Development of a Mitsuda-like antigen and its evaluation in multibacillary, Mitsuda-negative leprosy patients*

*Desenvolvimento de preparado antigênico Mitsuda-símile e sua avaliação em pacientes multibacilares Mitsuda-negativos**

Ruy Noronha Miranda[1] Luiz Carlos Pereira[2] Sérgio Fonseca Tarlé[3]
Rogério A. Nascimento[4] Sandra L. de Mello[5] Lismary A. F. Mesquita[6]
Ivone T. Dechandt[7]

Abstract: BACKGROUND - Leprosy, a disease caused by *Mycobacterium leprae*, manifests itself into two clinical forms: the paucibacillary form is benign, Mitsuda positive and immunocompetent; the multibacillary form is severe, Mitsuda negative and immunodeficient. Multibacillary affected individuals, who release bacilli, are postulated to maintain endemic leprosy.

OBJECTIVES - The authors used cultivated mycobacteria to test Mitsuda negative patients, with the objective to induce immune conversion.

METHODS - A Mitsuda-like antigen was prepared from cultivated mycobacteria and tested in 28 Mitsuda negative leprosy patients. All patients were evaluated and then submitted to a new Mitsuda test. A control group of 28 Mitsuda negative leprosy individuals were inoculated with placebo and later evaluated by a second Mitsuda test.

RESULTS - Patients inoculated with the experimental Mitsuda antigen had favorable responses: 25 presented positive macroscopic reactions and four were tuberculoid granuloma. When submitted to a second Mitsuda test, four individuals presented typical positive Mitsuda reactions, with tuberculoid granuloma. Among the control group inoculated with placebo, the responses were negative, and there was one positive response observed for the second Mitsuda test.

CONCLUSIONS - In Mitsuda negative leprosy patients tested with an experimental Mitsuda antigen, we observed 14.29% of favorable responses, with Mitsuda-like reactions induced by the experimental Mitsuda antigen. When tested again with the Mitsuda antigen, we observed 14.81% of favorable responses with positive reactions.

Keywords: Mitsuda antigen; Leprosy; Cellular immunity

Resumo: *FUNDAMENTOS - A hanseníase, causada pelo Mycobacterium lepræ, manifesta-se por forma clínica denominada paucibacilar, benigna, Mitsuda-positiva, imunocompetente, e por outra forma, denominada multibacilar, grave, Mitsuda-negativa, imunodeficiente. Doentes multibacilares, eliminadores de bacilos, são considerados os mantenedores da endemia hansênica.*

OBJETIVOS - Os autores testaram cultura de micobactérias, obtida em laboratório, em pacientes Mitsuda-negativos, em busca de possível viragem imunológica.

MÉTODOS - Com a cultura de micobactérias, foi preparado antígeno mitsudina-símile, que foi testado em 28 hansenianos Mitsuda-negativos, os quais, após avaliação desse teste, foram submetidos a novo teste de Mitsuda. Outros 28 Mitsuda-negativos receberam a inoculação de placebo e, posteriormente, foram avaliados por meio de novo teste de Mitsuda.

RESULTADOS - Nos pacientes inoculados com a mitsudina experimental houve respostas favoráveis: em 25 deles reações macroscópicas positivas, em quatro das quais, com granuloma tuberculóide. Avaliados por meio de novo teste de Mitsuda, quatro deles responderam com típicas reações Mitsuda-positivas, com granuloma tuberculóide. Nos pacientes testados com placebo, as respostas foram negativas; a um novo teste de Mitsuda, houve uma resposta positiva.

CONCLUSÕES – Em hansenianos Mitsuda-negativos, testados com mitsudina experimental, foram constatadas 14,29% de respostas favoráveis, com reações de Mitsuda-símiles provocadas por essa mitsudina; testados os pacientes, novamente, com o antígeno de Mitsuda, foram constatadas 14,81% de respostas favoráveis, com reações positivas.

Palavras-chave: Antígeno de Mitsuda; Hanseníase; Imunidade celular

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[1] MD. PhD. Emeritus Professor at UFPR. Dermatologist and specialist in leprosy. Chief researcher of the Fundação Pró-Hansen - Curitiba, Paraná (PR), Brazil.
[2] Full Professor of Dermatology at PUC-PR. Teaching and Research Director of the Fundação Pró-Hansen - Curitiba, Paraná (PR), Brazil.
[3] MD. Adjunct Professor of Dermatology at PUC-PR. Specialist in leprosy. Director of the Associação Médico-Social da Fundação Pró-Hansen - Curitiba, Paraná (PR), Brazil.
[4] Dermatologist (*in memoriam*).
[5] MD. Specialist in Dermatological Surgery. Medical residency preceptor of the Fundação Pró-Hansen - Curitiba, Paraná (PR), Brazil.
[6] MD. Pathologist of the Santa Casa de Curitiba - Curitiba (PR), Brazil.
[7] Accounting degree. Director-President of the Fundação Pró-Hansen - Curitiba, Paraná (PR), Brazil.

INTRODUCTION

Leprosy is a chronic infection that globally affects about 550000 individuals per year.[1] It is caused by the bacterium *Mycobacterium leprae* (*M. leprae*), it primarily involves the skin and the peripheral nervous system.[2] In the mid-1980's, the World Health Organization (WHO) launched a worldwide campaign with the purpose of eliminating leprosy as a public health problem (incidence < 1/10000 people) until the year 2000. However, in 2004, the disease is still present in many countries, with a high prevalence since 1996.[1] Because no natural reservoir of known biological importance is available for *M. leprae* and the fact that the current treatment furnished at no cost by the WHO to all patients is very efficient to cure the disease, the origin of these new cases seems obscure. Brazil, with 40000 leprosy patients, ranks second as most affected countries in the world, after India.[1]

Leprosy has very heterogenous clinical manifestations. According to the classical categorization of the disease's clinical forms proposed by Ridley and Jopling,[3] the affected patient may present a spectrum of manifestations varying from the polar tuberculoid form (milder and localized) to the Virchowian (systemic and more severe). Tuberculoid leprosy is characterized by few skin lesions, rarely detectable bacilli in skin and nerve biopsies, and the presence of a strong cell type Th1 anti*M. Leprae* immune response. Patients with Virchowian leprosy have numerous skin lesions, with abundant amount of bacilli and anergy to the *M. leprae* antigens.[3] Tuberculoid and Virchowian forms of the disease correspond to the paucibacillary and multibacillary forms of leprosy, respectively, according to the WHO for the purposes of drug therapy guidance.

A characteristic that distinguishes *M. leprae* from all the other pathogenic bacteria to humans is its incapacity to grow in artificial culture media. Nowadays, this feature can be analyzed according to recent data suggesting a marked evolutional reduction in *M. leprae* genoma.[4] Comparatively speaking, while the *M. tuberculosis* genome contains more than 4000 genes, *M. leprae*'s genome contains only 1600 open reading frames (ORFs). This comparison suggests that *M. leprae* has lost approximately 2000 genes since it evolutively diverted from a common ancestral bacterium, resulting in a highly specialized compulsory intracellular parasite. The loss of function in genes involved in crucial metabolic pathways offers a tentative explanation for the impossibility of cultivating this microorganism.

Miranda et al. obtained the growth of acid-alcohol resistant microorganisms by seeding of skin lymph of multibacillary leprosy patients in an original culture medium.[5] Supposedly, a culture of microorganisms is suggestive of *M. leprae* if it causes, in skin tests, identical responses to those caused by standard Mitsuda antigen in the classical Mitsuda reaction,[6] that is, positive responses in hyperergic paucibacillary patients (TT) and negative responses in anergic multibacillary (LL) patients. The use of an experimental bacillary Mitsuda antigen of the Dharmendra type,[7] called C12-004 and prepared from cultures obtained by Miranda et al., has caused a strong positive response in multibacillary patients not reagent to the Mitsuda test.[8] This result suggests that the bacterium cultivated was not *M. leprae*. In fact, typing of the cultures obtained by Miranda et al., performed at the Instituto Adolpho Lutz, in Sao Paulo and at the Biomanguinhos, in Rio de Janeiro, concluded that the mycobacterium cultivated belonged to the *M. kansasii* species, according to official reports released.

Previous studies used different antigen preparations to induce immune responses in anergic leprosy patients.[6,9,10] The author's data suggest that a Mitsuda-like antigen prepared from the microorganism cultivated could be efficient to induce an immune response to the Mitsuda antigen. To test this hypothesis, Mitsuda-like antigen C12-004 was prepared and inoculated in anergic leprosy patients, and its effects on the immune response induction were assessed by a subsequent Mitsuda test.

MATERIALS AND METHODS

1. Patients

Fifty-six adult individuals of both sexes, with no intercurrent diseases or pregnancy, affected with leprosy and tested as Mitsuda-negative, at different stages of treatment, were randomly recruited among patients receiving outpatient treatment at the Fundação Pró-Hansen.

The diagnosis was made by experienced leprosy specialists based on clinical, bacteriological, histopathological and immunological (Mitsuda reaction) criteria. The classification of the clinical types of leprosy was based on the criteria described by Ridley and Jopling.[3] Patients were divided into two groups of 28 individuals; the first group was called "experimental" and received intradermal inoculation of experimental Mitsuda antigen C12-004; the second group was called "control" and inoculated with a placebo. All the individuals enrolled were informed about the research project, without knowing to which group they belonged, invited to take part and, after agreeing, they signed an Informed Consent Form (ICF). The project was authorized by the Research Ethics Committee (REC) of the Fundação Pró-Hansen.

2. Mycobacterium cultures

As described,[5] the mycobacterium cultures used in this project were obtained from human plasma of healthy individuals as per an original technique. Plasma contained in 20 mL of venous blood from the donor, separated from the formed elements of the blood after a rest period or centrifugation, was seeded with bacillus-rich lymph of a multibacillary patient and maintained in an oven at 93.2ºF (34ºC) until, in case of a positive result, a whitish sediment would appear indicating the bacillary mass growth around the 45th day of incubation. The optical analysis of this sediment showed it was composed of microorganisms with all the morphological and staining criteria as those of the mycobacteria. It is important that the sequential analysis of this sediment did not recognize it as *Mycobacterium leprae*, but rather as *M. kansasii*. Transplant abbreviation 191/39FYA was selected for preparing the experimental Mitsuda antigen, which was labeled as C12-004. This culture was validated by means of the growth of its transplanted samples in a plasma broth medium.

3. Preparation of Mitsuda antigen C12-004

Experimental Mitsuda antigen used in this study was obtained using technology from the *Centro de Produção e Pesquisa em Imunobiológicos* (CPPI) [Center of Immunobiological Production and Research] of the State Secretariat of Health of Parana, which is accredited to produce and supply the Mitsuda antigen and has also performed all quality tests. The contents of culture 191/39FYA were transferred to an Erlenmeyer flask containing 100mL of plasma broth, in which it was homogenized by agitation. Four aliquots of 10mL of this bacterial suspension were distributed in centrifugation tubes and they were centrifuged until microorganism separation. The supernatant liquid was decanted and discarded; the sediment was suspended and washed with saline solution and it was obtained again by centrifugation. This procedure was performed once again and later the sediment was suspended in 100mL of 0.5% phenylated saline solution for bacillus counting, which revealed a total of $17x10^6$ microorganisms per milliliter. The suspension was bottled in flasks similar to the penicillin flasks, which were autoclaved at 248ºF (120ºC) for 20 minutes, and it was submitted to qualification tests: dosing of chemical components, sterility, innocuity, skin reactivity, necrosis test in guinea pigs and pH. All these qualification tests were satisfactory and they were released in official reports. The placebo used in this experiment was saline solution, which was also submitted to the above-mentioned tests.

4. Standard Mitsuda antigen

Standard Mitsuda antigen used in this study was gently supplied by the CPPI of the State of Parana.

5. Inoculation of patients

All individuals in the experimental group, after a confirmed negative Mitsuda reaction, received an intradermal inoculation of 0.1mL C12-004 antigen in the anterior surface of their right forearm. Individuals in the control group were inoculated with 0.1mL saline solution. The reading of inoculation results, in both groups, was performed according to the investigation schedule; macroscopically positive cases were those that responded with a tuberculoid granuloma $\geq$3mm in diameter, which was biopsied for histopathological confirmation. After excision biopsies of the macroscopic responses, all patients, as well as the control group, were submitted to a new Mitsuda test.

6. Statistical analysis

The differences in the ratio of individuals from the experimental and control groups who presented a positive response to the intradermal inoculation of standard lepromin following the exposure to experimental Mitsuda antigen (C12-004) were estimated by using the Fisher´s exact test.

RESULTS

The results related to 28 patients from the experimental group are shown in table 1. Twenty-five patients (89.28%) had positive macroscopic reaction to the experimental Mitsudina antigen C12-004 (Figure 1); four (patients 6, 13, 24 and 27) or 14.29% had Mitsuda-like tuberculoid histopathological images (Figure 2); eight patients (1, 5, 7, 10, 11, 14, 15 and 25) had histopathological responses containing epithelioid or giant cells (components of the positive Mitsuda reaction; Figures 3 and 4); 11 patients (5, 6, 12, 13, 14, 15, 18, 21, 24, 26 and 27) did not present any microorganisms in the histopathological sample. Afterwards, 27 patients in the experimental group were submitted to a new Mitsuda test and four of them (14.81%) responded with positive Mitsuda tests (Figure 5). In two other patients (01 and 13), who also had a positive macroscopic response, the histopathological analysis did not show formation of a tuberculoid granuloma and, therefore, they were considered as false Mitsuda reactions (Figure 6). Some patients who had a macroscopic response to the inoculation of Mitsuda antigen C12-004 presented discordant histopathological responses: Virchowian (LL), patient 04 (Figure 7); undetermined (I), patient 12 (Figure 8) (HE x 400).[11]

Patients in the control group, inoculated with placebo, did not present positive responses to the

TABLE 1: Results obtained from the experimental group

						Mitsuda antigen C12-004			Second Mitsuda test				
Nº	Name	Age	Sex	Clinical form	Mitsuda Reaction	Macro	Histop	Bacilli	Macro	Histop	Bacilli	Reactional states	Notes
01	BN	29	M	LL	0	+	BT	+	+	LL	+	Yes	False Mitsuda reaction
02	JNS	35	M	LL	0	+	LL	+	0			No	
03	RK	63	M	LL	0	+	LL	+	0			Yes	
04	HJP	35	M	LL	0	+	LL	+					Out of control
05	JMS	66	M	LL	0	+	BT	0	+	TT	0	No	Favorable response
06	MAC	29	M	LL	0	+	TT	0	0			Yes	
07	OA	55	M	LL	0	+	BT	+	0			Yes	
08	ACH	55	M	LL	0	+	LL	+	0			Yes	
09	DGF	59	M	LL	0	0		0				No	
10	ACN	18	M	LL	0	+	BT	+	0			Yes	
11	MLO	46	M	LL	0	+	BT	+	0			Yes	
12	JCS	31	M	LL	0	+	I	0	0			Yes	
13	GD	56	M	BB	0	+	TT	0	+	I	0	No	False Mitsuda reaction
14	SSK	42	M	LL	0	+	BT	0	0			No	
15	RM	36	F	LL	0	+	BT	0	0			No	
16	VLP	53	M	LL	0	+	I	+	0			No	
17	PS	75	M	LL	0	+	I	+	0			No	
18	CSC	46	F	LL	0	+	I	0	0			Yes	
19	RCF	28	M	LL	0	+	LL	+	0			Yes	
20	SAM	21	M	LL	0	+	LL	+	0			Yes	
21	JSM	59	M	LL	0	+	I	0	+	TT	0	No	Favorable response
22	DMC	30	F	LL	0	+	LL	+	0			No	
23	IB	61	M	LL	0	+	I	+	0			No	
24	JAR	48	M	LL	0	+	TT	0	0			Yes	
25	RB NS	39	F	LL	0	+	BT	+	0			No	
26	FK	58	M	I	0	+	I	0	+	TT	0	No	Favorable response
27	LAK	49	M	I	0	+	TT	0	+	TT	0	No	Favorable response
28	JO	56	M	I	0	0		0				Yes	

LL - multibacillary or Virchowian structure
TT - tuberculoid or granulomatous structure
I - clinical form or undetermined structure
BT and BB - combined TT and LL histopathological image

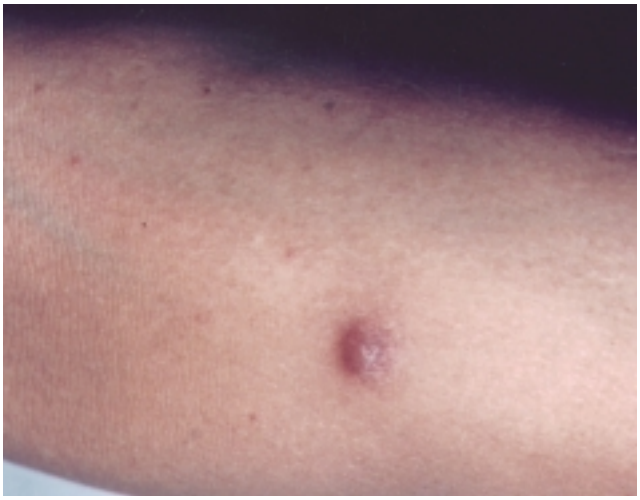

FIGURE 1: Response to Mitsuda antigen C12-004 at day 25

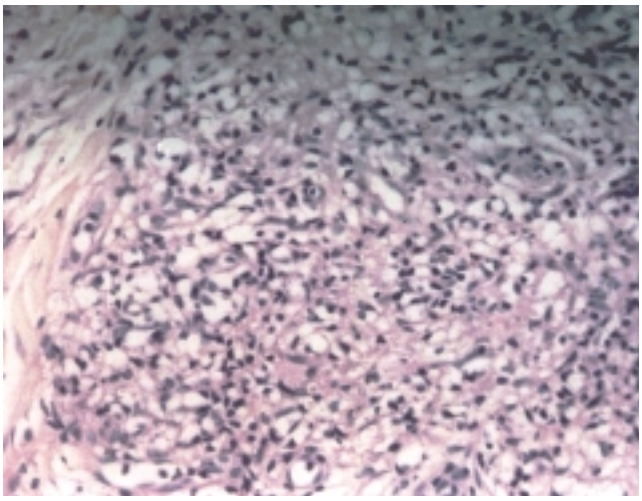

FIGURE 3: Granulomatous response to Mitsuda antigen C12-004 at day 45 (HE x 100)

inoculation. When subsequently submitted to a Mitsuda test, only one patient showed a positive macroscopic response, which was evaluated by histopathological analysis. No statistically significant difference was observed when a comparison was made in the numbers of individuals in both groups who showed a positive Mitsuda reaction after the inoculation of Mitsuda antigen C12-004 or placebo ($p = 0.351$).

The permanent clinical follow-up of patients throughout the research did not demonstrate any significant changes in the clinical course of the disease. All patients continued with the polychemotherapic treatment (PCT). Reactional outbreaks of leprosy, types I and II, manifested as a typical characteristic of the disease during the study project in individuals from the experimental group (48.14%) and in individuals from the control group (51.85%).

DISCUSSION

Mitsuda intradermal reaction is a test used to evaluate the type of immune response against *M. leprae* shown by an individual.[2] The result of Mitsuda reaction in healthy people has a predictive value regarding the type of clinical manifestation of the disease to be found in patients. A positive Mitsuda reaction indicates an intense type Th1 cellular immune response and is associated with a low risk of developing the Virchowian form of the disease. On the other hand, a negative Mitsuda reaction indicates a predominantly type Th2 humoral immune response and a high risk of manifesting the systemic and multibacillary forms of leprosy.[12,13] In this context, it is

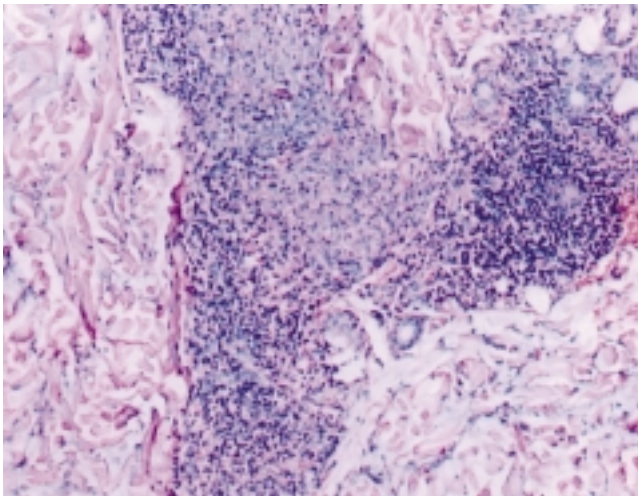

FIGURE 2: Granulomatous response to Mitsuda antigen C12-004 at day 45 (HE x 100)

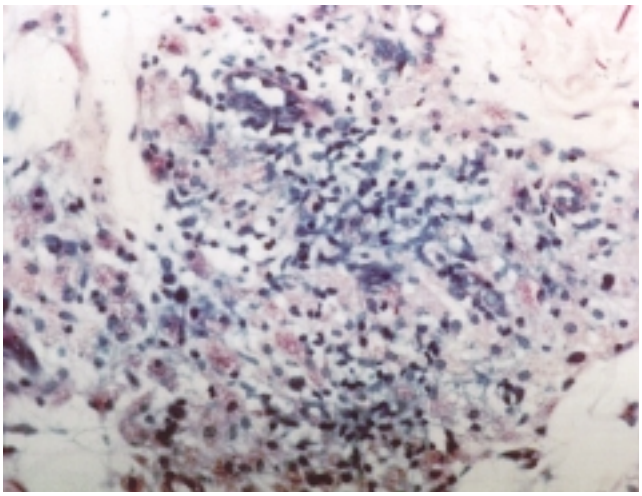

FIGURE 4: Late response to Mitsuda antigen C12-004, virchowian and tuberculoid images, BT (HE x 100)

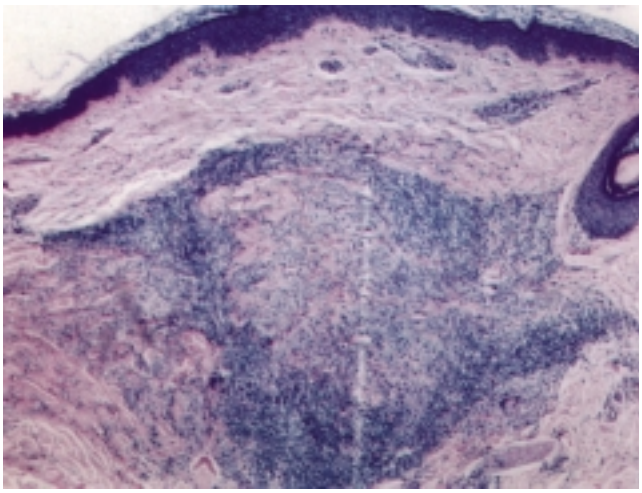

FIGURE 5: Positive Mitsuda reaction induced by Mitsuda antigen C12-004 (HE x 100)

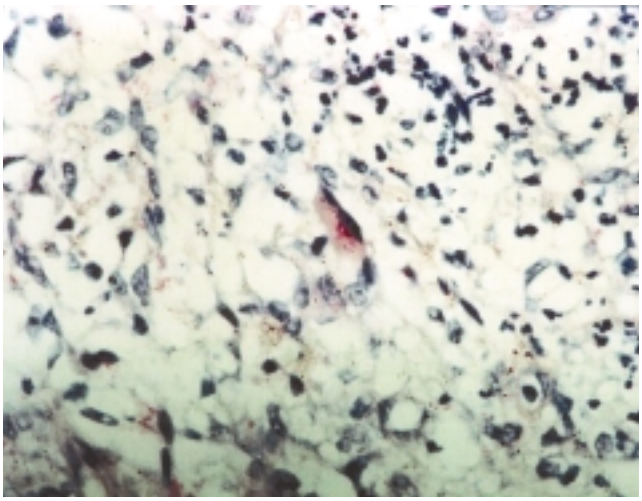

FIGURE 7: Virchowian response (LL) in a positive macroscopic response to the inoculation of Mitsuda antigen C12-004 (Zihel x 400)

considered reasonable to induce cellular immunity specific to *Mycobacterium leprae* in the anergic leprosy patient (multibacillary, LL). Induction of a positive result of the Mitsuda test has been performed by exposure of anergic patients to antigens other than those of *M. leprae*. In this current investigation, an experimental Mitsuda antigen (C12-004) was prepared from the culture of *Mycobacterium kansasii*, obtained from skin lesions in patients with confirmed multibacillary leprosy. The appearance of a culture of mycobacterium other than *M. leprae* from the lesion of a multibacillary leprosy patient is similar to the findings reported by other authors, according to the descriptions by Cochrane[14] and, more recently, by Chakrabarty.[6] Mitsuda antigen C12-004, when used as an intradermal inoculation in anergic patients, promoted specific cellular immune response in 14.29% of individuals studied. This result is suggestive of ability to induce cellular immune response with antigens present in the Mitsuda antigen C12-004. Immune conversion was demonstrated in four out of 27 patients submitted to a new exposure to specific *M. leprae* antigens, that is, in 14.81% of the population studied, who presented a classical positive Mitsuda reaction. Only one patient presented a coincidence of positive results obtained with Mitsuda antigen C12-004 and standard leprosin. Special attention should also be paid to other results of the histopathological analysis obtained with the inoculation of experimental Mitsuda antigen, either the partial tuberculoid response in 8 patients and the absence of inoculated bacillus with experimental Mitsuda

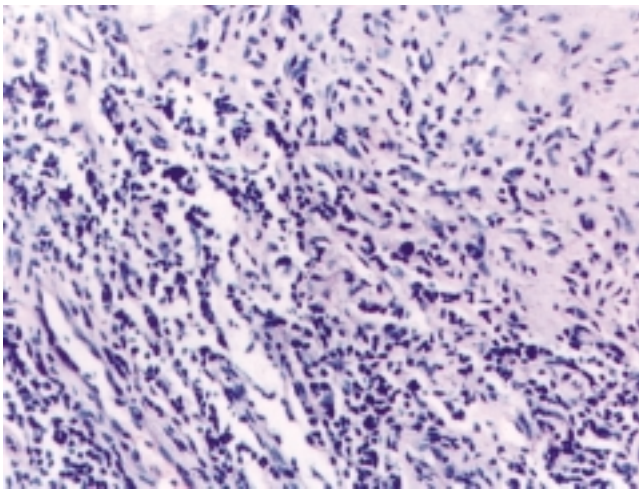

FIGURE 6: Histopathological structure of a false Mitsuda reaction (HE x 400)

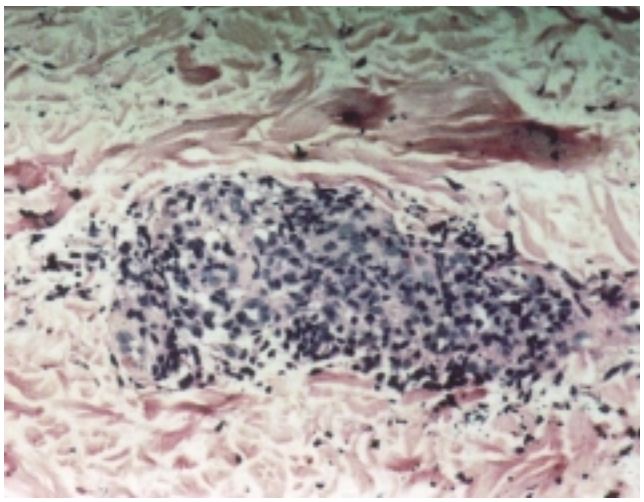

FIGURE 8: Undetermined response (U) in a positive macroscopic response to the inoculation of Mitsuda antigen C12-004 (HE x 400)

antigen in 11 patients. The authors' observations also indicate the occurrence of two false Mitsuda reactions confirmed in the final test, which were characterized by the non-correspondence of the tuberculoid granuloma to a positive macroscopic response. All 28 patients in the control group did not show any response to the inoculation of placebo. When they were tested with a second Mitsuda test, only one patient (patient 01) had a macroscopic response, and this response was not evaluated with a histopathological test.

The absence of statistical significance (p = 0.351) for the difference between the number of individuals who presented a positive Mitsuda reaction (1/28) is probably due to the small number of individuals tested and suggests replication of the results in an extended sample.

The specific treatment of patients was not discontinued during the course of experiments. The occurrence of reactive outbreaks in 48.14% of patients in the experimental group and 51.85% in the control group forced the use of corticosteroids and thalidomide, which may have had a negative influence on the results due to the immune modulator effect of these drugs.

CONCLUSIONS

The development of this current project enabled demonstrating signs of cellular immunity in multibacillary leprosy patients, with negative Mitsuda tests, by means of an intradermal injection of a bacillary leprosin prepared from a culture of mycobacteria other than *Mycobacterium leprae*.

The experimental Mitsuda antigen caused Mitsuda-like positive reactions in four (14.29%) out of 28 patients tested. A subsequent classical Mitsuda test showed positive reactions in another 4 out of 27 (14.81%) patients.

It is accepted that the percentage of favorable responses could be higher if the concentration of microorganisms in the experimental Mitsuda antigen were higher than 17 million microorganisms per mililiter.

The presence of epithelioid and giant cells in the histopathological tests of macroscopic responses to the experimental Mitsuda antigen, described as BT in table, in eight patients, was interpreted as an attempt of the respective organisms to develop cellular immunity.

The non-coincidence of the pattern of histopathological responses to the two antigens, experimental and standard leprosin, led us suppose that the antigen stimulus is different between them. The responses not showing the bacteria injected with experimental Mitsuda antigen suggest that the respective organisms may have acquired the ability to cause microorganism lysis.

Inoculation of placebo in other 28 patients of a group called "control" did not produce any responses, and a second testing with standard leprosin provided a positive macroscopic response in one patient.

The two groups of patients did not show any remarkable changes in the clinical course of the disease; they were affected with the usual leprosy reactions in the known rates. ❑

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MAILING ADDRESS:
Fundação Pró-Hansen
Fernando Amaro, 1116
80050-020 - Curitiba - PR
Tel./Fax: (41) 3263-2757
E-mail: fprohansen@onda.com.br