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Yersinia-hsp60-reactive T cells are efficiently stimulated by peptides of 12 and 13 amino acid residues in a MHC class II (I-A^b)-restricted manner

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SUMMARY

Heat shock proteins (hsp) are immunodominant antigens in microbial infections. Previous work from this laboratory demonstrated that *Yersinia*-hsp60 (Y-hsp60)-reactive $CD4^+$ $\alpha\beta$ T cells play an important role for resolution of *Y. enterocolitica* infections in mice. In the present study we identified two epitopes of Y-hsp60 recognized by $CD4^+$ Th1 cell clones. The epitopes comprise 12 (214–225) and 13 (74–86) amino acid (aa) residues of Y-hsp60, and are the first described for MHC class II (I-A^b) molecules. Both epitopes are also recognized by T cells isolated from mesenteric lymph nodes from mice orogastrically infected with yersiniae. Stimulation of T cells with peptides of 12 and 13 aa residues of Y-hsp60 caused highly efficient proliferation compared with longer peptides, full-length recombinant Y-hsp60, or heat-killed *Yersinia* (HKY). Incubation of antigen-presenting cells with chloroquine blocked both peptide and HKY-triggered T cell proliferation, whereas cytochalasin B only blocked HKY-induced proliferation and to a lesser extent peptide-induced proliferation. The identified epitopes reside in a region of Y-hsp60 that is conserved between *Enterobacteriaceae* but highly variable when compared with murine or human hsp60. Although both epitopes are identical to the related sequence of hsp60 (GroEL) of *Escherichia coli*, only weak T cell responses were observed upon stimulation with GroEL of *E. coli*, suggesting that other factors, e.g. flanking amino acid residues, might be important for antigen processing and T cell stimulation in a class II-restricted manner. Furthermore, these observations might be of significance for the rational design of subunit vaccines.

Keywords Yersinia T cells epitopes hsp MHC

INTRODUCTION

Yersinia enterocolitica is a common cause of diarrhoea in Europe and the USA [1]. Clinical manifestations caused by this pathogen range from enteritis, enterocolitis, and mesenteric lymphadenitis to systemic infection with abscesses and granulomatous lesions [1,2]. Furthermore, *Yersinia*-triggered reactive arthritis is a well known immunopathological complication of intestinal yersiniosis which is frequently observed in patients with HLA-B27 [3,4].

Expression of virulence factors which are encoded by the chromosome and by the *Yersinia* virulence plasmid confers resistance of *Y. enterocolitica* against phagocytosis and complement lysis [5,6]. Thus, *Y. enterocolitica* evades innate mechanisms of the host defence, and is mainly extracellularly located in infected tissues [7,8]. The host defence mechanisms required for elimination of *Y. enterocolitica* from infected tissues have been shown to be comparable to those required for control of intracellular pathogens, and comprise cell-mediated immune responses including CD4 and CD8 T cells as well as activated macrophages [9–15]. In keeping with this hypothesis, cytokines such as IL-12, tumour necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ) are essential key mediators of protective events against yersiniae [9,10,13]. Nevertheless, adoptive transfer experiments revealed that both T cells and antibodies can mediate immunity against yersiniae [14,16].

A significant number of *Yersinia*-specific T cells recognize the 60-kD heat shock protein (Y-hsp60) of *Y. enterocolitica* [17]. Hence, the adoptive transfer of Y-hsp60-specific T cell clones isolated from mice either after infection with *Yersinia* or after immunization with recombinant Y-hsp60 mediated protection against *Y. enterocolitica* infection in mice [17]. This was surprising, as microbial hsp were believed to account for autoimmune diseases [18–22], as hsp are immunodominant proteins with high sequence homology between mammalian and bacterial hsp [18–23]. Due to the fact that both the microorganisms and the host cells produce hsp upon interaction during an infection process, it was speculated that an immune response against microbial hsp might induce T cell and antibody responses against shared epitopes of hsp [18–22,24,25]. Subsequently, such processes might cause a breakdown of tolerance which might lead to autoimmune diseases such as arthritis [26–30]. Therefore, detailed studies of immune

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responses against microbial hsp are desirable in order to provide insight into the potential role of hsp in protective immunity, tolerance and autoimmunity.

Here we have identified two epitopes of Y-hsp60 which are recognized in the context of MHC class II (I-A^b) molecules by T cell clones and lines that mediate protection against infection with *Y. enterocolitica*. According to putative binding specificities of other MHC class II molecules and due to the characteristics of aa residues of the epitopes identified, the putative anchor positions of peptides binding to I-A^b molecules are discussed.

MATERIALS AND METHODS

Antigen preparations and peptides

Heat-killed *Yersinia* from *Y. enterocolitica* strain WA-314 of serotype O8, the recombinant hsp60 from *Y. enterocolitica* strain WA-314 of serotype O8 and a truncated hsp60 fragment (aa residues 90–286) were prepared and purified by affinity chromatography on metal chelate-adsorbent Ni-nitrilo-triacetic acid resin as recently described [17]. GroEL (hsp60) of *Escherichia coli* was kindly provided by H. Gaston (Cambridge, UK).

Y-hsp60 was cleaved by cyanogen bromide (CNBr; Sigma, Deisenhofen, Germany) or trypsin (Sigma) according to the manufacturer's instructions as described elsewhere. Briefly, samples of 6 mg lyophilized Y-hsp60 (104 nmol) were dissolved in 3 ml of 70% formic acid. CNBr was dissolved in acetonitrile (40 mg/ml; Merck, Darmstadt, Germany) and added to the protein in 1000-fold molar excess. The reaction was carried out overnight at room temperature. Thereafter, the solvents were removed by lyophilization. The dry pellet was dissolved in sterile PBS and the protein concentration determined. For trypsin digest, trypsin was dissolved in 0·01% trifluoroacetic acid (TFA) at a final concentration of 2 μg/ml. The protein was dissolved in reaction buffer (100 mM Tris–HCl, 1 mM $CaCl_2$, pH 7·6) with a final concentration of 1 μg/ml. Substrate/enzyme ratio was 50:1. The reaction was carried out overnight at 35°C.

The fragments obtained by CNBr cleavage were separated by reversed-phase chromatography using PepRPC HR5/5 column and fast performance liquid chromatography (FPLC; Pharmacia LKB, Uppsala, Sweden). The lyophilized peptides were dissolved in 70% formic acid. Peptides were eluted by a gradient with two buffers containing (i) 0·1% formic acid, and (ii) 0·1% formic acid in 70% acetonitrile.

The obtained fractions were purified, concentrated, analysed by tricine gel electrophoresis [31], and tested in T cell proliferation assays as described below. Peptide fractions causing T cell stimulation were further purified by a second and third reversed-phase chromatography in the presence of 0·1% TFA in 70% acetonitrile.

Finally, the peptides were separated by tricine gel electrophoresis and transferred on Selex 20 polypropylene membrane (Schleicher and Schüll, Dasssel, Germany) by Western blotting. The bands with appropriate molecular weight were cut out and processed for sequencing using sequencer 476 A (Applied Biosystems, Weiterstadt, Germany). Peptide sequencing was performed by J. Hoppe (Würzburg, Germany). According to the obtained sequence data synthetic peptides were produced and purified by HPLC (provided by D. Palm, Würzburg, Germany).

Animals and cells

Four to six-week-old C57Bl/6 and BALB/c mice were purchased from Charles River Wiga (Sulzfeld, Germany). Cloning procedures for T cell clones 4F7, K9.7.1C5 and K9.7.1D1 which were isolated from spleen of mice after i.v. *Yersinia* infection or Y-hsp60 immunization, respectively, were previously reported [17]. LK3.94 T cells were isolated from mesenteric lymph nodes of mice orogastrically infected with *Y. enterocolitica* and stimulated as previously described [14]. The phenotype of this T cell line and of the above T cell clones was: $CD3^+$, $CD4^+$, $CD8^-$, $\alpha\beta$ TCR^+, $CD25^+$. The T cell lines and clones produced IL-2 and IFN-γ, but no IL-4, IL-5 or IL-10 [17]. Antigen-presenting cells (APC) were prepared from spleens and irradiated (30 Gy) as recently described [14]. All cell cultures were carried out in Click/RPMI 1640 medium (Biochrom, Berlin, Germany) supplemented with 2 mM L-glutamine, 10 mM HEPES, 10^{-5} M 2-mercaptoethanol (2-ME), 10% fetal calf serum (FCS; Biochrom), and antibiotics.

Proliferation assays

Fourteen to 20 days after antigenic restimulation *in vitro* T cells were used for stimulation experiments. Triplicates of 5×10^4 purified T cells were cultivated with 2×10^5 syngeneic (I-A^b) irradiated APC in a volume of 0·2 ml in wells of microculture plates. Alternatively, APC from BALB/c mice (I-A^d) were used, or APC were treated with 50 μM chloroquine (Sigma), 50 μg cytochalasin B (Sigma) per ml culture medium. If not otherwise stated, antigens (HKY, Y-hsp60) were used at a concentration of 10 μg/ml, peptides were used at 1 μg/ml medium. Alternatively, 10 μl of the solvent-free fractions from reversed-phase chromatography were added to the cultures. In APC blocking experiments, APC were in parallel pretreated with inhibitors. After incubation for 5 h at 37°C, APC were washed and added to T cell cultures in the presence or absence of antigen and inhibitors. After 3 days, cultures were pulsed with ^{3}H-thymidine to determine T cell proliferation. ^{3}H-thymidine incorporation was measured using a liquid scintillation counter. Values are given as ct/min or as the stimulation index (SI) (= mean of antigenic proliferation/mean of non-antigenic spontaneous proliferation). All experiments were performed at least three times with comparable results.

RESULTS

CNBr cleavage of Y-hsp60 and identification of T cell epitopes

In order to identify the epitopes recognized by the Y-hsp60-specific T cell clones 4F7, K9.7.1C5, and K9.7.1D1, Y-hsp60 was cleaved with CNBr. According to the known aa sequence of Y-hsp60 containing 23 methionine residues, 21 peptides should result from this treatment. Stimulation of the above clones with CNBr-cleaved Y-hsp60 induced strong proliferation (Table 1), suggesting that the epitopes had not been destroyed by the cleavage. A number of synthetic peptides including aa residues 124–137, 154–169, 181–190, 188–209, 204–215, 224–244, and 246–254 of Y-hsp60 were produced according to the known aa sequences of Y-hsp60 O3 and O8, respectively, compared with those of other bacterial species and according to computer-predicted putative T cell epitopes [17]. However, none of these peptides was recognized by the T cell clones described here (Table 1).

Therefore, in a next step Y-hsp60/CNBr peptides were applied to reversed-phase chromatography. The pooled peak fractions (Fig. 1) were tested for their ability to stimulate the Y-hsp60-specific T cell clones. The data depicted in Table 1 show that clone 4F7 was stimulated by P3, P4 and to a minor extent by P5, whereas clones K.9.7.1D1 and K.9.7.1C5 were stimulated by P5 only.

Table 1. Proliferative responses of Y-hsp60-reactive T cell clones and lines upon stimulation with various hsp antigens*

	SI†			
	T cells			
Antigen‡	4F7	K9.7.1C5	K9.7.1D1	LK3.94
HKY	13·3	46·6	63·5	96·4
Y-hsp60	26·4	128·3	57·7	118·9
Y-hsp60 (aa 90–286)	27·6	1·1	1·0	36·0
Y-hsp60/CNBr	46·9	110·4	57·5	104·7
Escherichia coli GroEL	3·8	3·9	1·3	14·9
Y-hsp60 peptide residues:				
124–137	< 1	1·8	< 1	4·0
154–169	< 1	1·2	< 1	3·2
181–190	1·0	< 1	< 1	2·2
188–209	< 1	< 1	< 1	3·1
204–215	< 1	< 1	< 1	3·4
224–244	1	< 1	< 1	3·9
246–254	1·0	< 1	< 1	4·0
P1§	1·2	1·0	< 1	ND
P2	1·9	1·0	< 1	ND
P3	79·3	1·1	< 1	ND
P4	98·5	1·5	1·3	ND
P5	21·3	20·1	14·5	ND
P6	< 1	< 1	< 1	ND

*Proliferative responses were determined by ^{3}H-thymidine uptake of 5×10^4 T cells after antigenic stimulation in the presence of 2×10^5 irradiated syngeneic antigen-presenting cells (APC). T cells were incubated with 10 μg of HKY, full length, truncated (aa 90–286) or cyanogen bromide (CNBr)-cleaved Y-hsp60 per ml medium, or with 1 μg of the depicted synthetic peptides per ml medium.

†Proliferative response was measured as stimulation index (SI) and represents the quotient of ct/min (mean of triplicates): ^{3}H-thymidine uptake in the presence of the indicated antigen/ ^{3}H-thymidine uptake without antigen.

‡Synthetic peptides were selected according to sequence comparisons with hsp60 of other bacterial species and computer-based prediction of putative T cell epitopes. Numbers indicate aa residues of Y-hsp60.

§P1 to P6 represent the pooled fractions derived from reversed-phase chromatography of CNBr-cleaved Y-hsp60.

ND, Not determined.

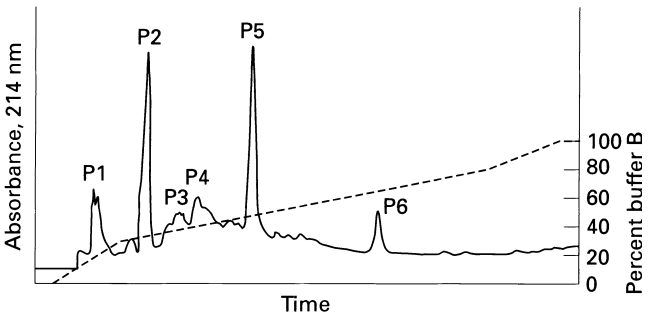

Fig. 1. Elution profile of Y-hsp60 cleaved by cyanogen bromide (CNBr) and separated by reversed-phase chromatography. The relevant part of the gradient is shown as a dashed line. P1 to P6 indicate the eluted pooled fractions used for T cell stimulation experiments shown in Table 1. Buffer B contained 0·1% formic acid in 70% acetonitrile.

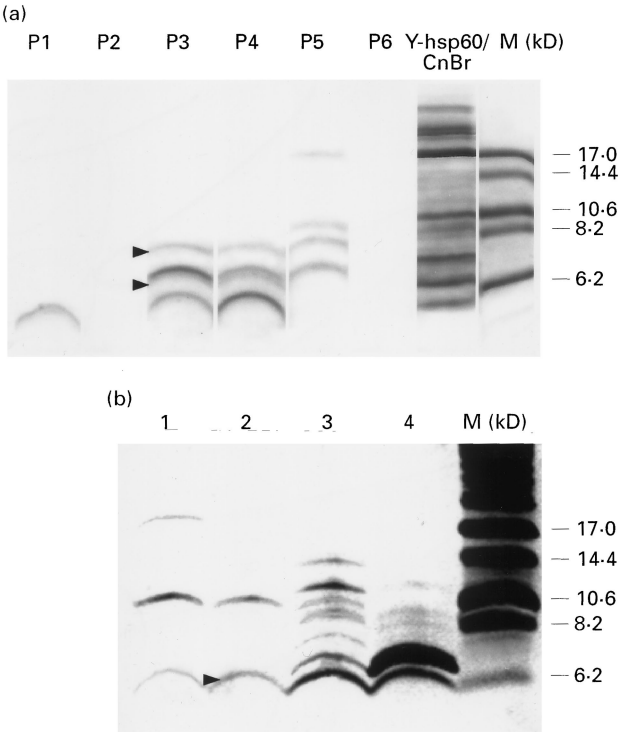

Fig. 2. (a) Tricine gel electrophoresis analysis of Y-hsp60 and eluted fractions P1 to P6 derived from cyanogen bromide (CNBr)-cleaved and reversed-phase chromatography-separated Y-hsp60. The upper arrowhead indicates aa 2–69 (≈ 7·5 kD), the lower arrowhead aa 194–233 (≈ 5·2 kD). M, Molecular weight marker. (b) Tricine gel electrophoresis of eluted fractions derived from P5 after two further purification steps by reversed-phase chromatography. The lower band (arrowhead) in lane 2 represents aa 74–111 of Y-hsp60.

Subsequently, the eluted peptide fractions were analysed by tricine gel electrophoresis. Figure 2a indicates that there were four major bands in P3 representing peptides of ≈ 7·5, 5·9, 5·2, and 4·7 kD. Western blotting and sequencing revealed that the upper 7·5-kD band of P3 represented an incompletely cleaved fragment comprising aa residues 2–69 of Y-hsp60. This peptide, however, could be excluded as a relevant antigen, since a truncated Y-hsp60 fragment (aa 90–286) was recognized by T cell clone 4F7 (Table 1). Sequencing of band 3 revealed that this band represented aa residues 194–233 of Y-hsp60.

Further processing of P5 finally revealed two bands in an elution fraction which stimulated clones K.9.7.1C5 and K.9.7.1D1 (Fig. 2b). Western blotting and sequencing data indicated that the lower band represented aa residues 74–111 of Y-hsp60. Further synthetic peptides were produced, and finally two epitopes of Y-hsp60 could be identified (Tables 2 and 3): aa residues 74–86 (recognized by clones K.9.7.1C5 and K.9.7.1D1) and 214–225 (recognized by clone 4F7).

In addition to splenic T cells after i.v. infection, Y-hsp60-reactive T cell line (LK3.94) isolated from *Yersinia*-infected mesenteric lymph nodes after intestinal *Y. enterocolitica* infection proliferated upon stimulation with either of the peptides, suggesting that Y-hsp60 is produced and presented as an immunodominant antigen to T cells in the gut-associated lymphoid tissue (Tables 1 and 2).

Amino acid sequence comparison revealed that aa residues 74–86 and 214–225 of Y-hsp60 are identical to the corresponding

Table 2. Proliferative responses of Y-hsp60-reactive T cell clones and lines upon stimulation with *Yersinia enterocolitica* hsp60-derived synthetic peptides*

	Stimulation index†			
	T cells			
Y-hsp60 peptide residues‡	4F7	K9.7.1C5	K9.7.1D1	LK3.94
194–232	30·9	< 1	< 1	36·9
203–232	36·0	< 1	< 1	25·6
208–220	1·6	< 1	< 1	5·4
212–225	20·4	< 1	< 1	28·7
212–224	25·6	< 1	< 1	15·5
214–225	32·2	< 1	< 1	15·0
214–222	1·6	< 1	< 1	ND
217–224	1·1	< 1	< 1	ND
74–86	1·8	135·3	197·1	131·8
74–82	1·4	1·2	1·3	ND
77–86	1·8	< 1	1·1	ND

*†See legend of Table 1.

‡Synthetic peptides were synthesized according to aa sequences after cyanogen bromide (CNBr) cleavage of Y-hsp60, reversed-phase chromatography and sequencing of single peptides.

ND, Not determined.

sequence of GroEL (hsp60) of *E. coli* (data not shown). However, surprisingly, both heat-killed *E. coli* as well as purified GroEL induced only weak proliferative responses of T cell clones 4F7, K.9.7.1C5 and K.9.7.1D1 (Table 1).

Blocking of peptide and Yersinia-induced T cell proliferation by cytochalasin B or chloroquine

Previous work indicated that long peptides induce more efficient T cell stimulation than do shorter peptides [32]. To test whether this relationship was also true for the T cell clones and lines described here, the cells were stimulated with various concentrations of peptides (aa residues 194–232, 212–225 or 74–86) in comparison with Y-hsp60 full length protein. The data depicted in Fig. 3 indicate that both the full length Y-hsp60 and the peptides induced efficient proliferative responses. However, for clone 4F7 or line LK3.94 a half-maximal T cell proliferation was observed at ≈ 1 μM with the short peptide (aa residues 212–225), at 3·6 μM and 14 μM for the longer peptide (aa residues 194–232), and at 14·5 μM and 50 μM for the full length Y-hsp60, respectively. *Vice versa*, half-maximal proliferation of clone K.9.7.1C5 was induced by the peptide (aa residues 74–86) at 120 μM, and at 31 μM for Y-hsp60.

In order to investigate the antigen-processing events required for T cell stimulation, the APC were treated with cytochalasin B or chloroquine. The results depicted in Fig. 4 show that chloroquine completely inhibited both peptide- and HKY-induced T cell proliferation, whereas cytochalasin B inhibited HKY-induced proliferation much more efficiently than peptide-triggered proliferative responses. Further, APC from BALB/c mice were used in controls. The results showed that APC from BALB/c mice which express I-A^d did not mediate peptide, Y-hsp60 or HKY-induced T cell proliferation (data not shown).

DISCUSSION

The discussion concerning the role of microbial hsp for immune responses of the host remains controversial [18–22]. Hence, protective immunity, tolerance as well as autoimmunity have been suggested to be induced by microbial hsp. The observation that Y-hsp60-reactive T cells mediate protection against a lethal *Y. enterocolitica* infection in mice was the first direct evidence for a protective host response triggered by microbial hsp [17]. Moreover, there was no significant cross-protection against other bacterial pathogens including *Y. pseudotuberculosis* and *Salmonella typhimurium* to be observed, although Y-hsp60 has extensive aa sequence homology with other bacterial species (e.g. 90% with *E. coli*, 59% with *Mycobacterium tuberculosis*) [17]. Furthermore, there is 50–52% aa sequence homology between Y-hsp60 and human or murine hsp60/P1. However, in both human and murine yersiniosis there are no data reported to date on Y-hsp60-induced autoimmunity [4,17,19,33]. *Yersinia*-induced reactive arthritis, which was believed to be the result of Y-hsp60-triggered autoimmune T cell reaction, appears to be a rather T cell-independent complication [34,35].

More recently, further evidence for a protective role of microbial hsp has been reported [36,37]. Silva & Lowrie showed that vaccination with *M. leprae hsp65*-transfected J774 cells induced

Table 3. Y-hsp60 synthetic peptides used for epitope mapping

Y-hsp60 aa residues*	aa sequence†	Length (aa)	Mol. wt (D)
194–232	QFDRGYLSPYFINKPETGSIELESPFILLADKKISNIRE	39	5209
203–232	YFINKPETGSIELESPFILLADKKISNIRE	30	3984
208–220	PETGSIELESPFI	13	1633
212–225	SIELESPFILLADK	14	1807
212–224	SIELESPFILLAD	13	1661
214–225	ELESPFILLADK	12	1571
214–222	ELESPFILL	9	1203
217–224	SPFILLAD	8	1000
74–86	VKEVAAKANDAAG	13	1474
74–82	VKEVAAKAN	9	1088
77–86	VAAKANDAAG	10	1064

*Numbers indicate aa residues of Y-hsp60.

†Amino acid sequences by single-letter code.

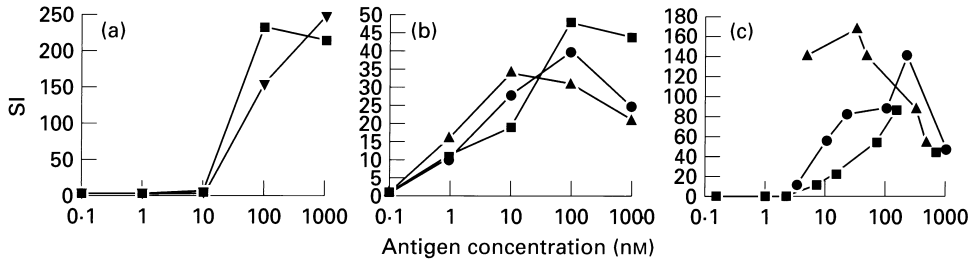

Fig. 3. Proliferative responses of T cell clones K9.7.1C5 (a), 4F7 (b) and T cell line LK3.94 (c) upon stimulation with various concentrations of Y-hsp60 full length protein (■), Y-hsp60 aa residues 194–232 (●), Y-hsp60 aa residues 212–225 (▲) or Y-hsp60 aa residues 74–86 (▼). Triplicates of T cells (5×10^4 per well) were stimulated in the presence of 2×10^5 irradiated syngeneic antigen-presenting cells. SI, Stimulation index.

CD8 T cell-mediated protection against a challenge with *M. bovis* bacille Calmette–Guérin (BCG) or *M. tuberculosis* H37Rv [36]. Conversely, however, endogenous hsp have been identified as antigen that triggers lysis of autologous IFN-γ-stressed macrophages by cytotoxic T lymphocytes (CTL), which argues for a potential role of hsp in the pathogenesis of autoimmune diseases [38]. Nevertheless, due to the proven protective role of microbial hsp in host immune responses, attempts should be made to identify the epitopes of microbial hsp that are recognized by T cells. Such studies are a prerequisite for the design of subunit vaccines that comprise protective hsp peptides but exclude peptides which may induce autoimmunity.

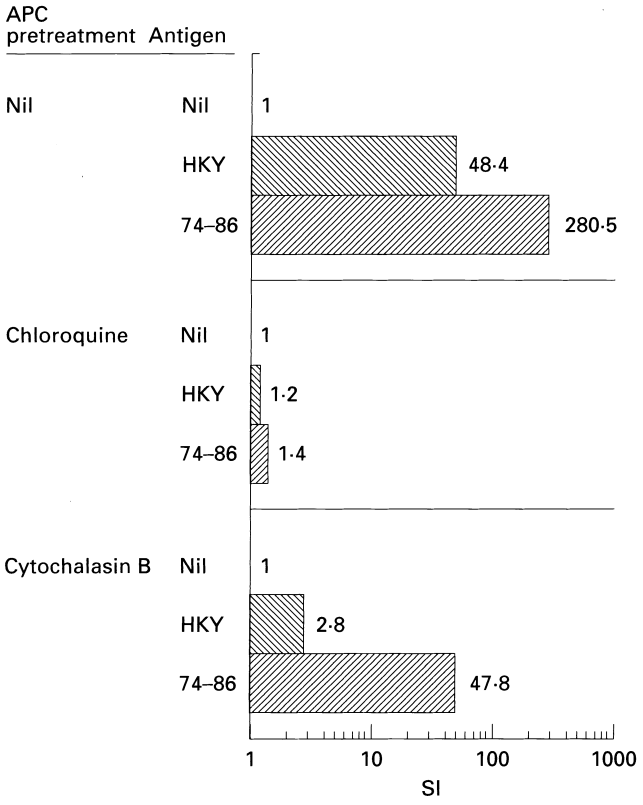

Fig. 4. Proliferative responses of T cell clone K.9.7.1C5 upon stimulation with HKY or a peptide comprising aa residues 74–86 of Y-hsp60 in the presence of chloroquine, cytochalasin B or PBS (nil). For proliferation assays, see legend of Fig. 4. Antigen-presenting cells (APC) were pretreated with or without inhibitor. After washing, T cells were added in the presence or absence of antigen and/or inhibitors. SI, Stimulation index.

In this study we have identified two epitopes of Y-hsp60 which may well be candidates to design a peptide vaccine against *Y. enterocolitica*. The epitopes comprise 12 (214–225) and 13 (74–86) aa residues of Y-hsp60, respectively, and are presented in a MHC class II-restricted manner by I-A^b molecules.

Preliminary data suggest that T cells derived from *Yersinia*-infected or Y-hsp60-immunized BALB/c mice (H-2^d) do not recognize peptides comprising the above aa residues of Y-hsp60 (our observation, unpublished). Since T cells from C57Bl/6 mice (I-A^b) do not recognize Y-hsp60 upon presentation by APC from BALB/c mice (I-A^d), it is not yet clear whether these epitopes can principally be presented by APC from BALB/c mice or not. Since not all MHC haplotypes respond to any one antigenic determinant [39], the above observation might argue against the use of Y-hsp60 or Y-hsp60 peptides as an efficient vaccine. Moreover, the above observation might provide a further clue to understanding the susceptibility of different strains of mice for *Yersinia* infections. BALB/c mice are highly susceptible to *Yersinia* infection, while C57Bl/6 mice are resistant [11]. We have claimed that the different *Yersinia*-triggered IL-12-dependent IFN-γ production may account for the different susceptibility for *Yersinia* [9]. However, we cannot yet exclude that the T cell repertoire or MHC haplotype in terms of Y-hsp60-triggered T cell responses might at least partially play a role in susceptibility or resistance against this pathogen. T cell cloning and immunization experiments with Y-hsp60 peptides will possibly elucidate a possible different role of

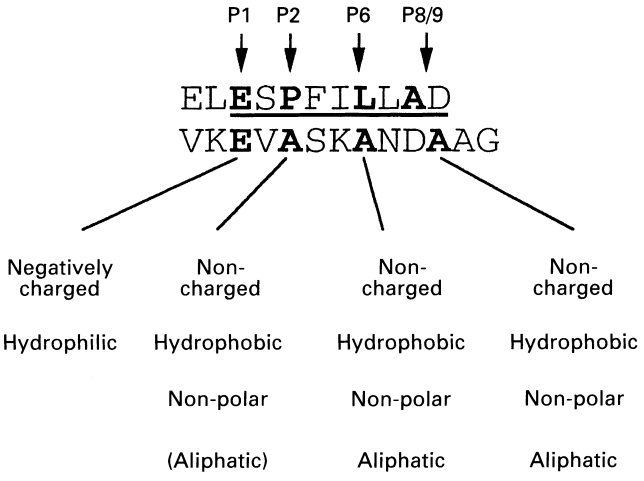

Fig. 5. Possible contact sites or anchor residues of the putative nonamer core (bold line) and characteristics of aa residues of Y-hsp60 epitopes presented by MHC class II I-A^b molecules (relative positions P1, 3, 6, and 8/9). Amino acid sequences by single-letter code.

Y-hsp60 in protective host responses against *Yersinia* in both mouse strains. In line with such a hypothesis, recent work from another laboratory provided evidence that the genetic background of different strains of mice is related to the development of hsp60-triggered autoimmune diseases [40]. Hence, non-obese diabetic (NOD) as well as C57Bl/6 but not BALB/c mice develop diabetes upon immunization with certain hsp60 peptides [40].

The epitopes described here are the first reported for I-A^b molecules. It is well established that MHC class II molecules present peptides of variable length, as the peptide binding pocket of class II molecules is open at both ends [41]. Although there is not such a close relationship for aa characteristics and MHC pocket binding specificities, typical peptide motifs which bind to certain class II molecules have been reported [41–43]. According to these results, it is conceivable that relative aa positions P1, 3, 6, and 8/9 might be crucial for I-A^b binding. The motif described by Rothbard & Taylor [42] in fact occurs twice in Yersinia-*hsp60* aa residues 74–86 (EVAS and DAAG) but not in aa residues 214–225. The proposed amino acid anchor residues of Y-hsp60 peptides which possibly bind to I-A^b are shown in Fig. 5. For both epitopes, putative P1 is negatively charged and hydrophilic. Nevertheless, due to the few existing data on I-A^b presented peptides, these motifs remain to be established by further studies.

Interestingly *E. coli* hsp60 (GroEL) did not induce T cell responses comparable to Y-hsp60, although aa residues of the related sequences are indentical. This might be explained by the different flanking aa sequences of GroEL and Y-hsp60, which might also modulate peptide binding to MHC class II molecules [32]. On the other hand, mutations leading to different hsp60 aa sequences in our particular *E. coli* strain cannot be excluded. Moreover, susceptibility to aminopeptidases and other proteases may be different for GroEL and Y-hsp60, which may account for different naturally processed peptides. Interestingly, GroEL-specific T cell clones recognize Y-hsp60 (H. Gaston, personal communication).

In keeping with the current view of cellular pathways involved in antigen processing in the context of MHC class I and II molecules, presentation of HKY was inhibited by cytochalasin B and chloroquine. The former inhibits glucose transport and phagocytic processes which require actin rearrangements, while the latter inhibits endosomal acidification and thus proteolysis [44]. Presentation of Y-hsp60 peptides was inhibited by chloroquine, but to a much lesser extent by cytochalasin B. Hence, both HKY and Y-hsp60 peptides were processed by the typical exogenous pathway, and uptake of HKY by APC occurred by phagocytosis. In contrast to recent published data, we found that longer peptides do not necessarily stimulate T cells more efficiently than shorter ones [32]. The concentration required for half-maximal T cell proliferation of some clones was lower for short peptides, and suggests that shorter peptides induce stronger or comparable T cell responses than do longer ones.

Taking all data into account, we have identified epitopes of Y-hsp60 presented by MHC class II molecules to $CD4^+$ T cells. Based on these results we are currently performing peptide vaccination experiments in order to evaluate the efficiency of a Y-hsp60 peptide vaccine in mice with different MHC haplotypes.

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