Multispecific CD4⁺ T Cell Response to a Single 12-mer Epitope of the Immunodominant Heat-Shock Protein 60 of Yersinia enterocolitica in Yersinia-Triggered Reactive Arthritis: Overlap with the B27-Restricted CD8 Epitope, Functional Properties, and Epitope Presentation by Multiple DR Alleles

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Multispecific CD4⁺ T Cell Response to a Single 12-mer Epitope of the Immunodominant Heat-Shock Protein 60 of Yersinia enterocolitica in Yersinia-Triggered Reactive Arthritis: Overlap with the B27-Restricted CD8 Epitope, Functional Properties, and Epitope Presentation by Multiple DR Alleles

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Yersinia heat-shock protein 60 (Ye-hsp60) has recently been found to be a dominant CD4 and CD8 T cell Ag in Yersinia-triggered reactive arthritis. The nature of this response with respect to the epitopes recognized and functional characteristics of the T cells is largely unknown. CD4⁺ T cell clones specific for Ye-hsp60 were raised from synovial fluid mononuclear cells from a patient with Yersinia-triggered reactive arthritis, and their specificity was determined using three recombinant Ye-hsp60 fragments, overlapping 18-mer synthetic peptides as well as truncated peptides. Functional characteristics were assessed by cytokine secretion analysis in culture supernatants after specific antigenic stimulation. Amino acid positions relevant for T cell activation were detected by single alanine substitutions within the epitopes. Fragment II comprising amino acid sequence 182–371 was recognized by the majority of clones. All these clones were specific for peptide 319–342. Th1 clones and IL-10-secreting clones occurred in parallel, sometimes with the same fine specificity. The 12-mer core epitope 322–333 is a degenerate MHC binder and is presented by the majority of clones. All these clones were specific for peptide 319–333. Th1 response to this epitope could play a dominant role in regulating the MHC-class I Ag HLA-B27. Among the organisms triggering ReA, Chlamydia trachomatis and Yersinia enterocolitica are frequent in western countries. The presence of bacterial Ag (2, 3) or DNA (4) in the joint suggests that local Ag may drive the immune response. The recent description of a Th2-cytokine secretion pattern in ReA (5–8) suggests that an ineffective Th1 response may contribute to bacterial persistence in vivo, because Th1 cytokines such as IFN-γ and TNF-α are crucial for the elimination of both Chlamydia (9, 10) and Yersinia (11).

The Yersinia heat-shock protein 60 (Ye-hsp60) has attracted increasing attention as an immunodominant Ag for T cells in ReA and other diseases. First, it has recently been found to be the major bacterial component recognized by synovial Th cells in a series of ReA patients (12). Second, nonamers derived from it stimulate synovial CD8⁺ T cells from patients with Yersinia-ReA in a HLA-B27-restricted way (13). Third, the Ye-hsp60 could be identified in peripheral blood cells of ReA patients years after onset of the arthritis (2). And finally, Ye-hsp60 belongs to the conserved hsp60 family (14) that has repeatedly been incriminated in induction of autoimmunity (15, 16) and in modulation of established inflammatory conditions (17, 18).

The present study was performed to determine the exact specificity and function of CD4⁺ T cells derived from the joint of a ReA patient in response to the Ye-hsp60. We identified an immunodominant CD4 T cell epitope within the Ye-hsp60 molecule, which was not restricted by a single DR haplotype and which showed plasticity in its capacity to elicit cytokines. Therefore, the response to this epitope could play a dominant role in regulating the pathogenesis of ReA.

Materials and Methods

Cell cultures, T cell cloning, Ag specificity determination, and HLA class II restriction

Synovial fluid from an 18-year-old male patient (HLA phenotype A2, 24; B27, 40; DR6(13), 52; DQ6; DPw1) suffering from ReA after a preceding diarrhea was obtained by arthrocentesis, performed for therapeutic reasons. The Yersinia-based etiology was confirmed by positive standard serology. Separation of synovial fluid mononuclear cells (SFMC) and lymphocyte proliferation assays were performed as described previously (19). SFMC...
were then cultured in the presence of Ye-hsp60 (10 μg/ml) for 6 days and cloned by limiting dilution in 20-μl Terasaki plates, at 1 cell/well, in the presence of 100 U/ml IL-2 (Eurocetus, Frankfurt, Germany), 3 μg/ml PHA (Sigma, Deisenhofen, Germany), and 10^5 irradiated allogeneic PBMC. Outgrowing T cell clones (TCC) were further expanded as described (12, 20) and then tested for Ag specificity in T cell proliferation assays (19) using irradiated (80 Gy) EBV-transformed autologous B lymphoblastoid cell lines (LCL) (10^5 T cells and 10^6 EBV-B-LCL). In some cases, irradiated (40 Gy) autologous PBMC (2 × 10^6) were used as APC. The HLA-restriction of the TCCs was determined using anti-DR, anti-DQ, and anti-DP mAbs (Beckton Dickinson, Heidelberg, Germany; each at 2.5 μg/ml).

For further specification of the HLA-DR restriction, PBMC from donors of the following DR haplotypes were tested at a final optimal concentration of 10 μg/ml as previously tested (12). Peptides were tested at a final concentration of 10 μg/ml. For investigation of DR-restricted presentation, irradiated PBMC were first preincubated with the candidate peptide (10 μg/ml) for 2 h, followed by two extensive washing steps, and TCCs were added subsequently. In addition, peptide titration experiments were performed (for details, see Results).

In addition, SFMC from seven other Yersinia-triggered ReA-patients (5 male, 2 female; mean age 25 (range, 18–39)) were stimulated with the Yersinia-specific 19-kDa protein (12), the Ye-hsp60, its three recombinant fragments, and overlapping synthetic peptides spanning the complete fragment II at a final concentration of 10 μg/ml over 6 days. Lymphocyte proliferation was measured as previously described (19).

Generation of recombinant proteins

Generation of recombinant Ye-hsp60, CT-hsp60, Bb-hsp60, and hu-hsp60 were prepared as previously (12) described. For the preparation of overlapping recombinant Ye-hsp60 fragments, appropriate parts of the open reading frames were amplified by PCR using the following primers pairs: 5'-GATC TCAGTA AACG TAAAG AGCTA-3' and 5'-GATC TACG TACT TGGT TCTG AG-3' for Ye-hsp60 amino acids (aa) 1–187; 5'-GATC GACT GAGC AGGCT GCAG-3' and 5'-GATC AGCCT GACG TCTG CTG-3' for Ye-hsp60 aa 182–371; and 5'-GATC TACG TAGC GCTG GCCG GCTG-3' and 5'-GATC CTGA CTGTA CTGTA CTGG CCGG CATG-3' for Ye-hsp60 aa 367–550. The annealing temperature was 60°C, and 30 cycles were performed. The amplified fragments were isolated from agarose gels with a Jet Sorb Kit (Genomed, Bad Oeynhausen, Germany). In addition, and ligated in frame with the maltose binding protein into the pMal vector (New England Biolabs, Beverly, MA) at the EcoRI/ HindIII site for the Ye-hsp60 (aa 367–550). Expression of the cloned genes was induced by 1 mM isopropyl-β-D-thiogalactoside (IPTG, Sigma, St. Louis, MO), and the cells were lysed with a French press. Recombinant proteins were affinity-purified on an amylase resin column according to the supplier’s instructions (New England Biolabs). The purified fragments were dialyzed against PBS and analyzed by SDS-PAGE.

Peptide synthesis

Peptides were synthesized by a robotic multiple peptide synthesizer (Syro; MultiSynTech, Bochum, Germany) using an F-moc/Bu solid-phase synthesis strategy (21). Wang resin (p-benzoxymethyl alcohol polystyrene) (Novabiochem, Bad Soden, Germany) was used as solid support. Side-chain-protected F-moc amino acids were obtained from Senn Chemicals (Bad Soden, Germany) and Novabiochem. Peptides were characterized by reversed-phase HPLC (M480 pump, UVD-320 S diode-array UV-detector, GINa 160 autosampler; Gynkotek, Germering/Munich, Germany) on Nucleosil C18, 100A, 5 μm (Macherey-Nagel, Düren, Germany) and electrospray mass-spectrometry (ESI-Quattro II; Micromass, Altrincham, U.K.).

HLA typing

Conventional HLA typing was performed by standard serological methods. In addition, cells from the ReA patient were HLA-typed at the DRB1 loci by PCR using sequence-specific primers.

Cytokine ELISA

Culture supernatants were harvested after 48 h of T cell stimulation for determination of Ag-induced cytokine secretion, and the concentration of cytokines in the supernatant was measured as described previously (5, 22). Briefly, levels of IFN-γ, TNF-α, IL-10, and IL-4 were analyzed by sandwich ELISA using purified anti-cytokine mAb (capture) and biotinylated anti-cytokine mAbs (detection) (PharMingen, San Diego, CA), with a detection level of 6 pg/ml for IL-4 and 20 pg/ml for the other three cytokines.

HLA-restricted T cell epitope prediction

The Ye-hsp60 protein sequence was run on a new T cell epitope prediction program, called TEPITOPE (23, 24). For prediction of HLA-DR-binding peptides derived from the Ye-hsp60 to identify “promiscuous” epitopes, the most frequent HLA-DR alleles in the Caucasian population (DRB1*0101, DRB1*0301, DRB1*0401, DRB1*0701, DRB1*0801, DRB1*1101 DRB1*1302, and DRB1*1501) were selected, and the prediction threshold was set at 4% (23). Peptide sequences predicted to bind at least three of seven DR molecules were selected.

HLA-DR-peptide binding assays

Peptide binding to detergent-solubilized DR molecules was measured using an ELISA-based high-flux competition assay (25). HLA-DR molecules were isolated from the following human LCLs: DR1 (DRB1*0101) from HOM-2, DR3 (DRB1*0301) from WT49, DR4 (DRB1*0401) from PREISS, DR5 (DRB1*1101) from SWEIG, DR6 (DRB1*1302) from the L cell transfectant L605.2, DR7 (DRB1*0701) from EKR, and DR8 (DRB1*0801) from BM9. DR2 (DRB1*1501) was isolated from the L cell transfectant L466.1. The molecules were affinity purified using the mAb 1-IC4 (26) as described (27).

Peptide competition assays were conducted to measure the ability of unlabeled peptides to compete with a biotinylated indicator peptide for binding to purified DR molecules. The following biotinylated indicator peptides were used: GFKA 7 for DR1 and DR7, GIRA 2 YA 4 for DR2, GIAAAT CTGCAGACATC (28) for DR3; UDA 11 for DR4 (28); TT; for DR5; and GYRA; for DR8. An optimal concentration of purified DR molecules (30–100 ng) was added to each well of a 96-well plate along with biotinylated peptide (0.1–5 mM) in binding buffer (25). The biotinylated indicator peptide and DR molecules were incubated with 10-fold dilutions (0.001–100 mM) of the unlabeled competitor peptide (Ye-hsp60 peptide). Following incubation overnight, the peptideclass II complexes were transferred to wells coated with anti-DR L243 (HB 55; American Type Culture Collection, Manassas, VA) mAb. After 2 h incubation at room temperature, the wells were washed with an appropriate buffer (25), and the specific degree of the biotinylated peptide bound to DR molecules was revealed by the addition of alkaline phosphatase-labeled streptavidin and 4-methylumbelliferyl phosphate. The amount of substrate hydrolyzed was assessed with a MC Multiscan ELISA reader (405 nm). To determine relative peptide binding affinity, the “promiscuous” PKYVKQLKLTLAT peptide from influenza hemagglutinin (HA) 307–319 (29) was included in each competition assay. The relative binding of the unlabeled competitor peptides was expressed as inhibitory concentrations (IC50).

Results

T cell cloning, efficacy, and gross specificity

The cloning procedure yielded 61 CD4+-TCCs specific for Ye-hsp60. All these TCCs responded also to whole heat-killed Yersinia. Fifty of these TCCs showed specific proliferation to Ye-hsp60 fragment II, spanning the aa residues 182–371, eight were specific for fragment III, spanning the aa residues 367–550, two reacted with fragment II and fragment III, and one was specific for fragment I, comprising the aa residues 1–187 (Fig. 1).

Fine specificity of TCCs

Eighty-nine 18-mer peptides, overlapping by 12 aa and covering the whole Ye-hsp60, were employed for initial epitope characterization. The clones responded preferentially to fragment II. Twenty-one of the 50 TCCs specific for fragment II could be grown sufficiently for epitope analysis. Fifteen of the 21 TCCs proliferated specifically to peptide p54/310–316 and the other six of the 21 TCCs responded to the adjacent peptide p55/325–342 (Fig. 1). Next, truncated peptide variants were constructed to identify the minimal length recognized. Five of the 15 clones specific for p54 and one of the six TCCs specific for p55 were characterized as shown in Table I. The 12-mer peptide p54/66-322–332 was the minimal epitope common to all clones responding to p54. Four types of TCCs could then be identified on the basis of a stepwise
truncation of peptide 322–336 by 1 aa at the N terminus. The first
type (clones 69 and 92) recognized the 10-mer p54-6g 322–331; the
second type (clones 83 and 85) recognized the 11-mer p54-6h 323–333;
the third type of clone (clone 98) could be stimulated by p55-8 324–337;
and the fourth type (clone 39) recognized p55-9 325–336. Further trun-
cations of the two latter peptides at the C terminus were not tested.

Stepwise truncations at the C terminus of p54-6b322–333 were of mi-
nor relevance and influenced only proliferation of clone 98.

**FIGURE 1.** Schematic representation of recombi-
nant Ye-hsp60, its recombinant fragments, and loca-
tion of the epitopes. A. Overview. B. Fragment II and
the epitope is shown in more detail. C. The peptide
epitope p54/55 from fragment II is shown in more
detail. The numbers below and beside the fragments
represent amino acid residues. The number of clones
recognizing each fragment is also indicated. A single
letter code is used for the amino acids.

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| aa requirements for
recognition by specific T
cell clone | RV-INKD-T- | -KD- | -INKDT-I- |

* The amino acid sequence of the peptides used is given in single letter code. The indices after the clone numbers indicate the type of clone as defined by its particular peptide response. The (+) semi-quantitatively summarizes the results of the proliferative responses as determined in three independent experiments. Although not shown, the proliferative response closely reflects and is confirmed by the amount of IFN-γ secreted into the culture supernatants. The alanine substitutions are given in bold.
The clones responding to fragment III were also investigated for their fine specificity (Table II). Two antigenic regions were identified on fragment III: one clone reacted with peptides p64 and p65 spanning 379–402, and six clones reacted with peptide p76451–468. The truncated variants p65-21 385–395 and p65-22 386–396 seemed to predominate, as also found by the alanine substitutions, because the T329 was also relevant for T cell recognition but was overlooked by the alanine substitutions, because the T→D substitution is a highly conserved hydrophobic one. In both the Chlamydia peptide and the human peptide, a nonconserved substitution (T329→D) is present (hydrophobic aa→negatively charged aa), whereas a relatively conserved T→N (hydrophobic aa→small polar aa) substitution present in the Borrelia peptide might be less important. Extension of the substitution experiments by introducing a negatively charged amino acid (aspartic acid (D)) instead of alanine could detect such a potentially critical aa. In addition, several substitutions of the C-terminal isoleucines present in the human peptide but not in the Borrelia peptide might result in a significant decrease of MHC binding, thus leading to a subsequent failure of T cell recognition.

There was no cross-reactivity of the fragment III peptide p76451–468 with the corresponding peptides from the other hsp60s. Although no essential aa residues were determined for this peptide, the substantial differences in the sequence between the Ye-hsp60-derived peptide and the corresponding peptide from the hu-hsp60, the CT-hsp60, or the Bb-hsp60 could easily explain this failure (Table II). In contrast, clone 68 specific for the second fragment III epitope responded both to the whole CT-hsp60 and to its derived peptide and the corresponding peptide from the hu-hsp60.

The amino acid sequence of the peptides is given in single letter code. Vertical lines (|) indicate identical amino acids, and the colons (:) indicate conserved amino acid substitutions. Amino acids given in bold represent the core peptide epitope as defined by truncated peptides. D→E substitution in the Chlamydia p54/6-h homologue is highly conserved. The T→D substitution at position 7 (underlined in the human peptide) and the Chlamydia peptide is a nonconserved hydrophobic→negatively charged aa substitution, while the T→N substitution (underlined) in the Borrelia peptide is a conserved one.

The effect of single alanine substitutions within peptide p54/6-h on T cell activation

Substitution of single positions by alanine was performed for the 12-mer peptide p54-6b to determine the aa residues that are crucial for stimulation of the TCCs, as shown in Table I for the three different types of TCCs (69/92, 83/85, and 98). Different determinants were identified. Interestingly, for the second type only two residues (K327 and D328) of the 10-mer peptide proved essential.

Cross-recognition of other hsp60s and hsp-derived peptides by TCCs

Cross-recognition of the Bb-hsp60, CT-hsp60, and hu-hsp60 by the TCCs was assessed using whole recombinant proteins as well as hsp60-derived 24-mer peptides homologous to the Ye-hsp60 epitopes (Table II) to circumvent possible “crypticity” of these epitopes. In the case of the Yersinia peptides p54 and p55, cross-reactivity was observed only with the homologous Bb-hsp60-derived 24-mer peptide but not with the CT-hsp60 or the hu-hsp60. Interestingly, the whole Bb-hsp60 was also not recognized. This indicates that the Borrelia peptide recognized was not generated under the experimental conditions used, i.e., it behaved as a “cryptic” epitope. The cross-reactivity was restricted to one clone, clone 83. Table II shows the appropriate sequence alignments. Clone 83 needs only K327 and D328 for activation. Both aa are indeed shared by the Borrelia peptide in the appropriate positions. In contrast, clones 92 and 98 needs several other positions for activation, which are not shared between the peptide p54-6b and the Bb-hsp60 peptide.

Surprisingly, the hu-hsp60 peptide and the Chlamydia-hsp60 peptide were not recognized by clone 83 although they shared the essential aa residues KD (human peptide) but showed a highly conserved substitution (D→E; Chlamydia peptide), which should be of minor relevance (Table II). This observation could be explained if T329 was also relevant for T cell recognition but was overlooked by the alanine substitutions, because the T→A substitution is a conserved hydrophobic one. In both the Chlamydia peptide and the human peptide, a nonconserved substitution (T329→D) is present (hydrophobic aa→negatively charged aa), whereas a relatively conserved T→N (hydrophobic aa→small polar aa) substitution present in the Borrelia peptide might be less important. Extension of the substitution experiments by introducing a negatively charged amino acid (aspartic acid (D)) instead of alanine could detect such a potentially critical aa. In addition, several substitutions of the C-terminal isoleucines present in the human peptide but not in the Borrelia peptide might result in a significant decrease of MHC binding, thus leading to a subsequent failure of T cell recognition.

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HLA restriction and “promiscuous” epitope recognition by TCC

We tested the effect of anti-DR-, anti-DP-, and anti-DQ-specific mAbs on proliferation and cytokine production on the TCCs. These experiments revealed that HLA-DR was the restricting class II molecule for all clones investigated. By using APC homozygous for various DR alleles from donors, effective epitope presentation to clone 92 could be shown for all DR alleles investigated, i.e., DR1, DR2, DR4, and/or DR53, DR5, and/or DR52, DR7, and/or DR53 (Fig. 2). Dose-response experiments identified autologous APC as the best presenters, particularly in the critical peptide concentration range between 1 μg/ml and 0.1 μg/ml (data not shown). In contrast, clone 69 recognized the peptide epitope only in the context of autologous DR6-positive APC, but not if presented by DR7,53- and DR4,53-positive APC (DR1-, DR2-, and DR5-positive APC were not tested) (Fig. 2).

“Promiscuous” T cell epitope prediction

The TEPITOPE program confirmed that peptides from the 24-mer peptide p54/5519–342 would bind to DR1, DR3, DR4, DR2b, and DR8 with a threshold of 3% and to DR6 with a 4% threshold and
that the homologous peptides derived from the Bb-hsp60, the CT-hsp60, and the hu-hsp60 had no significant affinity to DR6. The same “promiscuous” DR-binding properties were predicted for the truncated epitopes, i.e., the 15-mer p54-6 (RVVINKDTPITIIIDGV) and the 12-mer p54-6b (RVVINKDTHII).

A degenerate MHC binding was also predicted for p76/77 452–468, with a 1% threshold to DR3, a 2% threshold to DR1 and DR4, a 3% threshold to DR5 and DR6, and a 4% threshold to DR7.

**HLA-DR binding assays**

The competitive binding assays employing the eight most common HLA-DR binding assays with the epitopes of hsp60-fragment III (p64 and p76/77) were not performed.

**Comparison of the Ye-hsp 60 epitope 322–333 with other “promiscuous” epitopes**

We compared the predicted HLA-DR binding frame of the “promiscuous” Ye-hsp60 epitope p54-6b, which consists of the nonamer VINKDTTIII (324–332), with other microbial epitopes “promiscuous” for HLA-DR (Table III). Sequence alignment with these other T cell epitopes shows that the motif required for binding several DR molecules has similarities (30–33). The residues crucial for DR binding are position 1, 6, and 9. All the epitopes shown here including the Ye-hsp60 324–332 contain a hydrophobic residue (V for the Ye-hsp60) at position 1, a small hydrophobic residue (T for the Ye-hsp60) at position 6, and an aliphatic residue (I for the Ye-hsp60) at position 9.

**Cytokine profiles upon stimulation of T cell clones with Ye-hsp60 peptides**

The cytokine profile was determined for clones 69, 92, 83, and 98, which are all specific for p54-6b, and clone 55, being specific for p76/77 452–468. The data for clone 69 and clone 92 are shown in Fig. 3, and the data for clone 55 is shown in Table IV. IFN-γ is the prominent cytokine and closely reflects the magnitude of proliferation in response to the particular Ag. TNF-α is frequently secreted by all clones, except clone 55, in parallel with the level of activation as measured by proliferation.

In contrast, the amount of IL-10 secretion varied among the clones. Clone 92 (Fig. 3), clone 98, and clone 83 (data not shown) were potent IL-10 producers with IFN-γ:IL-10 ratios ranging from 10:1 to 5:1, depending on the stimulating peptide Ag. In contrast, clone 69, although recognizing the same peptide (Table I), failed to secrete substantial quantities of IL-10 (Fig. 3). Using the technique of intracellular cytokine staining of the TCCs with subsequent quantification of positive cells by flow cytometry, we could show that IL-10 was secreted by T cells and not by the non-T cells in the population of APC used for this experiment (data not shown). Moreover, the amount of IL-10 secretion differs not only among the clones but also for a single clone after stimulation with Ags of

**Table III. Promiscuous T cell epitopes with DR binding motifs**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ye-hsp60 324–332</td>
<td>Y</td>
<td>I</td>
<td>K</td>
<td>N</td>
<td>K</td>
<td>D</td>
<td>T</td>
<td>T</td>
<td>I</td>
</tr>
<tr>
<td>HA 307–319</td>
<td>Y</td>
<td>V</td>
<td>K</td>
<td>Q</td>
<td>N</td>
<td>T</td>
<td>L</td>
<td>K</td>
<td>L</td>
</tr>
<tr>
<td>TTS 330–343</td>
<td>I</td>
<td>A</td>
<td>K</td>
<td>M</td>
<td>E</td>
<td>K</td>
<td>A</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>CS 376–396</td>
<td>I</td>
<td>A</td>
<td>K</td>
<td>M</td>
<td>E</td>
<td>K</td>
<td>A</td>
<td>S</td>
<td>S</td>
</tr>
</tbody>
</table>

*Alignments of the DR binding motifs of Ye-hsp60 peptide epitope p54-6b 322–333 and various promiscuous T cell epitopes described in the literature.

*DR binding residues are shown in italic.

*DR binding region of Ye-hsp60 peptide p54-6b as predicted by the TEPITOPE program.

*Promiscuous T cell epitope (from Refs. 29 and 30).

*Promiscuous T cell epitope (from Ref. 31).

*Promiscuous T cell epitope (from Ref. 32).

*Promiscuous T cell epitope (from Ref. 33).
Ye-hsp60 (13), an almost complete overlap could be observed with stimulation. All values are given in picograms.

Comparison of the major Ye-hsp60 Th cell epitope 322–333 with the CTL epitope

When the sequence of the major CD4 T cell epitope p54-6b, RV-VINKDTTIII 322–333, identified in the present study was compared with the sequence of the recently discovered HLA-B27-restricted CTL epitope, KRVVINKDT321–329, which is also derived from the Ye-hsp60 fragment II favored the production of IL-10 (clone 92 is shown as an example).

Only 1 clone (clone 55) secreted a reasonable amount of IL-4 together with IFN-γ and IL-10 (Table IV).

Proliferation of other Yersinia-triggered ReA patients to Ye-hsp60-fragment II peptides

We tested SFMC derived from seven other patients for proliferation to various Yersinia-derived Ags and Ye-hsp60-fragment II-derived overlapping 18-mer peptides. All patients showed a good response to whole Yersinia, to the Yersinia-specific 19-kDa protein (data not shown), to the complete Ye-hsp60, and to the three overlapping Ye-hsp60 fragments. As shown in Fig. 4, the highest stimulation index (SI) could be seen in response to fragment II in the majority of patients (four of seven patients). However, we could not detect a proliferative response to any of the peptides (SI < 2), including p54/55, except in patient 7. This patient (DR1; DR2), with the highest SI (72.6) of all patients tested in response to fragment II, showed a slight proliferation to peptide p41_{241–258} (SI = 6.1).

The discrepancy between a good response to the whole fragment II and failure to respond to peptides derived from this protein indicates that stimulation of mononuclear cells with peptides might not be sensitive enough for epitope identification.

Discussion

Hsp60s are dominant Ags in the immune response to bacteria, despite or maybe because of their extraordinary high sequence conservation throughout the eukaryotic and prokaryotic kingdom (34). This has also been demonstrated for the two ReA-associated bacteria Yersinia and C. trachomatis. In a murine model of acute Yersinia infection, the Ye-hsp is an immunodominant T cell Ag with protective properties as shown in both adoptive transfer and vaccination experiments (35, 36). However, it seems to be also a relevant Ag in more chronic immune responses such as in ReA. We could recently show that the Ye-hsp60 is preferentially seen by both CD4+ and CD8+ T cells derived from synovial fluid of patients with Yersinia-induced ReA (12, 13). Synovial T cell responses to CT-hsp60 have also been described in Chlamydia-induced ReA (37), and the immune response to CT-hsp60 have been implicated in the immunopathology of trachoma of the eye (38). Furthermore, in ReA, Ye-hsp60 and CT-hsp60 are even preferentially expressed in the joint (39) or peripheral blood (2), compared with other bacteria-derived proteins such as membrane proteins. This has also been shown for Chlamydia in vitro under experimental conditions mimicking bacterial persistence (40). Thus, although bacterial hsp60 seems to be a preferential target of the acute immune response, its strong expression during bacterial persistence suggests that it might facilitate the bacterial survival of the host’s immune response.

In the present study, we provide a detailed analysis of the CD4+ T cell response to the Ye-hsp60 in one patient with ReA. We were able to identify one major epitope. A small antigenic region comprising the 24-aa 319–342 present on an Ag 550 aa long elicited the majority of TCCs in our study. This finding suggests that this part of the protein is central for induction of an immune response. We could also show that this peptide is presented by different

Table IV. Cytokine secretion of clone 55

<table>
<thead>
<tr>
<th>Peptide</th>
<th>IFN-γ</th>
<th>TNF-α</th>
<th>IL-10</th>
<th>IL-4</th>
<th>SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>367–550</td>
<td>1250</td>
<td>&lt;20</td>
<td>2175</td>
<td>690</td>
<td>24</td>
</tr>
<tr>
<td>451–468</td>
<td>7170</td>
<td>&lt;20</td>
<td>1750</td>
<td>810</td>
<td>34</td>
</tr>
<tr>
<td>454–471</td>
<td>6890</td>
<td>&lt;20</td>
<td>2210</td>
<td>880</td>
<td>39</td>
</tr>
<tr>
<td>454–468</td>
<td>7070</td>
<td>&lt;20</td>
<td>1790</td>
<td>590</td>
<td>44</td>
</tr>
</tbody>
</table>

* The cytokines were measured in culture supernatants after 48 h of antigenic stimulation. All values are given in picograms.

* Numbers indicate the amino acid position within the Ye-hsp60.

FIGURE 3. Cytokine secretion by clone 92 and clone 69 in response to various peptides. IFN-γ, IL-10, and TNF-α were measured in the supernatant by ELISA. The numbers in parenthesis indicate amino acid residues. Values are the mean of three measurements. [322–333]8A is 322–333 with a single alanine substitution at peptide position 10. #, The SI of clone 92 after stimulation with peptide [322–333]8A was reduced by 50%.

FIGURE 4. Proliferation of SFMC of seven Yersinia-triggered ReA patients to Ye-hsp60 fragments and overlapping 18-mer peptides, spanning the entire fragment II. *, None of the peptides stimulated SFMC of patients 1–6 (SI < 2). §, Only in patient 7, a single peptide p41_{241–258} was recognized.
HLA-DR alleles, underlining its possible importance beyond the single patient described here. This main epitope, in addition to the two others discovered in our study, differ from those identified in a mouse model of Yersinia infection (41), probably because of the interspecies MHC class II binding differences. Epitopes might also differ in acute infections from those in more chronic immune responses, such as in ReA.

In the context of ReA, an obvious question is whether the immune response to the up-regulated hs60 is not effective enough for the elimination of bacteria, leading to bacterial persistence. A Th1 response is crucial for successfully fighting microbes such as Yersinia and Chlamydia, and their elimination is inhibited by IL-10 (9–11). We have previously reported that a relative high amount of IL-4 and IL-10 and/or a low secretion of IFN-γ and TNF-α occurs in the joint (5, 6) or peripheral blood (42) of ReA patients, suggesting that such an ineffective Th1 response contributes to bacterial persistence. Therefore, the analysis of the cytokine secretion pattern of the TCCs upon stimulation with the immunodominant epitope was of great interest.

Among the inhibitory cytokines, IL-10 was secreted in considerable amount by several clones, while only one IL-4-secreting clone was identified (Table IV). Therefore, Ye-hsp60-specific IL-10 secretion might permit bacterial persistence and could be an explanation for the up-regulation of Ye-hsp60 in chronic inflammations such as ReA. We did not find TCCs secreting only IL-10, formerly described as Tr1 clones (43). However, it can be assumed that IL-10 produced together with IFN-γ and TNF-α acts also as an effective inhibitor of an antibacterial immune response. One interesting observation should be emphasized: stimulation of virtually all clones investigated with the whole Ye-hsp60 protein fragment II182–371 except clone 69 gave rise to high levels of IL-10, compared with stimulation by the short specific peptide epitope (Fig. 3). These results demonstrate that Ag-specific T cells with both high and low IL-10 production are present in the joint. The actual balance of these two types of cells could ultimately give rise to clearance or persistence of Yersinia. The relative contribution of these types of T cells for the in vivo situation has to be addressed in future studies.

In the context of the Ye-hsp60 T cell response, cross-reactivity has attracted a lot of interest in recent years. The hsp60s are highly conserved throughout evolution with an aa sequence identity of up to 50% between prokaryotic and eukaryotic species (14). The homology among enterobacteria can be as high as 90%. Cross-reactivity between Yersinia and other ReA-associated bacteria such as C. trachomatis might help to explain why such diverse bacteria induce a similar disease. However, in our study no cross-reactivity between the dominant Ye-hsp60 epitope 322–333 and the corresponding epitope from the CT-hsp60 was found. The identification of the aa critical for T cell recognition within the 12-mer epitope helps to explain why some hsp60s are recognized but not others. Indeed, the critical residues were not identical on both the Ye-hsp60 and the CT-hsp60. However, these residues were shared by the homologous hs60 peptide from Borrelia—a microbe not associated with ReA—which was readily recognized by this clone. This epitope was most probably a “cryptic” one, because no T cell activation was seen after stimulation with the whole Bb-hsp60 protein. A T cell response directed to CT-hsp60 has recently been described in a patient with Chlamydia-induced ReA (37). However, epitope analysis of one clone also revealed no cross-reactivity with either the hu-hsp60 or other enterobacterial hs60s.

We also identified one clone (clone 55) specific for the fragment III epitope p76<sub>354</sub>–368 that produced a relatively high amount of the Th2 cytokine IL-4 in addition to IL-10 (Table IV), a finding that could be confirmed by intracellular cytokine staining (data not shown). However, compared with IL-10, IL-4-secreting cells seemed to represent a minority. The experiments with the single alanine-substituted peptide variants are too limited to draw final conclusions regarding the effects of the so called “altered peptide ligands” to the functional outcome of the clones, a concept that has been extensively studied in recent years (44, 45). Substitutions with the tiny hydrophobic alanine, as used in our study, may result in effects different from those observed after substitution with positively or negatively charged amino acids. Nonetheless, substitution of T<sub>329</sub> by alanine at position 8 lead to a 50% reduction of the proliferation and to a 80% reduction in the secretion of the proinflammatory cytokines IFN-γ and TNF-α, while only slightly decreasing the secretion of IL-10, resulting in a markedly reduced IFN-γ/IL-10 ratio (Fig. 3).

A cross-reactive T cell response against bacterial and self-hsp60 has also been implicated in the induction of autoimmunity, as discussed previously (15, 16, 46, 47) and has even been described in Yersinia-induced ReA (48). It has recently been reported that an autoimmune response against self-hsp60 could be induced in mice by simultaneous immunization with both the self-hsp 60 and the CT-hsp60 but not with either of these molecules alone, suggesting that under certain circumstances autoimmunity against self-hsp60 with subsequent immunopathology can indeed occur (49). However, in contrast to animal models the evidence is less convincing in humans (17, 34). Rather, van Eden and colleagues suggest that a cross-reactive T cell response against self-hsp60 might be a relevant mechanism for down-regulating an autoimmune response by release of regulatory cytokines (17, 18, 50, 51). In our study, we could not detect a cross-reaction between human and Ye-hsp60 epitopes. However, in contrast to rheumatoid arthritis or experimental animal models such as adjuvant arthritis (46, 47), immunopathology in ReA is most likely caused by a bacteria-specific immune response and not by autoimmunity. Our results indicate that the T cell response to the hs60 is flexible enough to react with different cytokine patterns, even without cross-reactivity with the human counterpart.

After we had identified peptide p54<sub>322</sub>–333 as a relevant CD4 T cell epitope in the complete Ye-hsp60 molecule, it was a great surprise that it almost completely overlapped with the Ye-hsp60 nonamer KRVVINKD2<sub>321</sub>–329, which was recently described by us to be an immunodominant HLA-B27-restricted epitope for the cytotoxic CD8<sup>+</sup> T cell response in different patients with Yersinia-induced ReA. These findings are in accordance with earlier reports showing that CD4 T cell epitopes are often similar to CD8 epitopes, either covalently linked to or containing the CTL epitope (52, 53). In particular, this apparent overlap of epitopes for both the CD4 and the CD8 T cell responses might indicate a major immunogenic region within the Ye-hsp60 molecule that might have relevance beyond this particular patient.

Although peptide p54/55 could be presented to our T cell clones by different HLA-DR haplotypes, it is not clear whether this epitope is also seen by other patients with Yersinia-triggered ReA. Using whole SFMC instead of T cell lines and clones, lymphocyte proliferation assays were not sensitive enough for epitope mapping as described by others (33). This might be due to the relatively low peptide-specific T cell frequency among bulk SFMC and strengthens the current need for labor-intensive T cell cloning. More sensitive flow cytometric methods as a new technology for T cell epitope mapping (54) circumventing T cell cloning might be helpful in the future.

At present, there is no proof that the immunodominant peptides identified by T cell clones are relevant for the pathogenesis of ReA. Final confirmation would come from preventing or curing the disease using such peptides for vaccination or induction of
Ag-specific tolerance. However, for this purpose identification of immunodominant epitopes is a first and essential step.

In summary, we could define a multiclonal and multispecific CD4+ T cell response to a single epitope derived from Ye-hsp60 with no cross-reactivity to CT-hsp60 or hu-hsp60 in a patient with Yersinia-induced ReA. The promiscuity of this peptide indicates that it might also be important for the immune response of individuals with different MHC class II types. IL-10 secretion was prominent, but differed widely, dependent on the clones and the peptides tested. Future studies applying Ag-specific cytokine expression of nonseparated T cells will clarify the relative importance of Ag-specific IL-10 secretion in ReA (55). The cytokine secretion pattern could play a role for bacterial persistence in ReA and could be a target for future T cell manipulation in patients with ReA and related diseases. It is currently a subject of debate whether inflammation in ReA is the consequence of hypersensitivity to bacterial Ag (56), which could therapeutically be down-regulation of nonseparated T cells will clarify the relative importance of Ag-specific IL-10 secretion in ReA (55). The cytokine secretion pattern could play a role for bacterial persistence in ReA and could be a target for future T cell manipulation in patients with ReA and related diseases. It is currently a subject of debate whether inflammation in ReA is the consequence of hypersensitivity to bacterial Ag (56), which could therapeutically be down-regulated by Th2 cytokines, or alternatively, the consequence of an ineffective Th1 response necessary for effective elimination of bacteria. Our data favor the latter possibility.

Acknowledgments

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References


