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Protective Role for Heat Shock Protein-Reactive αβ T Cells in Murine Yersiniosis

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To investigate the role of heat shock proteins (HSP) of *Yersinia enterocolitica* for the host immune response against this pathogen, we cloned and expressed a 60-kDa HSP of *Y. enterocolitica* serotype O8. A fragment of *Y. enterocolitica* O8 HSP60 encoded by amino acids 90 to 286 was sequenced and showed more than 90% homology with HSP60 of *Y. enterocolitica* O3 and GroEL of *Escherichia coli* and 59% homology with HSP65 of *Mycobacterium bovis*. The arthritogenic T-cell epitope of mycobacterial HSP65 (amino acid residues 180 to 188) was not found on *Yersinia* HSP60. To determine whether *Yersinia* HSP60 is an immunodominant antigen, the immune responses of *Yersinia*-infected C57BL/6 mice were analyzed. *Yersinia*-infected mice evolved a significant serum antibody and splenic T-cell response against *Yersinia* HSP60. $CD4^+$ αβ T-cell clones which were generated from splenic T cells isolated from either *Yersinia*-infected or *Yersinia* HSP60-immunized mice, recognized both heat-killed *Yersinia* serotypes O3 and O8 as well as recombinant *Yersinia* HSP60 but not heat-killed *Yersinia pseudotuberculosis*, *Salmonella typhimurium*, or recombinant HSP65 of *Mycobacterium bovis*. The adoptive transfer of HSP60-reactive T-cell clones mediated significant protection against a lethal infection with *Y. enterocolitica* O8. These results indicate that HSP60 of *Y. enterocolitica* is an immunodominant antigen which is recognized by both antibodies and $CD4^+$ αβ T cells. Moreover, this is the first report providing direct evidence that microbial HSP may elicit a protective immune response which is not associated with autoimmunity.

Heat shock proteins (HSP) are a family of proteins with molecular masses ranging from 15 to 110 kDa which are produced by both eukaryotic and prokaryotic cells upon exposure to various stressful conditions such as temperature shift or low oxygen pressure (26). HSP have focused the interest of scientists and clinicans because they were identified as immunodominant antigens in a variety of microbial pathogens (12, 19, 22–24, 46). Due to the fact that HSP are used by both host and parasite during their interaction in infectious processes and due to the extensive sequence homology between mammalian and microbial HSP, it was suggested that HSP may account for autoimmune diseases (12, 19, 23, 46). Hence, T cells and antibodies might be directed against epitopes shared by host and parasite HSP and thus may break tolerance to self antigens. Indeed, in the rat adjuvant arthritis model, it was shown that both HSP65 of mycobacteria and cartilage proteoglycans are recognized by arthritogenic T cells (17, 38–41). More recently, synovial fluid T cells from patients with *Yersinia*-triggered reactive arthritis were found to recognize *Yersinia* antigens and both human and mycobacterial HSP65 (15). Similar observations have been reported for patients with rheumatoid or other forms of arthritis (11, 18). However, the significance of these findings for the pathogenesis of arthritis is still unclear. Moreover, it has not yet been shown whether T cells or antibodies reactive with microbial HSP may have protective properties.

Yersinia enterocolitica is a gram-negative bacterium causing intestinal diseases as well as immunopathological sequelae such as reactive arthritis (6, 8). In the experimental *Yersinia* mouse infection model, we demonstrated that T cells play an essential role in the resolution of primary *Y. enterocolitica* infection (1, 1a, 2, 4, 5, 13). The antigens recognized by these T cells have not been identified so far. Recently, a 60-kDa HSP60 protein of *Y. enterocolitica* serotype O3 has been cloned and sequenced (33a). However, the role of HSP in immunity to yersiniae has not yet been investigated. We used recombinant *Yersinia* HSP60 for screening of our T-cells clones and found that a significant number of clones recognized HSP60.

Therefore, in the present study, we wanted to determine whether HSP of *Y. enterocolitica* play a role as antigens for host immune responses. For this purpose, we have (i) cloned, sequenced, and expressed the *groEL* homolog *hsp60* of *Y. enterocolitica* serotype O8, and (ii) analyzed the cellular and humoral host immune responses against this antigen. The results indicate that *Yersinia* HSP60 is recognized by serum antibodies as well as by αβ T cells from *Yersinia*-infected mice. Moreover, *Yersinia* HSP60-reactive $CD4^+$ αβ T-cell clones mediate protection against *Yersinia* infection in mice. Finally, the obtained T-cell clones recognize conserved epitopes of HSP60 of *Y. enterocolitica* and are not associated with autoimmunity.

MATERIALS AND METHODS

Bacteria. *Y. enterocolitica* WA-314 serotype O8 (14), *Y. enterocolitica* Y-108 serotype O3, *Yersinia pseudotuberculosis* III pIBI, *Salmonella typhimurium* (clinical isolate 2943), and *Listeria monocytogenes* EGD 1/2a were passaged in mice and cultured as described previously (4, 5).

Cloning and sequencing of *Yersinia hsp60*. *Y. enterocolitica* O8 and O3 were used as sources of genomic DNA. The oligonucleotides (synthesized by MWG-Biotech, Munich, Germany) used in this study were deduced from *E. coli groEL* and used for construction of expressed *Yersinia* HSP60 (Table 1).

Plasmid pAN5 encodes HSP60 of *Y. enterocolitica* serotype O8 (HSP60-O8) and plasmid pAN18 encodes HSP60 from

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TABLE 1. Oligonucleotides used in this study

Oligonucleotide	Sequence (5′ → 3′)[a]	Restriction enzyme
ANV	CTC TCT CGC ATG CCA GCT AAA GAC GTA AAA TT	*Sph*I
ANR	CTC GAG CTC TTA CAT CAT GCC GCC CAT GC	*Sac*I
AR11a	CTC GGA TCC ATG GAC GGC ACC ACG ACG GCC ACC GTG CT	*Bam*HI
AR11b	CTC GTC GAC CTT GCG GCG GTC GCC GAA GCC	*Sal*I

[a] Restriction sites are underlined.

serotype O3 (HSP60-O3) (Table 2). Plasmid pAN11 encodes a truncated fragment of HSP60-O8. Sequencing of *hsp60-O3* will be described elsewhere (33a). The truncated *hsp* fragment encoded by pAN11 was sequenced according to the method described by Sanger et al. (34). The sequence data of *hsp60* fragment pAR11 are available from EMBL and GenBank data bases under accession number X59366.

For PCR, DNA from 1,000 CFU of *Y. enterocolitica* O8 or O3 (1 μg of chromosomal DNA) was suspended in 10 mM Tris-HCl (pH 8.5) containing 50 mM KCl, 2 mM $MgCl_2$, 0.01% (wt/vol) gelatin, 100 μM deoxynucleoside triphosphates (dNTPs) (Pharmacia LKB, Uppsala, Sweden), 100 pM (each) oligonucleotide, and 2.5 U of *Taq* DNA polymerase (Pharmacia LKB). The PCR mixture was overlaid with oil, heated to 94°C for 5 min, and then subjected to 33 PCR cycles (denaturation at 94°C for 30 s, annealing at 48°C for 1 min, and extension at 72°C for 2 min). PCR products were visualized and analyzed by ethidium bromide staining of 1% agarose gel and then transferred on DEAE-cellulose (Schleicher & Schuell, Dassel, Germany). Thereafter, the PCR products were eluted, purified, digested with restriction enzymes, and ligated by T4 ligase (New England Biolabs, Schwalbach, Germany) into expression vector pQE32 (Diagen, Hilden, Germany) before transformation into *Escherichia coli* M15 pREP4 (Diagen) according to the recommendations of the manufacturers. Cultures of transformed *E. coli* M15 were induced with 2 mM of isopropyl-β-D-thiogalactopyranosid (IPTG; Roth, Karlsruhe, Germany) and incubated for 5 h at 37°C. Finally, cells were collected, and recombinant HSP60 were purified by affinity chromatography on metal chelate-adsorbent Ni-nitrilotriacetic acid resin (Diagen). Chromatography was performed in the presence of 8 M urea or 6 M guanidinium hydrochloride according to the instructions of the manufacturer (Diagen). The expressed HSP60 was controlled by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting with a *Yersinia* HSP60-specific monoclonal antibody (MAb) (a kind gift from D. Pieridou, Würzburg, Germany) or a polyclonal rabbit anti-*Yersinia* HSP60 serum as will be described elsewhere (33a). According to a computer based epitope analysis of HSP60, oligopeptides (amino acid residues 124 to 137, 154 to 169, 188 to 209, and 224 to 244) of HSP60 were produced and provided by D. Palm (Institut für Physiologische Chemie, University of Würzburg).

Mice. Female C57BL/6 mice (6 to 8 weeks old) were purchased from Charles River Wiga (Sulzfeld, Germany) and kept under specific pathogen-free conditions.

TABLE 2. Plasmids used in this study

Plasmid	Oligonucleotide	HSP60 amino acid residues encoded (molecular mass [kDa])	*Yersinia* serotype
pAN5	ANV + ANR	1–550 (60)	O8
pAN11	AR11a + AR11b	90–286 (22)	O8
pAN18	ANV + ANR	1–550 (60)	O3

Infection of animals. Mice were intravenously infected with *Y. enterocolitica* O8 or O3, *Y. pseudotuberculosis*, or *S. typhimurium* as described previously (4, 5). The number of bacteria present in the spleens of infected mice were determined by homogenizing the spleens in 5 ml of phosphate-buffered saline (PBS) containing 0.1% Tergitol TMN 10 (Fluka, Buchs, Switzerland) and 0.1% bovine serum albumin, plating 0.2 ml of serial dilutions of the homogenate on Mueller-Hinton agar, and counting CFU after 2 days of incubation (4, 5).

Preparation of T cells and T-cell cloning. Spleens from mice 7 days after infection with *Y. enterocolitica* O8 were removed, single cell suspensions were prepared, and T cells were purified by Ficoll density gradient centrifugation and passage through a nylon-wool column (20). The purity of the eluted cell fraction was controlled by staining the cells with anti-CD3 MAb and analyzing them in a FACScan (Becton Dickinson, Heidelberg, Germany) (see below). The generation and cultivation of *Yersinia*-specific T-cell clones have been described previously (4). For generation of HSP60-reactive T cells, mice were immunized intraperitoneally with 0.2 ml of a solution containing 30 μg of recombinant HSP60-O8 dissolved in complete ABM 2 adjuvant (Sebak, Aldenbach, Germany). After 7 days, the mice were killed, their spleens were removed, single cell suspensions were prepared, and T cells were purified as described above. T cells were incubated with 10 μg of HSP60-O8 per ml in the presence of irradiated feeder cells and restimulated after 14 days. Three days later, T-cell lines were cloned by limiting dilution as previously described (4). In brief, 0.3 to 1 T cell was seeded per well of microculture plates. Fourteen days later, subcloning was performed for positive wells by repeating the limiting dilution procedure. Finally, the clones were expanded in 12-well macroculture plates. The cloning efficiency of this method was about 2 to 10%. The clones obtained were tested for specificity by proliferation assay, phenotypically characterized by immunostaining and FACScan analysis, and assayed for cytokine production (see below).

Proliferation assay. Purified T cells (5×10^4) were incubated with 2×10^5 irradiated syngeneic splenic feeder cells and antigen in 0.2 ml of Click-RPMI 1640 cell culture medium (Biochrom, Berlin, Germany) supplemented with 2 mM L-glutamine, 10 mM HEPES (*N*-2-hydroxyethylenepiperazine-*N*′-2-ethanesulfonic acid), 5×10^{-5} M 2-mercaptoethanol, 10 μg of streptomycin per ml, 100 U of penicillin per ml, and 10% heat-inactivated fetal calf serum (Biochrom) in microculture plates (Nunc, Wiesbaden, Germany). Heat-killed whole bacterial cells (10 μg/ml) of *Y. enterocolitica*, *Y. pseudotuberculosis*, *S. typhimurium*, *L. monocytogenes*, or *E. coli* M15), purified recombinant HSP60, or oligopeptides were used as antigens as described previously (4). After 3 days, cultures were pulsed with [^{3}H]thymidine, 16 h later samples were collected with a

cell harvester, and [^{3}H]thymidine uptake was determined in a liquid scintillation counter (4).

Cytokine assays. T-cell culture supernatants were collected 24 h after antigen stimulation and used in serial dilutions in the following assays as described previously (1, 4). Interleukin-2 (IL-2) was measured by bioassay using an IL-2-sensitive HT-2 cell line. Proliferation of HT-2 cells was measured by a colorimetric assay with an MTT solution (3-4,5-dimethylthiazol-2-μl)-2,5-diphenyl tetrazolium bromide (Sigma, Munich, Germany). IL-4, IL-5, gamma interferon, and tumor necrosis factor (TNF) were measured by capture enzyme-linked immunosorbent assay (ELISA) with MAbs AN-18.17.24 and R4-6A2 for gamma interferon, 11B11 and 24 G2 for IL-4, and TRFK4 and TRFK5 for IL-5. The purified immunoglobulin G (IgG) fraction of a rabbit anti-TNF serum (3) and the biotinylated MAb MP6-XT22 (Dianova, Hamburg, Germany) were used in the TNF specific ELISA. Finally, an avidin-alkaline phosphatase complex (Vectastain ABC-AP Kit; Camon, Wiesbaden, Germany) was used. Wells containing recombinant cytokines served as controls and to estimate the actual cytokine production by T-cell cultures in units per milliliter. *A* (optical density) was measured by an ELISA reader (Flow Labsystems, Solna, Sweden).

Flow cytometry. Cells were suspended in PBS containing 2% fetal calf serum and were incubated with the following MAbs: anti-CD3 (1452C11), anti-CD4 (YTS191), anti-CD8 (YTS169), anti-αβ T-cell receptor (TCR; H57-597), and anti-γδ TCR (GL3). All these MAbs were purified from hybridoma supernatants by fast protein liquid chromatography (Pharmacia) and were then coupled to *N*-hydroxy succinimido-biotin (Sigma) or fluorescein isothiocyanate (FITC; Sigma). Streptavidin-phycoerythrin (Becton Dickinson) was used as second step reagent. Labelling steps were carried out at 4°C for 30 min with 10^5 cells in 30 μl of PBS containing 2% fetal calf serum. From each sample, 10^4 cells were analyzed by FACScan. Dead cells were excluded from analysis by gating out propidium iodide (Sigma)-positive cells in parallel samples.

Detection of serum antibodies by ELISA and immunoblotting. Sera from *Yersinia*-infected mice were analyzed for the presence of *Yersinia*- and HSP-specific IgG antibodies by means of an ELISA as described previously (42). Either whole cells of *Y. enterocolitica* O8 or recombinant HSP60-O8 were used as the antigen. Sera were diluted 1:500 in PBS containing 0.5% Tween 20 (Merck, Darmstadt, Germany) and 1% fetal calf serum. Alkaline phosphatase-conjugated anti-mouse IgG (Sigma) was diluted 1:500. *p*-Nitrophenylphosphate disodium (Sigma) was used as a substrate. Optical density was measured with an ELISA reader. Five duplicates of sera from control mice were tested as negative controls to obtain cutoff values. The cutoff value was defined as the mean *A* value for the negative controls plus 2 standard deviations. Detection of HSP60-specific IgG antibodies in sera of mice by immunoblot technique was performed as described previously (42). Sera were diluted 1:100 as described above. As a positive control, we used an HSP60-specific MAb.

Adoptive transfer experiments. Two weeks after antigen stimulation and cultivation with irradiated feeder cells and IL-2, T-cell clones were collected, purified by Ficoll density gradient centrifugation, washed, and resuspended in PBS. Cell viability was controlled by trypan blue exclusion with a microscope. A total of 10^7 cells was administered intravenously to each mouse 1 day prior to the challenge with a lethal dose of bacteria. Control mice received an ovalbumin-specific Th1 T-cell clone or PBS (4). Each group consisted of five mice. The experiments were performed three times and revealed comparable results. The extent of protection against the bacterial challenge was assessed by homogenization and plating of the spleens as described above to determine splenic CFU. The extent of protection was calculated by substracting the mean $\log_{10}$ CFU per spleen for the test groups from the mean $\log_{10}$ CFU per spleen for the control group.

Statistics. The significance of the differences among the control and experimental groups was determined by the Student *t* test. *P* values of <0.05 were considered statistically significant.

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     AA 96                                                    143
Ye.0:8 AQAIITEGLKAVAAGMNPMDLKRGIDKAVAAAVEELKYLSVPCSDSK
Ye.0:3 ..S.........................I.......K.........
E.coli ............................T.......A.........
M.tub. ...LVR...RN....A..LG.....E...EKVT.T.LKGAKEVETKE

       144                                                    189
Ye.0:8 AIAQVGTISANSDETVGKLIAEAMEKVGKEGVITVEDGT GLQDELD
Ye.0:3 .............S...E..................E.P .......
E.coli .........................D.............. .......
M.tub. Q..ATAA...G...SI.D..........N.......ES.F...L..T

       190                                                    236
Ye.0:8 VVEGMQFDRGYLSPYFINKPETGAVELESPFILLADKKISNIREMLP
Ye.0:3 ........................SI......................
E.coli ..................................................
M.tub. .....R..L..I.G..VTD..RQEAVLED.Y...VSS.V.TVKDL..

       237                                                    280
Ye.0:8 VLEAVAKAGKPLLIIAEDVEGEALATLVVNTMRGIVKAAAVKAP
Ye 0:3 ....................................V......
E.coli ........................A....I.....V......
M.tub. L..K.IG.................S.....K...TF.SV.....
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FIG. 1. Comparison of deduced amino acid (AA) sequences of the GroEL HSP of several bacterial species including *Y. enterocolitica* O8 (Ye. O:8) and O3 (Ye. O:3), *E. coli*, and *M. tuberculosis* (M. tub.) (100% homology with *M. bovis*). Periods indicate identity. Single-letter designations represent amino acids. The underlined regions indicate known or putative B- and T-cell epitopes.

RESULTS

Cloning, expression and predicted amino acid sequence of *Y. enterocolitica hsp60*. The sequence of a *groEL* homolog *hsp* of *Y. enterocolitica* O3 will be described elsewhere (33a). This HSP60-O3 consisted of 550 amino acids with a molecular mass of 60 kDa. In this study we cloned *hsp60* of *Y. enterocolitica* O8, using the same PCR-based approach as that described for cloning of *hsp60*-O3. A truncated fragment of *hsp60*-O8 (putative amino acid residues 90 to 286) was sequenced and revealed more than 90% homology with the corresponding fragment of *hsp60*-O3, 90% homology with *hsp60* (*groEL*) of *E. coli*, and 59% homology with *hsp65* of *Mycobacterium bovis* (Fig. 1). Thus, *hsp60* is highly conserved within human pathogenic serotypes of *Y. enterocolitica*. The epitope of *M. bovis* HSP65 (encoded by amino acids 180 to 188), which is recognized by arthritogenic T cells in the rat adjuvant arthritis model, differed from the corresponding sequences of *Yersinia* HSP60-O3 and HSP60-O8. Both *hsp60*-O8 (encoded by pAN5) and *hsp60*-O3 (encoded by pAN18) as well as the truncated *hsp60* fragment from serotype O8 (encoded by pAN11) were cloned and expressed by the pQE vector, purified by affinity chromatography, and used in the immunological studies described below. Figure 2 shows the expressed (note the dominant 60-kDa band in whole-cell lysates of transformed *E. coli* pAN5 encoding HSP60-O8) and purified HSP60-O8. The expressed *Yersinia* HSP60 were confirmed by immunoblotting with a *Yersinia* HSP60-specific MAb and polyclonal rabbit anti-*Yersinia* HSP60 antibodies (data not shown).

Immune responses of *Yersinia*-infected mice against *Yersinia* HSP60. To determine whether HSP60 of *Y. enterocolitica* is an

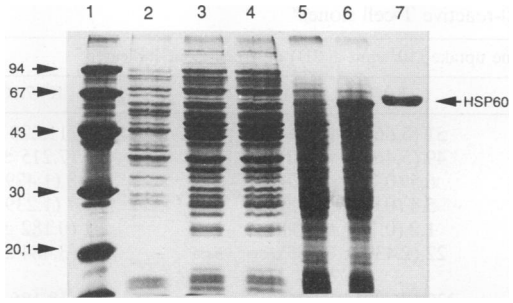

FIG. 2. Coomassie blue-stained SDS–12.5% PAGE of protein lysates of HKY-O8 (lane 2), *E. coli* transformed with control plasmid pQE32 from cultures without and with induction by IPTG (lanes 3 and 4, respectively), *E. coli* transformed with plasmid pAN5 encoding HSP60-O8 without and with induction by IPTG (lanes 5 and 6, respectively), and purified recombinant HSP60-O8 (lane 7). Lane 1, molecular weight markers (in thousands, as indicated on the left).

immunodominant antigen in yersiniosis, C57BL/6 mice were intravenously infected with *Y. enterocolitica* O8. At seven days postinfection, splenic T cells were isolated, purified, and assayed for their reactivity with both heat-killed whole cells of *Y. enterocolitica* (HKY) and recombinant HSP60 of *Y. enterocolitica*, respectively (Table 3). These T cells exhibited a significant proliferative response upon exposure to both HKY-O8 (stimulation index [SI; mean antigenic proliferation/mean nonantigenic spontaneous proliferation ratio] = 53.7) and HSP60-O8 (SI = 37.6), suggesting that HSP60 of *Y. enterocolitica* is an immunodominant antigen for T cells. However, it should be stressed that we have not yet determined the frequency of these T cells. Likewise, splenic T cells isolated from spleens of mice immunized with recombinant HSP60 recognized HSP60-O8 (SI = 24.0) as well as HKY O8 (SI = 42.3) (Table 3). In contrast, naive T cells from control mice did not exhibit a significant proliferative response upon stimulation with HKY or HSP60-O8 (Table 3).

To analyze the HSP60-specific humoral immune response, sera from infected mice were collected 2, 3, and 4 weeks after infection, and the presence of HKY, as well as HSP60-reactive IgG antibodies, was tested by an ELISA and immunoblotting. As depicted in Fig. 3, IgG antibodies in sera from infected mice exhibited a marked reactivity with recombinant *Yersinia* HSP60-O8, which was even higher than that against HKY. These results indicate that HSP60 of *Y. enterocolitica* elicited a strong humoral immune response during the infectious process.

TABLE 3. Proliferative responses of T cells from *Yersinia*-infected or HSP60-immunized mice[a]

Priming of T cells	SI [^{3}H]thymidine uptake (10^3 cpm ± SD) of T cells with antigen indicated[b]	
	HKY O8	HSP60-O8
Y. enterocolitica O8 infection	53.7 (5.422 ± 917)	37.6 (3.794 ± 613)
HSP60-O8 immunization	42.3 (9.704 ± 1.010)	24.0 (4.091 ± 1.060)
Control (naive T cells)	1.1 (0.171 ± 0.031)	<1 (0.092 ± 0.043)

[a] T cells were isolated either from spleens of C57BL/6 mice 7 days after infection with 0.5 50% lethal dose of *Y. enterocolitica* O8, from spleens of C57BL/6 mice 7 days after immunization with recombinant HSP60-O8, or from noninfected immunized naive control mice. For details, see Materials and Methods.

[b] Triplicates of 5×10^4 nylon-wool-purified T cells were cultured with 10 μg of HKY-O8, recombinant HSP60-O8, or without antigen in the presence of 2×10^5 irradiated feeder cells. Cultures were pulsed with [^{3}H]thymidine after 3 days of incubation.

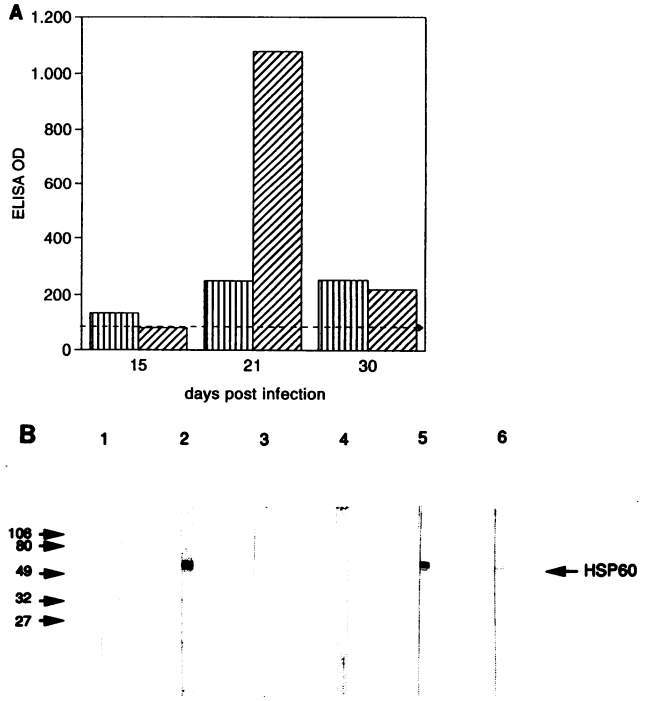

FIG. 3. Serum antibody responses of C57BL/6 mice 15, 21, and 30 days after *Y. enterocolitica* O8 infection. (A) The ELISA was performed, and the coating antigens (whole cells [HKY; ▥] and recombinant HSP60 of *Y. enterocolitica* O8 [▨]) were prepared and used as described in Materials and Methods. Serum was diluted 1:500. Bars represent the means for five animals. The dotted line indicates the cutoff value. OD, optical density. (B) IgG antibody responses against HSP60-O8 in sera from infected mice determined by immunoblotting. One representative immunoblot per experimental group is depicted. Lanes: 1, molecular weight markers (in thousands, as indicated on the left); 2, anti-HSP60-O8 MAb; 3, control serum (noninfected mouse); 4 to 6, sera from mice 15, 21, and 30 days postinfection, respectively.

Generation and characterization of HSP60-reactive T-cell clones. In order to investigate the cellular HSP60-specific immune response in more detail, we isolated and cloned T cells from the spleens of mice which had been either infected with *Y. enterocolitica* O8 or immunized with recombinant HSP60-O8. All T cells clones expressed an αβ TCR and were CD3$^+$ and CD4$^+$ but were negative for CD8 and γδ TCR as determined by FACScan analysis (data not shown). Three representative T-cell clones were chosen for further characterization and are described below. First, the proliferative responses of the clones against various antigens were analyzed. The data shown in Table 4 indicate that all three clones recognized HKY as well as HSP60 of *Y. enterocolitica* O8 and O3. The proliferative responses to HSP60-O8 of the clones is depicted in Fig. 4. Moreover, one clone (4F7) exhibited reactivity upon stimulation with the truncated HSP60-O8 fragment (amino acids 90 to 286). Furthermore, the T-cell clones recognize neither heat-killed *Y. pseudotuberculosis*, *S. typhimurium*, and *L. monocytogenes* nor HSP65 from *M. bovis*. However, all T-cell clones showed a significant cross-reaction with heat-killed *E. coli*. According to a computer-based prediction of putative HSP60 epitopes, oligopeptides of HSP60-O8 were produced and tested as antigens for the T-cell

TABLE 4. Proliferative responses of HSP60-reactive T-cell clones[a]

Antigen[b]	SI of [^{3}H]thymidine uptake (10^3 cpm ± SD) of T-cell clone indicated		
	4F7	K9.7.1D1	K9.7.1C5
HKY-O8	57 (15.970 ± 1.962)	51 (5.664 ± 0.799)	69 (11.220 ± 1.590)
HKY-O3	38 (10.665 ± 2.359)	49 (5.468 ± 0.140)	45 (7.215 ± 1.500)
Heat-killed *Y. pseudotuberculosis*	4.4 (1.226 ± 0.183)	6.5 (0.731 ± 0.207)	8.8 (1.429 ± 0.153)
Heat-killed *S. typhimurium*	2.4 (0.667 ± 0.177)	5.4 (0.602 ± 0.051)	7.7 (1.239 ± 0.168)
Heat-killed *L. monocytogenes*	<1 (0.248 ± 0.116)	1.2 (0.137 ± 0.034)	1 (0.182 ± 0.053)
Heat-killed *E. coli*	14.6 (4.098 ± 1.176)	22 (2.439 ± 0.185)	22 (3.484 ± 0.420)
HSP60-O8	193 (54.117 ± 6.672)	239 (26.821 ± 3.265)	112 (18.186 ± 2.047)
HSP60-O8 (90–286)	57.6 (18.244 ± 2.571)	1.7 (0.107 ± 0.060)	1.7 (0.372 ± 0.115)
HSP60-O3	163 (45.759 ± 2.813)	241 (27.041 ± 1.044)	141 (22.849 ± 3.148)
HSP65 (*M. bovis*)	<1 (0.192 ± 0.045)	1.5 (0.175 ± 0.024)	1.2 (0.152 ± 0.062)
aa 124–137	1.2 (0.398 ± 0.159)	<1 (0.096 ± 0.001)	<1 (0.109 ± 0.022)
aa 154–169	1.4 (0.337 ± 0.188)	<1 (0.089 ± 0.007)	<1 (0.124 ± 0.009)
aa 188–209	<1 (0.166 ± 0.014)	<1 (0.098 ± 0.024)	<1 (0.101 ± 0.021)
aa 224–244	<1 (0.188 ± 0.074)	<1 (0.078 ± 0.003)	<1 (0.101 ± 0.001)

[a] T-cell clone 4F7 was isolated from spleens of *Y. enterocolitica* O8-infected C57BL/6 mice. T-cell clones K9.7.1D1 and K9.7.1C5 were isolated from the spleens of mice immunized with recombinant HSP60-O8. For details, see Materials and Methods.

[b] For details, see Table 3, footnote *a*. HSP60-O8, HSP60-O3, and HSP60-O8 (90–286), recombinant full-length HSP60 of *Yersinia* O8 and O3 and truncated HSP60-O8 (amino acids 90 to 286), respectively. aa 124–137, aa 154–169, aa 188–209, and aa 224–244, amino acid (aa) residues (position numbers indicated) of synthesized peptides of HSP60-O8.

clones. However, none of the oligopeptides (amino acid residues 124 to 137, 154 to 169, 188 to 209, and 224 to 244 of HSP60-O8) tested induced a proliferative response of the clones. Although we do not yet know the epitopes of HSP60-O8 which are recognized by the T-cell clones, we can conclude that T cells isolated after *Yersinia* infection or HSP60 immunization recognize epitopes which are conserved in *Y. enterocolitica*. However, whether this pattern of antigen reactivity of T cells reflects the in vivo situation or whether it is due to the conditions of our experimental system (mouse strain and in vitro stimulation for generation of clones, etc.) remains to be investigated.

Determination of cytokine production by HSP60-reactive T-cell clones revealed that all clones exhibited a cytokine production pattern which is characteristic for Th1 cells. Hence, all clones produced minute quantities of IL-2 (~1 U/ml) and significant quantities of gamma interferon (41 to 448 U/ml) but not IL-4 and IL-5 (Table 5). Furthermore, TNF (22.5 to 24 U/ml) was detected in the supernatants of the T-cell clones.

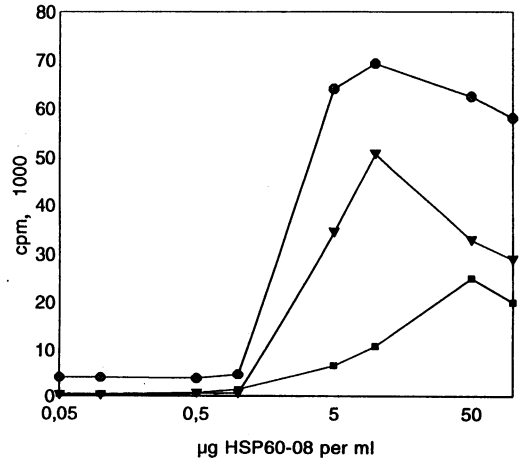

FIG. 4. Proliferative responses of T-cell clones 4F7 (square), K9.7.1D1 (inverted triangle), and K9.7.1C1 (circle) to various concentrations of HSP60-O8. For details see Materials and Methods.

Adoptive transfer of HSP60-reactive T-cell clones. To assess the protective properties of the T-cell clones in experimental yersiniosis, we transferred intravenously 10^7 T cells into naive C57BL/6 mice 1 day prior to the intravenous infection with a lethal dose of *Y. enterocolitica*. The data presented in Table 6 indicate that all clones mediated significant protection (log protection of 1.9 to 2.98 at P values of <0.02 for 4F7 and K9.7.1C5 and P of <0.03 for K9.7.1D1) against *Y. enterocolitica* O8. Thus, the T-cell clones mediated a 100- to 1,000-fold reduction of bacteria in the spleens 6 days after infection.

To determine the specificity of the T-cell-mediated protection, mice, after transfer of clone 4F7, were challenged with *Y. enterocolitica* O3, *Y. pseudotuberculosis*, or *S. typhimurium* (Table 6). The data show that T-cell clone 4F7 mediated protection against both serotypes of *Y. enterocolitica* (log protection, 2.2 for *Y. enterocolitica* O3; $P < 0.04$) but not against *Y. pseudotuberculosis* (log protection = 0). A minute protection was observed for *S. typhimurium* (log protection, 0.49; $P < 0.003$).

DISCUSSION

HSP of microbial pathogens have been identified as immunodominant antigens which share 50% and more homology in

TABLE 5. Cytokine production by HSP60-reactive T-cell clones[a]

T-cell clone	Cytokine activity (U/ml)[b]				
	IL-2	IL-4	IL-5	IFN-γ	TNF
4F7	~1	<1.5	<5	448	22.5
K9.7.1D1	~1	<1.5	<5	43	24
K9.7.1C5	~1	<1.5	<5	41	23

[a] Supernatants from T-cell cultures were collected 24 h after antigenic stimulation and tested for the presence of cytokines by capture ELISA (IL-4, IL-5, gamma interferon, and TNF and by bioassay (IL-2). For details, see Materials and Methods.

[b] Units per milliliter were estimated from the straight line portion of the respective standard curve.

TABLE 6. Adoptive transfer of HSP60-reactive T-cell clones[a]

T-cell clone	Protection (log_{10} CFU) against[b]:			
	Y. enterocolitica		*Y. pseudotuberculosis*	*S. typhimurium*
	O8	O3		
4F7	2.98	2.22	0.00	0.49
K9.7.1C5	2.30	ND	ND	ND
K9.7.1D1	1.98	ND	ND	ND

[a] T-cell clone (10^7 cells) 4F7, K9.7.1C5, or K9.7.1D1 was transferred into naive C57BL/6 mice (five mice per group) 1 day prior to infection with two times the 50% lethal dose of *Y. enterocolitica* O8, *Y. enterocolitica* O3, *Y. pseudotuberculosis*, or *S. typhimurium*.

[b] Mice were killed 6 days after infection, and the number of splenic CFU was determined as described in Materials and Methods. Log protection was calculated by substracting the mean log_{10} CFU of the T-cell groups from the mean log_{10} CFU of the respective control group (which received PBS or ovalbumin-specific T cells). The differences between the experimental and control groups were statistically significant ($P < 0.05$). ND, not determined.

their amino acid sequence with host HSP (12, 19, 23, 26, 46). Several lines of evidence suggested that HSP may trigger autoimmune diseases by overcoming tolerance to self antigens (12, 19, 22–24, 46). Hence, arthritogenic T cells reactive with both mycobacterial HSP65 and a cartilage proteoglycan have been described in a rat adjuvant arthritis model (17, 38–41), and $CD8^+$ T cells reactive with bacterial HSP were shown to lyse stressed macrophages (25). Furthermore, a delayed-type hypersensitivity-like reaction against chlamydial HSP seems to account for the pathogenesis of trachoma and other chlamydial infections (29, 37, 43). Moreover, T cells isolated from the synovial fluid of patients with reactive arthritis recognized both HSP65 of mycobacteria and human HSP60 and lysed heat-stressed autologous cells (11, 15). Likewise, T cells from healthy individuals were shown to be reactive with mycobacterial and human HSP (30). Finally, antibodies against *Borrelia burgdorferi* HSP60 were found in sera from patients with Lyme disease (35). Taken together, the majority of reports on the role of HSP in immunity have focused on and pointed out the putative harmful role of these proteins with regard to cross-reactions against self antigens of host tissues. Nevertheless, whether HSP-reactive antibodies and/or T cells do actually contribute to autoimmunity in humans remains to be elucidated.

On the other hand, if HSP are immunodominant antigens, it might well be that they induce protective immune responses. However, only few reports have addressed this question. It was shown that active immunization of neonatal mice with a *Bordetella pertussis* GroEL-like protein induced little, if any, protection against an aerosol challenge with *B. pertussis* (7). Furthermore, a putative protective role of γδ T cells reactive with HSP60 of *M. tuberculosis* (but not with heat-killed listeriae) was shown in the early stage of *Listeria* infection in mice (16, 28, 44). However, a study including intestinal γδ T cells could not demonstrate a specificity for HSP60 (44). More recently, it has been shown that resistance of mice against *Schistosoma mansoni* after vaccination with cercariae was associated with the appearance of antibodies against HSP70 of schistosomes (33). However, to date there is no direct evidence for a protective role of HSP-reactive antibodies or T cells in immunity to microbial pathogens.

To address this issue, we analyzed the HSP60-specific immune responses in an experimental *Yersinia* mouse infection model. The most salient findings of this study were that (i) HSP60 of *Y. enterocolitica* is an immunodominant antigen in murine yersiniosis for both humoral and cellular immune responses and (ii) *Yersinia* HSP60-reactive αβ TCR^+ $CD4^+$ Th1 cells mediate protection against infection by human pathogenic *Y. enterocolitica* serotypes O8 and O3. Thus, to our knowledge, this is the first report that provides direct evidence for a protective role for HSP-reactive αβ T cells in bacterial infections. Furthermore, the epitope recognized by one of the T-cell clones described resides on a fragment of HSP60 encoded by amino acids 90 to 286. Several reports indicated that a significant number of γδ T cells, which display a limited diversity in their TCR, are specialized to HSP (21, 31, 32). It was suggested that γδ T cells might represent a first line of defense against infectious agents (16, 21, 32, 36, 44). In contrast to the findings reported by these groups, we could not isolate γδ T cell clones reactive with *Yersinia* HSP60 from spleens of neither *Yersinia*-infected nor HSP60-immunized mice. Likewise, in bulk cultures with T cells isolated from spleens of either naive, *Yersinia*-infected, or HSP60-immunized mice, we did not observe a significant increase of γδ T cells after antigenic (HSP60 or HKY) stimulation (data not shown). Thus, an important role for γδ T cells in yersiniosis seems to be rather unlikely. However, we cannot exclude the fact that after infection via other routes (e.g., oral, intraperitoneal, or subcutaneous), γδ T cells may be involved in defense against yersiniae. Moreover, it may well be that infection by avirulent *Yersinia* strains may induce a response of γδ T cells, as has been recently reported for *Salmonella* infection (9). Furthermore, the ability to generate an HSP60-specific γδ T-cell response can be dependent on the presence of certain gene loci, e.g., *Ity* locus (10). Thus, it may be worthwhile studying *Yersinia* HSP60-specific T-cell responses in several mouse strains that differ in their *Ity* locus.

Although αβ T cells of *Yersinia*-infected mice exhibited a significant proliferative response upon exposure to HSP60, we do not yet know the frequency of these cells. To address this question, we currently perform limiting dilution assays in order to determine the frequency of HSP60-specific T cells of various mouse strains (C57BL/6 [*Yersinia* resistant] versus BALB/c [*Yersinia* susceptible]). Such studies may further elucidate the role of HSP60-reactive T cells in the protective host response against *Y. enterocolitica*.

In addition to the T-cell responses, *Yersinia*-infected mice developed high HSP60-specific serum IgG antibody titers. However, the protective role of these HSP60-reactive antibodies in yersiniosis is unknown so far. Preliminary data from adoptive transfer experiments, including *Yersinia* HSP60-specific MAbs (data not shown), indicate that these antibodies are not protective. Due to the fact that HSP, in general, are located predominantly intracellularly and in the periplasmic space (27, 45) but are not significantly expressed in the outer membrane of yersiniae (our observations), this finding was not surprising. Thus, a protective role of anti-HSP60 antibodies seems to be unlikely. By contrast, antibodies against abundantly expressed outer membrane proteins of yersiniae (e.g., YadA) have been shown to mediate significant protection (42). The epitopes which are recognized by *Yersinia*-specific T-cell clones are not yet known. However, one clone (4F7) was tested in terms of cross-protection. As expected from the proliferative responses to heat-killed *Y. pseudotuberculosis* and *S. typhimurium*, this clone did not mediate protection against infection by these pathogens. Thus, this clone probably recognizes a highly specific epitope of HSP60 of *Y. enterocolitica*. On the other hand, neither the protective role of T cells during infection by these pathogens nor the sequence of HSP60 of *Y. pseudotuberculosis* or *S. typhimurium* is known so far. Thus, detailed studies that focus on HSP60 T-cell epitopes are required. Such studies are a prerequisite for evaluating

whether HSP60 may induce protective or cross-protective host responses and elucidating the risk of *Yersinia* HSP60-induced self-reactive immune responses.

In summary, this is the first study that provides direct evidence for a protective role of HSP60-reactive T cells in experimental murine *Y. enterocolitica* infection. Vaccination studies including the recombinant *Yersinia* HSP60 are now required in order to further investigate the role of HSP for immunity to this pathogen.

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