BCG without antigen. Second row: same type of cells in the presence of purified cytoplasmic extract from N. asteroides (PCENa). Third row: same type of cells in the presence of purified cytoplasmic extract from N. brasiliensis (PCENb). Fourth row: same type of cells in the presence of PPD. These antigens did not inhibit the migration of peritoneal cells from normal animals.*

258 nm. The 280 to 260 ratio was 0.72 in the PCENa and 0.77 in the PCENb antigens.

When guinea pigs sensitized to *N. asteroides* or *N. brasiliensis* were skin-tested with PCENa and PCENb antigens, a cross-reaction was observed. The induration size, however, was larger when the homologous antigen was assayed (Table 2). The data obtained from the skin-test experiments were analyzed according to Magnusson's formula. Large differences are seen between PCENa and PCENb antigens. The SPD obtained was 7. Therefore by using these PCE antigens it was possible, at least with the strains utilized, to distinguish among animals sensitized to *N. asteroides* or *N. brasiliensis*. When both PCE antigens were assayed in guinea pigs sensitized to BCG, in doses ranging from 10.0 to 0.2 μg of protein, inflammatory activity was observed only with doses greater than 2.0 μg. The SPD values obtained were even larger than those observed in *Nocardia*-sensitized animals. Thus, the SPD value obtained when purified protein derivatives (PPD) and PCENb were compared was 12, and between PPD and PCENa was 13. Therefore, PCE antigens clearly showed differences in skin reactivity among *Mycobacterium* and *Nocardia* strains.

Guinea pigs sensitized to *N. asteroides* yielded exudate cells which were inhibited in migration to a significant degree only by the homologous PCE antigen. Although some inhibition was observed with the heterologous PCE antigen, it was not significant ($P > 0.2$ in *Nocardia* groups; $P > 0.05$ when *Mycobacterium* and *Nocardia* groups were compared) (Table 3). On the other hand, PPD

significantly inhibited the migration of macrophages from not only the BCG-sensitized animals, but the *Nocardia*-sensitized group as well (Fig. 1).

DISCUSSION

In this paper the isolation of cytoplasmic material from *N. asteroides* and *N. brasiliensis* was performed and its capacity to evoke delayed hypersensitivity was studied. When these materials were chemically analyzed, it was observed that significantly lower protein values were determined by the Lowry method than by the biuret technique (5). This finding suggests the presence of nonaromatic amino acids in the PCE materials. Similar extracts obtained from mycobacteria showed the same characteristic (1). In addition, an increase in the carbohydrate content is observed when ruptured with increased pressures. Therefore it is of importance to fix a psi value for disruption of the microorganisms.

The PCE from these microorganisms elicited a specific inflammatory reaction of the delayed-type of hypersensitivity in *Nocardia*-sensitized guinea pigs. The SPD were larger when the PCE species were assayed in *Nocardia*-sensitized animals, thus permitting the differentiation between animals sensitized with *N. asteroides* or *N. brasiliensis*. In addition, the SPD were even larger when *Mycobacterium*-sensitized animals were skin tested. The elicitation of delayed hypersensitivity by PCE species was also confirmed by an in vitro test. The cross-reactivity among the antigens of *Nocardia* and *Mycobacterium* could be due to the proteinaceous material, particularly in the amounts tested. We have recently found that polysaccharides from *Nocardia* showed cross-reactivity with similar preparations from mycobacteria. However, the skin reaction was positive only when doses of 5 μg or higher of carbohydrate were used (L. Ortiz-Ortiz, L. F. Bojalil, and M. F. Cantreras, J. Immunol., *in press*). On the other hand, the same carbohydrates did not elicit delayed hypersensitivity in BCG-sensitized animals (*unpublished observations*). The results obtained with PPD clearly demonstrated that the inhibition observed in cells from BCG- as well as *N. asteroides*- or *N. brasiliensis*-sensitized animals were significant. On the other hand, by the same in vitro technique, our PCE antigens did not cross-react when tested against cells from BCG-sensitized animals. The last finding, therefore, indicates a possible advantage of cytoplasmic antigens.

The differences observed between in vivo and in vitro techniques could well be explained in terms of the sensitivity of both techniques, particularly in using a single antigen dose in the in vitro technique or possibly taking different mediators into consideration.

Whereas an immunological test would not be essential for the diagnosis of mycetoma produced by *N. brasiliensis*, it could be very useful in the diagnosis of nocardiosis due to *N. asteroides*, particularly in its pulmonary localization when it is often confused with tuberculosis. The results reported in this paper suggest the possibility of using PCE species from *N. asteroides* and *N. brasiliensis*, not only in the diagnosis of infections produced by these microorganisms, but also in epidemiological studies.

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