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# Granulomagenic Activity of Serologically Active Glycolipids from *Mycobacterium bovis* BCG

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The granulomagenic properties of serologically active glycolipids $A_1$, $B_2$, $B_3$, and C isolated from *Mycobacterium bovis* BCG were studied. Glycolipid $A_1$, dissolved in olive oil and injected intradermally in guinea pigs, was able to elicit a granulomatous response that seemed to be of the nonallergic type. This granulomagenic activity was quite striking since only 2 $\mu$g was necessary to elicit the reaction. The B and C glycolipids were milder granulomagenic agents. Glycolipid $A_1$, dissolved in olive oil and injected intraperitoneally, was toxic for mice. Mice lost weight after the injection of as little as 10 $\mu$g of $A_1$, although not even a dose of 100 $\mu$g was lethal. Glycolipid $A_1$ failed to immunize mice against aerogenic infection with virulent tubercle bacilli.

Mycobacterial constituents have provided investigators with a seemingly unexhaustible number of biologically active components, among which lipids have attracted the most attention. Several lipid fractions of mycobacteria have been reported to induce granuloma formation: wax D (23), trehalose-6,6'-dimycolate (TDM; so-called "cord factor") (2, 4), cell walls (14), cell wall skeletons (14), and $P_3$, a trehalose mycolate (14, 15).

Three families of serologically active glycolipids (SAG), A, B, and C, have been isolated and purified from *Mycobacterium bovis* BCG in these laboratories and shown to be widely distributed in the genus *Mycobacterium* (19, 20). This paper describes the granulomagenic activity of these SAG in the skin of guinea pigs, especially glycolipid $A_1$, which also possesses the property of being toxic for mice.

## MATERIALS AND METHODS

**Preparation of SAG.** SAG were extracted from *M. bovis* BCG (Research Foundation, University of Illinois, Chicago, Ill.) and purified as previously described (19). Glycolipids $A_1$, $B_1$, $B_3$, and a mixture of $C_1$, $C_2$, and $C_3$ referred to as C were used. Glycolipid $A_1$ was also prepared from 2-week-old cultures of *Mycobacterium phlei*. SAG inocula were prepared by adding 1 ml of olive oil (Sclafani, Spain) or 1 ml of saline to different amounts of the dry glycolipids. The SAG were dissolved in olive oil at concentrations of 5, 20, 100, and 500 $\mu$g/ml or suspended in saline at 200 and 500 $\mu$g/ml. The inocula were heated at 56°C for 15 min before injection in the animals.

**Animals.** COBS-CD-1 male mice (Charles River, Wilmington, Mass.) and Hartley strain guinea pigs weighing 600 to 700 g were used.

**Inoculation of guinea pigs.** Guinea pigs were shaved at least 2 h before the intradermal injection of 0.1 ml of inoculum. The sites of injections were observed daily, the diameters of erythema and induration were recorded and averaged, and tissues were removed at different times. Each SAG was inoculated in duplicate, and 0.1 ml of olive oil was inoculated intradermally as a control. In one experiment four guinea pigs were vaccinated by the intraperitoneal (i.p.) route with $10^7$ viable units of BCG and 6 weeks later, together with four normal guinea pigs, were skin tested with 2 $\mu$g of $A_1$ in olive oil, 20 $\mu$g of $A_1$ suspended in saline, and 5 $\mu$g of purified protein derivative (PPD; NIH) diluted in saline containing 0.0005% Tween 80 and 0.3% phenol. In another experiment six guinea pigs were first inoculated intradermally with 10 $\mu$g of $A_1$ dissolved in olive oil and 6 weeks later received one intradermal injection of 2 $\mu$g of $A_1$ in olive oil, 20 $\mu$g of $A_1$ in saline, and 10 $\mu$g of PPD. Six normal guinea pigs were skin tested with 2 $\mu$g of $A_1$ in olive oil at the same time as the previously $A_1$-inoculated guinea pigs were tested. In both experiments the injection sites were observed for reactions, and the sites of the 2-$\mu$g $A_1$ injections were removed for histological studies after different intervals of time.

**Histological preparations.** Skin and subcutaneous tissues were removed and fixed in buffered formalin, and 5-$\mu$m sections were stained with hematoxylin-eosin.

**Inoculation of mice.** In one experiment 10 mice weighing 27 to 28 g were repeatedly injected i.p. with 10 $\mu$g of $A_1$ dissolved in 0.1 ml of olive oil. The 10 mice in the control group received 0.1 ml of olive oil. The injections were done at 0, 5, 10, and 13 days, and the mice were weighed daily. In another experiment, mice weighing 19 to 20 g received a single i.p. injection of 40 or 100 $\mu$g of $A_1$ in 0.1 ml of olive oil. The control group received 0.1 ml of olive oil. There were five mice in each group, and they were weighed daily.

**Aerogenic infection of mice with virulent tubercle bacilli.** Mice inoculated with $A_1$ or olive oil as described above were aerogenically infected 60 days after the first injection. *Mycobacterium tuberculosis* strain Erdman (TMCC 107) at a concentration of $10^6$ viable units/ml of nebulizer fluid was used, applying techniques already described (18). Mice were weighed weekly and the survival times were recorded.

## RESULTS

**Effect of the SAG in the skin of normal guinea pigs. (i) Macroscopic observations.** The sites of injections of 2 $\mu$g of glycolipid $A_1$ dissolved in olive oil showed 8 to 10 mm of pale erythema and weak induration at 24 h. At 48 h the erythema and induration had increased to 10 to 18 mm and central blanching was observed, with some of the reactions proceeding to necrosis. The erythema and induration began to decrease by day 3 to 4, and the reaction sites appeared normal by day 7 to 8. The reactions at the sites of 10 $\mu$g of $A_1$ in olive oil were more intense and lasted longer. At 24 h, 10 to 15 mm of pale erythema and weak induration were observed; by 48 h the erythema and induration reached 18 to 25 mm, with some of the reactions showing central blanching and others showing necrosis. At 72 h all the reactions showed necrosis and ulceration occurred by day 4 to 5, at which time the induration began to decrease. By day 7 to 9 the ulcers began to heal and were healed and only barely visible by day 13 to 15. Weak inflammatory reactions were elicited by 0.5 $\mu$g of $A_1$ in olive oil. They showed 4 to 6 mm of pale erythema at 24 h and 5 to 10 mm of erythema and weak induration at 48 h and disappeared by day 4 to 5. No central blanching or necrosis was observed. $A_1$ from *M. phlei* induced skin reactions similar to those just described for $A_1$ from BCG. Glycolipids $B_1$, $B_3$, and C dissolved in olive oil elicited very mild reactions: 50 $\mu$g produced only 3 to 5 mm of pale erythema, with minimal or no induration at 24 h; at 48 h the reactions were scarcely evident and they were negative by 72 h. Olive oil reactions consisted of 2 to 4 mm of erythema at 24 h and were negative or barely visible at 48 h. No visible skin reactions were observed at the sites of inoculation of 50 $\mu$g of $A_1$ or 50 $\mu$g of the other SAG suspended in saline.

**(ii) Histological observations.** The cellular reactions induced by 2 or 10 $\mu$g of glycolipid $A_1$ in olive oil were alike, except that the reactions to 10 $\mu$g were more intense. Twenty-four hours after the injection of 10 $\mu$g of $A_1$ there was edema along with an infiltrate consisting of polymorphonuclear granulocytes and a few macrophages. Histiocytes had increased in number at 48 h. By 72 h ulceration of the epidermis and necrosis were seen. Histiocytes, epithelioid cells, and a few multinucleated giant cells of the foreign body and Langhans types were evident in the subcutaneous tissue. At day 4 a distinct granulomatous response was present; lymphocytes were occasionally seen. From day 4 to 11 the chronic inflammatory reaction became more intense, with increasing numbers of epithelioid cells. A section taken 10 days after the injection of 10 $\mu$g of $A_1$ is shown in Fig. 1. By day 15 fibroblasts had appeared, and marked fibrosis was seen by day 20 together with epithelioid cells and giant cells. By day 35 healing was almost complete, and epithelioid or giant cells were no longer seen. No differences were observed in the cellular reactions induced by $A_1$ from *M. phlei* and $A_1$ from BCG.

The cellular reactions elicited by 50 $\mu$g of $B_1$, $B_3$, or C glycolipids in olive oil were about the same, except that the reactions induced by the B glycolipids were more intense. An acute inflammatory reaction was present at 24 h. By 48 h there was a chronic inflammatory infiltrate consisting mostly of histiocytes and some lymphocytes, with few polymorphonuclear granulocytes and multinucleated giant cells. By day 4 a granulomatous response was present, with epithelioid cells, histiocytes, multinucleated giant cells, and rare lymphocytes. By day 9 some fibroblasts had appeared, and by day 14 fibrosis had almost completely replaced the epithelioid cell reaction.

Olive oil induced a very mild granulomatous reaction. Acute inflammation was seen in the first 24 to 48 h, and by 72 h an inflammatory infiltrate composed of histiocytes, epithelioid cells, and occasional multinucleated giant cells and lymphocytes were present. The mild epithelioid reaction had increased by day 4 and then diminished, and it was minimal by day 8. By day 12 fibrous tissue had begun to replace the chronic infiltrate, but some epithelioid and giant cells were still seen. No histological studies were made of the sites of injections of the SAG suspended in saline.

**Effect of $A_1$ in the skin of BCG-vaccinated guinea pigs.** Tissues at the sites of injection of 2 and 10 $\mu$g of $A_1$ in olive oil were removed at 1, 2, 3, and 4 days after the inoculations. No differences, macroscopic (in size or time of appearance) or microscopic, were observed between the reactions developing in normal guinea pigs and BCG-vaccinated guinea pigs. Five weeks after BCG vaccination, the guinea pigs were skin tested with 5 $\mu$g of PPD and 20 $\mu$g of $A_1$ suspended in saline. PPD gave 10 mm of erythema at 24 h, and 20 $\mu$g of $A_1$ did not elicit any skin reactions.

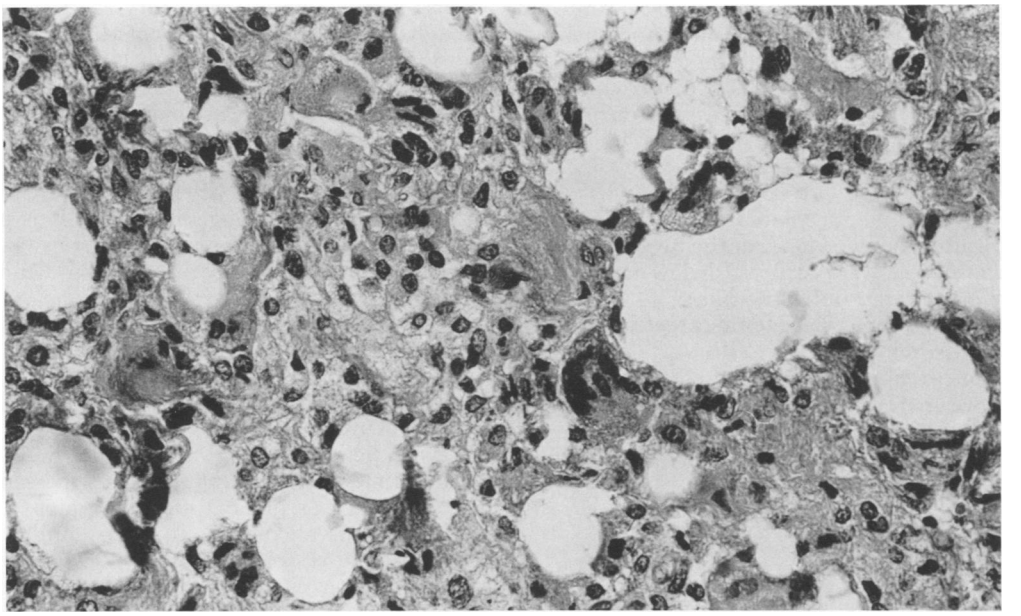

FIG. 1. *Granulomatous response in the skin of a guinea pig. Tissue section was taken 10 days after injection of glycolipid $A_1$. Hematoxylin and eosin; ×250.*

**Effect of $A_1$ in the skin of $A_1$-inoculated guinea pigs.** Tissues at the sites of 2-$\mu$g injections of $A_1$ in olive oil were removed at 1, 2, 3, 4, 15, and 23 days after the injections. The reactions to 2 $\mu$g of $A_1$ in olive oil observed in guinea pigs previously inoculated with $A_1$ were identical histologically, as well as in size and time of appearance, to the ones manifested by normal guinea pigs. All guinea pigs gave negative skin reactions to 10 $\mu$g of PPD and 20 $\mu$g of $A_1$ suspended in saline.

**Effect of repeated i.p. injections of $A_1$ in mice.** $A_1$ was toxic for mice as manifested by the weight loss that occurred in the 24 h after the injection of 10 $\mu$g of $A_1$ in 0.1 ml of olive oil. After the first injection the mice developed resistance against this toxicity and subsequent injections produced decreasing loss of weight, so that after the fourth injection no significant weight loss was observed. The changes in weight are presented in Fig. 2. The average weight losses observed in the 24 h after each $A_1$ injection were as follows: 12.5% after the first injection, 9.5% after the second, 3.8% after the third, and 0.6% after the fourth. These mice reached the same weight as the mice inoculated with olive oil at 24 to 25 days after the first injection.

**Effect of a single i.p. injection of $A_1$ in mice.** Both 40 and 100 $\mu$g of $A_1$ in 0.1 ml of olive oil were toxic for mice, although neither of these doses was lethal. After the injection of 40 $\mu$g of $A_1$, an average weight loss of 15.7% was

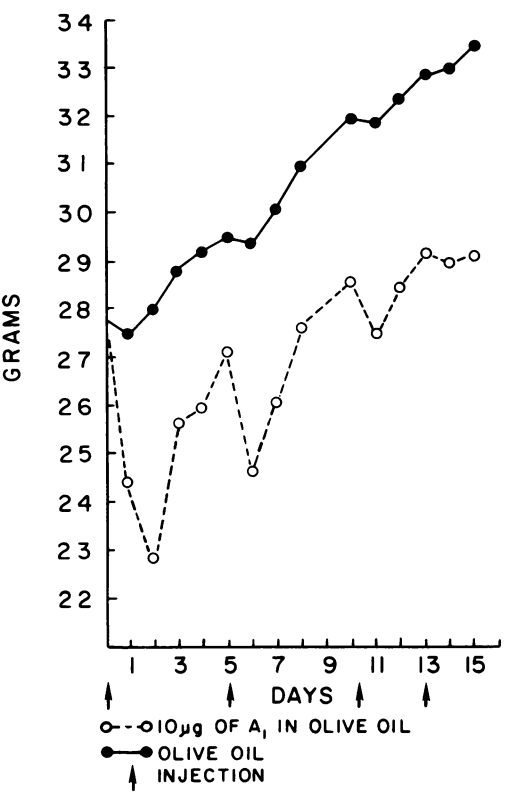


FIG. 2. *Average weight loss of mice after repeated i.p. injections of glycolipid $A_1$. Symbols: ○, 10 $\mu$g of $A_1$ in olive oil; ●, olive oil; ↑, injection.*

observed in 24 h; afterwards, mice continued to lose weight for the next 2 days and then recovered. After the inoculation of 100 μg of $A_1$, an average weight loss of 20% occurred in 24 h; the mice continued to lose weight during the next 3 days and began to recover by day 4. These results are presented in Fig. 3. Mice injected with 40 μg of $A_1$ reached the same weight as those inoculated with olive oil by 46 days after the injection, but the mice inoculated with 100 μg were still 5% below the control group at 59 days after the injection.

**Effect of $A_1$ on aerogenic infection of mice with virulent tubercle bacilli.** $A_1$ failed to protect mice against aerogenic tuberculosis; no difference in the survival time between the $A_1$-injected groups and the control group was observed. Mice died between week 4 and 5 after aerogenic challenge.

## DISCUSSION

The results of the experiments described in this paper show that BCG glycolipid $A_1$ when dissolved in olive oil is a very strong granulomagenic agent in the skin of guinea pigs; only 2 μg is necessary to elicit a granulomatous response and visible inflammatory reaction. Although $A_1$ from BCG and $A_1$ from *M. phlei* have the same antigenic determinant(s), it was not known whether they would have the same biological properties. It was found that both $A_1$ fractions have the same granulomagenic activity. Thus, *M. phlei* could provide an economical source of glycolipid $A_1$. Glycolipids $B_1$, $B_3$, and C were mild granulomagenic agents. It is unlikely that this granulomatous response was due to a contamination by $A_1$ since the B and C glycolipid fractions were serologically negative for $A_1$ by inhibition techniques, which can detect as little as 0.019 μg of $A_1$ (19). Since olive oil alone has a mild granulomagenic activity, it is possible that $A_1$ owes its activity to a marked enhancement of this property of olive oil. The same can be said of the B and C glycolipids.

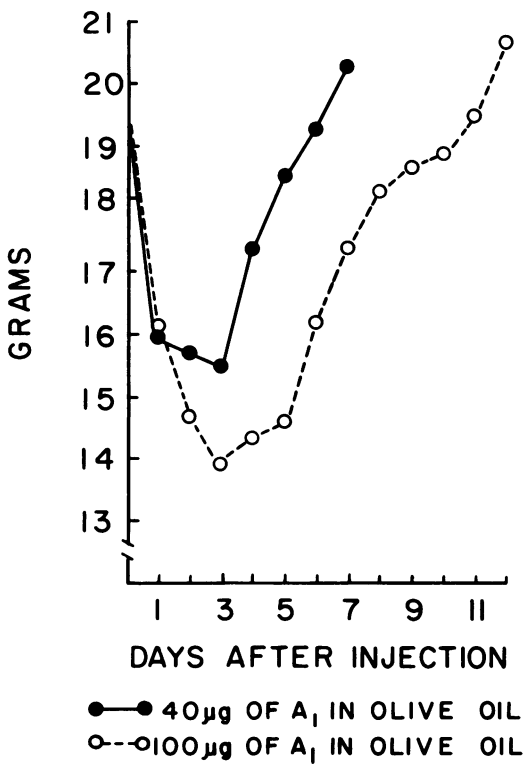


FIG. 3. *Average weight loss of mice after a single i.p. injection of glycolipid $A_1$. Symbols: ●, 40 μg of $A_1$ in olive oil; ○, 100 μg of $A_1$ in olive oil.*

Glycolipid $A_1$ did not induce skin hypersensitivity to itself or to PPD in guinea pigs. The skin reactions elicited by $A_1$ were similar in BCG-vaccinated, previously $A_1$-injected, and normal guinea pigs, which suggests that the granulomatous response to $A_1$ is of the nonallergic type. Langhans giant cells, which appeared in $A_1$ granulomas, have been observed in experimental nonallergic inflammatory reactions (13). Thus, their presence is not confined only to the classical tuberculo-granulomas and other infectious processes. It is of interest that different fractions isolated from BCG have distinctly different granulomagenic properties. For instance, the mycolates, TDM and $P_3$, induce nonallergic types, whereas BCG cell walls provoke an allergic type of granuloma (7, 15, 17). Although histologically the granulomas induced by different components of mycobacteria may appear to be the same, there is a real possibility that the cells are functionally diverse and contribute in a dissimilar way to host responses. In this regard the allergic granuloma type that develops in the lungs of BCG-sensitized rabbits is different from the hypersensitivity reaction induced by PPD. Rabbits injected with BCG and desensitized with tuberculin are still able to develop accelerated granulomatous responses in the lungs even though their capacity to react to tuberculin has been abrogated (11). Furthermore, antimacrophage serum is capable of suppressing the delayed-type hypersensitivity reactions but not the formation of allergic granulomas (16), suggesting additional differences in the mechanisms leading to the induction of allergic-type granulomas. It is reasonable to speculate that the granuloma found in active tuberculosis is a mixed granuloma, with both allergic and nonallergic components. Whether granuloma formation plays a role in the mechanisms of im-

munity in tuberculosis is not actually known, although it would seem that the mobilization of macrophages produced at the site of a granuloma probably contributes to the defense of the host (12).

In view of the overlapping biological activities shown by $A_1$, TDM, and $P_3$, it is worth pointing out the differences among these glycolipids. To begin with, they differ in their granulomagenic activity in guinea pigs and in their toxicity for mice. On the one hand, $A_1$ is a strong granulomagenic agent in the skin of guinea pigs, whereas the mycolates TDM and $P_3$ are only mild ones: 150 to 300 $\mu$g of $P_3$ in mineral oil is necessary to elicit a mild granulomatous reaction (5, 14), and a recent report showed that 500 $\mu$g of TDM in mineral oil emulsion did not induce inflammatory swelling in the skin of guinea pigs (22). On the other hand, $A_1$ seems to be much less toxic for mice than TDM. Repeated i.p. injections of 5 $\mu$g each (five injections in total) of TDM in mineral oil or olive oil killed 50% of mice between 1 and 4 weeks (6). Glycolipid $A_1$ has a characteristic toxicity for mice; mice lose weight after i.p. injection of 10 $\mu$g of $A_1$ dissolved in olive oil and then develop resistance against this toxicity after subsequent injections. The reasons for this peculiar toxicity and the resistance developed against $A_1$ are not known at the present time. It is possible that antibodies formed after the administration of $A_1$ neutralize the following $A_1$ injections. Another alternative speculation is on the basis of an increasing "tolerance" of a pharmacological nature such as seen in drug tolerance. It has been reported that in mice the $LD_{50}$ of $P_3$ is 40 $\mu$g (1). Further studies on $P_3$ toxicity are needed for evaluation of these results.

$A_1$ did not protect rabbits (19) or mice against aerogenic tuberculosis. Bekierkunst et al. have reported that TDM protects mice against tuberculosis (3), and Ribi et al. found that $P_3$ failed to immunize mice against tuberculosis (21). Whether the biological effects of TDM and $P_3$ are due to the same or different chemical components of BCG is not clear, as the TDM preparations used could have contained other mycobacterial components responsible for its effects.

Antibodies against $A_1$ have been found in the sera of tuberculosis and leprosy patients but not in the sera of tuberculous or BCG-vaccinated rabbits (19, 20). No serological activity has been reported for TDM or $P_3$. Antibodies against TDM have not been detected in the sera of tuberculous patients (10). Kato reported that he was able to make complexes of TDM and methylated bovine serum albumin and use the complexes to elicit antibodies against TDM in mice and rabbits (8). These mice were protected against the toxic effect of TDM and also showed an enhanced resistance against infection with virulent tubercle bacilli (9). It is of interest that species differences exist in the response to TDM (22). TDM induces a granuloma in mice but no inflammatory swelling in the skin of rats or guinea pigs, and it acts as an adjuvant for delayed-type hypersensitivity in rats but not in guinea pigs. TDM is not toxic for rats, whereas it is very toxic for mice.

Finally, it is reasonable to speculate that because of its granulomagenic activity $A_1$ may be endowed with immunological adjuvant effects and antitumor activity, both of which are presently under investigation. The chemical structure of $A_1$ is being studied.

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LITERATURE CITED

1. **Azuma, I., E. E. Ribi, T. J. Meyer, and B. Zbar.** 1974. Biologically active components from mycobacterial cell walls. I. Isolation and composition of cell wall skeleton and component P3. J. Natl. Cancer Inst. 52:95-101.
2. **Bekierkunst, A.** 1968. Acute granulomatous response produced in mice by trehalose-6,6'-dimycolate. J. Bacteriol. 96:958-961.
3. **Bekierkunst, A., I. S., Levij, E. Yarkoni, E. Vilkas, and E. Lederer.** 1969. Granuloma formation induced in mice by chemically defined mycobacterial fractions. J. Bacteriol. 100:95-102.
4. **Bekierkunst, A., I. S. Levij, E. Yarkoni, E. Vilkas, and E. Lederer.** 1971. Cellular reaction in the footpad and draining lymph nodes of mice induced by mycobacterial fractions and BCG bacilli. Infect. Immun. 4:245-255.
5. **Bekierkunst, A., L. Wang, R. Toubiana, and E. Lederer.** 1974. Immunotherapy of cancer with nonliving BCG and fractions derived from mycobacteria: role of cord factor (trehalose-6,6'-dimycolate) in tumor regression. Infect. Immun. 10:1044-1050.
6. **Bloch, H., and H. Noll.** 1955. Studies on the virulence of tubercle bacilli. The effect of cord factor on murine tuberculosis. Br. J. Exp. Pathol. 36:8-17.
7. **Granger, D. L., W. Brehmer, K. Yamamoto, and E. Ribi.** 1976. Cutaneous granulomatous response to BCG cell walls with reference to cancer immunotherapy. Infect. Immun. 13:543-553.
8. **Kato, M.** 1972. Antibody formation to trehalose-6,6'-dimycolate (cord factor) of *Mycobacterium tuberculosis*. Infect. Immun. 5:203-212.
9. **Kato, M.** 1973. Effect of anti-cord factor antibody on experimental tuberculosis in mice. Infect. Immun. 7:14-21.
10. **Kato, M.** 1973. Immunochemical properties of anti-cord factor antibody. Infect. Immun. 7:9-13.
11. **Kawata, H., Q. N. Myrvik, and E. S. Leake.** 1964. Dissociation of tuberculin hypersensitivity as mediator for an accelerated pulmonary granulomatous response in rabbits. J. Immunol. 98:433-438.

12. **Lurie, M. B.** 1964. Resistance to tuberculosis, p. 42. Harvard University Press, Cambridge, Mass.
13. **Mariano, M., and W. G. Spector.** 1974. The formation and properties of macrophage polykaryons (inflammatory giant cells). J. Pathol. 113:1-19.
14. **Meyer, T. J., E. Ribi, and I. Azuma.** 1975. Biologically active components from mycobacterial cell walls. V. Granuloma formation in mouse lungs and guinea pig skin. Cell. Immunol. 16:11-24.
15. **Meyer, T. J., E. E. Ribi, I. Azuma, and B. Zbar.** 1974. Biologically active components from mycobacterial cell walls. II. Suppression and regression of strain-2 guinea pig hepatoma. J. Natl. Cancer Inst. 52:103-111.
16. **Moore, V. L., and Q. N. Myrvik.** 1970. Effect of antimacrophage serum on dermal tuberculin sensitivity and allergic pulmonary granuloma formation in rabbits. Infect. Immun. 2:810-814.
17. **Moore, V. L., Q. N. Myrvik, and M. Kato.** 1972. Role of cord factor (trehalose-6,6'-dimycolate) in allergic granuloma formation in rabbits. Infect. Immun. 6:5-8.
18. **Reggiardo, Z., and G. Middlebrook.** 1974. Delayed-type hypersensitivity and immunity against aerogenic tuberculosis in guinea pigs. Infect. Immun. 9:815-820.
19. **Reggiardo, Z., and G. Middlebrook.** 1974. Serologically active glycolipid families from *Mycobacterium bovis* BCG. I. Extraction, purification and immunological studies. Am. J. Epidemiol. 100:469-476.
20. **Reggiardo, Z., and G. Middlebrook.** 1974. Serologically active glycolipid families from *Mycobacterium bovis* BCG. II. Serological studies on human sera. Am. J. Epidemiol. 100:477-486.
21. **Ribi, E., T. J. Meyer, I. Azuma, R. Parker, and W. Brehmer.** 1975. Biologically active components from mycobacterial cell walls. IV. Protection of mice against aerosol infection with virulent *Mycobacterium tuberculosis*. Cell. Immunol. 16:1-10.
22. **Saito, R., A. Tanaka, K. Sugiyama, I. Azuma, Y. Yamamura, M. Kato, and M. Goren.** 1976. Adjuvant effect of cord factor, a mycobacterial lipid. Infect. Immun. 13:776-781.
23. **White, R. G., and A. H. E. Marshall.** 1958. The role of various chemical fractions of *M. tuberculosis* and other mycobacteria in the production of allergic encephalomyelitis. Immunology 2:111-122.