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Studies on the pathogenesis of actinomycotic mycetoma in animals injected with fractions isolated from *Nocardia brasiliensis*

S.M. Ekizlerian, S.L. Brandão Filho, I. Tincani, L.M. Carareto Alves and C.L. Silva

Department of Parasitology, Microbiology and Immunology, School of Medicine of Ribeirão Preto, University of São Paulo, 14.049 Ribeirão Preto, SP, Brazil

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Summary. In the present study the participation was evaluated of isolated fractions of *Nocardia brasiliensis* in the genesis of the inflammatory response observed in actinomycotic mycetoma. Subcutaneous injection in mice of a suspension containing a polysaccharide fraction F_1 obtained by treating cell walls with sodium hydroxide induced an inflammatory response at the inoculation site which was characterized by a large influx of polymorphonuclear (PMN) and mononuculear (MN) leucocytes between the 2nd and 4th days. On the 8th day, a typical granulomatous reaction was observed involving large numbers of epithelioid cells. Intravenous injection of the lipid extract adsorbed to charcoal particles into mice induced an inflammatory reaction around the particles embolized in the pulmonary microcirculation which was similar to that described above. The kinetics of the inflammatory cell migration was studied by total and differential counts of leucocytes that migrated to the peritoneal cavity of rats inoculated intraperitoneally with the F_1 and lipid fractions. Both fractions initially induced intense PMN migration, which was later reduced, with a simultaneous increase in mononuclear cells. The present results demonstrate that a polysaccharide fraction (F_1) and the lipid fraction reproduce the fundamental lesion of actinomycotic mycetoma.

Keywords: *Nocardia brasiliensis*, inflammation, pathogenesis, polysaccharide, lipids, actinomycotic mycetoma

Nocardia brasiliensis is a soil-inhabiting aerobic actinomycetes and is a primary pathogen responsible for both acute and chronic suppurative disease in humans. There are several distinct clinical syndromes that may evolve. The primary cutaneous diseases that occur in patients following trauma and contact with soil (Satterwhite & Wallace 1979), may present as cellulitis, pustules, pyoderma, or a lymphocutaneous form which mimics sporotrichosis (Belliveau & Geiger 1977). The histopathology encountered in cutaneous infection is that of a mixed pyogenic and granulomatous reaction. If allowed to progress, it develops into actinomycotic mycetoma. This actinomycotic

Correspondence: Dr Célio Lopes Silva, Departamento de Microbiologia, Faculdade de Medicina de Ribeirão Preto, USP, 14049 Ribeirão Preto, SP. Brazil.

mycetoma is a chronic suppurative and granulomatous disease of the subcutaneous tissues and bones characterized by swelling, abscess formation and multiple draining sinuses containing pigmented granules (Connant *et al.* 1971). The granules of mycetoma lie within collections of polymorphonuclear leucocytes. Varying numbers of macrophages are detected in the pus. Giant cells may occasionally be seen lying on the surface of the granule or located at the periphery of the minute abscess. The abscess tends to ramify, producing fistulae to the skin and chronically draining sinuses. Often the bones of the foot are infected and suppurative osteomyelitis and periostitis develop. With long-standing infections, granulation tissue and scar tissue form and a true granuloma is produced. Caseation or a tuberculoid aspect are not characteristic of mycetoma (Rippon 1982).

Primary pulmonary and systemic nocardiosis also may be induced in humans by *N. brasiliensis*. This disease may be subclinical or pneumonic, chronic or rarely acute and may become systemic by hematogenous spreading, with the organism showing a predilection for the central nervous system (Causey & Sieger 1974).

In view of the lack of studies on the bacterial products involved in the biochemical process triggered by this chronic infection, the present investigation was undertaken to examine the *N. brasiliensis* fractions which induce chronic inflammation and inflammatory cell migration. The participation of these fractions in the pathogenesis of the fundamental damage caused by this microorganism is discussed.

Materials and methods

Bacterial strain and culture conditions. Nocardia brasiliensis strain 519 was the generous gift of Dr Carlos S. Lacaz School of Medicine, University of São Paulo (This number refers to Dr Lacaz's collection). This strain was originally isolated from an actinomycotic mycetomas patient and identified according to procedure described by Moore and Jaciow (1975). The microorganism was cultured at 37°C for 15 days in medium containing 10 g yeast extract, 10 g glucose and 1 g sodium chloride in 1 l distilled water. The cells were autoclaved, harvested by centrifugation at 5000 *g* for 5 min and washed seven times with 50 ml distilled water.

Lipid extraction. Lipids were extracted by soaking the cells (20 g wet weight) in a 300 ml chloroform-methanol mixture (2:1, v/v) and stirring at room temperature for 2 h. The extract was separated by centrifugation at 10 000 *g* for 5 min and the pellet, suspended in the same solvent mixture as described above, was reextracted three more times as described. The combined extracts were dried on a rotary evaporator and submitted to extraction with diethyl ether as described by Silva and Ioneda (1977*a*) to eliminate non-lipid residues.

Lipid analysis. The lipid extract was analysed by thin-layer chromatography (TLC) on plates coated with silica gel F254 (Merck). The following solvent systems were used; sol-vent A, *n*-hexane/diethyl ether/acetone/acetic acid (70:30:11:1, by vol.); solvent B, chloroform/acetone/methanol/water (50:60:2.5:3, by vol.). Lipids were detected on TLC plates by exposure to iodine vapour. Sugar-containing lipids were revealed with anthrone/sulphuric acid (Silva & Ioneda 1977*b*). The following standard lipids were used: palmitic acid and triglycerides (Sigma); nocardomycolic acid and trehalose dimycolate (purified from *Nocardia asteroides* as previously described, Silva *et al.* 1979).

Crude cell wall preparations. The lipid-extracted cell residues were disrupted by ultrasonic vibration at 200 w for 3 min (Labsonic 1510, B. Braun Melsungen AG). This process was repeated six times. Unbroken cells were removed by centrifugation for 10 min at 3000 *g*. Crude cell walls were obtained from the supernatant by centrifugation at 4°C for 1 h at 15 000 *g*.

Partial cell wall fractionation. The process was carried out by alkaline extraction (Kanetsuma *et al.* 1972). Briefly, walls were suspended in 1 M NaOH (10 mg/ml) and gently stirred at room temperature for 1 h. After centrifugation at 5000 *g* for 10 min, the supernatant was collected, and the procedure repeated four times combining all the supernatants. The alkali-insoluble sediment was washed with water until it reached pH 7 and then with ethanol, acetone, and diethyl ether, respectively. The resulting white powder was called fraction F_1. The pooled supernatants were neutralized with acetic acid and left to stand overnight at 4°C, after which a precipitate formed. The suspension was then centrifuged as before. The precipitate and the supernatant were collected, dialysed separately against distilled water and freeze-dried, yielding fractions F_2 (alkali-soluble and precipitable with acid) and F_3 (alkali-soluble and nonprecipitable with acid), respectively.

Animals. Male Swiss mice 4 to 6 weeks old weighing 19.0 to 21.0 g and male Wistar rats weighing 160.0 to 180.0 g were used.

Induction of pulmonary lesions by the lipid extract. The granuloma producing property of the extracted lipids was tested by intravenous injection of 5×10^5 lipid-coated charcoal particles (Silva & Ekizlerian 1985) through the retro-orbital venous plexus of mice. Uncoated particles were used as control. At different times after the intravenous injection of charcoal particles five animals per group were anaesthetized with ether and killed by exsanguination. Their lungs were fixed by the infusion of 2 ml Bouin's fixative into the exposed trachea. The fixed organs were sectioned through the hilus and histological sections of different areas of both lungs were prepared.

Subcutaneous lesions. Each suspension of heat-killed bacteria, crude cell wall preparation and cell wall fractions F_1, F_2 and F_3 (50 μg in 0.05 ml) was inoculated subcutaneously into the abdomen of mice. The subcutaneous lesions were observed both macroscopically and histologically after 2,4,8 and 16 days.

Evaluation of leucocyte influx into the peritoneal cavity. Groups of rats were inoculated intraperitoneally with 1 mg each of the following preparations in 1 ml of phosphate-buffered saline (PBS): heat-killed bacteria, crude cell-wall preparation, cell wall fractions F_1, F_2 and F_3, and lipid extract. At appropriate intervals thereafter the rats were killed with ether and injected intraperitoneally with 10 ml PBS-containing heparin. The abdomens were gently massaged and 5 ml of blood-free cell suspension was carefully withdrawn with a syringe. Abdominal washings were placed in plastic tubes and total cell counts were performed immediately in a Neubauer chamber. For differential counting a cell pellet was first obtained by centrifugation and resuspended in 0.4 ml PBS containing 3% bovine serum albumin. For counting cells, a piece of Whatman chromatographic paper was fixed to a glass slide using strong sellotape. A hole, 4 mm in diameter, was previously made through the sellotape and the chromatograhic paper. Two hundred microlitres containing 10^4 to 10^5 cells were then added to each well. The fluid was slowly absorbed and the cells evenly adhered to the glass surface. The paper was then allowed to dry and removed together with the sellotape. Cells were stained with May–Grunwald Giemsa and differential counts (100 cells) were made under the light microscope (Souza & Ferreira 1985).

Results

Subcutaneous inoculation of mice with different suspensions containing heat-killed bacteria, a crude cell wall preparation and an F_1 fraction obtained by treating cell walls with NaOH was effective in inducing an inflammatory reaction. On the 2nd and 4th days after inoculation, intense necrosis with

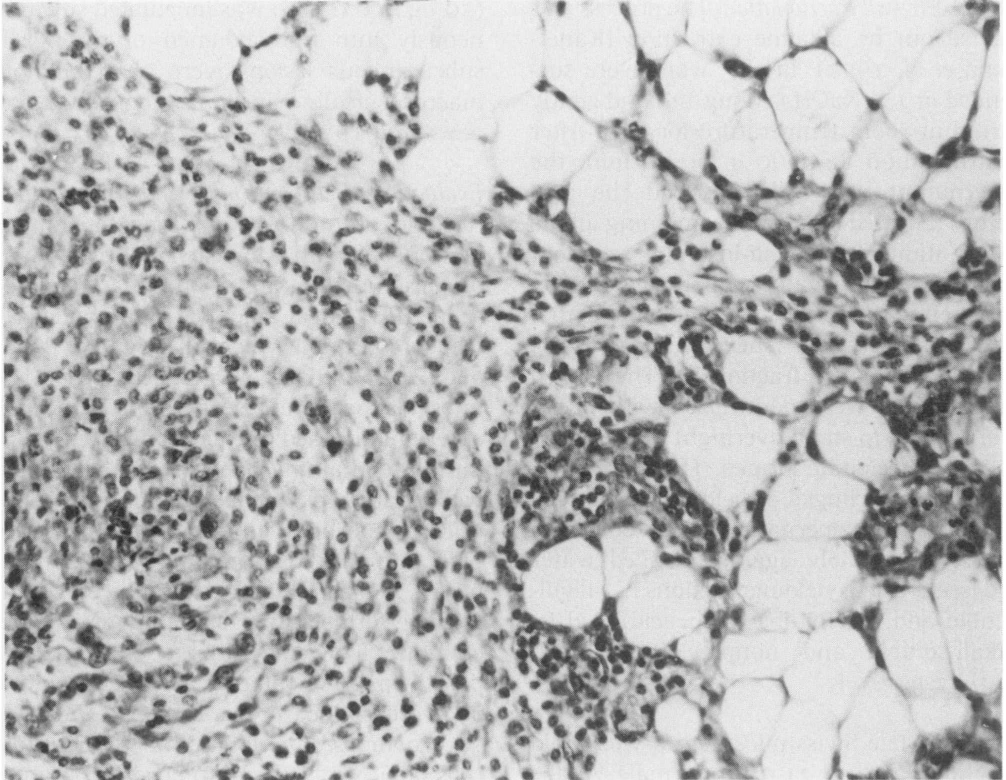

Fig. 1. Subcutaneous lesion induced on day 4 after inoculation of fraction F_1 isolated from *N. brasiliensis.* × 160.

large numbers of neutrophil granulocytes was observed at the site of injection. From the 8th day, the lesion was granulomatous with foci or suppuration and consisted of large numbers of macrophages at various levels of maturation and of epithelioid cells (Fig. 1). Between the 16th and 32nd day, the lesion disappeared, probably owing to detoxification of the irritating agent. The F_2 and F_3 cell wall subfractions were unable to induce an inflammatory reaction.

To demonstrate participation of the lipid extract in formation of the inflammatory lesion, the extract was adsorbed to charcoal particles and a suspension of these particles were injected intravenously into mice. Histological analysis disclosed the particles to be embolized in the pulmonary microcirculation and that a very intense inflammatory reaction had developed around them. On the 2nd day after inoculation, an intense neutrophil exudate was observed around the particles. On day 4, large numbers of histiocytes, surrounding the neutrophil crown around the charcoal particles, were observed (Fig 2). This cell distribution pattern was maintained up to the 8th day, after which the lesion regressed, with a predominance of mononuclear cells in the inflammatory focus.

The kinetic study of inflammatory cell migration was performed by total and differential counts of leucocytes migrating to the peritoneal cavity of rats inoculated intraperitoneally with the fractions that had induced an inflammatory response in mice. The results shown in Table 1 refer to experiments in which the total number of cells in the peritoneal fluid was determined 4 h after

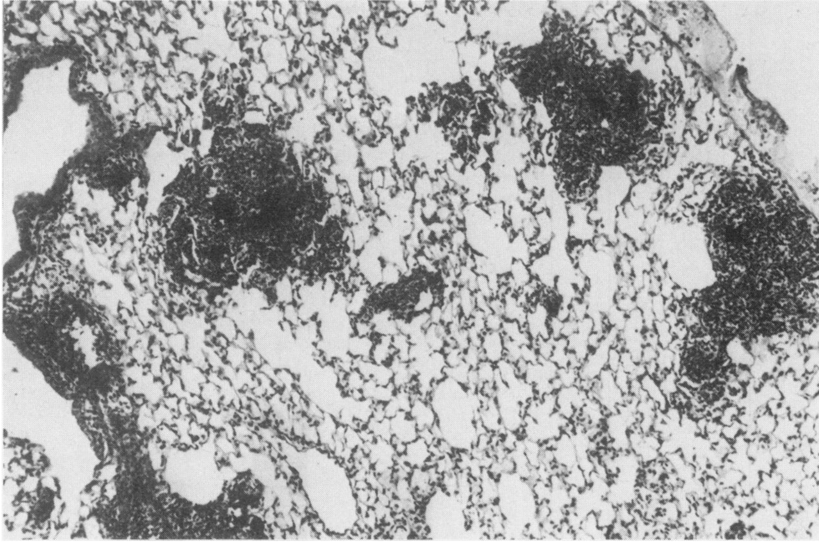

Fig. 2. Histology of lung from a mouse injected with charcoal particles coated with a lipid extract from *N. brasiliensis.* Note an intense inflammatory cell infiltrates around lipid coated charcoal particles 4 days after inoculation. ×63.

Table 1. Effect of dead *N. brasiliensis* and fractions thereof on the leucocyte cell influx into the peritoneal cavity of rats 4 h after intraperitoneal injection.

Preparations injected	Total cells ($\times 10^3$/mm^3)	PMN cells ($\times 10^3$/mm^3)	MN cells ($\times 10^3$/mm^3)
Control	4766±570	1117±310	2831±330
Dead bacteria	7875±640	3857±280	3467±380
Cell wall	10083±485	6030±585	3163±400
Fraction F_1	1158±1050	8161±610	2877±510
Fraction F_2	4775±550	920±442	2707±350
Fraction F_3	4075±420	1438±225	2084±332
Lipid extract	7208±710	3519±340	2655±278

Results are expressed as mean ± s.e.m.

inoculation of suspensions containing 1 mg/ml of killed bacteria, a crude cell wall preparation, fractions F_1 F_2, F_3, or the lipid extract. All preparations, except F_2 and F_3, induced a significant increase in the number of leucocytes present in the peritoneal cavity. When differential counts were performed on peritoneal washes, the increase was found to be in the number of polymorphonuclear leucocytes.

Figure 3 shows the cell migration kinetics induced by inoculation of 1 mg of fraction F_1 into the peritoneal cavity. A clear increase in the number of polymorphonuclear leucocytes (PMN) can be observed throughout the 4th hour after inoculation, followed by a

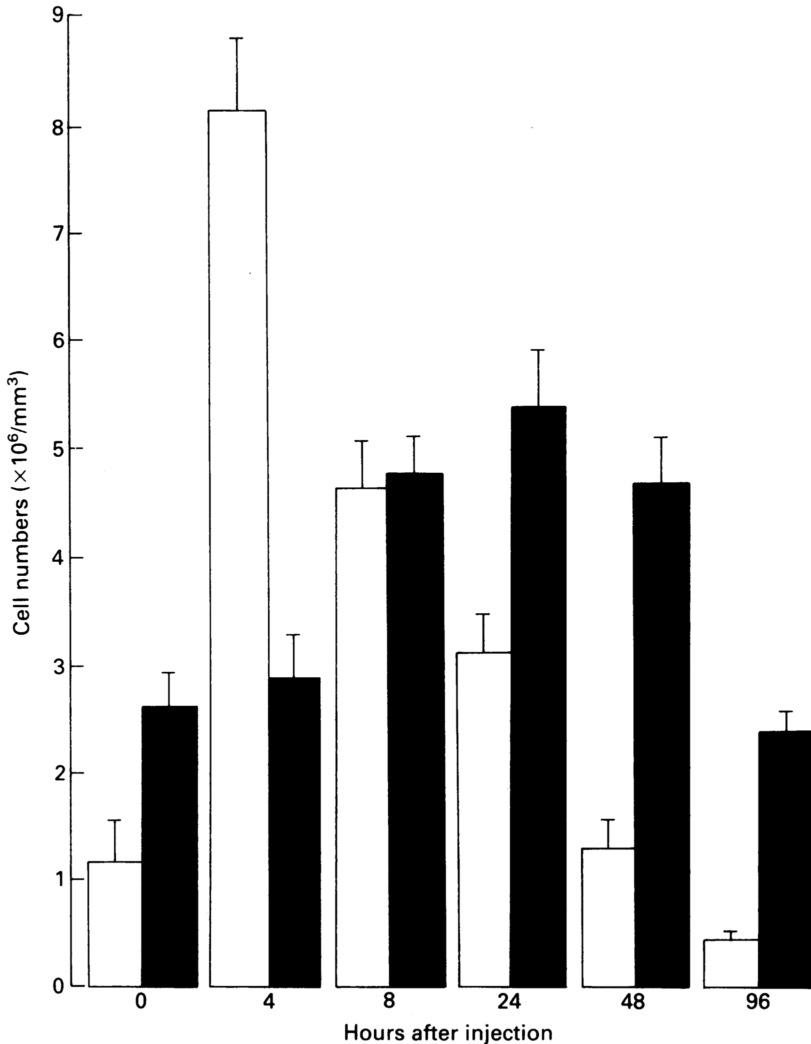

Fig. 3. Kinetics of PMN and MN cell influx into the peritoneal cavity of rats induced by an injection of 1 mg of fraction F_1 from *N.* brasiliensis. Results are expressed as mean ± s.e.m. □, PMN; ■, MN.

reduction (8–96 h) in parallel with an increase in mononuclear cells (4–24 h). The same response pattern of cell migration was obtained by inoculating the lipid extract (Fig. 4).

The lipid extract obtained from *N. brasiliensis* and a mixture of known standard lipids were chromatographed on TLC plates using solvent A. This solvent system was able to resolve the lipid mixtures, indicating the presence of ketone, menaquinone, triglycerides and plamitic and mycolic acids. When solvent B was used, a lipid component present in the extract migrated on TLC in a manner similar to trehalose dimycolate standard, and gave a positive reaction with anthrone/sulphuric acid suggesting a glycolipid nature.

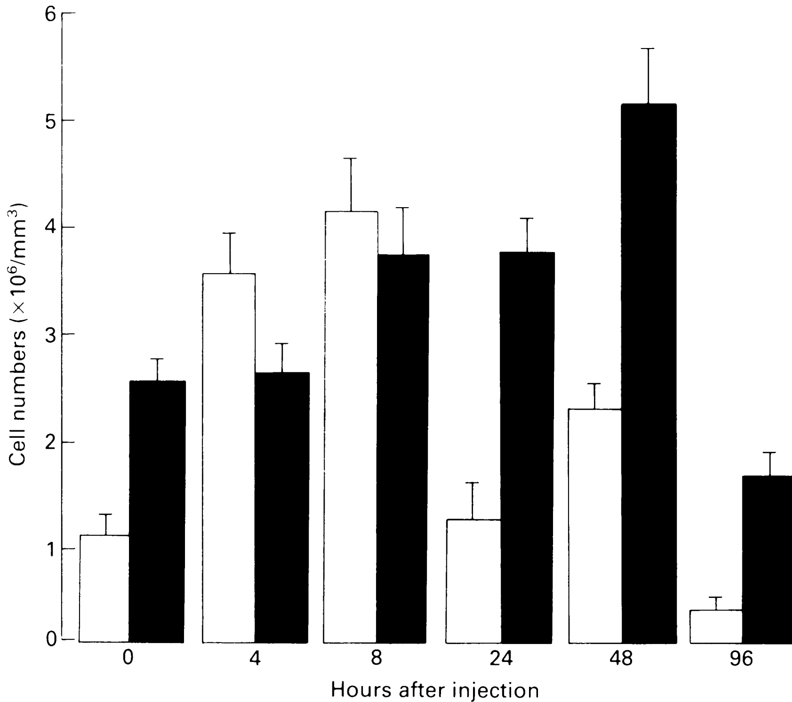

Fig. 4. Kinetics of PMN and MN cell influx into the peritoneal cavity of rats induced by an injection of 1 mg of lipid extract from *N. brasiliensis*. Results are expressed as mean ± s.e.m. □, PMN; ■, MN.

Discussion

The results obtained clearly indicate that heat-killed *N. brasiliensis* contain components with inflammatory properties, since they induced granuloma-like subcutaneous inflammation in mice and migration of leucocytes to the peritoneal cavity of rats. Delipidated preparation did not alter the inflammatory properties of cell walls when compared to the inflammatory response observed in animals inoculated with heat-killed microorganism. In contrast, when the lipid extract was injected intravenously, it induced severe pulmonary inflammation. TLC of this lipid extract showed the presence of a glycolipid characterized as trehalose dimycolate. This analogous glycolipid found in mycobacteria (cord factor), plays an important role in the modulation of mycobacterial infection (Silva *et al.* 1985).

Treatment of delipidated cell walls with sodium hydroxyde enabled a polysaccharide fraction (F_1) with granuloma-inducing activity to be obtained. Rats injected intraperitoneally with this fraction showed a greater influx of PMN and MN leucocytes compared with other polysaccharides present in fractions F_2, F_3 or with PBS.

The kinetics of the cell migration induced by the lipid extract and fraction F_1 permitted a gradual increase in PMN leucocytes to be characterized, which was later reduced, with a simultaneous increase in MN cells. These results agree with data showing an intense pulmonary inflammatory response around charcoal particles adsorbed with lipid extract and with data showing subcutaneous granuloma formation after injection of a suspension of fraction F_1. These inflammatory reactions were initially characterized by the massive presence of PMN leucocytes and later by MN cells.

Little has been published on mechanisms involved when acute inflammation becomes chronic in nature. It is not clear how mononuclear cells, in particular mononuclear phagocytes, come to replace PMN cells in inflammatory lesions. The transition from acute to chronic inflammation in actinomycotic mycetoma may be the result of the persistence of the lipid or polysaccharide irritant at the lesion site. The persistent presence of a poorly degradable substance in the tissue is a major mechanism for the induction of granulomatous inflammation (Sedgwick & Willoughby 1985).

The results presented here, taken as a whole, suggest that the active fractions isolated from *N. brasiliensis* are chemotactic for granulocytes. The influx of leucocytes to the inflammatory site of inoculation of any irritant has been attributed to chemotactic activity induced by products of complement activation, formyl-methionyl peptides, leukotriene B_4 (LTB_4) (Baggiolini 1985), and by a soluble low molecular weight factor produced by macrophages (Calich *et al.* 1985). Souza and Ferreira (1985) recently reported reduced PMN migration to the inflammatory site when the animals were previously treated with anti-macrophage serum. In this context, we believe that the lipid and polysaccharide constituents of *N. brasiliensis* probably participate in the inflammatory process of actinomycotic mycetoma by inducing the liberation of products of complement activation or by inducing macrophage to secrete potent mediators of the acute inflammatory response.

Present results also help some aspects of the inflammatory response observed in actinomycotic mycetoma caused by *N. brasiliensis* to be better understood, such as the neutrophil influx observed after bacterial penetration and multiplication (Connant *et al.* 1971; Rippon 1982), the presence of suppuration in the centre of the abscess formed (Rippon 1984) and the presence of exudate rich in MN cells around or throughout the granulomatous reaction (Connant *et al.* 1971; Rippon 1982; 1984). These features may be due, at least in part, to the presence of the lipid and polysaccharide constituents of the bacteria which may persist in the inflammatory focus as a function of local *N. brasiliensis* multiplication.

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