Lymphocyte Transformation Test in Leprosy: Decreased Lymphocyte Reactivity to *Mycobacterium leprae* in Lepromatous Leprosy, with No Evidence for a Generalized Impairment

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Received for publication 14 September 1978

Untreated leprosy patients were examined with respect to lymphocyte transformation in vitro after stimulation with mycobacterial and other microbial antigens, allogeneic lymphocytes, or nonspecific mitogens. Methods were used to circumvent technical variability. The results were compared with those obtained in controls matched for age, sex, race, and environment. No evidence was found for a generalized impairment of lymphocyte transformation in vitro, whereas a specific defect towards *Mycobacterium leprae* was demonstrable in lepromatous leprosy patients. The response to *M. leprae*, investigated in untreated and treated leprosy patients, decreased along the leprosy spectrum. Moreover, the results of the one-way mixed lymphocyte cultures showed that lymphocytes from leprosy patients had a normal stimulator and responder capacity, when they were tested against a panel of allogeneic lymphocytes. The influence of serum factors was investigated in untreated leprosy patients in the mixed lymphocyte culture. On average, tuberculoid as well as lepromatous sera showed a low-level depressive effect, but some sera showed a stimulatory effect. Therefore, a depressive effect of serum factors cannot be considered to be a general feature of leprosy. The correlation between the Mitsuda type of lepromin skin test and the lymphocyte reactivity in vitro to *M. leprae* was studied, and a positive correlation was found.

Although it is generally assumed that the type of leprosy from which a patient suffers is determined by the degree of cell-mediated immunity toward *Mycobacterium leprae*, it is still controversial to what extent, if at all, cell-mediated immunity in general is impaired in leprosy patients, notably in lepromatous leprosy patients. Evidence for such an impairment has been obtained in several studies in which different parameters for cellular immunity were used: delayed type hypersensitivity skin reaction (6, 7); induction of contact sensitization (7, 28, 40, 46, 49); skin allograft survival (21, 23); normal lymphocyte transfer tests (39, 40); and lymphocyte transformation test (LTT) with nonspecific mitogens and antigens (9, 14, 21, 24, 26, 27, 30, 42, 45) and the mixed lymphocyte culture (MLC) (20). However, the results obtained have not been consistent; in some studies no evidence for a generalized impairment has been found, and in several of the above-mentioned studies an impairment of the response to a particular antigen or mitogen was accompanied by a normal reactivity to other stimuli (7, 14, 15, 24, 28, 33, 34, 42, 47).

In the present study we have tested the in vitro reactivity of lymphocytes from leprosy patients as a parameter probably related to cellular immune reactivity. The response to a variety of mitogens and specific antigens (soluble and corpuscular bacterial antigens as well as allogeneic lymphocytes in the MLC) could be tested in one experiment.

Because a depressive effect of serum from leprosy patients has been described by some investigators, for instance on the reactivity in the MLC (20, 31), we have tested the influence of the serum of patients in the MLC.

Some studies indicate that a generalized impairment of lymphocyte reactivity in vitro tends to normalize upon treatment (22, 27, 30, 31); therefore, only patients who had not yet received treatment were accepted for these studies, with the exception of the investigation of the response to *M. leprae* across the leprosy spectrum, which was studied in untreated and treated patients, as
the response to *M. leprae* is reported not to be influenced by treatment (2, 16, 24).

The Mitsuda type of lepromin reaction is considered to be a measure of the resistance of the host to infection with *M. leprae* (35). The correlation between the lepromin reaction and the lymphocyte response in vitro to *M. leprae* has been studied by Myrvang et al. (29) in leprosy patients. However, in a number of leprosy patients, so-called “reversal reactions” may occur (36). During this type of reaction lymphocytes from these patients show increased reactivity in vitro to *M. leprae* (1, 17). This type of reaction is supposed to reflect a delayed-type hypersensitivity phenomenon (2). We studied the correlation between the lepromin skin test and the lymphocyte response in vitro to *M. leprae* in a few patients at the extremities of the leprosy spectrum (in whom reversal reactions do not occur), as well as in leprosy contacts.

**MATERIALS AND METHODS**

**Patients and controls.** All patients were classified by the criteria of Ridley and Jopling (37) with minor modifications by Leiker (25). Patients in or with a recent reactional phase of the disease were excluded from the study. All patients, except one untreated lepromatous patient, were living in The Netherlands for more than 1 year.

In the first set of experiments the tuberculoid patients consisted of seven untreated tuberculoid patients; the lepromatous patients consisted of four untreated borderline lepromatous and three untreated lepromatous patients. Age-, sex-, and race-matched controls, who all had been living in The Netherlands for more than 1 year, were selected.

The response to *M. leprae* across the leprosy spectrum was studied in 34 untreated and treated patients: 9 tuberculoid, 5 borderline tuberculoid, 13 borderline lepromatous, and 7 lepromatous.

The influence of serum factors in the MLC was examined in five untreated tuberculoid, one untreated borderline lepromatous, and four untreated lepromatous patients.

In the studies comparing the lepromin skin test reactivity with the lymphocyte transformation in vitro to *M. leprae*, the LTT was always performed either before or more than 6 months after the skin test.

**Lymphocyte isolation and preservation.** Lymphocytes were isolated from defibrinated blood by Ficoll-Isopaque density gradient centrifugation by the method of Böyum (5) and stored in liquid nitrogen (12).

**Lymphocyte cultures.** Lymphocyte cultures were performed as described by Du Bois et al. (13). Briefly, the procedure was as follows. After washing and cell counting, 4 × 10⁴ lymphocytes were cultured in Cooke round-bottom microtitre plates (220 M-24 AR) in 150 μl of medium RPMI 1640 which was buffered with bicarbonate and supplemented with 20% heat-inactivated human serum (pooled from 10 random donors, selected for low background activity), penicillin (100 IU/ml), and streptomycin (100 μg/ml). Incubation was performed at 37°C in a humidified atmosphere of 5% CO₂ in air.

The following stimulants were used: phytohemagglutinin (Wellcome Research Laboratories), 50 μg/ml; concanavalin A (Sigma Chemical Co.), 60 μg/ml; PWM (Grand Island Biological Co.), 50 μg/ml; purified protein derivative (RIV), 12 μg/ml; Candida albicans (Hollister Stier), 1:200 dilution; mumps (Eli Lilly & Co.), ±0.01 colony-forming units per ml; Trichophyton (HAL), 2.5 mg/ml; Variadase (streptokinase-strep- todornase; American Cyanamid Co., Lederle Laboratories Div.), 12 U/ml; BCG (a gift from J. L. Siks, RIV), 125 × 10⁶, 31 × 10⁶, and 8 × 10⁵ organisms/ml; *M. leprae* (prepared from untreated lepromatous leprosy skin and provided by R. F. M. Lai A. Fat, Paramaribo, Surinam), 125 × 10⁶, 31 × 10⁶ and 8 × 10⁵ organisms/ml. Control cultures consisted of lymphocytes cultured with medium alone.

In the one-way MLC 4 × 10⁴ nonirradiated lymphocytes in 75 μl of culture medium were mixed with 4 × 10⁴ γ-irradiated (2,000 rad) allogeneic lymphocytes in 75 μl of culture medium. Stimulator activity was determined against five different individuals (A, B, C, cryopreserved lymphocytes; D and E, freshly isolated lymphocytes); responder activity was determined against pools of γ-irradiated lymphocytes. Three pools were used, composed of lymphocytes from 35, 35, and 3 individuals.

Phytohemagglutinin- and concanavalin A-stimulated cultures were terminated after 3 days; all the other cultures were terminated after 6 days. At 24 h before harvesting the cells, 0.4 μCi of [3H]thymidine was added (specific activity, 200 mCi/mmol). The cells were collected on glass fiber filters and the [3H]thymidine incorporation was determined by counting the radioactivity in a liquid scintillation counter. All cultures were performed in quadruplicate; the median value was calculated. When any stimulant was tested with more than one concentration, the maximal reactivity was taken. The median value of the unstimulated cultures was subtracted, and the results were recorded as counts per minute.

In the first set of experiments, the cultures were initiated on 3 consecutive days; lymphocytes from each patient were always cultured parallel to the lymphocytes from the matched control. The influence of serum factors in the MLC was tested in an experiment in which the cultures were initiated on the same day.

**Statistical analysis.** The results of the first set of experiments were statistically analyzed by Wilcoxon's rank sum test. The influence of serum factors in the MLC was statistically analyzed by variance analysis of the differences. The results of the correlation studies comparing the lepromin skin test reactivity and the lymphocyte reactivity in vitro to *M. leprae* were statistically analyzed by a rank correlation test.

**RESULTS**

Table 1 shows the results obtained in the LTT with nonspecific mitogens (phytohemagglutinin, concanavalin A, PWM), corpuscular (BCG, *M. leprae*) and soluble (purified protein derivative) mycobacterial antigens, other antigens (mumps,
TABLE 1. Lymphocyte transformation to mitogens in vitro and in the one-way MLC of untreated leprosy patients and matched controls

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Median cpm in the following patient groups:</th>
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<tbody>
<tr>
<td></td>
<td>Tuber-</td>
</tr>
<tr>
<td></td>
<td>culoid</td>
</tr>
<tr>
<td>leprosy (n = 7)</td>
<td>(n = 7)</td>
</tr>
<tr>
<td>Day 3b</td>
<td>Unstimulated</td>
</tr>
<tr>
<td></td>
<td>Phytohemagglutinin</td>
</tr>
<tr>
<td></td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>Day 6b</td>
<td>Unstimulated</td>
</tr>
<tr>
<td></td>
<td>PWM</td>
</tr>
<tr>
<td></td>
<td>M. leprae</td>
</tr>
<tr>
<td></td>
<td>BCG</td>
</tr>
<tr>
<td></td>
<td>Purified protein derivative</td>
</tr>
<tr>
<td></td>
<td>Mumps</td>
</tr>
<tr>
<td></td>
<td>C. albicans</td>
</tr>
<tr>
<td></td>
<td>Trichophyton</td>
</tr>
<tr>
<td></td>
<td>Varidase</td>
</tr>
<tr>
<td></td>
<td>Patient (irradiated) + donor A</td>
</tr>
<tr>
<td></td>
<td>Patient (irradiated) + donor B</td>
</tr>
<tr>
<td></td>
<td>Patient (irradiated) + donor C</td>
</tr>
<tr>
<td></td>
<td>Patient (irradiated) + donor D</td>
</tr>
<tr>
<td></td>
<td>Patient (irradiated) + donor E</td>
</tr>
</tbody>
</table>

* Median values of the different groups are recorded; counts per minute of the stimulated cultures are recorded after subtraction of the counts per minute of the unstimulated control cultures.
* Lymphocytes harvested after 3 and 6 days.
* NT, Not tested.
* Stimulator capacity tested against lymphocytes from five different donors (A through E).
* Responder capacity was tested against a pool of lymphocytes from 35 individuals.

C. albicans, Trichophyton, Varidase), and it also shows the results of the one-way MLC of seven untreated tuberculoid and seven untreated lepromatous patients and their matched controls. The response to M. leprae was significantly lower in lepromatous patients compared with tuberculoid patients (P < 0.001). Lepromatous patients showed a significantly decreased response to BCG when compared with tuberculoid patients (P = 0.038), whereas no significant differences were found between the patients and their matched controls. With respect to the other antigens, no significant differences were found, except for a decreased response of lepromatous patients to mumps antigen when compared with their matched controls (P = 0.046); a similar difference was found between the lepromatous and tuberculoid patients (P = 0.012). This difference may possibly be due to a difference in sensitization to the various antigens. There were no significant differences with respect to the stimulation by nonspecific mitogens. Finally, stimulator and responder capacity in the one-way MLC showed no significant differences.

The response to M. leprae across the leprosy spectrum was investigated in 34 patients (Fig. 1). There was a significant difference between tuberculoid and borderline tuberculoid patients (P = 0.002), as well as between borderline tuberculoid and borderline lepromatous (P = 0.002) and lepromatous patients (P = 0.009). In borderline lepromatous and lepromatous patients marginal responses were observed, with the exception of one lepromatous patient. The latter patient also showed a strong LTT response to BCG and purified protein derivative, as well as a positive skin test to purified protein derivative indicating the possibility of cross-reactivity between the mycobacteria.

The results of the one-way MLC of lymphocytes from five untreated tuberculoid and five untreated lepromatous patients showed a possibly significantly lower response when they were cultured in autologous serum, as compared with cultures in pooled normal human serum (P = 0.04). In this respect there were no differences between tuberculoid and lepromatous sera; similar effects were found in MLC cultures when three different pools of irradiated stimulator cells were used. It appeared that some sera even had a stimulatory effect (Table 2). It should be emphasized, however, that no definite conclusions can be drawn, because cultures in autologous serum have, in general, a tendency to show decreased stimulation, as the pooled human serum batches are selected for optimal culture results.

The correlation between the lepromin skin test reactivity and the in vitro lymphocyte reactivity to M. leprae is shown in Fig. 2. The correlation found is 0.81, with a P value of <0.0005. For statistical analysis the results were grouped according to response level in the lymphocyte transformation test.
FIG. 1. Lymphocyte transformation in vitro to M. leprae in different types of leprosy patients. T, Tuberculoid patients (n = 9); BT, borderline tuberculoid patients (n = 5); BL, borderline lepromatous patients (n = 13); L, lepromatous patients (n = 7). Results are recorded in counts per minute after subtraction of the counts per minute of the unstimulated control cultures.

DISCUSSION

Several authors have found that in leprosy, especially of the lepromatous type, there is a generalized impairment of cellular immunity, when tested with delayed type hypersensitivity skin reactions (6, 7), induction of contact sensitization (7, 28, 40, 46, 49), skin allograft survival (21, 23) or normal lymphocyte transfer tests (39, 40). According to some reports, lymphocyte reactivity to nonspecific mitogens and antigens (9, 14, 22, 24, 26, 27, 30, 42, 45) and allogeneic lymphocytes (20) is also impaired. Our results, obtained in the present study, do not support the latter findings.

In the previous studies the response to one or a few stimulants was tested; therefore, in the present study the response to three mitogens, three mycobacterial antigens, and four other microbial antigens, as well as stimulator and responder capacity to a panel of allogeneic lymphocytes, was examined. Because variations in the different studies may reflect variations in the control groups (18), the patients were compared with controls matched for age, sex, race, and environment. To minimize technical variability, which inevitably occurs in a study performed over a prolonged period of time, we used cryopreserved lymphocytes. Moreover, the cultures were initiated on 3 consecutive days; as a result of these precautions, any variability in culture media and batches of stimuli was circumvented.

That there is a decreased response to M. lep-
Table 2. Influence of autologous serum compared with pooled human serum on the responder capacity of lymphocytes from untreated tuberculous and lepromatous leprosy patients in the one-way MLC

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Mean cpm in the following patient groups:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tuberculous leprosy</td>
</tr>
<tr>
<td>1</td>
<td>-4,432</td>
</tr>
<tr>
<td>2</td>
<td>-6,900</td>
</tr>
<tr>
<td>3</td>
<td>-1,663</td>
</tr>
<tr>
<td>4</td>
<td>+3,502</td>
</tr>
<tr>
<td>5</td>
<td>-7,191</td>
</tr>
<tr>
<td>Mean</td>
<td>-3,337</td>
</tr>
</tbody>
</table>

*Mean values of the differences of reactivity in autologous and pooled human serum against three pools of irradiated stimulator cells are recorded, expressed in counts per minute after subtraction of the counts per minute of the unstimulated control cultures. A minus sign indicates lower reactivity in autologous serum, and a plus sign indicates higher reactivity in autologous serum.

The possible occurrence of a suppressive effect on lymphocyte reactivity by serum factors from leprosy patients was investigated by testing the influence of such sera on the mixed lymphocyte reaction. No uniform effect was found, supporting the conclusions of Mehra et al. (27) that serum factors may play a role, but are not a general feature of leprosy.

The correlation between the Mitsuda type of lepromin reaction, considered to be a measure of the resistance of the host to *M. leprae* (35), and the in vitro lymphocyte reactivity to *M. leprae* has been studied in leprosy patients by Myrvang et al. (29). They found a correlation of 0.52. The higher correlation (0.81) found by us is most likely the result of the selection of the subjects and the sensitivity of the test. As the lymphocyte reactivity in vitro to *M. leprae* in leprosy patients is also correlated with the occurrence of reversal reactions (1, 17), a situation comparable to a delayed-type hypersensitivity reaction (3), it is important that leprosy patients investigated by cellular immunological in vitro techniques be exactly defined.

In the animal model the development of cell-mediated immune reactions after infection with mycobacteria is determined by genetic factors of the host (11, 48) and by the amount and route of administration of the antigen (3, 10, 38). Moreover, viable mycobacteria show more profound effects than do killed mycobacteria (3, 10). On the other hand, recent reports (4, 43, 44) seem to suggest that heat-killed *M. leprae* are as immunogenic as viable *M. leprae*. Bullock (8) found a disturbance of the circulation of thoracic duct lymphocytes in rats with an active infection with *Mycobacterium lepraemurium*. Ptak et al. (32) reported that mice infected with *M. lepraemurium* develop a generalized impairment of cell-mediated immune responses in the course of a massive infection.

It might be argued that in lepromatous leprosy a situation comparable to antigen overload in animals can only be obtained when the circumstances for continuous or repeated exposure to (viable) *M. leprae* are favorable, which is the case in leprosy patients living in leprosy-endemic areas. It should be noted in this respect that a high level of transmission in such an area is suggested by the work of Godal and Negassi (19), whereas in patients moved to nonendemic areas this exposure is discontinued. Concomitant other (parasitic) infections may also play a role in depressing cell-mediated immune responses (41).

From the present study it can be concluded that lepromatous leprosy patients show a strongly decreased lymphocyte response in vitro to *M. leprae*. However, the in vitro response in
general was found to be unimpaired in all leprosy patients tested, both of the lepromatous and the tuberculoid type. The latter observation is in contrast to findings of other authors; one explanation for this discrepancy may be that the present study was performed in a leprosy-nonendemic area.

ACKNOWLEDGMENTS

We are grateful to A. Hartman, formerly of the Department of Dermatology, University of Groningen, Groningen, The Netherlands, for providing individuals for the correlation studies. The statistical analysis was performed by M. F. M. Jansen.

This work was supported by a grant from the Praeventie Fonds.
LITERATURE CITED


