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Mapping of Multiple HLA Class II-Restricted T-Cell Epitopes of the Mycobacterial 70-Kilodalton Heat Shock Protein

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By combining a DNA subclone and synthetic-peptide approach, we mapped epitopes of the immunogenic mycobacterial 70-kDa heat shock protein (HSP70) recognized by human CD4$^+$ T-cell clones and lines. In addition, we identified the respective HLA-DR molecules used in antigen presentation. The donor groups used were healthy persons immunized with killed *Mycobacterium leprae* and tuberculoid leprosy patients. The results show that the N-terminal part of the HSP70 molecule contains three different T-cell epitopes, of which two were presented by DR7 (amino acids [aa] 66 to 82 and 210 to 226) and one was presented by DR3 (aa 262 to 274). The C-terminal part contains one epitope (aa 413 to 424) presented by HLA-DR2. The C-terminal epitope shows extensive homology to the corresponding region of the human HSP70 sequence. All of the T-cell epitopes identified were presented by only one particular HLA-DR molecule. We also found that HLA-DR5 and DRw53 can present HSP70 to T cells, demonstrating the presence of additional epitopes not yet defined at the peptide level. On the basis of the donors used in this study, recognition of HSP70 at the epitope level seems to be ruled by the restriction elements expressed by the donor rather than by any difference in reactivity between healthy individuals and patients. In conclusion, mycobacterial HSP70 is relevant to subunit vaccine design since it contains a variety of T-cell epitopes presented in the context of multiple HLA-DR molecules.

In leprosy, both protective immunity and immunopathology are dependent on antigen-specific major histocompatibility complex (MHC) class II-restricted T-cell responses (3). Identification and characterization of *Mycobacterium leprae* protein antigens with respect to epitopes and MHC restriction elements used in T-cell recognition are therefore important for both understanding the pathological conditions and development of subunit vaccines that induce protection.

By screening recombinantly expressed and Western blotted (immunoblotted) antigens for T-cell reactivity, a variety of different mycobacterial protein antigens have been identified and characterized (5, 10, 13, 18–22, 24–26, 30, 40). Among the well-characterized T-cell antigens, the heat shock proteins (HSP) are of special interest for the following reasons. HSP represent a highly conserved group of proteins in both prokaryotic and eukaryotic cell types which is strongly upregulated in response to a variety of different stress conditions, such as heat, irradiation, and the presence of oxidative and toxic agents (12). In addition, it is well known that the most important antigen-presenting cells (APC) for CD4$^+$ T cells, which belong to the monocyte-macrophage lineage, are also the main host cells for *M. leprae* and other mycobacteria (3). Infection of macrophages with *M. leprae* results in overexpression of HSP both from the pathogen itself and from the APC because of elevated temperature and induction of oxidative agents in the antimicrobial protection pathway (12). On the basis of this knowledge and recent experimental data, it has been proposed that HSP may represent one of the major targets of cell-mediated immune responses against mycobacteria (12, 29, 41), as well as intracellular bacterial and parasitic infections in general (41–43). However, because of the strong sequence homology between HSP from bacteria and human host cells (6, 11), these protein antigens also represent potential targets of autoimmune T-cell responses associated with infection. Cross-reactivity between mycobacterial and self HSP antigens has indeed been shown in both healthy individuals and patients with autoimmune diseases (12, 29, 32). In addition, mycobacterial HSP65 has been shown to directly regulate experimentally induced arthritis and diabetes in animal models (12). Hence, T-cell reactivity to the HSP antigens may represent key events in the balance between protective and pathological immune responses and therefore represent important model antigens for the study of the regulation of the outcome of infections in general.

Among the well-characterized mycobacterial HSP important to T-cell responses (10, 18, 65, and 70 kDa), only the 18- and 65-kDa HSP have been extensively mapped with regard to the primary structure of T-cell epitopes and MHC restriction elements used in T-cell recognition (4, 18, 27, 28, 37, 38). Although we have earlier identified HLA class II molecules involved in human T-cell recognition of the mycobacterial HSP70 antigen (23), this work represents the first mapping at the peptide level of immunogenic epitopes from this antigen as recognized by *M. leprae*-induced human CD4$^+$ T-cell clones and lines. In addition to defining T-cell epitopes, we have identified the HLA molecules presenting the individual peptide epitopes. The results show the presence of at least six different HSP70 T-cell epitopes presented by multiple HLA-DR molecules.

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MATERIALS AND METHODS

Whole-cell mycobacterial antigens. Armadillo-derived, killed *M. leprae* was kindly provided by R. J. W. Rees, of the World Health Organization (WHO)-IMMLEP Bank, Medical Research Council, Mill Hill, London, England. *M. tuberculosis* and *M. bovis* BCG were kindly supplied by Jan Eng, National Institute of Public Health, Oslo, Norway.

Recombinant *M. tuberculosis* HSP70 antigen. Purified recombinant HSP70 from *M. tuberculosis* was provided by J. D. A. van Embden with support from the UNDP-World Bank and WHO-TDR. The protein was obtained from *Escherichia coli* K-12 strain M1485, which carries plasmid Y3111/8 (17). After lysis of cells (lysozyme treatment and sonication) and centrifugation, the recombinant protein was precipitated from the supernatant with ammonium sulfate (60% saturation). HSP70 was then purified from the precipitate by DEAE anion-exchange chromatography and by ATP affinity chromatography and lyophilized (17).

Expression of the N-terminal 6 to 279-amino-acid (aa) sequence of the *M. leprae* HSP70 antigen in *E. coli.* The 5′ fragment of the *M. leprae* HSP70 gene was cloned from *M. leprae* DNA by PCR with selected primers as described in reference 10. The fragment was expressed as a polypeptide fused to β-galactosidase in *E. coli* by using plasmid pEX2. Expression of fusion proteins in *E. coli* was induced as described earlier (35). Induced cells were resuspended in 100 mM Tris/HCl (pH 8.0) containing 10 mM EDTA and 100 μg of lysozyme per ml to an optical density at 600 nm of 20. Cells were sonicated and then frozen and thawed to obtain complete lysis. The lysate was then pelleted, and the soluble fraction was resuspended in RPMI 1640 at a protein concentration of 500 μg/ml.

Expression of the C-terminal 278 to 621 aa of the *M. leprae* HSP70 gene in *E. coli.* The 3′ fragment of the *M. leprae* HSP70 gene (24) was kindly donated by A. Basten. The *Eco*RI fragment, called JKL2, was isolated, cloned into a Puc9 plasmid, and expressed in *E. coli* K-12 (POP2136). Expression of the C-terminal HSP70 polypeptide fused to β-galactosidase was obtained after induction with isopropyl-β-D-thiogalactopyranoside (IPTG) as judged by Western blot (immunoblot) analysis with mycobacterial HSP70-specific monoclonal antibody (MAb) L7.

Synthetic peptides. Peptides covering the N-terminal part (aa 6 to 278) of the *M. leprae* HSP70 sequence (44 17-mers overlapping by 10 aa) were synthesized by an ABIMED 422 peptide synthesizer (ABIMED, Lagenfeld, Germany) (7). Peptides covering the C-terminal part (aa 268 to 619) of the HSP70 sequence (69 12-mers overlapping by 7 aa) were synthesized by the PEPSCAN method (36).

T-cell donors. Donors of HSP70-reactive T-cell clones and lines were (i) healthy persons immunized with killed *M. leprae* (8) and (ii) tuberculoid leprosy patients (10).

HLA typing of T-cell and APC donors. *M. leprae*-vaccinated, healthy individuals, as well as APC donors from the hospital staff, were HLA typed serologically by the immunomagnetic method (39). Donors vaccinated with *M. leprae* were, in addition, typed for the presence of Dw4 and Dw14 by using alloreactive T-cell clones (34). APC donors were HLA class II typed genomically by hybridization of sequence-specific oligonucleotide probes to PCR-amplified DNA (33). The presence of the HLA-DRB4*0101 allele was confirmed genomically for selected individuals and the *M. leprae*-vaccinated T-cell donors. The tuberculoid leprosy patients were HLA typed as described earlier (31).

Mycobacterium-reactive T-cell lines. Human T-cell lines against *M. leprae* and *M. bovis* BCG were established as described earlier (21). Peripheral blood mononuclear cells (PBMC) (2×10^6/ml) from donors immunized with killed *M. leprae* were stimulated with optimal concentrations of *M. leprae* or BCG in complete medium (RPMI 1640 [GIBCO], 15% human AB serum, 1% penicillin-streptomycin) in 24-well Costar plates. After 6 days of incubation, antigen-reactive T cells were expanded by adding recombinant interleukin 2 (25 U/ml; Amersham) twice a week. After 4 weeks, the cells were restimulated with HSP70, APC, and interleukin 2 (25 U/ml). T-cell lines were maintained by supplementation of recombinant interleukin 2 twice a week.

Mycobacterium-reactive T-cell clones. Generation of *M. leprae*-reactive T-cell clones from subjects immunized with killed *M. leprae* was performed by the limiting-dilution technique (22). Establishment of *M. leprae*-reactive T-cell clones from the tuberculoid leprosy group, as described earlier (10), was also done by the limiting-dilution principle, but the primary antigen used was an *M. leprae* sonicate (1.0 to 100 μg/ml).

T-cell proliferation assays. Antigen-induced proliferative assays for T-cell lines and clones were performed by adding 10^4 T cells and 0.5×10^5 to 1×10^5 irradiated autologous or HLA-DR-typed allogeneic PMBC as APC in flat-bottom 96-well Costar plates together with antigen in triplicate at the following concentrations: whole *M. leprae*, 5×10^7 bacteria per ml; *M. leprae* sonicate, 5 μg/ml; *M. tuberculosis* sonicate, 40 μg/ml; purified *M. tuberculosis* HSP70, 0.4 to 10 μg/ml; *E. coli* lysate containing N- and C-terminal polypeptides from *M. leprae* HSP70, 0.01 to 0.5 μg/ml; synthetic peptides, 0.005 to 10 μg/ml. The total culture volume was adjusted to 200 μl. On day 3, cell cultures were pulsed with 0.045 MBq of tritiated thymidine (specific activity, 185×10^3 MBq/mM) and harvested after 4 h. Antigen-induced proliferation of *M. leprae*-induced T-cell clones from the tuberculoid leprosy patients was assayed as described in reference 10. Thymidine incorporation was determined by liquid scintillation counting or with a Packard Matrix 96 direct beta counter. A stimulation index (SI) of >10 was considered to represent positive proliferation.

Inhibition assays with MAbs. Inhibition assays with defined anti-HLA class I and II MAbs were performed by preincubation of irradiated APC (PBMC) in 96-well flat-bottom microtiter plates with the respective antibodies at different dilutions for 30 min. After preincubation, antigen-induced proliferation was assayed as described above. The following MAbs were used for inhibition studies: W6/32 (anti-HLA class I monomorphic, purchased from the American Type Culture Collection, Rockville, Md.), L243 (Anti-HLA-DR monomorphic, from ATCC), FN81 (anti-HLA-DQ monomorphic, a gift from S. Funderud, Oslo, Norway), 22C1 (anti-HLA-DP, a gift from S. Funderud), B7/21 (anti-HLA-DP monomorphic, a gift from F. Bach, Minnesota, Mo.), and 109 d6 (anti-HLA-DRw53, a gift from R. Winchester, New York, N.Y.).

RESULTS

Identification of *M. leprae* HSP70-reactive T-cell populations. *M. leprae* HSP70-reactive T-cell populations used in this study were derived mainly from two different donor groups.

In the first group, *M. leprae*-reactive T-cell clones and lines were raised from four HLA-typed healthy individuals immunized with killed *M. leprae* (8). To identify antigen specificity, these T-cell populations were tested for reactivity to known recombinant mycobacterial protein antigens (*M. leprae* 18- and 65-kDa antigens, *M. tuberculosis* 65- and 70-kDa antigens, and the *M. leprae* 13B3 antigen) in proliferative assays in vitro. The primary results showed that five T-cell clones recognized the

TABLE 1. Proliferative responses[a] of *M. leprae*-reactive T-cell populations to recombinantly expressed N- and C-terminal parts of the *M. leprae* HSP70 antigen

Cells	*M. leprae*	*M. tuberculosis* or *M. tuberculosis* PPD[b]	*M. tuberculosis* recombinant HSP70	*M. leprae* HSP70 N-terminal aa 6–279	*M. leprae* HSP70 C-terminal aa 278–621
Healthy subject lines					
ML-1-70K	349	338	102	56	77
ML-2-70K	297	200	17	2	12
BCG-3-TR 70K	125	172	391	41	1
ML-4-70K	668	528	149	2	115
Healthy subject clones					
ML-1-16 (DR2)	60	21	60	0	11
ML-2-15 (DR2)	77	13	55	0	12
ML-2-17 (DR2)	12	15	18	3	22
Tuberculoid leprosy patient line JIAW-TLEP	3	18	29	1	0
Tuberculoid leprosy patient clones[c]					
R3F7 (DR2)	119	344[b]	125	1	55
R1G6 (DR3)	174	2[b]	5	356	1
R1E4 (DR3)	398	4[b]	ND[d]	570	4
R1E3 (DR3)	397	3[b]	6	1,040	3
R1F3 (DR3)	21	1[b]	ND	33	2

[a] Results are expressed as an SI (defined as counts per minute obtained with T cells, APC, and antigen divided by counts per minute obtained with T cells, APC, and negative controls. The negative control for T-cell proliferation in response to *M. leprae*, *M. tuberculosis*, and purified recombinant *M. tuberculosis* HSP70 was medium only. The negative control for T-cell proliferation in response to the recombinantly expressed N- and C-terminal parts of the *M. leprae* HSP70 antigen added was *E. coli* lysate containing the wild-type pEX2 expression vector. Positive SIs (SI, >10) are underlined.

[b] In proliferative assays of tuberculoid leprosy patient clones, *M. tuberculosis* purified protein derivative (PPD) was used as the antigen.

[c] The reactivity patterns of these clones are the same as those published earlier by us (10), but the data shown here are based on new experiments with the same clones. The purpose of including these results here was to show the cross-reactivity patterns of all of the T-cell populations used in this work to map HSP70 epitopes in one table.

[d] ND, not done.

M. tuberculosis HSP70 antigen. When three of these T-cell clones (ML-1-16, ML-2-15, and ML-2-17) were tested for reactivity to the recombinantly expressed N- or C-terminal part of the *M. leprae* HSP70 molecule, all of the clones recognized the C-terminal sequence, comprising aa 278 to 621 (Table 1). As shown in Table 1, all of these T-cell clones were cross-reactive for *M. leprae* and *M. tuberculosis*. In addition, HSP70-reactive polyclonal T-cell populations were established from the same donor group by primary stimulation of PBMC with *M. leprae* and secondary stimulation with *M. tuberculosis* HSP70. The results of testing of these T-cell lines against the N- and C-terminal polypeptides of *M. leprae* HSP70 showed that both parts of the molecule contained T-cell epitopes (Table 1). One of the T-cell lines, ML-1-70K, contains T cells recognizing epitopes on both antigen fragments, whereas the other HSP70-reactive T-cell lines (ML-2-70K, BCG-3-70K, and ML-4-70K) recognize epitopes located on either the N- or C-terminal half of the HSP70 antigen (Table 1).

The second donor group for HSP70-reactive T cells in this study consisted of tuberculoid leprosy patients. Four of the *M. leprae*-reactive T-cell clones established from one tuberculoid leprosy patient were found earlier to respond to the recombinant *M. leprae* HSP70 antigen (13). When previously tested against the defined fragments of this protein antigen, one of the reactive T-cell clones (R3F7) recognized the C-terminal part (aa 278 to 621) (13). This T-cell clone was cross-reactive for *M. leprae* and *M. tuberculosis* (13). As reported in reference 13, all of the other T-cell clones (R1G6, R1E4, and R1E3), which, in contrast to clone R3F7, were *M. leprae* specific, showed a proliferative response to the N-terminal sequence (aa 6 to 279) of the HSP70 antigen. The cross-reactivity patterns of these T-cell clones (represented by newly obtained SI data) are included in Table 1 for comparison with those of the other T-cell clones and lines used to map HSP70 epitopes in this work. In contrast to the species specificity observed for these T-cell clones (cross-reactive to *M. leprae* and *M. tuberculosis* or *M. leprae* specific), we also identified T-cell lines from an HLA-DR3-positive tuberculoid patient which are specific to the *M. tuberculosis* HSP70 antigen. One example of this T-cell specificity pattern is shown in Table 1. In addition, the same recognition pattern has been observed by us in three HLA-DR3-restricted, *M. tuberculosis* HSP70-specific T-cell clones isolated from a coeliac disease patient (results not shown). However, any epitope mapping of these T-cell populations by the synthetic peptides available for this study was not possible since these peptides were derived from the *M. leprae* HSP70 sequence. An interpretation of these T-cell specificities in relation to the other results is given in the Discussion.

Mapping of T-cell epitopes by screening HSP70-reactive T cells against synthetic peptides. We mapped the individual T-cell epitopes recognized by screening the HSP70-reactive T-cell populations for reactivity against overlapping synthetic peptides covering the part of the *M. leprae* HSP70 sequence to which they respond.

First, the HSP70 N-terminal reactive T-cell populations (ML-1-70K, BCG-3-70K, R1G6, R1E4, and R1E3) were screened against a series of 44 synthetic peptides (17-mers overlapping by 10 aa) covering aa 6 to 278 of the *M. leprae* HSP70 sequence. The results (Fig. 1) showed that T-cell line ML-1-70K, established from a donor immunized with killed *M. leprae*, recognized both peptide 11, representing aa 66 to 82, and peptides 35 and 36, representing the overlapping sequences of aa 210 to 226 and 216 to 232, respectively (Fig. 1A). Thus, this T-cell line is able to recognize two different epitopes, defined by the sequences IRSVKRHMGSDWSIEID (peptide 11) and FKGTSGIDLTK (common sequence of peptides 35 and 36), located on the N-terminal half of the HSP70 molecule. Unexpectedly, the BCG-3-70K T-cell line, which also

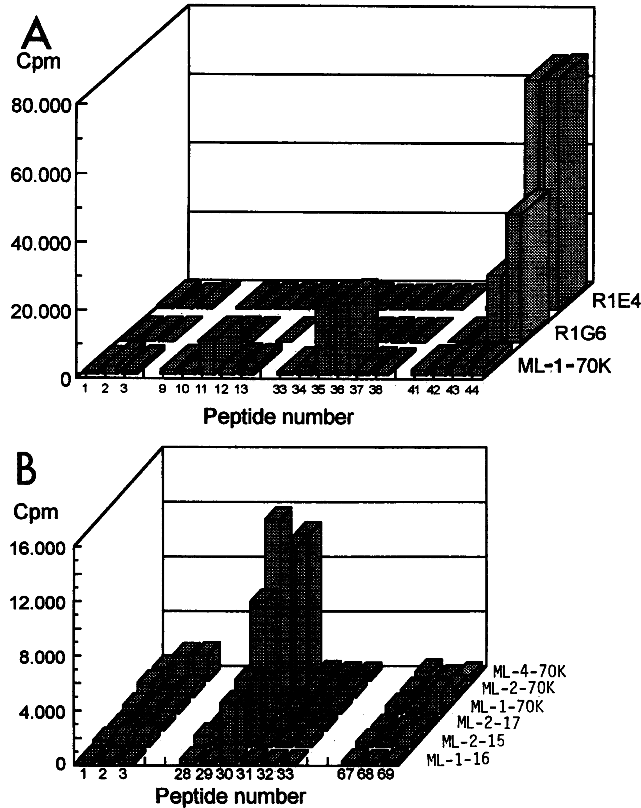
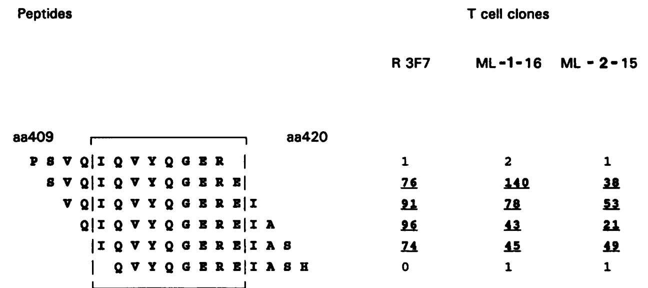


FIG. 1. T-cell proliferation in response to synthetic peptides covering the N- and C-terminal halves of the *M. leprae* HSP70 sequence. The results are expressed as 10^{-3} cpm when the optimal peptide concentrations for the indicated T-cell clones and lines were used. (A) Proliferation of the T-cell lines and clones indicated in response to 44 overlapping N-terminal HSP70 peptides. All positive peptide responses (peptides 11, 35, 36, 43, and 44) are shown, but only a selection of negative peptide responses are shown as examples. T-cell clone R1E3, which also responded to peptides 43 and 44, is not shown. (B) Proliferation of the T-cell lines and clones indicated in response to 69 overlapping C-terminal HSP70 peptides. Positive peptide responses (peptide 30) and some negative responses are shown. Results for clone R3F7, which also recognized C-terminal peptide 30, are shown in Fig. 2. All of the peptides not shown here (represented by gaps) were negative.

responded to recombinant HSP70 polypeptide aa 6 to 279, did not recognize any of the N-terminal peptides tested (data not shown), indicating the possibillity that the peptides employed did not encode all of the present epitopes. All three of the *M. leprae* N-terminal HSP70-reactive T-cell clones derived from the tuberculoid leprosy group responded to overlapping peptides 43 (aa 258 to 274) and 44 (aa 262 to 278), defining this epitope as the common sequence SDKNPLFLDEQLI (aa 262 to 274).

Epitope mapping of the C-terminal HSP70 sequence was performed by screening the reactive T-cell lines and clones for reactivity against a different series of synthetic peptides, covering aa 268 to 619, represented by 69 12-mer peptides overlapping by 7 aa. All of the C-terminal, HSP70-reactive T-cell lines and clones (ML-1-70K, ML-2-70K, ML-4-70K, ML-1-16, ML-2-15, and ML-2-17 from the immunized group and R3F7 from the tuberculoid leprosy group) showed a proliferative response to peptide 30 (aa 413 to 424), representing the sequence IQVYQGEREIAS (Fig. 1). Fine mapping,



Peptides (aa409 – aa420)	R 3F7	ML-1-16	ML-2-15
P S V Q|I Q V Y Q G E R |	1	2	1
S V Q|I Q V Y Q G E R E|	76	140	38
V Q|I Q V Y Q G E R E|I	91	78	53
Q|I Q V Y Q G E R E|I A	96	43	21
|I Q V Y Q G E R E|I A S	74	45	49
| Q V Y Q G E R E|I A S H	0	1	1

FIG. 2. Detail mapping of the N- and C-terminal boundaries of the HLA-DR2-restricted *M. leprae* HSP70 T-cell epitope. The results express the proliferative responses (positive SI values are underlined) of T-cell clones ML-1-16, ML-2-15, and R3F7 when stimulated with overlapping peptides covering the epitope region. The minimal epitope sequence necessary for recognition was defined as IQVYQGERE (aa 413 to 421).

performed by using overlapping peptides representing jumps of 1 aa, of this epitope recognized by T-cell clones R3F7, ML-1-16, and ML-2-15 revealed that the core sequence necessary for recognition by all of these clones is IQVYQGERE (Fig. 2).

Mapping of HLA restriction elements used in T-cell recognition of individual HSP70 epitopes. HLA restriction of the T-cell responses described was established by combining results from inhibition experiments with MAbs recognizing HLA class I, HLA-DR, HLA-DQ, and HLA-DP and panel studies using HLA-typed allogeneic PBMC as APC.

Antigen-induced proliferation of all of the T-cell clones described here was uniformly inhibited in a dose-dependent manner by MAbs recognizing HLA-DR and not by other MAbs recognizing HLA-DQ, HLA-DP, or HLA class I (Fig. 3 and data not shown). These experiments were performed with the relevant antigens for each T-cell clone or line; i.e., the antigens were stimulated in the presence of autologous APC with either HSP70 or individual peptides. The HLA-DR phenotypes of each donor were mapped as described in Materials and Methods and shown in Table 2 (1, DR2 and DR7; 2, DR2 and DR4; 3, DR4 and DR5; 4, DR1 and DR2). HLA-DR typing of tuberculoid leprosy donors is described in reference 10.

The panel studies, with HLA-typed, allogeneic APC, demonstrated that several HLA-DR molecules could present HSP70 to the T cells. Some of the data are shown in Table 2. For the polyclonal T-cell lines, these studies were performed

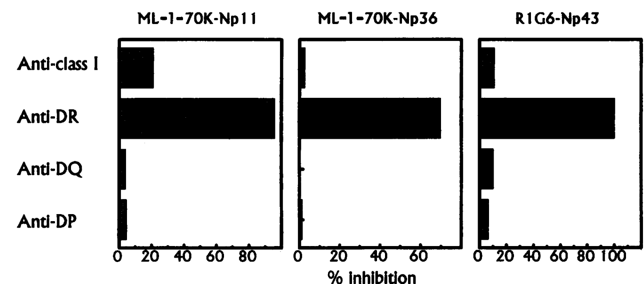


FIG. 3. Examples of proliferative T-cell responses to defined peptide epitopes in the presence of anti-class I, anti-DR, anti-DQ, or anti-DP antibodies (ascites 1:100). The results are expressed as percent inhibition compared with that of controls without blocking antibodies.

TABLE 2. Mapping of HLA-DR restricting elements on the basis of proliferation[a] of *M. leprae* HSP70-reactive T-cell lines and clones in response to synthetic peptides or recombinant HSP70 in the presence of HLA-typed allogeneic APC

T cells used (HLA-DR types)	SI with following synthetic peptide/antigen (HLA type[s] used):								
	ML-1-70K/ Np11 (DR7)	ML-1-70K/ Np35 (DR7)	ML-1-70K/ Cp30 (DR2)	ML-2-70K/ Cp30 (DR2)	ML-4-70K/ Cp30 (DR2)	BCG-3-70K/ N-terminal HSP70 (DR5, DRw53)	ML-1-16/ HSP70 (DR2)	ML-1-16/ HSP70 (DR2)	ML-2-15/ HSP70 (DR2)
Donor PBMC									
1 (2, 7, w53)	44	127	57	38	45	40	22	24	18
2 (2, 4, w53)	2	2	65	96	14	50	35	19	42
3 (4, 5, w53)	1	4	1	1	3	58	1	1	1
4 (1, 2)	1	0	125	55	41	1	31	37	49
Allogeneic PBMC									
1 (1, 4, w53)	1	7	1	ND[b]	1	ND	ND	1	1
2 (1, 2)	2	9	88	ND	ND	1	53	227	51
3 (1, 7, w53)	33	172	3	ND	ND	64	ND	ND	ND
4 (2, 3)	1	4	75	64	51	3	20	ND	ND
5 (4, 4, w53)	2	1	1	2	1	24	1	0	1
6 (5, 6)	1	5	ND	0	ND	37	ND	ND	ND
7 (4, 7, w53)	44	126	ND	ND	1	60	1	ND	ND
8 (7, 7, w53)	35	154	1	6	ND	ND	ND	ND	ND
9 (2, 5)	0	2	42	24	ND	82	31	63	42
10 (6, 7, w53)	34	84	ND	ND	1	ND	1	0	1
11 (3, 6)	1	5	ND	0	ND	1	1	1	0
12 (2, 9, w53)	2	1	65	51	38	35	34	35	53

[a] Proliferation is expressed as an SI. Positive SIs (SI, >10) are underlined.
[b] ND, not done.

by using synthetic peptides as antigens. For T-cell clones, the same studies were performed by using recombinant HSP70. Polyclonal T-cell line ML-1-70K was derived from a DR2-DR7 heterozygous donor and recognized peptides 11, 35 and 36 from the N-terminal part of HSP70, as well as peptide 30 from the C-terminal part. The panel study showed that the responses to peptides 11 and 35 and 36 were both DR7 restricted. However, the same T-cell line recognized peptide 30 only when presented by DR2. Furthermore, we investigated two other polyclonal T-cell lines (ML-2-70K and ML-4-70K) and three T-cell clones (ML-1-70K, ML-2-15, and ML-2-17), which all recognized C-terminal peptide 30. In all cases, these T-cell responses were restricted by DR2 and not by other HLA molecules. T-cell clone R3F7, which also recognized this peptide, has been shown to be HLA-DR2 restricted (10). These data establish that the C-terminal HSP70 epitope identified here (peptide 30) was presented predominantly by DR2. The three T-cell clones (R1G6, R1E3, and R1E4) derived from the tuberculoid leprosy group, which respond to N-terminal peptides 43 and 44, have previously been shown to be HLA-DR3 restricted (10, 31).

T-cell line BCG-3-70K, which responded to the N-terminal fragment of the *M. leprae* HSP70 molecule but not to any peptides, was also investigated with respect to MHC restriction. The results of the panel study showed that this line could recognize the N-terminal fragment of HSP70 when presented by APC expressing HLA-DR5, HLA-DR4, HLA-DR7, and HLA-DR9. This common presentation of APC expressing HLA-DR4, HLA-DR7, and HLA-DR9 indicates that HLA-DRw53 represents the restriction element used, since HLA-DRw53 is coexpressed with these three HLA-DR molecules. Blocking studies with anti-DRw53 antibodies (results not shown) confirmed that this was the case. We therefore conclude that both HLA-DRw53 and HLA-DR5 can function as restriction elements for N-terminal epitopes not yet defined at the peptide level.

In conclusion, we have shown that mycobacterial HSP70 can be recognized by human T cells in the context of HLA-DR2, HLA-DR3, HLA-DR5, HLA-DR7, and HLA-DRw53. In addition, these studies have shown a stringent correlation between T-cell epitopes and HLA restriction in the sense that each epitope was presented to the T cells by only one HLA molecule (Fig. 4).

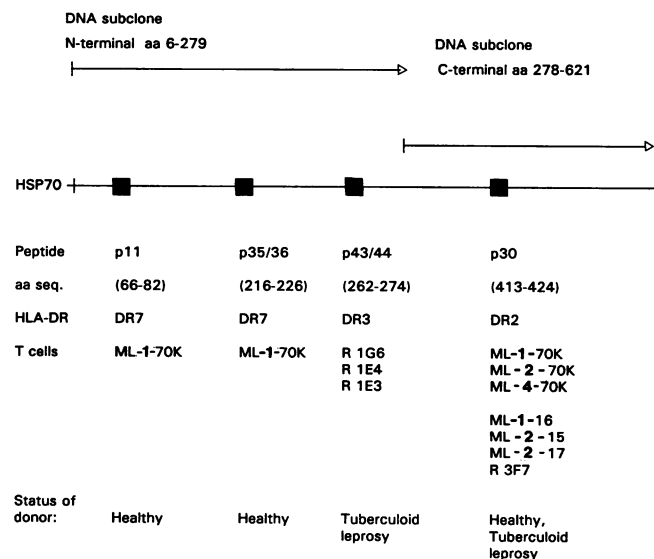


FIG. 4. Epitope map of the *M. leprae* HSP70 antigen. The peptide-defined T-cell epitopes identified, the restriction elements used in recognition, the responding T-cell lines and clones, and donor status are shown.

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DR7
aa (66-82)     M. leprae   I R S V K R H M G S D W S I E I D
               M. tub.     V - - - - - - - - - - - - - - - -
               Human       V F D A - - L I - R K V Q V S Y K

DR7
aa (216-226)   M. leprae   F K G T R G I D L T K
               M. tub.     - - - - S - - - - - -
               Human       - - R K H K K - I S Q

DR3
aa (262-274)   M. leprae   S D K N P L F L D E Q L I
               M. tub.     A - - - - - - - - - - - T
               Human       T L S S S T Q A S L E - -

DR2
aa (413-424)   M. leprae   I Q V Y Q G E R E I A S
               M. tub.     - - - - - - - - - - - A
               Human       - - - - E - - - A M T K
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FIG. 5. Sequence alignment of the *M. leprae* T-cell epitopes identified with the corresponding regions of *M. tuberculosis* (M. tub.) HSP70 and human HSP70. The sequence information is based on references 14 and 16.

DISCUSSION

In leprosy, both protective and pathological immune responses are dependent on antigen-specific HLA class II-restricted T cells (3). Mapping of *M. leprae* protein antigens with regard to epitopes and HLA class II molecules used in T-cell recognition is therefore important for understanding immunopathological conditions and designing subunit vaccines that will induce protection. Because of immunodominance and evolutionary sequence homology (12), the HSP group in this context represents interesting candidate antigens that might serve as model antigens to study the balance between protective and pathological T-cell responses at the molecular level. The biochemical role of HSP70, the N-terminal part of which contains ATPase activity, is to maintain proteins in an unfolded and membrane translocation-competent conformation (12). The *M. leprae* HSP70 sequence shows 95% homology with *M. tuberculosis* HSP70, 56% homology with *E. coli* HSP70, and 47% homology with the human HSP70 sequence (14, 16).

In this work, we used a combination of DNA subclones and overlapping synthetic peptides covering the *M. leprae* HSP70 sequence to identify the epitopes recognized by human T cells. In addition, the respective HLA molecules presenting the individual peptide epitopes were identified by combining results from HLA typing, inhibition with anti-class I and II MAbs, and panel studies employing HLA-typed allogeneic APC. By using this approach, we identified two different epitopes (aa 66 to 82 and 216 to 226), both of which were exclusively presented by HLA-DR7 to polyclonal T cells from *M. leprae*-immunized persons. The question of whether these two epitopes, containing one amino acid substitution each (Fig. 5), were recognized by the T-cell line as cross-reactive or specific was not possible to determine without using the homologous *M. tuberculosis* peptides, since the cross-reactivity actually observed for the ML-1-70K line against *M. leprae* and *M. tuberculosis* HSP70 (Table 1) could be due to reactivity to the peptide epitope (aa 413 to 424) alone. However, we assume that all of the T-cell specificities present in the ML-1-70K line were cross-reactive between the two species, since these *M. leprae*-induced T cells were expanded with *M. tuberculosis* HSP70 as the antigen before peptide screening. The three HLA-DR3-restricted T-cell clones from the tuberculoid leprosy group, which recognized a third epitope (aa 262 to 274) on the N-terminal half of the molecule, were shown to be *M. leprae* specific, as they did not recognize *M. tuberculosis* HSP70 (Table 1). As shown in Fig. 5, this *M. leprae*-specifc recognition can be accounted for by one or both of the two amino acid substitutions between the *M. leprae* and *M. tuberculosis* sequences: serine to alanine at position 262 and isoleucine to threonine at position 274 (13, 16). The fourth T-cell epitope (aa 413 to 424) defined by synthetic peptides was presented exclusively by HLA-DR2 molecules and was recognized both by the *M. leprae*-immunized and tuberculoid leprosy groups. Consistent with the cross-reactive nature of all of the T-cell clones responding to this epitope, the minimal amino acid sequence detected (aa 413 to 421) shows identity between *M. leprae* and *M. tuberculosis* (14, 16; Fig. 5). Compared with the human HSP70 sequence, the first three epitopes identified here showed no relevant homology, whereas the latter HLA-DR2-restricted epitope (aa 413 to 421) showed identity at seven of nine residues (14, 16; Fig. 5). Thus, we consider this HLA-DR2-restricted epitope to be only potentially cross-reactive with the corresponding region of the human HSP70 antigen. Similar to this observation, T-cell epitopes (located C terminal to aa 555) with extensive homology to the human HSP70 molecule have been identified in the *Plasmodium falciparum* HSP70 sequence (2). However, testing of peptides corresponding to the human HSP70 sequence is necessary to verify wether these epitopes are cross-reactive or not. However, no cross-reactivity of clone R3F7 with ATP agarose-purified human HSP70 from heat-shocked leukocytes was found (10).

We have shown that HSP70 antigen-induced proliferation of the BCG-3-70 K T-cell line was partly HLA-DR5 and partly HLA-DRw53 restricted. Although this T-cell line showed a proliferative response to the recombinant N-terminal *M. leprae* HSP70 polypeptide (aa 6 to 279), it did not recognize any of the overlapping peptides covering this sequence. Our interpretation of this result is that at least one additional T-cell epitope is present in this part of the molecule, but that the overlapping peptide system chosen did not allow it to be detected. With regard to additional epitopes not defined at the peptide level, we have also identified HLA-DR3-restricted, HSP70-reactive T-cell clones from a coeliac patient which, in contrast to the clones reported here, are *M. tuberculosis* specific. This observation suggests the presence of an *M. tuberculosis*-specific T-cell epitope within the mycobacterial HSP70 molecule. In support of this conclusion, the same recognition pattern was observed for *M. tuberculosis* HSP70-reactive T-cell lines established from DR3-positive tuberculoid leprosy patients (one example is shown in Table 1) and healthy contacts (results not shown). Identification of the epitope(s) recognized by such T cells was not possible, since the HSP70 peptide series used here covered the *M. leprae* sequence. However, from a theoretical point of view, one should expect species-specific epitopes from HSP70 to be localized within the last 60 C-terminal aa, since most of the amino acid differences between *M. leprae* and *M. tuberculosis* are clustered in this region (16).

In conclusion, both the present and previous results (23) demonstrate that the mycobacterial HSP70 antigen can be recognized in the context of multiple HLA-DR molecules, including HLA-DR2, HLA-DR3, HLA-DR5, HLA-DR7, and HLA-DRw53. In this study, four of the T-cell epitopes present were defined by reactivity to synthetic peptides but the results demonstrate the presence of at least two additional epitopes

on the mycobacterial HSP70 antigen that can be recognized by HLA class II-restricted $CD4^+$ T cells. Furthermore, HLA restriction analysis revealed that each peptide-defined epitope was presented by only one particular HLA molecule. This is in agreement with the general observation that individual T-cell epitopes are not promiscuous in relation to HLA binding and presentation to T cells (37). In the context of subunit vaccine design, the HSP70 antigen seems to fulfill some of the most important general criteria of a relevant candidate antigen: the presence of many T-cell epitopes presented by multiple HLA class II molecules. With regard to the development of antileprosy subunit vaccines, we consider HLA-DRw53-restricted epitopes to be specially relevant, since this HLA specificity is expressed by 40 to 80% of the populations in most of the regions where this disease is endemic (1).

In general, many factors are thought to determine the balance between protective and immunopathological T-cell responses: antigens-epitopes recognized, MHC restriction, lymphokine profiles, recognition of self-epitopes, and regulatory mechanisms. In leprosy, some epitopes may elicit the known T-cell-mediated immunopathological mechanisms associated with the different forms of the disease rather than contribute to protective immunity. Knowledge of the antigen-MHC elements recognized by such T cells, as well as their possible functions, may be especially important in the design of a subunit vaccine against this disease (9, 29). In this context, HSP represent attractive model antigens since they are potential autoantigens and are strongly upregulated during infection in both the pathogen and the host itself. However, the results obtained do not indicate any difference in recognition of HSP70 epitopes related to HLA expression between healthy and diseased subjects. On the basis of the few donors used in this study, recognition of this HSP at the epitope level seems to be determined by the restriction element expressed by the donor rather than by any difference in reactivity between the donor groups. Whether pathological T-cell responses in tuberculoid leprosy could be due to any overexpression of mycobacterial HSP, cross-reactivity to self HSP, or hyperfunctional T-cell activity (9, 29) is not known. However, on the basis of the strong homology (seven of nine residues are identical) between the *M. leprae* and human HSP70 sequence within the HLA-DR2-restricted epitope (aa 413 to 424) identified here, one could speculate that the T-cell response to this epitope is cross-reactive for the mycobacterial and human HSP70 antigens. In contrast, the HLA-DR3-restricted epitope (aa 262 to 274), also recognized by tuberculoid leprosy T cells, is unlikely to induce T-cell responses cross-reactive with self-HSP70, since the amino acid sequences in this region are completely different in *M. leprae* and humans. In general, a systematic comparison between T-cell epitopes recognized by healthy contacts and tuberculoid leprosy patients (representing a spectrum of HLA types) in relation to the restriction elements used should be helpful in distinguishing between possible protective or pathological *M. leprae* epitopes.

In conclusion, the results obtained in this work indicate that the immunogenic mycobacterial HSP70 antigen, like the HSP65 antigen, contains a variety of T-cell epitopes presented by multiple HLA-DR molecules, of which most should be useful in prophylaxis, whereas some epitopes could be susceptible to induction of T-cell-dependent immunopathology. From a vaccine design point of view, this challange could be met by recombinant engineering of HSP antigens to keep some parts and delete others.

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