Detection of shared antigenic determinants between *Mycobacterium leprae* heat shock protein 65 and human heat shock protein 60

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SUMMARY

In these studies, it was investigated whether M. leprae and man share antigenic determinants which may be located on Heat Shock Proteins (HSPs), and which may be responsible for tissue destruction. Using immunoperoxidase single-staining technique on cryostat sections it was observed that three antibodies which are directed against HSP 60 (polyclonal antibodies SPA 804 and SPA 805 and monoclonal antibody SPA 807) probably reacted specifically with macrophages and epitheloid cells in leprosy skin sections. On Western Blotting, it was observed that the antibodies against human HSP 60 and monoclonal antibodies (MoAbs) against M. leprae HSP 65 (F 47-10, F 67-18, F 88-1) all reacted strongly with sonicated M. *leprae* proteins with a molecular mass of 65 kDa indicating similarity of some antigenic determinants between human HSP 60 and M. *leprae* HSP 65. Subsequently, a comparative immunohistochemical study of the staining patterns of antibodies against human HSP 60 and antibodies against M. leprae HSP 65 using cryostat skin sections of paucibacillary (PB) leprosy, multibacillary (MB) leprosy and other granulomatous skin disorders revealed that the MoAbs F 47-10 and F 67-18 reacted only weakly with the granulomas in PB leprosy and in other granulomatous skin diseases, but stained MB leprosy granuloma strongly. The MoAb F 88-1 and the polyclonal antibodies SPA 804, SPA 805 and the MoAb SPA 807 stained granulomas of PB patients and of other granulomatous skin disorders with the same intensity as that MB patients. Using a double-staining technique, it was observed that the antigenic determinants recognized by the MoAb against human HSP 60 (SPA 807) and the MoAbs against M. leprae HSP 65 (F 67-18, F 47-10, F 88-1) were mostly located in the macrophages. These findings do not contradict our suggestion, that similarities between antigenic determinants on Heat Shock Proteins of M. leprae and the human host may be at least in part responsible for the induction of an autoimmune reaction causing granuloma formation with subsequent tissue damage in leprosy. The results of this study also indicated that some of these determinants are probably located on HSP 60. A similar explanation possibly applies to the findings in the other granulomatous diseases, e.g. sarcoidosis probably mycobacterial induced and necrobiosis lipoidica related to diabetis, in which antigenic similarities between bacterial HSP 65 and human HSP 60 are considered to play a part

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INTRODUCTION

The sharing of determinants by host and parasite was first described by Damian in 1964 (DAMIAN, 1964). This phenomenon, called "antigenic mimicry" has been suggested as a possibible explanation for the clinical and the immunopathological manisfetation of leprosy (NAAFS et. al. 1990; VAN DEN AKKER et a., 199; RAMBUKKANA et al., 1992)

Common antigenic determinants of *Mycobacterium* (M).Ieprae and host may lead either to a state of tolerance or to a state of autoimmunity. On the one hand, antigenic mimicry enables the parasite to survive in the human tissues because the immune system fails to recognize the antigens as "non-self". Such a situation may occur in Lepromatous (LL) Leprosy when the patient's tissues are teeming with mycobacteria, but a specific cell-mediated immunity (CMI) towards M. /eprae antigens is absent. On the other hand, shared antigenic determinants between M. leprae and the host may induce an autoimmune reaction against the host's own antigens. This could explain the immune responses observed in Tuberculoid (TT) Leprosy and during a Reversal Reaction (RR). A high CM! towards M. leprae antigens then can be shown, and a destructive granuloma formation with oedema in the dermis and in the superficial peripheral nerves occurs. However, the antigens responsible still remain unknown.

In previous studies it was reported that M. *leprae,* environmental mycobacteria and human tissue (skin and nerve) have antigenic determinants in common (NAAFS et al., 1990; VAN DEN AKKER et al., 1992). These findings supported the notion that autoimmunity may be involved in the tissue damage in leprosy Anti M. *leprae* monodonal antibodies (MoAbs) were first shown to cross-react with human skin using immunohistochemical methods (NAAFS et al., 1990). Subsequently, similarity between mycobacterial antigens and determinants in normal human epidermis were reported using an immunoblotting system (VAN DEN AKKER et al., 1992). It appeared that most of the anti-M. /eprae MoAbs used in those studies were directed against antigenic derminants belonging to the evolutionarily conserved group of proteins called the "Heat Shock Proteins" (HSPs). Heat Shock Proteins or 'stress proteins" are synthesized by eukaryotes and prokaryotes as a physiological response to stressful events. These proteins are believed to perform autoprotective functions which are essential for the survival of the cell (POLLA, 1991; 1991). Heat Shock Proteins can be classified into five major families based upon their apparent molecular mass: HSP 100, HSP 90, HSP 70, HSP 60 and HSP 27 (MAYTIN, 1995). Gene sequences of HSPs between man and (pathogenic) micro-organisms appear to show remarkable similarities (DUDANI et al., 1989; GARSIA et al., 1989). Moreover, highly immunodominant antigens of M. *leprae* have been recognized as homologues of stress proteins (YOUNG et al., 1987).

The aim of the present study was to extend the previous mentioned studies (NAAFS et al., 1990; VAN DEN AKKER et al., 1992) in order to investigate whether some of the antigenic determinants shared by M. *leprae* and the human host may be located on Heat Shock Proteins. Using a panel of commercially available antibodies against HSPs of 4 different families and a panel of antibodies against M. *leprae* antigenic determinants the following studies were undertaken:

A. a study on the specific reactivity of the anti-human HSP antibodies with antigenic determinants of M. leprae in multibacillary leprosy skin sections by means of immunoperoxidase staining technique on cryostat sections and by Western Blot technique using sonicated M. leprae as antigen;
B. a comparative study on the reactivity patterns of these antibodies with granuloma of paucibacillary and multibacillary leprosy and other granulomatous skin disorders;
C. the in situ detection of antigenic determinants of human HSP 60 and M. leprae HSP 65 in macrophages in skin tissues of paucibacillary (PB) and multibacillary (MB) leprosy and other granulomatous skin disorders using a double-staining technique.

The results of these studies showed that some of the antigenic determinants shared between M. /eprae and the human host which may be in part responsible for the leprosy pathology are located on the Heat Shock Protein 60.

MATERIAL AND METHODS

Biopsy specimens

Skin biopsy specimens from 9 patients with leprosy and 5 patients with granulomatous skin disorders were obtained from the outpatient clinics of the departments of Dermato-Venereology of the Academic Hospital Dijkzigt in Rotterdam (The Netherlands) and the Academic Hospital in Paramaribo (Surinam). Normal human skin was obtained from two patients undergoing breast reduction at the department of Plastic and Reconstructive Surgery of the Rotterdam hospital. The specimens were snap frozen in liquid nitrogen and stored at -70°C until use.

The diagnosis was confirmed histologically by examination of hematoxylin and eosin (H&E)-stained sections. The presence of acid fast bacilli in the leprosy skin sections was visualized by using the Fite-Faraco-Wade staining method in order to determine the bacillary index (B.I.).

Immunohistochemistry

Single-staining method.

Cryostat sections were stained using the Avidin-Biotin Complex method as described elsewhere (CATTORETTI et al., 1988). Briefly, 5pm cryostat sections were placed on glass slides, air dried and fixed in aceton for 10 minutes at room temperature. The sections were then rinsed in phosphate- buffered saline (PBS, pH 7.4) containing 0.05% Tween and were pre-incubated with 5% bovine serum albumine in PBS for 10 minutes to minimize non-specific staining. Subsequently, the sections were incubated for 60 minutes with an optimal dilution of the primary antibody. The sections were then rinsed twice with PBS containing 0.05% Tween and incubated for 30 minutes with 1:200 diluted goat anti- mouse biotin containing 3% normal goat serum (NGS) and 3% normal human serum (NHS). The sections were again rinsed twice in PBS containing 0.05% Tween and incubated for 30 minutes with the Avidin-Biotin complex diluted 1:1,200 (DAKO-kits, DAKO AIS, Denmark) and then rinsed twice with PBS (without Tween). The peroxidase reaction was developed by incubating the sections with 3,3- diaminobenzidine (DAB) at a concentration of 0.75 mg/ml and 0.25% hydrogen peroxide for 7 minutes in the dark. The sections were rinsed with tap water and counter-stained for 60 seconds in hematoxylin (Mayer) and rinsed again with tap water for 5 minutes. The sections were mounted in Malinol after a step-wise dehydration in alcohol and xylol.

Negative controls comprised the omission of primary antibody.

Double-staining method.

Cryostats sections of 5 pm were cut and fixed for 10 minutes in aceton, dried and rinsed in PBS. They were incubated for one hour with the first monoclonal antibody. After rinsing with PBS, they were incubated with 1:200 diluted biotinylated goat anti-mouse containing 3% normal human serum and 3% normal goat serum for 30 minutes. After rinsing in PBS, sections were incubated with 1:2 diluted streptavidin—Galactosidase (Biogenex Laboratories, San Ramon, USA) for 30 minutes. Then rinsing with PBS followed. The -Galactosidase reaction was developed with the -Galactosidase substrate (0.72% Ferry/Ferro cyanide, 2.5% 5-bromo-4-chloro-3-indoly1 -galacoside, 0.11% mgC12 in PBS) for 30-45 minutes at 37°C. The sections were rinsed in PBS for 15 minutes, and were incubated with the second monoclonal antibody for one hour. After rinsing with PBS, sections were incubated with 1:50 diluted rabbit anti-mouse containing 1:50 diluted normal human serum and 1:50 diluted normal rabbit serum for 30 minutes. Sections were rinsed with PBS and incubated with 1:100 dilution of alkaline phosphatase anti-alkaline phosphatase (APAAP) for 45 minutes. After rinsing with PBS, sections were placed in 0.2M Tris-HCI pH 8.0 for at least 5 minutes. The alkaline phosphatase reaction was developed by incubating the sections in the AP-substrate (150 pl New Fuchsine, 150 pl NaNO3, 18 mg Naphtol AS-MX phosphate and 15 mg Levamisol in 70 ml 0.2M Tris-HCI pH 8.0) for 30 minutes in the dark at room temperature. The sections were rinsed in PBS and embedded in Kaiser's glycerol-gelatine.

Negative controls comprised the omission of primary and secondary antibodies. The percentage of double-stained cells in the granuloma was microscopically estimated by two independent observers.

Primary antibodies

Monoclonal and polyclonal antibodies against human heat shock proteins were purchased from StressGen, Victoria, Canada. Their characteristics are listed in Table 1.

Optimal dilution for each of the anti-human HSP MoAbs was established using granuloma in the skin of multibacillary leprosy patients since antigenic determinants of *M. leprae* are most likely to be abundantly present at this end of the leprosy spectrum. By stepwise increments of the dilutions of each antibody, those antibodies which stained the granuloma positively but did not stain the epidermis were regarded as useful for the studies B and C.

Three mouse monoclonal antibodies raised against three different epitopes of the 65 kD M. *leprae* protein were available (KOLK et al., 1984) (Table 2). The monoclonal antibody against the macrophage marker CD68 (KP1) was purchased from DAKO, Denmark (PULFORD et al., 1989).

The specificity of the monoclonal antibody against the macrophage marker CD68 (KP1) was confirmed by titration on multibacillar leprosy skin tissues. The optimal dilution was 1:8,000.

The stainings of the granulomas and the epidermis were graded as follows:

(-) negative, (+/-) doubtful, (+) weakly positive, (+ +) moderately positive, H-++) strongly positive and H- +++1 very strongly positive.

M. leprae sonicate

Sonic extracts were prepared by breaking the bactena in a French press and sonicating them at 4°C. M. /eprae were purified from infected armadillo liver (DAS et al., 1982). Sonicated M. *leprae* (10mg/m1) was provided by Dr. RR. Klatser, N.H. Swellengrebel Laboratory of Tropical Hygiene, Royal Tropical Institute, Amsterdam, The Netherlands.

Table 1. Antibodies against Heat Shock Proteins (StressGen)

Product name	Immunoglobulin subclass	Specificity	Dilution in immunohistochemistry
SPA 820'	IgG1 mouse monoclonal	anti-human HSP 70 constitutive & inducible forms	1:500
SPA 815'	IgG1 mouse monoclonal	anti-human HSP 70 constitutive form	1:4000
SPA 8101	IgG1 mouse monoclonal	anti-human HSP 70 inducible form	1:500
SPA 830'	IgG1 mouse monoclonal	anti-human HSP 90	1:2000
SPA 8003	IgG1 mouse monoclonal	anti-human HSP 27	1:4000
SPA 804'	rabbit polyclonal	anti-HSP60 of cyanobacteria	1:4000
SPA 8055	rabbit polyclonal	anti-HSP60 of H. virescens	1:4000
SPA 807'	IgG1 mouse monoclonal	anti-human HSP60	1:500

[1]BEHNCKEet al., 1994;TRAUTINGERet al., 1993 [2]DALMAN et al., 1989. [3]CIOCCA et al., 1982. [4]WEBB et al., 1990.[5]MILLER et al., 1986.[6]800G et al., 1992.

Table 2. Specificities of mouse monoclonal antibodies against different epitopes of M. leprae HSP 65 (KOLK et al., 1984)

Product name	Iso-type	Dilution in immunohistochemistry
F47-1O	IgG1	1:3000
F 67-18	IgG1	1:1500
F 88-1	IgG2a	1:3000

Sodium dodecyi sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

M. leprae sonicate (10 mg/m1) at a dilution of 1:4 was subjected to SDS-PAGE using a 12% separating gel with 0.1% SDS in a discontinuous Tris-buffer system as described by Laemmli et al(LAEMMLI, 1970). Diluted M. leprae sonicate was treated with 5% 2- mercaptoethanol and 2% SDS and boiled in a water bath at 100°C for 4 minutes.

A mixture of pre-stained proteins consisting of phosphorylase B (94 kDa), bovine serum albumine (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30.1 kDa), soybean trypsin inhibitor (20.1 kDa) and -lactalbumin (14.4 kDa) was used as marker (Pharmacia Biotech, Uppsala, Sweden).

Western Blot

The separated M. leprae proteins in the gel were electrophoretically blotted (TOWBIN et al., 1979) onto a nitrocellulose membrane using a BioRad Mini Trans-Blot Electrophoretic Transfer Cell system (BioRad Laboratories, Venendaal, The Netherlands). After blotting, nitrocellulose strips were blocked for 30 minutes with PBS containing 0.3% Tween at 37°C. The strips were then washed three times for 5 minutes each with PBS containing 0.05% Tween at room temperature. Immunostaining was performed using the ImmunoGold Silver Staining method, according to the instructions by the manufacturer (Amersham Life Science, Amersham, UK). The strips were incubated with the primary

antibody and 1% normal goat serum diluted (1:200) in PBS containing 0.05% Tween for 2 hours. Then they were washed three times for 5 minutes each with PBS containing 0.05% Tween. Subsequently, the strips were incubated in a solution containing gold-labeled goat anti-mouse or gold-labeled anti-rabbit monoclonal antibody (AuroProbe BL plus, Janssen Life Science, Geel, Belgium) (1:100), Gelatin (1:20) and PBS with 0.05% Tween for 2 hours. After this, the strips were again washed three times for 5 minutes each with PBS containing 0.05% Tween. The strips were then washed for 2 minutes in distilled water. Finally, equal volumes of 'Enhancer" (Amersham Life Science, Amersham, UK) and 'Initiator" (Amersham Life Science, Amersham, UK) solutions were mixed and added to the strips to enhance the gold signal. Reaction took place between 15-20 minutes. The strips were then washed three times for 10 minutes each in distilled water and dried between filter papers.

Results

A. Specificity of the anti-Heat Shock Protein antibodies Immunohistochemistry

The reactivity of the granuloma and the epidermis of MB leprosy skin sections of five patients using the optimal dilutions of the antibodies are shown in Table 3.

No specific staining of the granulomas was observed using the MoAbs SPA 820, SPA 815, SPA 810, SPA 800, SPA 830. The staining intensity of the epidermis with all the MoAbs varied from weak to very strong.

Polydonal antibodies SPA 804, SPA 805 and MoAb SPA 807 reacted specifically with determinants present within the granuloma. Moreover, the staining patterns of these three intibodies were identical. The epidermis was not stained with any of these antibodies.

Western Blot

The specificity of the anti-human HSP antibodies to react with *M. leprae* antigenic determinants was further confirmed on Western Blot using sonicated *M. leprae* as antigen.

As a control, three MoAbs (F 47-10, F 67-18, F 88-1) against *M. leprae* were included which had specifically reacted with the granuloma and not with the epidermis and which were raised against the M. /eprae HSP65 (KOLK et al., 1984).

Immunoblot analysis shows that the polyclonal antibodies SPA 804, SPA 805 and the MoAbs SPA 807, F 47- 10, F 67-18 and F 88-1 recognized M. /eprae proteins with a molecular mass of 65 kDa. The results also indicate that the MoAbs F 47-10 and F-67-18 had stronger reacted with these 65 kDa proteins than the MoAbs F 88-1, SPA 807 and the polydonal antibodies SPA 804 and SPA 805 (Figure 1).

The MoAbs SPA 820, SPA 815, SPA 810, SPA 800 and SPA 830 showed no reactivity with *M. leprae* proteins on the blot (results not shown).

Based on these findings the MoAbs SPA 820, SPA 815, SPA 810, SPA **800** and SPA 830 were excluded from further investigations.

B. A comparison of the reactivity of the antibodies with granuloma of PB leprosy, MB leprosy and of other granulomatous skin disorders. Immunohistodiemistry

In this study the two polyclonal and the one monoclonal antibody against HSP 60 and the three

Table 3. Reactivity of the antibodies with granuloma and epidermis in multibacillary leprosy skin tissues (N=5)

Antibody (dilution)	Granuloma	Epidermis & Adnexae
SPA 820 (1:500)	+	+1-
SPA 815(1:4000)	+	++++
SPA 810 (1:500)	+/-	+/-
SPA 830 (1:2000)	ł	+ + +
SPA 800 (1:4000)	+/-	+
SPA 804 (1:4000)	++ +	-
SPA 805 (1:4000)	+ + +	-
SPA 807(1:500)	++ +	-

Intensity of staining: (-) negative; (+/-) doubtful, (+) weakly positive; (++) moderalBly positive; (+++) strongly positive; (++++) very strongly positive

monoclonal antibodies against M. *leprae* HSP 65 were selected. The objective was to investigate whether granuloma of other granulomatous skin disorders such as sarcoidosis and granuloma annulare showed staining patterns similar to those of the leprosy granuloma, both tuberculoid and lepromatous.

The results are shown in Table 4. It can be seen that specific staining of the granulomas was observed in all leprosy skin sections with all the antibodies used.

In the four paudbadllary cases, all the antibodies stained cells especially in the centre of the granuloma. Staining with the MoAb against the macrophage marker CD68 was also confined to the cells located in the same area (Figure 2).

The staining of the granulomas in PB- and MB leprosy skin sections with anti-HSP 60 mono and polyclonal antibodies and with MoAb F 88-1 was similar (moderately to strongly positive) (Figure 3).

Reactivity of the MoAbs F 47-10 and F 67-18 with the granulomas in PB leprosy lesions was doubtful to weakly positive. In contrast, the same MoAbs reacted strongly to very strongly positive with the granulomas in MB leprosy lesions.

The results as summarized in Table 5 show that granulomas in skin sections of other granulomatous skin disorders showed similar reaction-patterns as compared with those observed in the leprosy skin sections. A representative staining pattern is shown in Figure 4.

Anti-HSP 60 mono- and polyclonal antibodies and the MoAb F 88-1 stained the granuloma strongly to very strongly positive. The MoAbs F 47-10 and F 67-18 showed no staining or stained the granulomas only weakly.

C. In situ detection of antigenic determinants on HSP 60 in macrophages of paucibacillary and multibacillary leprosy and other granulomatous skin disorders. Double-staining immunohistochemistry

Double-staining was performed in order to demonstrate that the antigens recognized by both the anti- human HSP 60 mono and polyclonal antibodies and the anti-

Table 4. Single staining of granuloma in paucibadllary (N=4) and multibacillary (N=5) leprosy skin sections using antibodies against HSP 60 and MoAbs against M. leprae HSP 65.

Patient	FFW (B.I.)	anti CD 68	SPA 804/805/807	F 47-10	F 67-18	F 88-1
Paucibacillary						
TT 182-27	0	++ +	+ + +	+/+ +	+	+ ++
RR-BT 95522	0	++ +	+++	+	+/-	+++
BT 219-4	0	++ +	++	+	+/-	++
BT 88-6	0	+++	+++	++	+	+++
Multibacillary						
BL 250-11	3+	+++	+++	+++	+++	+~~I I~~
BL 22595	3+	++ +	++	++/+++	++/-i-++	+++
LL 3595	4+	++ +	++ +	++++	++++	++++
BL 152-15	2+	++	+++	++	++	+++
BL 19595	3+	+++	+++	++	++	++++

Intensity of staining: (-) negative; (+/-) doubtful; (+) weakly positive; (++) moderately positive; (+++) strongly positive; (++++) very strongly positive.
Bacterial index (BI) by FRN staining: (1+)1 to 10 bacilli in 100 fields; (2+) 1 to 10 bacilli in 10 fields; (3+) 1 to 10 bacilli in an average field; (4+) 10 to 100 bacilli in an average field; (5+) 100 to 1000 bacilli in an average field; (6+) many clumps or over 1000 bacilli in an average field.

M. leprae HSP 65 MoAbs are (mostly) located in the macrophages and to demonstrate that the anti-human HSP 60 mono- and polydonal antibodies and anti-M. *leprae* HSP 65 MoAbs may react with the same antigens.

The combination of MoAbs used for double-staining were as follows: F 88-1/anti-CD68, F 47-10/anti-CD68, F 67-18/anti-CD68, SPA 807/anti-CD68 and F 88-1/SPA 807.

One granuloma annulare, two paucibacillary leprosy and two multibacillary leprosy biopsies, were double-stained. The results are shown in Table 6.

Table 5. Single-staining of granuloma in skin sections of other granulomatous skin disorders (N=5) using antibodies against HSP 60 and MoAbs against M. *leprae* HSP 65

Patient	anti CD68	SPA 804/805/807	F 47-10	F 67-18	F 88-1
SA 173-24	+++	+++	+	+	+++
GA 157-01	+++	++	+/-	-	+++
NL 101-27	+++	++	+/-	+/-	+++
GA 288-14	+++	++	+/-	-	+++
LS 23595	+++	++	+/-	-	+++

Intensity of staining: (-) negative; (+/-) doubtful; (+) weakly positive; (+ +) moderately positive; (+++) strongly positive; (+ + + +) very strongly positive.
SA: Sarcoidosis; GA: Granuloma Annulare; NL: Necrobiosis Lipoidica; LS: Leishmaniasis cutanea.

Table 6. Immunohistochemical double-staining

Patient	F88-1 /CD68	F47-10 /CD68	F67-18 /C068	SPA807 /CD68	F88-1 /SPA807
Paucibacillary					
RR 95522	+ (70-80)	+ (60-70)	-	+ (60-70)	+ (70-80)
BT 88-6	+ (60)	+ (40)	-	+ (60)	+ (50-60)
7182-26	+ (60)	+ (40-50)	-	+ (60)	+ (50-60)
Multibacillary					
ENL 26995	+ (50-60)	+ (50)	+ (5-10)	+ (60)	+ (60-70)
BL 23595	+ (50-60)	+ (50-60)	+ (<5)	+ (60)	+ (70)
LL 22595	+ (40-50)	+ (50-60)	+ (20-30)	+ (50)	+ (50-60)
Granuloma annulare					
GA 288-14	+ (50)	+ (40)		+ (50-60)	+ (50)

Grades: + positive; - negative
(%) estimated percentage of double-stained cells in the granuloma.

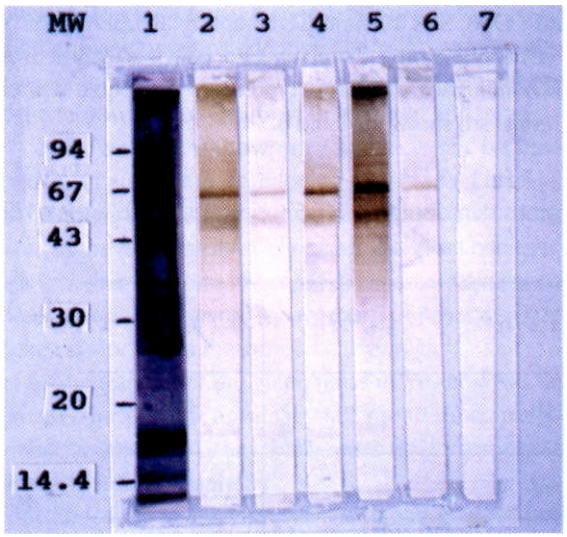
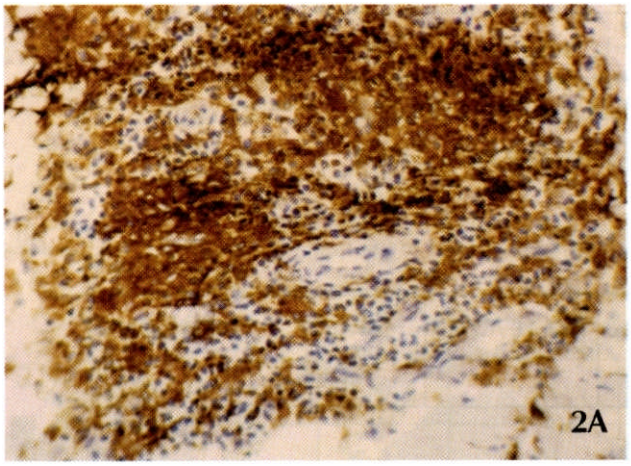
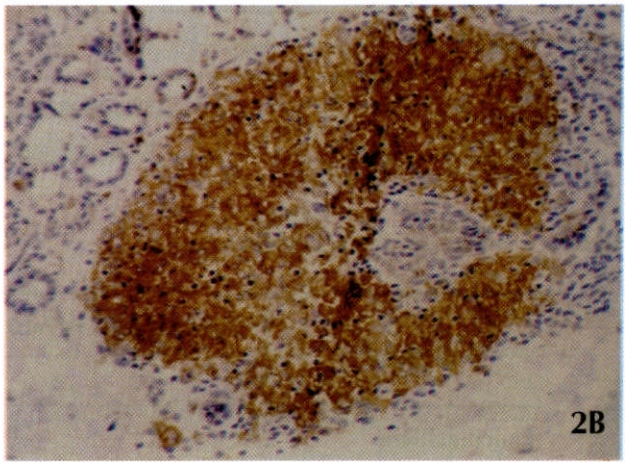


Figure 1. Immunoblot analysis showing the reactivity of anti-HSP 60 antibodies and anti- M. *leprae* HSP 65 antibodies with 65 kDa region proteins in sonicated M. *leprae* (using the ImmunoGold Silver Staining method); Lane 1, separated sonicated M. *leprae* proteins (10mg/m1) showing the presence of 65 kDa proteins; Lane 2, SPA 804; Lane 3, SPA 807; Lane 4, F 47-10; Lane 5, F 67-18; Lane 6, F 88-1; Lane 7, control (exclusion of primary antibody). Note that F 47-10, F 67-18 and SPA 804 reacted more strongly with these 65 kDa regions than SPA 807 and F 88-1.

Figura 1. *Análise do Imunoblot demonstrando a reatividade* dos *anticorpos anti-f ISP-60 e HSP-65 anti M. leprae sonicado* com 65 *kDa (usando o método de coloração ImmunoGold Silver); Fita 1: Proteínas do M. leprae sonicado (10mWm1) mostrando a presença das proteínas de* 65 *kDa; Fita 2:* SPA *804; Fita 3: SPA 807; Fita 4: F 47-10; Fita 5: F* 67-18; *Fita* 6: *F 88-1; Fita 7: controle* (exclusão *de anticorpo primário). Notar* que F *47-10, F 67-18 e SPA 804 reagem mais fortemente coin as regiões de* 65 *kDa do que SPA 807 e F 88-1.*





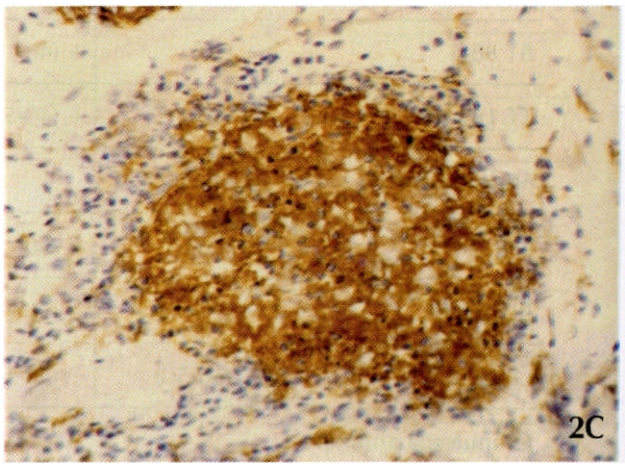


Figure 2. Immunoperoxidase single-staining of granuloma present in paucibacillary leprosy cryostat skin section. (A) Showing strongly positive staining with SPA 807 (BT 88-6), (B) Even strongly positive staining with F 88-1 (BT 95522), (C) Showing positively stained cells which are located mainly in the center of a granuloma using
anti-CD 68 (TT 182-27)

Figura 2. Método da imunoperoxidase com coloração única, em granulomas de pacientes com hansenfase paucibacilar:
(A) Reação fortemente positiva usando SPA 807 (BT 88-6), (B) Igual e fortemente positiva usando F 88-1 (BT 95522), (C) Notar células coradas positivamente, localizadas principalmente no centro do granuloma usando anti-CD 68 (TT 182-27)

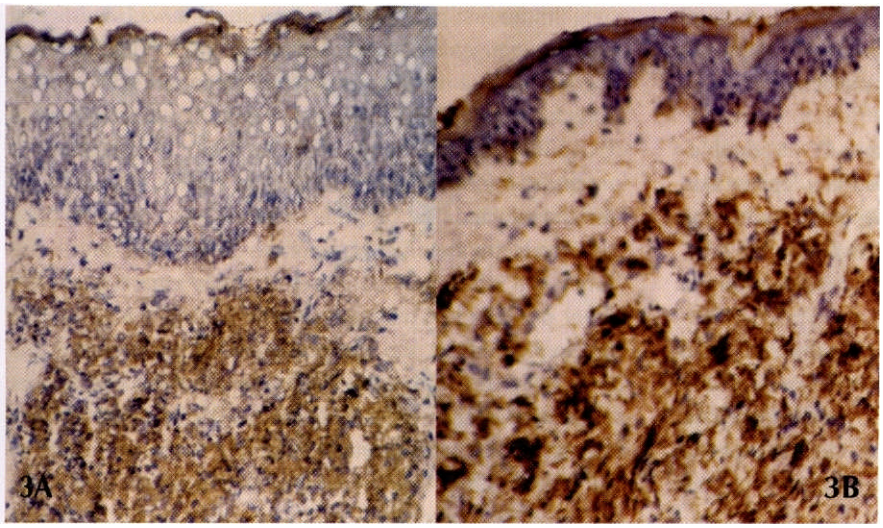
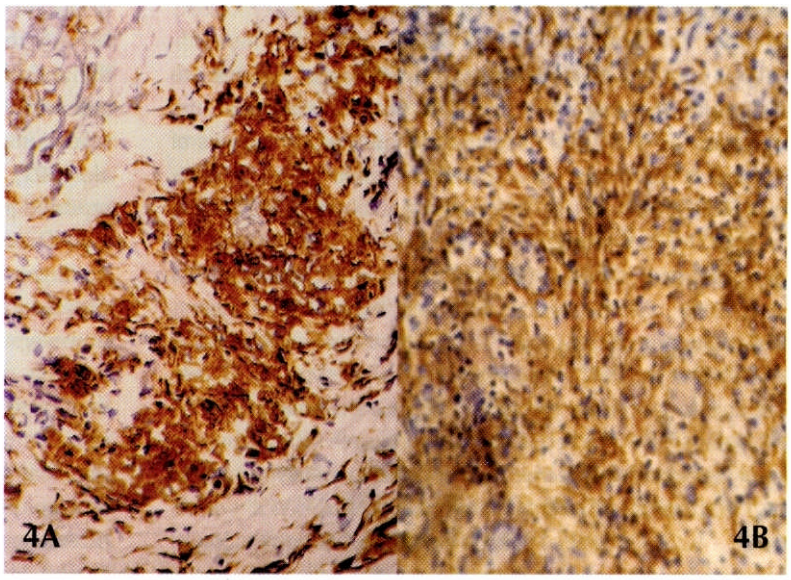


Figure 3. Immunoperoxidase single-staining of granuloma present in multi-bacillary leprosy cryostat skin section showing strongly positive staining with, **(A)** SPA 807 (BL 15215); **(B)** F 88-1 (LL 3595).

Figura 3. *Presença de granulomas demonstrando reação fortemente positiva corados pelo método da imunoperoxidase com coloração única, em secções de pele de pacientes com hanseniase multibacilar.* ***(A)*** *SPA 807 (BL 15215)* ***(B)*** *F88.1 (LL 3595)*

Figure 4. Immunoperoxidase single stainings of granuloma present in two different granulomatous skin disorders: **(A)** Showing strongly positive staining of granuloma of a granuloma annulare skin section (GA 157.01) using SPA 807, **(B)** Showing positive staining of granuloma of a Leishmaniasis cutanea skin section (LS) using F 88.1

Figura 4. *Método da imunoperoxidase com coloração única presente em duas diferentes doenças cutâneas granulomatosas:* **(A)** *Notar granuloma com reação fortemente positiva em secções de granuloma anular (GA 157.01) usando SPA 807.* ***(B)*** *Coloração positiva em granuloma da Leishmaniose cutânea (LC) usando F 88-1.*



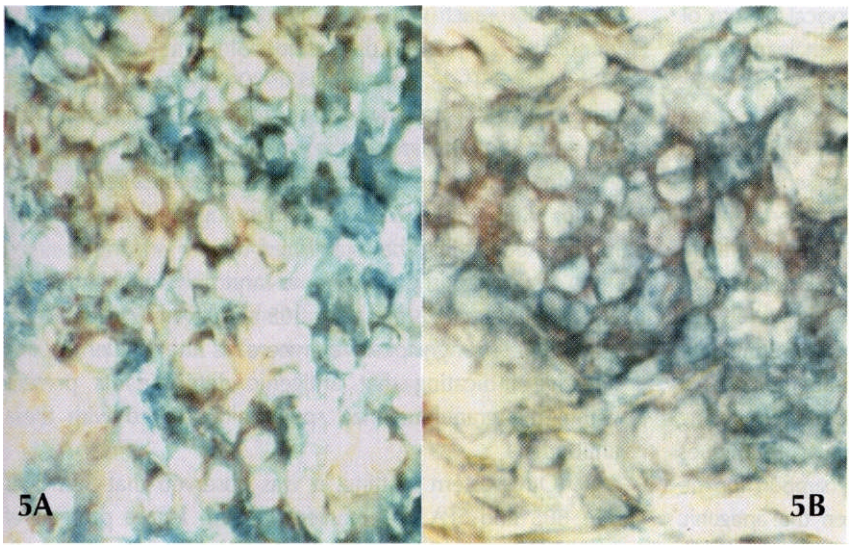


Figure 5. Immunoperoxidase double-staining of granulorna showing similar localization of determinants as detected by the following combinations of MoAbs: (double-stained cells are the purple coloured cells). **(A)** SPA 807 (red; human HSP 60)/F 88-1 (blue; M. *leprae* HSP 65) in a paucibacillary lesion (TT 182.27): 50-60% of the cells are double-stained; **(B)** SPA 807 (red; human HSP 60)/ F 88-1 (blue; *M. leprae* HSP 65) in a multibacillary lesion (ENL 26995): 50.60% of the cells are double-stained.

Figura 5. *Método da imunoperoxidase com coloração dupla presente em granuloma que mostra localização semelhante de determinantes detectados pelos seguintes MoAbs: (células coradas pela dupla coloração: púrpuras)* **(A)** *SPA 807 (vermelho: HSP-60/F 88-1 humano (azul:* FISP 65 *do M. leprae) em uma lesão paucibacilar (TT 182-27).* 50-60% *das células estão com dupla coloração*

The MoAbs F 88-1, F 47-10 and SPA 807 combined with the MoAb anti-CD68 showed many double-stained cells in the granuloma of all skin sections of patients with leprosy and other granulomatous skin disorders.

With the MoAbs F 67-18 and anti-CD68 double- stained cells were only seen in the multibacillary leprosy skin sections. In the paucibacillary leprosy and granuloma annulare sections there was no staining with MoAb F 67-18. These results were comparable with the results obtained upon single-staining with the same MoAb.

In all the skin sections of both paucibacillary and multibacillary leprosy stained with the combination F 88-1 and SPA 807 double stained cells were observed (Figure 5). Using the same combination of MoAbs, double staining pattering similar to those in PB-leprosy were seen within the granuloma annulare sections.

DISCUSSION

About 2 detades ago, immunologists started to investigate the role of heat shock proteins (HSPs) in the pathogenesis of leprosy Highly immunodominant antigens of M. leprae have been recognized as homologues of stress proteins (COHEN et al., 1991; KAUFMANN, 1990 YOUNG et al., 1987). More important, M. /eprae HSPs show up to 50% gene sequence identity with their human analogues (DUDANI et al., 1989; GARSIA et al., 1989). Sera from infected patients react strongly especially against HSP 60 antigenic determinants (YOUNG et al., 1987). A subdass of T lymphocyte, the IT cells are able to specifically recognize antigenic determinants of the M. *leprae* HSP 65 molecule. Activation of such T cells results in cytokine release and recruitment of more specific lymphocytes and macrophages to the infected area (HAREGEWOIN et al., 1991; MODLIN et al., 1989). It is known that aggregation of infected and activated macrophages and T cells leads to the formation of a so called "granuloma" causing tissue (skin and nerve) damage, the characteristic histopathological picture of leprosy. However, the exact mechanisms, which lead to the activation and the aggregation of these cells in lesional areas are not well understood. Moreover, the antigens involved in these processes still remain unknown especially since M. *leprae* itself often can not be detected. Since the gene sequences of the HSPs of M. *leprae* and man are so similar, it is tempting to postulate that autoimmunity against human HSPs is involved in granuloma formation and the subsequent tissue damage in leprosy

In continuation of previous work to demonstrate the existence of similarities between M. *leprae* and human skin antigens, the present study was undertaken using immunohistochemical single- and double-staining techniques and Western Blot technique to obtain further support for the notion that there may be similarities between the antigenic determinants of human HSP 60 and M. *leprae* HSP 65.

In the first part of the present study it was investigated whether (antigenic determinants of) HSPs could be detected in granuloma of multibacillary leprosy skin, using mono- and polyclonal antibodies against HSPs. It was suggested (COLSTON et al., 1989; POLLA, 1991; 1991) that the process of infection "stresses" not only the invading bacilli but also the infected host cells (i.e. macrophages, Schwann cells). This leads to the over-production of HSPs in these cells during the infection. Therefore, one may expect that HSPs either produced by macrophages or by M. *leprae* or by both are expressed in the macrophages. The attention was specifically focussed on macrophages because beside their function as antigen-presenting cells, activated macrophages appear to perform important "effector" functions leading to cell lysis. Furthermore, macrophages represent the majority of the cell population within a granuloma, either as epitheloid or as giant cells in paucibacillary leprosy or as histiocytic "foamy cells" in multibacillary leprosy.

In the present study, the finding of positive staining with the anti-HSP 70 monoclonal antibodies (SPA 820, SPA 815 and SPA 810) in the epidermis, was reported previously by several investigators (BOEHNCKE et al., 1994; EDWARDS et al., 1991; TRAUTINGER et al., 1993). Khanolkar et al., 1994, reported that HSP 70 was expressed significantly more in granuloma in skin and nerve tissue of leprosy patients undergoing a reversal reaction (RR) than in those patients without a RR. Using the same MoAbs (SPA 810 and SPA 820) however, we failed to observe any specific difference in staining of the granuloma in either paucibacillary, multibacillary or reversal reaction leprosy skin. Recently, using immunohistochemistry, Aroni et al. (1996) observed a significantly higher expression of HSP 70 in the granuloma of lepromatous leprosy skin than in the granuloma of tuberculoid leprosy skin. However, the polyclonal antibody against HSP 70 used in their study also reacted with the epidermis, so that the ability of this polyclonal antibody to specifically react with HSP 70 determinants within the granuloma is a point of discussion.

Anti-HSP 27 and anti HSP-90 MoAbs also failed to specifically stain the granuloma. This may indicate that F1513 27 and HSP 90 are not expressed in cells within the granuloma. Whether this finding also indicates that HSP 27 and/or FISP 90 are not of immunological importance in leprosy needs still to be established.

In contrast, the three mono- and polyclonal antibodies against HSP 60 (SPA 804, SPA 805 and SPA 807) stained the granuloma strongly positive, where as the epidermis was negative indicating that antigenic determinants present within the granuloma had specifically reacted with these antibodies.

On Western blotting, it was observed that SPA 804, SPA 805 and SPA 807 reacted with M. *leprae* proteins of molecular mass 65 kDa. As a control, three MoAbs (F 47-10,

F 67-18 and F 88-1), each reacting with a different antigenic determinant on the 65 kDa antigen of *M. leprae*, were included. On the blots, these three MoAbs also reacted with a 65 kDa M. *leprae* protein in a pattern which was similar to that previously reported by several other investigators (BUCHANAN et al., 1987; THOLE et al., 1987). Monoclonal antibodies against human HSP 70, HSP 27 and HSP 90 showed no significant reactions on the blots confirming the findings of immunohistochemistry. It is suggested that the 65 kDa protein of M. *leprae* belongs to the HSP 60 family (DUDANI et al., 1989). Therefore, it is not surprising that antibodies against human HSP 60 and antibodies against M. /eprae 65 kDa protein produce similar banding pattern on Western Blot. Nevertheless, it was noticed, that the bands at the 65 kDa level produced by the polyclonal antibody SPA 804 and the MoAbs SPA 807 and F 88-1 were less prominent than those which were produced by the MoAbs F 47-10 and F 67-18, which seemed to be more M. *leprae* specific.

In the second study the staining patterns of anti-HSP 60 antibodies were compared with those of anti-M. *leprae* HSP 65 antibodies using cryostat sections of paucibacillary and multibacillary leprosy and other granulomatous skin disorders. This study also demonstrated similarities between human HSP 60 and M. leprae HSP 65. The polydonal antibodies SPA 804 and SPA 805 and the MoAbs SPA 807 and F 88-1, showed very similar staining of the granuloma, with regards to the intensity of staining and the localisation of the positive cells, in paucibacillary and multibacillary leprosy as well as in other granulomatous skin disorders. Positive immunoperoxidase stainings of granuloma in sarcoidosis lesions in the skin and in lung tissues using anti-HSP antibodies have been demonstrated before (STATON et al., 1995). Our finding that anti-HSP antibodies also stained granuloma in skin sections of granuloma annulare, necrobiosis lipoidica and leishmaniasis cutanea may indicate that high expression of HSPs could represent a characteristic feature of any granulomatous inflammatory disease (infectious and non-infectious).

In contrast, the MoAbs F 47-10 and F 67-18 stained the granuloma of multibacillary leprosy very strongly, but did not or only weakly stained the granuloma of paudbacillary leprosy and of other granulomatous skin disorders.

These findings, together with the differences in the intenstity of the reactivity of the MoAbs in the blot, on the one hand, showed that MoAb F 88-1 probably had cross- reacted with antigenic determinants which are not specific for M. *leprae*, but are common to determinants on the human HSP 60. On the other hand, the results of the Western blotting also indicated that the MoAbs F 47-10 and F 67-18 reacted with regions on the 65 kDa M. leprae protein which are probably species-specific for *M. leprae* (resulting in a stronger reactivity) and which, therefore, share less similarities with human HSP 60. It would thus be interesting to perform absorption studies using recombinant or purified HSP 60 protein and their individul peptides to exactly define the reacting antigenic determinants (ANDERSON et al., 1988; MEHRA et al., 1986; THOLE et al., 1988).

Double-staining methods further revealed not only that it was very likely that the human HSP 60 monoclonal antibody (SPA 807) and M. /eprae HSP 65 (F 88-1) monoclonal antibodies reacted with the same "structures", but that these "structures" are mostly expressed in macrophages. Further studies, however, are needed to elucidate the link between HSPs and macrophages and the possible role of shared antigenic determinants on HSPs of different origin in generating an (auto)immune response leading to the formation of granulomata in leprosy and other granulomatous diseases. Sarcoidosis for instance, is seen by some as the tuberculoid form of tuberculosis (ROOK et al., 1992; STANFORD, 1994). Necrobiosis lipoidica is often related to diabetis, a condition in which an antigenic similarity between bacterial HSP 65 and human HSP 60 seems to play a role (HORVATH et al., 2002). Interesting is to note recent developments in the understanding of arteriosclerosis where infection induced autoimmunity against human HSP 60 seems to play a major part (PERSCHINSKA et al., 2003).

Nevertheless, the results of this study showed that the concept of autoimmunity in leprosy that may be triggered on the basis of similarities in HSPs between M. *leprae* and man is appealing and worthy of further investigations.

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