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Adoptively transferred reactivity to *M. leprae* in nude mice infected with *M. leprae*

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SUMMARY

Reversal reactions are manifestations of delayed hypersensitivity to *M. leprae* and are thought to be usually accompanied by manifestations of effective cell-mediated immunity (CMI) as measured by bacterial clearing. These experiments were designed to study the induction of reversal reactions in *M. leprae*-infected, congenitally athymic nude mice using adoptive transfer of CMI. Splenic cell suspensions derived from unimmunized heterozygous nu/+ mice, and those vaccinated with heat-killed *M. leprae*, viable BCG and a mixture of the two antigens were diluted to contain 10^4, 10^5, 10^6, 10^7 lymphocytes/0·1 ml and infused intravenously into multibacillary nude mice. The production of reversal reactions in leprous nude mice in response to adoptively transferred CMI was studied in a quantitative fashion. Dose responsive induction of reversal reactions, apparent by footpad inflammation and swelling, decreased morphological indices (MI) of the bacteria and mononuclear cell infiltrations, histopathologically, were observed. For nude mice receiving cells primed with $3{\cdot}9 \times 10^5$ living BCG alone, the effective dose 50% (ED_{50}) was $1{\cdot}0 \times 10^6$ lymphocytes to induce reversal reactions. For those receiving cells primed with 10^7 *M. leprae* the ED_{50} was $3{\cdot}7 \times 10^5$ lymphocytes. For nude mice receiving cells primed with a mixture consisting of $\frac{1}{2}$ the above dose of BCG + $\frac{1}{2}$ the above dose of *M. leprae*, the ED_{50} was $6{\cdot}8 \times 10^4$ lymphocytes.

Keywords leprosy nude mice vaccination

INTRODUCTION

BALB/c mice are susceptible to localized infections with *Mycobacterium leprae* when inoculated in the footpads (Shepard, 1960). They can be immunized against *M. leprae* infection with vaccines prepared from killed *M. leprae* (Shepard, 1976) and viable BCG (Shepard, Walker & Van Landingham, 1978a, b). In contrast to the localized footpad infection in immunologically intact BALB/c mice, athymic BALB/c nude mice, when injected in the footpad, develop disseminated leprosy and support the growth of large numbers of *M. leprae* at the site of injection in a lepromatous granuloma (Kohsaka, Mori & Ito, 1976; Chehl *et al.*, 1985).

A vaccine regimen against *M. leprae* in man proposed by Convit *et al.* (1974; 1979) has been of great interest to leprosy researchers. Convit and associates have advocated the use of heat-killed armadillo-derived *M. leprae* in combination with live BCG (*Mycobacterium bovis* strain of Calmette and Guerin) (Convit, Ulrich & Aranzazu, 1980).

This study assessed the degree of sensitization to *M. leprae* induced by *M. leprae* and BCG separately and in combination. In order to accomplish this, heterozygote (nu/+) mice were

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vaccinated with heat-killed armadillo-derived *M. leprae,* or live BCG, or a combination of heat-killed armadillo-derived *M. leprae* and live BCG. Single cell suspensions were prepared from the spleens of these vaccinated heterozygote donor mice, diluted to contain the desired numbers of lymphocytes, and injected intravenously into nude mice (nu/nu) harbouring approximately 2×10^8 *M. leprae* in the inoculated footpad. We postulated that the more potent the vaccine, the greater will be the clonal expansion *in vivo* of immunocompetent cells in the vaccinated donor mice, and the lower will be the dose of leucocytes to induce delayed-type hypersensitivity reactions in *M. leprae*-infected nu/nu recipient mice.

MATERIALS AND METHODS

Infection of recipient nude mice. Six-week-old, female, homozygous nude mice ((BALB/c AN BOM) nu/nu DF) from Harlan Sprague-Dawley, Madison, Wisconsin, were inoculated in the left hind footpad with 1×10^6 armadillo-derived *M. leprae* (morphological index (MI) = 8%) prepared from freshly necropsied armadillo spleen (Prabhakaran, Harris & Kirchheimer, 1976). The nude mice were maintained in a pathogen-free environment as described before (Chehl *et al.*, 1985).

Immunization of donor mice. Retired breeder heterozygous (nu/+) female mice (SCH (BALB/c/ANE/+dF)) from Harlan Sprague-Dawley, Madison, Wisconsin, were divided into four groups with five mice in each group. These mice received intradermal injections in the shaved flank in volumes of 0·1 ml. Group one received Medium 199 and served as an unimmunized control. Group two received 10^7 heat-killed armadillo-derived *M. leprae*. Group three received 15·6 μg dry weight BCG containing approximately $3{\cdot}9 \times 10^5$ viable units (ATCC 19015, Batch 11-82). Group four received a mixture of 7·8 μg dry weight BCG containing approximately $1{\cdot}95 \times 10^5$ viable units and 5×10^6 heat-killed armadillo-derived *M. leprae*. The dose ratio of heat-killed *M. leprae* and BCG was based on *M. leprae* dosage suggested by Shepard, Walker and van Landingham (1978a, b) and adjusted to BCG equivalent according to the *M. leprae*: BCG ratio used by Convit *et al.* (1979). The doses of *M. leprae* and BCG in the mixture given to Group four were arbitrarily chosen to be $\frac{1}{2}$ the doses of *M. leprae* and BCG given individually to groups two and three respectively. It was reasoned that a mixture containing full doses of each component might be expected to produce additive effects. A mixture containing $\frac{1}{2}$ doses of each component might be more likely to be suggestive of a potentiation effect of the mixture over the individual components separately.

Donor leucocyte suspensions. Three immunized donor mice from each group were sacrificed 4 weeks after immunization, and splenic leucocyte suspensions were prepared (Shannon *et al.*, 1981). The leucocyte suspensions were enumerated using a Coulter Counter (Coulter Electronics, Inc., Hialeah, Florida) and the number of viable lymphocytes per unit volume was calculated using the percentage of peroxidase negative mononuclear cells, and the percentage of mononuclear cells excluding 0·4% trypan blue.

Cell infusion. One hundred and seventy nude mice, approximately 5 months after being inoculated in the footpad with 1×10^6 *M. leprae* (MI = 8%), were divided into groups of 10. Control animals received 100 μl of Medium 199 intravenously. The experimental nude mice received 100 μl suspensions containing 1×10^4, 1×10^5, 1×10^6 or 1×10^7 viable, peroxidase negative, naive or sensitized, histocompatible leucocytes diluted in Medium 199.

Harvest. The recipient nude mice were observed daily for gross morphological changes and were killed 28 days after the cell transfer. The *M. leprae*-inoculated footpads were removed, cut into approximate halves and weighed. One-half of the footpad tissue was processed for enumeration of acid-fast bacteria (AFB) by standard acid-fast staining techniques (Shepard & McRae, 1968). The other half was prepared for histological studies as follows: the footpad tissue was fixed in 10% buffered formalin and processed for paraffin sections; 5 μm sections prepared from the paraffin blocks were stained with haematoxylin and eosin and a modified Fite-Faraco stain for AFB.

Statistical analysis. The 50% effective dose (ED_{50}), i.e. the number of lymphocytes required to induce reversal reactions in 50% of the recipient mice, with 95% confidence intervals, was calculated using the method of Litchfield and Wilcoxon (Tallarida & Murray, 1981). The Duncan test was used for comparison among the control and treated mice (Tallarida & Murray, 1981).

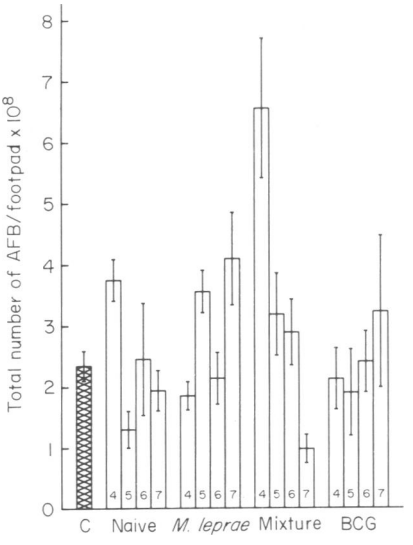

Fig. 1. Number of acid-fast bacteria in infected footpads of nu/nu mice 4 weeks after receiving 10^4 (4), 10^5 (5), 10^6 (6), 10^7 (7) splenic cells from nu/+ heterozygote donors injected with Medium 199 (naive), *M. leprae*, *M. leprae* + BCG, and BCG. Control mice (c) were infected with *M. leprae* and received Medium 199 only. Values are means ± s.e.m. of 8–10 animals in each group.

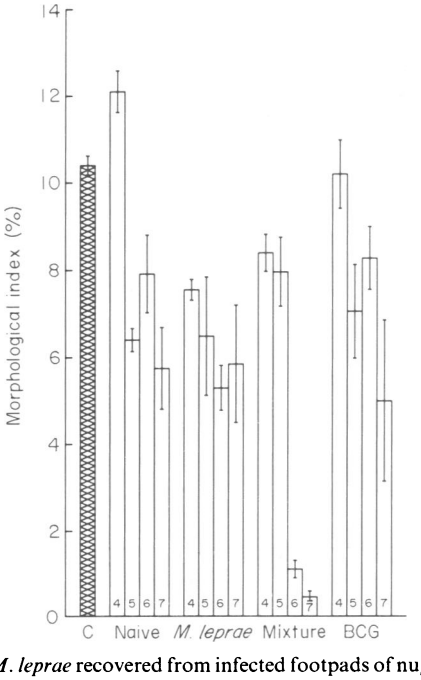

Fig. 2. Morphological index of *M. leprae* recovered from infected footpads of nu/nu mice 4 weeks after receiving 10^4 (4), 10^5 (5), 10^6 (6), 10^7 (7) splenic cells from nu/+ heterozygote donors injected with Medium 199 (naive) *M. leprae*, *M. leprae* + BCG, and BCG. Control mice (c) were infected with *M. leprae* and received Medium 199 only. Values are means ± s.e.m. of 8–10 animals in each group.

RESULTS

For 28 days following the leucocyte infusions, the recipient nude mice were observed daily for erythema and oedema of the infected footpads. These observations were scored on an arbitrary scale of '0' to '6+'. Cells from heterozygote mice immunized with the mixture of BCG plus *M. leprae* induced the most marked and persistent oedema and erythema in the recipient animals, followed by *M. leprae*-primed cells, followed by BCG-primed cells, followed by naive cells. Significant footpad enlargement, as measured by weighing the infected footpads 4 weeks after cell transfer, was seen among animals receiving 1×10^7 cells from donors immunized with all three vaccines.

Bacterial enumerations showed considerable variations. When compared with untreated controls, the number of AFB per footpad significantly increased in animals receiving 10^4 leucocytes from donors immunized with a mixture of BCG plus *M. leprae* (Fig. 1). Otherwise, no statistically

Table 1. Histopathologic evaluation of left hind footpads of nude mice harbouring *M. leprae* and having received nude/BALB/c heterozygote splenic leucocytes 4 weeks earlier

	Grade of reversal reaction*			
Group	0	1+	2+	3+
Control	(10)†	—	—	—
Naive cells				
10^4	(9)	—	—	—
10^5	(10)	—	—	—
10^6	(9)	—	—	—
10^7	(3)	(4)	(1)	(1)
M. leprae primed cells				
10^4	(9)	—	—	—
10^5	(8)	(2)	—	—
10^6	(2)	—	(6)	—
10^7	—	(1)	(6)	—
BCG primed cells				
10^4	(9)	—	—	—
10^5	(6)	(2)	(1)	—
10^6	(5)	(1)	(1)	—
10^7	(1)	(3)	(4)	—
M. leprae + BCG primed cells				
10^4	(10)	—	—	—
10^5	(3)	(3)	(4)	—
10^6	—	(3)	(3)	(2)
10^7	—	—	(5)	(5)

* The following criteria were used to assess the biopsy grades: 0, No change (macrophages + bacilli 'solids'); 1+, Scattered lymphocytes + marked granularity of bacilli + oedema; 2+, Scattered lymphocytes + marked granularity of bacilli + some reduction in bacillary density + some epithelioid cells + oedema; 3+, Scattered lymphocytes + marked granularity of bacilli + much reduction in bacillary density + many epithelioid cell collections + oedema.

† (N), number of animals graded.

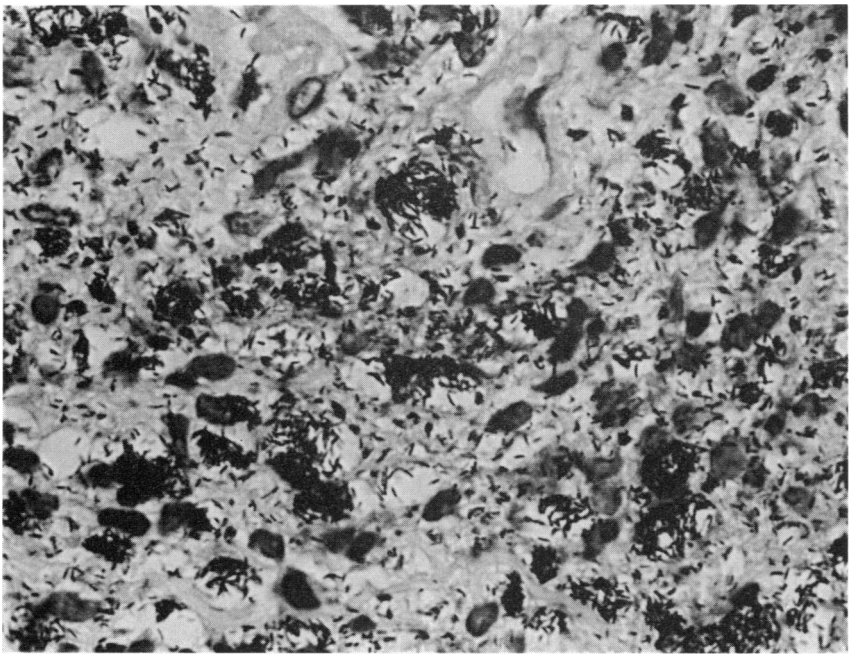

Fig. 3. Acid-fast staining of a lepromatous granuloma in a nude mouse receiving Medium 199. Numerous bacilli are visible, many of which were solidly stained, elongated and visible inside macrophages, striated muscle cells and nerve bundles. Grade 0. × 1100.

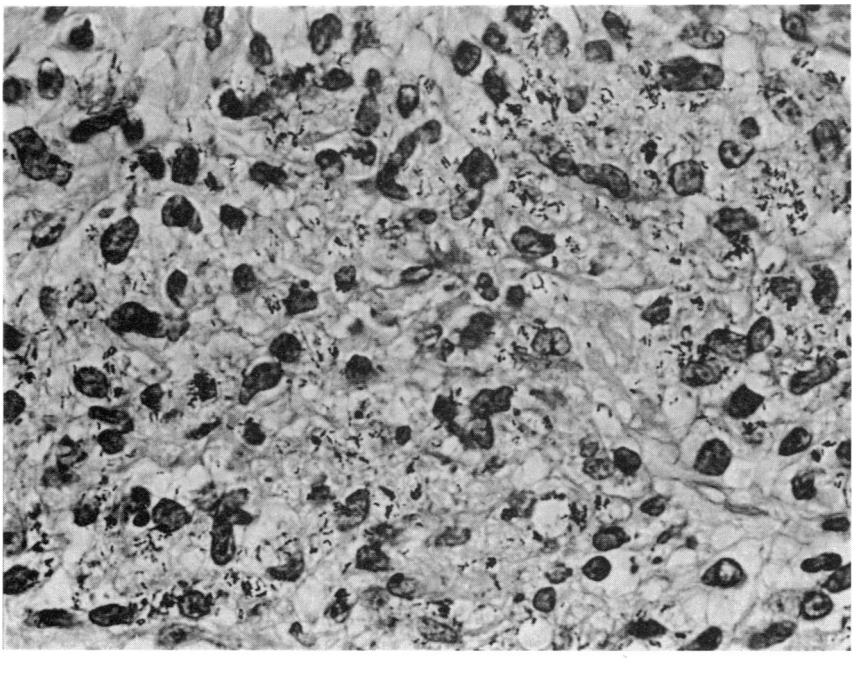

Fig. 4. Acid-fast staining of the granuloma of an adoptively vaccinated nude mouse. The bacilli are inside macrophages and are granular organisms. × 1100.

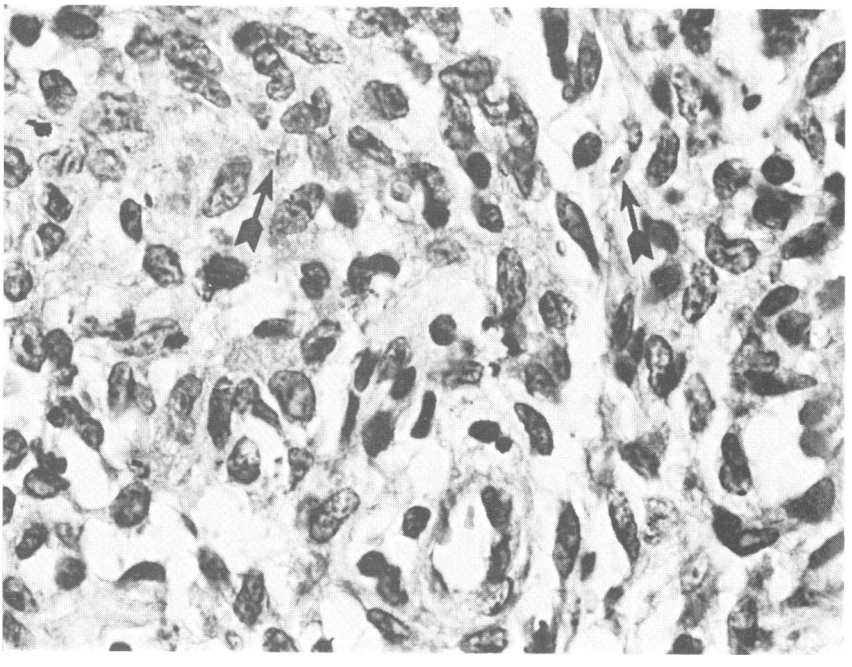

Fig. 5. Acid-fast staining of the granuloma shows only a few granular bacilli. × 1100.

significant differences were seen in the number of AFB in infected footpads of the control and experimental animals according to the Duncan test (Tallarida & Murray, 1981).

In general the MI of *M. leprae* decreased in nude mice receiving leucocytes from unimmunized donors or donors immunized with all three mycobacterial vaccines (Fig. 2). The most dramatic fall in MI occurred among the animals which received cells from donors immunized with the mixture of BCG and *M. leprae*. None of the recipient nude animals showed any weight loss or any other evidence of graft vs host reactions (data not shown).

Histopathological changes in the footpad tissues of the leucocyte-treated, *M. leprae*-infected nude mice were graded on an arbitrary scale of '0' to '3+' (Table 1). The histological specimens from the unimmunized control mice, which received Medium 199 only, showed granulomas composed mostly of macrophages; a few scattered lymphocytes and plasma cells were also present. Acid-fast staining of these specimens showed macrophages packed with bacilli which included many solid-staining organisms (Fig. 3). These specimens showed no indication of reversal reactions and were graded '0'.

Infusion of immunocompetent leucocytes into *M. leprae*-infected nude mice produced changes in the granulomas of (a) accumulations of lymphocytes, (b) loss of solid-staining bacilli, and (c) development of epithelioid cells. Biopsies showing macrophage granulomas with diffuse lymphocyte infiltrations and marked granularity of organisms were graded 1+ (Fig. 4). Those showing marked increase in lymphocyte infiltration, granular degeneration of bacilli and some epithelioid cells were graded as 2+. Specimens with marked lymphocytic infiltration, granularity of bacilli and numerous epithelioid cells were graded 3+ (Fig. 5).

The granuloma consisted almost entirely of macrophages among control mice. In treated mice the granulomas were gradually replaced by a mixture of epithelioid cells, macrophages and lymphocytes. The epithelioid cells did not usually contain any organisms, however when present, they were very few and were scattered (Fig. 5). In addition to these three major pathological changes, in some of the mice receiving 10^5 or more immunocompetent leucocytes, areas of focal necrosis in the granuloma with infiltrating neutrophils were also seen.

A summary of the effectiveness of the vaccine regimens as measured by the histopathologic changes is illustrated in Figure 6. Of the three vaccines tested, for nude mice receiving cells primed

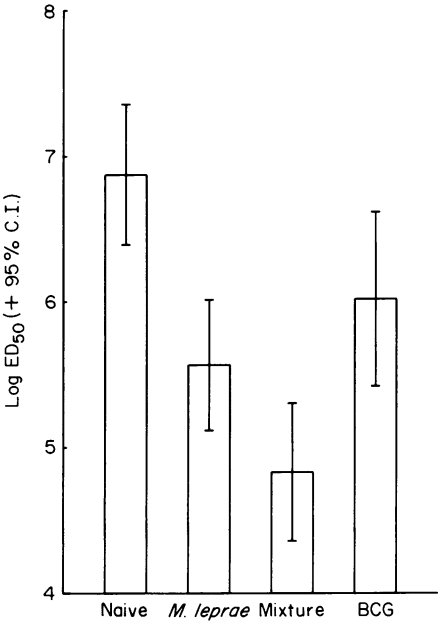

Fig. 6. The number of lymphocytes with 95% confidence interval required to induce histopathological changes of reversal reactions in 50% of the footpads of *M. leprae*-infected nude mice.

with a combination of live BCG and killed *M. leprae*, the ED_{50} was $6{\cdot}8 \times 10^4$ ($2{\cdot}3$–$20{\cdot}1 \times 10^4 = 95\%$ confidence interval) or over 110-fold less than naive cells whose ED_{50} was $7{\cdot}5 \times 10^6$ ($2{\cdot}5$–$22{\cdot}8 \times 10^6$). For those receiving cells primed with *M. leprae* alone, the ED_{50} was $3{\cdot}7 \times 10^5$ ($1{\cdot}3$–$10{\cdot}3 \times 10^5$) or over 20-fold less than the naive cells. For those receiving BCG primed cells, the ED_{50} was $1{\cdot}0 \times 10^6$ ($0{\cdot}26$–$4{\cdot}1 \times 10^6$) or only 71/2-fold less than that with naive cells.

DISCUSSION

In vaccine studies, experimental animals are customarily challenged after the introduction of a vaccine in order to assess the prophylactic value of the vaccination against the infecting agent. In order to carry out such a study in leprosy, and to estimate the relative potency of different vaccines, large numbers of immunologically-intact mice would be required. We reasoned that a more practical approach would consist of challenging *M. leprae*-infected nude mice with various dilutions of mycobacterial-primed or naive histocompatible splenic leucocytes. The ability of adoptively-transferred splenic leucocytes from naive and *M. leprae*-immunized heterozygote BALB/c (nu/+) mice to induce reversal reactions in recipient nude mice has been described previously (Chehl *et al.*, 1983). Those data showed that *M. leprae*-sensitized histocompatible leucocytes were some 140 times more effective than equivalent numbers of naive leucocytes in inducing reactivity to *M. leprae* in recipient nude mice.

An agreement on a vaccine strategy against leprosy is difficult until there is a better understanding of the immunological complexities associated with the leprosy spectrum. However, field trials for vaccination against leprosy are not awaiting a complete understanding of the pathogenesis of the disease. BCG has been used as a vaccine against leprosy (Kinnear-Brown, Stone & Sutherland, 1968; Russel, Scott & Wigley, 1968; Bechelli *et al.*, 1968).

Using adoptive transfer experiments, others have shown that *M. leprae*-sensitized murine lymphocytes increase resistance to *M. leprae* (Lowe, Brett & Rees, 1985) and inhibit the growth of *M. tuberculosis* and BCG (Patel & Lefford, 1978). In the present model, nude mice harbouring *M.*

leprae developed reversal reactions when infused with donor leucocytes primed with *M. leprae* alone or BCG alone. In this study, BCG alone induced clonal expansions of cells with immunoreactivity to *M. leprae*. These observations parallel those of Kaufmann (1984), who developed a murine T cell clone to *M. leprae* which exhibited reactivity to BCG. The clone responded to BCG *in vitro*, indicating cross-reactivity between BCG and *M. leprae* at the clonal level.

Leucocytes from donor mice primed with heat-killed *M. leprae* plus viable BCG were considerably more effective in inducing a response to *M. leprae* than were cells from animals primed with killed *M. leprae* alone, or with live BCG alone. The potential value of BCG plus killed *M. leprae* as a vaccine against leprosy was suggested by Hanks and Fernandez as early as 1956 (Hanks & Fernandez, 1956) and is the basis for field trials initiated in 1984 in Venezuela by Convit and his associates (Tropical Disease Research, WHO, 1985). Whether the apparent synergistic effect of BCG plus killed *M. leprae* is due to the non-specific effects of BCG or to T cell mediated responses to antigens which are cross-reactive between BCG and *M. leprae* cannot be determined from the present data. The data demonstrate that either BCG or *M. leprae* or a combination of the two are capable of inducing immunoreactive splenic lymphocytes which recognize *M. leprae* and induce reversal reactions when adoptively transferred into *M. leprae*-infected nude mice. In the absence of full dose-response relationships, synergism between BCG and *M. leprae* cannot be proven. However, the data are suggestive that such synergism may exist since a mixture of half-doses of BCG and half-doses of *M. leprae* is considerably more potent than either full doses of BCG alone or full doses of *M. leprae* alone.

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