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Immunological Profile of Treated Lepromatous Leprosy Patients[1]

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Leprosy continues to be a major health problem in some countries ([24]). The disease manifests in a clinical and immunological spectrum that represents perhaps the most striking example of a varied cellular immune response pattern to *Mycobacterium leprae* in different individuals ([27]). At one end of the spectrum are the tuberculoid (TT) type of patients who have high cell-mediated immunity (CMI) and on the other end are lepromatous patients with no evidence of CMI to *M. leprae*. Although both *in vivo* and *in vitro* CMI to *M. leprae* are lowered in lepromatous leprosy (LL) patients, their humoral response to *M. leprae* remains rather elevated. This CMI unresponsiveness has been found to be specific to *M. leprae* ([20]).

It may be expected that after the clearance of bacilli following treatment there could be an upregulation of antigen-specific CMI responses in borderline lepromatous/polar lepromatous (BL/LL) patients. Available reports on the immune response of BL/LL patients after long-term and short-term treatment are equivocal. Some authors have reported enhancement in both the skin delayed-type hypersensitivity (DTH) response and *M. leprae*-induced lymphoproliferative responses ([7, 33]); whereas others have found a continued suppression of lymphoproliferation to *M. leprae* in long-term treated, smear-negative (LTTSN) patients ([25]). With regard to the humoral response in long-treated patients, a reduced antibody response to phenolic glycolipid-I (PGL-I) with no change in the antibody response to whole *M. leprae* has been observed ([1]).

Cytokine level measurements are currently being used to determine an individual's immune status. In the T helper 1 (TH1)-type response, interleukin 2 (IL-2) and gamma interferon (IFN-γ), which are CMI and DTH mediators, are secreted. Conversely, in the T helper 2 (Th2)-type response, IL-4, IL-10, IL-5, and IL-13, which help B cells for antibody production, are secreted ([15]). In some situations no particular type of T-helper cells are found to be associated with LL or TT type of leprosy ([10, 13, 18]). It also has been noted that in LL patients the predominant response is of the Th2 type secreting IL-4, IL-5, IL-10, while in TT patients the response is of the TH1 type, secreting IL-2 and IFN-γ ([34]). Reports on cytokine response of LTTSN LL patients are scanty ([6]).

In the present study, we have investigated the skin DTH response, lymphocyte transformation (LT) response, antibody responses to the 35-kDa peptide and PGL-1 of *M. leprae* in LTTSN lepromatous (BL/LL) patients. In these patients, the cytokine pattern of peripheral blood mononuclear cells (PBMC) in response to *M. leprae* stimulation was also seen and compared with the results obtained from active lepromatous patients.

MATERIALS AND METHODS

Patients and blood samples. Nineteen treated lepromatous patients (individually detailed in Table 1) who had been released from treatment for a long time (2–20 years) and had remained skin-smear negative for *M. leprae* were the subjects selected for the

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TABLE 1. *Details of smear-negative treated patients.*

Patient no.	Age	Sex	Clinical form of disease	Duration of disease (yrs.)	Initial BI	Treatment (drugs)	Length of treatment (yrs.)	Duration of smear negativity (time of becoming negative to assessment) (yrs.)
T1	63	M	LL	20	5+	DDS[c] → MDT[d]	13	8
T2	53	F	LL	20	3+	DDS + TCT + INH	9	12
T3	69	M	LL	11	5+	DDS → MDT	19	4
T4	48	M	LL	5	5+	DDS → MDT	8	8
T5	35	F	LL	12	2+[a]	DDS → MDT	7	10
T6	53	M	BL	5	NA[b]	MDT	5	4
T7	68	M	LL	25	4+	DDS	14	15
T8	37	M	LL (R)	11	5+	MDT	4	4
T9	52	M	LL	5	NA[b]	MDT	7	6
T10	72	M	LL	8	4+	DDS → MDT	20	9
T11	54	M	LL	30	4+	MDT	6	14
T12	48	M	LL	1	6+	MDT	6	2
T13	49	F	LL	10	4+	MDT	5	4
T14	42	M	LL	10	4+	MDT (irregular)	7	5
T15	46	F	LL	3	5+	MDT	6	6
T16	57	M	LL	2	3+[a]	DDS → MDT	10	13
T17	35	M	LL (R)	5	4+	MDT	7	4
T18	30	M	LL	8	3+	MDT	3	7
T19	35	M	LL	6	5+	MDT	4	7

[a] BI of all cases was determined by Ridley's method ([26]) except patients T5 and T16 whose BI was determined by the method of Dharmendra and Chatterjee ([5]).

[b] NA = not available.

[c] DDS = Dapsone.

[d] MDT = Multiple drug therapy.

study. These patients had received diaminodiphenol sulfone (dapsone; DDS) or DDS followed by multidrug therapy (MDT) for varying lengths of time (3 to 20 years) until they became smear negative. All 19 patients were on follow up. Another 10 lepromatous patients who were smear positive (Table 2) and had recently been initiated on treatment formed the control group. Patients were clinically examined and classified according to the Ridley-Jopling scale ([27]). Blood samples were taken with and without heparin for PBMC culture and sera, respectively. Sera were stored at –20°C until used.

Lepromin test. The 4-week lepromin skin reaction was noted using standard Mitsuda antigen ([8]). For this, 0.1 ml of antigen was injected intradermally in the forearm of patients and the induration was measured with a standard caliper after 4 weeks.

Antigens. *M. leprae* soluble antigen (MLSA) was obtained from IMMLEP-(WHO) Bank through Dr. M. J. Colston, National Institute of Medical Research, London, U.K. Natural disaccharide octyl bovine serum albumin (ND-O-BSA), a synthetic sugar of PGL-I *M. leprae,* was kindly provided by Dr. Delphi Chatterjee, Colorado State University, Fort Collins, Colorado, U.S.A. (contract no 1-AI-55262). Purified protein derivative (PPD) was procured from the Central Veterinary Laboratory, New Haw, Weybridge, Surrey, U.K.

Serum antibody competition test (SACT). The SACT assay was performed according to the method of Sinha, *et al.* ([29]). In brief, 50 µl of MLSA (50 µg/ml) in sodium carbonate bicarbonate buffer, pH 9.6, was coated onto rows 2–12 of an ELISA plate (Maxisorp; Nunc, Napierville, Illinois, U.S.A.) at 4°C overnight. In the first row only phosphate buffered saline (PBS) was added. The plate was washed with PBS Tween (PBS-T) three times before blocking with 1% bovine serum albumin (BSA) in PBS for 2 hr. Duplicate wells in rows 3–12 of the plate were incubated with 25 µl of serial dilutions (1:10–1:1250 in PBS-T) of each serum. The first row and the E to H wells of row 2 were

TABLE 2. *Details of patients undergoing treatment.*

Patient no.	Age	Sex	Clinical form of disease	Duration	Initial BI	Treatment status[a]
A1	30	M	LL	16 yrs.	5+	Untreated
A2	42	M	LL	10 yrs.	5+	Treated
A3	40	M	LL	1 yr.	5+	Treated
A4	30	M	BL	1 yr.	3+	Untreated
A5	25	M	BL	10 yrs.	3+	Treated
A6	22	M	BL-LL	4 yrs.	5+	Treated
A7	40	M	LL	2 yrs.	4+	Untreated
A8	38	M	LL	7 yrs.	4+	Treated
A9	45	M	LL (R)	9 mos.	5+	Untreated
A10	25	M	BB-BL	1 yr.	2+	Untreated

[a]Patients receiving treatment for more than 3 months were classified as treated; those receiving less than 3 months of treatment were classified as untreated. All patients received MDT.

incubated with 25 µl of PBS BSA, and wells A to D of row 2 were incubated with 25 µl of a positive serum sample (having antibody to 35 kDa for 90 min; 25 µl of 1:500 diluted peroxidase conjugated monoclonal antibody, MLO4 was added in all wells followed by incubation at 37°C for 2 hr. After washing the plate three times color was developed by incubating the plate with 50 µl of o-phenylene diamine (Sigma; 5 mg tablet dissolved in 10 ml of distilled water) for 20 min at 37°C. Reaction was stopped by adding 7% H_2SO_4, and the optical density (OD) was read at 492 nm using anELISA reader (Titertek Multiskan Plus; Flow Laboratories, Rockville, Maryland, U.S.A.). The dilution which would cause 50% inhibition of the maximum binding (100% OD value) of peroxidase-MLO4 (100% value) is referred to as the ID_{50} titer of the serum. A serum with an ID_{50} titer of >1:5 was regarded as SACT positive.

ELISA for detection of antibody to ND-O-BSA. The ELISA to detect antibody to ND-O-BSA was carried out according to the method described by Sinha, *et al.* ([29]). Briefly, 100 µl of 4 µg/ml of ND-O-BSA in sodium carbonate bicarbonate buffer 0.05 M, pH 9.6, was coated onto two alternate rows of an ELISA plate (Polysorp; Nunc) for 18 hr at 37°C. Other wells were coated with coating buffer only. After washing the plate three times with PBS-T, all wells were blocked with 200 µl of 1% BSA in PBS for 2 hr at 37°C; 1:300 diluted sera were incubated in duplicate in buffer as well as antigen-coated wells for 2 hr at 37°C. The plate was washed three times with PBS-T and anti-human IgM (Sigma) diluted 1:10,000 in PBS-BSA was added to each well. After incubation for 2 hr at 37°C the plate was washed three times and the remaining step (color development, reading) was performed as described in the SACT ELISA. On the basis of reactivity obtained with nonleprosy healthy controls, a serum dilution of 1:300 was taken for all the tested sera. For each serum the mean OD of the buffer-coated wells was subtracted from the mean OD of the antigen-coated wells. The mean ± 2 S.D. (0.056) of the OD values of healthy controls was taken as the cut-off value.

PBMC culture. PBMCs were separated from heparinized blood by Ficoll hypaque density gradient centrifugation. PBMCs were washed three times with PRMI 1640 and cell concentration was adjusted to 2×10^6 cells/ml. Cells were cultured in PRMI 1640 with 10% AB serum with and without MLSA and PPD antigens (2–6 µg per ml) for 6 days at 5% CO_2 at 37°C. For the cytokine assay, supernatants were collected from antigen and control wells after 48 hr and 5 days of setting up of culture; 1 µCi of ^{3}H methyl thymidine (Amersham Life Sciences, Arlington, Illinois, U.S.A.) was added to each well 18 hr before the cultures were harvested on day 6 onto fiberglass filters using a Skatron harvester. Incorporation of ^{3}H thymidine into proliferating cells was measured by a scintillation counter (LKB Rackbeta).

Cytokine assay. The levels of IL-2 and

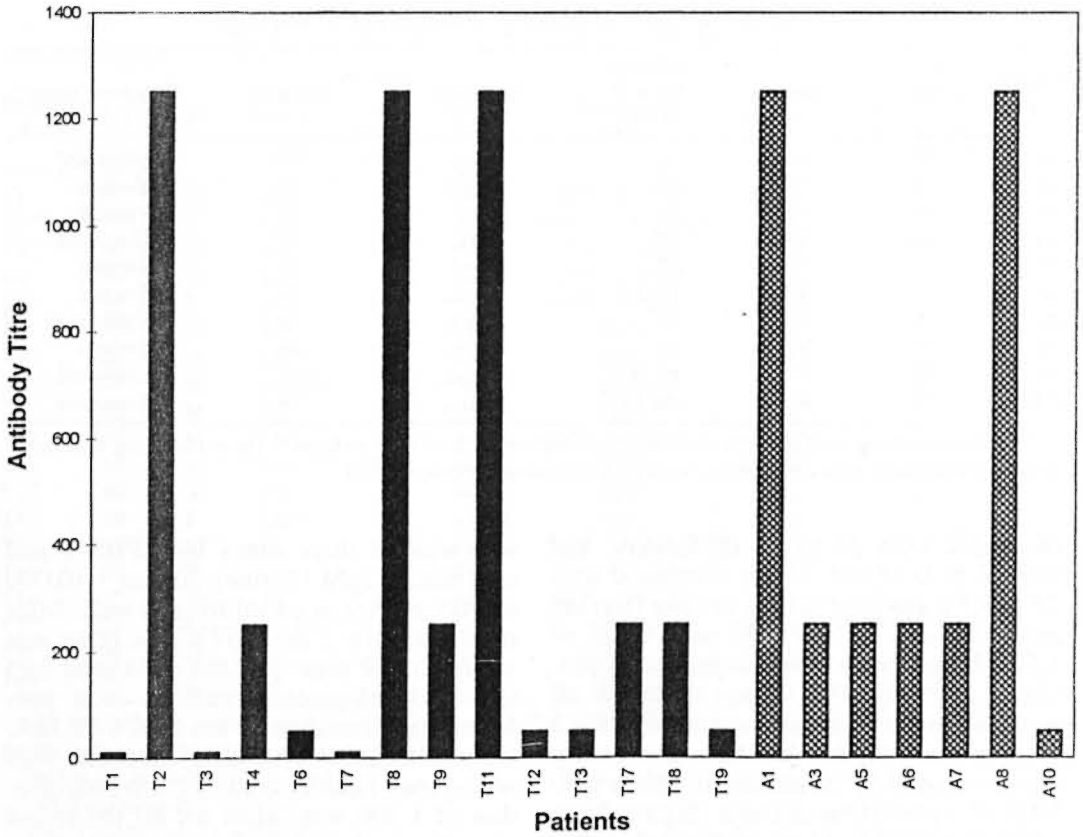

FIG. 1. Antibody responses of LTTSN and active LL/BL to 35-kDa antigen of MLSA. Each bar represents antibody titer of a single patient. The Y axis shows serum dilution at which the antibody reactivity was seen. Patients nos. T5, T10, T14, T15, T16, A5 and A9 were negative for antibody. Antibody could not be measured in the serum of patient A2. ■ = LTTSN; ▩ = active LL/BL.

IFN-γ were estimated in the supernatants collected from the PBMC cultures after 48 hr of stimulation with MLSA using Predicta kits procured from Genzyme, San Carlos, California, U.S.A.). IL-10 was measured in the supernatant collected after 5 days by using Quantikine kits procured from R & D Systems, Minneapolis, Minnesota, U.S.A.

Statistical analysis. Statistical analysis was done to compare LTTSN and active LL/BL patients using Epi-info Computer Software and Fisher's exact test.

RESULTS

Lepromin skin DTH response. For the lepromin response, an induration of <3 mm was taken as negative, from 3–4 mm as doubtful and >4 mm as positive. None of the LTTSN patients or active patients undergoing treatment showed a positive response to lepromin 4 weeks after its intradermal inoculation.

Antibody response. The antibody response was determined in all patients except patient A2. No significant difference in the antibody response to the 35-kDa antigen of *M. leprae* between LTTSN (73.6% antibody positivity) and active patients (77.7% antibody positivity) undergoing treatment was observed. However, only 31% of LTTSN patients showed a high titer of 1:250 or more as compared to 66% of untreated patients having such titers. The difference in the antibody level was not significant ($p > 0.05$) (Fig. 1). While 77.7% of active LL patients were positive for IgM antibody to ND-O-BSA, only 26% of the LTTSN patients were positive for the same ($p < 0.02$) (Fig. 2).

Lymphocyte transformation (LT) test.

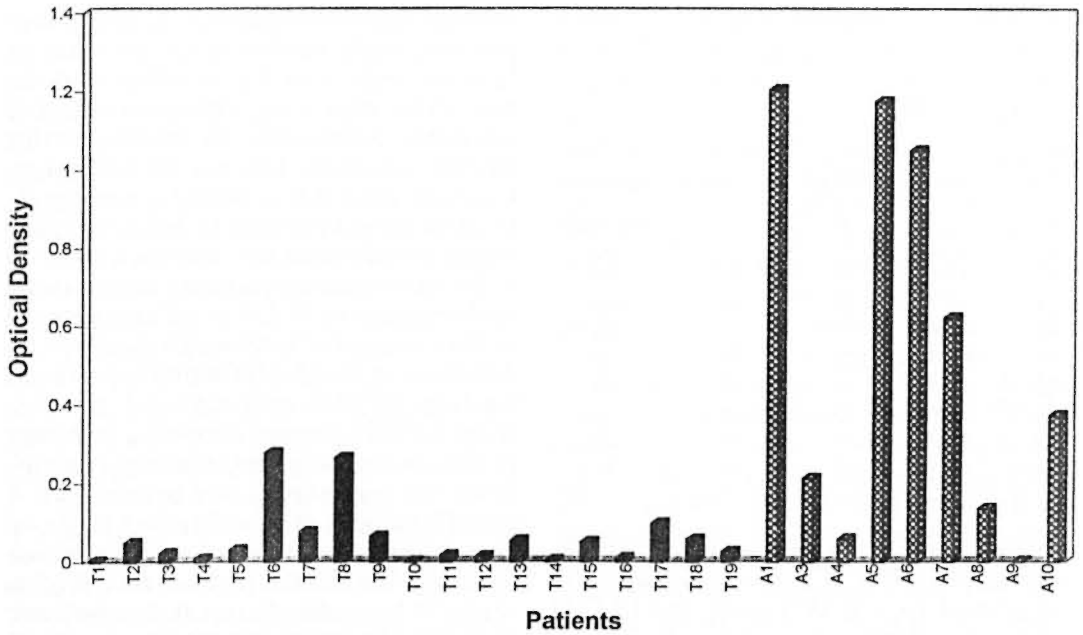

FIG. 2. IgM antibody responses of LTTSN and active LL/BL patients to ND-O-BSA. Each bar represents antibody reactivity of a single patient. Optical density of reaction at wavelength 492 nm is shown on the Y axis. Antibody could not be measured in the serum of patient A2. ■ = LTTSN; ▩ = active LL/BL.

The stimulation index (SI) of stimulated PBMCs was calculated using the formula, SI = mean cpm of experimental wells/ mean cpm of control wells. Patients showing a SI of >2 were taken as responders. Only 2 (10.5%) of the 19 LTTSN patients and 1 of the 10 active patients responded to MLSA (Tables 3 and 4); whereas 94% of LTTSN and 90% of active patients were responsive to PPD. Although some of the LTTSN patients showed a very high response (SI >80) to PPD, there was no difference in the mean SI between the two groups (data not shown).

IFN-γ assay. The minimum detection limit of the IFN-γ assay was 16 pg. Eight (42%) of the LTTSN patients produced IFN-γ (>16 pg) after stimulation with *M. leprae* (Table 3). One of the patients (T6) produced a level as high as 1800 pg. Interestingly, patients nos. T1, T3 and T6 produced high levels of IFN-γ but had a low titer (1:10) of antibody to 35 kDa. On the other hand, only one active patient produced IFN-γ *in vitro* (Table 4). However, there was no significant difference in the levels between the LTTSN groups and the active group of patients ($p > 0.05$).

IL-2 assay. The PBMCs of none of the active patients produced IL-2 after stimulation with *M. leprae* (Table 4); whereas 6 of the 19 (31%) LTTSN patients produced IL-2 upon stimulation (Table 3). Four of these

TABLE 3. *Lymphoproliferative response of PBMC, IFN-γ, IL-2 and IL-10 levels in culture supernatant of LTTSN patients to MLSA.*

Patient no.	LT test	IFN-γ (pg/100 µl)	IL-2 (pg/100 µl)	IL-10 (pg/100 µl)
T1	0.69	740	0	80
T2	1.16	740	620	88
T3	0.6	980	1100	90
T4	1.43	115	94	120
T5	0.39	0	45	0
T6	1.24	1800	850	0
T7	0.5	0	0	0
T8	1.88	140	0	0
T9	0.62	37	0	55
T10	0.82	45	0	190
T11	1.4	0	0	55
T12	1.21	0	0	0
T13	3.19	0	16	118
T14	1.62	0	0	70
T15	1.24	0	0	0
T16	0.73	0	0	0
T17	10.8	0	0	65
T18	1.53	0	0	0
T19	0.85	0	0	360

TABLE 4. *Lymphoproliferative response of PBMC, IFN-γ, IL-2 and IL-10 levels in culture supernatant of active LL/BL patients to MLSA.*

Patient no.	LT test (SI)	IFN-γ (pg/100 µl)	IL-2 (pg/100 µl)	IL-10 (pg/100 µl)
A1	2.27	0	0	Not done
A2	4.59	0	0	160
A3	1.2	0	0	16
A4	1.7	0	0	180
A5	1.76	0	0	500
A6	0.87	235	0	50
A7	1.6	0	0	120
A8	1.09	0	0	0
A9	1.06	0	0	320
A10	0.51	0	0	52

patients were those who produced significant levels of IFN-γ. The p value was nearly significant (p = 0.057) when the LTTSN and active groups were compared.

IL-10 assay. In some of the patients IL-10 production was seen in the control wells where no antigen was added. Therefore, the values shown in Tables 3 and 4 are after the deduction of the values of control (medium without antigen) wells from the values of wells where antigen was added. Eleven of 19 (57.8%) LTTSN patients produced IL-10 in response to MLSA (Table 3); whereas 88% of active patients produced IL-10 (p >0.05) (Table 4).

DISCUSSION

We have assessed the CMI, antibody and cytokine responses to various antigens in LTTSN and active smear-positive BL/LL patients. Anergy to lepromin has been noted in all LTTSN patients similar to earlier findings ([7]). This suggests that despite treatment and a "clinical" bacterial clearance lasting several years (mean 7.89 ± 4.25) the patients continue to be anergic to *M. leprae*. This may be because of the fact that most of the cases belonged to the lepromatous pole. However, a positive Mitsuda response in some treated lepromatous patients has also been reported ([3, 32]) after over 20 years of smear negativity ([33]). In the present study, *in vitro* lymphoproliferation to *M. leprae* antigen was seen in only 10% of the patients. Similarly, suppression of proliferation of PBMCs in response to *M. leprae* in bacillary negative, treated LL patients also has been shown by others ([6, 21]). Such a suppression might be due to the presence of large amounts of *M. leprae* antigens in the host even after long-term treatment ([11]) since this length may be insufficient for antigen clearance. This is corroborated by the observation that in some LL patients after 30 years of treatment *M. leprae*-induced lymphoproliferation has been seen ([6]).

We have noted a significant decline in the IgM response to PGL-I in LTTSN patients as have others ([1, 19]). Although there was no difference in the 35-kDa antibody positivity between LTTSN and untreated patients, fewer LTTSN patients showed a high titer (>250) as compared to untreated patients. Since the majority of our patients had a high BI initially, presumably they might be having initial high titers of antibody which remains a long time after treatment. Roche, *et al.* ([28]) have already reported persistence of the 35-kDa antibody even after 2 years of chemotherapy in patients who had an initial high titer of antibody. The presence of the 35-kDa reactive memory B cell could explain the persistence of this antibody. Moreover, since the 35-kDa antibody is of the IgG type the persistence of the antibody in the host is longer than the ND-O-BSA antibody which is of IgM nature.

It has been reported that IFN-γ plays an important role in the clearance of mycobacteria by activation of macrophages ([22]). IL-10 is known to negatively regulate Th1 cells and has been shown to inhibit antimycobacterial activity of macrophages *in vitro* by overriding the signals delivered by IFN-γ, and this could account for the ability of mycobacteria to survive intracellularly ([17]). In response to *M. leprae* stimulation, IFN-γ has been shown to downregulate the release of IL-10 ([12]). Monocyte-derived IL-10 and PGE_2 have been reported to be associated with the absence of Th1 cells and *in vitro* suppression of the cellular response in lepromatous leprosy ([14]). LL patients have been shown to produce low levels of IFN-γ after stimulation of the PBMC with *M. leprae* ([23]). It also has been suggested that lymphocytes of LL patients fail to make IL-2 and that the addition of a crude supernatant containing IL-2 restored their proliferative response to specific antigen ([9]).

We studied IL-2, IFN-γ and IL-10 production by PBMC of LTTSN BL/LL pa-

tients to show whether these patients have regained a Th1-type of response or continue to show suppression. Our data demonstrated that PBMC of a small number of LTTSN patients elicit better IFN-γ response than those of untreated patients. As expected, these patients also have shown a rise in IL-2 production. In contrast, none of the active patients produced IL-2 *in vitro.* Further, fewer LTTSN patients were found to be producing IL-10 as compared to active patients. This Th1 response seen in some of the LTTSN patients was not found to be related to the duration of disease, initial BI, duration of treatment or length of smear negativity. Although a similar rise in IFN-γ had been reported earlier ([6]) in treated LL patients, we have not come across any report on increased IL-2 and reduced IL-10 production in treated lepromatous patients. However, reduction in serum IL-10 of multibacillary patients and high levels of IL-2 and IFN-γ after both chemotherapy and chemo-immunotherapy have been reported ([2, 16]). IL-10 has been shown to suppress the proliferation of T cells ([4]). The low levels of IL-10 produced by most of the LTTSN patients in our present study might be enough to suppress the proliferation of PBMC. However, we have noted IL-10 production by two of the LTTSN patients who also responded to *M. leprae* by the LT test, and one of these patients showed significant production of IL-2. These variations in cytokine responses could be easily explained from the proliferation of bystander cells (Th0) which are known to produce cytokines of both types ([31]). We have recently noted TGF-β production by PBMCs of most of the LTTSN as well as active BL/LL patients (data not shown). The suppression thus seen in the lepromatous patients could as well be due to TGF-β since this cytokine has been reported to be inhibitory for T-cell activation in leprosy patients ([30]).

This study gives insight into the immunological profile of long-treated, bacillary negative lepromatous patients and suggests that there is an upregulation of CMI in some of these lepromatous patients. However more work on the cytokine profile in relation to *M. leprae* is required in the future in order to understand the exact mechanism of anergy in lepromatous patients.

SUMMARY

The immune responses of 19 treated lepromatous patients who had remained smear negative for a long period were assessed for specific cell-mediated immunity (CMI), anti-*Mycobacterium leprae* antibodies and cytokine release in response to challenge with *M. leprae* soluble antigen (MLSA). All of these patients remained anergic to Mitsuda lepromin. Lymphoproliferation in response to *M. leprae* antigen was noted in only two patients. Significant reduction in the phenolic glycolipid I (PGL-I) antibody response in treated patients with no difference in the *M. leprae* 35-kDa antibody response was observed when these responses were compared with those of active lepromatous patients. More treated patients produced interleukin-2 (IL-2) and interferon gamma (IFN-γ) than did active patients. On the other hand, fewer treated patients produced IL-10 than did active patients. These limited findings suggest that the host immune response makes an attempt toward upregulation of CMI in some treated LL/BL patients.

RESUMEN

Se midió le respuesta inmunitaria celular y humoral, y la producción de citocinas en respuesta a un antígeno soluble de *Mycobacterium leprae* (MLSA), en 19 pacientes lepromatosos tratados, que han permanecido baciloscopicamente negativos por períodos prolongados de tiempo. Todos los pacientes permanecieron anérgicos en la reacción de Mitsuda. La linfoproliferación en respuesta al antígeno de *M. leprae* sólo se observó en dos pacientes. En comparación con los pacientes con lepra lepromatosa activa, se observó una reducción significativa en los niveles de anticuerpos anti-glicolípido fenólico I (PGL-I) en los pacientes tratados, sin diferencia en los niveles de anticuerpos contra la proteína de 35 kD de *M. leprae.* La producción de interleucina 2 (IL-2) e interferón gamma (IFNγ) fue más frecuente entre los pacientes tratados que entre los no tratados. Por otro lado, se encontró menor producción de IL-10 en los pacientes tratados que en los no tratados. Estos resultados sugieren que los macanismos reguladores del sistema inmunitario del huésped tienden a mejorar la respuesta inmune celular en algunos de los pacientes LL/BL tratados.

RÉSUMÉ

Les réponses immunitaires de 19 patients lépromateux auparavant traités, qui sont restés bactérioscopiquement négatifs pendant une longue durée, furent évaluées pour l'immunité à médiation cellulaire

spécifique (IMC), les anticorps anti-*Mycobacterium leprae* et la libération de cytokines secondairement à l'injection d'un antigène soluble de *M. leprae* (MLSA). Tous ces patients ont gardés leur état anergique lors du test de Mitsuda à la lépromine. Une réaction de blastogenèse (lymphoprolifération) en réponse aux antigènes de *M. leprae* ne fut enregistrée que chez 2 patients. Une réduction significative de la réponse sérique des anticorps dirigés contre le glycolipide phénolique de groupe I (PGL-I) était présente chez les patients traités, par rapport à celle de patients souffrant de lèpre lépromateuse active. Cette réduction significative n'a pas éte observée concernant la réponse sérique en anticorps dirigés contre la protéine de 35 kDa de *M. leprae*. Une proportion plus importante de patients traités que de patients en phase active de la maladie libéraient de façon importante de l'interleukine 2 (IL-2) et de l'interféron gamma (IFN-γ), tandis qu'une proportion moins importante de patients traités que de patients en phase active produisaient de l'IL-10. Ces données, bien que limitées, suggèrent une tentative de réactivation de l'IMC chez certains patients LL/BL.

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