# *Role of Cord Factor in the Modulation of Infection Caused by Mycobacteria*

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**The subcutaneous, intradermal, and pulmonary inflammatory lesions induced in mice by viable *Mycobacterium bovis* (BCG) with no glycolipid cord factor (CF) on the outer cell wall (delipidated BCG, dBCG) was drastically different from that induced by inoculation with intact bacteria. The reaction caused by dBCG was of an acute nature: the cells making up the inflammatory infiltrate exhibited polymorphonuclear-like (PMNs) morphologic characteristics, there was a decrease on delayed hypersensitivity response, and the lesion was resolved around the 16th day after inoculation. Complete disappearance of viable organisms from the lungs, liver, and spleen of these animals occurred in parallel with the dissipation of the dBCG-induced inflammatory infiltrate, showing that CF plays an important role in the host-parasite relationship that takes place in infections caused by mycobacteria. In addition, when deprived of this glycolipid component, bacilli lose their immunostimulant ability. (Am J Pathol 1985, 118:238-247)**

AMONG THE various factors isolated from mycobacteria that may participate in the pathogeny of the infections caused by these microorganisms, lipids deserve special mention. These components, which make up about 40% of bacterial dry weight,[1] confer to the bacilli their alcohol-acid-fast dying property,[2] highly hydrophobic nature, and the tendency to adhere to each other and to float on the surface of liquid media.[3]

As far back as in 1947, Middlebrook et al[4] utilized these properties of lipids to distinguish virulent *Mycobacterium tuberculosis* strains from most attenuated and avirulent strains. These investigators observed that virulent strains were able to grow on the surface of liquid media and had the appearance of ropes or coils, whereas avirulent strains were smooth or wrinkled. In 1948, Dubos[5] proposed that this growth characteristic and the virulence may depend on the same substance, or substances present on the surface of bacilli. Searching for this substance, Bloch,[6] in 1950, submitted virulent *M tuberculosis* strains to light petroleum ether extraction and observed that the bacilli continued to be viable but lost their virulence as well as the ability to form "cords" on the surface. This factor, isolated with petroleum ether and denoted "cord factor" (CF), when adsorbed to nonvirulent bacilli, invested the bacilli with the ability to inhibit leukocyte migration (only possessed by virulent bacilli)[7,8] and was toxic to laboratory animals when administered in small doses.[6]

The structure of CF isolated from *M tuberculosis* was fully elucidated by Noll et al,[9] who concluded that CF was a glycolipid consisting of two mycolic acid molecules (an $\alpha$-branched and $\beta$-hydroxylated acid with 90 carbon atoms) linked with trehalose by the hydroxyl groups of carbons 6 and 6', ie, 6,6'-trehalose dimycolate.

Several other effects of CF in addition to its toxicity, such as granulomatous reaction in mouse lung after intravenous administration,[10,11] adjuvant and immunostimulant properties,[13] and clear antitumoral activity,[14,16] suggest its participation in the host-parasite relationship at different levels.

The objective of the present study was to show that CF plays a fundamental role in the modulation of chronic infection caused by mycobacteria.

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## Materials and Methods

### Preparation of Bacterial Suspensions

Mycobacterium bovis

*Mycobacterium bovis* (BCG, Moreau strain) suspensions were prepared by the method of Forget.[17] Briefly, the bacilli were grown as a pellicle on Sauton medium at 37 C for 14 days. Live bacteria were washed six times in deionized water, lyophylized, and kept at 4 C (stock preparation). Before use, the BCG preparation was filtered through a 5-$\mu$ membrane to eliminate bacterial clumps. This monocellular suspension was used for infection of mice and for cord factor extraction.

*Live CF-Depleted BCG (Delipidated BCG)*

The depletion of cord factor from live BCG was made by treating the bacteria with petroleum ether (boiling point, 35–60 C) according to the procedure proposed by Bloch.[6] Sixty percent of CF was removed from the bacilli after this procedure in relation to that obtained with treatment of microorganisms with chloroform/methanol (1:1 by volume) mixture. The viability of BCG and the solvent-treated bacteria (dBCG) was estimated by plating serial fivefold dilutions of the final bacterial suspension on Middlebrook 7H10 agar medium (Difco), followed by incubation at 37 C for 14 days.

*CF Purification and Characterization*

The residual material extracted with petroleum ether contained several lipid components such as glycolipids, free mycolic acid glycerydes, menaquinones, and hydrocarbons and were therefore fractionated by column chromatography on silicic acid/silica gel as previously reported.[18] The purified glycolipid accounting for 95% of the petroleum ether extract had $(\alpha)_D^{25^\circ}$ + 47 (c = 0.5, $CHCl_3$), a melting point of 58 C, 11% sugar content determined by the phenol–sulfuric acid method,[19] and an infrared spectrum similar to that described for dimycoloyl trehalose isolated from *M tuberculosis*.[20] After alkaline hydrolysis,[21] trehalose and mycolic acid were identified in the aqueous and ethereal phases, respectively. Physical and chemical analysis of the isolated mycolic acid[22] showed that the carbon chain length centered on $C_{84}$, in agreement with previously reported results.[23]

*Coating of dBCG With CF (CFdBCG)*

Delipidated BCG (dBCG) were coated with CF by suspending approximately 0.01 g of dried bacilli in 5 ml of a 0.01% petroleum ether solution of the glycolipid and evaporating the solvent at 30 C. The dry bacilli were resuspended in saline containing 0.5% bovine albumin. Control was prepared by treating the bacilli with solvent alone and suspending them in the same way.

### Animals

Male Swiss mice weighing 25–30 g were used.

### Hind Footpad Injections and Thickness Measurements

Suspensions of live BCG, dBCG, and CFdBCG ($10^6$ bacilli and a CF emulsion in saline, 50 $\mu$g/0.05 ml) were injected into the left footpad (FP) located just distal to the hock. This was done by passing the needle proximally through the skin of the plantar FP and then engaging the needle point into the dermis of the FP area mentioned. A 0.05-ml volume was injected in all cases with plastic tuberculin syringes equipped with 25-gauge needles. FP thickness was measured with a Schnell-Taster (Systems Kröplin, Schluchtern, West Germany) at various intervals after injection. Baseline measurements before injection (Day 0) were always made to assure group uniformity. At least five animals were used per group, and the variations in FP thickness were observed up to 30 days after inoculation. Delayed hypersensitivity (DH) responses to purified protein derivative (PPD) were assessed 12 days after immunization by the inoculation of 2 $\mu$g of PPD in 0.05 ml saline into the right FP. Thickness measurements were made after 24, 48, and 72 hours.

### Intradermal Lesion

A suspension of BCG, dBCG, CFdBCG ($10^5$ viable bacilli) and a CF emulsion (50 $\mu$g/0.05 ml) were inoculated into the ventral intraderma of mice. The dermonecrotic lesions were detectable both macroscopically and histologically after 2, 4, 8, and 16 days.

### Evaluation of Lung Granulomatous Reaction

The granulomatogenic property of BCG, dBCG, CFdBCG, and CF was tested by recording the histologic analysis and by recording the lung index after intravenous injection of $10^6$ bacilli or injection of 100 $\mu$g of CF through the retroorbital venous plexus. For histologic analysis, the animals were anesthetized with ether and killed by exsanguination. The lungs of the animals were fixed with 2 ml of Bouin's injected into the exposed trachea. The fixed organs were sectioned through the hilus, dehydrated in graded ethyl alcohol, sectioned in sequence, and embedded in paraffin. The sections obtained were stained with hematoxylin and eosin (H&E). For recording the lung index, the animals were killed by cervical dislocation. They were weighed; and their

lungs were removed, trimmed of extraneous tissue, rinsed in saline, blotted, and weighed. The lung index was calculated by the method of Allen et al.[24]

### Bacterial Enumeration *in Vivo*

Groups of five randomly selected mice were killed by cervical dislocation at regular intervals over a 30-day period; and the lungs, livers, and spleens were removed aseptically and homogenized separately in cold saline. The homogenates were suitably diluted with saline and plated onto Lowenstein-Jensen medium (GIBCO Diagnostic). The plates were incubated in sealed plastic bags at 37 C for 2–4 weeks before counting. The data are expressed as the $\log_{10}$ mean number of viable organisms per organ (standard error of the mean always <18%).

### Evaluation of Macrophage Stimulation

*Spreading*

Peritoneal cells were collected from mice pretreated or not with an intraperitoneal injection of 3 ml saline. Peritoneal fluid was withdrawn with a Pasteur pipet. After harvesting, the cells were washed and seeded at a concentration of $5 \times 10^5$ cells in 0.25 ml of growth medium in the glass coverslips (18 by 22 mm). The culture medium was 199 medium (Flow Laboratories) containing 5% heat-inactivated fetal serum and 1% glutamine. After cells were allowed to adhere for 20 minutes at 34 C, the nonadhering cells were removed by intense washing of monolayers with 199 medium. Then the glass cover was added to a plastic Petri dish (30 mm), covered with the medium described above, and incubated at 34 C for 40 minutes. After incubation the slides were washed three times with saline, fixed with glutaraldehyde (2.5%), and water-mounted for phase microscopy (×400). Macrophage stimulation was evaluated by the percentage of adhering peritoneal cells that spread onto glass coverslips.

*Acid Phosphatase*

Acid phosphatase activity of peritoneal macrophages was determined as follows. Peritoneal fluid collected as described above was placed in a test tube and allowed to stand at 34 C for 20 minutes to permit macrophage adherence to the glass surface. The tube was washed three times with PBS, 2 ml water was added, and sonication was performed for 30 seconds at 100 watts to promote cell lysis. To the cell lysate thus obtained 1.5 ml 0.3 M citrate buffer, pH 4.9, and 0.2 ml 0.04 M *p*-nitrophenylphosphate substrate was added. The mixture was incubated at 37 C for 1 hour, and 0.5 ml 1 M Tris-(hydroxymethyl)-aminomethane, pH 8.5, containing 0.4 M $K_2HPO_4$ was added. Readings at 420 nm were then carried out with a Zeiss spectrophotometer. Protein determination in the cell lysate was carried out by the method of Lowry,[25] modified. Enzyme activity was defined as units of optic density of nitrophenol released per milligram of protein per hour.

### Statistics

All results are expressed as the mean or mean ± SD. Statistical significance was determined by the unpaired Student *t* test.

## Results

BCG and dBCG suspensions containing $10^6$ viable bacilli and a 100 μg/0.05 ml suspension of CF were inoculated subcutaneously into the left FP of mice. The inflammatory reaction induced by the preparations, measured by the increase in thickness of the animal's paw, showed different kinetics, as illustrated in Figure 1. The inflammation caused by BCG followed the characteristic pattern reported in the literature,[26] ie, increased thickness of the paw between the 2nd and the 4th day, a period of time characterized by the presence of mononuclear cells and polymorphonuclear leukocytes (PMNs), as revealed by analysis of the histologic preparations. The inflammatory infiltrate decreased between

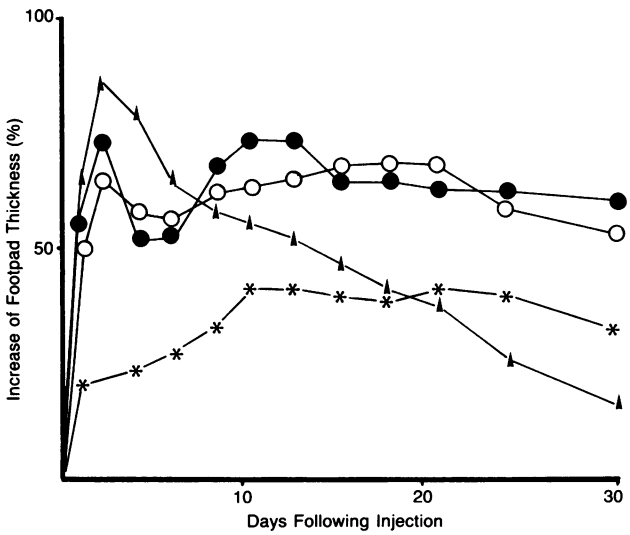



**Figure 1**—Kinetics of the local inflammatory reaction in response to injection of live BCG (●), live dBCG (▲), live dBCG recomposed with CF (CFdBCG) (○), and CF (*) into the left hind footpad of normal mice. The reaction was measured as the difference in thickness between the treated and untreated foot. Each point represents the median of 5 animals. The increase of footpad thickness induced by BCG was significantly different from that induced by dBCG on Day 4 and after Day 10.

Table 1—Intensity of Skin Lesions After Inoculation of Suspensions Containing BCG, dBCG, CFdBCG, and an Emulsion of the CF Glycolipid Into Mice

| Inflammatory agent | Intensity of the lesion (+) | | | |
|---|---|---|---|---|
| | 2 days | 4 days | 8 days | 16 days |
| BCG | ++* | ++++ | ++++† | ++++† |
| dBCG | ++++* | +++* | ++ | + |
| CFdBCG | +++* | ++++ | ++++† | ++++† |
| CF | ++ | ++++ | ++++† | +++ |

* Intense PMN infiltrate.
† Granulomatous lesion.

the 4th and the 8th day and again increased after the 10th day of the experiment, this time with the massive presence of mononuclear cells, denoting a delayed hypersensitivity response to the inoculated agent. The inflammation caused by dBCG was more intense between the 2nd and the 8th day after inoculation, with a 70–90% increase in thickness in the affected paw in relation to the contralateral one given an injection of saline. During this time, the cell infiltrate consisted mainly of PMNs and lesser numbers of macrophages and lymphocytes. After this intense initial reaction, a gradual decrease in inflammation occurred up to the 30th day, when the experiment was concluded. The kinetics of paw thickening in the animals inoculated with the CF emulsion was different from that induced by BCG and dBCG. The highest intensity of reaction to CF was obtained around the 10th day, and the cells in the inflammatory infiltrate were almost all macrophages, with large vesicular nuclei and indistinct cytoplasmic borders. These cells are so numerous as to form a monotonous pattern throughout the dermis. In addition, scattered lymphocytes and some PMNs could be seen. An interesting fact was that the inflammatory reaction induced by CFdBCG was similar to that obtained after inoculation with BCG.

### Intradermal Inflammatory Reaction

The intradermal inflammatory reaction induced by BCG, dBCG, CFdBCG ($10^5$ viable bacilli), and 50 μg/0.05 ml CF was followed for 2, 4, 8, and 16 days, and the results are shown in Table 1. It can be observed

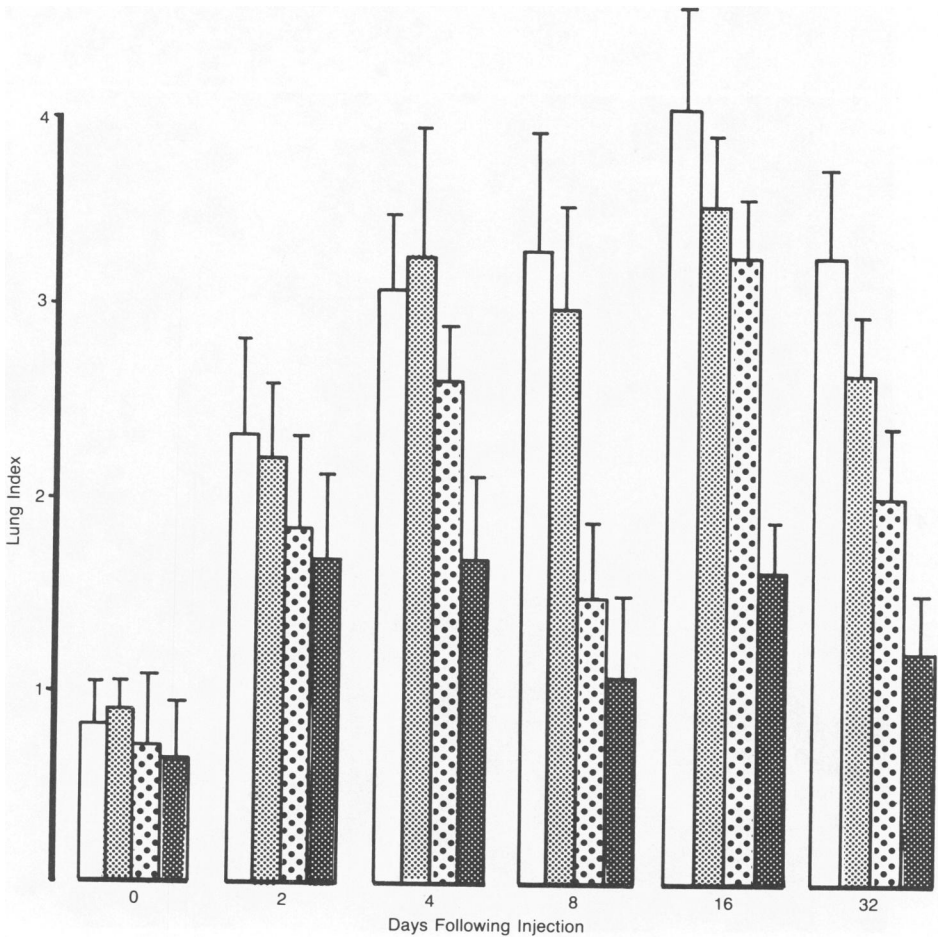
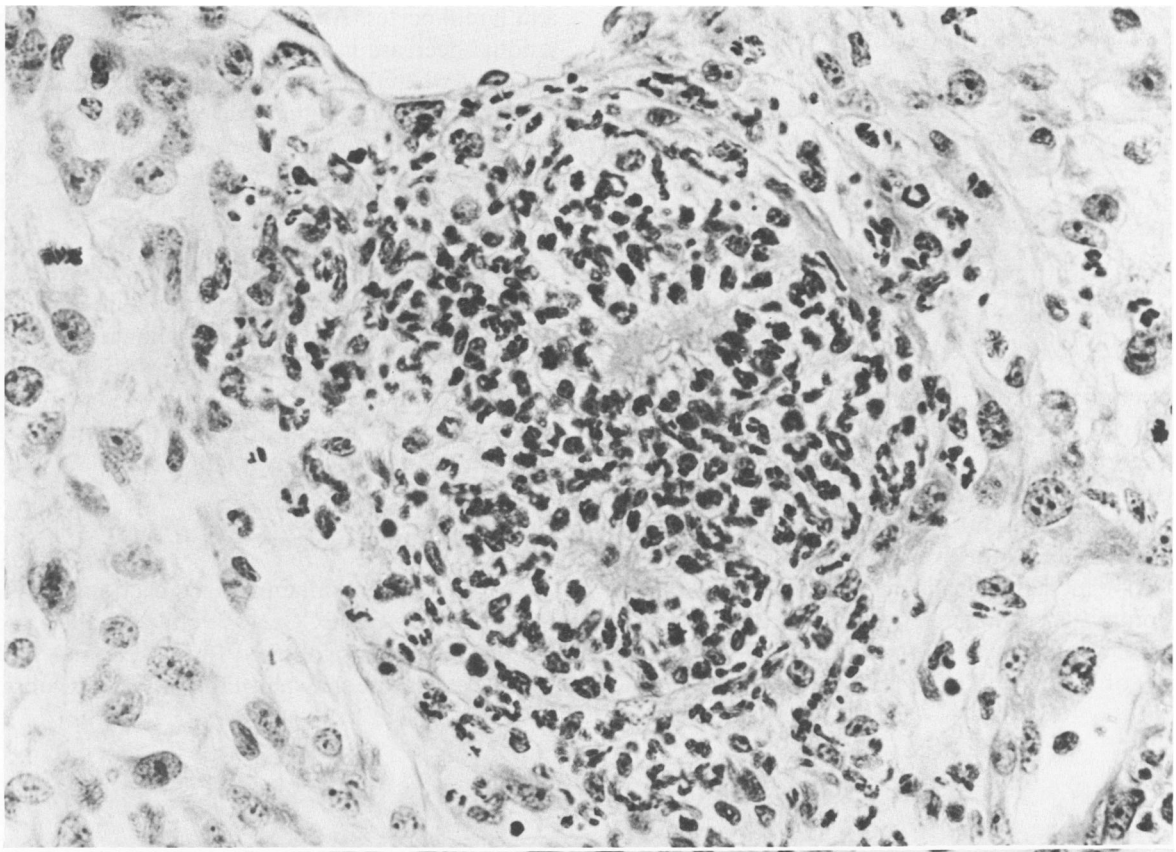



**Figure 2**—Lung index of mice given intravenous injections of live BCG (□), live BCG with no glycolipid CF on the outer cell wall (dBCG) (▩), live dBCG recomposed with CF (CFd BCG) (▨), and CF (⊡). Median and range for groups of 5 mice. The lung index recovered from animals inoculated with BCG, CFdBCG, and CF was significantly different from that inoculated with dBCG after Day 4 (except for CF on Day 8).



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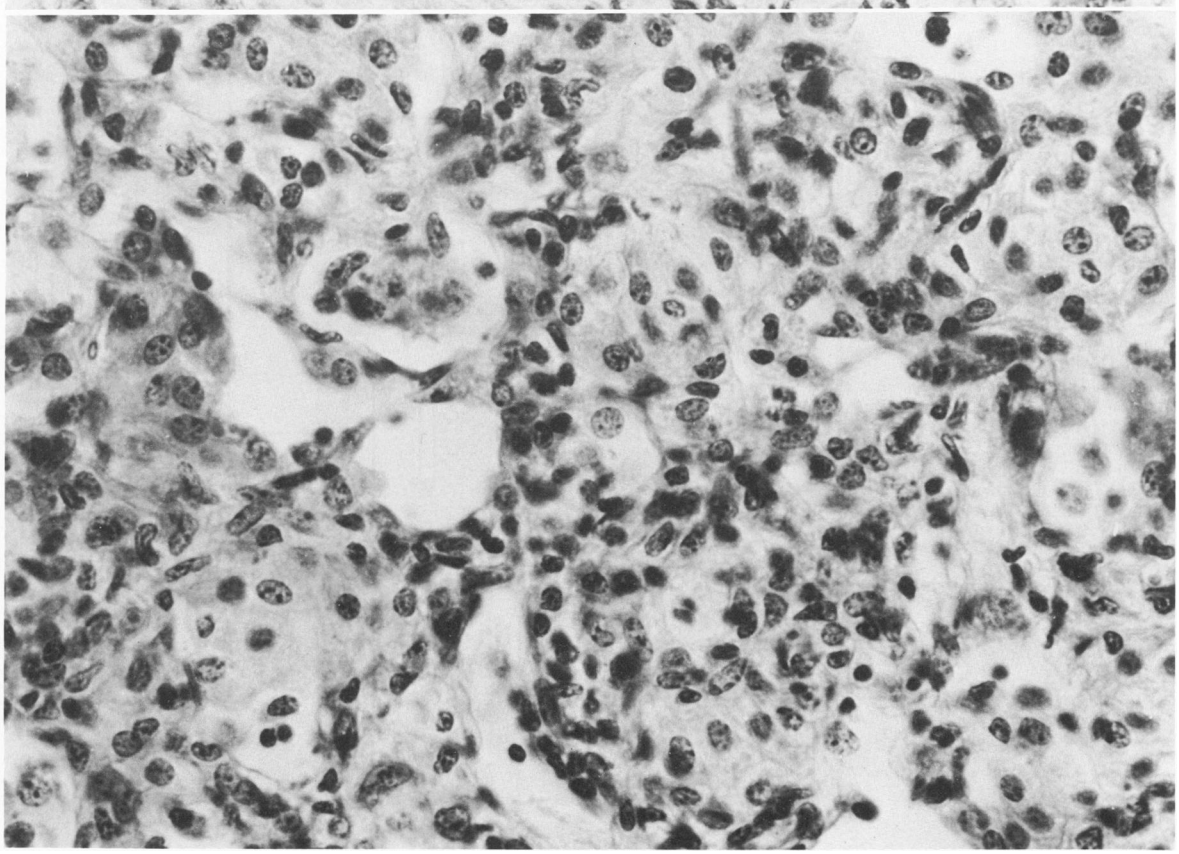

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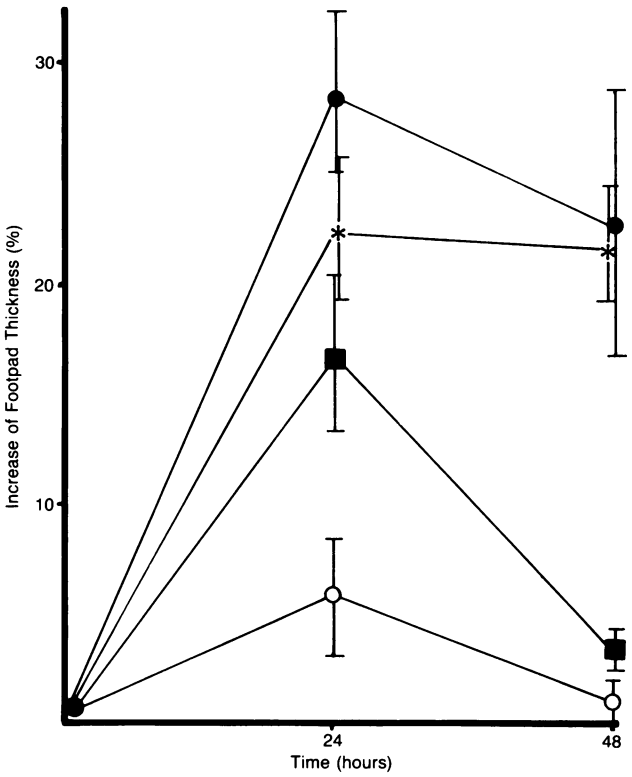


**Figure 5**—Kinetics of the footpad reaction in mice given 2 μg of PPD 12 days after a single immunizing dose of BCG (●), dBCG (■), dBCG recomposed with CF (✳), and CF (○) subcutaneously. The inflammatory reaction was measured as the difference in thickness between the treated and untreated (hind) foot. Values given are the median and range for groups of 5 mice. The hypersensitivity response to PPD in animals immunized with BCG was significantly greater than that from dBCG immunized animals for all time points ($P < 0.04$ at 24 hours and $P < 0.01$ at 48 hours).

that there was a sharp difference in the intensity of the lesion and in the type of cells making up the inflammatory infiltrate as a function of the different agents inoculated.

## Lung Inflammatory Reaction

The pulmonary inflammatory reaction induced by BCG, dBCG, CFdBCG, and CF was determined by calculating the lung indexes and by histologic analysis of the respective tissues 2, 4, 8, 16, and 32 days after intravenous inoculation. Figure 2 shows that by the second day of the experiment a significant increase occurred in lung indexes for all bacillus preparations inoculated as well as for the CF emulsion in relation to the indexes obtained for animals killed immediately after inoculation (time zero). During this same period, however, differences were observed between the indexes obtained for animals inoculated with BCG, CFdBCG, and CF and those obtained for animals inoculated with dBCG. Histologic analysis of the lungs of animals inoculated with dBCG revealed intense infiltrates of PMNs (Figure 3), whereas the lungs of animals inoculated with BCG, CFdBCG, and CF showed infiltrates predominantly consisting of mononuclear cells (Figure 4). Stabilization of the cell infiltrate occurred on the 2nd day in the lungs of animals inoculated with dBCG, and large amounts of PMN cells were still present between the 4th and the 8th day. On the 16th day, however, the small infiltrate observed was diffuse throughout the pulmonary parenchyma, and on the 32nd day no inflammatory reaction was observable in the lung. The pulmonary lesion of the animals that received BCG and CFdBCG was characterized by constant growth of the cell infiltrate, reaching a peak about 16 days after inoculation. Histologic examination showed an intense focal inflammatory reaction consisting of mononuclear cells with morphologic traits similar to those of macrophages and epithelioid cells. The chronic and granulomatous lesion induced by the two preparations was still quite evident by the 32nd day, with clusters of alcohol-acid-fast bacilli being visible in the center of each lesion. Intravenous inoculation of the CF emulsion induced a chronic diffuse inflammatory reaction consisting of mononuclear cells in the animals' lungs after about 8 days.

## Delayed Hypersensitivity

Different groups of mice immunized subcutaneously with BCG, dBCG, CFdBCG ($10^6$ viable bacilli), and CF (50 μg/005 ml) and challenged 12 days later with 2 μg PPD in the FP showed different delayed hypersensitivity responses, as shown in Figure 5.

## Viability of Bacilli in the Animals' Organs

dBCG viability inside the host and the effect of the lipid layer in protecting the bacilli were assessed by determining the number of colony-forming units in the lungs, liver, and spleen of mice after intravenous inoculation of $10^6$ viable bacilli (BCG, dBCG, and CFdBCG). As shown in Figures 6, 7, and 8, the viability of microorganisms having no external lipid layer was drastically reduced during the first 2 weeks after inoculation, and no viable organisms were observed in any

**Figure 3**—The lung inflammatory response 2 days after the intravenous injection of live BCG with no glycolipid CF on the outer cell wall (dBCG). Note the large numbers of neutrophils surrounding the bacilli foci as seen in the center of this photomicrograph. (H&E, ×400) **Figure 4**—The lung inflammatory reaction 2 days after the intravenous injection of live BCG. The alveolar interstitium is densely infiltrated with mononuclear cells forming loosely organized granulomas. (H&E, ×400)

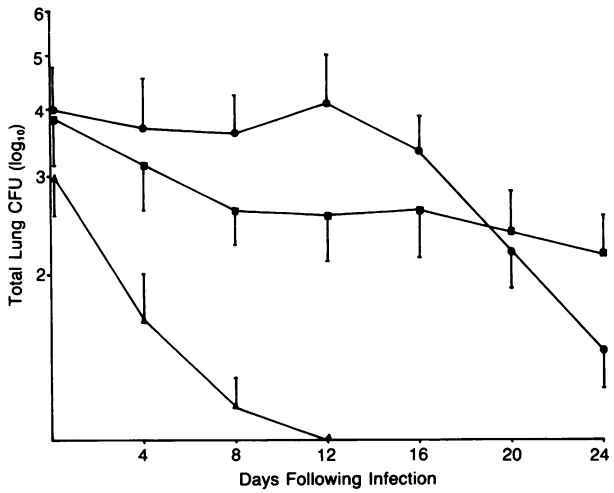


**Figure 6**—Growth of $10^6$ viable BCG (●), $10^6$ viable BCG with no glycolipid CF on the outer cell wall (dBCG) (▲), and $10^6$ viable dBCG recomposed with CF (CFdBCG) (■) in the lungs following intravenous inoculation into mice. The number of bacilli recovered from animals inoculated with BCG and CFdBCG was significantly greater than that from dBCG-treated animals after Day 4.

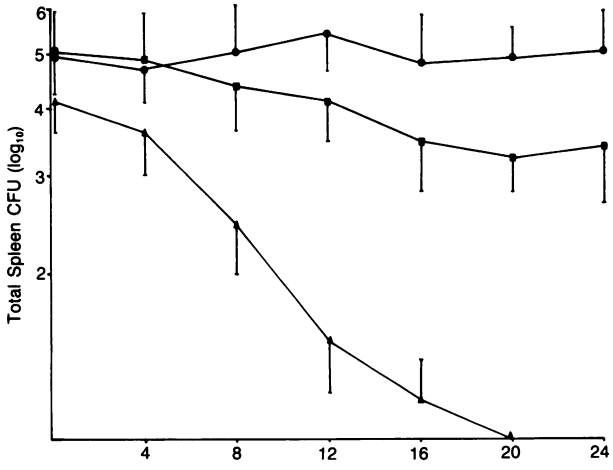


**Figure 8**—Growth of $10^6$ viable BCG (●), $10^6$ viable BCG with no glycolipid CF on the outer cell wall (dBCG) (▲), and $10^6$ viable dBCG recomposed with CF (CFdBCG) (■) in the spleens following intravenous inoculation into mice. The number of bacilli recovered from animals inoculated with BCG and CFdBCG was significantly greater than from dBCG-treated animals after Day 4.

of the organs studied from the 20th day on. Microorganisms that had been previously delipidated and then recomposed with CF (CFdBCG) were able to survive and multiply in the animals' organs in a manner similar to that observed for the intact bacilli (BCG).

## Macrophage Stimulation

BCG and dBCG showed different ability to stimulate macrophages in the peritoneal cavity of mice after intraperitoneal inoculation of $10^6$ viable bacilli (Figure 9). Highest macrophage activation by BCG occurred about 8 days after inoculation, when 90% of the cells spread on the slide. In contrast, highest activation by dBCG occurred around the fourth day of the experiment and was much lower than observed for BCG on the eighth day. Intraperitoneal inoculation of a 10 μg/0.05-ml emulsion of CF was highly effective in stimulating adhering macrophages to spread on the slide surface.

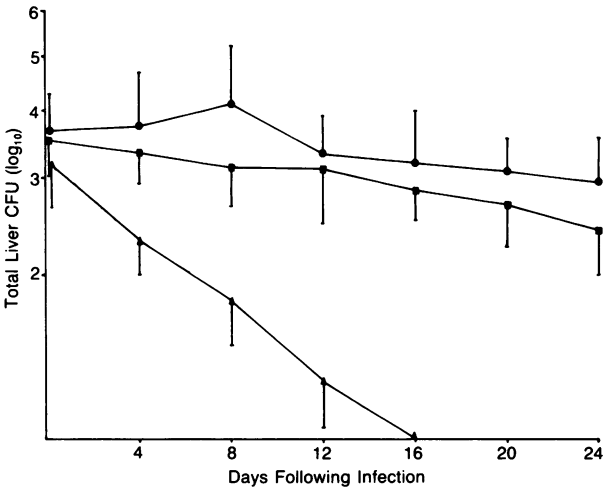


**Figure 7**—Growth of $10^6$ viable BCG (●), $10^6$ viable BCG with no glycolipid CF on the outer cell wall (dBCG) (▲), and $10^6$ viable dBCG recomposed with CF (CFdBCG) (■) in the livers following intravenous inoculation into mice. The number of bacilli recovered from animals inoculated with BCG and CFdBCG was significantly greater than from dBCG-treated animals after Day 4.

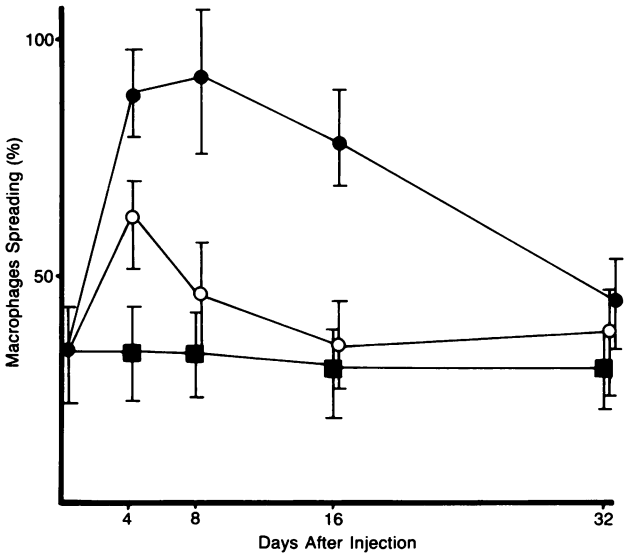


**Figure 9**—Percentage of spreading peritoneal macrophages collected from mice given live BCG (●), dBCG (○), and saline (■) intraperitoneally. Values are the means ± SD of 5 experimental and 5 control animals for each time point ($P < 0.04$ compared with values from the dBCG group).

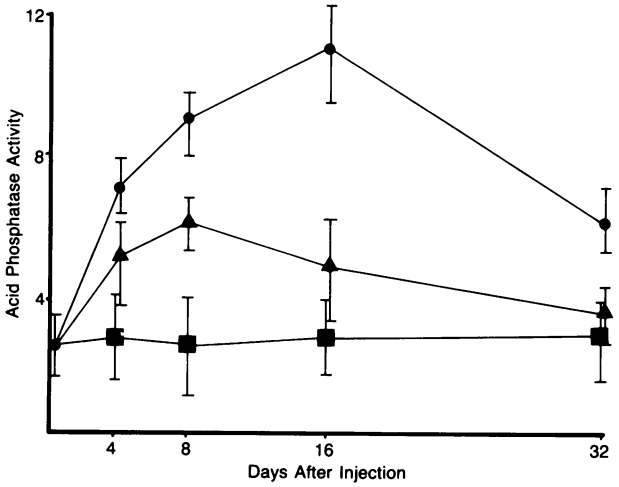


**Figure 10**—Peritoneal macrophage acid phosphatase activity from mice given live BCG (●), live dBCG (▲), and saline (■) intraperitoneally. Enzyme activity was defined as units of optical density of nitrophenol released per milligram of protein per hour. Values are the means ± SD of 5 experimental and 5 control animals for each time point ($P < 0.05$ compared with values from the dBCG group).

The determination of the specific activity of peritoneal macrophage acid phosphatase was also used as a parameter for evaluating the level of macrophage stimulation by BCG and dBCG. As shown in Figure 10, the two preparations stimulated the enzyme at different levels. Maximum acid phosphatase activation by BCG occurred around the 16th day of inoculation and was threefold that induced by dBCG.

## Discussion

Treatment of viable BCG with petroleum ether solvent removed lipid substances from the outer cell wall, and 90–95% of all bacilli continued to be viable after the extraction procedure. The lipid extract was fractionated by column chromatography,[18] and a component was obtained that, when analyzed by physical and chemical techniques,[18,22] proved to be a trehalose dimycolate similar to the CF isolated by Noll et al[9] from *M tuberculosis*.

On the basis of the number of bacteria submitted to the petroleum ether extraction procedure and of the amount of CF isolated, we may speculate that approximately $1.3 \times 10^{-5}$ μg CF was removed from each bacillus. This amount represents 60% of the total CF present in BCG, 0.32% of bacterial dry weight, or roughly $2.5 \times 10^9$ molecules per cell. Total depression of CF from BCG was realized with chloroform–methanol (1:1, by volume), and the bacilli lose their viability after this treatment.

The subcutaneous, intradermal, and pulmonary inflammatory picture induced by inoculation of delipidated BCG (dBCG) was drastically altered when compared with that induced by intact BCG, especially in terms of the nature of the reaction, types of cells involved, development of delayed hypersensitivity, and onset and duration of the lesion. Thus, the lesion induced by dBCG was characterized as an acute inflammatory reaction, mainly because of the constant and massive presence of PMNs. The secondary inflammatory response of the delayed hypersensitivity type occurred to a lesser degree, which is unexpected for reactions produced by mycobacteria.[27] As to the duration of the lesion, a gradual decrease of the cell infiltrate occurred, generally from the 4th day after inoculation by any route, followed by disappearance between the 16th and 32nd day.

The changes that occurred during the course of the infection caused by dBCG in relation to that caused by BCG, especially in terms of persistence of the lesion and type of cells involved, were a consequence of the presence or absence of CF at the reaction site.

We studied previously the role of CF in the induction of granulomatous inflammation (unpublished observations). Preliminary experiments showed that when CF was adsorbed to charcoal particles (50–100 mμ in diameter) and was 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 days after inoculation and in view of the absence of lesions on the 32nd day, we postulated the occurrence of progressive elimination of CF from these particles. If the duration of the inflammatory process were related to CF clearance, we could speculate that the chronic process denoted granuloma, which contains live bacilli, may derive its chronic property from the molecules of this glycolipid, which may be constantly synthesized and feed the inflammatory process by being transferred to the surface of the bacilli.

As far as the multiplication of the organism is concerned, we wish to emphasize that the tubercle bacillus can grow in culture media even without CF on its surface. Thus, CF is not essential for bacterial multiplication *in vitro*, although it is constantly synthesized by the bacillus. On the other hand, CF is essential for bacillus multiplication in the host, because it may protect microorganisms against destruction by phagocytes. Unprotected bacilli, ie, bacilli without the outer glycolipid layer, may be phagocytized by leukocytes and may be eliminated by not having enough time to resynthesize a new lipid layer for protection against the destroying mechanisms of these leukocytes. If the bacilli can escape from these destruction systems, however, they can form a new protective lipid layer and behave as normal

bacilli, triggering the entire process of chronic tubercular lesion.

Other reports in the literature have indicated that CF plays an active role in the infections caused by mycobacteria. Intravenous inoculation of a CF oil emulsion is able to induce the formation of granulomas in the lungs of mice,[10,11] and these granulomas increase the resistance of the animals to challenge with live bacteria.[10] Furthermore, Kato,[28,29] by immunizing rabbits with CF complexed with methylated bovine albumin, obtained antibodies of the IgM class specific for CF. These antibodies were able to protect the animals not only from the toxic effects of the factor per se, but also from infection with virulent tubercle bacilli. This protection against infection also appeared in animals that received passive transfer of serum from immunized animals. In addition, in several experimental models, cord factor proved to have adjuvant activity comparable to that of intact bacilli.[12]

The data reported in the present paper show that this adjuvant activity of cord factor is important for the development of delayed hypersensitivity to the tubercle bacillus, since it is responsible for maintaining a large number of macrophages around the lesion, thus permitting these cells to present cell-wall antigens (PPD, for example) to T-lymphocytes. Delayed hypersensitivity did not occur when dBCG was inoculated, because these delipidated bacilli induce an acute inflammatory reaction, and the cells present at the lesion site, composed predominantly of PMNs, are unable to present antigens to lymphocytes, and therefore no animal sensitization to mycobacterial antigens occurs.

The immunostimulating properties of BCG are well known, and activation occurs at the level of the mononuclear phagocytic system.[30] Among the various components present in mycobacteria is the CF, which has the ability to stimulate macrophages nonspecifically[31] and highly increases host resistance against infection caused by mycobacteria and organisms unrelated to these alcohol-acid-fast bacilli, such as *Lysteria monocytogenes* and *Klebsiella pneumoniae*.[13] Notable among the different properties of activated macrophages are spreading on glass surfaces and large increase of lysosome enzyme activities.[32] Intraperitoneal administration of dBCG drastically reduces the number of macrophages that spread on slides *in vitro* and considerably decreases acid phosphatase activity in relation to BCG. Therefore, macrophage activation by BCG is due at least in part to the constant presence of CF on the bacillus surface.

The data presented here clearly show that CF plays a fundamental role in the genesis and persistence of the chronic and granulomatous lesions induced by mycobacteria.

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