Lepr Rev (2003) **74,** 263–274

Comparison between anti-PGL-I serology and Mitsuda reaction: clinical reading, microscopic findings and immunohistochemical analysis

SOLANGE M. MAEDA*, OSMAR ROTTA*, NILCEO S. MICHALANY**, ZOILO P. CAMARGO***, CORD SUNDERKÖTTER+ & JANE TOMIMORI-YAMASHITA*

**Department of Dermatology, **Department of Pathology, ***Department of Microbiology, Immunology and Parasitology, Federal University of São Paulo, Brazil*

+Institute of Experimental Dermatology, University of Muenster, Germany

Accepted for publication 21 May 2003

Summary The lepromin test, serum IgM antibodies against *Mycobacterium leprae* and *in situ* observations of T cell subsets in biopsies of Mitsuda reaction using monoclonal antibodies were performed on 44 untreated leprosy patients belonging to various classifications of the disease. The Mitsuda reaction was accessed clinically and histologically after 28 days. Clinical reading and histological analysis of Mitsuda reaction showed good agreement. The high positivity in clinical reading correlated with compact granulomas in histology. There is a graduation of Mitsuda reaction that follows the immunological spectrum of the disease. The histological study of Mitsuda reaction is valuable to confirm the immunological condition in doubtful clinical reaction. Anti-PGL-I IgM levels correlated with disease classification, increasing from the tuberculoid towards the lepromatous pole of the disease spectrum. There was an inverse correlation between serum IgM antibody levels and clinical and histological reading of Mitsuda reaction. There were no statistical difference in quantities and distribution of CD4+ and CD8+ T cells in all Mitsuda reactions. The pattern of cellular content of Mitsuda reaction could not be related to the T cells.

Introduction

Leprosy is an infectious disease that presents polymorphism as a major characteristic, both under the clinical and laboratory aspects.

The structure of its aetiological agent, *Mycobacterium leprae*, has been largely studied. However, clinical manifestation depends on the relationship between the bacillus and the host immune response.

Correspondence to: S. M. Maeda, Department of Dermatology, Federal University of São Paulo, Rua Botucatu, 740, CEP: 04023–900 São Paulo/SP, Brazil. (Tel/Fax: +55–11–55712947; e-mail: jane.derm@uol.com.br)

0305-7518/03/064053+12 $1.00

The Mitsuda reaction is the classical lepromin test and although of little diagnostic value, it is generally accepted as a useful tool in accessing the immunological status and the prognosis of patients with leprosy.[1,2] The positive reaction consists of a nodular infiltration which begins after the first week following the injection, reaches the maximum size about the fourth week and later regresses, frequently leaving atrophy or scarring.[1,3,4] The histological evaluation of the Mitsuda reaction gives an indication of the state of tissue reactivity to antigens of *M. leprae* and hence it is an index of resistance of the patient to the disease.[5,6]

It has been demonstrated that mycobacteria can stimulate specific antibody production. A specific antigen PGL-I (phenolic glycolipid I) from *M. leprae* was described by Brennan and Barrow[7] and it has been used for serological studies in leprosy diagnosis. High levels of immunoglobulin G and M (IgG and IgM) against PGL-I have been detected in multibacillary patients, and some authors reported higher titres of IgM compared to IgG.[8,9] The use of anti PGL-I specific serology has shown to be useful in the disease classification and detection of new cases.[10,11]

The morphology and cellular composition of the infiltrate of the cutaneous lesions in leprosy depends on the intensity of cell-mediated immunity. The immunohistochemical study of T-cell subsets in granulomatous inflammation added new information to the pathophysiological mechanism of tuberculoid granuloma formation. In tuberculoid leprosy, CD4+ lymphocytes are present in the centre of the aggregates of epithelioid cells. The CD8+ cells are predominantly present in the lymphocytic mantle surrounding the granuloma. In lepromatous leprosy, CD4+ and CD8+ cells are both diffusely distributed among the mononuclear phagocytes without any discernible mantle.[12–14]

The aim of the present study was to evaluate the cellular and humoral immunity in untreated patients, by studying both histological and immunohistochemical aspects of Mitsuda reaction and the specific serologic analysis by the IgM anti PGL-I titres.

Materials and methods

PATIENTS

Forty-four untreated leprosy patients [18 (LL/BL), 5 (BB), 11 (BT) and 10 (TT)] classified according to Ridley–Jopling criteria[15] were included in this study from the out patients of the Department of Dermatology, UNIFESP–Escola Paulista de Medicina, São Paulo, Brazil. Their ages ranged from 16 to 86 years (mean age = 40·86), and 19 females and 25 males were included. In addition, sera from 38 normal blood donors from the same institution in São Paulo were included as control for IgM anti PGL-I (36 males and 2 females).

The diagnosis was made by clinical and histopathological features, and Mitsuda reaction was done in all patients to access their immunological condition.

All patients were enrolled after signing an informed consent that was approved by a medical ethics committee.

MITSUDA REACTION

Each patient was administered a 0·1 ml intradermal injection of lepromin (40×10^6 bacillus/ml). The lepromin was prepared 1 or 2 years before the utilization in our patients, and kindly provide by Dr Maria Alice da Silva Telles, Adolfo Lutz Institute, São Paulo, Brazil. After 4 weeks, clinical reading and cutaneous biopsy of the reaction were performed.

Clinical evaluation was made by measuring two diameters of the Mitsuda reaction (longitudinal and transverse). The arithmetic mean was calculated for each patient. A 6 mm punch biopsy was performed centrally on the injection site under local anaesthesia (0·1–0·2 ml lidocaine 2%).

The tissues were divided into two pieces. Half of the specimen was fixed in 10% formalin for haematoxylin and eosin (H&E) staining. The other part was stored in liquid nitrogen for immunohistochemical analysis.

The histological classification of Mitsuda reaction was based on six criteria:

Score zero: No inflammatory infiltrate
Score I: Non-granulomatous mononuclear infiltrate
Score II: Infiltrate composed of scarce epithelioid cells, without granulomatous formation
Score III: Incomplete tuberculoid granuloma with aggregates of epithelioid cells
Score IV: Loose tuberculoid granuloma
Score V: Tuberculoid granuloma without nodular pattern
Score VI: Tuberculoid granuloma with nodular pattern

ANTIGEN

A native lipidic antigen, the phenolic glycolipid-I (PGL-I) of *M. leprae* was used. This antigen was kindly provided by Mr Philippe Cruaud, Hôpital Jean-Verdier, Bondy, France.

SERA

Serum was collected from all patients before leprosy treatment, aliquoted and stored at -70°C until the laboratory procedure. All sera were tested at the same time.

ELISA PROCEDURE

An enzyme-linked immunosorbent assay (ELISA) was performed, using the same method for glycolipid mycobacterial antigens, as described by Cruaud *et al*.[16] with few modification using goat antihuman IgM peroxidase (Sigma®, EUA) as conjugate. All sera were tested in duplicate at dilutions of 1:20 in PBS (phosphate buffer saline) in both PGL-I-coated and blank wells. Results were expressed as the difference between the mean absorbance in the PGL-I coated wells (OD = optical density) and the mean absorbance of the uncoated wells. The positive standard control consisted of pooled human leprosy sera. The negative control consisted of pooled sera from healthy subjects to determine the cut-off value at mean $\pm$ 3 SD.

IMMUNOHISTOCHEMICAL ANALYSIS

The immunohistochemical analysis was performed in 39 patients. The tissues obtained from the Mitsuda reaction were rapidly frozen in liquid nitrogen and were stored at -70°C until the analysis were performed. We used anti CD4 (DAKO®, Denmark) and anti CD8 antibodies (DAKO, Denmark) by GaRat-peroxidase. The CD4 and CD8 expression was analysed semi-quantitatively. The following score was used:

Score zero: No immuno-labelled cells
Score I: Rare immuno-labelled cells (0–50 cells)

Score II: Few immuno-labelled cells (50–200 cells)
Score III: Moderate immuno-labelled cells (200–1000 cells)
Score IV: Numerous immuno-labelled cells (> 1000 cells)

STATISTICAL ANALYSIS

The Kruskal–Wallis non-parametric test was performed for statistical analysis of the clinical and histological reading of Mitsuda reaction and antibody levels of CD4+ and CD8+ cells expression in respect to the different forms of leprosy. The relationship between the antibody levels and Mitsuda reaction, histological features of Mitsuda reaction and semi-quantitative analysis of CD4+ and CD8+ cells was tested by Spearman correlation coefficient.

The cut-off level of the test, determined by mean value + 3 SD of IgM anti PGL-I obtained in healthy control was 163·57 ($OD \times 10^3$) at a 1:20 dilution of the sera.

Results

CLINICAL READING OF MITSUDA REACTION

At 28 days, a clinical reaction was observed in all TT patients (10) and it varied in size from 6·0 to 26·5 mm. In three of the 11 BT patients, the nodules were smaller than 5·0 mm in diameter. In the remaining eight patients, nodular reactions 7–16 mm in diameter were observed. No ulceration was observed in this group.

In BB group (5), clinical reading was smaller than 5·0 mm in three patients; in one patient it measured 6·0 mm and no clinical reaction was observed in one of the five patients.

No reaction was observed in 12 of the 18 multibacillary patients (LL + BL group). In the remaining six patients there was minimal induration, varying from 1 to 4 mm in size.

Higher values in clinical reading of Mitsuda reaction were observed in paucibacillary patients as compared with multibacillary forms ($P \leq 0{\cdot}1$). The results of clinical reading of the Mitsuda reaction are shown in Table 1.

HISTOLOGICAL ANALYSIS

Tuberculoid group

The histology of the Mitsuda reaction was characterized by the presence of large and often confluent granuloma composed of organized epithelioid cells and Langhans' giant cells.

Ulceration of the epidermis was observed in one patient. The real evaluation of the extent of the granuloma reaction in this patient was difficult due to the presence of an abscess in the centre of the lesion.

Borderline tuberculoid group

In seven of the 11 patients the histological study demonstrated complete tuberculoid granulomas composed predominantly of epithelioid cells with variable number of giant cells and lymphocytes. The granulomas were generally looser than those seen in the tuberculoid group. Incomplete granuloma presenting as aggregates of epithelioid cells was

Table 1. Mean value of Mitsuda reaction clinical reading (mm), Mitsuda reaction histological analysis (score) and anti PGL-I levels using ELISA (Δ492 nm × 103) in leprosy patients' sera. The results are expressed as the mean ± SD in clinical classes of leprosy (and control for serology)

Leprosy classification	No. of patients	Clinical reading (mm) ± SD	Histological analysis (score) ± SD	Anti PGL-I serology
TT	10	12·05 ± 5·96	4·10 ± 1·29	74·10 ± 60·86
BT	11	7·86 ± 3·63	3·73 ± 1·10	238·90 ± 248·86
BB	5	3·40 ± 2·19	1·80 ± 0·45	806·80 ± 430·69[b]
BL + LL	18	1·50 ± 1·50[a]	0·94 ± 1·51[a]	914·39 ± 374·39a
Control serology	38	–	–	38·42 ± 41·72

[a] Significant difference from BT and TT group ($P < 0·01$) , Kruskal–Wallis test.
[b] Significant difference from TT group.

present in two patients and in the remaining two patients scarce epithelioid cells, without granuloma formation was observed.

Borderline group

Infiltrate composed of scarce epithelioid cells without granuloma formation was observed in four of the five biopsies and non-granulomatous mononuclear infiltrate was noted in one of the five biopsies.

Borderline lepromatous and lepromatous leprosy group

A variety of histological patterns were noted in this group. Twelve of the patients demonstrated no inflammatory infiltrate (score zero). In one patient, there was a non-granulomatous mononuclear infiltrate; five patients presented different scores in histological analysis: scarce epithelioid cells without granulomatous formation (1); incomplete tuberculoid granuloma with aggregates of epithelioid cells (2) and a loose tuberculoid granuloma (2).

The results of histological analysis of Mitsuda reaction are presented in Table 1.There was significant difference between the mean of histological score of the TT and BT groups compared to BL and LL ($P < 0·01$).

There is a significant association comparing clinical reading and histological score of Mitsuda reaction. Clinical reaction $> 5·0$ mm presented tuberculoid granuloma in 68·5% of cases; in 31·5% the histological analysis showed incomplete tuberculoid granuloma or scarce epithelioid cells without granuloma formation. Negative reaction showed no inflammatory infiltrate in 83·3% and mononuclear infiltrate in 8·3% and incomplete tuberculoid granuloma with aggregates of epithelioid cells in 8·3%. Clinical reaction ranging from 1·0 to 5·0 mm in diameter showed a variety of histological patterns including negative reaction to complete tuberculoid granuloma formation (Table 2).

Correlation between clinical and histological readings of Mitsuda reaction was statistically significant. The Spearman coefficient of correlation was 0·7893 ($P < 0·05$) (Figure 1).

Table 2. Distribution of patients according to clinical reading of Mitsuda reaction (mm) and histological analysis (score)

	Histology (score)						
Clinical reading (mm)	Zero	I	II	III	IV	V	VI
Negative (12)	10	1	–	1	–	–	–
0–3 mm (1)	–	–	–	1	–	–	–
3–5 mm (11)	2	1	6	–	1	1	–
> 5 mm (19)	–	–	2	4	6	6	1
Ulcerated reaction (1)	–	–	–	1	–	–	–

DETECTION OF ANTI-PGL-I USING ELISA

Patients showed a characteristic pattern of anti PGL-I IgM level according to Ridley–Jopling classification (Table 1). Multibacillary forms (BL + LL) showed higher IgM antibody levels compared to paucibacillary (BT and TT) and control group ($P < 0{\cdot}01$). BB group showed higher IgM anti-PGL-I compared to TT group. Considering a cut-off value of a titer of 163·57 ($OD \times 10^3$) for IgM anti-PGL-I, 100% and 80% of BL + LL and BB group, respectively, were positive for this antibody. TT group showed only 10% positivity. TT group showed some antibody activity, when compared with the control group, but there was no statistical difference between these two groups ($P > 0{\cdot}01$) (Table 1).

Correlation between IgM anti PGL-I titres and clinical reading of Mitsuda reaction showed a negative correlation in these parameters. The increase in values of anti PGL-I titers resulted in decreased values of induration of the Mitsuda reaction, and *vice versa*. The Spearman correlation coefficient was 0·6457 ($P < 0{\cdot}05$) (Figure 2).

Similar results were obtained on comparing IgM anti PGL-I titres and histological reading of Mitsuda reaction. The Spearman correlation coefficient was 0·6863 ($P < 0{\cdot}05$) (Figure 3).

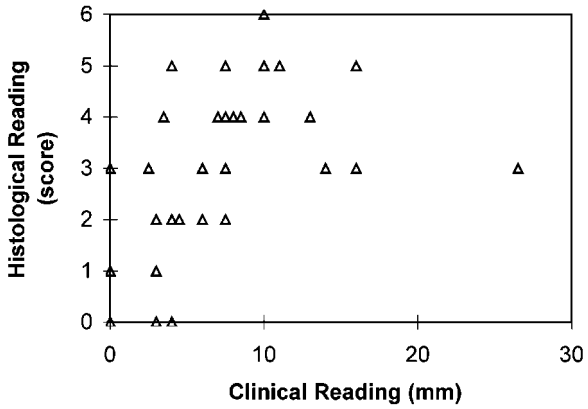

Figure 1. Correlation between clinical reading (mm) and histological analysis (score) of Mitsuda reaction. Spearman coefficient correlation = 0·7893 ($P < 0{\cdot}05$).

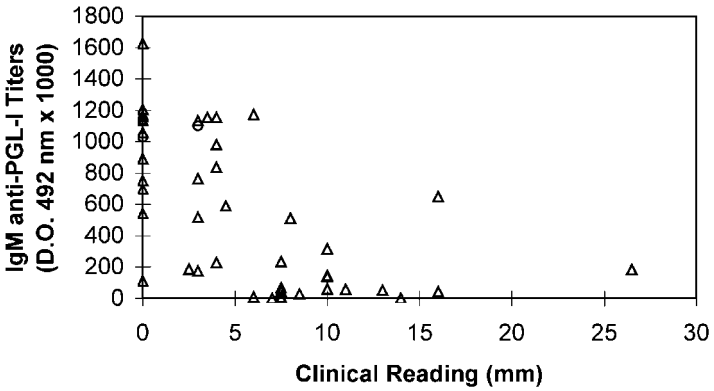

Figure 2. Correlation between IgM-anti PGL-I titres (OD $\times$ 10^3) and clinical reading of Mitsuda reaction (mm). Spearman coefficient correlation = $-0{\cdot}6475$ ($P < 0{\cdot}05$).

IMMUNOHISTOCHEMICAL ANALYSIS

Immunohistochemical analysis using monoclonal anti-CD4 and anti-CD8 antibodies showed no statistical difference in number of cells staining for the suppressor/helper phenotype in the spectrum of the disease ($P > 0{\cdot}01$). No immunohistological pattern of distribution of these cells could be noted in the spectrum of Mitsuda reaction.

There was no correlation between histological and clinical presentation and quantification of lymphocyte T CD4+ and CD8+ in the Mitsuda reaction. The Spearman correlation coefficient was 0·2291 and 0·2374 ($P > 0{\cdot}05$), respectively.

CD4+ and CD8 + lymphocytes are represented in Figures 4 and 5.

Discussion

Leprosy is a chronic infectious disease and can be considered an immunological disease because it presents with a spectrum of clinical manifestations that correlate with immune responses against the pathogen. At one end of the spectrum, patients with tuberculoid leprosy

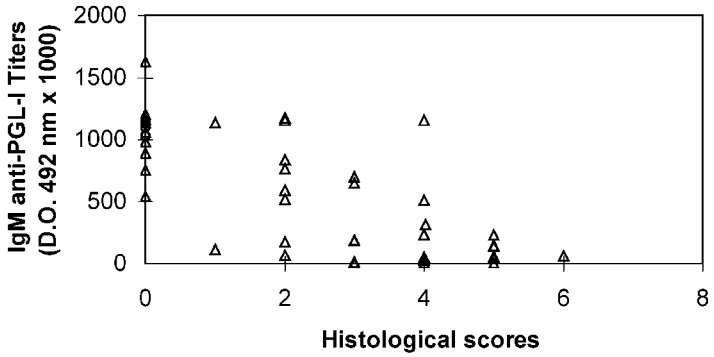

Figure 3. Correlation between IgM anti PGL-I titres (OD $\times$ 10^3) and histological analysis of Mitsuda reaction (score). Spearman coefficient correlation = $-0{\cdot}6863$ ($P < 0{\cdot}05$).

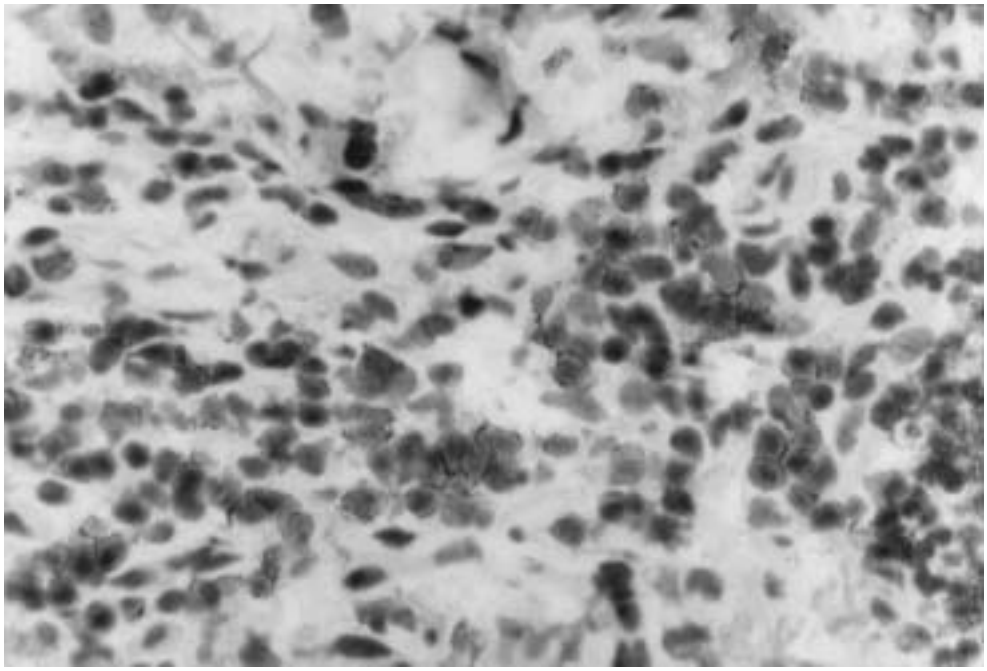

Figure 4. Expression of CD4+ lymphocytes—score III (×400).

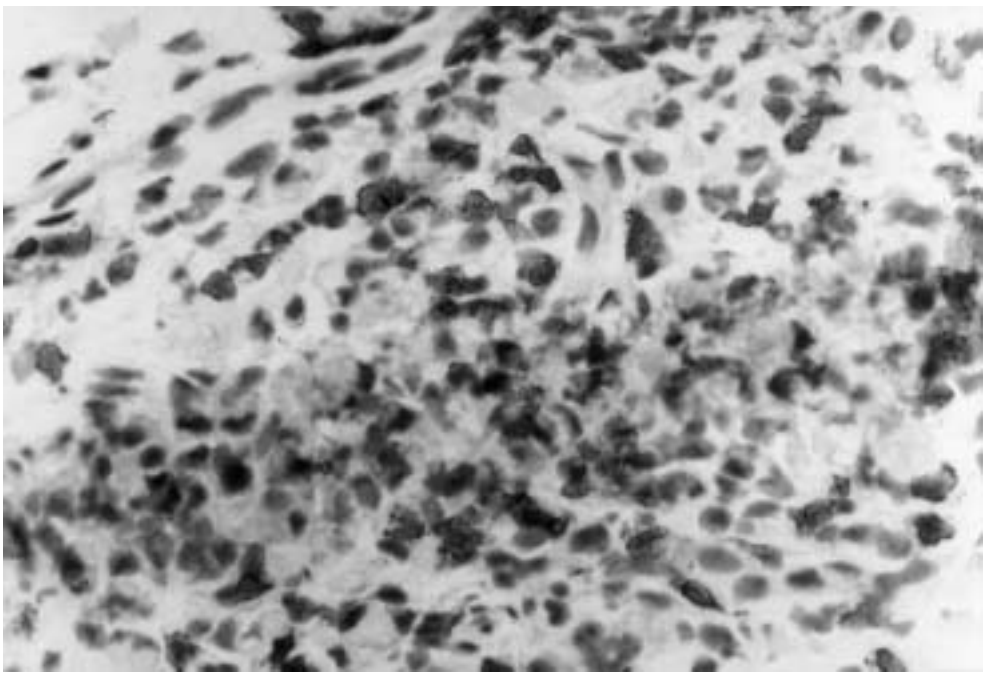

Figure 5. Expression of CD8+ lymphocytes—score III (×400).

represent the resistant response that restricts the growth of the agent. The lesions are few. Opposite end patients with lepromatous leprosy represent extreme susceptibility to *M. leprae* infection. In lepromatous leprosy, the skin lesions are numerous and the growth of the pathogen is continuous, resulting in proliferation of viable bacillus in the skin lesions. Clinical presentations are related with the level of cell-mediated immunity (CMI) against *M. leprae*. The standard measure of CMI to the pathogen is the Mitsuda reaction. It is widely agreed that T cells involved in cell-mediated immunity are pivotal in determining the outcome of infection with *M. leprae*. There is an interesting paradox, wherein CMI and humoral responses exhibit a negative correlation.[11,17]

The results of this study confirm earlier reports. Clinical and histological analysis of Mitsuda reaction showed good agreement;[3,18–21] all patients belonging to the polar tuberculoid form demonstrate a positive reaction and typical tuberculoid granuloma formation in histological evaluation of the nodules.

In our study, only one patient showed an ulcerating reaction. The histological picture in this case consisted of aggregates of epithelioid cells with no granuloma formation and necrosis in the centre of the reaction with difficulty in assessing the real extent of the tuberculoid granuloma. The reaction in borderline tuberculoid group was essentially similar to that observed in tuberculoid patients with a few minor differences.

In the polar borderline-lepromatous and lepromatous leprosy group, the majority of patients showed clinically negative reactions, although induration was observed in some patients (33%). In the literature, Rotberg[18] and Hayashi[3] found 38% and 27·3%, respectively, of clinically positive reactions in lepromatous patients. This phenomenon could be due to non-specific causes: foreign body irritation, sensitivity to the protein contents of the antigen or lipids antigens presents in lepromin.[18] In our patients, the histological reaction was negative in the majority of the clinically negative reactions.

False-positive reactions (minimal induration with no cellular infiltrate) were observed in 27·2% similar to other studies.[20–22] False-negative reaction (Mitsuda reaction clinically negative with tuberculoid granuloma structure with aggregates of epitheliod cells) was observed in 8·3%. This could have happened because of deep inoculation of the lepromin in these cases.

This study showed a graduation of Mitsuda reaction that follows the immunological spectrum of the disease. The clinical reading of lepromin reaction although reliable, may occasionally be misleading and a histological evaluation is useful to confirm the immunological condition in doubtful clinical reaction.

The results of serological analysis in this study confirm earlier reports on the pattern of anti PGL-I IgM in patients grouped according to the Ridley–Jopling classification, increasing from the tuberculoid toward the lepromatous pole of the disease spectrum.[23–26] Anti PGL-I and clinical reading of Mitsuda reaction showed a negative correlation in agreement with previous work.[11] Although a wide variation in antibody content in individual sera from patients with similar clinical classification was observed, the present data demonstrated three distinct groups: patients with negative lepromin test with antibody levels >500 (OD$\times$1000) corresponding to the multibacillary group; patients with a positive lepromin test (>5·0 mm of induration) and antibody levels <500 (OD$\times$1000) corresponding to the tuberculoid or borderline tuberculoid group; and patients with lepromin test between 1 and 5 mm and various antibody levels. Most of these patients belonged to the mid-borderline group.

The negative correlation between cell-mediated and humoral immunity was not applied

strictly to individual patients as each one represents a much more complex response. The serum antibody levels and skin test results showed the expected correlation.[27]

On comparing the antibody levels with histological analysis of Mitsuda reaction, a negative correlation was observed between these two parameters. Increasing antibody levels was associated with low histological scores (low capacity of tuberculoid granuloma formation). Low antibody anti PGL-I titres correlated with tuberculoid granuloma or tuberculoid element in histology showing a better cellular response. There was a strong positive correlation between the degree of cell mediated immunity (expressed by capacity of tuberculoid granuloma formation) and antibody titres against PGL-I. These findings suggest that the development of protective cell-mediated immunity results in the suppression of B-cell responses against specific antigens of *M. leprae* and *vice versa*.

Multibacillary and paucibacillary groups could be easily characterized by cellular aspects (measured by clinical and histological analysis of Mitsuda reaction) and humoral aspects (serology). The borderline group showed uncharacteristic immunological behavior. In this study mid-borderline group's behavior was similar to the multibacillary group.

It is well accepted that lepromatous leprosy patients show deficient cell-mediated immunity (CMI) to *M. leprae* antigens while the humoral response is present. This is peculiar because T helper lymphocytes are necessary to generate efficient antibody response. This phenomenon could be explained by many speculative propositions:

1. Balance in favour of either a Th1 or the Th2 subpopulation: the immunodominant epitopes of *M. leprae* trigger the Th2 cells rather than the Th1 cells in the lepromatous group, resulting in Th1 suppression (low cellular response-CMI) and stimulation of B cells and antibody response. In the tuberculoid group the Th1 cells are activated preferentially, resulting in good CMI and no humoral response. Additionally, other factors might also participate in the differential activation of each Th1 or Th2 cells: inadequate participation of APC (antigen presenting cells); inadequate secretion of cytokines and genetic factors.[28]
2. CD4+ cells could be inhibited by glycolipids isolated from *M. leprae, M. bovis and M. kansasii*. Mycobacteria could inhibit lymphocyte proliferation by switching off CD4+ cells directly or by preferencially activating CD8+ cells. This inhibition may be a general property of mycobacterial phenolic glycolipids. Despite the lack of specificity *in vitro*, this may occur *in vivo* by virtue of the localization of glycolipids in the leprosy lesions.[29]
3. Relationship between B cell and T cell *M. leprae* antigens must be equally processed by macrophages. For every specific B cell clone there is a specific twin T cell clone, strictly regulated and in subclinical phase of the disease whichever the clone is primed to proliferate the other must be repressed.[11]

This inverse relationship of Mitsuda reaction and antibody titres against *M. leprae* cannot be applied in normal subjects or in treated patients with negative bacillary index because the antibody behavior is dynamic and depends on the bacterial load. Therefore, this kind of relationship can be found only in untreated leprosy patients.

Immunohistochemical study of T cell subsets in dermal granulomas showed CD4+ T lymphocyte diffusely distributed in the granuloma and CD8+ cells confined at the periphery of the granulomas with a typical ring like pattern in tuberculoid lesions.[12,13] This pattern can be observed not only in tuberculoid leprosy but also in other granulomatous conditions such as sarcoidosis and positive Kveim reaction.[12,13] In lepromatous leprosy the T cells are present in the dermis in a diffuse pattern, it is not possible to delineate a typical distribution such as observed in tuberculoid lesions.[12,14,30]

Dugan *et al*.[31] and Narayanan *et al*.[32] studied dermal T cell subsets in Mitsuda reaction and they found typical distribution of CD4+ in the granuloma and CD8+ surrounding the epithelioid tubercle. The present study searched CD4+ and CD8+ cells in all positive and negative lepromin reactions. In only seven cases could the typical distribution of these cells, i.e. CD4+ in the centre and CD8+ in the periphery, be noted: five patients with lepromin reaction $> 5{\cdot}0$ mm and two patients with $< 5{\cdot}0$ mm.

There were no statistically significant difference in the quantities of CD4+ and CD8+ cells in all reactions studied. We observed similar quantities of helper and suppressor cells in Mitsuda reaction of all the leprosy forms.

The two polar groups of leprosy that showed statistical differences in clinical and histological readings did not exhibit any discrepancy in CD4+ and CD8+ quantities. We also could not observe any increase in some subset of T cells with the tuberculoid granuloma. These findings do not agree with other studies in tuberculoid granuloma reaction.[12,14,30]

The following possibilities could explain this findings:

1. Artificially induced antigenic stimulated granuloma (Mitsuda reaction) do not reproduce immuno-histochemically the same pattern seen in specific lesions of leprosy, and this kind of granuloma could be independent of T cell influence in its development.
2. CD4+ and CD8+ cells sensitized to *M. leprae* can be sequestered in other lesions or parts of the body and so we may not find any significant increase or decrease in these cells at the site of Mitsuda reaction.
3. The capacity of development of tuberculoid granuloma in Mitsuda reaction is not directly related with the sub population of T lymphocyte and this type of reaction is inconsistent with the immuno pathogenesis of a delayed-type hypersensitivity (DHT).

Although many studies of Mitsuda reaction (histological and immunohistochemical) do not clarify the pathophysiology of tuberculoid granuloma formation in the patients, this intradermal reaction still continues to be a valuable and useful method to evaluate the immunity against *M. leprae* and for leprosy classification and disease prognosis.

Acknowledgements

This study was partially supported by the 'Fundação Paulista contra a Hanseníase', São Paulo, Brazil. We would like to thank Professor Philippe Cruaud, Hôpital Jean-Verdier, France for providing the antigen PGL-I and active help in the early stages of this research. We are grateful to Dr Maria Alice Silva Telles, Instituto Adolfo Lutz, Brazil for kindly providing the lepromin antigen.

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