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# Superior efficacy of secreted over somatic antigen display in recombinant *Salmonella* vaccine induced protection against listeriosis

**(antigen compartmentalization/aroA⁻ *Salmonella typhimurium*)**

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**ABSTRACT** **Vaccination provides the most potent measure against infectious disease, and recombinant (r) viable vaccines expressing defined pathogen-derived antigens represent powerful candidates for future vaccination strategies. In a new approach we constructed r-aroA⁻ *Salmonella typhimurium* displaying p60 or listeriolysin (Hly) antigen of *Listeria monocytogenes* in secreted or somatic form in the host cell. Vaccination of mice with r-aroA⁻ *S. typhimurium* induced protection against the intracellular pathogen *L. monocytogenes* only with secreted and not with somatic antigen. Secreted Hly was slightly more potent in inducing protective immunity than secreted p60. Both r-aroA⁻ *S. typhimurium* secreting p60 in the endosome and r-aroA⁻ *S. typhimurium* secreting Hly in the cytosol induced protective CD4⁺ and CD8⁺ T-cells suggesting CD8⁺ T-cell stimulation independent from intracellular residence of r-aroA⁻ *S. typhimurium* carriers. Hence, not only the type of antigen but also its display by the r-carrier within the host cell critically influences vaccine efficacy.**

Although efficacious vaccines exist for numerous viral and bacterial pathogens, several infectious agents of medical importance have evaded their satisfactory control by conventional vaccination strategies (1). Because efficient protection against intracellular pathogens crucially depends on T lymphocytes, administration of soluble proteins may be insufficient and live vaccines are considered essential (2). Expression of defined antigens from pathogens in recombinant (r)-form by live vectors provides an innovative strategy for inducing T-cell-mediated protection (3, 4). Identification of T-cell antigens and selection of appropriate carriers are generally accepted as necessary prerequisites for rational design of efficacious r-vaccines. In contrast, the impact on protective efficacy of (*i*) antigen compartmentalization in and (*ii*) intrahost cell localization of r-vaccine carriers has not been studied satisfactorily.

As long as intracellular microorganisms resist phagolysosomal killing and degradation, secreted proteins are the first stimulatory antigens in infection (5). Hence, the concept emerged that secreted antigens are potent vaccine candidates against intracellular pathogens, including *Mycobacterium tuberculosis, Listeria monocytogenes*, and *Legionella pneumophila*, the causative agents of tuberculosis, listeriosis, and Legionnaires disease, respectively (6–9). CD4⁺ as well as CD8⁺ T cells are required for efficient protection against various intracellular bacterial pathogens (2). Therefore, microbial antigens must have access to both major histocompatibility complex (MHC) class II and class I pathways. Originally, it was thought that antigens from microbial pathogens that remain in the endosome stimulate MHC class II-restricted CD4⁺ T cells only (2). The unusual capacity of *L. monocytogenes* to escape from the phagocytic vesicle into the cytosol of the infected cell (10), therefore, seemed to be a unique feature of this pathogen, which led to the preferential stimulation of MHC class I-restricted CD8⁺ T cells. However, more recently, pathogens such as *M. tuberculosis* and *Salmonella typhimurium*, which persist in the endosome, have also been found to stimulate MHC class I-restricted CD8⁺ T cells (2).

Two secreted proteins of *L. monocytogenes* have been identified as protective T-cell antigens (11, 12). These are the p60 protein, which participates in listerial invasion (13, 14), and listeriolysin (Hly), which is central to listerial egression from the endosome into the cytosol (15). We expressed these two listerial proteins in r-aroA⁻ *S. typhimurium* either in secreted or in somatic form and compared vaccine efficacy of the two modes of antigen display. In this model, we identified secretion as essential and somatic antigen display as insufficient prerequisite for protection. Moreover, we found only a minor, if any, influence of r-carrier escape from the endosome into the cytosol on activation of protective CD8⁺ T lymphocytes.

## 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. Breeding pairs of mutant mice with $\beta_2$-microglobulin deficiency ($\beta_2m^{-/-}$) and their heterozygous littermates ($\beta_2m^{+/-}$) were kindly provided by R. Jaenisch (Massachusetts Institute of Technology, Boston) and breeding pairs for the H-2I-A$\beta$-deficient mutants (A$\beta^{-/-}$)and their heterozygous littermates (A$\beta^{+/-}$) were kindly obtained from D. Mathis (INSERM, Strasbourg, France).

**Bacteria.** *L. monocytogenes* EGD Sv 1/2a was originally obtained from G. B. Mackaness. The aroA⁻ *S. typhimurium* SL7207 strain (16) was kindly provided by B. Stocker (Department of Medical Microbiology, Stanford University, Palo Alto, CA). The r-*S. typhimurium* strain SL7207 pMOhly1 (termed SL7207 control) expresses a secreted peptide (95 aa) consisting of N-terminal (34 aa) and C-terminal (61 aa) *Escherichia coli* hemolysin (HlyA)-derived fusion components. These HlyA components have no significant homology to Hly or p60 as assessed by sequence comparison [computer program WORDSEARCH (Genetics Computer Group)]. The *S. typhimurium* SL7207 control and SL7207 p60s constructs have been described (14). The strain *S. typhimurium* SL7207 Hlys is analogous to the aroA⁻ *Salmonella dublin*/pILH-1 construct (17).

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Abbreviations: $\beta_2$m, $\beta_2$-microglobulin; CFU, colony-forming unit(s); MHC, major histocompatibility complex; MLN, mesenteric lymph node; p.i., postinfection; p.o., per os; r, recombinant.

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**Construction and Characterization of r-*S. typhimurium*.** The previously described plasmids pIPH-1 and pILH-1, which encode the secreted fusion proteins p60–HlyA (66 kDa) and Hly–HlyA (64 kDa) each carrying 34 aa (N-terminal) and 61 aa (C-terminal) specific for HlyA amino acid sequence, were used for the following genetic modifications (14, 17): By means of *Apa* I digestion [position 7601 (18), T4 polymerase (Boehringer Mannheim)] reaction and religation, the correct reading frame of the *hlyD* gene was altered to inactivate the pIPH-1- or pILH-1-encoded HlyB/HlyD/TolC export apparatus (19, 20). The resulting plasmids pIPH-10 and pILH-10 were isolated from *E. coli* JM109, analyzed, and transformed in *S. typhimurium* LB5000, a restriction-negative and modification-proficient strain, by a standard electroporation protocol for *E. coli* (Bio-Rad). Subsequently, plasmids purified from *S. typhimurium* LB5000 were introduced into *S. typhimurium* SL7207 by electroporation. The resulting r-*S. typhimurium* SL7207 strains harboring the plasmid pIPH-10 or pILH-10 were termed SL7207 p60c or SL7207 Hlyc, respectively. The supernatants and bacterial lysates were obtained from bacterial cultures grown under logarithmic conditions at 37°C ($A_{600}$ = 0.6) in 2× yeast tryptone medium (Difco) supplemented with 10 μg each of *p*-aminobenzoic acid (PABA; Sigma) per ml, 10 μg of 2,3-dihydroxybenzoic acid (DHB; Sigma) per ml, and 100 μg of ampicillin (Sigma) per ml. The colony-forming units (CFU) of the different bacterial cultures were determined. Bacterial pellets were directly lysed in Laemmli buffer (21) and bacterial supernatants were concentrated (22). Protein concentrations of these bacterial lysates were determined under reducing conditions by a modified bicinchoninic acid test (23). The following samples were applied to SDS/10% polyacrylamide gel (21): (*i*) r-aroA$^-$ *S. typhimurium* lysates [5 μg of protein (equivalent of $3.5 \times 10^9$ lysed bacteria) per lane]; (*ii*) concentrated supernatants harvested from $7 \times 10^9$ r-*S. typhimurium* SL7207. For immunodetection of proteins on RAD-FREE transfer membranes (24) (Schleicher & Schuell), the monoclonal antibody H14-3 against Hly (1 μg/ml) (25) and polyclonal rabbit anti-p60 antiserum (final dilution, 1:1000) (14) were used. Immunostainings were performed with alkaline phosphatase-conjugated goat anti-mouse or anti-rabbit IgG (Jackson ImmunoResearch). The washing procedure and the chemiluminescent immunodetection protocol were according to the manufacturer's descriptions (Schleicher & Schuell). The signal development on x-ray film (Kodak, XO-MAT-AR) was performed for 1 min.

**Infection Studies.** Mice at 8–10 weeks of age were infected per os (p.o.) with *S. typhimurium* SL7207 r-strains at $5 \times 10^9$ bacteria per 200 μl of PBS. CFU in mesenteric lymph nodes (MLNs) of infected mice were determined by plating serial dilutions of organ homogenates on nutrient agar containing 10 μg of PABA per ml, 10 μg of DHB per ml, and 100 μg of ampicillin per ml at 37°C. Previous studies on *in vivo* plasmid stability showed that all r-*S. typhimurium* SL7207 strains (SL7207 control, SL7207 p60s, SL7207 p60c, SL7207 Hlys, and SL7207 Hlyc) still harbored their plasmids pMOhly1, pIPH-1, pIPH-10, pILH-1, and pILH-10, respectively, by day 21 postinfection (p.i.).

**p.o. Immunization of Mice with r-aroA$^-$ *S. typhimurium*.** C57BL/6 mice were immunized p.o. three times with $5 \times 10^9$ r-*S. typhimurium* SL7207 p60s, SL7207 Hlys, SL7207 p60c, SL7207 Hlyc, and SL7207 control at 5-day intervals. Ten weeks after the last injection, vaccinated mice were completely cured from r-*S. typhimurium* SL7207 infection as verified by CFU analysis on brilliant green *Salmonella* selective agar (Difco). At day 100 after the last vaccination, these mice were challenged i.v. with $10^5$ *L. monocytogenes* EGD bacteria ($10 \times LD_{50}$). *L. monocytogenes* CFU in spleens of r-*S. typhimurium* SL7207 Hly or SL7207 p60s vaccinated mice challenged with *L. monocytogenes* EGD were determined by plating serially diluted organ homogenates on PALCAM *Listeria* selective agar (Merck).

**i.v. Vaccination of Mice.** Unless otherwise indicated, five mice per group were immunized i.v. with $5 \times 10^5$ r-*S. typhimurium* SL7207 Hlys, SL7207 p60s, SL7207 Hlyc, SL7207 p60c, or SL7207 control. After 7 wk, mice were treated twice with 10 mg of ampicillin at weekly intervals, and after another 2 wk these mice were challenge infected i.v. with the indicated doses of *L. monocytogenes* EGD. Ten days after challenge, CFU were determined by plating serial dilutions of spleen homogenates on PALCAM *Listeria* sp. selective agar (Merck) and on brilliant green *Salmonella* sp. selective agar (Difco). The r-*S. typhimurium* SL7207 strains persisted in A$\beta^{-/-}$ mice, unless ampicillin-treated, whereas $\beta_2$m$^{-/-}$ mice were capable of clearing salmonellosis without antibiotic treatment (J.H. and S.H.E.K., unpublished results).

**Adoptive Transfer of Selected T Lymphocytes.** C57BL/6 mice were immunized i.v. once with $5 \times 10^5$ r-*S. typhimurium* SL7207 Hlys, SL7207 p60s, or SL7207 control. After 10 wk, mice were completely cured from r-*S. typhimurium* SL7207 infection as shown by CFU determination of liver organs on brilliant green *Salmonella* sp. selective agar (Difco). Spleen cells of these mice were passed over nylon wool columns. Afterwards, $1 \times 10^7$ nylon wool-purified spleen cells were labeled with either Lyt-2-MACS beads or L3T4-MACS beads and depleted of the respective subset by MACS sorting (Miltenyi Biotech, Sunnyvale, CA). Depletion of CD4$^+$ and CD8$^+$ T cells was >98%. Ten mice per experimental group received i.v. $5 \times 10^5$ unsorted cells as a source of CD4$^+$/CD8$^+$ T cells or MACS-sorted cells as a source of selected CD8$^+$ and CD4$^+$ T cells. Immediately thereafter, mice were challenged with 1.5 $LD_{50}$ *L. monocytogenes* EGD ($1.5 \times 10^4$ CFU). Mortality was recorded daily.

## RESULTS

**Characterization of r-*S. typhimurium* SL7207 p60c and SL7207 Hlyc.** The r-pBR322 based vectors pIPH-1 from r-*S. typhimurium* SL7207 p60s and pILH-1 from r-*S. typhimurium* SL7207 Hlys carry the gene fusions *iap–hlyA* and *hly–hlyA* from *L. monocytogenes* EGD and *E. coli* pHly152 plasmid-encoded hemolysin system (Table 1; Fig. 1*A*), respectively. During their logarithmic growth r-*S. typhimurium* SL7207 p60s and SL7207 Hlys secrete ≈30% of the expressed p60–HlyA and Hly–HlyA fusion antigens into the supernatant by means of the HlyB/HlyD/TolC hemolysin export machinery (J.H. and I.G., unpublished results). The uncleaved C-terminal HlyA fusion component (61 aa) (26) of the chimeric proteins represents the signal peptide directing transport across the bacterial cell wall of *E. coli* or *Salmonella* ssp. This HlyA-derived transport signal is recognized by the plasmid-encoded inner membrane proteins HlyB, a member of the ABC (ATP-binding cassette)-transporter superfamily, and HlyD (19, 27, 28). The chromosomally encoded membrane protein TolC completes this export complex (20). The C-terminal part of HlyD is required for secretion, suggesting that this protein conformation may contain contact sites for HlyB or TolC recognition (28). The plasmids pIPH-1 and pILH-1 were used for the construction of the *hlyD*-deficient plasmid derivatives to retain the corresponding and otherwise secreted fusion

Table 1. Antigen expression by and microbial host-cell localization of the r-*S. typhimurium* constructs used for vaccination against *L. monocytogenes*

| *S. typhimurium* | Plasmid | Listerial antigen display | Preferential bacterial host-cell localization |
|---|---|---|---|
| SL7207 control | pMOhly1 | — | Endosomal |
| SL7207 Hlys | pILH-1 | Secreted/somatic | Endosomal/cytosolic |
| SL7207 Hlyc | pILH-10 | Somatic | Endosomal |
| SL7207 p60s | pIPH-1 | Secreted/somatic | Endosomal |
| SL7207 p60c | pIPH-10 | Somatic | Endosomal |

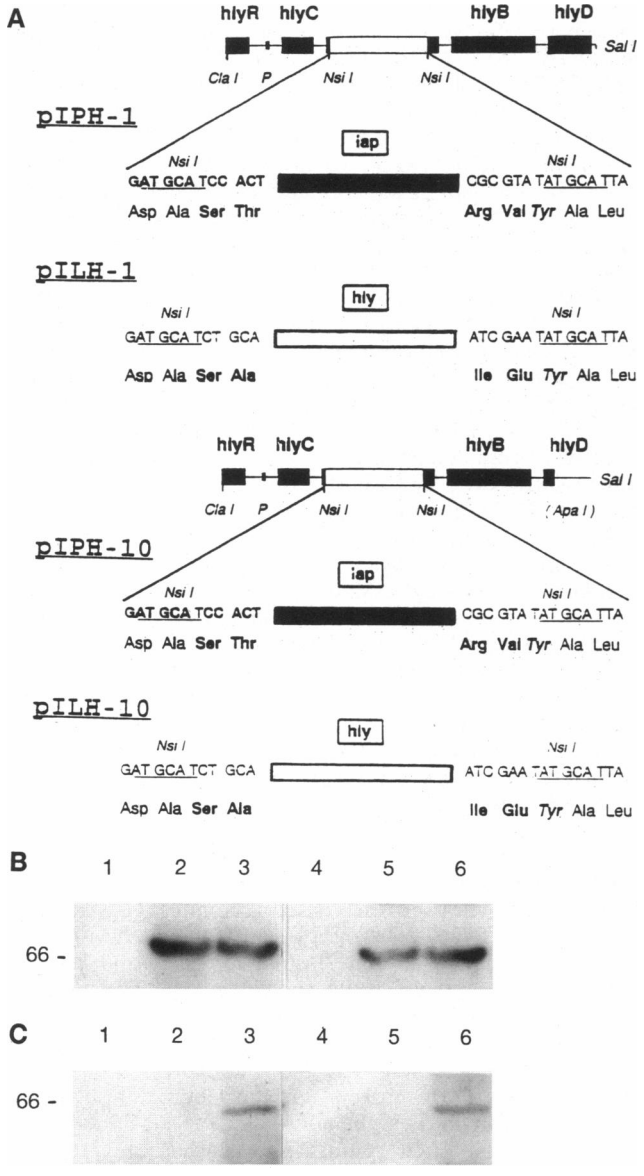


FIG. 1. Hly and p60 expression by and localization in r-*S. typhimurium* SL7207 constructs. (*A*) Schematic illustration of pBR322-based expression vectors pILH-1 and pIPH-1 or pILH-10 and pIPH-10 for secretion or somatic display of Hly–HlyA or p60–HlyA fusion proteins by r-*S. typhimurium* SL7207 Hlys, SL7207 p60s, SL7207 Hlyc, or SL7207 p60c, respectively. hlyR, hlyC, hlyB, and hlyD, *E. coli* pHly152 hemolysin gene cluster (18); P, *E. coli hly* promoter; *iap*, p60 coding gene; *hly*, Hly coding gene. (*B*) Identification of Hly–HlyA and p60–HlyA protein by chemiluminescent immunostaining of r-*S. typhimurium* SL7207 lysates: lane 1, SL7207 control harboring plasmid *pMOhly1* (17); lane 2, SL7207 Hlyc; lane 3, SL7207 Hlys; lane 4, SL7207 control; lane 5, SL7207 p60c; lane 6, SL7207 p60s. Lysates of $3.5 \times 10^9$ r-*S. typhimurium* SL7207 organisms were separated on SDS/10% polyacrylamide gel and transferred to nylon. Primary antibodies used for protein detection were (*i*) anti-Hly mAb to detect Hly-HlyA fusion proteins (lanes 1–3) and (*ii*) rabbit anti-p60 antiserum to identify p60-HlyA antigens (lanes 4–6). (*C*) Chemiluminescence detection of corresponding samples of concentrated r-*S. typhimurium* SL7207 supernatants with anti-Hly mAb (lanes 1–3) and rabbit anti-p60 antiserum (lanes 4–6). Lane 1, SL7207 control; lane 2, SL7207 Hlyc; lane 3, SL7207 Hlys; lane 4, SL7207 control; lane 5, SL7207 p60c; lane 6, SL7207 p60s (lane 6). Fusion proteins p60–HlyA and Hly–Hly are 66 and 64 kDa.

proteins p60-HlyA and Hly-HlyA in the cytosol of r-*S. typhimurium* SL7207. By destroying the *Apa* I site [position 7601 (18)], the correct reading frame of the *hlyD* gene was altered to inactivate the pIPH-1- and pILH-1-encoded HlyB/HlyD/TolC export apparatus as described for the hemolysin gene cluster of *E. coli* (29). The resulting constructs were termed pIPH-10 and pILH-10 and the corresponding r-carriers were *S. typhimurium* SL7207 p60c and SL7207 Hlyc, respectively (Table 1; Fig. 1*A*). As shown in Fig. 1*B*, chemiluminescent immunodetection of equalized amounts of extracellular and somatic proteins indicates that the somatic amounts of the fusion proteins p60-HlyA and Hly-HlyA in all r-vaccine strains were comparable. Moreover, the chimeric antigens p60-HlyA and Hly-HlyA were not detectable in culture supernatants of r-*S. typhimurium* SL7207 p60c and SL7207 Hlyc (Fig. 1*C*). We conclude that the four r-antigen delivery systems represented valuable tools to study the relevant impact of antigen display (secreted vs. somatic) and of carrier localization (endosomal vs. cytosolic) on protective immunity *in vivo*.

**Superior Vaccine Efficacy of Secreted over Somatic Antigen Display.** C57BL/6 mice were vaccinated p.o. with the r-aroA$^-$ *S. typhimurium* constructs (Table 1) and then challenge infected with a lethal inoculum of *L. monocytogenes* ($10 \times LD_{50}$). All r-*S. typhimurium* SL7207 strains showed similar CFU in MLNs of immunized mice at days 1, 3, and 7 p.i. (Fig. 2). *In vivo* stability of the appropriate plasmids harbored by the r-carrier strains were identical (data not shown). Vaccination with r-*S. typhimurium* SL7207 p60s and SL7207 Hlys displaying the two listerial antigens in secreted form rendered mice fully protected (Fig. 3*A*). In contrast, the r-*S. typhimurium* strains SL7207 p60c and SL7207 Hlyc expressing the same listerial antigens in somatic form failed to induce protection against listeriosis (Fig. 3*A*). Control mice all succumbed to this high challenge inoculum. The CFU in spleens of survivors vacci-

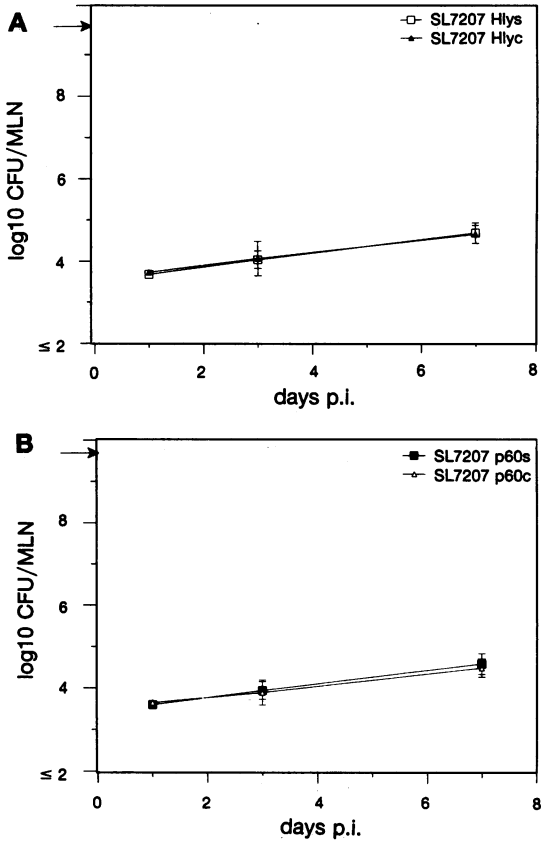


FIG. 2. Oral infection of mice with r-aroA$^-$ *S. typhimurium* SL7207 p60s, SL7207 Hlys, SL7207 p60c, and SL7207 Hlyc. (*A*) SL7207 Hlys (□), SL7207 Hlyc (▲). (*B*) SL7207 p60s (■), SL7207 p60c (△). Determination of CFU in MLNs of infected C57BL/6 at days 1, 3, and 7 p.i. Results represent geometric means of five mice per group from one of three representative experiments (±SD).

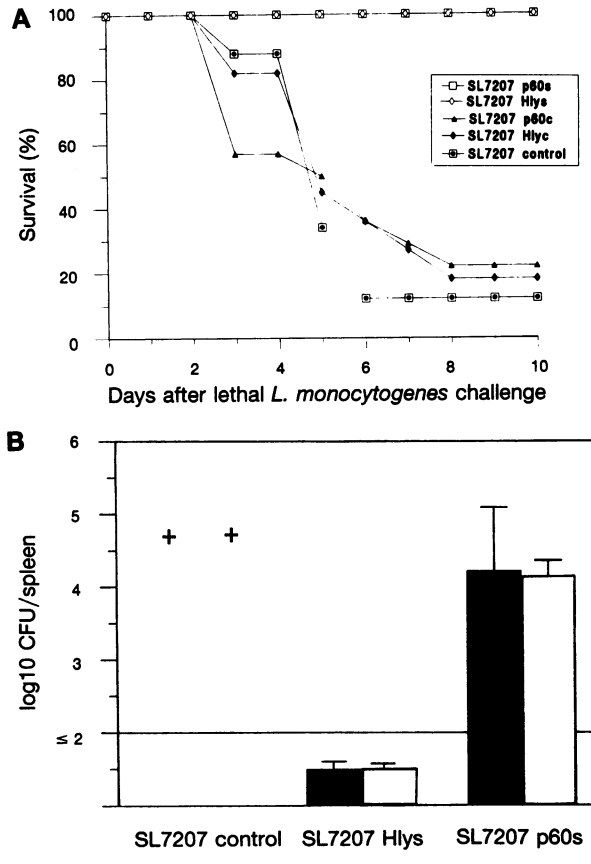


FIG. 3. Vaccine efficacy against listeriosis of r-*S. typhimurium* strains SL7207 p60s, SL7207 Hlys, SL7207 p60c, SL7207 Hlyc, and SL7207 control in C57BL/6 mice. (*A*) Survival curves of eight mice per group vaccinated p.o. with r-*S. typhimurium* SL7207 p60s (□), SL7207 Hlys (◇), SL7207 p60c (▲), SL7207 Hlyc (◆), and SL7207 control (⊡) after lethal *L. monocytogenes* EGD challenge (10 × $LD_{50}$). (*B*) CFU in spleens of survivors at day 6 (■) and day 10 (□) after *L. monocytogenes* challenge in mice that had been vaccinated with r-*S. typhimurium* SL7207 Hlys and SL7207 p60s. All mice immunized with r-*S. typhimurium* SL7207 control succumbed to listeriosis (+). Each column represents geometric mean of eight animals per group. Vertical bars indicate SD of the geometric mean.

nated with r-*S. typhimurium* SL7207 p60s and SL7207 Hlys was determined at days 6 and 10 after challenge (Fig. 3*B*). Mice vaccinated with r-*S. typhimurium* SL7207 Hlys cleared listeriosis, whereas a significant number of listeriae still persisted in mice vaccinated with r-*S. typhimurium* SL7207 p60s by day 10. As few as 5 × $10^3$ microorganisms of r-aroA$^-$ *S. typhimurium* secreting listerial antigens p60 or Hly caused effective protection against lethal *L. monocytogenes* challenge after i.v. vaccination (Table 2, experiment A). Protection was more profound with r-*S. typhimurium* SL7207 Hlys compared with SL7207 p60s. In contrast, a 100-fold higher i.v. inoculum of 5 × $10^5$ r-*S. typhimurium* SL7207 p60c and SL7207 Hlyc did not induce protective immunity against listeriosis (experiment A). We conclude that both proteins were protective only in secreted and not in somatic form and, moreover, that secreted Hly was slightly superior to p60.

**Impact of MHC I- and II-Dependent Immunity on Vaccine-Induced Protection.** To define the contribution of MHC class I- and II-dependent immune responses to vaccine-induced protection, MHC class I-deficient $\beta_2m^{-/-}$ (30) and MHC class II-deficient $A\beta^{-/-}$ (31) gene disruption mutant mice were vaccinated with the r-*S. typhimurium* constructs SL7207 Hlyc, SL7207 p60c, SL7207 Hlys, SL7207 p60s, or SL7207 control. Due to 100% *in vivo* loss of plasmid constructs by these r-carriers within 7 wk p.i., mice were treated twice weekly with

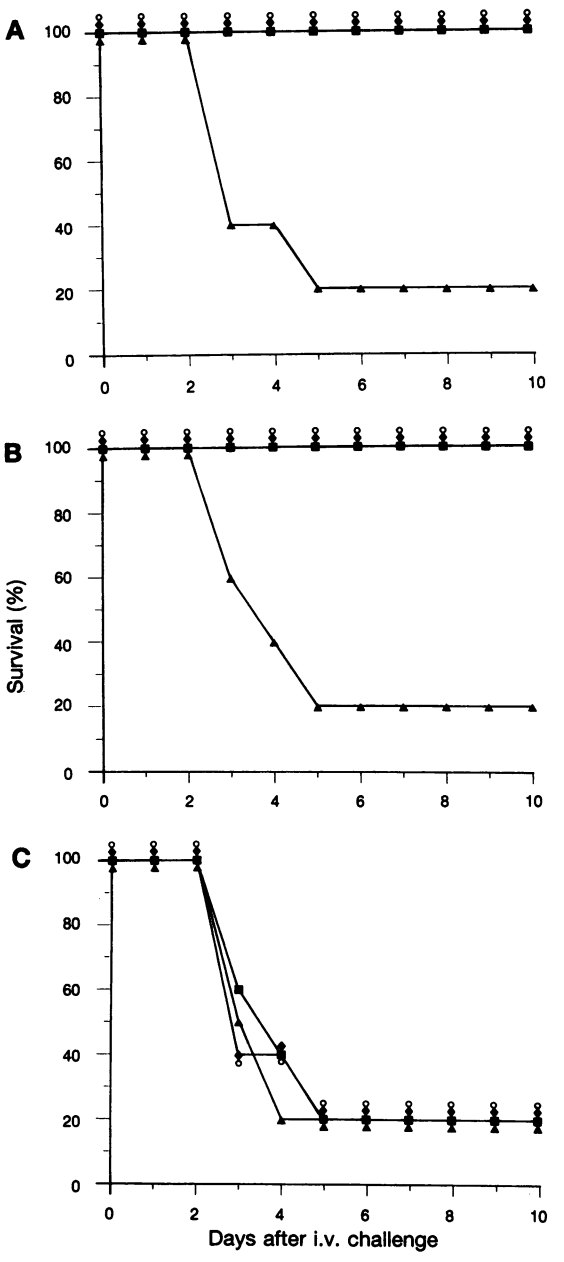


FIG. 4. Protection against *L. monocytogenes* after adoptive transfer of selected CD4$^+$ and CD8$^+$ T cells from mice vaccinated with r-*S. typhimurium* SL7207 Hlys and SL7207 p60s. (*A*) T-cell transfer from r-*S. typhimurium* SL7207 Hlys vaccinated mice. Unselected T cells (■), CD4$^+$ T-cell depleted (◆), CD8$^+$ T-cell depleted (○), and T cells from naive mice (▲). (*B*) T-cell transfer from r-*S. typhimurium* SL7207 p60s-vaccinated mice. (*C*) T-cell transfer from r-*S. typhimurium* SL7207 control-vaccinated mice. Mortality of 10 recipient mice per group was recorded daily after adoptive transfer and subsequent challenge of *L. monocytogenes* (1.5 $LD_{50}$).

ampicillin and after 2 wk these mice were challenged i.v. with *L. monocytogenes*. Ten days after challenge, CFU were determined. A significant proportion of the nonvaccinated and r-*S. typhimurium* SL7207 Hlyc, SL7207 p60c, and SL7207 control immunized mice succumbed to listeriosis (Table 2, experiment B). In contrast, all mice vaccinated with the r-*S. typhimurium* constructs SL7207 Hlys and SL7207 p60s secreting Hly and p60 survived. Determination of CFU revealed no differences between the $\beta_2m^{-/-}$ and $A\beta^{-/-}$ mutants and their respective heterozygous control littermates or between the two antigens (experiment B). These findings suggest that both MHC class I- and II-dependent immune responses contributed to vaccine-

Table 2. Influence of dose of immunization and MHC presentation on vaccine efficacy of r-*S. typhimurium* SL7207 Hlyc, SL7207 p60c, SL7207 Hlys, and SL7207 p60s against listeriosis

| Mouse strain | Nonvaccinated mice (proportion of survivors) | r-SL7207 vaccinated mice [proportion of survivors ($log_{10}$ CFU of *L. monocytogenes* per spleen)] | | | | |
|---|---|---|---|---|---|---|
| | | Control | Hlyc | p60c | Hlys | p60s |
| | | | Experiment A | | | |
| C57BL/6* | 0/5† | 0/5* | 2/5* | 0/4* | 5/5 (2.74 ± 0.2)‡ | 5/5 (3.63 ± 0.1)‡ |
| C57BL/6§ | 0/5† | 0/5§ | 1/5§ | 0/5§ | 5/5 (2.28 ± 0.3)‡ | 5/5 (4.21 ± 0.5)‡ |
| | | | Experiment B | | | |
| $A\beta^{-/-}$¶ | 1/5¶ | 1/5¶ | 1/5¶ | 0/5¶ | 5/5 (<2)‖ | 5/5 (<2)‖ |
| $A\beta^{+/-}$** | 2/5** | 1/5** | 2/5** | 1/5** | 5/5 (2.12 ± 0.3)‖ | 5/5 (2.53 ± 0.2)‖ |
| $\beta_2m^{-/-}$¶ | 1/5¶ | 0/5¶ | 0/5¶ | 1/5¶ | 5/5 (2.87 ± 0.4)‖ | 5/5 (2.67 ± 0.1)‖ |
| $\beta_2m^{+/-}$** | 1/5** | 1/5** | 1/5** | 1/5** | 5/5 (2.87 ± 0.7)‖ | 5/5 (2.36 ± 0.3)‖ |

Unless otherwise indicated, five mice per group were immunized i.v. with $5 \times 10^5$ r-*S. typhimurium* SL7207 Hlys, SL7207 p60s, SL7207 Hlyc, SL7207 p60c, and SL7207 control. After 7 weeks, heterozygous and homozygous $\beta_2m^{-/-}$ and $A\beta^{-/-}$ mutant mice were treated twice with 10 mg of ampicillin at weekly intervals, and after another 2 weeks these mice were challenged i.v. with the indicated doses of *L. monocytogenes* EGD. Vaccinated C57BL/6 mice were challenged together with the immunized mutant mice without antibiotic treatment. Ten days after *L. monocytogenes* challenge, CFU in spleens were determined.

*Vaccination inoculum, $5 \times 10^5$ r-*S. typhimurium* SL7207 i.v.; challenge dose, $10^5$ *L. monocytogenes* EGD ($10 \times LD_{50}$).

†Challenge inoculum, $10^5$ *L. monocytogenes* EGD ($10 \times LD_{50}$).

‡All mice survived. Results are means ± SD. Only CFU differences between *S. typhimurium* SL7207 Hlys and SL7207 p60s immunized mice are statistically significant ($P < 0.05$; Student's *t* test).

§Vaccination inoculum, $5 \times 10^3$ r-*S. typhimurium* SL7207 i.v.; challenge dose, $10^5$ *L. monocytogenes* EGD ($10 \times LD_{50}$).

¶Challenge inoculum, $7 \times 10^3$ *L. monocytogenes* EGD ($1 \times LD_{50}$).

‖All mice survived. Results are means ± SD. CFU differences are not statistically significant ($P > 0.05$; Student's *t* test).

**Challenge inoculum, $10^4$ *L. monocytogenes* EGD ($1 \times LD_{50}$).

induced protective immunity. Because r-*S. typhimurium* SL7207 p60s induced protection in $A\beta^{-/-}$ mice lacking $CD4^+$ T cells, antigen delivery from the endosome into the cytosolic MHC class I pathway, independent from bacterial egression, appears likely.

**Adoptive Protection by Selected $CD4^+$ and $CD8^+$ T Cells.** To directly determine activation of these two T-cell subpopulations by r-aro$A^-$ *S. typhimurium* secreting p60 or Hly in the endosome or cytosol, respectively, adoptive transfer experiments were performed (Fig. 4). Selected $CD4^+$ and $CD8^+$ T cells ($5 \times 10^5$) from donor mice vaccinated with r-*S. typhimurium* SL7207 p60s and SL7207 Hlys secreting p60 and Hly, respectively, transferred protection against *L. monocytogenes* at a dose of $1.5 \times 10^4$ bacteria ($1.5\ LD_{50}$). In contrast, T lymphocytes from naive mice or selected and unselected T cells from r-*S. typhimurium* SL7207 control-immunized donor mice did not confer protection and a significant proportion of recipient mice succumbed to listeriosis. We conclude that both vaccine constructs induced $CD4^+$ and $CD8^+$ T lymphocytes, which were capable of mediating antilisterial protection by themselves in the absence of the alternative T-cell subset. Consistent with our finding, selected or cloned $CD8^+$ and $CD4^+$ T lymphocytes from *L. monocytogenes* immune mice have been shown to adoptively transfer antilisterial protection (32–35). Induction of protective $CD4^+$ T cells by the r-aro$A^-$ *S. typhimurium* constructs secreting Hly and p60 does not argue against a superior role of $CD8^+$ over $CD4^+$ T cells in naturally acquired resistance against listeriosis (36, 37).

## DISCUSSION

The present report describes efficient protection against the intracellular pathogen, *L. monocytogenes* by vaccination with r-aro$A^-$ *S. typhimurium* constructs secreting two defined listerial antigens, p60 and Hly. First, our findings reveal a crucial impact of antigen display on protection. Antigen secretion was an essential, and somatic expression was an insufficient, prerequisite for successful vaccination. Second, our data reveal that carrier localization was not critical for stimulation of $CD8^+$ T cells, because r-constructs residing in either compartment were capable of introducing secreted antigens into the MHC class I pathway.

The r-aro$A^-$ *S. typhimurium* system described here allows comparison of different expression modes of identical proteins by the same plasmid with the exception of a minor manipulation in the *hlyD* gene. In contrast to surface-associated epitope localization (38), the hemolysin-derived export machinery allows secretion of full-length antigenic proteins by r-aro$A^-$ *S. typhimurium*, thus avoiding limitation to single epitopes. For both secreted and somatic display in r-aro$A^-$ *S. typhimurium*, copy number and *in vivo* stability of the plasmids, regulation of the hemolysin operon as well as synthesis and stability of heterologous mRNA were comparable (J.H., unpublished results). In addition, p60-HlyA and Hly-HlyA fusion proteins retained inside r-*S. typhimurium* SL7207 p60c and SL7207 Hlyc, respectively, were probably equally abundant as their secreted cognates in r-*S. typhimurium* SL7207 p60s and SL7207 Hlys. The impact of the *Apa* I mutation in the *hlyD* gene on protein export has been thoroughly studied in the hemolysin system of *E. coli* (29). These data suggest that the proteins are not exported into the bacteria-free supernatant or exposed on the bacterial surface, although deletion of the C-terminal part of HlyD allows initiation of secretion. Therefore, we are confident that p60 and Hly were not available to antigen processing prior to *in vivo* killing and degradation of r-*S. typhimurium* SL7207 p60c and SL7207 Hlyc, respectively.

The major finding of our experiment is that secreted antigens rather than somatically displayed ones induced protective immunity. We do not know whether differential antigen display influenced protein conformation. Because both p60 and Hly are secreted naturally, it is possible that the conformation of the fusion proteins resembles that of their natural cognates more precisely in the secreted form than in the somatic one. Conformational differences could influence antigen processing and availability of protective T-cell epitopes. Two lines of indirect evidence argue against this assumption. First, hemolysin when retained in the cytoplasm of *E. coli*, maintains its biological activity, suggesting similar protein folding in the somatic and secreted form (29). Second, processing and presentation of T-cell antigens are generally less conformation dependent than antigen recognition by antibodies (39). Although we cannot formally exclude an impact of conformational differences, we consider this possibility less

likely. Rather, we assume direct influence of antigen display on vaccine efficacy in this system.

The second important finding is that p60 secretion by r-aroA$^-$ *S. typhimurium* in the endosome of infected host cells induced CD8$^+$ T cells, which mediated protection against *L. monocytogenes*. Thus, localization of r-aroA$^-$ *S. typhimurium* inside the host cell had a minor influence on protection. Induction of protective CD8$^+$ T cells by viable *L. monocytogenes* requires bacterial release from the phagosome into the cytosol of host cells (40). Nonhemolytic, Hly-deficient *L. monocytogenes* strains fail to induce protection against listeriosis. These avirulent listeriae are rapidly killed in the phagolysosomal vacuole, suggesting that they fail to introduce their antigens into the MHC class I processing pathway. In contrast, the aroA$^-$ *S. typhimurium* persist in the phagosome for a considerable period of time, and we assume that this longevity promoted introduction of secreted antigens from the endosome into the MHC class I processing pathway. As a corollary, induction of protective MHC class I-dependent immune mechanisms by r-*L. monocytogenes* strains depends on endosomal escape of the carrier (41), whereas endosomal persistence of r-aroA$^-$ *S. typhimurium* allows MHC class I presentation. Recent evidence suggests the existence of alternative processing pathways (42), which allow endosomal loading of empty MHC class I molecules with antigenic peptides (43) or release of free antigens from the endosome into the cytoplasm with subsequent processing and presentation by the classical MHC class I pathway through the endoplasmic reticulum (44).

The r-aroA$^-$ *S. typhimurium* secreting Hly expressed slightly improved vaccine efficacy compared to the p60-secreting construct. At least two possibilities could account for this difference. First, we have shown recently that Hly not only promotes egression of *L. monocytogenes* from the endosome into the cytosol but also confers this capacity to r-aroA$^-$ *S. typhimurium* and aroA$^-$ *S. dublin* (17). Hence, it is possible that the cytosolic residence of r-aroA$^-$ *S. typhimurium* improved vaccine efficacy against *L. monocytogenes*, which primarily replicates in the cytosol. Alternatively, it is possible that p60 is a weaker immunogen than Hly and, therefore, less efficacious in inducing protective immunity independent from carrier localization. Experiments with constructs secreting biologically inactivated Hly, which is still immunogenic, could help decide between these two alternatives.

Secreted antigens produced by metabolically active microbial pathogens are available to the immune system at the outset of infection, whereas presentation of somatic antigens is delayed because it depends on prior microbial killing and degradation. Recently, the importance of secreted proteins as protective antigens has been emphasized by successful vaccination of guinea pigs against tuberculosis and Legionnaires disease (6, 9). Antigen secretion by r-carriers mimics the natural infection more precisely and hence should be considered an important criterium of "protective T-cell antigens" in the rational design of future vaccines against intracellular microbial pathogens.

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