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Lysis of autologous macrophages pulsed with hsp10 from *Mycobacterium leprae* is associated to the absence of bacilli in leprosy

Silvia de la Barrera [a], Susana Fink [a], Marta Finiasz [a], Mercedes Alemán [a], María Helena Fariña [b], Graciela Pizzariello [b], María del Carmen Sasiain [a,*]

[a] *Departamento de Inmunología, Instituto de Investigaciones Hematológicas, Academia Nacional de Medicina, Pacheco de Melo 3081, 1425 Buenos Aires, Argentina*

[b] *Servicio de Dermatología, Hospital F.J. Muñiz, Buenos Aires, Argentina*

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Abstract

Peripheral blood mononuclear cells from leprosy patients and normal individuals were analysed for their ability to lyse autologous macrophages pulsed with the *Mycobacterium leprae* 10 kDa heat shock protein (hsp10), an antigen considered to have an important role in the protective responses in leprosy. Strong cytotoxic responses, with an involvement of γδ T and class-I and class-II restricted αβ T cells and/or CD16 + 56 + cells, were observed in normal individuals, paucibacillary (PB) and those multibacillary (MB) patients with undetectable bacillary load. On the contrary, only a weak class-II restricted cytotoxic response was observed in those MB patients with positive bacillary load (MB(+)). Simultaneous addition of IFNγ plus TNFα and IL-12 during hsp10 stimulation could partially upregulate the low cytotoxic response observed in MB(+) by enhancing class-II restricted T cell activity and by development of γδ T and/or CD16 + 56 + cell activity. Our results suggest that the ability to mount an effective cytotoxic response against hsp10-pulsed macrophages in leprosy patients is closely related to the patient's bacterial load and not to the clinical form of the disease.

Keywords: Leprosy; hsp10; CTL

1. Introduction

Mycobacterium leprae is an obligate intracellular bacterium that resides and replicates within macrophages and Schwann cells. Cellular immune response leading to elimination of the intracellular pathogen is considered to have an important role in determining either the development of protective immunity in healthy individuals, or the evolution of symptoms and complications driving to the outcome of clinical disease. *M. leprae* specific cytotoxic T cells with capacity to lyse mycobacterial antigen-pulsed macrophages have been demonstrated in leprosy [1–4]; these cells may participate in the elimination of mycobacteria and, at the same time, in adverse responses observed in leprosy patients. *M. leprae* specific T cell response is elicited by recognition of different mycobacterial antigens. Several *M. leprae* antigens have been identified and include proteins implicated in bacterial response to stress – heat shock (hsp) which play important biochemical and regulatory functions [5].

M. leprae 10 kDa heat shock protein (hsp10) is a homologue of the GroES gene product of *Escherichia coli* [6], it belongs to the human chaperonin 10 family [7] that presents a sequence phylogenetically conserved, and has a 90% amino acid homology with *M. tuberculosis* hsp10 [6,8,9], which is expressed in the cytosol and cell wall [8]. GroES is necessary for the optimal activity of GroEL, a member of the chaperonin 60 family that includes *M. leprae* hsp65. Both chaperonin families act coordinately in protein folding. The 10 kDa hsp is the major cytosolic protein of *M. leprae* representing 1% of

* Corresponding author. Tel.: + 54-11-48055695; fax: + 54-11-48039475.

E-mail address: isturiz@mail.retina.ar (M. del Carmen Sasiain).

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the bacterial mass. It is recognized by most *M. leprae* reactive human T cell lines and cell-wall reactive T cell clones [6] and it has been shown to introduce strong proliferative T cell responses in cells from tuberculoid leprosy patients, close contacts and healthy individuals [6,10,11]. Furthermore, this hsp elicited a strong delayed-hypersensitivity reaction in guinea pigs sensitised with killed *M. leprae* [8].

The aim of this study was to analyse the development of hsp10-specific cytotoxic effector cells across the leprosy spectrum. Our results demonstrate that hsp10-induced cytotoxic activity against autologous antigen-pulsed macrophages is closely related to the bacterial load of leprosy patients. Newly diagnosed or under treatment multibacillary (MB) patients with positive bacillary load did not develop a cytotoxic response to hsp10. On the contrary, MB patients undergoing treatment who negativized their skin and nose blow smears, were able to mount an effective cytotoxic response to this hsp.

2. Materials and methods

2.1. Patients

Thirty four leprosy patients, diagnosed on the basis of clinical and bacteriological criteria and classified according to Ridley and Jopling [12], were studied: 22 lepromatous (LL), four borderline lepromatous (BL), two tuberculoid (TT) and five borderline tuberculoid (BT) patients. They were divided into two groups: paucibacillary (PB: TT and BT) (three women, five men; 33–72 years) and multibacillary (MB: LL and BL) (ten women, 16 men; 19–76 years) patients. Skin and nose-blow smears were made routinely and the presence of acid-fast bacilli was analysed to assess the effect of treatment. MB patients were then divided in two groups: 12 patients with negative (MB(−)) and 14 with positive smears (MB(+), two newly diagnosed and 12 under treatment). All the patients included in this study were free of other infectious diseases and patients under treatment received multidrug therapy according to the recommendations of the WHO. Most of the patients came from or were residing in endemic areas at the moment of the study. Fourteen BCG-vaccinated normal controls (N) (eight women, five men; 24–60 years) were studied simultaneously.

2.2. Mononuclear cells

Heparinized blood was collected and peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque gradient centrifugation [13]. Cells were collected from the interface and resuspended in RPMI 1640 tissue culture medium (Gibco Lab, NY) containing gentamycin (85 μg/ml) and 15% heat inactivated fetal calf serum (FCS) (Gibco Lab) (complete medium).

2.3. Effector cells for cytotoxicity assays

PBMC (2×10^6 cells/ml) were cultured in Falcon 2063 tubes at 37°C in humidified 5% CO_2 atmosphere, in complete medium with or without the 10 kDa (10 μg/ml) recombinant protein from *M. leprae* (hsp10), in the presence or absence of, (a) IL-12 (10 ng/ml), (b) TNFα (10 ng/ml), (c) IFNγ (100 U/ml), and (d) combinations of these cytokines with or without anti-IL4 monoclonal antibody (10 ng/ml). Hsp10 (batch ML10-2) was kindly provided by Dr M. Singh, GBF, Braunschweig, Germany, through the UNDP/World Bank/WHO; the endotoxin concentration was 603 U/mg of protein according to Limulus amoebocyte lysate assay. IFNγ and TNFα were purchased from Genzyme (USA) and IL-12 from Pharmingen (USA) and were recombinant proteins. Monoclonal anti-human IL-4 antibody was purchased from R&D (USA). On day 7, treated and control cells were washed three times with RPMI 1640, resuspended in complete medium (2×10^6 cells/ml) and tested for cytotoxic activity.

2.4. Isolation of CD4-, CD8-, γδ T- or NK-depleted effector cells

After seven days of culture, PBMC were depleted of lymphocytes bearing the CD4, CD8 or CD16 antigens as well as of those expressing the γδ TCR, by a magnetic method. Briefly, $2–4 \times 10^6$ control and hsp10 stimulated PBMC resuspended in 100 μl of PBS containing 2% FCS, were treated (a) with anti-γδ TCR (Pan γδ, IgG1, clone Immun 510, Immunotech, France), anti-CD16 Mab (Leu 19, IgG1, clone MY31, Becton Dickinson) or anti-CD56 (Leu 11a, IgG1, clone 3G8, Becton Dickinson) during 30 min at 4°C and then with goat anti-mouse IgG-coated beads (Dynal, USA) (30 min), or (b) with anti-CD8 or anti-CD4-coated beads (Dynal) (30 min). Treatment with magnetic beads was performed on an ice-cold bath. Then cells were resuspended with PBS in a final volume of 2 ml and placed in a magnetic particle concentrator for 3 min to collect the unbound cells. The purity of the depleted populations as well as the percentage of the different lymphocyte subsets present in the whole PBMC cultured was determined by flow cytometry. Purity of the isolated cell populations ranged from 85 to 98% in each case. The depleted lymphocyte populations were resuspended in complete medium ensuring that the proportion of the cells present in the depleted cell suspensions was the same as in total cultured PBMC in order to compare their lytic activity.

2.5. Target cells

Monocytes were allowed to adhere to the bottom of 24 well flat-bottom Falcon plates by incubation of PBMC (5×10^6 ml^{-1}) for 2 h at 37°C. After removing non-adherent cells, cells remaining in the plates (10% of the original cell suspension) were incubated at 37°C in a humidified 5% CO_2 atmosphere for seven days. For the cytotoxic assays, on day 6 of incubation the cells were pulsed with hsp10 (10 μg/ml). Macrophages kept under the same conditions but without addition of antigen were used as controls. Plates were cooled for 2 h at 4°C to facilitate the detachment of adherent cells by vigorous pipetting using ice-cold medium. These cells were washed and pellets of $5–7 \times 10^5$ cells were labelled with 100 μCi of $Na_2^{51}CrO_4$ (NEN, USA) by incubation for 1 h at 37°C. Then the cells were washed three times and resuspended in complete medium at 1×10^5 cells/ml.

2.6. Cytotoxic assay

4×10^4 target cells were seeded into each well of 96 well microtitre plates (Corning, USA). Effector cells (1×10^6 cells/ml) were added in triplicate at an effector to target cell ratio = 40:1 (E/T) in 0.2 ml final volume. The plates were centrifuged at $50 \times g$ for 5 min and incubated at 37°C in 5% CO_2 for 4 h. After centrifugation at $500 \times g$ for 5 min, 100 μl of supernatants was removed from each well. The radioactivity of supernatants and pellets was measured in a gamma counter. Results were expressed as percentage of cytotoxicity:

$$\%\ \mathrm{Cx} = \frac{\mathrm{cpm\ exp} - \mathrm{cpm\ spontaneous\ release}}{\mathrm{cpm\ total} - \mathrm{cpm\ spontaneous\ release}} \times 100.$$

Spontaneous release is the radioactivity released from target cells incubated with complete medium alone. It ranged from 8% to 15%. In all cases, the cytotoxic assays performed with PBMC cultured in the absence of hsp10 or with macrophages not pulsed with antigen rendered negligible cytotoxicity (0–6%), even if cytokines were included in the cultures (2–7%). Data presented in Tables 1 and 2 were obtained by subtracting the cytotoxicity against non-hsp10 pulsed macrophages from the experimental values determined using hsp10-pulsed targets. PBMC were incubated during 7 days with LPS (*Escherichia coli* 011:B4, Sigma), at the same concentration as endotoxin is present contaminating the hsp10 preparation. A negligible (1–2%) cytotoxicity against hsp 10-pulsed macrophages was observed.

Table 1
Modulation of hsp10-induced cytotoxic activity by Th1 cytokines[a]

PBMC inc with	Cytokine treatment	MB (+) % Cx	MB (−) % Cx	PB % Cx	N %Cx
–	–	2 ± 1	1 ± 1	3 ± 2	1 ± 1
–	IFNγ	2 ± 1	2 ± 2	2 ± 1	2 ± 1
–	IFNγ+TNFα	2 ± 1	2 ± 1	3 ± 2	2 ± 2
–	IFNγ+TNFα+IL−12	2 ± 2	3 ± 1	3 ± 1	1 ± 1
hsp10	–	8 ± 1	35 ± 4	49 ± 5	34 ± 3
hsp10	IFNγ	10 ± 2	60 ± 4 #	64 ± 3*	44 ± 3*
hsp10	IFNγ+TNFα	16 ± 4	66 ± 5 #	65 ± 2*	47 ± 3*
hsp10	IFNγ+TNFα+IL−12	26 ± 2*	68 ± 3 #	70 ± 4*	50 ± 4*

[a] PBMC from 14 MB (+), 7 MB (−), 7 PB patients and 14 N were stimulated during 7 days with or without hsp10 in the presence or absence of Th1 cytokines and were then used as effector cells in the cytotoxic assay. Results are expressed as percentage of cytotoxicity ($x \pm$ SEM). Statistical differences between% Cx from hsp10-induced CTL with and without cytokines: $^*P<0.05$; $^{\#}P<0.005$.

Table 2
Differential hsp10-induction of effector cells in leprosy patients and N[a]

PBMC from	Cytotoxicity (%)				
	No MoAb	a-class-I	a-class-II	a-γδTCR	a-CD16/CD56
MB (+)	10 ± 3	9 ± 2	4 ± 2*	10 ± 2	11 ± 3
MB (−)	36 ± 3	22 ± 4*	20 ± 4*	25 ± 4*	26 ± 5*
PB	50 ± 2	27 ± 3**	36 ± 2*	26 ± 3**	21 ± 4**
N	32 ± 3	20 ± 3	16 ± 3	25 ± 2*	23 ± 2*

[a] PBMC from 14 MB(+), 10 MB(−), 7 PB patients, and N were stimulated with hsp10 during 7 days. Previous to the cytotoxic assay, control and hsp10-induced effector cells were treated or not with anti-γδTCR or anti-CD16 plus anti-CD56 (a-CD16/CD56) MoAb while control and hsp10-pulsed macrophages (previously labelled with ^{51}Cr) were incubated with anti-class-I or anti-class-II MoAb. After 1 h of incubation at 37°C and two washes, effector and target cells were incubated during 4 h at E/T ratio of 40:1. Results are expressed as percentage of cytotoxicity. Statistic differences between% Cx from treated effector or target cells and% Cx from non-treated cells: $^*P<0.05$; $^{**}P<0.01$.

In order to analyse the nature of hsp10-induced cytotoxic effector cells, (a) effector cells (control and hsp10-pulsed) were incubated with anti-CD16 Mab (Leu 19, IgG1, clone MY31, Becton Dickinson), anti-CD56 (Leu 11a, IgG1, clone 3G8, Becton Dickinson), anti γδ TCR (Pan γδ, IgG1, clone Immun 510, Immunotech, France), and (b) target cells were incubated with anti-class-I MHC (monoclonal antibody HLA-ABC, IgG2κ, clone B9.12.1, Immunotech, France) or anti-class-II MHC (anti-HLA-DR, IgG2a, clone L243, Becton Dickinson) monoclonal antibodies. After 1 h incubation at 37°C, cells were washed twice and used in the cytotoxic assay. Isotype matched non-relevant antibodies were also employed and they had no significant effect on hsp10 cytotoxic values.

2.7. Natural killer activity

Fresh PBMC or 7-days cultured PBMC were employed as effector cells in order to test their natural killer cytotoxic activity. ^{51}Cr-labelled K562 target cells (5×10^5 cells/ml) were mixed with the effector cells (1×10^6 cells/ml) in a total volume of 0.2 ml into each well of 96-well microtitre plates (Corning, USA) at an E/T ratio of 40:1. Plates were centrifuged ($200 \times g$) for 1 min and incubated at 37°C for 4 h in a CO_2 incubator. Then they were centrifuged at $500 \times g$ for 5 min, 100 μl of supernatant were removed and counted for radioactivity. Results were expressed as percentage of cytotoxicity. Spontaneous release from K562 target cells did not exceed 10%.

2.8. NK cells, αβ and γδ T cells in fresh and 7 days cultured peripheral blood mononuclear cells

The presence of lymphocytes expressing the CD16 + /CD56 + antigens, CD3 + T cells bearing αβ or γδ TCR was determined in fresh PBMC as well as in mononuclear cells cultured with or without hsp10 during 7 days, by flow cytometry. Briefly, 1×10^6 cells resuspended in PBS + 0.1% sodium azide were incubated with 3 μl of pure FITC or PE conjugated monoclonal antibodies, specific for the human CD16 (Leu 11-a-FITC, Becton Dickinson), CD56 (Leu 19-PE, Becton Dickinson), CD3 antigens (CD3-PE, Immunotech, France) anti-αβ TCR (Pan αβ TCR-FITC Immunotech), or anti-γδ TCR (Pan γδ-FITC, Immunotech) during 30 min at 4°C. Non-specific binding was analysed by incubation of the cell suspension with FITC or PE labelled $F(ab')_2$ anti-mouse IgG1 (Becton Dickinson or Immunotech according to the specific MoAb employed). After three washes stained cells were analysed using a dual colour analysis (CELLQuest software) on a FACScan (Becton Dickinson) 20 000 events were acquired for each sample, gates were set with respect to the forward and side scatter to exclude cell debris and apoptotic cells. Results are expressed as percentage of positive cells.

2.9. Statistics

Comparisons of MB and PB and N were performed using the Student's test. Cytotoxicity values obtained from the different subsets of effector cells of each individual were compared using the Wilcoxon signed rank test.

3. Results

3.1. Heat shock protein 10 induces differential specific-cytotoxic T cell activity in leprosy patients and normal controls

PBMC from six normal individuals were tested for their cytotoxic activity against autologous hsp10-pulsed macrophages at different E/T ratios. As shown in Fig. 1A, hsp10 was able to induce cytotoxic activity in normal individuals (N) and a dose-response curve was obtained. Further studies were performed incubating effector and target cells at a 40:1 ratio. As shown in Fig. 1B, under these conditions hsp10 was able to induce hsp10-specific cytotoxic activity in PBMC from MB and PB patients and N. While 4/7 PB patients presented higher cytotoxic activity than N, in MB patients different levels of lytic activity were observed depending on their bacillary load at the moment of the study. MB patients with positive skin and/or nose-blown smears, MB(+), presented a marked deficiency to mount an hsp10-induced cytotoxic response, while in those MB patients under treatment, with negative smears, MB(−), a cytotoxic activity similar to N controls was observed (Fig. 1B).

3.2. Modulation of heat shock protein 10-induced CTL activity by IFNγ, TNFα and IL-12

In order to evaluate whether hsp10-induced CTL activity from MB(+) patients could be modulated by cytokines, IFNγ, TNFα and IL-12 were added at the onset of the antigen stimulation. As shown in Table 1 IFNγ was able to modulate by itself hsp10-CTL activity in MB(−), PB and N, and no enhancement over these values was observed either by the coaddition of TNFα and IL-12 even when IL-4 was neutralized in MB(−) (% $Cx = 70 \pm 5$). In contrast, IFNγ alone could not revert the low cytotoxic activity observed in MB(+) patients; evenmore the co-addition of TNFα did not modify significantly the lytic activity. The

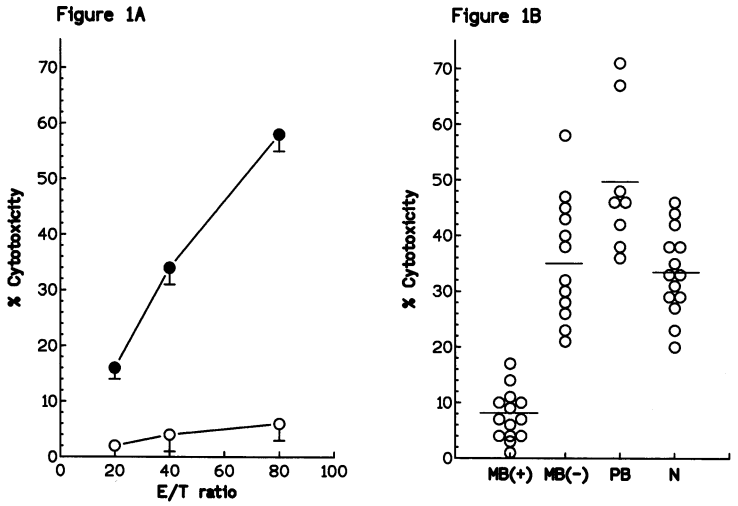

Fig. 1. Hsp10 was able to induce cytotoxic effector cells in leprosy patients and normal individuals: (A), Effector PBMC from six normal individuals were incubated with hsp10 from *M. leprae* during 7 days at 37°C. Then they were tested for their lytic activity against hsp10-pulsed (●) or unpulsed (○) autologous macrophages at different effector to target cell ratios (E/T ratio) as mentioned in Section 2. Results are expressed as percentage of cytotoxicity and (B), PBMC from 12 MB with negative smears for acid-fast bacilli (MB(−)), 14 MB with positive smears (MB(+)), 8 PB patients and 14 normal individuals (N) were stimulated with hsp10 during 7 days. Then, they were tested for their lytic activity against hsp10-pulsed- and non-pulsed-macrophages at a 40:1 E/T ratio and percentage of cytotoxicity was calculated. Individual data were obtained by subtracting the cytotoxicity against non-pulsed macrophages from the experimental values obtained employing hsp10 pulsed target cells.

simultaneous presence of IFNγ, TNFα and IL-12 was necessary to obtain a partial increase in the cytotoxic response, while neutralization of IL-4 and co-addition of the three Th1 cytokines produced a marked enhancement of lytic values in MB(+) (% $Cx = 43 \pm 4$, $P < 0.05$). However, the hsp10-CTL activity even with the three Th1 cytokines added was lower than that of MB(−) cells.

3.3. Differential profile of heat shock protein 10-induced effector cells in leprosy patients and normal individuals

In order to analyse the nature of the hsp10-effector cells, blocking assays were performed by treating the effector or target cells with different monoclonal antibodies, previous to the cytotoxic assay. As shown in Table 2, significant inhibition of hsp10-CTL activity by treatment of target cells with anti-class-I, anti-class-II, or effector cells with anti-CD16/56 and anti-γδ TCR MoAb was observed in PB and MB(−) patients and N. On the contrary, in MB(+) patients only anti-class-II inhibited the CTL activity. Depletion experiments were also carried out on effector cells and confirmed the results obtained in the blocking assays (Fig. 2). These results suggested that hsp10 was able to induce both class-I- and class-II-restricted effector αβ cells as well as γδ T and/or CD16 + 56 + cells in PB, MB(−) and N, while in MB(+) only a class-II-restricted CTL response was observed.

3.4. Determination of CD16 + /CD56 + and αβ and γδ T cells in fresh and (7 days) cultured cells

In order to determine whether the differences observed between MB(+) and MB(−) hsp10-CTL activity could be ascribed to differences in the percentage of responding cells, we evaluated the percentage of CD3 + αβ TCR + T cells as well as CD3 + γδ TCR + T cells or NK cells in fresh PBMC and cells

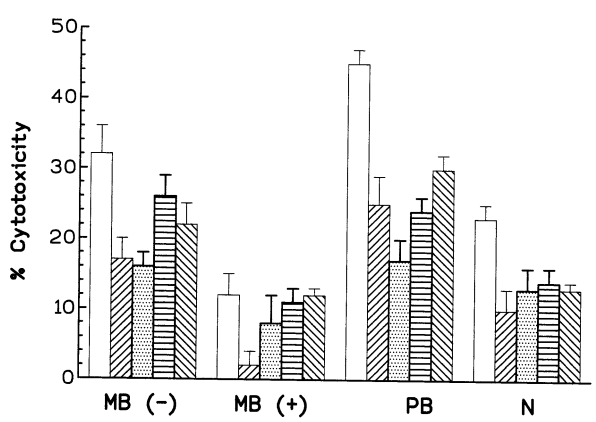

Fig. 2. Differential hsp10-induced cytotoxic activity in CD4-, CD8-, γδ T- or NK-depleted populations in leprosy patients: PBMC from 3 MB(−), 3 MB(+) and 3 PB patients as well as 3 N controls were or not stimulated with hsp10 during 7 days. Then, cells were depleted of CD4 (), CD8 (), γδ T () or CD16 + cells (▨) by a magnetic method as described in Section 2. Total PBMC (□) or depleted populations were employed as effector cells in the cytotoxic assay against hsp10-pulsed target cells (E/T rati-40:1). Results are expressed as percentage of cytotoxicity.

Table 3
Percentage of CD16+/CD56+ or γδTCR+ lymphocytes in fresh or cultured PBMC[a]

PBMC from	CD16+/56+			γδTCR+			αβTCR+		
	Fresh	Control	hsp10	Fresh	Control	hsp10	Fresh	Control	hsp10
MB (+)	4 ± 1*,**	3 ± 1*,**	4 ± 1*,**	2 ± 1	2 ± 1	3 ± 1	69 ± 4	76 ± 5	70 ± 4
MB (−)	8 ± 2	7 ± 1	9 ± 2	6 ± 2	4 ± 1	6 ± 1	60 ± 5	67 ± 5	59 ± 2
N	6 ± 1	5 ± 2	8 ± 1	4 ± 1	3 ± 1	6 ± 1	65 ± 3	60 ± 2	69 ± 3

[a] Fresh PBMC as well as hsp10-stimulated (hsp10) and non-stimulated (control) PBMC from 7 MB(+), 6 MB(−) and 6 N were labelled with anti-CD16-FITC, anti-CD56-PE, anti-CD3-PE, anti-αβ TCR-FITC or γδ TCR-FITC monoclonal antibodies during 30 min and stained cells were analysed by flow cytometry as mentioned in Section 2. Results are expressed as percentage of positive cells. Statistical differences: patients vs. N: *$P<0.05$ and MB(+) vs. MB(−): **$P<0.05$.

Table 4
NK cytotoxicity from fresh and hsp10-stimulated PBMC[a]

PBMC from	Fresh PBMC% NK-Cx	Control PBMC% NK-Cx	hsp10-PBMC% NK-Cx	hsp10+12 TI PBMC% NK-Cx
MB (+) ($n=6$)	13 ± 1**	3 ± 1*	10 ± 3*[b]	21 ± 3**[b]
MB (−) ($n=5$)	23 ± 4*[a]	10 ± 2*[a]	30 ± 3*	50 ± 3
PB ($n=4$)	30 ± 4	15 ± 3	35 ± 3	49 ± 2
N ($n=7$)	28 ± 3	18 ± 4	40 ± 2	56 ± 3

[a] Fresh PBMC as well as hsp10-stimulated PBMC with or without IL-12 plus TNFα and IFNγ (12 TI) from MB(+), MB(−) and PB patients and N were tested for their lytic activity against ^{51}Cr-labelled K562 cells as mentioned in Section 2. Results are expressed as% of NK-cytotoxicity (% NK-Cx). Statistical differences: patients vs. N: *$P<0.05$, **$P<0.005$; MB(+) vs. MB(−): [a]$P<0.05$, [b]$P<0.005$.

cultured for 7 days. As shown in Table 3, similar percentages of CD3 + αβ TCR + cells were observed in fresh PBMC as well as in control or hsp10-stimulated PBMC from the three groups studied. No differences were detected in CD16 + /CD56 + cells between MB(−) and N controls while in MB(+) patients the number of these cells was lower. On the other hand, the number of CD3 + γδ TCR + T cells was lower in MB(+) patients.

3.5. Determination of NK cytotoxic activity in fresh and 7 days cultured PBMC

Fresh, control (cells cultured without antigen stimulation) and hsp10-stimulated PBMC were tested for their NK activity. As shown in Table 4, a lower NK activity was observed in fresh and control PBMC from MB(−) patients than in those from PB and N while hsp10 stimulation made no differences in NK lytic activity between these experimental groups. On the contrary, an impairment of NK activity was observed in MB(+), fresh as well as control and hsp10-stimulated PBMC, when compared with MB(−), PB and N. An enhancement of NK activity in all the groups evaluated was observed by addition of IFNγ plus TNFα and IL-12 to hsp10-cultures, although the NK activity of MB(+) had the lowest value.

4. Discussion

Both *M. leprae* and *M. tuberculosis* hsp10 have been reported as dominant T cell antigens in leprosy and tuberculosis. *M. leprae* hsp10 has been shown to be a strong T cell antigen in patients with tuberculoid disease and in lepromin-positive contacts [6,8,10]. Furthermore, in studies of precursor frequency analysis of *M. leprae* stimulated PBMC, one-third of reactive T cells were hsp10-specific [6,10]. Although poor proliferative responses had been observed in lepromatous patients, a much higher frequency of hsp10-reactive lepromatous patients' T cells was found to be respondent to this antigen [11]. Our results showed that those MB patients with MB(+) did not develop hsp10-CTL activity, this result is in accordance with those obtained in proliferation assays [6,11]. On the other hand, CTL activity observed in MB(−) patients did not agree with data obtained from those proliferation assays. Even taking the MB patients as a whole, we found a significantly higher cytotoxic response to hsp10 (33%) with a number of patients similar to those studied by Hussain et al. [11]. In this sense, and opposite to the result of Hussain [11], we evaluated MB patients under specific chemotherapy, hence the differences observed could probably be ascribed to the multidrug treatment. Thus, the hsp10-CTL activity recovered in those MB patients

under treatment that negativized their skin or nasal-blow/smear may suggest that these patients became able to recognize the reactive epitopes present on the 10 kDa antigen after clearence of the leprosy bacillus. It has been demonstrated that lepromatous patients recognize common epitopes in the 10 kDa preparations of *M. leprae* and *M. tuberculosis* [11], while in tuberculoid patients both common and species-specific epitopes were recognized when proliferation assays were carried out. Thus, the higher hsp10-CTL activity observed in PB patients could be due to differences in the epitopes recognized by cells obtained from these patients. On the other hand, the CTL activity observed in the control group may be related to the recognition of cross-reactive epitopes from *M. bovis*-BCG and/or to *M. tuberculosis*, not to *M. leprae* exposure.

The impaired CTL activity observed in MB(+) patients could be ascribed either to a disbalance in cytokine production and/or to poor antigen-presenting capability of stressed macrophages. To test the first possibility we generated CTL effector cells in the presence of IFNγ. No enhancement of hsp10-CTL activity could be observed in MB(+) although this cytokine did enhance the cytotoxic activity in the other groups studied (Table 1). It is well known that IL-12 is able to activate antigen-specific αβ and γδ T cells as well as NK cells to proliferate and secrete higher amounts of IFNγ and TNFα [14,15]. mRNA coding for IL-12 has been detected in tuberculoid lesions and the ability of this cytokine to modulate the proliferation of cells isolated from lesions or PBMC has been demonstrated [16,17]. The addition of IFNγ, TFNα and IL-12 to hsp10-cultures from MB(+) enhanced the CTL activity, but even in this case the cytotoxic activity generated was lower than that of MB(−). On the other hand, data obtained by neutralization of IL-4 during CTL generation in MB(+), suggest that MB(+) cells are able to release IL-4 (even in trace amounts) that could account for the low CTL activity in MB(+). As a matter of fact, it has been well established that IL-4 antagonizes IL-12 for high IFNγ production by αβ and γδ T cells as well as NK cells by downregulating IL-12 receptor expression [18].

Activation of both, CD4+ and CD8+ T cells in response to *M. leprae* and *M. tuberculosis* hsp has been reported [4,19,20]. In our study we demonstrated that hsp10 induced class-I and class-II restricted hsp10-CTL in PB, MB(−) and N. Involvement of class-I restricted cytotoxic T cells was observed in PB patients while neither class-I nor class-II restricted T cells were preferentially induced in MB(−) and N. It has been shown that γδ T cells recognize preferentially small peptides from proteins of low molecular weight, although proliferative and cytotoxic responses to high molecular weight hsp, such as hsp65 and hsp70, have been reported [21,22]. The in vivo accumulation of γδ T cells in the skin lesions of leprosy [23] and tuberculosis [24] suggests a role for these cells in human disease by contributing to granuloma formation [23]. Upon activation γδ T cells produce IL-2 and lyse *M. tuberculosis*-target cells [25], while they require either activated CD4+ αβ T cells or exogenous IL-2 for in vitro expansion [26]. Therefore, the higher capacity to lyse hsp10-pulsed macrophages elicited by γδ T cells in PB and N may suggest an in vitro activation of cells capable of releasing soluble factors for γδ T cell stimulation. On the other hand, NK cells exhibited non-MHC restricted cytotoxic activity against macrophages infected with live BCG or *M. tuberculosis* [27]. A role in the modulation of immune response has been ascribed to both NK and γδ T cells as early producers of IFNγ. NK activity could be generated from PBMC of human leprosy patients upon stimulation with bacterial antigens such as hsp65 [22]. We can not distinguish by our blocking assays whether the effector cells induced by hsp10 are γδ T or NK effector cells. Both populations express the NK CD56 antigen on their surface [28]. Hence the inhibition by anti-CD16/CD56 could be due to blockade of NK cells and/or γδ TCR/CD56+ T lymphocytes. Thus, in our system hsp10 is able to stimulate specific and non-specific cytotoxic cells that contribute to the overall cytotoxic response (Fig. 2). Moreover, the low CTL activity observed in MB(+) could be due to a low NK and γδ T cell biological function and/or the presence of IL-4 that down-modulates IFNγ production.

It is well known that mycobacterial components that are able to stimulate T cells can be broadly divided into somatic and secreted antigens. Somatic antigens belonging to the hsp group [29] are mainly derived from destroyed bacilli during the late phase of infection, while secreted antigens are produced by metabolically active organisms and are related to the early or active phase of infection [30]. Furthermore, secreted antigens are readily accessible for MHC processing as long as the bacteria survive inside macrophages. On the other hand, somatic proteins are only released and processed by MHC after the bacteria are killed by macrophages or by specific chemotherapy. Besides, although it is well known that lepromatous leprosy patients are poor responders to *M. leprae* antigens, it would seem that in MB(+) the lack of hsp10 specific CTL activity and the production of IL-4 during the generation of effector cells, were linked to the presence of the bacillus. Thus, our results suggest that in MB(+) patients, the low hsp10-CTL activity might be related to an impaired T cell stimulation caused by the absence of free hsp10 and/or the incapacity of stressed antigen presenting cells to process hsp10 antigen. On the other hand, in MB(−), the in vivo exposure by T cells to hsp10 as a consequence of bacilli destruction allows for recall recognition during CTL development.

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