Clin. exp. Immunol. (1972) **12,** 225–229.

DELAYED SKIN REACTIONS TO CYTOPLASMIC EXTRACTS OF *NOCARDIA* ORGANISMS AS A MEANS OF DIAGNOSIS AND EPIDEMIOLOGICAL STUDY OF *NOCARDIA* INFECTION

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(*Received* 16 *November* 1971)

SUMMARY

Purified cytoplasmic extracts were prepared from *Nocardia asteroides* and *Nocardia brasiliensis* micro-organisms. They contained protein, carbohydrate and nucleic acid material. When these extracts were utilized as skin test antigens in individuals infected with *Nocardia*, a delayed hypersensitivity reaction was observed. Cross reactivity occurred between the *Nocardia* antigens, but the inflammatory reaction was usually larger with extracts obtained from the infecting micro-organism. On the other hand, low reactivity, if any, was observed when the same antigens were assayed in patients with tuberculosis and leprosy or in healthy individuals. In addition, the cytoplasmic extract from *N. brasiliensis* appeared useful in epidemiological studies, since skin reactivity was shown by individuals working or living in areas in which *N. brasiliensis* was isolated from the soil.

INTRODUCTION

Few attempts have been made to isolate biologically active fractions from *Nocardia* that could be used in man and animals for identification of *Nocardia* infections (González-Ochoa & Baranda, 1953; González-Ochoa *et al.*, 1962). Most workers have isolated these materials from the culture filtrates of *Nocardia* (Arêa Leão, 1928; Bojalil & Magnusson, 1963; Bojalil & Zamora, 1963), and presumably used mixtures of heat denatured and undenatured proteins. The results obtained with such filtrates have not been consistent, and we thought that a more controlled method of antigen preparation might yield a material more suitable for the detection of hypersensitivity states in individuals infected with *Nocardia* microorganisms.

The present paper describes a procedure for the preparation of purified cytoplasmic extracts from *Nocardia brasiliensis* and *Nocardia asteroides*. These cytoplasmic materials elicited delayed skin reactions in humans infected with these micro-organisms. Evidence is

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put forward that these extracts are useful, not only as a diagnostic aid, but also as a tool for epidemiological studies.

MATERIALS AND METHODS

Antigens

The antigens were isolated from *Nocardia asteroides* IP-766 and *Nocardia brasiliensis* UPHG-24. These strains were grown in a modified Proskauer and Beck medium (Youmans & Karlson, 1947). After incubation for 21 days at 37°C the microorganisms were harvested and washed five times with warm 0·15 M sodium chloride solution. The Nocardia suspension was then defatted by repeated treatment with an ethyl alcohol-diethyl ether (1:1) solution and vacuum dried. Fifty grams of dried micro-organisms were resuspended in 200 ml of a tris (hydroxymethyl)- aminomethane buffer, 0·01 M, pH 7·4, containing 0·01 M magnesium acetate and 2 μg/ml of deoxyribonuclease. The organisms were broken at 15000–30000 psi in a Sorvall Ribi Refrigerated Cell fractionator (model RF-1). The disruption of the micro-organisms was checked by morphological examination of smears stained with carbolfuchsin in the usual manner. To permit extraction of cytoplasmic components the treated suspension was placed at 4°C overnight. It was then centrifuged at $3020 \times g$ for 15 min to deposit unbroken whole cells and the cell wall material. The supernatant fluid was recentrifuged at $12\,100 \times g$ for 30 min. The sediment was discarded and the supernatant was centrifuged at $48\,200 \times g$ for 15 min. Finally, the supernatant obtained from the last centrifugation was again centrifuged for 3 hr at $144\,000 \times g$ in a Spinco ultracentrifuge (model L) using a type 40 fixed-angle rotor. The above centrifugation steps removed most of the highly polymerized DNA and ribosomes. The supernatant was then dialysed against water, lyophilized, and stored at −20°C until used.

Chemical and physical analysis

The product, a purified cytoplasmic extract (PCE), was chemically and physically analysed. Protein content was determined by the method of Lowry *et al.* (1951), carbohydrate by the anthrone technique (Hassid & Abraham, 1957) and nucleic acids by the formula of Warburg and Christian (Layne, 1957). The absorption of the *Nocardia* PCEs was measured from 220 to 300 mμ.

Biological assay

Patients with tuberculosis, leprosy and mycetoma, and healthy individuals were skin tested with the *Nocardia* extracts. In additon, tests were performed on individuals living in areas in which *Nocardia* infections were frequently found. The skin tests were made by injecting into the forearm 0·1 ml of PCE, obtained from *N. asteroides* (PCENa) and *N. brasiliensis* (PCENb), in doses ranging from 5·0 to 2·0 μg of protein/0·1 ml. Reactions were read 48 hr later and evaluated according to the diameter of induration (in mm). Tests with readings of less than 6 mm of induration were arbitrarily considered negative.

Bacteriological studies

The culture of *N. brasiliensis* from the soil of endemic areas was carried out by taking soil samples and processing them according to the method described by González-Ochoa (1962). In brief, 5 g of soil samples were diluted in 50 ml of 0·15 M NaCl. One-tenth of the

mixture was poured out into a Petri dish containing Czapek's medium and incubated at 37°C for 4 days. Colonies resembling *N. brasiliensis*, according to their macroscopic morphology, were transferred into Mycosel's agar medium. Further selection was carried out from this medium using the same criterion. Organisms from these colonies were then examined for fragmentation and acid-fastness and identified by their biochemical properties, such as decomposition of casein and hydrolysis of gelatin.

RESULTS

Chemical analysis of PCENa and PCENb showed that the extracts contained approximately 65% protein, 27% carbohydrate and 8% nucleic acid.

When individuals known to be infected with *N. brasiliensis* were skin tested with the *Nocardia* extracts, a delayed-type hypersensitivity reaction was observed. An inflammatory reaction characterized by induration and a delayed onset occurred when PCENb antigen, in doses of 5·0 and 2·0 μg, was used. The size of induration was usually larger than 8 mm (Table 1). PCENa also elicited an inflammatory reaction in those individuals with mycetoma.

TABLE 1. Skin test with purified cytoplasmic extracts of *N. asteroides* (PCENa) and *N. brasiliensis* (PCENb) in patients with mycetoma due to *N. brasiliensis**

Case No.	PCENb		PCENa	
	5 μg (mm)	2 μg (mm)	5 μg (mm)	2 μg (mm)
1	14	8	7	−ve
2	22	20	11	7
3	18	10	9	−ve
4	26	ND†	12	ND
5	15	ND	11	ND
6	20	10	10	−ve
7	8	8	−ve	−ve
8	11	11	−ve	−ve
9	50	ND	14	ND
10	18	20	7	−ve

* Skin reactions were measured at 48 hr after the injection and represent the diameter of induration in mm. When they were ≤6 mm were arbitrarily considered negative.

† ND = not done.

In these cases, however, the size of the induration with the 5·0 μg dose was generally less than that elicited by the homologous antigen and induration was usually absent when 2·0 μg was used (see Table 1). When normal individuals and patients with tuberculosis and leprosy were skin tested with the 5·0 μg dose of PCENa or PCENb, induration less than 6 mm in diameter was usually observed (Table 2).

In order to test the potential of these antigens for epidemiological studies, we selected a rural zone in a tropical region of the State of Morelos (México) where actinomycotic myce-

toma is prevalent (González-Ochoa & Sandoval, 1960). The predominant agricultural activity is sugar cane production. Most of the individuals that were skin tested were farmers, and ten out of fifteen tested subjects were positive to PCENb (see Table 2). Five of the group of reactive individuals also reacted to PCENa.

TABLE 2. Skin tests with purified cytoplasmic extracts from *N. asteroides* (PCENa) and *N. brasiliensis* (PCENb) in various groups of individuals*

Group	PCENb (5 μg/0·1 ml)		PCENa (5 μg/0·1 ml)	
	(> 6 mm)	(⩽ 6 mm)	(> 6 mm)	(⩽ 6 mm)
1. Patients with mycetoma due to *N. brasiliensis*	10	0	8	2
2. Patients with tuberculosis (PPD positives)	2	23	1	24
3. Patients with lepromatous leprosy	1	15	0	16
4. Healthy individuals from non-endemic zones	2	20	1	21
5. Healthy individuals from endemic zones	10	5	5	10

* Skin reactions were measured at 48 hr after the injection and represent the diameter of induration in mm. When they were ⩽ 6 mm were arbitrarily considered negative.

This result suggested that the soil in the endemic areas must contain *N. brasiliensis*, and analyses were carried out in order to determine whether these micro-organisms were present. The studies indicated that the soil was in fact contaminated. We were able to isolate 500 colonies of *N. brasiliensis* per 0·1 g of soil examined.

DISCUSSION

The experiments reported here show that purified cytoplasmic extracts obtained from *Nocardia* organisms can elicit delayed hypersensitivity reactions, not only in those individuals infected with *Nocardia*, but also in those living or working in areas contaminated with the same micro-organisms. This last observation raises the possibility of using these PCE antigens in epidemiological studies. Although a cross-reaction between the *Nocardia* extracts was observed, the intensity of the skin response to the homologous PCE, obtained from the infecting microorganism, was greater than that of the heterologous PCE antigen.

We were unable to test individuals infected with *N. asteroides* and to assess the specificity of PCENa antigen. In experimental animals, however, the behaviour of both extracts permits differentiation between infections induced by the two types of *Nocardia* on the basis of degree of reaction (to be published). If PCENa proves to be as useful in the diagnosis of human infection as it is in that of animal infection a better understanding of the physiopathology of the disease, as well as a better clinical management of the patients, might be possible in individuals infected with *N. asteroides*.

ACKNOWLEDGMENTS

We thank Dr Roberto Rivas, Dr Rigoberto Gómez, Dr Oscar Velasco and Dr Magdalena Contreras for their valuable help.

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