INTERNATIONAL JOURNAL OF LEPROSY

Volume 67, Number 4
Printed in the U.S.A.
(ISSN 0148-916X)

Serologic Response to Mycobacterial Proteins in Hansen's Patients During Multidrug Treatment[1]

Elsa Rada, Nacarid Aranzazu, Marian Ulrich, and Jacinto Convit[2]

Serologic studies have been widely done in leprosy using the bacterial cell wall component phenolic glycolipid (PGL-I) for purposes of diagnosis, classification in the disease spectrum, chemotherapy monitoring, detection of subclinical infection, prognosis and response to vaccines ([1, 4, 10, 16, 23]). The role which might be played by other structural components, individual *Mycobacterium leprae* proteins among them, could be very interesting for follow up of the behavior of antibodies directed to those proteins, both in serologic reactions and in cell-mediated immunity.

In previous studies we have reported on a group of 150 patients followed over 5–10 years through repeated determinations of anti-*M. leprae* PGL-I IgM antibody levels and studies of the lymphoproliferative responses toward various *M. leprae* antigens (whole bacilli, complete protein extract). Multidrug therapy (MDT) plus immunotherapy (IT) or MDT alone resulted in a statistically significant decrease in antibody levels in the multibacillary (MB) group at the end of 2 years of treatment, and those values continued decreasing in later evaluations ([15, 16]).

With the introduction of molecular biology technology and with monoclonal antibodies, it has been possible to obtain individual proteins from mycobacterial antigens ([2, 3, 8, 11, 20, 23]). A good knowledge of the humoral response of the host directed to specific *M. leprae* mycobacterial antigens, especially heat shock proteins, could be useful in vaccine preparation and epidemiological studies ([5]).

In the present study, we monitored IgG antibodies directed to mycobacterial proteins from *M. tuberculosis* (Mt 70), *M. bovis* (Mb 65), *M. leprae* (Ml 36, Ml 28, Ml 18, Ml 10) and the complete protein *M. leprae* antigen (MlSA) before starting MDT (year 0) and during and after completing treatment (years 2–3). The recombinant antigens used in these assays were obtained from the Recombinant Protein Bank of the World Health Organization.

MATERIALS AND METHODS

Patients. Patients were examined at the Clinical Section of the institute of Biomedicine, Caracas, Venezuela. The form of the disease in each patient was classified according to clinical, histopathological and bacteriological criteria as defined in the Ridley-Jopling scale ([18]). Originally, a sample of 15 multibacillary (MB) patients and 17 paucibacillary (PB) patients were evaluated. The follow-up process included 12 MB patients and 12 PB patients who were sampled before, during and after MDT. Only the data from the groups of 12 with all samples are reported in the results. All MB patients (80% men and 20% women) and PB patients (30% men and 70% women) were adults.

Six Mitsuda-positive and 10 Mitsuda-negative contacts were also evaluated. The positive contacts were personnel of the Institute of Biomedicine in frequent contact with patients over a period of 10 years or more. Negative contacts were household contacts of both MB and PB patients.

Antigens. The antigens used for this study were: soluble *M. leprae* extract MLSA obtained by rupturing bacilli with a

[1] Received for publication on 22 April 1999. Accepted for publication in revised form on 5 August 1999.

[2] E. Rada, M.Sc.; N. Aranzazu, M.D.; M. Ulrich, Ph.D.; J. Convit, M.D., Instituto de Biomedicina, Apartado 4043, Caracas 1010A, Venezuela.

Reprint request to Elsa Rada, M.Sc., at the above address or FAX: 58-2-861-1258; e-mail: erada@telcel.net.ve

TABLE 1. *Pre-treatment IgG antibody levels directed toward various mycobacterial proteins in the different forms of the disease and in contacts. (Serum diluted 1:200.)*

	No.	Mycobacterial protein antigens						
		Mt70	Mb65	Ml36	Ml28	Ml18	Ml10	MlSA
Multibacillary	12	0.629[a]	0.446	0.662	0.431	0.279	0.828	1.114
Paucibacillary	12	0.427	0.448	0.642	0.274	0.118	0.149	0.508
Mitsuda-positive contacts	6	0.883	0.343	0.052	0.384	0.193	0.099	0.202
Mitsuda-negative contacts	10	0.265	0.095	0.155	0.153	0.181	0.141	0.086

[a] Average OD at 490 nm.

French Press ([6]); recombinant 70 kDa *M. tuberculosis* antigen, 65 kDa *M. bovis* antigen and 36 kDa, 28 kDa 18 kDa and 10 kDa *M. leprae* antigens. All recombinant antigens were used at a 5-μg/ml concentration and the complete soluble *M. leprae* at 2 μg/ml according to standardization established in previous studies ([14]).

Serologic tests. IgG antibodies directed to the total *M. leprae* protein and the recombinant antigens mentioned above were determined by an ELISA ([14]). Each patient's serum, diluted 1:200, was tested individually during treatment. Circulating IgG values were expressed in optical density (OD) at 490 nm. Positive and negative control sera were included in each plate in order to control for possible nonspecific contaminants in the antigen preparations. Specific tests with an *Escherichia coli* control were not done because negative sera gave very low values and because standardization with an appropriate concentration would be somewhat empirical.

Data analysis. Data were expressed in percentage of positives considering as positive IgG antibodies that produced an OD 0.2 higher than the value established using MlSA in previous studies. This value is high for some of the recombinant proteins, but does not alter the interpretation of the data with respect to changes in antibody levels during follow up.

RESULTS

We used the ELISA technique to evaluate the antibody response toward the various mycobacterial proteins. All MB patients in the initial samples (N = 12) showed antimycobacterial protein antibodies, mainly and with greater intensity against MlSA, Ml20, Mt70, Ml36 kDa and, to a lesser degree, Ml65, Ml18 and Ml28 kDa, but always demonstrating a difference in response in relation to the PB group (Table 1). The optical densities and numbers of positive samples are shown in Tables 2 and 3.

The results shown in this study reveal a clear decline in IgG antibodies directed toward mycobacterial proteins in the 12 MB patients when they were treated with MDT. Initially, we found strong reactivity toward complete cytosolic protein and *M. leprae* membrane. The most reactive recombinant proteins in MB patients were Ml10, Ml36, Mt70 kDa and, finally, Ml18 kDa when compared to the PB group (Fig. 1). After treatment was completed, all LL and BL patients showed low or zero levels as compared with the initial values before starting treatment (Fig. 2). The 12 PB patients were

TABLE 2. *IgG positivity toward mycobacterial proteins before and after MDT in 12 MB patients.*

OD (490 nm)	Mycobacterial protein antigens													
	Mt70		Mb65		Ml36		Ml28		Ml18		Ml10		MlSA	
	i[a]	f[b]	i	f	i	f	i	f	i	f	i	f	i	f
>1	2/12	0	1/12	0	1/12	0	0	0	0	0	4/12	2/12	5/12	0
0.2–0.99	5/12	2/12	8/12	0	9/12	4/12	12/12	0	9/12	3/12	3/12	1/12	7/12	5/12
0.0–0.19	5/12	10/12	3/12	12/12	2/12	8/12	0	12/12	3/12	9/12	5/12	9/12	0	7/12

[a] i = OD at beginning of treatment.
[b] f = OD at end of treatment (2 years).

TABLE 3. *IgG positivity toward mycobacterial proteins before and after MDT in 12 PB patients.*

OD (490 nm)	Mycobacterial protein antigens													
	Mt70		Mb65		Ml36		Ml28		Ml18		Ml10		MlSA	
	i[a]	f[b]	i	f	i	f	i	f	i	f	i	f	i	f
>1	2/12	0	0	0	0	0	0	0	0	0	0	0	2/12	1/12
0.2–0.99	8/12	6/12	9/12	9	11/12	8/12	7/12	2/12	2/12	6/12	2/12	3/12	4/12	6/12
0–0.19	2/12	6/12	3/12	3/12	1/12	4/12	5/12	10/12	10/12	6/12	10/12	9/12	6/12	5/12

[a] i = OD at beginning of treatment.
[b] f = OD at end of treatment (1 year).

followed up for only 1 year since the duration of their treatment is shorter than that of patients with a high bacterial load (Fig. 3).

DISCUSSION

According to WHO treatment guidelines, MB patients were followed up for a 2- to 3-year treatment period and PB patients were evaluated for a 1-year treatment period. All 12 patients from each group were followed individually until completion of treatment, contrary to a previous study ([14]) in which we evaluated a pool of sera from patients belonging to each type of the disease. IgG antibodies directed toward heat shock protein (hsp) Ml10, Mt70, Mb65 and Ml28 showed high levels. Other workers have reported that two of these hsp, Mt70 and Ml65, are not good specific markers in the disease spectrum ([9]). We also detected IgG antibodies directed toward these proteins in MB and PB patients and in healthy Mitsuda-positive contacts, but not in Mitsuda-negative contacts.

Heat or stress shock proteins are induced by a variety of stimuli and one of them is

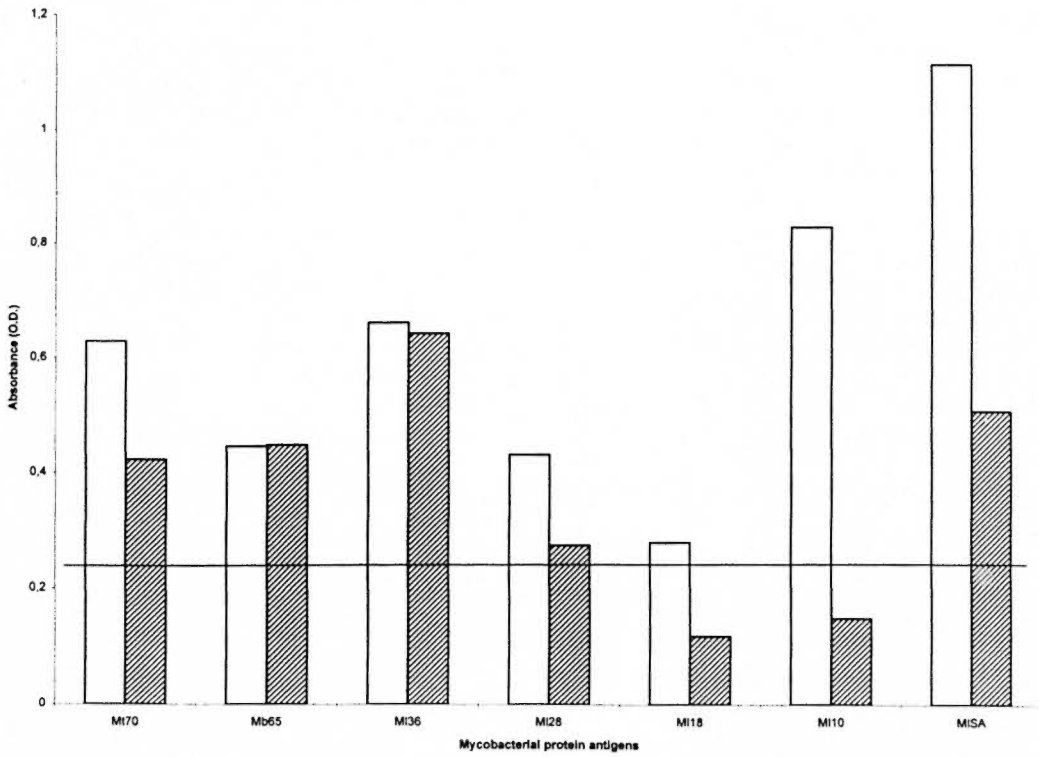

FIG. 1. Comparison of the initial serologic responses (year 0) in multibacillary (□) and paucibacillary (▨) patients.

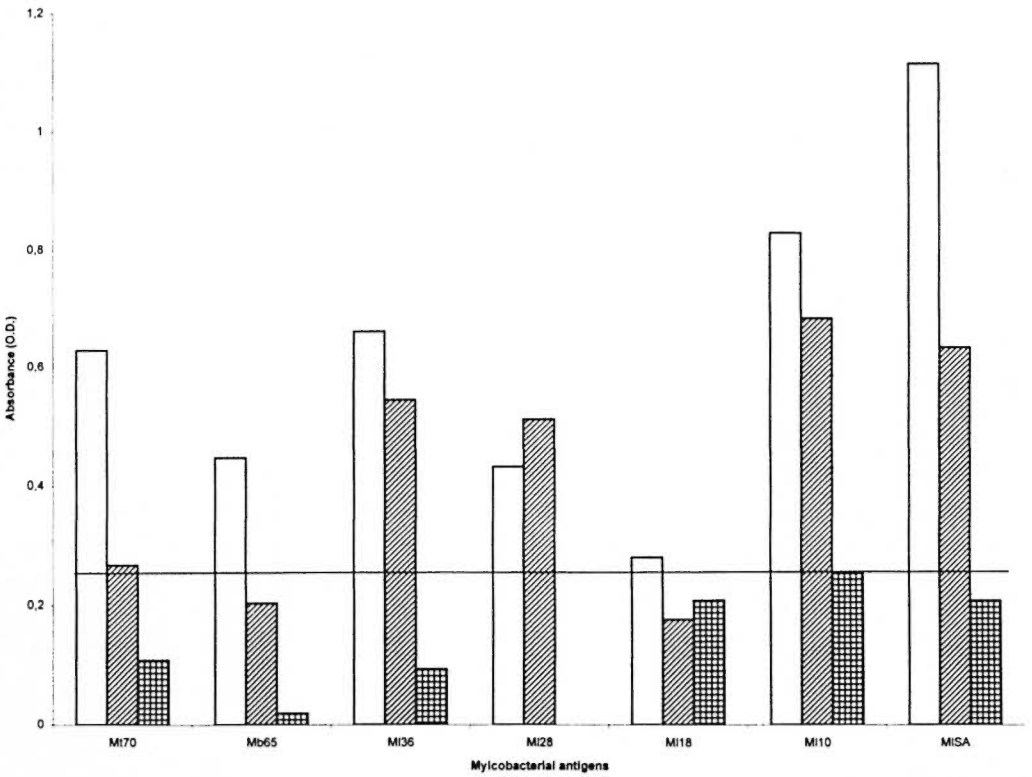

FIG. 2. Serologic follow up in multibacillary patients treated with multidrug therapy. □ = year 0; ▨ = year 1; ▦ = year 2.

mycobacterial infection ([12]). They also have great homology with gene sequences that code for these proteins in other phylogenetically separated species such as *E. coli* and *Homo sapiens* ([7]). These proteins are constitutively expressed, and one of the functions in which they are involved is protein translocation and folding at an intracellular level. The average decrease in antibody levels against heat shock proteins during therapy is almost twice as accelerated in MB patients as compared with the levels of antibodies directed toward other proteins (MI10, MI36 and MI28 kDa).

Only the MI10 kDa protein establishes differences in the group of patients studied, on the basis of the difference in bacillary load between the two groups (MB and PB). It is considered a promising antigen for skin testing and the most adequate for measuring leprosy incidence. Quantitatively, it is the most abundant, representing around 1% of the bacterial mass ([19]). The MI10 protein, together with the MI36 and MI28 proteins, are the ones against which IgG antibodies persist for longer periods in MB patients during MDT.

It is notable that the only serologic response directed toward recombinant proteins which increased during treatment was the one directed toward Mb65, and this was observed in PB patients with low bacillary loads. This protein could be involved in the immunopathological processes which characterize this group of patients, among them peripheral nerve damage, which is the most important cause of deformities in leprosy.

MI36 protein is a disease marker; it is a specific immunodominant protein with proline-rich regions where, according to other authors ([8, 22]), 100% of MB and 91% of PB patients present positive serology. In our study, MB patients showed a high percentage of positives (83%). Among PB patients, 91.6% were positive. This protein could be used to detect high-risk persons, which could be very useful for leprosy control programs. The antibody decrease against MI36 is much faster in the PB group compared with the high bacillary

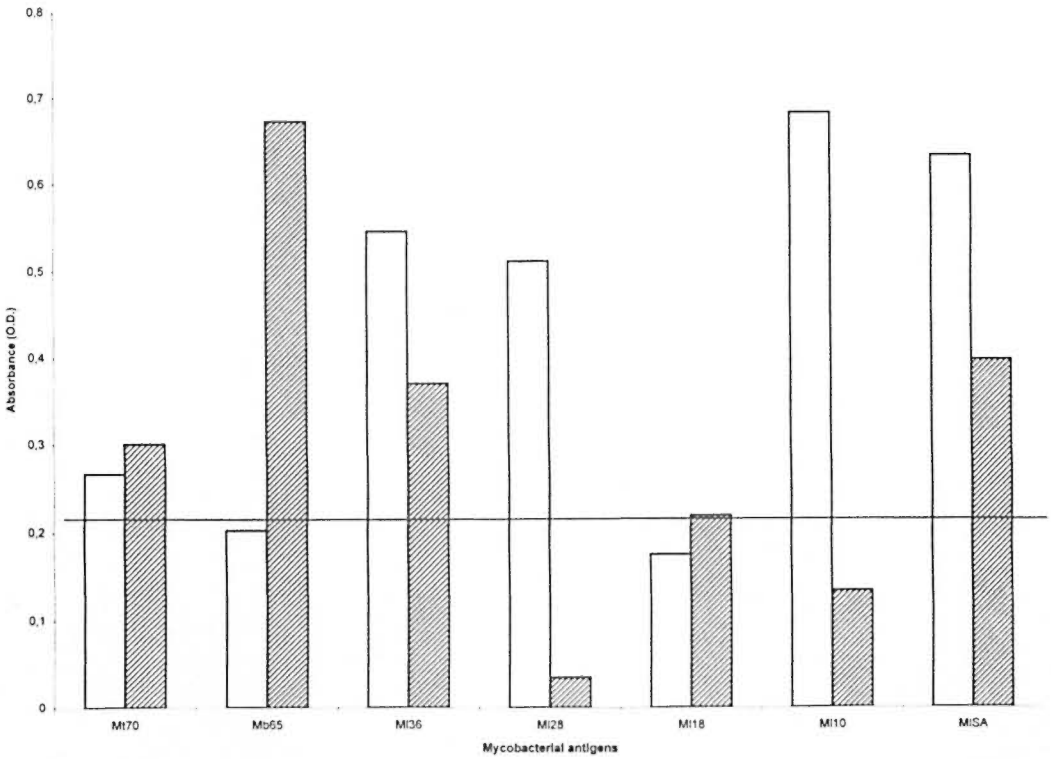

FIG. 3. Comparison of serologic responses after 1 year of treatment in multibacillary (□) and paucibacillary (▨) patients.

load in MB patients. This is the only protein which does not show crossreactivity with autoimmune processes; whereas the other proteins tested in this study reflect the presence of antibodies against kDa 70 (mean OD = 0.873), 65 (0.823), 36 (0.060), 28 (0.387), 18 (0.519) and 10 (0.782) mycobacterial proteins in sera from patients with autoimmune diseases (collagen type diseases, lupus erythematosus, scleroderma).

The Ml28 kDa protein is an enzyme, superoxide dismutase (SOD), secreted in culture medium by growing bacilli, and there is preliminary evidence that the 28 kDa protein can be secreted in infected tissues ([20, 24]) in which the bacterium occupies an environment in which it is submitted to severe oxidative stress. The importance of this enzyme as a cytoprotective immunodominant antigen lies in maintaining mycobacterial survival within the macrophage. Antibodies against this protein were not detected in lupus erythematosus but were present in other autoimmune processes, such as scleroderma. Mitsuda-positive contacts showed 0.384 OD IgG antibody levels toward Ml28 as compared with Mitsuda-negative contacts who only showed 0.153 OD. It is interesting to note that detectable excretion of this protein by the presence of antibodies was zero in MB patients after completing 2 years of MDT. Mitsuda-positive contacts also showed high levels of antibodies directed toward the 28 kDa protein compared with Mitsuda-negative contacts.

Once more we would stress the high homology with human heat shock proteins where stress proteins protect cells from the toxic effects of oxidative intermediates and mitochondria are their selective target ([13]). SOD is a bacterial defense component against the intracellular attack of phagocyte cells against mycobacteria ([20]). The amino-acid sequence of this protein shows 67% homology with human SOD.

Regarding Ml18 kDa protein, the antibody response is much higher in MB as compared with PB patients; it does not disappear completely but, rather, maintains low levels. This protein has been consid-

ered as an important antigen for T lymphocyte presentation ([25]).

Total proteins represent the combination of all the individual proteins and, therefore, reflect a high value in antibody detection, even though used at a lower (2 µg/ml) concentration. Thus, the variability of the responses may be due to the role that each of the individual proteins plays.

In summary, we found that IgG antibody levels toward mycobacterial proteins (MlSA, Ml36, Ml28, Ml10, Ml18) in most patients are expressed in relation to the size of the bacillary load, and the response to the 70 and 65 kDa proteins reflects the high homology they show with human 70 and 65 kDa proteins in patients previously sensitized to mycobacterial antigens. The marked decrease in antibody levels once more demonstrates the efficacy of MDT administered to these patients.

We cannot overlook certain other mycobacterial proteins, such as those belonging to the group of proteins secreted by *M. bovis*, among them 30, 31 and 32 kDa proteins, which could be modulating the host response since MB patients presented high levels of IgG antibodies directed to this group of proteins compared with PB patients and controls using the Western blot technique ([17]).

SUMMARY

Humoral immune responses were studied in 24 leprosy patients treated with multidrug therapy (MDT) and 16 contacts. The patients were monitored for 2 to 3 years with repeated determination of IgG antibody levels directed to different mycobacterial proteins (*Mycobacterium tuberculosis*, Mt70; *M. bovis*, Mb65; *M. leprae*, Ml36, 28, 18, 10 kDa, and the complete protein *M. leprae* extract, MLSA). All recombinant antigens were used at 5 µg/ml concentration and the complete soluble *M. leprae* extract at 2 µg/ml. The results shown in this study reveal a clear decline in IgG antibodies directed toward mycobacterial proteins in the 12 multibacillary (MB) patients when they were submitted to MDT. Initially we found strong reactivity toward complete cytosolic protein and *M. leprae* membrane protein. The most reactive recombinant proteins in MB patients were Ml10, Ml36, Mt70 kDa and, finally, Ml18 kDa when compared to the paucibacillary (PB) group. After treatment was completed all lepromatous and borderline lepromatous patients showed low or undetectable levels as compared with their initial values before starting treatment.

RESUMEN

Se estudiaron las respuestas humorales en 24 pacientes con lepra tratados con poliquimioterapia (PQT) y en 16 contactos sanos. Los pacientes fueron estudiados durante 2 ó 3 años con relación a la presencia de anticuerpos IgG contra diferentes proteinas microbacterianas (*Mycobacterium tuberculosis*, Mt70; *M. bovis*, Mb65; *M. leprae*, Ml36, 28, 18, 10 kDa, y un extracto total de *M. leprae*, MLSA). Todos los antigenos recombinantes fueron usados a la concentración de 5 µg por ml y el extracto soluble MLSA a 2 µg por ml. Los resultados de este estudio mostraron una clara disminución en los anticuerpos IgG contra las proteinas micobacterianas en los 12 pacientes multibacilares (MB) sujetos al tratamiento con PQT. Inicialmente se encontró una fuerte reactividad hacia las proteinas citosólicas y de membrana de *M. leprae*. Las proteinas recombinantes más reactivas en los pacientes multibacilares fueron Ml10, Ml36, Mt70 y finalmente Ml18. En comparación con los niveles de anticuerpos encontrados al inicio, los niveles de anticuerpos encontrados después de completar el tratamiento fueron bajos o no detectables.

RÉSUMÉ

La réponse immunitaire à médiation humorale fut étudiée chez 24 patients traités par la polychimiothérapie (PCT) et chez 16 personnes en contact avec ces derniers. Les patients furent suivis pendant au moins 2 à 3 années, période au cours de laquelle les niveaux d'anticorps de type IgG dirigés contre des protéines variées d'origine mycobactérienne (*Mycobacterium tuberculosis*, Mt 70; *M. bovis*, Mb 65; *M. leprae*, Ml 36, 28, 18, 10 kDa, et l'extrait complet soluble de *M. leprae* MLSA) furent déterminés de façon répétée. Tous les antigènes recombinants furent utilisés à une concentration de 5 (µg/ml et l'extrait complet soluble de *M. leprae* à 2 (µg/ml. Les résultats présentés dans cette étude ont montré une nette diminution des anticorps de type IgG dirigés contre les protéines d'origine mycobactérienne chez les 12 patients multibacillaires (MB) lorsqu'ils débutèrent la PCT. Au début, nous avons trouvé une forte réactivité contre la protéine cytosolique complête et la protéine membranaire de *M. leprae*. Les protéines recombinantes les plus réactives chez les patients MB étaient Ml 10, Ml 36, Mt 70 kDa et, finalement Ml 18 kDa, comparé au groupe des patients paucibacillaires (PB). Après la fin du traitement, tous les patients lépromateux et lépromateux borderline ont montré des niveaux faibles ou non-détectables, comparés aux valeurs initiales mesurées avant le traitement.

Acknowledgment. We wish to thank the WHO Recombinant Protein Bank for supplying the proteins used in this study.

REFERENCES

1. Bach, M.-A., Wallach, D., Flageul, B., Hoffenbach, A. and Cottenot, F. Antibodies to phenolic glycolipid-I and to whole *Mycobacterium leprae* in leprosy patients: evolution during therapy. Int. J. Lepr. **54** (1986) 256–267.
2. Booth, R. J., Harris, D. P., Love, J. M. and Watson, J. D. Antigenic properties of *Mycobacterium leprae,* complete sequence of the gene for the 18 kDa protein. J. Immunol. **140** (1998) 597–601.
3. Buchanan, T. M., Nomaguchi, H., Anderson, D. C., Young, R. A., Gillis, T. P., Britton, W. J., Ivanyi, J., Kolk, A. H., Closs, O., Bloom, B. R. and Mehra, V. Characterization of antibody-reactive epitopes on the 65-kilodalton protein of *Mycobacterium leprae.* Infect. Immun. **55** (1987) 1000–1003.
4. Chanteau, S., Cartel, I. L., Celerier, P., Plichart, R., Desforges, S. and Roux, J. PGL-I antigen and antibodies detection in leprosy patients: evolution under chemotherapy. Int. J. Lepr. **57** (1989) 735–743.
5. Choudhuri, K. The immunology of leprosy; unraveling an enigma. Int. J. Lepr. **63** (1995) 430–447.
6. Convit, J., Aranzazu, N., Ulrich, M., Pinardi, M. E., Reyes, O. and Alvarado, J. Immunotherapy with a mixture of *Mycobacterium leprae* and BCG in different forms of leprosy and Mitsuda-negative contacts. Int. J. Lepr. **50** (1982) 415–424.
7. Garsia, R., Hellqvist, L., Booth, R., Radford, A., Britton, W., Astbury, L., Trent, R. and Basten, A. Homology of the 70-kilodalton antigens from *Mycobacterium leprae* and *Mycobacterium bovis* with the *Mycobacterium tuberculosis* 71-kilodalton antigen and with the conserved heat shock protein 70 of eucaryotes. Infect. Immun. **57** (1989) 204–212.
8. Klatser, P. R., de Witt, M. Y. and Kolk, A. H. J. An ELISA-inhibition test using monoclonal antibody for the serology of leprosy. Clin. Exp. Immunol. **62** (1985) 468–473.
9. Launois, P., Niang, M. N., Drowart, A., Van Vooren, J. P., Sarthou, J. L., Lalu, T., Millan, J. and Huygen, K. IgG response to purified 65- and 70-kDa mycobacterial heat shock proteins and to antigen 85 in leprosy. Int. J. Lepr. **62** (1994) 48–54.
10. Meeker, H. C., Shuller-Levis, G., Fusco, F., Giardina-Becket, M.-A., Sersen, E. and Levis, W. R. Sequential monitoring of leprosy patients with serum antibody levels to phenolic glycolipid-I, a synthetic analog of phenolic glycolipid-I, and mycobacterial lipoarabinomannan. Int. J. Lepr. **58** (1990) 503–511.
11. Mehra, V., Bloom, B. R., Bajardi, A. C., Grisso, C. L., Sieling, P. A., Alland, D., Convit, J., Fan, X. D., Hunter, S. W., Brennan, P. J., Rea, T. H. and Modlin, R. L. A major T-cell antigen of *Mycobacterium leprae* is a 10 kD heat-shock cognate protein. J. Exp. Med. **175** (1992) 275–284.
12. Mistry, Y., Young, D. and Mukherjee, R. Hsp70 synthesis in Schwann cells in response to heat shock and infection with *Mycobacterium leprae.* Infect. Immun. **60** (1992) 3105–3110.
13. Polla, B. S., Katengwa, S., Francois, D., Salvioli, S., Franceschi, C., Marsac, C. and Cossarizza, A. Mitochondria are selective targets for the protective effects of heat shock against oxidative injury. Proc. Natl. Acad. Sci. U.S.A. **93** (1996) 6458–6463.
14. Rada, E., Aranzazu, N. and Convit, J. Immunological reactions to mycobacterial proteins in the spectrum of leprosy. Int. J. Lepr. **65** (1997) 497–500.
15. Rada, E., Ulrich, M., Aranzazu, N., Rodríguez, V., Centeno, M., González, I., Santaella, C., Rodríguez, M. and Convit, J. A follow-up study of multibacillary Hansen's disease patients treated with multidrug therapy (MDT) or MDT + immunotherapy (IMT). Int. J. Lepr. **65** (1997) 320–327.
16. Rada, E., Ulrich, M., Aranzazu, N., Santaella, C., Gallinoto, M. E., Centeno, M., Rodríguez, V. and Convit, J. A longitudinal study of immunologic reactivity in leprosy patients treated with immunotherapy. Int. J. Lepr. **62** (1994) 552–558.
17. Rada-Schlaefli, E., Santaella, C., Aranzazu, N. and Convit, J. Detection of antibodies toward secreted mycobacterial antigen 85 in untreated leprosy patients' sera. (Letter) Int. J. Lepr. **67** (1999) 167–170.
18. Ridley, D. S. and Jopling, W. H. Classification of leprosy according to immunity; a five-group system. Int. J. Lepr. **34** (1966) 255–273.
19. Rivoire, B., Pessolani, M. C., Bozic, C. M., Hunter, S. W., Hefta, S. A., Mehra, V. and Brennan, P. Chemical definition, cloning, and expression of the major protein of the leprosy bacillus. Infect. Immun. **62** (1994) 2417–2425.
20. Thangaraj, H. S., Lamb, F. I., Davis, E. O., Jenner, P. J., Jeyakumar, L. H. and Colston, M. J. Identification, sequencing, and expression of *Mycobacterium leprae* superoxide dismutase, a major antigen. Infect. Immun. **58** (1990) 1937–1942.
21. Thole, J. E. R., Janson, A. A. M., Kifle, A., Howe, R. C., McLean, K., Nurilygn, A., Filley, E., Shannon, E. J., Bulla, G. J., Hermans, J., de Vries, R. R. P., Frommel, D. and Rinke de Wit, T. Analysis of T-cell and B-cell responses to recombinant *M. leprae* antigens in leprosy patients and healthy contacts: significant T-cell responses to antigens in *M. leprae* nonresponders. Int. J. Lepr. **63** (1995) 369–380.

22. THOLE, J. E. R., STABEL, F. E. M., SUYKERBUYK, M. E. G., DE WITT, M. Y. L., KLATSER, P. R., KOLK, A. H. J. and HARTSKEERL, R. A. A major immunogenic 36,000-molecular-weight antigen from *Mycobacterium leprae* contains an immunoreactive region of proline-rich repeats. Infect. Immun. **58** (1990) 80–87.
23. ULRICH, M., SMITH, P., SAMPSON, C., ZUÑIGA, M., CENTENO, M., GARCÍA, V., MANRIQUE, X., SALGADO, A. and CONVIT, J. IgM antibodies to native phenolic glycolipid-I in contacts of leprosy patients in Venezuela; epidemiological observations and a prospective study of the risk of leprosy. Int. J. Lepr. **59** (1991) 405–415.
24. WATSON, J. D. Leprosy: understanding protective immunity. Immunol. Today **10** (1989) 218–221.
25. YOUNG, D. B., KAUFMANN, S. H. E., HERMANS, P. W. M. and THOLE, J. E. R. Mycobacterial protein antigens. Mol. Microbiol. **6** (1992) 133–145.