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Immunity against *Yersinia enterocolitica* by Vaccination with *Yersinia* HSP60 Immunostimulating Complexes or *Yersinia* HSP60 plus Interleukin-12

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Microbial heat shock proteins (HSP) are dominant antigens for the host immune response. Because of the high sequence homology between mammalian and microbial HSP, their value as component of a subunit vaccine has been the subject of controversy. Previous work from this laboratory, however, demonstrated for the first time that the adoptive transfer of HSP60-reactive $CD4^+$ αβ T-cell clones confers protection against bacterial infection in mice but does not induce autoimmunity. In the present study, we have therefore evaluated the potential role of *Yersinia* HSP60 (Y-HSP60) as a vaccine in the *Yersinia enterocolitica* mouse infection model. For this purpose, immunostimulating complexes (ISCOM) which included Y-HSP60 were constructed. Parenteral administration of this vaccine induced high Y-HSP60-specific serum antibody responses as well as T-cell responses. This reaction was parallelled by immunity against a lethal challenge with *Y. enterocolitica*. In contrast, mucosal application of Y-HSP60–ISCOM failed to induce systemic Y-HSP60-specific T-cell responses and thus failed to induce immunity against yersiniae. Likewise, vaccination with purified recombinant Y-HSP60 induced antibody responses but only weak T-cell responses. Therefore, this vaccination protocol was not protective. However, when interleukin-12 was used as an adjuvant, purified Y-HSP60 induced significant Y-HSP60-specific T-cell responses and thus induced protection against subsequent challenge with yersiniae. These studies suggest that (i) microbial HSP might be promising candidates for the design of subunit vaccines and (ii) interleukin-12 is an efficient alternative adjuvant to ISCOM particles for induction of protective CD4 Th1-cell-dependent immune responses against bacterial pathogens.

Infection with *Yersinia enterocolitica* causes a broad spectrum of clinical manifestations including enteritis, enterocolitis, and mesenteric lymphadenitis (12, 14). Moreover, both systemic infections with abscesses and granuloma-like lesions in the liver and spleen and immunopathological sequelae such as reactive arthritis and erythema nodosum are associated with *Y. enterocolitica* infections (1, 58, 62). The pathogenicity of *Y. enterocolitica* for humans and rodents depends on plasmid-encoded (e.g., Yops, YadA) and chromosomally encoded (e.g., *Yersinia* bactin, Inv) virulence factors (13). Several of these virulence factors mediate efficient resistance against phagocytosis and complement lysis and thus promote the extracellular survival of *Y. enterocolitica* in infected host tissues (13, 26, 37). In fact, electron-microscopic studies confirmed that *Y. enterocolitica* is extracellularly located in Peyer's patch and liver tissue of experimentally infected mice (4, 24, 37).

An overwhelming body of evidence suggests that a specific T-cell-mediated host response is required for resolution of *Y. enterocolitica* infections (3, 7, 8). Both CD4 Th1 and CD8 T cells confer resistance against this pathogen when transferred into T-cell-deficient athymic nude mice or naive syngeneic mice (3, 7, 8). As the cytokines interleukin-12 (IL-12), tumor necrosis factor alpha, and gamma interferon are essential mediators of the protective events against yersiniae, macrophages are possibly the final (T-cell-activated) effector cells in the infection process (2, 5, 10, 11). The integrins Mac-1 and VLA-4 are involved not only in phagocytosis of *Yersinia* cells by macrophages in vitro and in vivo but also in host cell interactions; thus, they play a multifunctional role in host defense mechanisms against *Y. enterocolitica* (6).

The 60-kDa *Yersinia* heat shock protein (Y-HSP60) is a major antigen for murine T and B cells (49). Protection of mice against *Y. enterocolitica* infection can be transferred by Y-HSP60-reactive T cells but not by Y-HSP60-reactive antibodies (49). Moreover, immunodominant 12- and 13-amino-acid T-cell epitopes of Y-HSP60, which are presented by MHC class II (I-A) molecules, have been recently identified (48). Whether Y-HSP60 might be useful as a vaccine, however, remained questionable, since microbial HSP were believed to be involved in autoimmune responses (23, 27, 29, 32, 33, 63). Indeed, there is extensive sequence homology between mammalian and microbial HSP (23, 27, 29, 33, 38, 63). Hence, an immune response against shared epitopes of microbial and mammalian HSP might destroy the tolerance for self-antigens (HSP) and subsequently might cause autoimmune diseases (23, 27, 29, 31, 33, 63). In keeping with this hypothesis, T cells isolated from the synovial fluid of patients with *Yersinia*-triggered reactive arthritis recognize mycobacterial HSP65 (27). Further, the cell surface of gamma interferon-stressed macrophages contains endogenous HSP exposed to autologous cytotoxic T cells, which subsequently can lyse the stressed macrophages (36).

The primary aim of the present study was to evaluate whether Y-HSP60 might be a useful vaccine. Since T cells have been identified as major protective component of the host response against *Y. enterocolitica*, a Y-HSP60 vaccine should efficiently stimulate T cells. Immunostimulating complexes (ISCOM) have been used as immunization vectors for the induction of a wide range of immune responses to a variety of protein antigens (41, 43, 45). ISCOM contain Quil A, an ex-

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tract from the bark of the South American tree *Quillaja saponaria* Molina with adjuvant properties (34). Further elements of ISCOM are cholesterol, phosphatidylcholine, and the desired antigen in equimolar amounts (39). In this study, we produced and used Y-HSP60–ISCOM as a vaccine. Alternatively, pure Y-HSP60 with recombinant IL-12 as an adjuvant was used for vaccination in a second approach, since cytokines turned out to be useful as adjuvants for vaccines (57, 59). The results reported herein indicate that both vaccination strategies conferred protection against *Y. enterocolitica* infection and argue for a possible role of HSP as a component of an efficient (subunit) vaccine.

MATERIALS AND METHODS

Bacteria and infection of animals. *Y. enterocolitica* WA-314 serotype O8 was cultured as described previously (7). Female C57BL/6 and BALB/c mice, 6 to 8 weeks old, were purchased from Charles River Wiga, Sulzfeld, Germany, and kept in positive-pressure cabinets under specific-pathogen-free conditions. The mice were intravenously infected with 5 to 10 50% lethal doses (LD_{50}) of *Y. enterocolitica* WA-314 as described previously (7). At various intervals after infection, mice were killed, their spleens were removed, and serum was collected. The number of bacteria present in the spleens of infected mice was determined by homogenization of the spleens in phosphate-buffered saline (PBS; pH 7.4) containing 0.5% bovine serum albumin (Biomol, Hamburg, Germany) and 0.5% Tergitol TMN10 (Fluka, Buchs, Switzerland) and plating of 0.1 ml of serial dilutions of the homogenate on Mueller-Hinton agar. CFU were counted after 2 days of incubation at 27°C. All experiments were repeated at least three times for verification.

Antigen preparations. Y-HSP60 was cloned and purified as described elsewhere (49). Briefly, *Escherichia coli* M15(pAN5) (49) was grown, and expression of recombinant Y-HSP60 was induced with 2 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma, Deisenhofen, Germany). Y-HSP60 was extracted and purified by affinity chromatography on metal-chelating Ni resin (Pharmacia LKB, Uppsala, Sweden) in the presence of 8 M urea. After renaturation, the protein was sterilized, its concentration was measured by the bicinchoninic acid assay (Pierce, Oud-Beijerland, The Netherlands), and aliquots were frozen at −20°C until further use. The purity of Y-HSP60 preparation was controlled by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting (49). Heat-killed Yersiniae were prepared as described elsewhere (7). Ovalbumin (OVA; Sigma) was used as the control antigen.

Preparation of ISCOM by incorporation of acid-treated proteins. Lyophilized Y-HSP60 or ovalbumin (OVA) (20 mg) was dissolved in PBS at a final concentration of 2 mg of protein per ml. To each 1 ml of protein solution was added 100 μl of 1 M glycine buffer (pH 2.5), which exposed hydrophobic amino acid residues by reducing the pH of the protein solution. The 20 μl of a lipid mix consisting of 8.3 mg of cholesterol (Sigma) per ml, 8.3 mg of phosphatidylcholine (Sigma) per ml, 167 mg of decanoyl-*N*-methylglucamide (Mega10; Sigma) per ml, and 0.17% (vol/vol) chloroform was added. Afterwards, Quil A (Roth, Karlsruhe, Germany) was added at a final concentration of 1 mg/ml. Then the mixture was sonicated for 15 min in a 20°C water bath to disrupt any protein aggregates. After incubation for 60 min at 20°C, the solution was extensively dialyzed against 0.1 M glycine buffer (pH 2.5) for 20 h at 20°C and then against PBS for 20 h at 20°C and for at least 20 h at 4°C. Formation of the typical cage-like structures of ISCOM was determined by transmission electron microscopy. For this purpose, Y-HSP60-ISCOM and OVA-ISCOM were negatively stained with 0.5% uranyl acetate (Merck) at 4°C for 16 h and analyzed by electron microscopy. Typical ISCOM particles form cage-like structures 30 to 40 nm in diameter. Furthermore, incorporation of antigen into ISCOM was analyzed by density sucrose gradient centrifugation as described elsewhere (47).

Vaccination protocols. (i) Immunization with ISCOM. To induce proliferative T-cell responses in mice (four animals per group), immunizations with either Y-HSP60–ISCOM or OVA-ISCOM were performed by using three different protocols. (i) On days 0, 10, and 21, the mice were given intraperitoneal (i.p.) injections of 10 μg of Y-HSP60–ISCOM or OVA-ISCOM in 0.1 ml of PBS. (ii) On days 0, 10, and 21 the mice were given injections in the footpads with 10 μg of Y-HSP60–ISCOM or OVA-ISCOM in 0.05 ml of PBS. (iii) On days 0, 7, 14, and 21, 100 μg of Y-HSP60–ISCOM or OVA-ISCOM was orally administered 0.1 ml of PBS via a 0.86-mm-diameter polypropylene tube connected to a syringe fitted with a 20-gauge 1/2 needle.

Eight days after the final injection, the mice were killed, sera were collected, and splenic or lymph nodes T cells were prepared to characterize humoral and cellular immune responses induced by the immunization procedures.

For protection assays, mice (four animals per group) were given i.p. injections on days 0, 14, and 28 with 10 μg of Y-HSP60–ISCOM or OVA-ISCOM in 0.1 ml of PBS or with 0.1 ml of PBS. Ten days later (day 38), the mice were infected intravenously (i.v.) with a lethal dose (5 to 10 LD_{50}) of *Y. enterocolitica*. Seven days after the infection, the mice were killed and sera were collected. Spleens were removed and homogenized, and serial dilutions of the homogenates were plated on Mueller-Hinton agar as described above. Bacterial numbers in the spleens of mice are given as $\log_{10}$ CFU.

(iii) Immunization with Y-HSP60 plus IL-12. To induce proliferative T-cell responses, mice were given injections of 0.2 μg of recombinant murine IL-12 (r-IL-12; kindly provided by M. Gately, Hoffmann-La Roche Inc., Nutley, N.J.) and 50 μg of purified Y-HSP60 i.p. on days 0 and 3. Control mice received Y-HSP60 alone (50 μg), r-IL-12 alone (0.2 μg), or 0.1 ml of PBS. Five days later, splenic T cells were isolated, purified, and assayed for their reactivity with different antigens.

For protection assays, mice (three or four animals per group) were given i.p. or subcutaneous (s.c.) injections in the footpads with (i) 25 μg of Y-HSP60 plus 0.5 μg of murine r-IL-12, (ii) 25 μg of Y-HSP60, (iii) 0.5 μg of r-IL-12, or (iv) PBS on days 0 and 14. Fourteen days later, the mice were infected i.v. with a lethal dose of *Y. enterocolitica* (~10 LD_{50}). Six days after infection, the mice were killed, sera were collected, spleens were homogenized, and serial dilutions were plated on Mueller-Hinton agar to determine bacterial counts in the spleens.

Flow cytometry. To characterize the purity of isolated T cells, the cells were stained with fluorescein isothiocyanate-coupled anti-CD3ε (145 2C11) monoclonal antibody. Staining procedures were carried out as previously described (49). Finally, fluorescence staining of cells was analyzed with a FACScan (Becton Dickinson, Heidelberg, Germany). Dead cells were excluded from analysis by gating out propidium iodide (Sigma)-positive cells. At least 10,000 cells per sample were analyzed.

T-cell preparation and proliferative responses. At 5 to 8 days after immunization of mice, spleens and/or popliteal lymph nodes were removed and single-cell suspensions were prepared as described previously (7). T cells were isolated and purified by Ficoll density gradient centrifugation and passaged through a nylon wool column (30). The purity of eluted T cells was controlled by flow cytometry as described above. The final cell fraction usually contained 90% $CD3^+$ T cells.

For proliferation assays, 1×10^5 to 2×10^5 freshly prepared and purified T cells were incubated with 2×10^5 irradiated (3,000 rads) syngeneic splenic cells as antigen-presenting cells and 1 to 10 μg of antigen per ml of culture medium in a total of 0.2 ml in 96-well microtiter plates (Nunc, Wiesbaden, Germany). The culture medium was Click-RPMI 1640 (Biochrom, Berlin, Germany) supplemented with 2 mM L-glutamine, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*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). Heat-killed whole bacterial cells of *Y. enterocolitica* O8 (7), recombinant purified Y-HSP60, truncated recombinant Y-HSP60 (amino acid residues 90 to 286) (49), and cyanogen bromide-cleaved Y-HSP60 peptides (48) were used at 10 μg/ml of culture medium. Synthetic Y-HSP60 peptides (amino acid residues 194 to 232 and 74 to 86) (48) were used at 1 μg/ml. Concanavalin A (3 μg/ml) was used as an indication of maximal T-cell stimulation, and background activity was determined in the absence of antigen (medium, antigen-presenting cells, and T cells).

After 3 days of incubation, microcultures were pulsed with [^{3}H]thymidine. Eight hours later, samples were collected with a cell harvester (Pharmacia) and [^{3}H]thymidine uptake was determined with a liquid scintillation counter (Pharmacia) (7). Proliferative responses were expressed as stimulation index (SI), which was calculated as follows: SI = [^{3}H]thymidine uptake (cpm) in the presence of the indicated antigen/[^{3}H]thymidine uptake (cpm) without antigen. Standard deviations are not included in the tables for the sake of clarity. All experiments were repeated at least three times for verification.

Detection of serum antibodies by ELISA. Sera of immunized and control mice were prepared and analyzed for the presence of Y-HSP60 or OVA-specific immunoglobulin G (IgG) antibodies as described previously (61). Recombinant Y-HSP60 or OVA (50 μl; 10 μg/ml of PBS) was used as the antigen for coating. Sera were diluted 1:100 in PBS containing 0.5% Tween 20 (Merck, Darmstadt, Germany). Alkaline phosphatase-conjugated anti-mouse IgG (Sigma) was diluted 1:1,000 with PBS containing 0.5% Tween 20. *p*-Nitrophenylphosphate disodium (Sigma) was used as the substrate. Optical density was measured with an enzyme-linked immunosorbent assay (ELISA) reader (Flow Laboratories, Meckenheim, Germany) at a wavelength of 405 nm. Five duplicates of sera from uninfected nonimmunized control mice were tested as negative controls to obtain cutoff values. The cutoff value was defined as the mean value for the negative controls plus 2 standard deviations. As a positive control, a Y-HSP60-specific monoclonal antibody was used (49).

Statistics. Data from different experimental groups were compared by Student's *t* test. $P < 0.05$ were considered statistically significant.

RESULTS

Vaccination of mice with Y-HSP60–ISCOM particles. C57BL/6 mice were immunized i.p. or s.c. three times with 10 μg of Y-HSP60–ISCOM (Fig. 1) or OVA-ISCOM. Alternatively, mice were immunized by four oral administrations of 100 μg of Y-HSP60–ISCOM or OVA-ISCOM. Eight days after administration of the final boost, the mice were killed, serum and splenic or lymph node T cells were prepared and

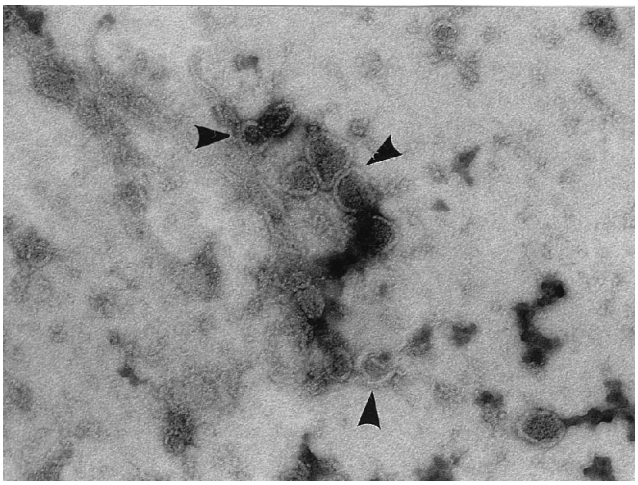

FIG. 1. Transmission electron micrographs of ISCOM containing Y-HSP60. The particles have formed the typical cage-like structure (arrows). Magnification, ×40,000.

TABLE 1. Proliferative T-cell responses after immunization with ISCOM particles[a]

Antigen[b]	SI[c]			
	Y-HSP60–ISCOM			OVA-ISCOM (i.p.)
	i.p.	s.c.	p.o.[d]	
HKY	4.8	5.0	1.8	1.9
Y-HSP60	5.0	1.9	<1	1.1
Y-HSP60 (aa 90–286)	6.2	2.3	1.6	1.9
Y-HSP60/CNBr	7.3	2.9	1.7	1.5
Y-HSP60 peptides				
aa 194–232	5.0	1.7	1.0	1.3
aa 74–86	4.0	1.8	1.1	1.6
OVA	1.0	1.1	<1	2.1

[a] Preparation of ISCOM and immunization of mice were performed as described in Materials and Methods.

[b] T cells were incubated with 10 μg of HKY; full-length, truncated (aa 90 to 286), or CNBr-cleaved Y-HSP60 or OVA per ml of medium; or 1 μg of the depicted synthetic peptides per ml of medium.

[c] Proliferative responses were determined by [^{3}H]thymidine uptake of 2×10^5 T cells after antigenic stimulation in the presence of 2×10^5 irradiated syngeneic antigen-presenting cells. Proliferative responses are expressed as the SI.

[d] p.o., oral administration.

serum antibody responses and T-cell responses against Y-HSP60 or OVA were determined. Both i.p. (Fig. 2) and s.c. (data not shown) immunization induced a strong antigen-specific serum IgG antibody response against Y-HSP60 or OVA, respectively (Fig. 2). In contrast, orally or nasally immunized mice did not show an antigen-specific response (data not shown).

Furthermore, proliferative T-cell responses upon stimulation with various *Yersinia* antigens or OVA were determined. T cells from mice immunized with Y-HSP60–ISCOM exhibited a significant proliferative response upon stimulation with either heat-killed yersiniae or various Y-HSP60 preparations (Table 1). Likewise, Y-HSP60 peptides (amino acid residues 74 to 86 and 194 to 232) which have been identified as dominant T-cell epitopes for *Yersinia*-reactive CD4 Th1 cells from C57BL/6 mice were recognized by T cells after i.p. immunization with Y-HSP60–ISCOM. In contrast, T cells isolated from mice after orogastric immunization did not show significant reactivity upon antigenic stimulation. Furthermore, T cells isolated from mice immunized with OVA-ISCOM responded upon stimulation with OVA only but not with Y-HSP60 (Table 1).

To determine whether the above immunization procedure induced protection against *Yersinia* infection, mice that had been immunized i.p. with Y-HSP60–ISCOM or OVA-ISCOM were challenged i.v. with 10 LD_{50} of *Y. enterocolitica*, and 7 days later, the bacterial counts in their spleens were determined. The data depicted in Fig. 3 indicate that immunization with Y-HSP60–ISCOM induced significant protection against subsequent challenge with yersiniae whereas administration of OVA-ISCOM did not. Hence, bacterial counts in the spleens of control mice (administration of PBS or OVA-ISCOM) were ~1,000-fold higher ($P < 0.05$). Taken together, these results demonstrate that Y-HSP60–ISCOM immunization (footpad or i.p.) induced Y-HSP60-specific T- and B-cell responses, the former of which conferred subsequent protection against a lethal challenge with yersiniae.

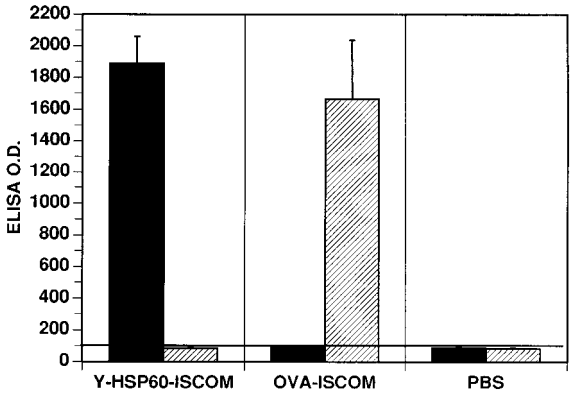

FIG. 2. Serum IgG antibody responses of immunized C57BL/6 mice 10 days after the final i.p. injection with 10 μg of Y-HSP60–ISCOM or OVA-ISCOM or 100 μl of PBS buffer on days 0, 14, and 28. The ELISA was performed, and the coating antigens (Y-HSP60 [solid bars] or OVA [hatched bars]) were prepared and used as described in Materials and Methods. Sera were diluted 1:100. Bars represent means and standard deviations of results obtained with four animals. The horizontal line indicates the cutoff value. O.D., optical density.

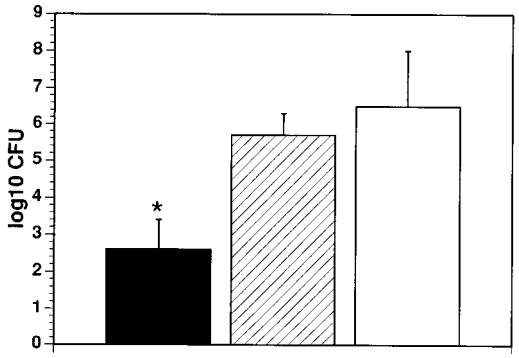

FIG. 3. Bacterial numbers in spleens of immunized C57BL/6 mice after infection with *Y. enterocolitica*. For immunization, mice were given i.p. injections on days 0, 14 and 28 with 10 μg of ISCOM preparations (Y-HSP60–ISCOM [solid bars] or OVA-ISCOM [hatched bars]) or with PBS (open bars) as a control. The mice were infected i.v. with 2.2×10^4 CFU of *Y. enterocolitica* 10 days later. Seven days after infection, the mice were killed, spleens were removed and homogenized, and bacterial counts were determined. Bacterial numbers in the spleens are expressed as $\log_{10}$ CFU. Bars represent means and standard deviations of results obtained with four animals. The asterisk indicates statistically significant differences between experimental and control groups.

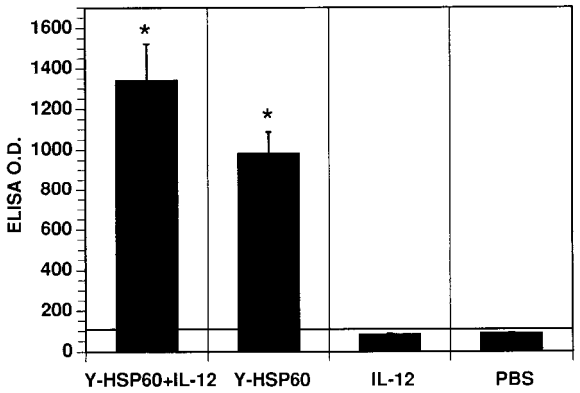

FIG. 4. Serum IgG antibody responses of BALB/c mice 14 days after the final s.c. immunization with Y-HSP60 plus r-IL-12, Y-HSP60, r-IL-12, or PBS on days 0 and 14. The ELISA was performed, and the coating antigen (Y-HSP60) was prepared and used as described in Materials and Methods. Sera were diluted 1:100. Bars represent means and standard deviations of results obtained with three animals. The horizontal line indicates the cutoff value. O.D., optical density. The asterisk indicates statistically significant differences between experimental and control groups.

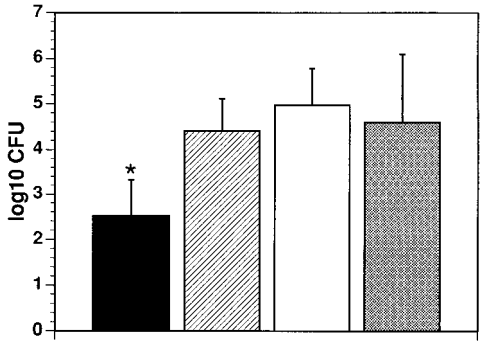

FIG. 5. Bacterial numbers in spleens of immunized BALB/c mice after infection with *Y. enterocolitica*. For immunization, the mice were given injections into the footpads on days 0 and 14 with 25 μg of Y-HSP60 plus 0.5 μg of IL-12 (solid bar), 25 μg of Y-HSP60 (hatched bar), 0.5 μg of IL-12 (open bar), or PBS (stippled bar). The mice were infected i.v. with 4.1×10^3 CFU of *Y. enterocolitica* 14 days later. Six days after infection, the mice were killed, spleens were homogenized, and bacterial counts were determined. Bacterial numbers in the spleens were expressed as $\log_{10}$ CFU. Bars represent means and standard deviations of results obtained with three or four animals. The asterisk indicates statistically significant differences between experimental and control groups.

Vaccination with Y-HSP60 plus IL-12 as adjuvant. Recent studies suggested that ILs might be useful as adjuvant for immunization (57, 59). Since IL-12 turned out to be a crucial cytokine for the development of *Yersinia*-specific T-cell responses in mice, pure recombinant Y-HSP60 was administered with IL-12 as adjuvant. For this purpose, mice were given two i.p. or s.c. injections of (i) 0.2 to 0.5 μg of r-IL-12 plus 25 to 50 μg of Y-HSP60, (ii) 25 to 50 μg of Y-HSP60 without r-IL-12, or (iii) r-IL-12 only. As described above, serum antibody responses and T-cell responses were determined 5 days after the boost injection. Immunization with Y-HSP60 plus IL-12 or with Y-HSP60 only induced significant Y-HSP60-specific serum IgG antibody responses, whereas injection of IL-12 alone or PBS buffer did not (Fig. 4). However, Y-HSP60-specific IgG antibody responses were significantly higher in mice which received the combination of Y-HSP60 plus IL-12 than in those which received Y-HSP60 only ($P < 0.05$).

Analysis of proliferative T-cell responses revealed that immunization with Y-HSP60 plus IL-12 induced significant proliferative responses upon stimulation with heat-killed yersiniae or various Y-HSP60 preparations (Table 2). However, there was only a weak response upon stimulation with Y-HSP60 peptides (data not shown). Likewise, T cells from mice immunized with Y-HSP60 without IL-12 as adjuvant exhibited Y-HSP60-specific proliferative responses, although the responses were weaker than those induced by the combination of Y-HSP60 with IL-12.

TABLE 2. Proliferative T-cell responses of after immunization with Y-HSP60 plus IL-12 as an adjuvant[a]

Antigen[b]	SI[c] for:		
	Y-HSP60 + IL-12	Y-HSP60	Control
HKY	6.8	4.8	ND[d]
Y-HSP60	3.1	1.6	1.2
Y-HSP60/CNBr	5.6	5.2	3.1

[a] Immunization of mice was performed as described in Materials and Methods. Controls were given injections with PBS.
[b] As described in the legend to Table 1.
[c] Proliferative responses were determined by [^{3}H]thymidine uptake by 1×10^5 T cells after antigenic stimulation in the presence of 2×10^5 irradiated syngeneic antigen-presenting cells. For details, Table 1, footnote *c*, and Materials and Methods.
[d] ND, not done.

Subsequently, mice were challenged i.v. 14 days after two footpad immunizations with viable *Y. enterocolitica* to determine whether this immunization protocol induced protection. The data depicted in Fig. 5 indicate that only administration of Y-HSP60 plus IL-12 induced significant protection whereas immunization with Y-HSP60 or injection with IL-12 alone did not confer protection. Hence, bacterial counts in spleens were reduced ~100-fold in the former group only ($P < 0.05$). Comparable results were obtained after i.p. immunization (data not shown). These results show that IL-12 can be used as an alternative adjuvant to induce Y-HSP60-specific protective immune responses against *Y. enterocolitica* in mice.

DISCUSSION

In the past decade, efforts have been made toward the development of subunit vaccines. The rational basis of such vaccines is to include only proteins or peptides which efficiently elicit protective immune responses and exclude components of the antigen that account for deleterious immune responses. The limited antigenic composition of a subunit vaccine, however, might be inefficient in individuals with certain major histocompatibility complex MHC haplotypes according to the MHC restriction of peptide presentation for T cells (52).

Since microbial HSP have been recognized as immunodominant antigens for the host response, a controversial discussion about their significance as vaccines arose for two major reasons. First, HSP-specific immune responses may induce autoimmune diseases by generating immune responses against shared epitopes in the conserved HSP sequences and hence cross-reactions between foreign (microbial) and self (endogenous) HSP (23, 27, 29, 32, 33, 63). Second, on the other hand, there is evidence that because of their chaperone properties, HSP themselves may have immunostimulating properties (9). For instance, HSP may act as carrier molecules for other antigens (55), accelerate the formation of compact class II molecules (15), and channel exogenous antigens into the endogenous pathway (55). Furthermore, there is some evidence that

bacterial HSP might directly induce cytokine production in macrophages (50). Hence, HSP may principally contribute to the induction of efficient immune responses.

There is experimental evidence for both of the above-mentioned aspects. First, CD8 T cells reactive with bacterial HSP lyse stressed macrophages which present endogenous HSP at their cell surface (36), and autoimmune diabetes can be elicited by immunization with HSP peptides (17). Second, on the other hand, recent studies have demonstrated that immune responses against microbial HSP mediate protection against infection (49, 54).

In the experimental *Y. enterocolitica* mouse infection model, Y-HSP60 was identified as the dominant antigen for T cells which mediate immunity against yersiniae in adoptive transfer experiments (49). Therefore, we have used Y-HSP60 as a model antigen for vaccination studies. As $CD4^+$ $\alpha\beta$-TCR^+ gamma interferon-producing T cells are the crucial protective component against *Yersinia* infections (7, 49), a vaccine against *Y. enterocolitica* should induce Th1 responses. Keeping this prerequisite in mind, we selected two approaches for the design of a Y-HSP60 vaccine. First, Y-HSP60–ISCOM were produced. ISCOM, which were first described by Morein et al. (44), are multimeric three-dimensional cage-like structures with a mean diameter of 30 to 40 nm (44). ISCOM have been applied mainly to membrane proteins, because it is difficult to introduce cytoplasmic proteins such as HSP into ISCOM (42). Immunization with ISCOM induces a broad range of immunological responses involving both B and T cells (43). They direct antigens into the major histocompatibility complex class I pathway and thereby induce cytotoxic T-lymphocyte responses (25, 46). Moreover, ISCOM particles induce strong antibody responses, and at least in some models, ISCOM induce mucosal immune responses upon oral or nasal administration (45, 46).

To date, ISCOM have been used only for parasitic and viral antigen preparations, which, after immunization, induced protective immune responses (40). This report is the first to demonstrate that ISCOM can induce immunity against bacterial pathogens, even when applied to cytoplasmic proteins. Thus far, however, it is not clear why nasal or oral administration of Y-HSP60–ISCOM failed to induce systemic immune responses. Whether this is due to an inappropriate immunization protocol (e.g., larger amounts may be required for oral application) or to the immunological events operating in the mucosa-associated lymphoid tissue upon exposure to Y-HSP60 remains to be elucidated. The latter are currently being investigated at our laboratory. On the other hand, we cannot completely exclude that the Y-HSP60 and ISCOM matrix might be in separate particles and that the ISCOM matrix without antigen induced an adjuvant effect, although the small amounts of Y-HSP60–ISCOM required for protective immunity may argue against this possibility.

IL-12 is produced by macrophages and B cells upon stimulation with microorganisms or their products (16, 28, 51). IL-12 promotes growth and cytolytic activity of cytotoxic T lymphocytes and NK cells and stimulates gamma interferon production (20, 21, 35). IL-12 enhances Th1 responses in *Yersinia* infections and, when administered during the acute phase of *Yersinia* infection, renders *Yersinia*-susceptible BALB/c mice resistant to this pathogen (10). Beside its potential therapeutic effect, recent studies suggested that IL-12 can be used as an adjuvant (19, 22). Hence, IL-12 is an essential component of a (subunit) vaccine against *Leishmania major* (56), *Listeria monocytogenes* (60), *Toxoplasma gondii* (22), and *Mycobacterium tuberculosis* (19), all of which are intracellular pathogens. The present study demonstrates that IL-12 can be used as an adjuvant to promote protective immune responses against enteropathogenic bacteria by using a single protein for immunization. However, we believe that ISCOM might act better than IL-12 as adjuvant in our model.

Even highly *Yersinia*-susceptible BALB/c mice could be protected against a normally lethal *Yersinia* challenge when IL-12 was coadministered with Y-HSP60. Previous studies demonstrated that the susceptibility of BALB/c mice for *Y. enterocolitica* is due to the inability of these mice to generate a rapid and efficient *Yersinia*-specific Th1 response (2, 10). Therefore, we assume that the protective effect induced by Y-HSP60 plus IL-12 is due to the promotion of a Y-HSP60-specific Th1 response in BALB/c mice.

Two further studies which demonstrate that microbial HSP can be used as a protective vaccine without inducing deleterious immune responses have been published. In the first, immunization with *Helicobacter pylori* GroES homolog (small ~10-kDa HSP family) and GroEL-like proteins conferred protection against mucosal infection in mice (18). In this model, IgG1 antibodies are believed to represent the protective component of the HSP-specific immune response because *H. pylori* colonizes the mucosal surface rather than invading the tissues of the host. In an experimental *M. tuberculosis* infection, immunization of mice with J774.G8 cells transfected with HSP65 resulted in presentation of HSP65 fragment by both major histocompatibility complex class I and II molecules (53). Protection induced in the latter model, however, was most efficiently transferred by $\alpha\beta$ TCR^+ $CD4^-$ $CD8^+$ cytotoxic T cells (54). This is in contrast to the observations in the *Yersinia* mouse model, in which CD4 Th1 cells represent the most important cell type that confers immunity (49), and reflects the different habitats of the two pathogens. While *M. tuberculosis* is intracellularly located, *Y. enterocolitica* is a predominantly extracellular pathogen (24). *Y. enterocolitica*, however, evades innate host defense mechanisms such as phagocytosis and complement by expression of certain virulence factors such as YadA and Yops (13, 26). Therefore, a Th1-promoted host response is required to overcome *Yersinia* infection.

Taken together, our results demonstrate that vaccination with Y-HSP60 in ISCOM or plus IL-12 induces immunity against *Y. enterocolitica*. However, more detailed studies are now required to fully establish the immunological events involved in these protection mechanisms.

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