

## Mouse Toxicity Induced by Lipids and Cell Walls Isolated from Actinomycetes

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The possibility was examined that the toxicity induced in mice by *Actinomadura madurae*, '*Streptomyces pelletieri*' and *Nocardia brasiliensis* was due to lipid and cell-wall constituents. Mice were inoculated intraperitoneally with heat-killed bacteria, lipid extracts and cell-wall preparations emulsified in mineral oil: toxicity was evaluated by recording weight loss and deaths. Killed cells and cell-wall preparations of all three actinomycetes produced a pronounced loss of body weight, tissue necrosis, splenomegaly, a granulomatous inflammation and sometimes death. Mice inoculated with lipid extracts from *A. madurae* and '*S. pelletieri*' neither died nor showed toxic effects, but mice injected with lipids isolated from *N. brasiliensis* did suffer toxic effects. They showed more marked wasting symptoms than observed after inoculation of heat-killed bacteria or of the cell-wall preparation.

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### INTRODUCTION

*Actinomadura madurae*, '*Streptomyces pelletieri*' and *Nocardia brasiliensis* are actinomycetes which cause a chronic granulomatous disease in man, commonly called mycetoma and characterized by the presence of localized swollen and hard lesions similar to tumours. The aetiological agent penetrates the host by traumatic inoculation, and develops in the subcutaneous tissue into numerous filaments approximately 1 µm in diameter, which proliferate in the form of microcolonies (grains or granules) and form conduits and sinuses that can reach connective and bone tissue. The granules detected in the lesions are associated with the formation of pus and with tissue necrosis (Conant *et al.*, 1971; Rippon, 1982). The mycetoma granules are normally surrounded by inflammatory cell infiltrates, with polymorphonuclear cells encountered in the more adjacent region. Mononuclear cells characterized as macrophages are observed at the periphery of the lesion (Rippon, 1982). Giant cells may occasionally be observed near the surface of these abscesses. In infections of long duration there is granulation of peripheral tissue, in which abundant lymphocytes and other plasma cells are detected. The abscesses tend to branch out, producing fistulae in the skin and chronic draining sinuses. Characteristically, mycetomas are more frequently encountered in the feet or legs, but they can also appear in other parts of the body exposed to the contaminated environment. In general, they occur in rural workers, especially in hot climates, with cases reported in the tropical and subtropical regions of India, Mexico, USA, Sudan, Senegal, Nigeria, Abyssinia, Venezuela and Brazil (Conant *et al.*, 1971; Rippon, 1982; Cross *et al.*, 1976).

In view of the lack of studies on the bacterial products involved in the biochemical process triggered in these chronic infections, the present investigation was done to examine the possible correlation between the lipid and cell-wall constituents of *N. brasiliensis*, '*S. pelletieri*' and *A. madurae* and their pathogenicity.

## METHODS

**Bacterial strains and culture conditions.** *Actinomadura madurae* 956, '*Streptomyces pelletieri*' 293 and *Nocardia brasiliensis* 519 were obtained from Dr Carlos S. Lacaz, Faculty of Medicine, University of São Paulo, Brazil. *A. madurae* and *N. brasiliensis* were cultured at 37 °C for 15 d in a medium containing 10 g yeast extract, 10 g glucose and 1 g sodium chloride in 1 litre distilled water. '*S. pelletieri*' was cultured under the same conditions except that the medium contained 10 g Bacto-Peptone (Difco) and 20 g glucose in 1 litre distilled water. The cells were autoclaved, harvested by centrifugation at 5000 g for 5 min and washed seven times with distilled water.

**Lipid extraction.** Lipids were extracted by soaking the cells in chloroform/methanol (2:1, v/v), with stirring, at room temperature for 2 h. The extracts were separated by centrifugation at 10000 g for 5 min and the pellet, suspended in the same solvent mixture, was re-extracted three more times. The combined extracts were reduced to dryness on a rotary evaporator and extracted with diethyl ether as described by Silva & Itoneda (1977a) to eliminate non-lipid residues.

**Lipid analysis.** Lipid extract was analysed by thin-layer chromatography (TLC) on plates coated with silica gel F<sub>254</sub> (Merck). The following solvent systems were used: solvent A, n-hexane/diethyl ether/acetone/acetic acid (70:30:11:1, by vol.), and solvent B, chloroform/acetone/methanol/water (50:60:2.5:3, by vol.). Lipids on TLC plates were detected by exposure to iodine vapour. Sugar-containing lipids were revealed with anthrone/sulphuric acid (Silva & Itoneda, 1977b). The following standard lipids were used: palmitic acid and triglycerides (Sigma); noncardiomycolic acid and trehalose dimycolate (purified from *Nocardia asteroides* as described by Silva *et al.*, 1979).

**Cell-wall preparations.** The lipid-extracted cell residues were disrupted by ultrasonic vibration at 200 W for 3 min (Braun Labsonic 1510). This process was repeated six times. The walls were collected and washed with distilled water by centrifugation at 10000 g for 10 min.

**Partial fractionation of walls.** This was done by alkaline extraction as described by Kanetsuna *et al.* (1972). Briefly, walls were suspended in 1 M-NaOH (10 mg ml<sup>-1</sup>) and gently stirred at room temperature for 1 h. After centrifugation at 5000 g for 10 min, the supernate was collected, the procedure was repeated four times and all the supernates were pooled. The alkali-insoluble sediment was washed several times with water until the washings reached pH 7, and then once with ethanol, followed by acetone, and diethyl ether. The resulting white powder was called fraction F1. The pooled supernates were neutralized with acetic acid and left to stand overnight at 4 °C, after which a precipitate had formed. The suspension was centrifuged as before. The precipitate and the supernate were collected, dialysed separately against distilled water and freeze-dried, yielding fractions F2 (alkali-soluble and precipitable with acid) and F3 (alkali-soluble and nonprecipitable with acid).

**Animals.** Male Swiss mice 4 to 6 weeks old weighing 19.0 to 21.0 g were used.

**Toxicity.** The material to be tested for toxicity was dissolved in mineral oil (Nujol). Before use, the clear solutions were heated to 56 °C and subjected to ultrasonic vibration for 5 min using a probe of 9 mm diameter at 100 W. The toxicity of heat-killed bacteria, lipid extracts and cell-wall preparations was evaluated by inoculating mice intraperitoneally at 2 d intervals with five 200 µg doses in 0.1 ml mineral oil and by recording weight loss and deaths. Dead mice were examined by autopsy and the lungs, spleen and liver inspected for gross lesions. Control groups were given corresponding amounts of mineral oil alone. Experiments were carried out over a period of 30 d.

## RESULTS

Heat-killed *N. brasiliensis*, *A. madurae* and '*S. pelletieri*', and their respective lipid extracts and cell-wall preparations, were inoculated into mice intraperitoneally (5 × 200 µg) in 0.1 ml mineral oil and the variations in body weight and mortality were observed for 30 d. Results from a typical experiment are shown in Fig. 1 and Table 1.

In the case of *A. madurae* (Fig. 1c, Table 1), no significant variations in body weight were observed in the animals inoculated with the lipid extract when compared to the controls injected with mineral oil alone, and all animals survived the experimental period. In contrast, the animals injected with cell-wall preparations or heat-killed bacteria stopped gaining weight during the first two weeks of the experiment, appeared unwell, with ruffled fur, and were lethargic. When inoculation of the irritants ceased, the animals started to regain weight and appeared healthy. Both the heat-killed bacteria and their cell-wall preparations, in addition to inducing these characteristics, also caused the death of 40% of the animals. Autopsy of the animals injected with these toxic preparations showed the presence of peritonitis, with an inflammatory exudate, massive adhesions of the organs in the peritoneal cavity, numerous granulomas and splenomegaly.

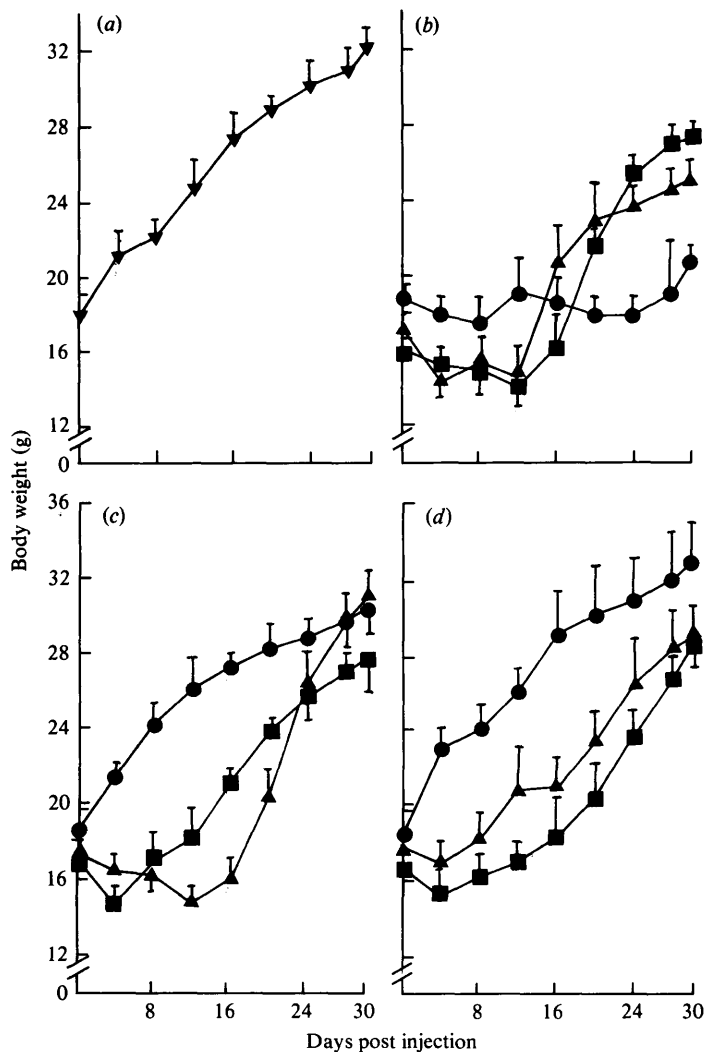


Fig. 1. Mean body weight ( $\pm$ SD) of groups of ten mice at various times after intraperitoneal injection (in mineral oil) of five doses of 200  $\mu$ g of killed bacteria (■), cell walls (▲) and lipid extract (●), from *N. brasiliensis* (b), *A. madurae* (c) and '*S. pelletieri*' (d), at 2 d intervals starting at day 0. The control group (a) was injected with mineral oil alone. It is not possible to examine the results statistically because of the decreasing number of animals in each group as seen in Table 1. Results are from a typical experiment.

A similar response pattern to that obtained after the inoculation of animals with various *A. madurae* preparations was also observed after inoculation of '*S. pelletieri*', i.e. no toxic effects with the lipid extract and induction of weight loss and mortality by heat-killed micro-organisms or cell walls (Fig. 1d, Table 1).

Mice injected with the lipid extract, cell walls and heat-killed bacteria of *N. brasiliensis* showed an initial decrease in body weight after inoculation and stopped gaining weight for 16 d, slowly recovering thereafter (Fig. 1b). The survival rate of the animals injected with cell walls or heat-killed bacteria was 60%, whereas the rate for animals inoculated with the lipid extract was 30% after 30 d (Table 1).

Table 1. *Lethal effect of killed bacteria, cell-wall preparations and lipid extracts in mice given intraperitoneal inocula*

Organism	Material injected*	Deaths in groups of 10 mice on day†:							Total
		8	12	16	20	24	28	30	
<i>N. brasiliensis</i>	Control	0	0	0	0	0	0	0	0
	Killed bacteria	0	2	1	0	0	1	0	4
	Cell walls	0	1	0	1	1	0	1	4
	Lipid extract	1	2	2	0	0	1	1	7
<i>A. madurae</i>	Killed bacteria	0	1	2	0	1	0	0	4
	Cell walls	0	2	1	1	0	0	0	4
	Lipid extract	0	0	0	0	0	0	0	0
<i>'S. pelletieri'</i>	Killed bacteria	0	1	1	0	0	0	0	2
	Cell walls	0	0	1	0	0	0	0	1
	Lipid extract	0	0	0	0	0	0	0	0

\* Mice were given five doses of 200 µg of each preparation at 2 d intervals starting at day 0. The control group was injected with mineral oil alone.

† For convenience, the data were recorded at 4 d intervals. Results are from a typical experiment.

The results obtained were reproducible from experiment to experiment: the values varied slightly from one experiment to the next but the general pattern of body weight curves and survival rate were very similar. It was not possible to examine the results statistically because of the decreasing numbers of animals in each group as seen in Table 1.

Fractions F1, F2 and F3 obtained from cell walls of '*S. pelletieri*', *A. madurae* and *N. brasiliensis* after alkali treatment were also assayed for toxicity in mice by the procedure described above. The alkali-insoluble fractions F1 were toxic, causing wasting symptoms and rapid loss of body weight. The initial chemical analysis of these toxic fractions showed that they were composed mainly of polysaccharide. Mice injected with the alkali-soluble fractions F2 and F3 showed no signs of illness and gained weight normally.

The lipid extracts obtained from the three micro-organisms and a mixture of known standard lipids were chromatographed on TLC plates using solvent A. This solvent system resolved the lipid mixtures, indicating the presence of ketone, menaquinone, triglycerides and palmitic acid in the lipid extracts obtained from all three micro-organisms. On the other hand, mycolic acid was only detected in the lipid extract of *N. brasiliensis*. When solvent B was used, a lipid component present in the extract obtained from *N. brasiliensis* migrated on TLC in a similar manner to the trehalose dimycolate standard. A similar compound was not detected in the lipid extracts from *A. madurae* and '*S. pelletieri*'.

#### DISCUSSION

The results obtained here clearly indicate that heat-killed *A. madurae*, '*S. pelletieri*' and *N. brasiliensis* contain components with toxic and inflammatory properties, since they produced loss of body weight, formation of inflammatory exudates, granulomas and death when inoculated into mice. Removal of the lipids of these micro-organisms by solvent treatment did not alter the toxicity of the cell wall when compared to that observed in animals inoculated with heat-killed micro-organisms. On the other hand, when the lipid extracts were inoculated, only that from *N. brasiliensis* was highly toxic to mice.

Among the lipid constituents found in *Nocardia*, the most characteristic one is trehalose dimycolate (Silva *et al.*, 1979; Ionedá *et al.*, 1981). This substance, initially demonstrated in *Mycobacterium tuberculosis* (Bloch, 1950; Noll *et al.*, 1956) and later in *Corynebacterium* (Ionedá *et al.*, 1963) and *Nocardia* (Ionedá *et al.*, 1970), consists of two molecules of mycolic acid ( $\alpha$ -branched and  $\beta$ -hydroxylated acid-containing carbon chains which vary in length from organism to organism) bound to the disaccharide trehalose. The glycolipid isolated from mycobacteria is directly involved in the formation of granulomas in tuberculosis (Bekierkunst, 1968; Bekierkunst *et al.*, 1969) and has adjuvant (Saito *et al.*, 1976), immunostimulant (Yarkoni *et al.*, 1977; Parent *et al.*, 1977) and antitumour properties (Bekierkunst *et al.*, 1971). These

effects, in addition to its toxicity, suggest its participation in the host-parasite relationship at different levels.

In previous studies it was demonstrated that *Mycobacterium bovis* BCG without the glycolipid trehalose dimycolate on the external part of their cell walls were more susceptible to phagocytosis and were eliminated more rapidly by the host. They did not induce granuloma formation, and had a low capacity for nonspecific immunostimulation and a reduced ability to induce a delayed hypersensitivity response (Silva *et al.*, 1984, 1985). In view of these considerations, and especially considering our work with BCG, the pathogenic mechanism of nocardia species may well be similar, since they possess the glycolipid trehalose dimycolate. Species of the genera *Streptomyces* and *Actinomadura*, which cause infections similar to those induced by *Nocardia* and *Mycobacterium*, have no lipid components similar to mycolic acid or trehalose dimycolate. Thus, the toxic components present in these organisms must be different.

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