Protective immunity against herpes simplex virus (HSV) type 1 following oral administration of recombinant Salmonella typhimurium vaccine strains expressing HSV antigens

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Salmonella typhimurium strains expressing foreign antigens of various pathogens are capable of eliciting antigen-specific humoral and cellular immune responses. Attenuated S. typhimurium strain χ4550 (Δcya Δcrp Δasd) was used as an expression vector for herpes simplex virus (HSV) antigens. Genes encoding glycoprotein D (gD) and the immediate-early protein ICP27 of HSV-1 were cloned and expressed in plasmid pYA292 (asd"") and subsequently placed into χ4550. Following two oral immunizations, the protective efficacy of recombinant strains against zosteriform challenge with HSV-1 was measured in 3–4-week-old BALB/c mice. Levels of protection observed were 77% with the ICP27 construct but only 31% with the gD construct. Zosteriform protection correlates with a CD4+ mediated delayed-type hypersensitivity (DTH) reaction against HSV. Accordingly, significant DTH was observed only in mice immunized orally with the S. typhimurium ICP27 construct. ELISA analysis of antigen-specific humoral responses failed to detect serum antibody responses following oral administration although recombinant S. typhimurium were isolated from spleens of orally dosed mice up to day 30. Intravenous (i.v.) immunization with the gD-expressing construct did, however, induce detectable serum antibody responses. Some humoral IgA responses against gD in faecal samples were detected as early as 3 weeks post-oral immunization while those induced by the i.v. route were slightly lower. These data suggest that recombinant S. typhimurium HSV antigens are capable of inducing immunity against HSV, some aspects of which are protective against HSV challenge.

Introduction

Herpes simplex virus (HSV) infection typically occurs via a mucosal surface, after which virus latency is established in the nervous system. The majority of vaccine approaches against HSV have emphasized injectable routes of immunization with attenuated or inactivated HSV, or subunit or recombinant vectors such as vaccinia virus and plasmid DNA (Burke, 1992; Nash et al., 1987; Manickan et al., 1995a). To date there has been little focus on the potential role of mucosal immunizations in protecting against HSV infections. Desired aspects of mucosal immunizations include low cost, non-invasive administration, potential for single-dose immunity, and ease of preparation, storage and transport. In pursuit of attaining these attributes, multiple protocols and vectors have been developed. Of these, the use of live attenuated bacterial carriers, particularly attenuated Salmonella, has been extensive. Salmonella vectors are capable of inducing a broad spectrum of mucosal and systemic immune responses to expressed heterologous antigens including cell-mediated responses (Cárdenas & Clements, 1992; Chatfield et al., 1994; Roberts et al., 1994; Karem et al., 1995).

In this study, we have analysed the use of live recombinant Salmonella typhimurium as mucosal vaccines to induce HSV specific immunity and protection. Vaccine vectors such as Salmonella hold promise in overcoming limitations seen with other methods of mucosal vaccination. Delivery of protein alone to mucosal surfaces generally fails to produce humoral or cellular responses and at certain doses may result in immune tolerance (Weiner, 1994; Challacombe & Tomasi, 1980;
Richman, 1979). Although the use of adjuvant proteins such as cholera toxin allows induction after oral peptide delivery, these methods are often limited in their ability to stimulate cellular responses, attributes attainable with *Salmonella* vectors (Brown et al., 1987; Flynn et al., 1990). Live-replicating vectors such as *Salmonella* may also be advantageous over microencapsulation due to the immunogenicity of the bacteria which may aid in priming inductive tissues (i.e. Peyer’s patches). We have combined these assets to target inductive sites with the expression of HSV antigens from *Salmonella* vectors.

Protective mechanisms against systemic HSV-1 infection include both cell-mediated and humoral responses (Mester et al., 1991). Live virulent HSV-1 given orally at sublethal levels protected mice against lethal dose challenge (Irie et al., 1992). This protection was correlated with Th1-type responses in the form of humoral IgG2a sera as well as proliferative T cell responses. The majority of HSV studies have emphasized the form of humoral IgG2a sera as well as proliferative T cell responses. The expression of HSV antigens from *Salmonella* may also be advantageous over microencapsulation due to the immunogenicity of the bacteria which may aid in priming inductive tissues (i.e. Peyer’s patches). We have combined these assets to target inductive sites with the expression of HSV antigens from *Salmonella* vectors.

Immunizations. Three- to four-week-old female BALB/c mice were obtained from Harlan Sprague-Dawley and housed in filter-top cages for the duration of the study. For inoculum preparation, S. *typhimurium* was grown overnight in 1 ml LB broth and the next day diluted 1:50 in fresh LB broth. Cultures were grown at 37 °C, with shaking at ~180 r.p.m. After 4 h growth, cultures were centrifuged at 6000 g at 4 °C to pellet the cells. Cells were maintained on ice and resuspended in 1/10 the original volume of cold sterile 1× PBS. Following 3–4 h starvation of food and water, 4–5-week-old mice (five or six mice per group) were dosed orally with 100 μl (~1 × 10^6 c.f.u.) S. *typhimurium* using a feeding needle inserted to the stomach. Intravenous (i.v.) immunizations were performed by tail vein injections of 100 μl culture dilutions containing 5 × 10^6 c.f.u. followed by a single boost 1 week later of 10^6 c.f.u. Plate counts were performed on all inocula to verify the dose given to the animals. When required, mice were anaesthetized using Metofane (methoxyflurane, Pitman-Moore). Following immunization, mice were observed daily for signs of illness. Few mice showed any such signs; however, several in both oral- and i.v.-immunized groups showed brief periods of ‘ruffled’ fur on days 2–5 post-immunization.

Detection of recombinant *S. typhimurium* in vivo. Spleens were removed from animals under sterile conditions and minced using fine wire screen in DMEM media (Gibco). Aliquots of the mixture were then plated on Brilliant Green agar and resulting colonies were screened by Western blot analysis to ensure expression of the antigen. Intravenous (i.v.) immunizations were performed by tail vein injections of 100 μl culture dilutions containing 5 × 10^6 c.f.u. followed by a single boost 1 week later of 10^6 c.f.u. Plate counts were performed on all inocula to verify the dose given to the animals. When required, mice were anaesthetized using Metofane (methoxyflurane, Pitman-Moore). Following immunization, mice were observed daily for signs of illness. Few mice showed any such signs; however, several in both oral- and i.v.-immunized groups showed brief periods of ‘ruffled’ fur on days 2–5 post-immunization.

Zosteriform challenge. Mice immunized orally or i.v. with two doses of *S. typhimurium* KRI (vector), KR8 (gD) or KR10 (ICP27) were challenged on day 21 post-initial dosing with a lethal dose of HSV-1 (strain 17; 1.25 × 10^5 TCID_{50} on the mid-abdomen (Simmons & Nash, 1984). Briefly, 1 day before challenge, mid-abdominal to flank regions of the mice were shaved using an animal hair clipper (Oster) and depilated with Nair (Carter-Wallace). On the day of challenge, mice were anaesthetized using Metofane and mid-sections abraded with a 28 gauge needle to disrupt the cutaneous layer of skin. HSV-1 was then applied in PBS plus 0.1% sodium azide and vortexing for 15 min. Mice were then centrifuged in a microfuge for 5 min to pellet debris and the supernatant collected for ELISA analysis. ELISA was performed using plates coated with purified...
Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td><em>Escherichia coli</em>&lt;br&gt; χ6097</td>
<td>araB(lac–pro) rpsL ΔasdA4 [zhf-2::Tn10] thiφ 80d lacZ Δm15</td>
<td>Nakayama et al. (1988)</td>
</tr>
<tr>
<td>XL-1-Blue</td>
<td>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB lacF ZAM15 Tn10(tef1)]</td>
<td>Stratagene</td>
</tr>
<tr>
<td><em>Salmonella typhimurium</em>&lt;br&gt; χ3730</td>
<td>ΔasdA1 Δ[zhf-4::Tn10] metE551 metA22 hsdSA hsdSB ilv/pStSR100&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Curtiss &amp; Kelly (1987)</td>
</tr>
<tr>
<td>χ4550</td>
<td>gyrA1816 cya-1 crp-1 asdA1/pStSR100&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Nakayama et al. (1988)</td>
</tr>
<tr>
<td>KR1</td>
<td>χ4550/pYA292 gD</td>
<td>This paper</td>
</tr>
<tr>
<td>KR8</td>
<td>χ4550/pYA292 gD</td>
<td>This paper</td>
</tr>
<tr>
<td>KR10</td>
<td>χ4550/pYA292 ICP27</td>
<td>This paper</td>
</tr>
<tr>
<td><strong>Plasmids</strong>&lt;br&gt; pYA292</td>
<td>asd&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Curtiss &amp; Kelly (1987)</td>
</tr>
<tr>
<td>pJB1</td>
<td>pUC19 gD</td>
<td>Bowen et al. (1990)</td>
</tr>
<tr>
<td>pJB3</td>
<td>pUC19 ICP27</td>
<td>This paper</td>
</tr>
<tr>
<td>pJB9</td>
<td>pYA292 gD</td>
<td>This paper</td>
</tr>
<tr>
<td>pJB12</td>
<td>pYA292 ICP27</td>
<td>This paper</td>
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Fig. 1. Schematic representation of the subcloning strategies used for construction of plasmids expressing HSV-1 antigens. (a) DNA encoding gD was subcloned from pJB1 into pYA292 asd<sup>+</sup> as described in Methods to create pJB9. (b) DNA encoding ICP27 was subcloned from pJB3 into pYA292 asd<sup>+</sup> as described in Methods to create pJB12.

gD (kindly provided by Phillip Berman, Genentech, USA) at 2 µg/ml or HSV antigen at 2 µg/ml (Advanced Biotechnologies). For ICP27 antibody detection, plates were coated with extracts from SF9 insect cells infected with recombinant baculoviruses containing ICP27 which was kindly supplied by Sandri Golden, University of California, Irvine, Calif., USA. Enhanced chemiluminescense Western blotting (ECL-WB) was also performed to detect antibodies to ICP27 using a Lumi-Phos Alkaline Phosphatase Gene Images Kit (United States Biochemical). *S. typhimurium*
antigen was used at 100 μg/well for detection of anti-S. typhimurium antibodies. S. typhimurium antigen was prepared by growth of KR1 overnight in LB broth, pelleting of cells by centrifugation and resuspending in 1 × PBS. Cell resuspending were then sonicated five times each with 10 s bursts using a microtip sonicater (Cell Disrupter, Model W-220F, Heat Systems-UltraSonic). Cell debris was removed after centrifugation and the protein concentration of lysates determined with a Bradford assay kit (Bio-Rad). Plates were blocked with 3% skim milk in 1 × PBS for 1–2 h at 37 °C. After blocking, plates were frozen at −20 °C until used. Plates were thawed at room temperature and rinsed three times with 1 × PBS-Tween 20 (0.05%) solution. Samples were then added, with serum dilutions starting at 1:25 and faecal extracts at 1:1. Samples were then serially diluted in PBS-Tween in 96-well microtitre plates. Plates were incubated with samples for 2 h at 37 °C, then rinsed three times with PBS-Tween. Secondary antibodies were used at 1:3000 dilutions and included goat anti-mouse IgG–horseradish peroxidase conjugate (HRP) and goat anti-mouse IgA–HRP (Southern Biotechnology). Secondary antibodies were incubated for 1–2 h at 37 °C, washed three times and plates developed using ABTS solution [8 mg 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), Sigma], 20 ml 0·1 M citric acid, 20 ml 0·1 M sodium phosphate (dibasic) and 8 μl hydrogen peroxide (H₂O₂). Absorbance was read at 405 nm using an automated ELISA plate reader (BIO-TEK, model EL310). Titres are expressed as the log₁₀ of the highest dilution giving A₄0₅ values at least twice those obtained in mice immunized orally or i.v. with control stain KR1 (e.g. for KR1 A₄0₅ of 0·03 at 1:50 dilution, the titre of recombinants at 1:50 would equal ≥ 0·6). All titres are representative of duplicate experiments where titres of pooled samples for each group were the same or within one dilution of each other.

Delayed-type hypersensitivity (DTH). Immunized mice were anaesthetized, then injected in the left ear pinnae with 1 × 10⁶ TCID₅₀ UV-inactivated HSV-1 (KOS) diluted in 1 × PBS to a total volume of 50 μl. HSV virions contain significant amounts of gD as well as low levels of ICP27 (Nahmias & Josey, 1968). As a negative control, the right ear pinna of each mouse was injected with 20 μl Vero cell extract since HSV-1 was grown on Vero cells. Mice were checked for ear swelling using an Oditest (H. C. Kroepelin) spring-loaded caliper at 24, 48 and 72 h. The left and right ear thicknesses were recorded for individual mice and the difference measured as a measure of the DTH response. The significance of these differences was determined using the paired two-tailed Student’s t-test.

Results

Construction of recombinant S. typhimurium HSV strains

Genes encoding gD and ICP27 of HSV-1 were subcloned from pJB1 and pJB3, respectively (see Methods and Fig. 1). The resultant plasmids, pJB9 (gD) and pJB12 (ICP27) were subsequently analysed for the ability to express gD and ICP27 from the trc promoter of pYA292 in E. coli strain BL21. Western blot analysis indicated that both constructs constitutively express the recombinant proteins (data not shown). Subsequently, both pJB9 and pJB12 were placed in S. typhimurium vaccine strain χ4550, resulting in strains KR8 and KR10, respectively, as described in Methods (see Table 1). Expression of recombinant proteins in χ4550 was determined by Western blot (Fig. 2). As indicated, KR8 and KR10 expressed significant amounts of gD and ICP27, respectively. KR10 also expressed multiple lower molecular mass products, most likely the result of degradation or processing by S. typhimurium.

Immunizations and challenge

Recombinant S. typhimurium strains KR8 and KR10 were assessed for their ability to afford protection to mice following oral immunization. S. typhimurium constructs were grown as described in Methods and administered to female BALB/c mice orally on day 1 and day 14. On day 21, mice were challenged with HSV-1 (17) (1·25 × 10⁶ TCID₅₀) on the mid-abdomen as described in Methods. Mice were observed on a daily basis for lesions and signs of encephalitis. Lesion development in all groups began by day 6 (Fig. 3). By day 8 post-challenge, 7/8 (87·5%) of KR1 (vector) immunized mice developed severe (+) lesions with 8/8 by day 9 (Fig. 3a). Encephalitic death of KR1 control animals began by day 8 and 7/8 (87·5%) had succumbed to encephalitis by day 13, with the survivor recovering from encephalitis but maintaining a +4 lesion. In contrast, of mice receiving KR10 (ICP27) group 0/12 developed +4 lesions by day 8 and only 3/12 (25%) by day 13. Encephalitic death was not observed in KR10 mice until day 9 and only 4/12 (33%) died by day 13 (Fig. 3b). Of mice receiving KR8 (gD), 5/13 (38·5%) had +4 lesion scores by day 8 with 8/13 (61·5%) by day 9 (Fig. 3c). A total of 9/13
Table 2. ELISA analysis of gD-specific antibody responses following two doses of i.v. or orally administered S. typhimurium KR1 (vector) or KR8 (gD) as described in Methods

<table>
<thead>
<tr>
<th>Strain</th>
<th>Serum anti-gD IgG</th>
<th>Faecal anti-gD IgA</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>i.v.</td>
<td>oral</td>
</tr>
<tr>
<td>KR1</td>
<td>&lt; 1:50</td>
<td>&lt; 1:50</td>
</tr>
<tr>
<td>KR8</td>
<td>1:400</td>
<td>&lt; 1:50</td>
</tr>
</tbody>
</table>

(69%) of KR8-immunized mice died of encephalitis, with death occurring as early as day 8 as was observed for KR1 vector controls.

**Humoral responses following oral immunization**

Serum and faecal samples of each group were collected at weekly intervals to evaluate HSV-specific antibody responses following oral administration of recombinant S. typhimurium. Faecal IgA titres of 1:32 against gD were evident by week 3 (Table 2). However, no serum responses were detectable following oral dosing although recombinant S. typhimurium were recoverable from spleen tissue at day 30 (approximately 330 ± 60 c.f.u./g tissue). It is significant that these isolates maintained their ability to express gD in vitro (data not shown). Humoral responses to ICP27 were undetectable in serum or faecal samples of KR10 orally dosed animals by standard ELISA or ECL–WB (data not shown); however, detection of anti-ICP27 responses remains technically difficult (see Discussion).

**Humoral responses following i.v. immunization**

Since low humoral responses were detected following oral administration of recombinant S. typhimurium, i.v. immunization was performed to establish their ability to induce serum antibody responses. As indicated in Table 2, serum IgG titres of 1:400 against gD were detected following i.v. immunization. Faecal IgA responses against gD, though present, were lower than those following oral immunization (Table 2). As described for oral immunization, humoral responses to ICP27 were undetected in serum or faecal samples following i.v. administration of KR10 by ELISA or ECL–WB (data not shown).

Asterisk above the score symbols represents a single animal which died of encephalitis on that particular day with a lesion of the score indicated. HSV-immunized animals (sublethal infection) remained resistant to zosteriform challenge (see Methods).
Lymphoid cells then interact with antigen to evoke immune responses. Specific antigen (and some pathogens such as Salmonella) with epithelium or microfold (M) cells function in transport of antigens. Lymphoid tissues are covered by specialized follicle-associated epithelium. These lymphoid patches are the Peyer's patch of the small intestine. These areas are normally induced by mucosal antigens that may play a role in Th1 proliferative responses has recently emerged from studies on recombinant ICP27 vaccinia virus and ICP27 encoding naked DNA (see Discussion).

DTH

Protection against zosteriform challenge is thought to result from CD4+ cell-mediated responses (Nash et al., 1987; Simmons & Tscharke, 1992). Since protective responses were observed following oral immunization, orally dosed mice were examined for DTH reactions against HSV-1 antigen. Immunized mice were injected in the ear pinnae with UV-inactivated HSV-1 as described in Methods. Mice were observed for ear swelling at 24, 48, and 72 h after injections (Fig. 4). As shown, statistically significant ear swelling was observed only in mice immunized with KR10 (ICP27). S. typhimurium. Induction of a DTH response in the ICP27-immunized animals suggests that CD4+ T cells could account for the protection seen upon zosteriform challenge (Fig. 3). Additional evidence that ICP27 may play a role in CD4+ T cell protective responses has recently emerged from studies on recombinant ICP27 vaccinia virus and ICP27 encoding naked DNA (see Discussion).

Discussion

Vaccine vectors such as Salmonella hold promise in overcoming limitations seen with other methods of mucosal vaccination. One primary site of induction for mucosal immunity is the Peyer's patch of the small intestine. These lymphoid tissues are covered by specialized follicle-associated epithelium or microfold (M) cells which function in transport of antigen (and some pathogens such as Salmonella) from the lumen of the gastrointestinal tract to basolateral surfaces. Lymphoid cells then interact with antigen to evoke immune responses. The use of Salmonella as a delivery vehicle for heterologous antigens exploits these aspects of mucosal immune induction. In an attempt to develop HSV vaccines using Salmonella vectors, antigens gD and ICP27 were chosen since infected patients have high anti-gD serum titres (Burke, 1992) and ICP27 has been identified as a major CTL target (Banks et al., 1991, 1992). In the present study, recombinant S. typhimurium administered orally elicited no detectable serum IgG response and little faecal IgA humoral response to gD and none to ICP27. It is not clear why humoral responses to S. typhimurium-delivered HSV antigens are so low. However, variation in responses to an antigen in different expression systems is not unlikely. For example, some proteins which normally induce humoral responses may not be immunogenic when expressed from Salmonella constructs. In fact, although extremely immunogenic in HSV infections, gD has also failed to induce significant responses in other Salmonella systems. The expression of gD using the nirB promoter, a highly promoted system to generate high in vivo antigen levels (Chatfield et al., 1992; Fayolle et al., 1994; Karem et al., 1995) also failed to induce elevated responses (data not shown). Indeed, lysates of S. typhimurium gD constructs grown in vitro which contain gD protein, as measured by Western blot analysis, failed to induce serum anti-gD responses when injected subcutaneously with Freund's complete adjuvant followed by a boost with lysate and Freund's incomplete adjuvant (data not shown). Thus, although some levels of gD-specific antibodies are detectable following KR8 oral and i.v. immunization, it is apparent that in this context, gD is limited immunologically. This limitation of S. typhimurium-expressed gD may reflect a deficiency in glycosylation of this protein, thereby altering its immunogenicity. Similar results were recently reported by Fouts et al. (1996) using S. typhimurium expressing HIV-1 gp120. In this study, gp120, normally a high inducer of antibodies, failed to induce humoral responses. Despite this, significant gp120-specific Th1 proliferative responses were detected in splenocytes in vitro. These data, along with our analysis of gD and ICP27 constructs, suggest that some antigens expressed in Salmonella vectors may fail to induce detectable humoral responses. However, the ability to induce Th1 proliferative responses against gp120 (Fouts et al., 1996) and DTH following S. typhimurium ICP27 administration (this work) in the absence of humoral responses is apparent. As regards to ICP27, detection of humoral responses to this IE HSV protein have been rare at best. Immunization with live or inactivated HSV, as well as naked DNA expressing ICP27, have failed to induce detectable antibody responses (Manickan et al., 1995a). In fact, only vaccinia virus vectors expressing ICP27 have been found to induce detectable humoral responses (Manickan et al., 1995b). The few reports of anti-ICP27 humoral responses may also reflect difficulties in developing reliably sensitive antibody assays due to lack of availability of purified ICP27 protein. In the light of these observations, it is not surprising that S. typhimurium ICP27 constructs failed to induce detectable humoral responses in the present study.

Despite the absence of antibodies, protection against lethal HSV challenge was apparent in KR10 (ICP27)-immunized animals (Fig. 3). Severe lesion development (+ 4 score) in mice
receiving KR10 orally was delayed until day 10, while both KR1 vector control and KR8 (gD) animals showed +4 lesions by day 7. In addition, encephalitic death occurred in only 4/12 (33%) of KR10 animals while 7/8 of KR1 and 9/13 of KR8 animals died by day 12 post challenge. Both the delay and lower rate of encephalitic death indicate protective responses in mice receiving KR10 orally. It is known that zosteriform protection involves antigen-specific CD4+ cell activity (Nash et al., 1987; Manickan et al., 1995b), and detection of DTH responses in KR10-immunized animals supports a role for CD4+ T cells. Additionally, the DTH reaction is mediated predominantly by Th1 cells, further supporting the role of CD4+ Th1 cells in mediating ICP27-induced protection (Cher & Mosmann, 1987). Thus it appears that development of T cell (Th1) responses against ICP27 occurs in the absence of humoral responses, as observed by Fouts et al. (1996) using gp120 expressed in S. typhimurium. The mechanisms of CD4+-mediated protection may include antiviral cytokines (Smith et al., 1994) and/or MHC II-restricted killing (Doymaz et al., 1991). Oral dosing of Salmonella attenuated vectors leads to acute phase gut-associated lymphoid tissue cytokine expression in the form of both Th1 and Th2 cytokines (Karem et al., 1996). These responses may greatly influence the subsequent immune induction against heterologous antigens used and enhance development of cellular responses. Along these lines, the challenge route used here was cutaneous while the immunization was mucosal (oral). Perhaps mucosal HSV challenge models will reveal additional attributes of mucosal vaccines. In addition, variations in responses to specific heterologous antigens may also depend on the antigen in question as not all proteins are equally immunogenic.

These data suggest that the use of Salmonella vectors holds potential for use as mucosal carriers of HSV antigens to induce protective prophylactic responses against HSV challenge. Successful utilization of Salmonella vectors for some antigens may require modifications such as secretion of recombinant proteins to improve T cell induction (Hess et al., 1996) or expression of multiple copies of peptide-encoding sequences. In fact, the latter approach has been successfully employed in recent work by Chabalgoity et al. (1996). These authors used an araA Salmonella vector expressing multiple copies of a peptide from HSV gD as a fusion to fragment C of tetanus toxin. This system resulted in a reduction in virus load in HSV-challenged mice following a single i.v. immunization. In addition, the use of cytokine therapy to enhance humoral and cellular responses against the antigen may strengthen the use of Salmonella vectors. In fact, the development of cytokine-expressing Salmonella vectors has been performed and constructs tested in vivo with encouraging results (Denich et al., 1993; Ianaro et al., 1995) and will most likely have a dramatic effect on the use of these vectors. In addition, multiple antigens expressed in one Salmonella strain or the use of synthetic multi-epitope genes could potentially afford greater protective responses. Experiments to analyse the effects of these modifications are in progress and may prove beneficial in the use of Salmonella vectors for HSV vaccine development.

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