Clin. exp. Immunol. 36, 214–220.

Immunological changes observed in indeterminate and lepromatous leprosy patients and Mitsuda-negative contacts after the inoculation of a mixture of *Mycobacterium leprae* and BCG

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(*Accepted for publication* 12 *October* 1978)

SUMMARY

This investigation was carried out to study the possibility of eliciting favourable immunological changes in small groups of Mitsuda-negative patients with indeterminate leprosy, lepromatous patients who were bacteriologically negative after prolonged treatment with sulphones, and in Mitsuda-negative contacts by means of stimulation with a mixture of autoclaved tissues from *Mycobacterium leprae*-infected armadillos and living BCG.

A radical change was observed in the specific immunological activity of the indeterminate group, all of whom initially had occasional bacilli in cutaneous nerves in biopsies taken from hypopigmented spots, and in the persistently Mitsuda-negative contacts. The 48 hr and 30 day reactions to lepromin, the 48 hr reaction to supernatant antigen from lepromin, the test for bacillary clearence and *in vitro* lymphocyte transformation (LTT) to *M. leprae* from human and armadillo lesions all became positive.

Of the lepromatous patients studied, only one became positive to all the criteria mentioned above. In the others, the 48 hr reaction to supernatant antigen, the LTT to antigen from a human source, and the clearance test remained negative, while the Fernandez and Mitsuda reactions became positive.

These results are discussed in terms of the possible use of this stimulation procedure in the prevention and immunotherapy of leprosy.

INTRODUCTION

There is considerable evidence that partial or complete resistance to infection by the intracellular microorganism *Mycobacterium leprae* requires an effective cell-mediated immune response. The concepts which have arisen with regard to an immunological defect in leprosy, particularly evident in the lepromatous and borderline lepromatous forms of the disease, arise from two different interpretations concerning the nature of this defect. When research on the immunology of leprosy was initiated, the idea prevailed that there was a generalized, non-specific depression of cell-mediated immune reactions (Turk & Waters, 1968; Sorres & Israel, 1954). Thereafter, evidence began to accumulate which indicated that the basic immunological defect in lepromatous leprosy was specific for *M. leprae*; a certain degree of generalized cell-mediated immunological depression observed in some patients was probably a secondary effect of the disease itself, since this situation largely disappeared when the patient improved with treatment (Sheagren *et al.*, 1967; Convit, Pinardi & Arias Rojas, de Salas & Convit, 1971; Ulrich, 1972). It is important to keep these two concepts in mind when attempting to correct the immunological defect.

In this study, we report the immunological changes induced in individuals previously non-reactive

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0099-9104/79/0050-0214$02.00

to *M. leprae* by various criteria. Several earlier investigations provided data which constitute the bases for this study. Firstly, the injection of relatively large numbers of heat-killed *M. leprae* (competency in clearing bacilli or CCB test) produces characteristic reactions in lepromatous patients which clearly demonstrate the specific inability of the lepromatous macrophage to remove *M. leprae* (Convit *et al.*, 1972). Secondly, the local activation of lepromatous macrophages by injecting the patient intradermally with a mixture of *M. leprae* and another mycobacterium, such as BCG, stimulates the macrophage into removing the inoculated *M. leprae* (Convit *et al.*, 1974).

In an experimental model using murine leprosy, it has been observed that a mixture of attenuated tubercle bacilli plus specific *M. lepraemurium* antigen produced a greater cell-mediated immunological response than either antigen alone (Hanks & Fernandez, 1956).

The investigation reported here evaluates the effect produced on the immunological defect seen in certain forms of leprosy by the intradermal inoculation of autoclaved *M. leprae* obtained from the tissues of experimentally infected armadillos, together with viable BCG.

MATERIAL AND METHODS

The study was carried out in twelve patients with leprosy and two Mitsuda-negative contacts. The group of patients with leprosy was composed of six patients with the indeterminate form and six with lepromatous leprosy.

The six lepromatous leprosy patients were cases of more than 20 years of evolution, all of whom had suffered one or more episodes of reactional phenomena (erythema nodosum leprosum) and who presented typical Mitsuda-negative lepromatous leprosy by histopathological and immunological criteria. When the study was initiated, these patients were completely free of clinical lesions and had been bacteriologically negative for nearly 20 years, after regular sulphone treatment for about 10 years. Their general state of health was good.

The six patients classified as having indeterminate leprosy presented the following characteristics: clinically, they had one or two hypopigmented spots with sensory loss, over a period of from 2 to 5 years. They were Fernandez- and Mitsuda-negative and had been negative throughout the course of their disease; this response had not been modified by repeated vaccination with BCG. Histopathologically, their lesions showed discrete perivascular infiltration by lymphoid cells and isolated bacilli within dermal nerves. *In vitro* tests of lymphocyte transformation in the presence of *M. leprae* were negative. All of these patients had received sulphone treatment for 3–5 years, with no modification of their clinical, bacteriological or immunological characteristics. During the trial chemotherapy was discontinued.

The Mitsuda-negative contacts, aged 12 and 18 years, were contacts of patients with lepromatous leprosy. They remained Mitsuda-negative in spite of three and four vaccinations, respectively, with BCG.

At the beginning of the investigation, both the patients and the contacts were evaluated according to the following criteria: (a) complete and detailed dermatological and neurological examination. (b) Skin test with a supernatant antigen (Convit *et al.*, 1975) obtained by centrifuging integral human lepromin of a concentration of $1{\cdot}6\times10^8$ acid-fast bacilli (AFB) per ml for 2 hr at 192,000 *g*, then filtering the supernate with a Millipore filter, pore size 0·45 μm. The test was read at 48 hr. (c) Skin tests with human integral lepromin at two concentrations, 4×10^7 and $1{\cdot}6\times10^8$ AFB/ml, with readings at 48 hr (Fernandez reaction) and 30 days (Mitsuda reaction). (d) *In vitro* lymphocyte transformation tests (LTT) with a suspension containing *M. leprae* from experimental leprosy lesions produced in armadillos and from human lepromas, standardized at a concentration of $1{\cdot}5\times10^8$ AFB/ml and with BCG (Statens Seruminstitut, Copenhagen) at a concentration of 1·5 mg/ml. Lymphocyte transformation was measured at 7 days by the incorporation of ^{3}H-thymidine, and results are expressed as the stimulation index (ct/min test per ct/min control).

The indeterminate patients and the Mitsuda-negative contacts were also given a CCB test with human *M. leprae* at a concentration of $6{\cdot}4\times10^8$ AFB/ml. The local reaction was biopsied at 30 days and studied histopathologically from sections stained with hematoxylin-eosin and by the Fite–Faraco method.

Everyone included in this study gave negative reactions to the supernatant antigen and to both concentrations of lepromin, in both the Fernandez and Mitsuda reactions. Their LTTs were also negative to both *M. leprae* suspensions but were usually positive to BCG. All of these subjects gave a negative CCB test, i.e. there was no clearance of *M. leprae* 1 month after intradermal injection.

The procedure used to stimulate the immunological apparatus of these patients was as follows: each was given six or eight 0·1 ml intradermal injections, in various sites, of a mixture of armadillo *M. leprae* at a concentration of $6{\cdot}4\times10^8$ AFB/ml plus lyophilized BCG at a final concentration of 1·0 mg/ml. This concentration of BCG was used when the patient's reaction to 2 units of PPD was negative; in those individuals with a positive reaction, the amount of BCG injected was lowered to 0·2 or 0·5 mg/ml.

Both patients and contacts were seen every 2 or 3 days during the first 2 weeks after stimulation and the local and generalized responses produced by the injection of the mixture of *M. leprae* plus BCG were noted. A dermatological examination was carried out every 1 or 2 weeks to detect possible modifications of the existing lesions in the indeterminate cases, or appearance of new lesions in all the groups. They were tested with PPD (Statens Seruminstitut, Copenhagen) every 4–6

weeks after the first stimulation to determine the intensity of their sensitization to BCG. Since this test was usually positive, the concentration of BCG used later on was decreased according to the intensity of the PPD reaction. Repeated stimulations were tolerated well by all patients, although local reactions consisting of erythematous nodules with small areas of necrosis were seen; two lepromatous patients had brief periods of moderate fever.

The tests with supernatant antigen and the LTTs were repeated at variable intervals, according to the observations in each case. When the tests were negative, the stimulation was repeated every 2 or 3 months. According to the results of these tests, the stimulation procedure was carried out from one to three times in indeterminate patients, three to four times in lepromatous patients, and once in Mitsuda-negative contacts. If, during the course of the stimulations there was any modification of the lesions or appearance of new ones in the indeterminate and lepromatous patients, the lesions were biopsied for histopathological study.

Before closing the study, each person was evaluated according to the following scheme: (a) dermatological and neurological clinical examination; (b) test with supernatant antigen, read at 48 hr; (c) test with human lepromin at a concentration of 4×10^7 AFB/ml, to study the Fernandez and Mitsuda reactions. In the indeterminate cases and the Mitsuda-negative contacts a biopsy was taken of the 30-day Mitsuda reaction for histological and bacterial clearance studies; in the lepromatous cases biopsies of the Mitsuda reaction were taken at 30 and 60 days. (d) LTT using armadillo and human *M. leprae* antigens.

The study lasted 4 years and 4 months, at the end of which the first observation period was closed.

RESULTS

The results for each group are analysed separately.

Indeterminate leprosy patients

All these patients overcame their immunological defect after one to three stimulations. The test with supernatant antigen became positive, varying in size from 12 to 18 mm. A typical pattern of the cutaneous reaction to supernatant antigen is shown in Fig. 1, which compares this response in the

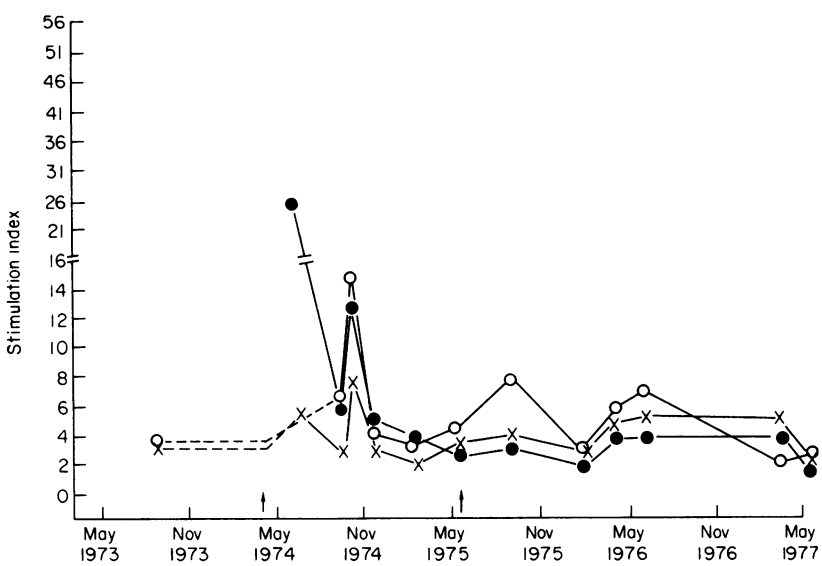

FIG. 1. Lymphocyte transformation tests in response to suspensions containing *M. leprae* of human origin (×———×), of armadillo origin (●———●), and BCG (○———○) in a representative patient with indeterminate leprosy during the course of stimulation with a mixture of *M. leprae* and BCG. Arrows indicate dates of stimulation.

indeterminate and lepromatous groups. The test with human lepromin was positive at 48 hr, between 12 and 16 mm, and at 30 days with formation of a nodule of 8–12 mm (see Table 1). Bacillary clearance was positive at 30 days, with complete elimination of the injected *M. leprae* and formation of a tuberculoid granuloma. The LTT became positive in one patient after the first stimulation; the others required two or three stimulations for this test to become positive.

After the first stimulation, dermatological examination of one patient revealed about five plaques of a

TABLE 1. Reactions to human lepromin in Mitsuda-negative lepromatous patients and Mitsuda-negative contacts before and after stimulation with a mixture of *M. leprae* and BCG

Group (number of patients)	Fernandez reaction (48 hr) Induration (mm)		Mitsuda reaction (30 days) Nodule (mm)	
	Initial	Final	Initial	Final
Indeterminate leprosy (6)	0	15·2 (12–16)*	0	9·6 (8–12)
Lepromatous leprosy (6)	0	14·6 (12–18)	0	7·1 (7–10)
Mitsuda-negative contacts (2)	0	13·0 (12–14)	0	8·0 (7–10)

* Number in parentheses indicate the range of reactions.

tuberculoid structure with abundant lymphoid cells and very few bacilli, located over the two hypopigmented spots or on distant normal skin; these lesions were classified as borderline tuberculoid leprosy. In another patient, an erythematous infiltrated lesion appeared in the border of the hypopigmented spot on the left cheek. A biopsy of this lesion showed a tuberculoid granuloma with no demonstrable bacilli. There was also thickening of a superficial nerve at the level of the hypopigmented spot on the left elbow. In two more patients, a very fine papulous eruption developed on the upper and lower limbs and back; biopsy of one of the papules showed a small focus of epithelioid cells with moderate infiltration by lymphoid cells but with no demonstrable bacilli. All the lesions disappeared after 4 or 5 months. In some cases, regional lymph nodes appeared enlarged after the second stimulation, but they had regressed completely within 2 weeks.

Fig. 2 shows the pattern of responses in the LTT of stimulated indeterminate patients to *M. leprae* of human and armadillo origin and BCG during the course of the trial. In this case the tests became positive after one stimulation, with variations in intensity during the trial.

Lepromatous leprosy patients

Of the six lepromatous cases, only one (SR) presented dermatological lesions more than 2 years after

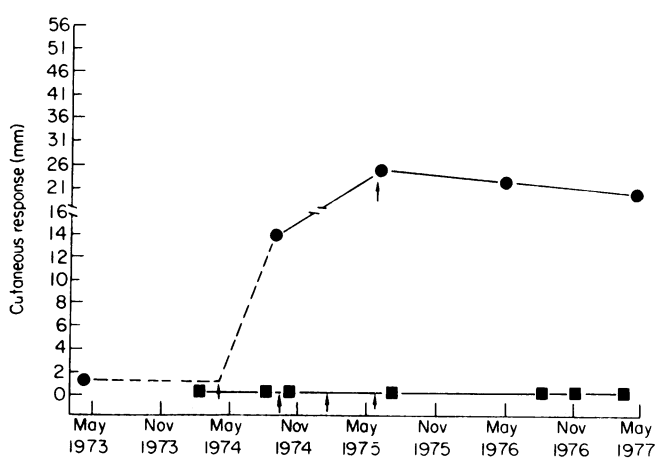

FIG. 2. Representative cutaneous responses to supernatant antigen at 48 hr in indeterminate leprosy (●——●) and lepromatous leprosy (■——■) after stimulation with a mixture of *M. leprae* and BCG. The portion of the curve represented by dashes was not determined experimentally.

the start of the trial. These lesions were characterized by erythematous infiltrated plaques on the abdomen, whose structure corresponded to a borderline type lesion (centre of the leprosy spectrum) with few bacilli, which regressed completely in a period of 2–3 months.

From an immunological viewpoint, all the patients showed a positive response to human lepromin (4×10^7 AFB/ml), with erythema and induration between 12 and 18 mm at 48 hr and a nodule between 7 and 10 mm at 30 days (Table 1). The test with supernatant antigen was negative in five (see Fig. 1); the exception was the case mentioned previously (SR) who gave a positive reaction of 12 mm.

The bacillary clearance read at 30 days was negative in five patients; again the exception was SR who gave a positive clearance of *M. leprae*. The histological appearance of the clearance test in all six patients was of a tuberculoid granuloma, with abundant giant cells and infiltration by lymphoid cells. Clearance was observed in these patients at 60 days.

The LTT was negative to *M. leprae* of human origin, but positive to armadillo-derived *M. leprae* (Fig. 3). However, patient SR gave positive responses to both bacillary antigens.

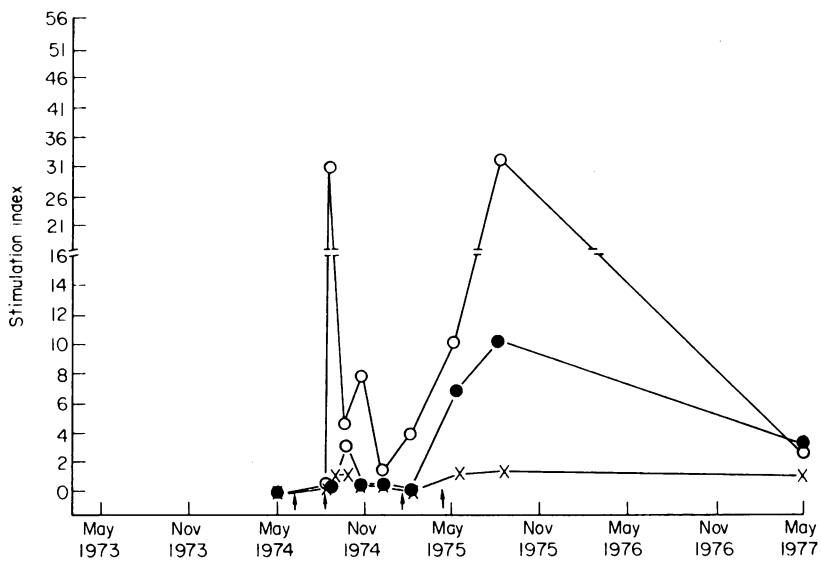

FIG. 3. Lymphocyte transformation tests in response to suspensions containing *M. leprae* of human origin (×——×), of armadillo origin (●——●), and BCG (○——○) in a representative case of lepromatous leprosy during the course of stimulation with a mixture of *M. leprae* and BCG.

Mitsuda-negative contacts

These two persons developed positive responses to the supernatant antigen after one stimulation, with an infiltrated reaction of 12–14 mm. The lepromin tests became positive, with a response of 12–14 mm at 48 hr and a nodule of 7–10 mm at 30 days (Table 1). The bacillary clearance was positive at 30 days. The LTT was also positive for *M. leprae* of human and armadillo origin.

At the completion of this study, none of the patients showed dermatological lesions and their bacteriological examinations were negative. Sulphone treatment was re-initiated.

DISCUSSION

The results of this investigation show that radical immunological changes occurred in all the indeterminate leprosy patients and Mitsuda-negative contacts studied. If we consider the widely accepted evidence of the importance of cell-mediated immune reactions in resistance to *M. leprae*, the conversion of the parameters studied in these two groups of previously non-reactive individuals is of great interest.

The characteristics already described of the group of patients with indeterminate leprosy, including

the persistence of a negative Mitsuda reaction and the lack of modification of their clinical and histopathological picture, in spite of regular treatment with dapsone (DDS) during more than 3 years and repeated vaccinations with BCG, clearly suggests that these cases had a great potential for eventual transformation towards lepromatous leprosy. The radical change in the immunological reactivity of these cases after stimulation implies an important change in the prognosis of their disease.

Contacts of patients with lepromatous leprosy who do not respond to repeated injections of lepromin usually do not convert to lepromin-positive after repeated BCG vaccination. This may be due to subclinical infection or may reflect an inherent defect in the capacity to respond to specific antigens of *M. leprae*. Only two persons with these characteristics were studied; nevertheless, the positive conversion of all the parameters of cell-mediated immunity studied suggests that this condition can be modified by the stimulation procedure described. Since positive cutaneous reactions to lepromin are clearly related to resistance, this procedure would seem to have potential as a preventive vaccine.

Anti-leprosy campaigns in the future will undoubtedly be concentrated precisely in these two groups and be directed towards the prevention of the disease in the susceptible population and the identification and effective treatment of early cases. The possible use of preventive and immunotherapeutic stimulation therefore appears to be of singular importance.

The interpretation of the response of the lepromatous group to the stimulation is more complex. Some of the parameters of cell-mediated immunity became positive, while others were not modified. It would seem to be useful to analyse each of these parameters separately.

We consider that the failure to respond to the supernatant antigen, except in one, case, reveals a lack of development of specific delayed hypersensitivity towards *M. leprae*.

The discordance between the positivity of the Fernandez response, which is obtained by injecting integral lepromin with whole bacilli, and the supernatant antigen, suggests significant antigenic differences between these two preparations. Stimulated lepromatous patients show strong hypersensitivity to BCG. Studies (Ribi *et al.*, 1958; Goihman-Yahr, Raffel & Ferraresi, 1969) have shown cross-reactivity between the bacterial cell-wall antigens of several mycobacteria, while cytoplasmic preparations show a high degree of specificity. Therefore, we suggest that the positive Fernandez and Mitsuda reactions reflect a cross-reaction with BCG in the lepromatous stimulated group; the supernate, which may contain predominantly specific cytoplasmic antigens, remains negative.

The clearance tests, which present the characteristics of a tuberculoid granuloma of the 'immune' type, but with clearance of the bacilli only at 60 days, could have several explanations. Firstly, we want to emphasize previous observations that clearance in lepromatous patients shows various degrees of competence; some show elimination at 90 days, others at 120 days, and in still others bacilli are still present at 120 days. Clearance at 60 days in lepromatous patients has only been observed in this stimulated group. This accelerated clearance, together with the tuberculoid structure of the lesions at 1 month, suggest a real but inefficient immune reaction. There are two possibilities: the immune reaction reflected by the histology may be due to cross-reactivity with BCG, and efficient macrophage activation may require, additionally, elements specific for *M. leprae*; another possibility, which has been studied in numerous investigations but never proven, is that macrophages in lepromatous patients are defective in their production of the enzymes necessary to digest *M. leprae*, even under adequate conditions of lymphocyte stimulation, they do not respond optimally.

The gamut of clearance responses of the lepromatous macrophage could explain the different potential of responses to treatment, thus explaining why some patients become bacteriologically negative after 5 years of treatment, while others remain positive in spite of regular treatment for more than 8 or 10 years.

This spectrum of competence of the lepromatous macrophage would be the active element in the digestion of *M. leprae* killed by sulphone treatment, without the participation of a lymphocytic element. The present investigation suggests the possibility of stimulating this lymphocytic element, at least to a certain degree. Prolonged observation will be necessary to evaluate the persistence of the lymphocytic activity and its possible beneficial action.

The lepromatous potential of an individual may be based on as yet undefined genetic factors or on the development of a state of tolerance. It seems possible that the incompetence of the lepromatous macro-

phage could be an important factor in the determination of this potential, due to a limited capacity to present the antigen to the lymphocyte in adequate amounts, from a qualitative and quantitative viewpoint, for lymphocyte sensitization.

Another interesting observation in the lepromatous group concerns the positive LTT to the antigen-containing *M. leprae* from the armadillo, but not to the *M. leprae*-containing antigen from human material. It seems probable that the reaction to the first antigen may be due to sensitization to components of armadillo tissue which, even though denatured by autoclaving, produced a reaction due to the potent adjuvant action of BCG. This sensitization disappeared some time after the last stimulation.

The negativity of the LTT towards human-derived antigen, which contains bacillary bodies, contrasts with the positivity of the Fernandez reaction *in vitro*. This suggests that the LTT is less sensitive than the intradermal reaction in detecting low levels of sensitivity. This is also suggested by the persistence of strong cutaneous reactivity at the end of the trial, when LTTs were generally weak.

The immunological changes observed in the groups studied clearly demonstrate the importance of using a combination of autoclaved armadillo *M. leprae* plus BCG and its potential as a preventive and therapeutic vaccine. We should, however, point out that this type of vaccine would be limited to highly susceptible individuals or those already infected, both requiring careful selection. The dose of both components, *M. leprae* and BCG, has still to be determined in order to obtain the maximal response with minimal side effects.

This work was supported by grants from the World Health Organization, the Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICIT), Caracas, Venezuela and the Consejo de Desarrollo Científico y Humanístico, Universidad Central de Venezuela.

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