Cord Factor is Associated with the Maintenance of the Chronic Inflammatory Reaction Caused by Mycobacteria

By CELIO L. SILVA,* SEBASTIÃO L. BRANDÃO FILHO, IZAÍRA TINCANI AND LÚCIA M. CARARETO ALVES

Department of Parasitology, Microbiology and Immunology, School of Medicine of Ribeirão Preto, University of São Paulo, 14 100 Ribeirão Preto, SP, Brazil

(Received 12 November 1985; revised 14 February 1986)

The distribution of an aqueous suspension of cord factor (CF) from Mycobacterium bovis BCG in several mouse organs was examined after intravenous injection, and the correlation between evolution of the inflammatory granulomatous reaction and the presence of CF in these organs was determined. CF was preferentially deposited in the lungs and liver, and the kinetics of the pulmonary and hepatic inflammatory reaction, evaluated by determining the indices for these organs, showed a gradual increase on day 2 after injection, reached a peak around the fifth day, and declined thereafter. Histological analysis showed that on day 5 both the lungs and the liver were diffusely damaged by a mononuclear inflammatory infiltrate arranged in a granulomatous manner and consisting predominantly of histiocytes. CF elimination was more marked in the liver than in the lungs: 2 d after injection 76% of the material deposited in the liver had been eliminated. Little or no CF was detected in the liver and lungs by day 16, when the inflammatory reaction was also substantially decreased. A second CF dose administered 8 d after the first exacerbated the inflammatory process in both the lungs and the liver, indicating that the intensity of this process depends on CF concentration in the lesion site.

INTRODUCTION

Cord factor (CF) is a glycolipid (6,6'-dimycoloyltrehalose) present in mycobacteria (Noll et al., 1956) and it plays an important role in the pathogenesis of these micro-organisms (Silva et al., 1985). In a previous study it was demonstrated that Mycobacterium bovis BCG depleted of CF in the external part of the cell wall did not induce formation of pulmonary granuloma, was eliminated more rapidly by the host, had lower macrophage-stimulating activity and perceptibly reduced the delayed hypersensitivity response (Silva et al., 1985). The role of CF in the induction of granulomatous inflammation was studied in this laboratory (Silva et al., 1986). When CF was adsorbed to charcoal particles and inoculated intravenously into mice, embolization of these particles occurred in the pulmonary circulation, and typical epithelioid granulomas developed around them. In view of the decreased inflammatory reaction observed around the charcoal particles in material collected 16 d after inoculation, and the absence of lesions on day 32, we postulated the occurrence of progressive elimination of CF from these particles. The objective of the present study was to investigate the relationship between the disappearance of CF in the host and the resolution of granulomas.

METHODS

Preparation of 14C-labelled CF. BCG (Moreau strain) was grown for 6 weeks in Long's medium (Long, 1958) containing 0.3 μCi sodium [1-14C]acetate ml−1 (specific activity 56 mCi mmol−1, 2.07 GBq mmol−1). The bacilli were collected on a sintered glass filter and washed with distilled water until the filtrate was free of radioactivity.

Abbreviation: CF, cord factor.

0001-3068 © 1986 SGM
CF was purified by the method of Silva et al. (1979). Briefly, the lipid extract (chloroform/methanol extract) dissolved in a small volume of chloroform was loaded on a column (21 x 25.0 cm) of silicic acid/silica gel H (1:1, w/w) and eluted with 500 ml of each of the following solvents: chloroform; 5% (v/v) ethanol in chloroform; 60% (v/v) acetone in chloroform; acetone; 20% (v/v) methanol in chloroform; and methanol. The lipid fractions were analysed by thin-layer chromatography (TLC) on plastic sheets coated with silica gel F254 (Merck), with the solvent system chloroform/acetoacetone/methanol/water (50:60:2.5:3, by vol.). Repurification of the 60% (v/v) acetone in chloroform fraction by preparative TLC on silica gel H plates yielded a purified glycolipid that had $[^{13}C] + 46 (\delta = 0.5, \text{CHCl}_3)$, a melting point of 56°C, 11-5% sugar content determined by the phenol-sulphuric acid method (Dubois et al., 1956) and an IR spectrum similar to that described for diminocyclohexalose isolated from Mycobacterium tuberculosis (Kato & Asselineau, 1971). After alkaline hydrolysis (Isoneda et al., 1963), trehalose and mycolic acid were identified in the aqueous and ethereal phases, respectively. Physical and chemical analysis of the isolated mycolic acid showed that the carbon chain length centred on C8*, in agreement with previously reported results (Minnikin et al., 1983). The radioactivity of $^{14}$C-labelled CF was measured in an aqueous emulsion with Bray's reagent (Bray, 1960) in a liquid scintillation spectrometer (Beckman); the calculated specific activity was 0.02 µCi µg$^{-1}$.

Preparation of an aqueous suspension of CF. The procedure was that used by Kato (1967). CF was dissolved in ether (5 mg ml$^{-1}$) and warmed to just below the boiling point. Equal volumes of ethanol and phosphate-buffered saline (per litre: 8 g NaCl, 0.2 g KCl, 1.15 g Na$_2$HPO$_4$, 0.2 g KH$_2$PO$_4$, pH 7.2; PBS), in that order, were added. The resulting fine suspension of CF was evaporated under reduced pressure and gentle heating until the volume was reduced to that of the added PBS. This aqueous suspension was stable to sterilization at 100°C and to prolonged storage at 0°C.

Animals. Female or male Swiss mice 6 to 8 weeks old were used.

CF distribution in tissues. Mice were carefully injected intravenously through the retro-orbital venous plexus with 100 µg of $^{14}$C-labelled CF (specific activity 0.02 µCi µg$^{-1}$) in 0.2 ml PBS. At different times after injection, mice were anaesthetized with ether and killed by exanguination, and the liver, lungs, spleen, kidneys, mesenteric lymph nodes and brain were removed. The amount of CF in each tissue was determined as described below, and expressed as mg CF in tissue per g tissue. The results presented are from a single experiment; each value represents the mean ± standard deviation for seven mice at a determined time after injection. The brain (where CF is not expected to accumulate) was used as a control.

Lipid extraction from tissues. The mouse tissues were washed in ice-cold PBS, blotted on filter paper, weighed individually and homogenized in 5 vols water. The homogenized tissues were suspended in 10 vols chloroform/methanol (1:1, v/v) and stirred with a magnetic stirrer for 10 min. The extracts were separated by centrifugation at 5000 g for 5 min and the insoluble residue was resuspended in the same solvent system and re-extracted twice more. The extracts were combined and reduced to dryness on a rotary evaporator.

CF determination. A sample of lipid extract from each tissue was analysed by TLC on plastic sheets coated with silica gel F$_{254}$ (Merck), with the solvent system chloroform/acetoacetone/methanol/water (50:60:2.5:3, by vol.). Lipids on TLC plates were detected by exposure to iodine vapour. Visualized spots of lipid were scraped off the plate and the lipids were eluted from the gel with chloroform/methanol (2:1, v/v). The eluted fractions were dried in a rotary evaporator and the amount of $^{14}$C-labelled CF was determined with a scintillation counter.

Measurement of lung and liver granulomas. The extent of granuloma formation was evaluated by the increase in lung or liver weight at different times after CF injection and calculated using the organ index (organ weight/body weight) × 100 as previously described (Silva et al., 1985).

Histological analysis. The lungs and livers were fixed in Bouin's fixative, and histological sections of different areas were made and stained with haematoxylin/eosin.

RESULTS

The results in Table 1 show that intravenously administered $^{14}$C-labelled CF was preferentially deposited in the lungs and liver of the mice; 60-7 and 8.5 mg CF per g tissue were detected, respectively, in these organs 4 h after inoculation. The amount of CF detected in the spleen, lymph nodes, brain and kidney was much lower. The concentration of CF in the tissues decreased with increasing time after administration (Table 1). Elimination of CF from the liver occurred gradually between 4 h and 24 h after administration, and very intensively by day 2, when 76% of the material deposited in this organ had been eliminated. After this time, the remaining CF was gradually eliminated, until only traces were detected on day 16. CF elimination during the first 2 d after administration was slower from lung tissue than from the liver, but by day 16 the CF concentration in the lungs was very low (Table 1).

Fig. 1 shows the CF concentration in the lungs as a function of time after administration, in
Inflammation caused by cord factor

Fig. 1. Changes with time in CF concentration (■) and granuloma evolution [lung index, i.e. (lung weight/body weight) \times 100, ●] in the lungs of mice injected intravenously with 100 μg CF. ■----■ and ●-----● represent the CF concentration and lung index, respectively, after a second dose of CF on day 8 (arrow). The bars represent SD (n = 7).

Table 1. Distribution of an aqueous suspension of CF in tissues of mice after intravenous administration of a 100 μg dose

The results are means ± SD (n = 7).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>4 h</th>
<th>8 h</th>
<th>1 d</th>
<th>2 d</th>
<th>5 d</th>
<th>8 d</th>
<th>16 d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td>60.7 ± 6.7</td>
<td>55.9 ± 3.5</td>
<td>51.4 ± 13.4</td>
<td>34.5 ± 8.4</td>
<td>13.9 ± 1.6</td>
<td>7.7 ± 2.8</td>
<td>3.6 ± 2.2</td>
</tr>
<tr>
<td>Liver</td>
<td>8.5 ± 4.9</td>
<td>6.5 ± 0.8</td>
<td>5.9 ± 1.3</td>
<td>2.0 ± 0.8</td>
<td>1.8 ± 1.1</td>
<td>0.5 ± 0.1</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>Spleen</td>
<td>1.2 ± 0.5</td>
<td>0.9 ± 0.2</td>
<td>0.9 ± 0.3</td>
<td>0.8 ± 0.1</td>
<td>0.7 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.8 ± 0.4</td>
<td>0.8 ± 0.3</td>
<td>0.7 ± 0.2</td>
<td>0.6 ± 0.1</td>
<td>0.7 ± 0.2</td>
<td>0.5 ± 0.1</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>Lymph node</td>
<td>0.5 ± 0.2</td>
<td>0.5 ± 0.1</td>
<td>0.4 ± 0.2</td>
<td>0.3 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>0.3 ± 0.1</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>Brain</td>
<td>0.3 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>0.3 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>0.3 ± 0.1</td>
</tr>
</tbody>
</table>

relation to the kinetics of the inflammatory reaction induced by CF in this organ. The results show that the inflammatory lung infiltrate had already significantly increased by day 2, when 57% of the CF deposited there still remained. The maximum pulmonary index was observed on day 5, after which a gradual decrease occurred with stabilization at normal levels on day 16. Histological analysis of the lungs on day 2 showed an intense diffuse inflammatory infiltrate distributed throughout the pulmonary parenchyma, mainly consisting of mononuclear cells. Between days 5 and 8 the lungs still showed an intense infiltrate of inflammatory histiocytic cells, which at times formed compact arrangements very similar to granuloma. This inflammatory infiltrate had significantly decreased by day 16. To confirm that the intensity of the pulmonary inflammatory reaction was directly related to the presence of CF in the organ, a second 100 μg dose of 14C-labelled CF was injected 8 d after the first. The amount of CF deposited in the lung was slightly greater than that observed after the first injection and its rate of elimination was more rapid. The inflammatory response was exacerbated by the second dose of CF, and higher concentrations of CF were maintained than those induced by the first dose (Fig. 1).

Intravenous injection of CF also induced a significant increase in the hepatic index immediately after day 1 of the experiment. The time course of the hepatic inflammatory reaction was similar to that found in the lung. On day 2 the liver showed several clusters of mononuclear cells, at times containing neutrophils and diffusely distributed throughout the parenchyma.
Between days 5 and 8, the liver was diffusely damaged by mononuclear inflammatory infiltrates arranged as granulomas and consisting predominantly of histiocytes, which differed in size, at times converged and were often located in the perivascular spaces. Localized lesions were rare after day 16. A second CF dose 8 d after the first induced a significant increase in the organ index, as observed in the lungs.

DISCUSSION

After intravenous administration to mice as an aqueous suspension, CF was deposited preferentially in the lungs and liver and produced a marked chronic inflammatory reaction in both organs. Persistence of the inflammatory process depended on the presence of CF at the lesion site. When the level of inflammatory reaction had declined, and only a small amount of CF was present in the tissue (by day 8 after the first injection) a second dose of CF exacerbated the reaction, which persisted for more than two weeks.

Other investigators have produced pulmonary granuloma by intravenous or intraperitoneal injection of CF. Bekierkunst et al. (1969) found maximal granulomatous responses at day 7 and noted that intraperitoneal was less effective than intravenous administration. McLaughlin et al. (1978) also reported that the granulomatous response was maximal at day 7 and that the lesions had nearly resolved by day 28. Moore et al. (1972) reported intense microscopic granulomatous responses in rabbits 3 weeks after intravenous injection of 100 μg CF, although the response was much milder than that elicited by BCG.

Silva et al. (1985) demonstrated that M. bovis BCG depleted of CF in the external layer of the cell wall had a reduced capacity to induce a chronic inflammatory reaction. It was concluded that the inflammatory process caused by this micro-organism was related to CF clearance and that the chronic granuloma, which contains live bacilli, may derive its chronicity from this glycolipid, which may be constantly synthesized and feed the inflammatory reaction by being transferred to the surface of the bacilli. The participation of CF in the maintenance of mycobacterial inflammation is further suggested by the following line of reasoning. CF is a glycolipid consisting of two mycolic acid molecules (an α-branched and β-hydroxylated fatty acid with 90 carbon atoms) linked to trehalose by the hydroxyl groups of carbons 6 and 6', i.e. 6,6'-dimycolyltrehalose. Takayama et al. (1975) stated that isoniazid, a potent tuberculostatic drug, inhibited mycolic acid synthesis and consequently reduced the CF layer around the bacilli. Bacilli without CF in the outer layer may be phagocytosed by leucocytes and eliminated because the unprotected bacilli have insufficient time to resynthesize a new lipid layer to protect them against leucocyte activity. If treatment with isoniazid is withdrawn the bacilli can form a new protective lipid layer and trigger the process of chronic granuloma formation. These observations support our hypothesis that CF plays an important role in the infection caused by mycobacteria.

This research was supported by grants from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, process no. 84/0387-8).

REFERENCES


Inflammation caused by cord factor


