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# Cross Reactivity of *Mycobacterium leprae* and BCG
## A Report on Further Studies[1,2]

**Mauricio Goihman-Yahr and Jacinto Convit[3]**

In previous studies ([1, 2, 3]) we were able to show cross reactivity between *Mycobacterium leprae* and other mycobacteria. We used an experimental guinea pig system and a purified, enzyme-digested suspension of *M. leprae.*

Considering delayed hypersensitivity only, cross reactivity existed among suspensions of mycobacteria (whole bacilli), including BCG, and *M. leprae*; as well as between the latter and BCG cell-wall fraction. Protoplasmic fraction of BCG and *M. leprae* cross-reacted only to a small degree. Even this was attributed to contamination of protoplasmic fractions with cell-wall material.

We concluded that the relationship between Hansen's bacillus and BCG was similar to that reported by Larson *et al* ([5, 6, 7]) and Ribi *et al* ([8, 9]) when cell walls and protoplasms from distantly related mycobacteria were tested in reciprocally-sensitized animals.

The question remained open as to whether or not cross reactivity between *M. leprae* and BCG protoplasm was indeed non-existant. Further, it could be argued that a purified suspension of *M. leprae,* processed as described ([2, 3]) might be considered as equivalent to a cell-wall suspension, rather than to one of unaltered bacilli. Thus, sensitizing animals to it would produce only cross reactivity to cell walls.

The following experiments were designed to shed light on these and related points.

## MATERIALS AND METHODS

Crude (whole) lepromin was prepared as recommended by Hanks *et al* ([4]). It contained 157 x $10^6$ bacilli per milliliter as determined by the method of Shepard *et al* ([11]). Lyophilized BCG cell-wall and protoplasmic fractions were kindly provided by Dr. Edgar Ribi, Hamilton, Montana. They were obtained by disrupting bacilli and separating the fractions by differential ultracentrifugation and were reported to be free of reciprocal contamination ([10]). To check this further, lyophilized materials were suspended or dissolved in water and droplets were stained by Gram, Ziehl-Neelsen and Wright-Giemsa methods. No acid-fast particles or other bacteria were seen in protoplasmic fractions by light microscopy. Acid-fast structures were abundant in cell-wall suspensions.

Droplets were also examined using a Hitachi HS-7S electron microscope. They were dried in 100-mesh parlodion-coated grids, and examined without further treatment, or after carbon coating, employing a Hitachi HUS-3B vacuum evaporator. No structures were observed in protoplasmic fractions. Broken mycobacteria were easily discerned in the cell-wall preparations.

Heat-stable freeze-dried glutamate BCG vaccine (Japan BCG Laboratory) was grown in Loewenstein-Jensen medium at 37°C. When abundant growth was obtained, bacteria were dislodged, washed, heat-killed and ground using a Tenbroeck apparatus. Wet weight was then determined. Processing was carried out as described previously ([2, 3]).

Albino guinea pigs of the Rockefeller strain were fed rabbit pellets and water *ad libitum,* supplemented twice weekly with fresh carrots and cabbage.

After the experiments were completed, representative experimental lesions were

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3 Mauricio Goihman-Yahr, M.D., Ph.D., Department of Dermatology, Vargas Hospital, Vargas School of Medicine, Central University of Venezuela, Caracas, Venezuela; Jacinto Convit, M.D., Division of Sanitary Dermatology, M.S.A.S., Venezuela.

biopsied. Specimens were fixed in buffered formalin, processed routinely and stained by the hematoxylin-eosin and Fite-Faraco methods.

## RESULTS

**Preliminary experiments.** These were done to determine appropriate concentrations of BCG fractions to be employed later. Six guinea pigs (500 gm mean weight) were sensitized with four milligrams, wet weight, of heat-killed BCG, emulsified in incomplete Freund's adjuvant (Difco). The emulsion was injected into the four foot pads and the neck of each animal. Three weeks later, each animal was injected intradermally with the following: 100 $\mu$g, 10 $\mu$g or 1 $\mu$g of cell-wall suspension or 500 $\mu$g, 50 $\mu$g or 5 $\mu$g of protoplasmic fraction. All fractions were dissolved or suspended in sterile phosphate-buffered saline, pH 7.2, and each was given in a volume of 0.1 ml. Six untreated guinea pigs were similarly injected, as controls, at the same time and in the same fashion.

Diameters of induration were measured at one, two, four, and eight days after injection. Mean values obtained are shown in Figures 1 and 2. Based on these, and on results obtained previously ([3]), it was decided to use 100 $\mu$g of cell-wall fraction, and 500 $\mu$g and 50 $\mu$g of protoplasmic fraction respectively in the experiments described below.

**Definitive experiments.** Nineteen guinea pigs (475 gm mean weight) were alternately injected with lepromin emulsified (1:1 v/v) in incomplete Freund's adjuvant or intradermally with lepromin alone. Animals were injected on five different occasions during a period of five months. Each guinea pig received a total of 1.1 ml of lepromin (approximately 173 x $10^6$ bacilli). At the same times and intervals, 18 guinea pigs (mean weight 486 gm) received phosphate-buffered saline, pH 7.2 with 0.5% of phenol, and 0.05% of Tween-80 (PBSTP) alone, or emulsified in incomplete Freund's adjuvant. These animals formed the "negative control group."

Thirteen guinea pigs (mean weight 456 gm) received 3 mg wet weight of heat-killed BCG, emulsified in incomplete adjuvant, five weeks before testing. They re-

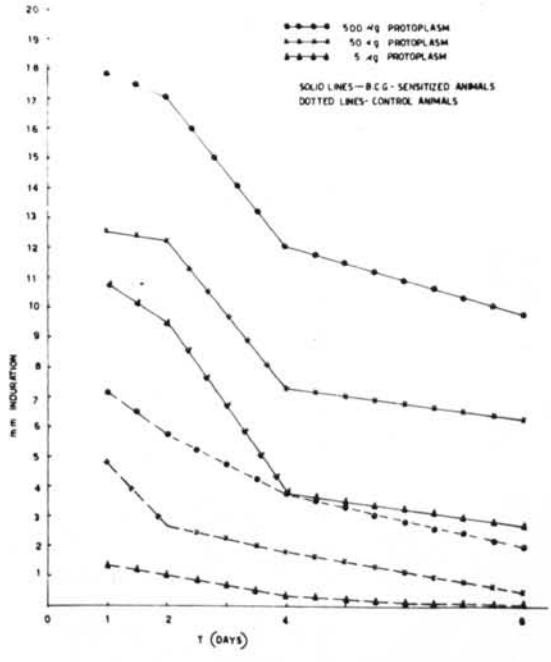


FIG. 1. Reactions to BCG protoplasm. Preliminary experiments. Values shown for the different groups are the means of diameters of induration (in mm).

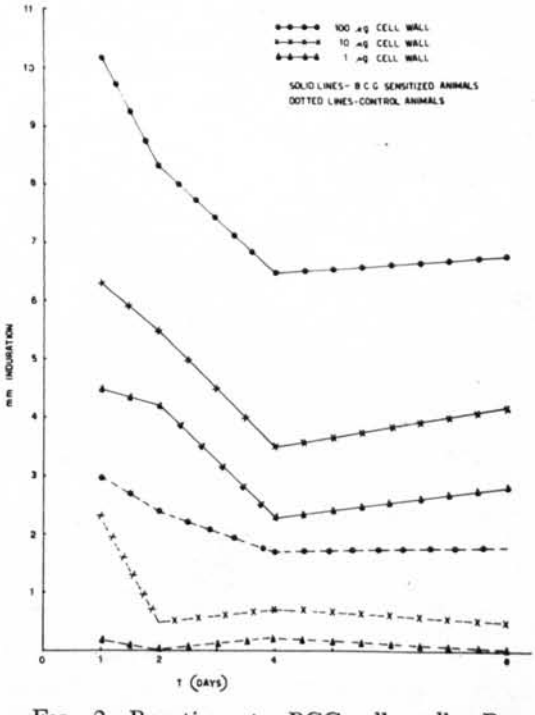


FIG. 2. Reactions to BCG-cell walls. Preliminary experiments. Values shown for the different groups are the means of diameters of induration (in mm).

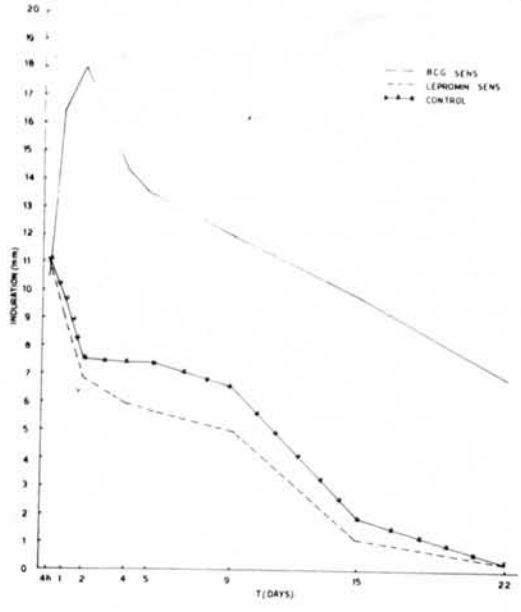


FIG. 3. Reactions to 500 $\mu$g of BCG protoplasm. Definitive experiments. Values shown for the different groups are the means of diameters of induration (in mm.).

Note lack of significant differences between lepromin-sensitized and negative control groups.

ceived a booster of 0.8 mg of BCG, emulsified in incomplete adjuvant, 18 days before testing. These animals constituted the "positive control group."

Every guinea pig received intradermal injections of BCG cell-wall and protoplasmic fraction as mentioned before. Diameters of induration were measured at four hours, and at 1, 2, 4, 5, 9, 15 and 22 days after injection. Results were statistically evaluated by the t-test. At the end of the experiment selected sites were biopsied.

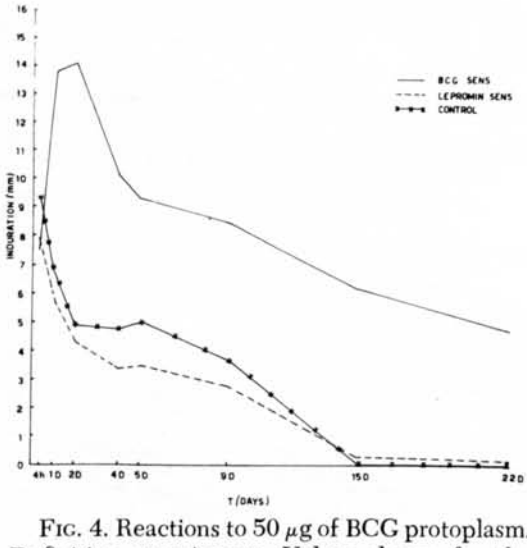


FIG. 4. Reactions to 50 $\mu$g of BCG protoplasm. Definitive experiments. Values shown for the different groups are the means of diameters of induration (in mm).

Note lack of significant differences between lepromin-sensitized and negative control groups.

Mean values obtained from readings are depicted in Figures 3, 4, 5, and 6. These show that BCG-sensitized animals reacted markedly to BCG protoplasm. There was no significant difference in reactions to this fraction between normal and lepromin-sensitized guinea pigs at any of the concentrations employed.

Ten micrograms of cell-wall material elicited reactions which were more marked in lepromin-sensitized guinea pigs than in negative controls. Differences were statistically significant at 1, 4, 5, 9 and 15 days after testing (values of P were less than 0.001, except at 15 days when $0.001 < P < 0.01$).

One hundred micrograms of cell walls induced a noticeable inflammatory response even in non-sensitized animals and thus results were less demonstrative.

**Histopathologic findings.** Microscopic features correlated very well with gross appearances. Five hundred micrograms of BCG protoplasm induced in normal controls and lepromin-sensitized animals, a mild to moderate inflammatory infiltrate. This infiltrate was formed mainly by lymphocytes and histiocytes. It affected the dermis (particularly around hairs and blood vessels), and the dermal-subcutaneous junction. There was no appreciable difference between sections from animals of these two groups. Fifty micrograms of protoplasm induced a similar, but much milder response in animals from such groups.

In contrast, the inflammatory response was massive in BCG-sensitized guinea pigs. There were isolated or confluent macrophages (epithelioid cells) and typical giant cells. There was necrosis. The infiltrate affected the dermis and dermis-subcutaneous junction. These features were present using both concentrations of protoplasm, with a difference in degree only.

Cell-wall material produced a similar picture in BCG-sensitized and lepromin-sensitized animals. With both concentrations, there was a typical tuberculoid

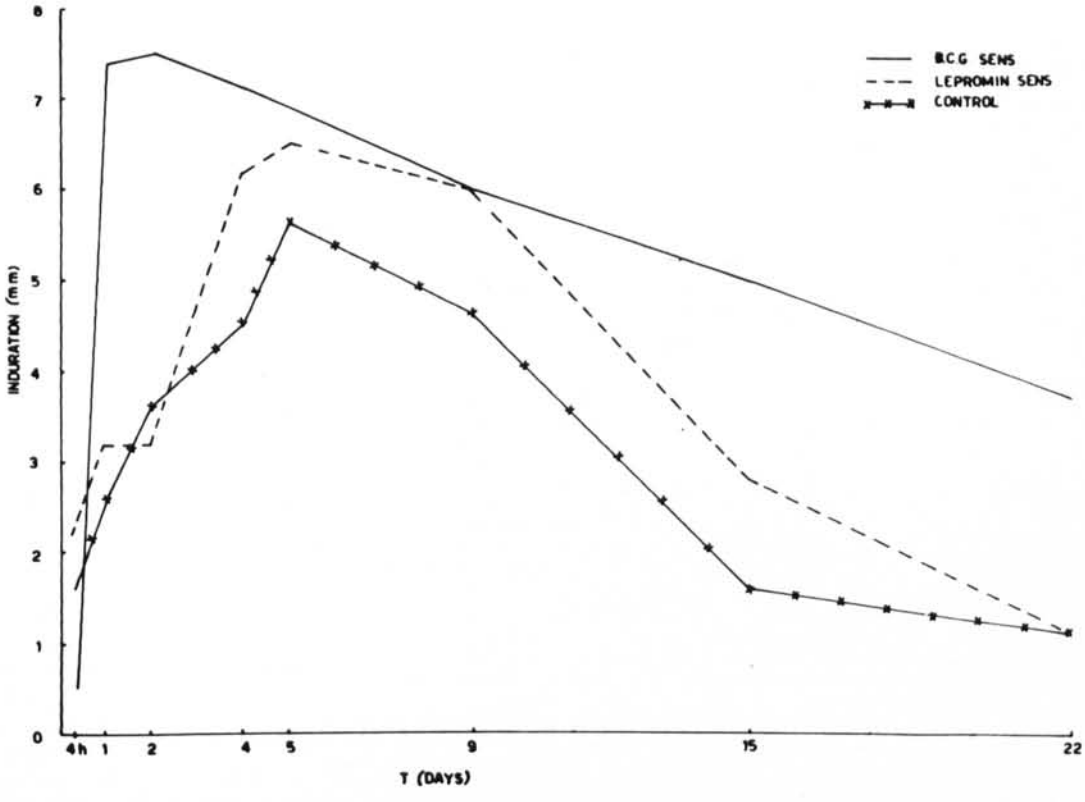


FIG. 5. Reactions to 100$\mu$g of BCG-cell walls. Definitive experiments. Values shown for the different groups are the means of diameters of induration (in mm). Notice appreciable inflammatory response in all groups.

infiltrate with giant cells and confluent macrophages (epithelioid cells) present in mid-dermis and at the junction of dermis and subcutaneous tissue.

In normal guinea pigs, one hundred micrograms of cell walls induced a moderate inflammatory response. In one instance there was a tuberculoid focus. In others, there was only a rather diffuse infiltrate where macrophages predominated. Ten micrograms of this fraction induced in normal guinea pigs a mild infiltrate formed by lymphocytes and some macrophages.

Acid-fast stains were not demonstrative since acid-fast particles were scarcely or not at all, seen. No difference could be detected, by the use of these stains, between experimental groups.

It should be noted that in BCG-sensitized guinea pigs, responses to protoplasm were of greater intensity than reaction to cell walls, and had tuberculoid features. This means that in exquisitely-sensitized animals, BCG protoplasm may induce tuberculoid responses. It is not absolutely necessary to assume contamination with cell-wall material to account for this phenomenon.

## DISCUSSION

Using purified BCG cell walls and protoplasm in intradermal testing, the existence of clear-cut antigenic relationships between BCG cell walls and *Mycobacterium leprae* were confirmed. No cross reactivity was detected between the latter and BCG protoplasm. These results clarified questions left open from previous work [1, 2, 3].

Findings such as these help in explaining the seeming paradox of BCG being able to convert a previously-negative Mitsuda reaction, while no relationship is apparent between a positive Mitsuda and Mantoux reactions to *Mycobacterium tuberculosis* tuberculin.

We submit that the Mitsuda reaction is a response to cells walls. Tuberculin reactions are equivalent to a response to protoplasm.

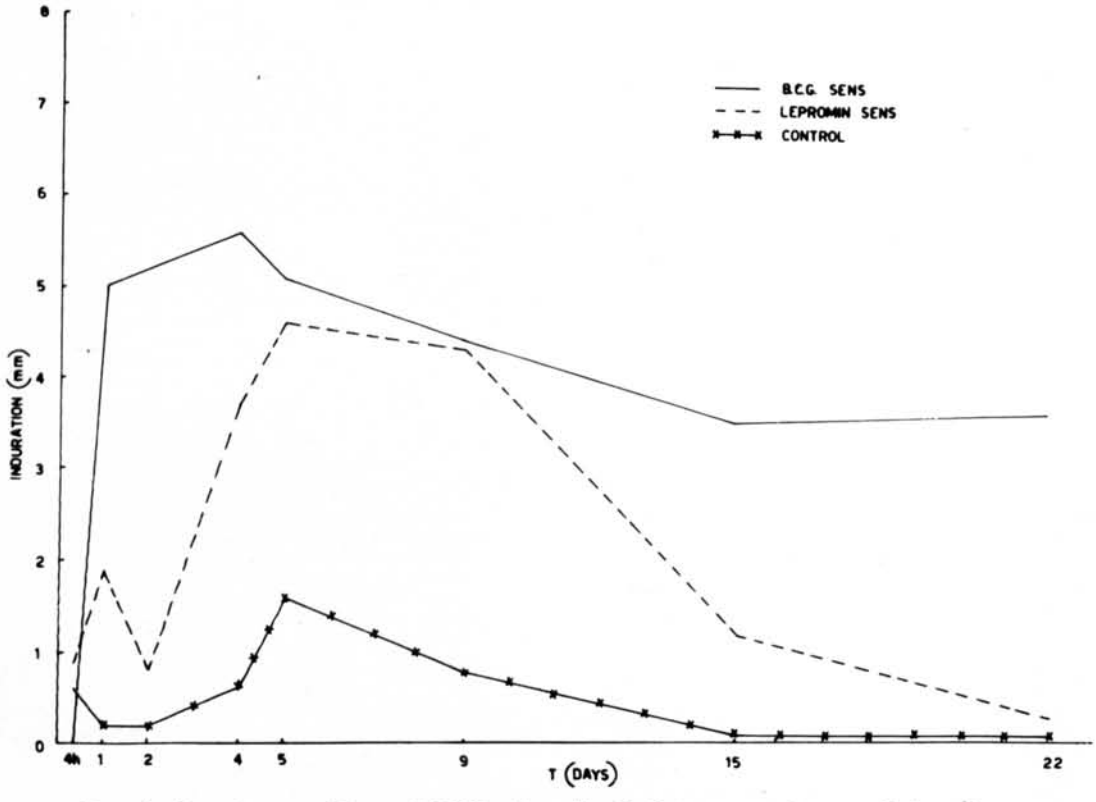


FIG. 6. Reactions to 10 $\mu$g of BCG-cell walls. Definitive experiments. Values shown for the different groups are the means of diameters of induration (in mm). Note significant difference between lepromin-sensitized and negative control groups.

We suggest that *M. tuberculosis* and *M. leprae* are related, but distantly. Their cell walls cross react, but not their protoplasms or tuberculins. There may exist other mycobacteria more closely related to *M. leprae* whose protoplasms and tuberculins would cross react with the latter. The influence which infection with such organisms may have on naturally occurring immunity to *M. leprae* and on the additive effect on BCG vaccination is worth thinking about, and has been commented upon ([1, 2, 3]).

## SUMMARY

Guinea pigs were sensitized with crude lepromin. Animals were tested with BCG cell wall and protoplasmic fractions. There was cross reactivity between *M. leprae* and BCG cell walls. No relationship was found between BCG protoplasm and *M. leprae*. These results confirm and clarify previous observations where an enzyme digested suspension of *M. leprae* was used as sensitizer.

Reported findings help in explaining the lack of correlation between Mitsuda and *M. tuberculosis* tuberculin reactions. They also pose interesting questions concerning possible relationships between mycobacterial ecology, immunity to leprosy and effectiveness of BCG vaccination.

## RESUMEN

Usamos esta vez a la lepromina integral como agente sensibilizante en el cobayo. Confirmamos resultados anteriores con preparaciones sometidas a la acción enzimática, los cuales mostraban relaciones antigénicas entre el *M. leprae* y la pared celular del BCG. No encontramos reactividad cruzada entre *M. leprae* y la fracción protoplasmática del BCG.

Los resultados que presentamos ahora y los anteriores, ayudan a explicar la ausencia de relación aparente entre las reacciones de Mitsuda y Mantoux. Así misno, plantean interesantes relaciones en tre la ecología micobacteriana, la inmunidad contra la lepra y la efectividad de la vacunación BCG en la lucha antileprosa.

## RÉSUMÉ

Enemployant la lépromine brute comme préparation sénsitizante. Nous avons confirmé qu'il-y-a une rélation antigenique entre les parois céllulaires du BCG et *Mycobacterium leprae*. En revanche, nous ne pouvons pas détecter aucune réactivité croisée entre le protoplasme de BCG et *M. leprae*.

Ces résultats, avec des autres déjà publies, aident en expliquer l'absence de rélation entre les réactions de Mitsuda et Mantoux. Ils posent aussi des questions interessantes concernant les rélations possibles entre écologie mycobacterienne, immunité contre lèpre et éfficacité de la vaccination par le BCG.

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