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# Protection against Murine Listeriosis by an Attenuated Recombinant *Salmonella typhimurium* Vaccine Strain That Secretes the Naturally Somatic Antigen Superoxide Dismutase

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**A recombinant (r)-*Salmonella typhimurium aroA* vaccine strain was constructed which secretes the naturally somatic protein of *Listeria monocytogenes*, superoxide dismutase (SOD), by the HlyB/HlyD/TolC export machinery. Vaccine efficacy of the SOD-bearing carrier strain was compared with that of the p60-secreting construct, *S. typhimurium* p60s (J. Hess, I. Gentschev, D. Miko, M. Welzel, C. Ladel, W. Goebel, and S. H. E. Kaufmann, Proc. Natl. Acad. Sci. USA 93:1458–1463, 1996). Vaccination of mice with both constructs induced protection against a lethal challenge with the intracellular pathogen, *L. monocytogenes*. While the somatic listerial antigen, SOD, is immunologically uncharacterized, the naturally secreted protein of *L. monocytogenes*, p60, is known to be highly immunogenic. Our data emphasize the high vaccine potential of r-*Salmonella* constructs secreting antigens of somatic or secreted origin. Moreover, they suggest that the HlyB/HlyD/TolC-based antigen delivery system with attenuated *Salmonella* spp. as the carrier is capable of potentiating the immune response against foreign proteins independent from their immunogenicity in and display by the natural host.**

Intracellular bacteria including *Salmonella* spp., *Listeria monocytogenes*, and *Mycobacterium tuberculosis*, the causative agents of salmonellosis, listeriosis, and tuberculosis, respectively, are controlled by T-cell-mediated immune mechanisms (21). Satisfactory control of these pathogens would be best achieved by vaccines which efficiently stimulate protective T cells. Frequently, however, antigens of intracellular microbial pathogens are insufficiently presented to T lymphocytes at critical stages of disease (26). Factors which influence the protective strength of an immune response against a microbial protein include the following: antigen display by the microbe (somatic or secreted), subcellular localization of the antigen in the host cell (cytosolic or endosomal), abundance and rate of antigen degradation, efficacy of antigen processing, and presence of appropriate amino acid motifs in the protein sequence for allele-specific major histocompatibility complex (MHC) class I presentation. Due to this complexity, a general strategy for development of vaccines against intracellular pathogens is not available to date. Evidence that secreted proteins are preferred antigen candidates for vaccines against intracellular bacteria is, however, accumulating (3, 16, 17, 25). Some intracellular pathogens have developed mechanisms which result in rapid escape from the phagosome of host cells, while others partially block phagolysosomal killing (26). These evasion mechanisms limit delivery of somatic bacterial antigens to MHC processing (26). In contrast, proteins secreted by intracellular microorganisms are available for T-cell recognition from the early stages of infection onwards (26).

The unique property of *L. monocytogenes* to escape from the phagosome by pore formation and to grow subsequently in the less hostile environment of the host cytosol is responsible for the strong dependency on CD8 T cells for eradication of *L. monocytogenes* (1). In the host cell cytosol, two secreted listerial antigens are highly abundant. These are the listeriolysin (Hly) and p60 antigens (32, 35, 40, 41). Both antigens are efficiently presented by MHC class I molecules to protective CD8 T cells. Hly is a SH-dependent cytolysin which is responsible for listerial egression from the endosome into the cytosol (11). The p60 protein represents a bifunctional protein which contributes to listerial invasion and exhibits murein hydrolase activity necessary for bacterial septation (19, 27, 46). We have utilized p60 and Hly for studying the influence of antigen display on vaccine efficacy. We found that p60 or Hly antigen expressed by recombinant *Salmonella typhimurium* (r-*S. typhimurium*) strains induced protection against listeriosis only in secreted and not in somatic form (22). Immunized animals were protected independently from the putative localization of the r-*Salmonella* vaccine strain in the cytosol or phagolysosome of the host cell (21). These findings suggested superior vaccine efficacy of secreted over somatic antigen display. However, because both Hly and p60 are secreted by their natural host, *L. monocytogenes*, it remained to be determined whether naturally somatic proteins are also protective when displayed as secreted antigens by the vaccine. In an attempt to answer this question, we chose as antigen superoxide dismutase (SOD) of *L. monocytogenes* (4). This manganese-associated enzyme converts superoxide into hydrogen peroxide and hence may contribute to listerial resistance against attack by reactive oxygen intermediates (26). To assess whether the HlyB/HlyD/TolC-mediated secretion of the naturally somatic SOD protein by r-*S. typhimurium* is capable of inducing protective immunity against *L. monocytogenes* infection, we constructed the r-*S. typhimurium* strain SODs, which secretes the SOD-hemolysin (HlyA) fusion protein.

Our experiments reveal that the SOD-secreting r-*S. typhimurium* vaccine strain protects against listeriosis. These data demonstrate that an antigen secreted by a heterologous r-vaccine can induce protective immunity against the homologous pathogen which displays the same antigen in somatic

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TABLE 1. Antigen expression by the r-*S. typhimurium aroA* strains used for vaccination against *L. monocytogenes* EGD

| *S. typhimurium* SL7207 strain | Plasmid | Listerial antigen display |
|---|---|---|
| Control | pMOhly1 | |
| p60s | pIPH-1 | Secreted-somatic |
| SODs | pGD4Sod | Secreted-somatic |
| SODc | pGD40Sod | Somatic |

form. These findings extend our hypothesis that antigen secretion by this vaccine is of major importance for protective efficacy. As a corollary, it is conceivable that as-yet-uncharacterized protein antigens of somatic or secreted origin can induce protective immunity against listeriosis when secreted by r-*Salmonella* strains.

## MATERIALS AND METHODS

**Mice.** C57BL/6 mice were kept under specific-pathogen-free conditions in isolators and fed autoclaved food and water ad libitum at the central animal facilities of the University of Ulm. In a given experiment, mice were age and sex matched.

**Bacteria and cells.** *L. monocytogenes* Sv1/2a EGD was originally obtained from G. B. Mackaness. Listeriae were cultured in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.) at 37°C with aeration. The *S. typhimurium* SL7207 strain [2337-65 (WRAY) *hisG46* Δ*aroA407* (Tc$^s$ *aroA544*::Tn*10*)] was kindly provided by B. Stocker (Department of Medical Microbiology, Stanford University, Palo Alto, Calif.) (24). The r-*S. typhimurium* strains pMOhly1 (termed control) and p60s have been described previously (Table 1) (19, 22). The *hlyA*$_s$-specific open reading frame upstream of the *Nsi*I site of plasmid pMOhly1 (13) differs from the originally used pMOhly6. Instead of 34 amino acids at the N-terminal end of the fusion proteins, pMOhly6 codes for only 4 amino acids.

**Construction of recombinant plasmids pGD4Sod and pGD40Sod.** A vector system for direct cloning of the PCR-amplified heterologous genes in frame to the last 183 bp of the *Escherichia coli* hemolysin gene (*hlyA*$_s$) was developed. This 3′ part of *hlyA*$_s$ encodes the C-terminal 61-amino-acid secretion signal of *E. coli* hemolysin (HlyA$_s$) which is recognized by the HlyB/HlyD/TolC secretion machinery required for HlyA protein export in *Salmonella* spp. and *E. coli*. Into the single *Nsi*I site of plasmid pMOhly6, an *Srf*I linker consisting of the oligonucleotide *Srf*I 5′-GCCCGGGCTGCA-3′ (underlined sequence indicates the *Srf*I recognition site) was inserted. The *Cla*I-*Bgl*II fragment of the resulting plasmid pMO6SrfI was inserted in *Acc*I- and *Bam*HI-digested pUC18. The resulting plasmid, pGD4, was used for direct cloning of PCR products. The *lmsod* gene of *L. monocytogenes* Sv1/2a EGD was amplified by PCR with primers G1 (5′-TA ATGACTTACGAATTACCTA-3′) and G2 (5′-TAGCTGCGTCAAAGCGTT TGT-3′). PCR was carried out in a Thermal Cycler 60 (Bio-Med, Theres, Germany) for 30 cycles at 94°C for 1 min, 45°C for 45 s, and 72°C for 1 min. The 0.6-kb amplicon, encoding the *lmsod* gene, was gel purified with the Geneclean Kit (Bio 101, Inc., La Jolla, Calif.) and cloned into the *Srf*I site of pGD4. This led to plasmid pGD4Sod, which carries the *lmsod-hlyA*$_s$ gene fusion and the functional *hlyB* and *hlyD* genes required for secretion of the fusion protein. To inactivate the transport function of the *hlyD* gene, pGD4Sod was digested with *Apa*I, treated with mung bean nuclease, and religated. The resulting plasmid pGD40Sod encodes a truncated *hlyD* gene which cannot mediate the transport function and is therefore secretion deficient for the *lmsod-hlyA* gene product. The plasmids pGD4Sod and pGD40Sod were isolated from *E. coli* JM109, analyzed, and electroporated in *S. typhimurium* LB5000, a restriction-negative and modification-proficient strain, by a standard protocol for *E. coli* (Bio-Rad, Richmond, Calif.). Subsequently, plasmids purified from ampicillin-resistant r-*S. typhimurium* LB5000 colonies were introduced into *S. typhimurium* SL7207 by electroporation. The resulting r-*S. typhimurium* strains, SODs and SODc, harboring plasmids pGD4Sod and pGD40Sod (Table 1), respectively, were grown in 2× yeast tryptone medium supplemented with 100 μg of ampicillin (Sigma, Deisenhofen, Germany) per ml and 10 μg each of 2,3-dihydroxybenzoic acid (Sigma) and *p*-aminobenzoic acid (Sigma) per ml at 37°C. They showed similar growth rates in broth and on agar.

**Characterization of r-*S. typhimurium* SODs and SODc.** Bacteria of strains SODs and SODc were grown in 2× yeast tryptone medium and BHI broth, respectively, to a cell density of $5 \times 10^8$ cells per ml. Culture supernatant proteins were precipitated with trichloroacetic acid (final concentration, 7%) on ice for 1 h, washed with acetone, dissolved in Laemmli sample buffer, and heated at 95°C for 5 min (29). For whole-cell lysates, the bacteria were centrifuged, redissolved in Laemmli buffer, and treated like supernatant proteins. *L. monocytogenes* bacteria were treated with 10 U of mutanolysin (Sigma) per ml at 37°C for 15 min prior to the addition of Laemmli buffer. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 12% acrylamide gels as described by Laemmli (29). Gels were stained with Coomassie brilliant blue R-250. Proteins were transferred to nitrocellulose membranes as described previously (37), incubated with polyclonal anti-SOD antibodies, and visualized by the horseradish peroxidase reaction with 0.015% hydrogen peroxide and 4-chloro-1-naphthol.

**Plasmid stability in vivo.** Previous studies on in vivo plasmid stability revealed that the r-*S. typhimurium* control strain and p60s still harbored their plasmids pMOhly1 and pIPH-1, respectively, by day 21 postinfection (p.i.) (19). The plasmids pGD4Sod and pGD40Sod of *S. typhimurium* SODs and SODc, respectively, were equally maintained in vivo (23).

**Vaccination of mice.** Five C57BL/6 mice per group were immunized intravenously (i.v.) with $5 \times 10^5$ r-*S. typhimurium* SODs, SODc, p60s, or control bacteria (Table 1). After 100 days, these mice were challenge infected i.v. with $10^5$ (10× 50% lethal dose [LD$_{50}$]) of *L. monocytogenes* EGD. Ten days after challenge, vaccinated survivors were sacrificed and the numbers of CFU of remaining *L. monocytogenes* bacteria were determined by plating serial dilutions of spleen or liver homogenates on PALCAM-*Listeria* sp. selective agar (Merck, Darmstadt, Germany) and on brilliant green *Salmonella* sp. selective agar (Difco). We considered the possibility that persistent r-*S. typhimurium* bacteria could be reactivated in response to the listerial challenge. However, we could not detect any *Salmonella* sp. microorganisms in the liver or spleen by plating organ homogenates on selective agar (23).

**Antigen preparation.** Viable bacterial counts of *L. monocytogenes* EGD samples were determined, and bacteria were subsequently killed by heat treatment at 70°C for 1 h (heat-killed listeriae [HKL]). *S. typhimurium* SODs, p60s, or control microorganisms were cultured to an $A_{600}$ of 1.2 in supplemented medium as described above. Single supernatants were harvested by centrifugation at 780 × *g*, passed through sterile 0.2-μm-pore-size filters (Sartorius, Göttingen, Germany), and enriched fivefold by microconcentrators (Centricon 10; Amicon Division, Beverly, Mass.).

**T-cell proliferation assay.** T cells were enriched from spleens of infected C57BL/6 mice 6 weeks p.i. as described previously (6). Antigen-presenting cells (APC) were prepared from naive, syngeneic mice and irradiated with 3,000 rad. Cell concentrations were separately adjusted to $1 \times 10^6$ T cells and $3 \times 10^6$ APC per ml. T cells (100 μl), APC (100 μl), and 25 μl of each antigen preparation were cultured in the presence of 200 μg of gentamicin per ml to kill the remaining *S. typhimurium aroA* microorganisms. T cells were stimulated for 2 days (0.5 μg of concanavalin A per well) or 5 days (antigen preparations). [$^3$H]thymidine (0.5 μCi; Amersham-Buchler, Braunschweig, Germany) was added to each well, and the plates were frozen at −20°C 6 h later. Cells were thawed and harvested onto glass fiber filter mats, and radioactivity was determined in a Betaplate counter (Skatron, Lier, Norway). Data are presented as a stimulation index equal to the [$^3$H]thymidine uptake in antigen-containing samples divided by the [$^3$H]thymidine uptake in the medium control.

**Stabilization assay of H-2K$^b$ molecules by peptides on RMA-S cells.** Previous reports have focused on immunodominant epitopes of the p60 or Hly protein which are efficiently presented by H-2K$^d$ MHC class I molecules (35, 40, 41). CD8 T cells specific for the p60 protein epitope at amino acids 217 to 225 ($p60_{217–225}$) or the $Hly_{91–99}$ epitope suffice for adoptively transferring protective immunity to naive BALB/c mice (16, 17). The amino acid sequences of these highly immunogenic epitopes of *L. monocytogenes* correspond to H-2K$^d$ peptide binding motifs (9). Similarly, we identified a $SOD_{8–15}$ peptide motif which matches the consensus sequence for H-2K$^b$ binding (9). The binding of the $SOD_{8–15}$ peptide to H-2K$^b$ molecules was studied with cells of the transporter mutant cell line RMA-S. Cells were cultured overnight at 26°C (30, 36), after which a 100 μM concentration of peptides of $SOD_{8–15}$, i.e., LPYTYDAL, or of chicken ovalbumin ($OVA_{257–264}$), i.e., SIINFEKL, as a control, was added and the cells were incubated for an additional 30 min at 26°C. After three washings with ice-cold phosphate-buffered saline, $10^5$ cells were collected. Subsequently, the peptide-pulsed cells were subjected to 37°C and aliquots of $10^5$ cells were collected at intervals of 20 min. The decay of H-2K$^b$ molecules was monitored by labeling the cells with fluorescein isothiocyanate-conjugated monoclonal antibody (MAb) specific for H-2K$^b$ (AF6-88.5; Pharmingen, Hamburg, Germany). Stained cells were analyzed with a FACScan (Becton Dickinson, Heidelberg, Germany) at 5,000 cells per sample.

## RESULTS

**Construction and characterization of r-*S. typhimurium* displaying SOD from *L. monocytogenes* in secreted or somatic form.** The *S. typhimurium* SL7207 strain was used as an antigen delivery system for the somatic SOD protein of *L. monocytogenes* EGD (4) as was previously described for the naturally secreted listerial p60 protein (19). Analogous to the p60-secreting *S. typhimurium* p60s vaccine strain (19, 22), *S. typhimurium* SODs microorganisms were constructed which harbored the r-pUC18-based plasmid pGD4Sod carrying the *lmsod-hlyA* gene fusion of *L. monocytogenes* EGD and of the *E. coli* pHly152 plasmid-encoded hemolysin (HlyA) transport sig-

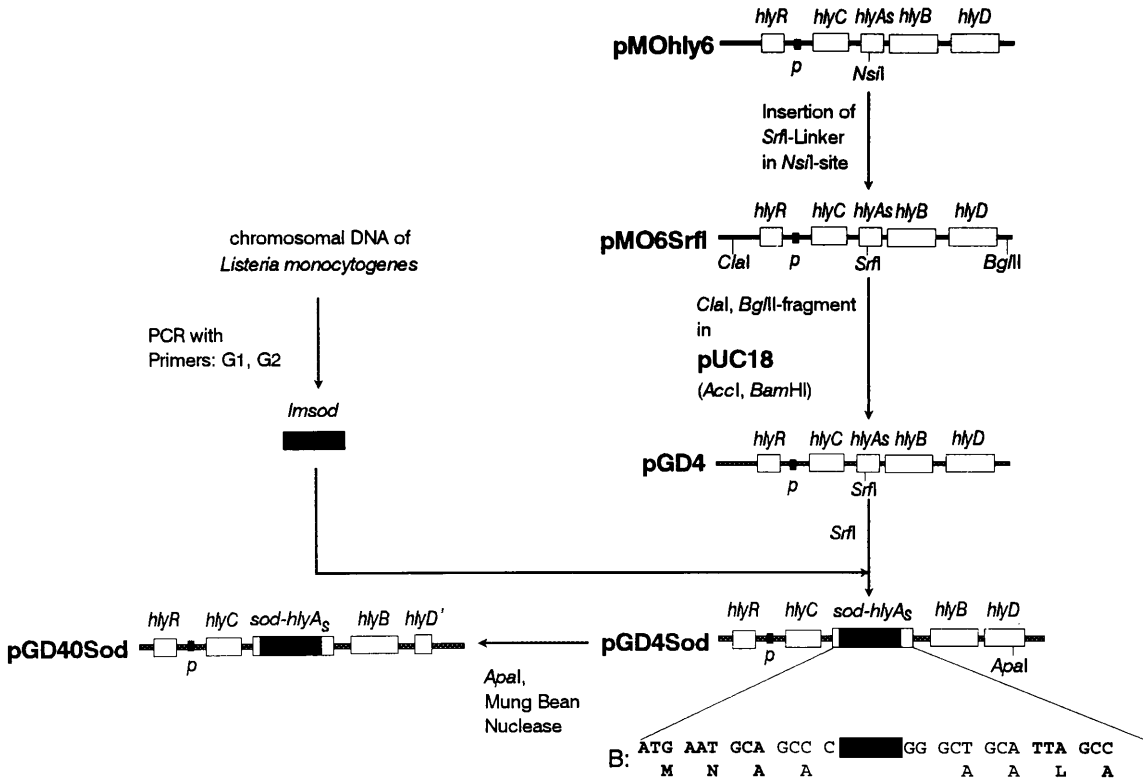


FIG. 1. Construction of recombinant plasmids pGD4Sod and pGD40Sod. The *lmsod* gene of *L. monocytogenes* EGD is represented by a black box; the genes and sequences (*hlyR*, *hlyC*, *hlyAs*, *hlyB*, and *hlyD*) necessary for hemolysin expression in *E. coli* and *S. typhimurium* SL7207 are represented by open boxes. Abbreviation: p, promoter. B: designates the $hlyA_s$-derived amino acid sequence (in boldface type) and unrelated amino acid sequence derived from the *Srf*I linker (in light face type).

nal (Fig. 1). The HlyA-derived signal peptide directs transport of the expressed SOD-$HlyA_s$ fusion protein across the bacterial cell wall of *E. coli* and *Salmonella* spp. This HlyA transport signal interacts with the inner membrane proteins HlyB and HlyD encoded by the same plasmid (15, 42). The chromosomally encoded membrane protein TolC represents an additional component of the HlyA export complex (43). At the N-terminal end, the hybrid protein SOD-$HlyA_s$ contains four unrelated amino acids, and at the carboxy terminus, it contains 62 amino acids representing the transport signal of *E. coli* hemolysin (HlyA) (18). The pGD4Sod-derived plasmid pGD40Sod exclusively directs somatic expression of the SOD-$HlyA_s$ fusion because the *hlyD* gene was partly deleted (22) and thus inhibited secretion of the hybrid protein by *S. typhimurium* SODc (Fig. 1). Large amounts of SOD-$HlyA_s$ fusion protein of 30 kDa were detected in supernatants of *S. typhimurium* SODs by immunostaining with a specific anti-SOD antiserum (Fig. 2). During logarithmic growth, *S. typhimurium* SODs secreted 80 to 90% of the expressed SOD-$HlyA_s$ fusion protein into the supernatant by means of the HlyB/HlyD/TolC export machinery. In contrast, SOD-$HlyA_s$ chimeric antigens were not secreted into the supernatants of *S. typhimurium* SODc (Fig. 2B). After equalizing bacterial counts, the amounts of the somatically expressed SOD-$HlyA_s$ fusions by *S. typhimurium* SODs

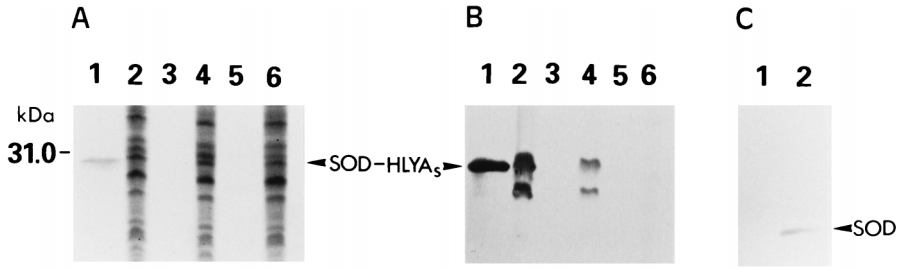


FIG. 2. Identification of the SOD-$HlyA_s$ fusion protein by immunostaining. Cultures of *S. typhimurium* SL7207 carrying the plasmids pGD4Sod (lanes 1 and 2), pGD40Sod (lanes 3 and 4), and pMOhly1 (lanes 5 and 6) were grown in 2× yeast tryptone medium to a cell density of $5 \times 10^8$ cells per ml. Supernatant proteins from 1 ml of bacterial culture were loaded in lanes 1, 3, and 5; cellular proteins from $5 \times 10^7$ cells were loaded in lanes 2, 4, and 6. (A) Coomassie brilliant blue R-250-stained acrylamide gel. (B) Immunoblot of proteins developed with polyclonal anti-SOD antibodies. Samples were prepared and analyzed as described in Materials and Methods. The position of the SOD-$HlyA_s$ fusion protein is indicated by arrowheads. The SOD-$HlyA_s$ fusion protein could also be detected with anti-$HlyA_s$ antiserum (data not shown). (C) Detection of SOD in *L. monocytogenes* by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis *L. monocytogenes* Sv1/2a EGD was grown in BHI broth to a cell density of $5 \times 10^8$ cells per ml. Supernatant proteins from 1 ml of bacterial culture were loaded in lane 1; cellular proteins from 0.1 ml of culture were loaded in lane 2. An immunoblot of proteins was developed with polyclonal anti-SOD antibodies.

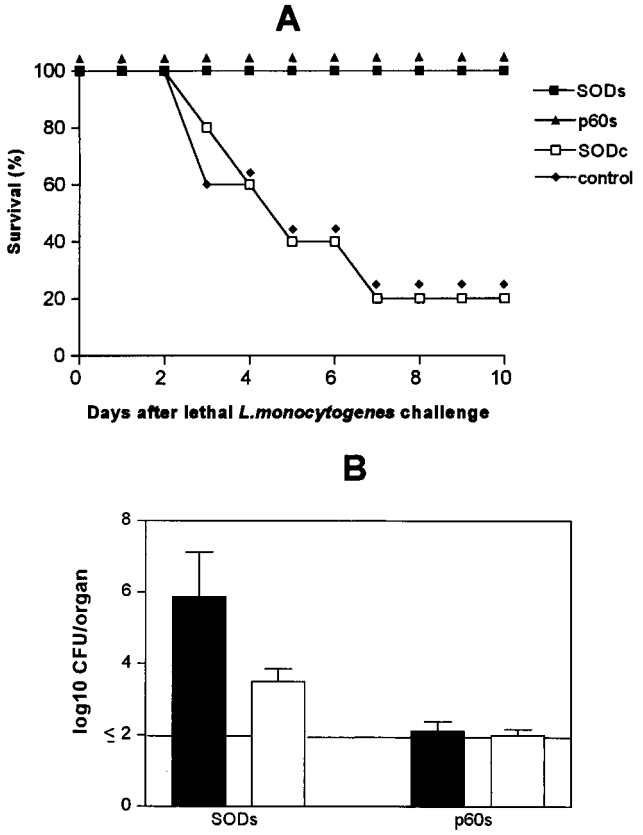


FIG. 3. Protection against *L. monocytogenes* after lethal challenge of C57BL/6 mice vaccinated with r-*S. typhimurium* SODs, p60s, or control. (A) Survival curves of five mice per group immunized i.v. 100 days previously with r-*S. typhimurium* SODs, p60s, SODc, or control after lethal *L. monocytogenes* EGD challenge (10× $LD_{50}$). Mortality was recorded daily. (B) CFU in spleens (■) and livers (□) of survivors at day 10 after *L. monocytogenes* challenge in mice which had been immunized i.v. 100 days previously with r-*S. typhimurium* SODs or p60s. Each column represents the geometric mean of five animals per group. Vertical bars indicate the standard deviations of the geometric means.

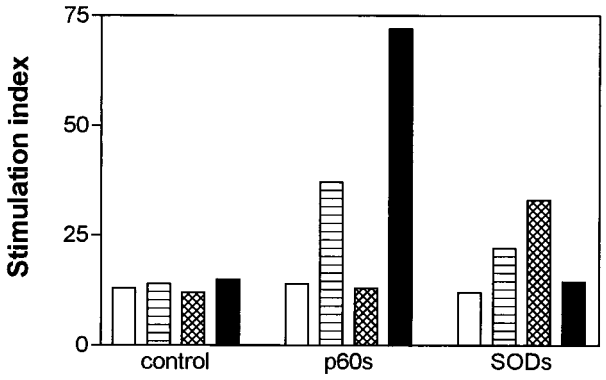


FIG. 4. Proliferative response of T cells from mice immunized with r-*S. typhimurium* control, p60s, or SODs ($5 \times 10^5$ bacteria given i.v.). Data are represented as a stimulation index (mean of triplicate antigen-pulsed cultures of pooled cells from three mice after dividing the background value of the same cultured cells in the presence of medium). The antigens of *S. typhimurium* control supernatant (□), $10^7$ HKL (▤), SODs supernatant (▨), or p60s supernatant (■) were prepared as described in Materials and Methods. The results are representative of two individual experiments. Medium controls: *S. typhimurium* control, 280 cpm; p60s, 155 cpm; SODs, 346 cpm.

and SODc were not comparable due to the weak expression by SODc microorganisms (Fig. 2B). We assume that the instability of the retained SOD-$HlyA_s$ protein depends on the nonfunctional conformation of the HlyB/HlyD/TolC pore. Therefore, *S. typhimurium* SODs represented the only useful carrier for further in vivo studies and direct comparison of its vaccine potential with that of *S. typhimurium* p60s.

**Vaccine efficacy of *S. typhimurium* SODs and p60s against listeriosis.** C57BL/6 mice were vaccinated i.v. with r-*S. typhimurium* p60s, SODs, SODc, or control constructs (Table 1) and then challenge infected with a lethal inoculum of *L. monocytogenes* (10× $LD_{50}$). In vivo stability of the appropriate plasmids harbored by the r-carrier strains were comparable (data not shown). Vaccination with r-*S. typhimurium* p60s and SODs displaying the two listerial proteins in secreted form rendered mice fully protected (Fig. 3A). In contrast, 80% *S. typhimurium* control- and SODc-immunized mice succumbed to this high challenge inoculum (Fig. 3A). To assess whether SODs- and p60s-vaccinated mice had been able to achieve sterile clearance of listeriosis, CFU numbers in spleens and livers of survivors were determined at day 10 after challenge infection (Fig. 3B). Although neither vaccination regime resulted in sterile clearance at day 10 p.i., mice vaccinated with r-*S. typhimurium* p60s controlled listeriosis more efficiently than animals vaccinated with r-*S. typhimurium* SODs. However, we conclude that both p60 and SOD proteins secreted by viable r-carriers induced protective immunity because vaccination regimes had protected mice against lethal listeriosis. Thus, a protein naturally displayed in somatic form possesses protective activity when secreted by an r-vaccine. In contrast, SOD-$HlyA_s$ fusion protein retained in the bacterial cytoplasm did not induce protection against *L. monocytogenes* infection (Fig. 3A).

**Antigen-specific T-cell responses in vitro.** To compare the T-cell responses induced by the two vaccine strains, C57BL/6 mice were vaccinated by a single i.v. administration of r-*S. typhimurium* control strain, SODs, or p60s. At 6 weeks p.i., spleens were removed, and T cells were enriched and cultured in vitro with antigen-pulsed syngeneic spleen cells. Fivefold-concentrated supernatants of (i) r-*S. typhimurium* control, (ii) SODs containing SOD-$HlyA_s$ fusion protein, and (iii) p60s containing p60-$HlyA_s$ chimeric antigen were used as antigen preparations. HKL were used as a positive control. *S. typhimurium* SL7207-induced T cells showed low responses against *S. typhimurium* control supernatant, suggesting that *Salmonella* antigens were weak T-cell stimulators. Cells from mice immunized with r-*S. typhimurium* p60s or SODs showed proliferative responses to the homologous antigen preparation which were significantly higher than responses of T cells from control immune mice (Fig. 4). SOD-$HlyA_s$-specific proliferative responses of T cells from *S. typhimurium* SODs-immune mice were lower than responses of p60-immune T lymphocytes to p60-$HlyA_s$.

We conclude that *S. typhimurium* secreting SOD-$HlyA_s$ or p60-$HlyA_s$ protein induced specific T-cell responses to the homologous antigen. Although our findings suggest significant cross-reactivity between the applied antigen preparations, a stronger antigen-specific response was elicited by SOD-$HlyA_s$ and p60-$HlyA_s$.

**H-2K$^b$ binding of $SOD_{8-15}$ peptide.** Protective immunity against *L. monocytogenes* infection mainly requires CD8 T cells (26). In contrast, protection against the *S. typhimurium aroA* carrier strain is primarily CD4 T-lymphocyte dependent (20). These differences probably reflect a cytosolic rather than a phagolysosomal habitat of *L. monocytogenes* or *S. typhimurium* in APC, respectively. Recent adoptive transfer experiments suggest that protective CD8 T cells were induced by r-*S. typhi-*

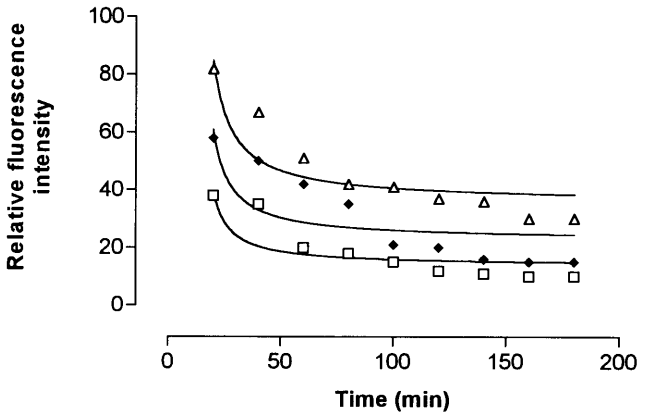


FIG. 5. Kinetic analysis of the decay of surface-expressed H-2K$^b$ molecules of peptide-pulsed RMA-S cells. Empty H-2K$^b$ molecules were charged at 26°C with peptide $SOD_{8–15}$ (LPYTYDAL) (◆) or $OVA_{257–264}$ (SIINFEKL) (△) or without peptide (□). After washing away unbound peptides, the pulsed cells were exposed to 37°C, and $10^5$ cells were collected in 20-min intervals. Surface expression of H-2K$^b$ was revealed by the H-2K$^b$-specific, fluorescein isothiocyanate-conjugated MAb AF6-88.5. Values represent the percentages of relative fluorescence intensity as compared to starting levels. The hyberbolas are calculated by nonlinear regression of values by use of the one-binding-site parameter of GraphPad Prism (GraphPad Software, San Diego, Calif.).

*murium aroA* vaccine strains (22). We conclude from these findings that antigens secreted by r-*S. typhimurium* in the phagolysosome are introduced into the MHC class I pathway and presented to CD8 T cells. Successful CD8 T-cell activation depends on two major prerequisites, (i) introduction of peptides into the MHC class I presentation pathway and (ii) peptide binding to MHC class I molecules with sufficiently high avidity. We wondered whether SOD-derived peptides can be presented by MHC class I molecules in the mouse strain used in these studies (*H-2$^b$* haplotype). The SOD protein contains the epitope $SOD_{8–15}$, LPYTYDAL (amino acid residues for MHC I [H-2K$^b$] anchoring are underlined), which fulfills the criteria required for a dominant anchor motif and strong binding to H-2K$^b$ MHC class I gene products (9). To verify H-2K$^b$ binding of peptide $SOD_{8–15}$, we used the peptide transporter-deficient RMA-S cell line. Surface expression of MHC class I molecules by these mutant cells depends on stabilization by peptide binding (30). To determine stabilization by $SOD_{8–15}$ peptide and of $OVA_{257–264}$ control peptide, a kinetic assay which quantifies the decay of peptide-pulsed H-2K$^b$ molecules on the cell surface was employed (34). Surface expression of H-2K$^b$ was determined cytofluorimetrically by using the specific MAb AF6-88.5. Our experiments reveal that the $SOD_{8–15}$ peptide binds to H-2K$^b$ molecules. Although the stability of the $SOD_{8–15}$ peptide-MHC class I complex was less stable than the known H-2K$^b$ peptide $OVA_{257–264}$ with high binding activity (Fig. 5), the avidity of $SOD_{8–15}$ peptide to H-2K$^b$ molecules was sufficiently high. This peptide, therefore, is a likely candidate epitope of *L. monocytogenes*-reactive CD8 T cells induced by r-*S. typhimurium* SODs in C57BL/6 mice (*H-2$^b$* haplotype).

## DISCUSSION

Our report describes protection against the intracellular bacterial pathogen *L. monocytogenes* by immunization of mice with an r-*S. typhimurium* vaccine strain secreting the naturally somatic antigen SOD. In contrast, the SOD protein which was exclusively retained in the cytoplasm of the r-*S. typhimurium* SODc construct did not induce protective immunity against lethal listerial infection. Our data reveal that somatic localization of SOD in the natural microbial host *L. monocytogenes* does not impair successful vaccination. This finding is consistent with the assumption that antigen display by the pathogen in secreted or somatic form has a minor impact on the protective potential of a given antigen. On the contrary, our findings are consistent with the hypothesis that antigen secretion by the r-*Salmonella* vaccine strain is central to vaccine efficacy even for a naturally somatic antigen. Moreover, the data presented here confirm our previous finding that residence of r-*S. typhimurium* strains in the phagosome of APC is sufficient for induction of protective immunity against listeriosis (22). These data emphasize that a *Salmonella* sp. vaccine carrier can induce efficient protection against *L. monocytogenes* infection. In this regard, it may be noteworthy that salmonellosis is primarily controlled by CD4 T cells (20), whereas listeriosis is controlled by CD4 and CD8 T cells (28).

The r-*S. typhimurium* strain combined with the plasmid-encoded HlyB/HlyD/TolC secretion system of hemolytic *E. coli* isolates has been previously described as a successful antigen carrier for vaccination against murine listeriosis (22). In our previous study, the naturally secreted proteins p60 and Hly of *L. monocytogenes* were delivered in secreted form by r-*Salmonella* constructs for vaccination. The versatile secretion system in r-*Salmonella* spp. not only allows export of naturally secreted proteins into the extracellular environment but also can be used for export of proteins which are membrane or soma localized in the natural microbial host (14, 31). This feature of the HlyB/HlyD/TolC export machinery has important implications for vaccine design because it allows secretion of nearly any protein independent from its natural form of display. Consequently, we extended our analyses and constructed r-*S. typhimurium* vaccine strains which express the somatic listerial MnSOD protein in two different bacterial compartments (somatic and secreted).

Although SOD expression of *L. monocytogenes* was shown to depend on different growth conditions (39, 44), a clear correlation between different SOD activities and virulence among certain *L. monocytogenes* strains was not found (45). However, inactivation of the MnSOD-coding gene *lmsod* of *L. monocytogenes* EGD impaired listerial survival in vivo. The course of infection by this listerial MnSOD mutant strain differs significantly from that of wild-type *L. monocytogenes* EGD as indicated by a decreased recovery of the MnSOD-deficient bacteria from murine organs (5). These data suggest that MnSOD may protect *L. monocytogenes* from oxidative damage. Thus, the possibility that MnSOD secreted by r-*S. typhimurium aroA* not only acted as an antigen but also interfered with nonspecific host resistance mechanisms cannot be formally excluded. However, because r-*S. typhimurium* SODs-immunized mice were challenged with *L. monocytogenes* 100 days after vaccination, at a time when sterile clearance of *S. typhimurium* had been achieved, we consider a major impact of biologically active MnSOD unlikely. The MnSOD protein of *L. monocytogenes* was selected as the vaccine antigen for the following reasons. First, analogous enzymes contribute to intracellular survival of *S. typhimurium* and *Shigella flexneri* (10, 38). Second, MnSOD increases resistance to oxidative stress of mucoid *Pseudomonas aeruginosa* strains in cystic fibrosis patients (33). Third, vaccination with purified somatic 28-kDa MnSOD of *Mycobacterium leprae* inhibited *M. leprae* multiplication in mouse footpads (12). Fourth, a recent report supported the importance of FeSOD or MnSOD as a major T-cell-inducing antigen of *Mycobacterium habana*, a candidate vaccine strain against leprosy and tuberculosis (2). Fifth, the 23-kDa SOD protein of *Mycobacterium tuberculosis* is one of the major released proteins of *M. tuberculosis* and is a protective antigen

(25). Taken together, these properties argue for MnSOD as a potential vaccine antigen.

The SOD-HlyA$_s$ fusion protein was efficiently secreted by r-*S. typhimurium* SODs. In contrast, SOD-HlyA$_s$ proteins were not detected in supernatants of the r-*S. typhimurium* SODc strain. Due to the genetically engineered truncation of the HlyD protein, the HlyB/HlyD/TolC transport pore was inactivated and therefore the SOD-HlyA$_s$ fusions were completely retained in the bacterial cytoplasm. Unexpectedly, the intracellular SOD-HlyA$_s$ protein pool of this strain was not comparable to the entire amount of the same fusion product in the r-*S. typhimurium* SODs strain. Since transcription of the *lmsod-hlyA*$_s$ gene fusion was unaffected by the absence of *hlyB*/*hlyD* genes (7), we assume that the expressed SOD-HlyA$_s$ protein was rapidly degraded in the absence of a functional HlyB/HlyD/TolC export machinery. Because normalization of these different SOD-HlyA$_s$ antigen doses was impossible, we focused on protective properties of the r-*S. typhimurium* SODs construct. It is noteworthy that immunization of mice with *S. typhimurium* SODc against lethal *L. monocytogenes* challenge failed to induce protective immunity, which underlines the superiority of antigens secreted by *Salmonella* spp.

Both the p60s and SODs vaccine strains induced specific T-cell responses against the homologous antigen. We assume that antigen-specific T cells were induced by both the r-*S. typhimurium* p60s and the SODs strains, but the accessibility of SOD and p60 proteins for MHC class I or class II presentation derived from the natural antigen carrier, *L. monocytogenes*, may differ during secondary listerial infection. The secreted antigen p60 is available to the immune system at the outset of listerial infection (40). In contrast, SOD is apparently not secreted by *L. monocytogenes* bacteria (Fig. 2C). No putative N-terminal signal peptide in the deduced amino acid sequence could be detected by computer analysis (4), which could suggest SOD release by viable *L. monocytogenes* bacteria inside APC. Therefore, during natural infection, presentation of somatic SOD should be delayed because it depends on prior killing and degradation of *L. monocytogenes*. It appears that this delay of somatic SOD antigen presentation is efficiently compensated by the prompt induction of T-cell-mediated immunity in primary infection of the *S. typhimurium* SODs carrier which secretes the SOD antigen. Although formal proof is still missing, we consider it likely that both CD4 and CD8 T lymphocytes were induced by *S. typhimurium* SODs because of the potent protection against listeriosis. An additional immune mechanism could contribute to host protection induced by *S. typhimurium* SODs. CD4 T cells primed by recognition of antigenic SOD fragments subsequent to degradation of killed *L. monocytogenes* could exert a bystander effect against host cells infected with live *L. monocytogenes*. It is possible that this bystander effect was elicited more efficiently by r-*S. typhimurium* SODs immunization.

Because CD8 T cells are considered important for antilisterial protection, it is gratifying that we could identify a peptide motif in the SOD sequence with high binding affinity for MHC class I on the H-2K$^b$ background (C57BL/6). Our future efforts are therefore directed at identifying SOD-specific CD8 T lymphocytes in *S. typhimurium* SODs-immunized mice. Our data emphasize the high vaccine potential of r-*Salmonella* constructs secreting antigens of somatic or secreted origin. Therefore, this system appears to be a suitable device for potent stimulation of protective T-cell responses even by proteins which have not been characterized immunologically. This powerful delivery system may even be useful for potentiating antigens which are weakly immunogenic in the natural pathogen and consequently may allow successful vaccination with otherwise insufficiently protective antigens.

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