INFECTION AND IMMUNITY, Oct. 2004, p. 5535–5547 Vol. 72, No. 10
0019-9567/04/$08.00+0 DOI: 10.1128/IAI.72.10.5535–5547.2004

MINIREVIEW

Enteric Pathogens as Vaccine Vectors for Foreign Antigen Delivery

Camille N. Kotton* and Elizabeth L. Hohmann

Infectious Diseases Division, Massachusetts General Hospital, Boston, Massachusetts

Live attenuated bacteria are attractive vaccine vectors that can elicit host immune responses to foreign antigens. Bacterial vectors may mimic natural infection and therefore interact with the mucosal, humoral, and cellular compartments of the immune system. A plethora of foreign (heterologous) antigens derived from bacterial, fungal, viral, or parasitic organisms have been expressed within bacterial vectors. Delivery of contraceptive and tumor antigens, cytokines, and adjuvants has also been explored. The result is a type of "vaccine factory" (29) in which the bacterial vector produces a foreign protein. Since bacterial vectors replicate within the host, it is hoped that they provide sustained exposure to the antigen, potentially augmenting the type and level of immune response. In addition, bacteria naturally possess immunostimulatory molecules such as lipopolysaccharide (LPS) and flagella that can stimulate immune responses (56). Internationally, there are three live bacterial vaccines commercially available and in clinical use: *Salmonella enterica* serovar Typhi Ty21a, *Vibrio cholerae* CVD 103-HgR, and *Mycobacterium bovis* BCG. These strains plus many other attenuated strains have been used as vectors to express heterologous antigens. Live bacterial vector vaccines have been evaluated in numerous animal and human studies, but there are currently no licensed live bacterial vector vaccines being used clinically.

The most compelling rationales for the development of live bacterial vectors are the possibility of oral delivery and protection at mucosal surfaces. This review will cover attenuated bacterial enteric pathogens used as vectors to express foreign (heterologous) antigens, with emphasis on those that have reached clinical study, including *Salmonella*, *Shigella*, *V. cholerae*, and *Listeria*. This review will not cover bacterial vectors used to deliver DNA vaccines or subunit and conjugate vaccines.

LIVE BACTERIAL VECTORS IN HUMANS

Live bacterial vectors offer many potential clinical advantages. They are easy and relatively inexpensive to produce; they are also well suited to large-scale manufacture and potentially stable without refrigeration (via lyophilization), are able to carry large or multiple antigens or adjuvants, and can be eradicated with antibiotics should the need arise. These attributes may make these vaccinations more available to the developing world. Compared to injectable vaccines, oral delivery should result in increased compliance, safety, and ease of administration. Eliciting mucosal, cellular, and humoral immunity is desirable for protection against many of the organisms that infect at mucosal surfaces, such as human immunodeficiency virus (HIV) and other important gastrointestinal, genital, and respiratory pathogens. Attenuation can be accomplished through the deletion of different genes, including virulence, regulatory, and metabolic genes. Some attenuated bacterial vectors have already been evaluated in humans, as discussed below.

Potential pitfalls of live bacterial vectors include possible systemic dissemination, transmission to contacts of the recipient, unanticipated or undesirable immune responses, and environmental contamination. Systemic dissemination and illness could occur in either healthy or immunocompromised individuals. Postinfectious reactions are possible, including arthritis, uveitis, and urethritis, that are similar to those seen after natural enteric infections such as campylobacteriosis (108) or salmonellosis (144), although these are not necessarily limited to live vaccines, as exemplified by a recent description of oculorespiratory symptoms after immunization with inactivated split virus influenza vaccine (125). Environmental contamination or inadvertent spread of the live bacterial strain necessitates evaluation of these possibilities during the development of live vectors.

STIMULATION OF THE IMMUNE SYSTEM

Parenteral vaccines elicit primarily a humoral (antibody) immune response (86). Although successful for a variety of systemic pathogens (111), it has been hoped that mucosal vaccines may provide a more physiological and effective protective immune response, since it is estimated that 90% of human infections are initiated at mucosal surfaces (73). Bacterial vaccine vectors induce the production of multiple cytokines, including tumor necrosis factor alpha (TNF-α), gamma interferon (IFN-γ), and interleukin-12 (IL-12), and proinflammatory mediators such as nitric oxide, which enhance early innate immunity and create a local environment favorable to antigen presentation (31). An ideal vector should stimulate both strong primary and lasting memory immune responses.

Mucosal immunity collectively accounts for more than two-thirds of the activity of the entire immune system, based on considerations of the numbers of immunocompetent cells, the extent of the mucosal tissues, and the quantities of immunoglobulins produced at these sites (114). Mucosal immunity is distinguished from systemic immunity by the abundance of secretory immunoglobulin A (IgA) and a dedicated cellular

* Corresponding author. Mailing address: Infectious Diseases Division GRJ504, Massachusetts General Hospital, 55 Fruit St., Boston, MA 02114. Phone: (617) 724-7532. Fax: (617) 726-7416. E-mail: ckotton@partners.org.

TABLE 1. Human studies of *S. enterica* vectors expressing a foreign antigen[a]

Serovar	Parent strain	Vaccine strain	Genetic mutation(s)	Foreign antigen expressed	No. of volunteers	No. of volunteers with IgG response to:		Reference
						Vector	Foreign antigen	
Typhi	Ty21a	5076-1C (different lots)	*galE*, *rpoS*, Vi antigen	*S. sonnei* LPS	60	NA	12/56	15
Typhi	Ty21a	5076-1C	*galE*, *rpoS*, Vi antigen	*S. sonnei* LPS	16	NA	3	58, 136
Typhimurium	ATCC14028	LH1160	*phoP*, *phoQ*, *purB*	*H. pylori* urease	6	5	3	3
Typhi	Ty21a	EX645	*galE*, *rpoS*, Vi antigen	*V. cholerae* LPS O antigen	10	7	3	43
Typhi	Ty21a	EX645	*galE*, *rpos*, Vi antigen	*V. cholerae* LPS O antigen (with challenge)	14	14	1	130
Typhi	Ty21a	EX645	*galE*, *rpoS*, Vi antigen	*V. cholerae* O antigen	61	NA	NA	5
Typhi	Ty21a	EX880	*galE*, *rpoS*, Vi antigen *rfb*	*V. cholerae* O antigen	80	NA	NA	5, 7
Typhi	Ty2	CVD908	*aroC*, *aroD*, *rpoS*	*P. falciparum* CSP	10	10	2	53
Typhi	Ty2	CVD908-htrA	*aroC*, *aroD*, *htrA*, *rpoS*	Tetanus toxin	20	4/9[b]	3	131
Typhi	Ty2	χ4632	*cya*, *crp*, *cdt*, *asd*, *rpoS*	Hepatitis B	40	24	0	132
Typhi	Ty2	χ4632	*cya*, *crp*, *cdt*, *asd*, *rpoS*	Hepatitis B (oral)	7	6	0	94
Typhi	Ty2	χ4632	*cya*, *crp*, *cdt*, *asd*, *rpoS*	Hepatitis B (rectal)	6	1	1	94

[a] Only IgG responses in serum are shown, since other immunological tests were not consistently performed and IgG responses in serum to vector and foreign antigen were usually determined. A denominator in the response column implies that not all volunteers had test results. These studies differ greatly with regard to methodologies and study designs, and this table is presented only as a compilation of study references without implied comparisons.
[b] Only data from highest inocula given.

response. In the gut lumen, the most important protective antibodies include those that inactivate soluble toxic protein products of bacteria (antitoxins), facilitate phagocytosis and intracellular digestion of bacteria (opsonins), interact with components of serum complement and lysozyme to damage the bacterial membrane with resultant bacteriolysis (lysins, as with gram-negative infections), prevent proliferation or invasion of infectious virus (neutralizing antibodies, as with polio), or interact with components of the bacterial surface to prevent adhesion to mucosal surfaces (anti-adhesins, as with *Escherichia coli* or *V. cholerae*) (91).

Development of immunity to *Salmonella* infections relies on the cellular, humoral, and mucosal arms of the immune system (88). Although the role of humoral immunity has been long appreciated, recent work has explored the specifics of cellular responses. Separate studies in primates with serovar Typhi Ty21a and with *Salmonella* vectors expressing simian immunodeficiency virus antigens show that $CD8^+$ cellular immunity is increased after immunization with *Salmonella*, specifically in the intestinal T-cell population that is positive for the intestinal homing receptor $\alpha_4\beta_7$ (39, 87), and "homes" to and resides within the intestinal compartment (Table 1). Other studies in humans with serovar Typhi Ty21a demonstrate that immunization elicits a specific $CD8^+$ cytotoxic-T-lymphocyte (CTL) response (122). Serovar Typhi CVD908-*htrA* also elicits both a $CD4^+$ and a $CD8^+$ cellular immune response in humans (123). Attenuated serovar Typhi vaccines delivered intranasally in mice also stimulate $CD8^+$ major histocompatibility complex (MHC) class I-restricted CTLs (103).

The intracellular pathogen *Listeria monocytogenes* also induces both mucosal and systemic immunity. When *L. monocytogenes* was used in early work in mice to express *E. coli* β-galactosidase, a $CD8^+$ cellular immune response to the foreign protein was found (124). A murine study showed that oral inoculation of *L. monocytogenes* expressing HIV-1-Gag induced mucosal and systemic CTL-mediated immunity to the viral antigen (107). In the only published human trial to date, humoral, mucosal, and cellular immune responses to attenuated *L. monocytogenes* were all detected in individual volunteers (4).

The type of immunity that is elicited by bacterial vectors depends on both the vector used and the "biological details" of foreign antigen expression. For example, *V. cholerae* is noninvasive and elicits mostly a secretory IgA and serum vibriocidal antibody response, whereas the more invasive salmonellae will elicit musosal, humoral, and systemic immunity, and *Listeria* tends to elicit a predominantly cellular response. Although it is presumed that the immunity a host engenders against the foreign antigen will be similar to that raised by the vector, this may not always be true, since host responses may be strongly influenced by factors such as method of foreign antigen expression and secretion.

EFFECT OF PREVIOUS EXPOSURE TO BACTERIAL VECTORS

Recipients of live attenuated vaccines may have had prior exposure to enteric pathogens that are used as vectors (sometimes this prior exposure is called "vector priming"). Whether this previous exposure increases or decreases the immune response to a vectored antigen remains to be determined and may be specific to vector and antigen. Preexisting immunity against the vaccine vector could enhance presentation of the foreign antigen to T cells by mechanisms such as enhanced uptake of antibody-coated recombinant bacteria by antigen-presenting cells, by altering the cytokine milieu during presentation, or by direct cell-cell interactions. Studies of vector priming in animals and humans have produced conflicting data (73).

Most work on vector priming in animals has been done in

BALB/c mice, a line with genetically determined susceptibility to intracellular pathogens. Using *S. enterica* serovar Dublin *aroA* vectors, two studies have demonstrated some improvement in response to foreign antigen delivered orally or systemically to vector-primed hosts (8, 141). Other groups, using *S. enterica* serovar Typhimurium *aroA* or the naturally attenuated *S. stanley*, concluded that prior exposure to the vector compromises both serum and intestinal antibody responses to a range of foreign antigens (6, 74, 112, 138, 139). Bouwer et al. showed that previous exposure of BALB/c mice to *L. monocytogenes* did not inhibit the development of a primary CTL response to an epitope delivered by an *L. monocytogenes* vector (17).

Human data on vector priming is also conflicting. When serovar Typhi Ty21a is given as a vaccine, the immune response inversely correlates with the preimmunization IgA anti-LPS antibody titer (86). Similarly, when serovar Typhi Ty21a was used to express *V. cholerae* O antigen in a large human study, volunteers with higher prevaccine titers against *V. cholerae* were less likely to develop significant bactericidal responses against *V. cholerae* after immunization (5). However, in a study of women who were orally and rectally vaccinated with serovar Typhi Ty21a, researchers found that *Salmonella*-specific antibodies in the human female genital tract induced by primary vaccination could be enhanced by subsequent rectal administration of these vaccines (82). In a recent trial with serovar Typhi Ty21a expressing *H. pylori* ureases A and B, one volunteer with prior immunity to the carrier appeared to have an enhanced immune response to the foreign antigen (19). Further human studies are needed to clarify the significance of vector priming.

BALANCE BETWEEN IMMUNOGENICITY AND ATTENUATION IN ATTENUATED BACTERIAL STRAINS

Balancing immunogenicity and attenuation of vectors may be difficult. Excessive attenuation of the bacterial vector can render it poorly immunogenic, as seen in our human study of Ty445, a serovar Typhi Ty2 strain with *phoP*/*phoQ aroA* deleted (only 2 of 14 volunteers receiving Ty445 seroconverted) (62). Inadequate attenuation may result in excessive reactogenicity or symptomatic infection, especially in immunocompromised hosts.

Vectors that seem highly attenuated in animals do not always result in a lack of reactogenicity in humans. In trying to improve upon Ty21a, Hone et al. constructed a *galE* mutant in serovar Typhi Ty2 (Ty2H1) which was serum sensitive and highly attenuated in the mouse hog gastric mucin virulence assay compared to the Ty2 parent strain (64). When four human volunteers ingested 7×10^8 CFU of this strain, two became ill and developed a typhoid fever-like illness with fever and bacteremia. The authors of that study concluded that the *galE* mutation alone did not adequately attenuate serovar Typhi Ty2 for human use. Similar results occurred with a *Shigella* vaccine strain CVD1203, derived from wild-type *S. flexneri* 2a by introducing deletions in chromosomal *aroA* and invasion plasmid *virG*. This strain was highly attenuated in the guinea pig conjunctival sac challenge (Serény) model. Two 10^9 CFU orogastric doses (2 weeks apart) stimulated production of secretory IgA antibodies to *S. flexneri* 2a and protected against conjunctival challenge with virulent *S. flexneri* 2a (99). When given to humans, a single dose of CVD1203 at 10^6, 10^8, or 10^9 CFU resulted in self-limited (<48-h duration), objective reactogenicity (fever, diarrhea, or dysentery) in 0, 18, and 72% of subjects, respectively, and in no placebo recipients. The majority of volunteers in each group did develop IgA-producing anti-LPS antibody-secreting cells (ASCs), but the reactogenicity of the vaccine was not predicted by the animal model (78) data.

The specific attenuation(s) chosen for a vaccine vector strain can have a significant impact on its immunogenicity (13, 27, 36, 134, 137, 142). For example, *Salmonella* PhoP-null mutants are more efficiently processed by macrophages in vitro than wild-type bacteria; salmonellae constitutively expressing PhoP (PhoPc) are processed even less efficiently than wild-type salmonellae (142). The altered processing and presentation efficiency is not due to differences in the level of antigen expressed by the bacteria or differences in the level of bacterial uptake by the macrophages. Paradoxically, *Salmonella* PhoPc mutants have been shown to be highly immunogenic vectors for delivery of papillomavirus antigens in mice (11, 95). Although the *Salmonella* PhoPc mutant was more efficient at infecting dendritic cells in vitro than the wild type and the *phoP* and *aroA* mutants, PhoPc mutants induced significantly less IL-10, suggesting that this mutant suppressed the secretion of IL-10 by dendritic cells and may result in a stronger cell-mediated immune response than the other attenuations (33). These data not only demonstrate a role for virulence loci in directing induction of specific immune responses (142) but also highlight the idiosyncratic nature of antigen-vector combinations.

Recent work with a panel of purine (*purEK* and *purHD*) and guanine (*guaAB*) auxotrophs in *S. flexneri* 5 was undertaken to maximize immunogenicity and minimize reactogenicity. The best vaccine candidate in mice was the *S. flexneri* 5 *guaAB purHD* strain, which was markedly attenuated but still induced proinflammatory cytokines and factors such as IL-1β, IL-6, tumor necrosis factor alpha, and inducible nitric oxide synthase. This strain upregulated cytokines such as IL-12 and IFN-γ and also highly stimulated MHC class II expression, suggesting activation of innate immunity (21). A detailed comparison of rationally designed attenuated mutants would be ideal, but a lack of good animal models makes vector development an empirical business requiring human clinical studies.

OPTIMIZING EXPRESSION: CODONS, PLASMIDS, AND CHROMOSOMES

Expression of foreign antigens in live bacterial vectors can be complicated. The possibility of improper protein folding, lack of posttranslational modification, degradation of foreign proteins by bacterial proteases, inclusion of toxic protein sequences, lack of proper secretion, or ineffective presentation to the immune system can lead to the induction of nonprotective immune responses or the complete abolition of immune responses. Choice of promoter, localization within the vector, construction of protein fusions to promote secretion, codon optimization for a bacterial host, and plasmid copy number can all affect foreign antigen expression.

Differences in codon usage between organisms can have a significant impact on heterologous protein production. A gene with a codon usage different from the targeted bacterial host

can have poor expression in that particular host. The presence of rare codons in cloned genes can affect protein expression level and mRNA and plasmid stability. In some cases, rare codons can inhibit protein synthesis and cell growth. Although more often cited for DNA vaccine work, codon optimization of tetanus toxin fragment C expressed by serovar Typhimurium *aroA* resulted in protection of orally immunized mice against an otherwise-lethal tetanus toxin challenge (40).

Foreign antigens may be expressed from a chromosomal integration or from a plasmid. Chromosomal integration of single gene copies generally increases the stability of the foreign antigen genes but reduces antigen mass compared to multicopy plasmids. A single chromosomal gene copy may be adequate when stable proteins are expressed from strong promoters in a highly immunogenic vector, as in the case of an *L. monocytogenes* strain expressing HIV gene products under the control of the hemolysin promoter (89). This *Listeria* construct was able to induce Th1-type HIV-Gag specific $CD4^+$ T cells in mice. Cells were of the Th1 phenotype and produced IFN-γ at levels similar to $CD4^+$ T cells directed against an endogenous listerial antigen. However, another group reported 50-fold less antigen production and no immune responses in mice after receipt of serovar Typhimurium bearing a chromosomal integration of the HIV-1 gp120 gene compared to a similarly attenuated strain expressing the same antigen from a multicopy *asd*-stabilized plasmid (44). Other investigators reported 10-fold-lower production of a fusion protein composed of the cholera toxin B subunit (CT-B) and the serine-rich *Entamoeba histolytica* protein in vitro in a *V. cholerae* chromosomal integration strain compared to their *glnA* balanced lethal plasmid strain (121). Decreased antigen expression correlated with a decreased immune response in mice. In a different study, these authors reported 100- to 1,000-fold-greater expression of the foreign antigen from a plasmid-bearing strain compared to the chromosomal integration strain, as well as a greater immune response in mice (118); another group showed similar findings with *E. coli* mutant heat-labile enterotoxin in serovar Typhimurium in mice (27).

For ease of manipulation and copy number effects, plasmids are often used in bacterial vectors. An optimal plasmid is stable in vivo, has a copy number which supports vigorous antigen production but does not compromise the growth rate or metabolism of the vector (48), is nonconjugative, and has a narrow host range. Antibiotic resistance genes are in general proscribed in clinical products. Attempts to optimize plasmid-based expression systems have resulted in several clever ideas that are discussed below.

PLASMID STABILITY

To be effective, an antigen-encoding plasmid must be retained by the vector microorganism. Although antibiotic pressure works well for plasmid maintenance in vitro, this selective pressure is not present in vivo. Balanced lethal host-vector systems ensure that plasmid is maintained in the bacterial vector by requiring that the proteins expressed by the plasmid are essential for survival of the vector. A classic example is the *asd* system. A deletion of the chromosomal aspartate-semialdehyde dehydrogenase (*asd*) gene is made in the bacterial vector, resulting in an absolute requirement for diaminopimelic acid, an essential component of the peptidoglycan of the cell wall of gram-negative bacteria that is not found in mammalian hosts. The *asd* gene is supplied on a plasmid and complements the mutation. In the original work describing this system in serovar Typhimurium, the plasmid was stable in the absence of any exogenous selective pressure in vitro or in vivo (30). The system has since been used to express varied foreign antigens in both murine and human studies (94). A similar system is the *glnA* balanced lethal system in *V. cholerae* (121).

The *hok-sok* system, initially developed for use in serovar Typhi, is similar to the balanced lethal system but does not require genetic manipulation of the vector organism. It was developed for use in serovar Typhi, since the *asd*-based plasmids, successfully used in serovar Typhimurium, were less successful at eliciting immunity to the foreign antigen in these strains (94, 132). This two-component toxin-antitoxin system or "killer locus" is based on the naturally occurring *hok-sok* postsegregational killing system on the *E. coli* antibiotic resistance factor pR1. Synthesis of Hok (a lethal pore-forming protein) is blocked by binding of a *sok* mRNA to *hok* mRNA. However, *sok* mRNA is highly susceptible to degradation, and its intracellular concentration must be maintained by constitutive transcription from plasmids carrying *hok-sok*. Thus, bacteria that lose these plasmids are postsegregationally killed when the levels of the protective *sok* mRNA rapidly drop, and the levels of the more stable toxin-encoding *hok* mRNA quickly lead to Hok synthesis and cell death (52). Unfortunately, some work showed that >50% of bacteria that lost the plasmid were still viable, suggesting that the presence of a *hok-sok* postsegregational killing system is not sufficient to ensure vector death (49). The highest levels of sustained synthesis of the test heterologous antigen were detected from expression plasmids carrying the full complement of maintenance functions, including at least one partition function that mediates pairing of plasmids at mid-cell and subsequently moves them to the cell poles before cell division. Although less successful than the *asd* balanced lethal system, this system is conceptually appealing. Other plasmid-based mechanisms that result in carefully choreographed, time-limited antigen secretion and/or programmed vector death can also be envisioned.

HOST FITNESS AND PLASMID BURDEN: POOR HOUSEGUESTS?

Some part of a bacterial vector's metabolism is usurped by maintenance of an antigen-bearing plasmid, potentially compromising the vector's growth rate, fitness, persistence, and immunogenicity. Studies with *E. coli* have clearly established that plasmid-bearing bacteria grow more slowly than plasmid-less bacteria (16, 90). Since mucosal vaccines are sometimes designed to not replicate extensively in vivo, this is not always detrimental. Plasmid copy number has a significant impact on the survival and success of the bacterial vector. As the copy number increases, the toxicity to the vector increase as well (49) and the growth rate of such strains decreases. Similarly, as expression of heterologous genes increases, for example, by manipulation of promoter strength or promoter induction, the growth rate decreases further (14, 104, 143). Enterotoxigenic *E. coli* (ETEC) antigens expressed in *Shigella* resulted in a slowing of the intracellular growth rate and a decrease in

invasiveness in HeLa cells (1). Covone et al. found that a serovar Typhimurium strain expressing mutant *E. coli* heat-labile enterotoxin with a high-copy-number plasmid underwent lysis in stationary phase, a phenomenon which was not observed with a medium-copy-number plasmid (27). Thus, there is a fine balance between adequate antigen production, toxicity, invasiveness, and immunogenicity.

LOCATION, LOCATION, LOCATION

Expression of a foreign antigen on the outer surface of a bacterial vector or secretion of an antigen into the extracellular milieu (51, 59, 71) appears to be immunologically advantageous in many instances. Recent work in mice with serovar Typhimurium expressing the recombinant *Streptococcus pneumoniae* antigen PspA compared expression in the cytoplasm with secretion into the periplasm and culture supernatant. There was a 10^4 increase in IgG titer in animals that received the strain bearing a secreted antigen (70).

Foreign antigen genes may be fused with those of naturally occurring secreted bacterial protein(s). A popular system is the hemolysin A (HlyA) export-expression system of uropathogenic *E. coli*. Heterologous antigens are inserted into a truncated version of the HlyA protein and secreted by the hemolysin type I secretion apparatus. More than a dozen foreign antigens have used this secretion system successfully (50, 117).

The Lpp-OmpA system can successfully target foreign antigens to bacterial outer membrane. A fusion between the signal sequence and first nine N-terminal amino acids of the mature major *E. coli* lipoprotein (Lpp) and five transmembrane segments of the outer membrane protein A (OmpA) effectively exposes homologous and heterologous proteins on the external side of the *E. coli* outer membrane in vitro (45–47). This system has been used in attenuated serovar Typhimurium to express an immunodominant epitope of HIV transcriptase, resulting in mucosal IgA and cellular immune responses in mice (20).

The type III secretion system of *Salmonella* and *Yersinia* spp. has also been exploited for foreign antigen delivery (39, 55, 116). Upon bacterial contact with a eukaryotic cell, the *Salmonella* type III secretion system creates a hypodermic needle-like apparatus that spans the bacterial envelope and "injects" proteins from the bacteria into the host cell. Type III secretion systems have been used as delivery mechanisms for vaccine antigens because effector proteins such as SopE directly access MHC class I-restricted, cytosolic antigen-processing pathways. SopE fusion proteins with H-2-restricted influenza virus epitopes expressed by *Salmonella* can effectively present influenza virus antigens to MHC class I-restricted T-cell hybridoma cells in vitro (3, 39, 116). *Salmonella* vectors expressing SopE fusion proteins with lymphocytic choriomeningitis virus (LCMV) epitopes protected mice from fatal intracerebral challenge with LCMV (116).

The autotransporter secretion pathway of gram-negative bacteria has been used for the surface display of antigenic determinants (81). Autotransporters are expressed as a single polypeptide chain containing all of the features necessary to translocate an N-terminal passenger domain to the cell surface, which makes them attractive candidates for antigen display. For example, the autotransporter domain of the *E. coli* adhesion AIDA-I (for adhesin involved in diffuse adherence) was fused to a *Yersinia* heat shock protein and expressed in *E. coli* and serovar Typhimurium. This vaccine strain had high genetic stability in vivo and resulted in a pronounced cellular immune response in mice (81) vaccinated intragastrically. In a similar study, the same autotransporter domain was used to express both nearly full-length *H. pylori* UreA and defined T-helper-cell epitopes on the surface of attenuated serovar Typhimurium, which were much more protective vaccines in subsequent challenge experiments than a similar vaccine strain that expressed UreA in the cytoplasm (110).

The ice nucleation protein of *Pseudomonas syringae* is another cell surface display system that has been used for display of foreign antigens (84). The ice nucleation protein is an outer membrane protein that accelerates ice crystal formation in supercooled water and has been used to express large proteins at the cell surface. When used to express the foreign antigens hepatitis B surface antigen and the core protein of hepatitis C in serovar Typhi strain Ty21a, this expression system was able to elicit a much higher antibody response in mouse serum than a system that expressed antigens intracellularly (84).

Other methods to improve presentation to the immune system include fusions to the *E. coli* heat-labile LT-B (66), the MalE export signal (72), the *Salmonella* autotransporter MisL (113), and insertion of antigen into several cell surface proteins, including the main *Salmonella* flagellar component FliC (96) and the *E. coli*-derived P87 fimbriae (24), LamB (57), TolC (128), and PhoE (67).

PROMOTERS

Choice of promoter can have a significant impact on the amount of antigen produced and on the immunogenicity of the vaccine. In early studies, strong constitutive promoters were often used with the expectation that "more is better." Since constant antigen synthesis may result in decreased vector fitness, increased attenuation, and decreased immunogenicity (29), some recent work has focused on promoters that are in vivo inducible, such as the anaerobically inducible *nirB* (23) and *dmsA* (101) promoters. A recent comparison of seven different promoters in *Salmonella* used flow cytometry to quantify in vivo antigen levels and to simultaneously monitor the early steps of antigen-specific T-cell responses in mice. The present study demonstrated that in vivo expression levels for an antigen can be rationally selected, perhaps allowing one to fine-tune and time the expression of foreign antigens (18) to direct specific immune responses. The *pagC* promoter was found to have especially high in vivo activity with low in vitro activity, which may explain why it has generated strong humoral immune responses in prior studies (18). John et al. evaluated the strong constitutive *tac* promoter, the in vivo-induced *htpG* promoter, and the in vivo-induced iron-regulated *irgA* promoter to express CT-B in *V. cholerae* strain Peru 2 (69). In vitro expression was greatest under the control of the *tac* promoter and the *irgA* promoter when under low-iron conditions. Interestingly, although the gut is a low-iron environment, the immune response in mice was much higher when the *tac* promoter was used. The failure of in vitro testing to predict in vivo responses is not unique to live bacterial vectors, but the

complexity of living vectors interactions' with mammalian hosts adds to the challenge of developing this type of vaccine.

ALTERNATIVE DELIVERY SITES

Although the majority of mucosal vaccines have been delivered orally, other mucosal sites, such as the nares, vagina, and colon, could also be used as delivery sites. Inoculation at one mucosal surface can confer immunity at other sites (56, 68, 82, 94), and the site of administration can have a significant impact on the type of immune response (68). Some studies evaluating live vectors for cancer immunotherapy have used intravenous administration of attenuated bacteria (22). Intranasal administration of mucosal vaccines has been popular in multiple animal models. Some studies report higher antibody titers via the intranasal route compared to oral inoculation (98). To our knowledge, there have been no published human trials in which live attenuated bacteria have been administered intranasally. Given the proximity of the central nervous system to the nares, complications of direct spread of an infection into the brain, i.e., "vector sinusitis," could be of concern and might not be predictable from animal models. Although not a live vaccine, a recent inactivated intranasal influenza vaccine given with *E. coli* heat-labile toxin as a mucosal adjuvant was associated with Bell's palsy in humans, resulting in the vaccine being removed from the market (93).

Rectal vaccination has also been evaluated for attenuated enteric pathogens as vaccine vectors, with the hope that this route might be more immunologically relevant for important sexually transmitted pathogens. In 1996, Nardelli-Haefliger et al. showed for the first time that it was possible to induce specific secretory IgA in the genital and rectal tracts of women by using an attenuated serovar Typhi χ4632 *cya crp-cdt asd* expressing a gene encoding a hepatitis B virus core-pre-S protein vaccine strain given orally or rectally (94). These investigators found that the secretory IgA response was predominantly against the vector rather than the foreign antigen and that oral immunization was more effective than rectal immunization in inducing a systemic humoral response: while oral immunization induced seroconversion against *Salmonella* LPS in six of seven volunteers, only one of six women developed anti-LPS and anti-pre-S1 after rectal immunization. In another study of 26 women who underwent oral and/or rectal immunization with Ty21a, *Salmonella*-specific IgG and IgA antibodies were measured periodically in vaginal lavage and cervical mucus. *Salmonella*-specific antibodies (especially IgA) were significantly increased in vaginal fluids and cervical mucus in seven women who were given an oral priming dose followed by rectal booster at 6 months (82). These results suggest that specific antibodies in the human female genital tract induced by primary oral vaccination can be boosted by subsequent rectal administration of vaccines. Human studies with CT-B-subunit conducted by Holmgren and coworkers (68) showed that a combination of nasal and vaginal immunization was the most effective way to induce genital tract IgA; results obtained with this unique adjuvant molecule may not extend generally to bacterial vectors.

SPECIFIC ENTERIC PATHOGENS USED AS VECTORS

Salmonellae. Salmonellae were among the first bacteria used as recombinant vectors for antigen delivery (29). In their seminal study in 1981, Formal et al. demonstrated that live, attenuated serovar Typhi Ty21a expressing a *Shigella* surface antigen protected mice against subsequent serovar Typhi and *S. sonnei* challenges (42). Salmonellae are the most commonly studied vectors, perhaps in part because of the ease with which they are genetically manipulated, the existence of the serovar Typhimurium BALB/c ($NRAMP1^{-/-}$) mouse model for preclinical work, and favorable prior human experience with the live attenuated vaccine U.S. Food and Drug Administration-approved vaccine for typhoid fever, Ty21a. Ty21a serves as an important safety benchmark for researchers undertaking clinical trials. Ty21a was created from Ty2, which has been maintained in laboratories since its isolation in 1918. Ty2, which is known to have a frameshift mutation in *rpoS* (28), underwent chemical mutagenesis with nitrosoguanidine to create Ty21a. Ty21a is known to be Vi antigen negative and harbors a *galE* mutation that results in altered biosynthesis of smooth LPS. Other mutations are suspected but not identified thus far; sequencing of Ty21a is currently under way (D. J. Kopecko and D. Q. Xu, unpublished data). Because Ty21a requires three or four doses to induce a significant protective immunity in two-thirds of people immunized (86), work has focused on developing a live attenuated serovar Typhi derivative that survives longer in the gut to trigger both a priming and booster immune response with a single dose. Recent work has shown that immunization of human volunteers with Ty21a elicits specific $CD8^+$ CTL (87, 103, 122) and specific circulating mucosa-homing $CD4^+$ and $CD8^+$ T cells, almost all of which express the gut-homing integrin $\alpha_4\beta_7$ (87).

Serovar Typhi Ty21a has been used for the delivery of foreign antigens (42, 58). When *Helicobacter pylori* urease was expressed in serovar Typhi Ty21a (19), 10 of 12 human volunteers developed humoral immune responses to the *Salmonella* carrier, as detected by examining antigen-specific ASCs, but only two volunteers seroconverted to serovar Typhi LPS, as measured by enzyme-linked immunosorbent assay (ELISA). Five volunteers had positive responses in at least one of three assays for cellular responses to the vector (i.e., lymphoproliferation, IFN-γ secretion, and IFN-γ enzyme-linked immunospot [ELISPOT] assays), and three volunteers showed a weak but significant T-cell response to *H. pylori* urease. No volunteer had detectable humoral responses to urease.

Since Ty21a was constructed by general chemical and UV mutagenesis techniques (i.e., the strain contains uncharacterized mutations) and requires multiple doses, recent serovar Typhi vaccine development efforts have focused on creating defined mutations that retain immunogenicity but allow for a more effective attenuation. Common attenuations in serovar Typhi include mutations that render the bacteria auxotrophic for nutrients that are scarce or absent in mammals (e.g., *purA* or *aro* mutations), mutations that alter important regulatory genes (e.g., *cya*/*crp*, *phoP*/*phoQ*, and *rpoS*), mutations in secretory function (e.g., *ssaV*), and temperature-sensitive mutations (e.g., *ts*) (56, 60). Often more than one defined deletion mutation has been introduced to minimize the chance of reversion to a wild-type virulent phenotype.

Defects in the aromatic amino acid synthesis pathway (including the *aroA*, *aroC*, and *aroD* genes) were one of the first attenuating mutations investigated, after an *aroA* mutant of serovar Typhimurium was shown to elicit protective immunity against fatal salmonellosis in a mouse model (63). Human studies of serovar Typhi Ty2 *aroC aroD* (CVD908) expressing the *Plasmodium falciparum* circumsporozoite protein (CSP) provided the first report of attenuated serovar Typhi eliciting a human serologic or a CTL response to a foreign protein. This investigational strain was well tolerated by 10 volunteers given two doses of 5×10^7 organisms 8 days apart. All 10 subjects developed serologic responses to O and H antigens of the live serovar Typhi vector, whereas three vaccinees responded to the foreign antigen: one developed an 80-fold rise in serum anti-sporozoite antibody, another had a 4-fold rise in antibody to a recombinant portion of CSP, and a third developed CSP-specific $CD8^+$ CTL activity. Unfortunately, positive blood cultures and fevers were seen in some volunteers when early *aro* strains were administered to humans (65). Introduction of another mutation in the stress response gene *htrA* (133) appears to have eliminated this problem. The serovar Typhi strain CVD 908-*htrA*, with *aroC aroD* and *htrA* deleted, has been used in multiple human and animal studies. CVD908-*htrA* was used as a vector to deliver nontoxic fragment C of tetanus toxin to human volunteers. Although hampered by preexisting immunity to tetanus in healthy adults, increases in the levels of IgG antitoxin in serum were elicited in three of nine volunteers who received $\geq 10^8$ CFU doses of CVD908-*htrA*(pTETlpp) construct (131). Subjects who received the *S. enterica*-expressing C fragment had a decreased cellular and humoral response to serovar Typhi antigens compared to volunteers who ingested the parent strain without foreign antigen.

The *phoP*/*phoQ* locus provides an interesting target for vaccine design. This two-component regulatory locus is necessary for serovar Typhimurium survival within macrophages, defensin resistance, acid resistance, and murine typhoid fever pathogenesis (92). Serovar Typhi Ty2 *phoP*/*phoQ* mutants may have a more favorable side effect profile because of these phenotypes. Single doses of serovar Typhi Ty2 *phoP*/*phoQ* (Ty800) induced high-level immune responses in humans with a single dose and without adverse effects other than diarrhea in a small number of volunteers (61). Serovar Typhi Ty2 *phoP*/*phoQ* (Ty800) was engineered to express *H. pylori* urease from a *purB*-based balanced lethal plasmid system and evaluated in a human study. The majority of volunteers developed mucosal immunity (eight of eight by IgA ELISPOT) and humoral immunity to the vector (seven of eight by serum ELISA), but no volunteer had detectable mucosal or humoral immune responses to the urease antigen after immunization with single doses. A subset of three volunteers received an oral booster vaccination consisting of recombinant purified *H. pylori* urease A/B and *E. coli* heat-labile toxin B subunit 15 days after immunization with the live vector. None of the three volunteers had detectable humoral or mucosal immune responses to urease (32). In subsequent work, an identically engineered serovar Typhimurium *phoP*/*phoQ purB*-expressing urease was given to human volunteers (3). Five of six volunteers seroconverted to serovar Typhimurium antigens and had strong evidence of anti-*Salmonella* mucosal immune responses by ELISPOT assay. Three of six (three of five who seroconverted to *Salmonella*) had immune responses in the most sensitive assay of urease-specific immunoglobulin production by blood mononuclear cells in vitro. Although not compared contemporaneously, attenuated serovar Typhimurium appeared more effective than identically attenuated serovar Typhi in engendering immune responses to urease. The data suggested that this could be due to a greater stability of antigen-expressing plasmid in serovar Typhimurium and/or prolonged intestinal colonization. Specific factors unique to nontyphoidal salmonellae may also have been important for stimulation of the gastrointestinal immune system, and raise the interesting question as to whether attenuated nontyphoidal salmonellae can be utilized as vectors for human vaccines.

Although most *Salmonella* vaccine strains have mutations in metabolic or regulatory genes, Hindle et al. (60) mutated a specific type III secretion system virulence factor *ssaV* to attenuate their strains of serovar Typhi and serovar Typhimurium. Both strains were well tolerated at escalating doses. The serovar Typhi strain provided better immune responses than the serovar Typhimurium strain, with six of nine volunteers developing anti-serovar Typhi LPS IgA ASC responses and three of three vaccinees receiving doses of 10^8 and two of three receiving 10^9 CFU, which elicited high-titer LPS-specific IgG levels in serum. Only volunteers immunized with 10^9 CFU of the serovar Typhimurium strain mounted detectable anti-serovar Typhimurium LPS-specific ASC responses, and serum antibody responses were variable. These data indicate that mutations in type III secretion systems may provide a route to the development of live vaccines in humans.

Shigellae and hybrids. Shigellae spread cell to cell and do not usually result in deeper tissue spread and/or septicemia. Like listeriae, once internalized and free from the primary vacuole, shigellae move and reproduce in the host cytosol. This allows bacterial proteins to directly bind MHC class I molecules not normally available to intravacuolar bacteria. This results in the generation of $CD8^+$ cells, which are necessary to control intracellular pathogens and viruses (56).

There are four clinically significant *Shigella* serogroups. Only *S. dysenteriae* serotype 1 causes severe dysentery due to Shiga toxin production. *S. flexneri* and *S. sonnei* are the most common agents of endemic shigellosis in the developing world, whereas *S. sonnei* is the most common serogroup found in industrialized countries (80). *S. boydii* and *S. dysenteriae* are less common agents of shigellosis. Limited animal models (predominantly the Serény guinea pig conjunctivitis model and primates) make preclinical studies of *Shigella*-vector vaccines difficult (56).

S. flexneri 2a is the most commonly used strain in vaccine work and, to our knowledge, is the only one that has been used to express foreign antigens. Thus far, there have been no published human trials involving *S. flexneri* expressing foreign antigens. *Shigella* strains have been attenuated by introducing mutations that alter the cytosolic proliferation (*aroA*, *aroD*, *thyA*, *pur*, *iuc*, and *guaBA*), the ability to spread from cell to cell (*kcpA* and *virG*/*icsA*) (56), or the ability to produce enterotoxin (*sen* and *set*). Multiple mutations have been combined to try to maximize delivery of antigen to mucosal lymphoid tissues but minimize tissue damage and diarrhea. This has been difficult, perhaps in part because of the low infectious dose and high virulence of this organism.

The most experience has been with the CVD series, derived from *S. flexneri* 2a strain 2457T. *aroA* and *virB*/*icsA* are deleted in CVD1203, and CVD1203 has been shown to be immunogenic by stimulating secretory IgA antibodies and protective in the conjunctival sac challenge (Serény test) in guinea pigs (99). When used to deliver ETEC colonization factor antigen I (CFA/I) and colonization factor antigen CS3 in guinea pigs and mice, CVD1203 was able to elicit high titers of anti-*Shigella* LPS secretory IgA and anti-CS3 secretory IgA, as well as a strong serum IgG response against the ETEC antigens (but not against *Shigella* LPS) (98). In humans, CVD1203 (without a foreign antigen) was immunogenic, as measured by IgG LPS by ELISA and IgA-producing anti-LPS ASCs, and yet this strain was excessively reactogenic at higher doses (self-limited, objective reactogenicity [fever, diarrhea, or dysentery] developed in 0, 18, and 72% of subjects receiving, respectively, 10^6, 10^8, or 10^9 CFU) (78), indicating a need for further attenuation.

CVD1204 is the most extensively evaluated *Shigella* vaccine strain. It is a *guaBA* attenuated strain of *S. flexneri* 2a and thus has limited proliferation capacity in vivo due to the inactivation of purine metabolic pathway enzymes. It has been used to deliver foreign antigens in guinea pigs, including fragment C of tetanus toxin (2) and different ETEC antigens (1, 9, 75). CVD1204 was used to express fragment C of tetanus toxin either as a polypeptide expressed in the bacteria or as a DNA vaccine. Guinea pigs immunized intranasally with either recombinant strain mounted a IgG response in serum against fragment C of tetanus toxin and a secretory immune response against *S. flexneri* 2a LPS. The animals were protected against ocular challenge with wild-type *S. flexneri* 2a, demonstrating the ability of CVD1204 to serve both as a vector for foreign antigen delivery and as a mucosal *Shigella* vaccine (2).

With a goal of creating a multivalent *Shigella*-ETEC vaccine, a series of ETEC antigen-containing CVD1204 strains were created (1, 9, 75). The first strains expressed both ETEC CFA/I and one of two mutant heat-labile toxins (75). These strains elicited significant humoral immunity against *Shigella* and CFA/I but less immunity against either heat-labile toxin in guinea pigs. Challenge studies with wild-type *S. flexneri* 2a demonstrated protection in all animals. The present study demonstrated the feasibility of expressing multiple ETEC antigens on a single plasmid and the ability to raise immunity against both the vectored antigen and the bacterial host (75). Subsequent work with CVD1204 to express ETEC *E. coli* surface antigens CS2 and CS3 showed similar results (1). Finally, when guinea pigs were intranasally inoculated with five different CVD1204 strains containing plasmids expressing the ETEC colonization factors CFA/I, CS2, CS3, CS4, and LThK63, respectively, serum IgG and mucosal IgA immune responses were observed against each ETEC antigen, as well as the *Shigella* vector (9). The goal is to make a vaccine that is protective against both *Shigella* spp. and ETEC; Barry et al. propose using a mixture of five different *Shigella* strains, each expressing one or more ETEC antigens.

Strain SC602 (*S. flexneri* 2a *virB*/*icsA iuc iut*) is attenuated both in its capacity to move intra- and intercellularly and in its survival in tissues. SC602 has been found to be safe and protective in the macaque monkey model and in human volunteers. Seven volunteers who had been vaccinated 8 weeks earlier with a single dose of 10^4 CFU and 7 control subjects were challenged with 2×10^3 CFU of virulent *S. flexneri* 2a organisms (26). Six of the control volunteers developed shigellosis with fever and severe diarrhea or dysentery, whereas none of the vaccinees had fever, dysentery, or severe symptoms. Three vaccinees had mild diarrhea; these subjects had lower antibody titers than did the asymptomatic volunteers. SC602 is an example of an attenuated *S. flexneri* 2a candidate vaccine that provides protection against shigellosis in a stringent, human challenge model. In mice, SC602 has been successfully used to express a foreign antigen (the C3 neutralizing epitope of the poliovirus VP1 protein within the IpaC carrier protein) and to induce a serum and local antibody response (10). Significant mouse-to-mouse variability of the IgG immune response to C3 and IpaC was seen, and it was not felt that this provided an optimal mucosal vaccine.

Additional strains of *S. flexneri* 2a have been attenuated, but none have been used for foreign antigen expression thus far. CVD1205 is a *guaBA virG*/*icsA* deleted strain that has been evaluated in guinea pigs (97). CVD1207 is a *virG*/*icsA sen set guaBA* attenuated strain that has been evaluated in humans (79). CVD1208 is a *sen set guaBA* attenuated strain that has been used as a DNA vaccine vector in an animal model (102).

An epithelial cell-invasive *E. coli* strain, K-12, was used as a carrier for *Shigella* antigens and tested in humans. This live, oral *Shigella* hybrid vaccine was constructed by transfer of the 140-MDa invasiveness plasmid from *S. flexneri* 5 and the chromosomal genes encoding the group- and type-specific O antigen of *S. flexneri* 2a to *E. coli* K-12 (76). Designated EcSf2a-1, this vaccine produced adverse reactions (fever, diarrhea, or dysentery) in 4 (31%) of 13 subjects who ingested a single dose of 1.0×10^9 CFU, while at better-tolerated doses (5.0×10^6 to 5.0×10^7 CFU), it provided no significant protection against challenge with *S. flexneri* 2a. A further-attenuated *aroD* mutant derivative, EcSf2a-2, was then developed. Rhesus monkeys that received EcSf2a-2 tolerated the vaccine well, and the vaccinated monkeys were protected against shigellosis after challenge with *S. flexneri* 2a (60% efficacy). In humans, EcSf2a-2 was well tolerated at lower inocula. After a single dose of 2.5×10^9 CFU, however, 4 (17%) of 23 subjects experienced adverse reactions, and after a single dose of 1.8×10^{10} CFU, 2 of 4 volunteers developed dysentery. Recipients of three doses of 1.2 to 2.5×10^9 CFU showed significant increases in the levels of antibody to LPS (61%) in serum and invasiveness plasmid antigens (44%) and in gut-derived IgA ASCs specific for LPS (100%) and invasiveness plasmid antigens (60%). Despite its immunogenicity, the vaccine conferred only 36% protection against illness induced by experimental challenge. In subsequent work with 21 adults, four doses of 7×10^8 CFU EcSfla-2 spaced over 17 days were well tolerated and elicited an ASC response to *Shigella* LPS in all subjects but resulted in a vaccine efficacy of only 27% after experimental challenge with virulent *S. flexneri* 2a (77).

***V. cholerae*.** Unlike many of the other enteric pathogens, *V. cholerae* does not invade the mucosa. *V. cholerae* colonizes the surface of the small intestine and secretes cholera toxin, resulting in potentially severe diarrhea. Natural disease induces a Th2-type immune response, and recent data suggest more of an inflammatory response than previously understood (106). The fact that it adheres to M cells and other epithelial cells

without further invasion may be viewed as a strength (decreased pathogenicity) and a weakness (decreased interaction with deeper immune compartments (140) from the perspective of vector development. Cholera might be best suited for delivery of antigens relevant to luminal organisms rather than infections that invade systemically and/or require a vigorous cellular immune response for clearance.

Many attenuated strains of *V. cholerae* have been evaluated in human studies, but few have shown low reactogenicity and protective immunity. CVD 103-HgR is a derivative of *V. cholerae* O1 classical strain 569B that does not express the enzymatically active subunit of cholera toxin, CtxA, and contains a mercury resistance gene (*hgR*) that permits identification of the vaccine strain; in volunteer studies, it provided 62 to 100% protection against cholera caused by classical and El Tor *V. cholerae* O1 for at least 6 months (119) but was poorly protective in a field trial involving 67,500 people in Indonesia (109). Peru 15 is a well-tolerated and immunogenic oral cholera vaccine derived from *V. cholerae* O1 El Tor Inaba that affords protective efficacy against life-threatening cholera diarrhea in a human volunteer challenge model (25). *V. cholerae* 638 (El Tor, Ogawa), a CTXϕ-negative hemagglutinin/protease-defective strain was safe and immunogenic in a trial of 42 volunteers (12); 638T, the thymidine auxotrophic version of 638, is nonproliferative and may offer vaccination without the environmental contamination of a proliferative strain (135). Additional live attenuated *V. cholerae* O1 El Tor and *V. cholerae* O139 vaccines are in various stages of analysis (119). Many of these vaccines have undergone removal of the entire cholera toxin genetic element (the filamentous phage Ctxφ), including the *attRS*1 site (the site of reintegration), by molecular genetic techniques. This last modification decreases the likelihood that the vaccine strains will reacquire Ctxφ in the environment and become toxigenic again. In an attempt to decrease reactogenicity, a number of these strains are also deficient in motility.

V. cholerae has been used to express a variety of foreign antigens in animal models (56, 73), including *Shigella* O polysaccharide (41), heat-labile enterotoxin of *E. coli* (120), *Clostridium difficile* toxin A (117), the serine-rich *E. histolytica* protein (118), and the spherule-associated antigen 2/proline-rich antigen (Ag2/PRA) of *Coccidioides immitis* (126). There are no published human trials with *V. cholerae* expressing foreign antigens. Recent work with foreign antigens in *V. cholerae* has examined their export to the cell surface or to the extracellular milieu (73). Protein fusions between foreign antigens and the nonenzymatic CT-B are able to induced high-titer immune responses against the foreign epitope in mice (118). This system is limited to smaller epitopes that do not interfere with the function of CT-B (73). To enhance the capacity to secrete large foreign antigens, the *E. coli* HlyA secretion system has been used successfully in *V. cholerae* to express toxin A of *C. difficile* genetically fused to HlyA (117). Other work with foreign antigens in *V. cholerae* has focused on optimizing the promoter used to express the foreign antigen (69), comparing expression from a plasmid with expression from a chromosomal integration (118, 121), and developing a balanced lethal plasmid system (121).

Although not live vaccines, *V. cholerae* organisms have recently been used as "bacterial ghosts," a novel vaccine delivery system with intrinsic adjuvant properties that may become an effective vaccine delivery vehicle (38). Bacterial ghosts are nonliving bacterial cell envelopes devoid of cytoplasmic contents that maintain their cellular morphology and native surface antigenic structures. Since both native and foreign antigens can be expressed in the envelope complex of ghosts, multiple antigens of various origins can be presented to the immune system simultaneously.

Listeriae. Similar to salmonellae, listeriae invade the intestinal epithelium and disseminate to systemic lymphoid tissues. Rather than entering into the gut-associated lymphoid tissues via M cells and Peyer's patches, however, *L. monocytogenes* invades by binding the bacterial surface protein internalin with E-cadherin on eukaryotic enterocytes (83). Like *Shigella*, *Listeria* spreads from cell to cell by propelling itself with host filamentous actin. It escapes a membrane-bound vacuole and synthesizes and secretes proteins within the cytoplasm, which can then be processed by the MHC class I pathway of the infected cell. Both $CD4^+$- and $CD8^+$-T-cell responses are needed for clearance of a listerial infection in mice. Peptides derived from *L. monocytogenes* in the phagolysosome can be presented via both MHC class I and class II pathways and thus induce both $CD4^+$- and strong $CD8^+$-T-cell responses (105).

L. monocytogenes is an attractive vaccine vector for viral antigens since it can stimulate a $CD8^+$ response to foreign antigens. Antigens studied in mice LCMV nucleoprotein, influenza virus NP, HPV-16, and HIV Gag (4, 56). In addition, *L. monocytogenes* has been shown to elicit a $CD4^+$ response to a *Leishmania* antigen in mice (127) and to HIV-1 Gag in mice (89). Since the bacteria replicate within the cytoplasm without coming into contact with the extracellular environment, *L. monocytogenes* does not elicit a strong humoral immune response and thus may be better used to prevent diseases that do not require significant humoral immunity. *Listeria* has also been evaluated as an anti-tumor vaccine and as a DNA vaccine vector (73).

Although it is a pathogen commonly found in many foods, *Listeria* is a rare cause of disease in immunocompetent hosts; it is more likely to cause disease in immunosuppressed individuals and pregnant women. Mutations have been introduced to limit bacterial spread, including *actA* (actin nucleator, necessary for cell to cell spread but still able to escape the phagosome), *plcB* (phospholipase B, needed for escape from secondary vacuoles), *Da* (alanine racemase, rendering the strain auxotrophic for D-alanine), and *dat* (D-amino acid aminotransferase, necessary for cell wall biosynthesis).

L. monocytogenes has been successfully used to express HIV Gag from a chromosomally integrated copy of *gag* behind the strong *Listeria hly* promoter (105). Recent work in mice explored the ability of this construct to induce $CD8^+$ T cells against HIV Gag in the spleen, mesenteric lymph nodes, and Peyer's patches and its ability to provide effector Gag-specific $CD8^+$ T cells to the lamina propria after intravenous, oral, or rectal administration of the vaccine. Both primary and secondary oral immunization resulted in abundant Gag-specific $CD8^+$-activated T cells in the lamina propria that constituted ~35% of the CD8 compartment. To achieve significant levels of Gag and listeriolysin O-specific $CD8^+$ T cells in mucosal lymphoid tissues, two immunizations were required. These data suggest that mucosally administered *L. monocytogenes* can

prompt a vigorous cellular response at mucosal sites and that booster doses can maintain this response (105).

In the only published human safety study to date (4), an attenuated *L. monocytogenes* strain bearing deletions in the *actA* and *plcB* virulence genes was evaluated in 20 healthy adult men. Single escalating oral doses of 10^6 to 10^9 CFU were administered; in the highest-dose cohort (10^9 CFU), humoral, mucosal, and cellular immune responses to the investigational organism were detected in individual volunteers. It would appear from the limited human studies conducted thus far that attenuated *L. monocytogenes* can be studied in healthy adult volunteers without serious long-term health sequelae.

OTHER BACTERIAL VECTORS

In addition to the enteric pathogens discussed above, other bacteria being considered as live vectors include *Yersinia enterocolitica* (55), *Y. pseudotuberculosis* (115), *Bordetella bronchiseptica*, and *B. pertussis* (129). The mucosal commensals such as *Streptococcus gordonii* (85) and the lactic acid bacteria (54, 100) such as the *Lactococcus* and *Lactobacillus* spp. have also been shown to raise immunity to foreign antigens in animal models. Recombinant bacillus Calmette-Guerin (BCG) has also been used in animal models to elicit a cellular immune response to foreign antigens. In 24 human volunteers, intradermal BCG expressing the outer surface protein A (OspA) of *Borrelia burgdorferi* had a safety profile comparable to that of licensed BCG, but it did not elicit primary humoral responses to the vectored antigen (37). Although not a bacterial vector, endospores from *Bacillus subtilis* are also being used as an oral vaccine delivery system (34, 35) The advantages of using the *B. subtilis* spore as a vaccine vehicle include nonpathogenicity, advanced cloning tools, hardiness of the spores, cost-effective manufacturing, long-term storage properties, and its current use as a probiotic for both humans and animals (34).

SUMMARY

Live attenuated enteric bacterial vectors expressing foreign antigens have the potential to provide protection against a multitude of diseases, including HIV, bioterrorism agents, and enteric pathogens. Although the animal data have been encouraging, thus far there is no bacterial vector containing a foreign antigen shown to be effective in reliably eliciting high-level immune responses in a significant proportion of human vaccinees. Further human studies of improved bacterial vectors with more vigorous or elegant antigen expression systems will be needed to further develop these tools.

ACKNOWLEDGMENTS

This study was supported by NIAID grants K08 AI001701 to C.N.K. and R01 51206 to E.L.H.

We thank Katherine Raschke for review of the manuscript.

REFERENCES

1. **Altboum, Z., E. M. Barry, G. Losonsky, J. E. Galen, and M. M. Levine.** 2001. Attenuated *Shigella flexneri* 2a Δ*guaBA* strain CVD 1204 expressing enterotoxigenic *Escherichia coli* (ETEC) CS2 and CS3 fimbriae as a live mucosal vaccine against *Shigella* and ETEC infection. Infect. Immun. **69:** 3150–3158.
2. **Anderson, R. J., M. F. Pasetti, M. B. Sztein, M. M. Levine, and F. R. Noriega.** 2000. Δ*guaBA* attenuated *Shigella flexneri* 2a strain CVD 1204 as a *Shigella* vaccine and as a live mucosal delivery system for fragment C of tetanus toxin. Vaccine **18:**2193–2202.
3. **Angelakopoulos, H., and E. L. Hohmann.** 2000. Pilot study of *phoP*/*phoQ*-deleted *Salmonella enterica* serovar Typhimurium expressing *Helicobacter pylori* urease in adult volunteers. Infect. Immun. **68:**2135–2141.
4. **Angelakopoulos, H., K. Loock, D. M. Sisul, E. R. Jensen, J. F. Miller, and E. L. Hohmann.** 2002. Safety and shedding of an attenuated strain of *Listeria monocytogenes* with a deletion of *actA*/*plcB* in adult volunteers: a dose escalation study of oral inoculation. Infect. Immun. **70:**3592–3601.
5. **Attridge, S.** 1991. Oral immunization with *Salmonella typhi* Ty21a-based clones expressing *Vibrio cholerae* O-antigen: serum bactericidal antibody responses in man in relation to pre-immunization antibody levels. Vaccine **9:**877–882.
6. **Attridge, S. R., R. Davies, and J. T. LaBrooy.** 1997. Oral delivery of foreign antigens by attenuated *Salmonella*: consequences of prior exposure to the vector strain. Vaccine **15:**155–162.
7. **Attridge, S. R., C. Dearlove, L. Beyer, L. van den Bosch, A. Howles, J. Hackett, R. Morona, J. LaBrooy, and D. Rowley.** 1991. Characterization and immunogenicity of EX880, a *Salmonella typhi* Ty21a-based clone which produces *Vibrio cholerae* O antigen. Infect. Immun. **59:**2279–2284.
8. **Bao, J. X., and J. D. Clements.** 1991. Prior immunologic experience potentiates the subsequent antibody response when *Salmonella* strains are used as vaccine carriers. Infect. Immun. **59:**3841–3845.
9. **Barry, E. M., Z. Altboum, G. Losonsky, and M. M. Levine.** 2003. Immune responses elicited against multiple enterotoxigenic *Escherichia coli* fimbriae and mutant LT expressed in attenuated *Shigella* vaccine strains. Vaccine **21:**333–340.
10. **Barzu, S., J. Arondel, S. Guillot, P. J. Sansonetti, and A. Phalipon.** 1998. Immunogenicity of IpaC-hybrid proteins expressed in the *Shigella flexneri* 2a vaccine candidate SC602. Infect. Immun. **66:**77–82.
11. **Baud, D., J. Benyacoub, V. Revaz, M. Kok, F. Ponci, M. Bobst, R. Curtiss III, P. De Grandi, and D. Nardelli-Haefliger.** 2004. Immunogenicity against human papillomavirus type 16 virus-like particles is strongly enhanced by the PhoPc phenotype in *Salmonella enterica* serovar Typhimurium. Infect. Immun. **72:**750–756.
12. **Benitez, J. A., L. Garcia, A. Silva, H. Garcia, R. Fando, B. Cedre, A. Perez, J. Campos, B. L. Rodriguez, J. L. Perez, T. Valmaseda, O. Perez, M. Ramirez, T. Ledon, M. D. Jidy, M. Lastre, L. Bravo, and G. Sierra.** 1999. Preliminary assessment of the safety and immunogenicity of a new CTXϕ-negative, hemagglutinin/protease-defective El Tor strain as a cholera vaccine candidate. Infect. Immun. **67:**539–545.
13. **Benyacoub, J., S. Hopkins, A. Potts, S. Kelly, J. P. Kraehenbuhl, R. Curtiss III, P. De Grandi, and D. Nardelli-Haefliger.** 1999. The nature of the attenuation of *Salmonella typhimurium* strains expressing human papillomavirus type 16 virus-like particles determines the systemic and mucosal antibody responses in nasally immunized mice. Infect. Immun. **67:**3674–3679.
14. **Betenbaugh, M. J., and P. Dhurjati.** 1990. Effects of promoter induction and copy number amplification on cloned gene expression and growth of recombinant cell cultures. Ann. N. Y. Acad. Sci. **589:**111–120.
15. **Black, R. E., M. M. Levine, M. L. Clements, G. Losonsky, D. Herrington, S. Berman, and S. B. Formal.** 1987. Prevention of shigellosis by a *Salmonella typhi-Shigella sonnei* bivalent vaccine. J. Infect. Dis. **155:**1260–1265.
16. **Boe, L., K. Gerdes, and S. Molin.** 1987. Effects of genes exerting growth inhibition and plasmid stability on plasmid maintenance. J. Bacteriol. **169:** 4646–4650.
17. **Bouwer, H. G., H. Shen, X. Fan, J. F. Miller, R. A. Barry, and D. J. Hinrichs.** 1999. Existing antilisterial immunity does not inhibit the development of a *Listeria monocytogenes*-specific primary cytotoxic-T-lymphocyte response. Infect. Immun. **67:**253–258.
18. **Bumann, D.** 2001. Regulated antigen expression in live recombinant *Salmonella enterica* serovar Typhimurium strongly affects colonization capabilities and specific $CD4^+$-T-cell responses. Infect. Immun. **69:**7493–7500.
19. **Bumann, D., W. G. Metzger, E. Mansouri, O. Palme, M. Wendland, R. Hurwitz, G. Haas, T. Aebischer, B. U. von Specht, and T. F. Meyer.** 2001. Safety and immunogenicity of live recombinant *Salmonella enterica* serovar Typhi Ty21a expressing urease A and B from *Helicobacter pylori* in human volunteers. Vaccine **20:**845–852.
20. **Burnett, M. S., N. Wang, M. Hofmann, and G. Barrie Kitto.** 2000. Potential live vaccines for HIV. Vaccine **19:**735–742.
21. **Cersini, A., M. C. Martino, I. Martini, G. Rossi, and M. L. Bernardini.** 2003. Analysis of virulence and inflammatory potential of *Shigella flexneri* purine biosynthesis mutants. Infect. Immun. **71:**7002–7013.
22. **Chabalgoity, J. A., G. Dougan, P. Mastroeni, and R. J. Aspinall.** 2002. Live bacteria as the basis for immunotherapies against cancer. Expert Rev. Vaccines **1:**495–505.
23. **Chatfield, S. N., I. G. Charles, A. J. Makoff, M. D. Oxer, G. Dougan, D. Pickard, D. Slater, and N. F. Fairweather.** 1992. Use of the nirB promoter to direct the stable expression of heterologous antigens in *Salmonella* oral vaccine strains: development of a single-dose oral tetanus vaccine. Bio/Technology **10:**888–892.
24. **Chen, H., and D. M. Schifferli.** 2000. Mucosal and systemic immune re-

sponses to chimeric fimbriae expressed by *Salmonella enterica* serovar Typhimurium vaccine strains. Infect. Immun. **68:**3129–3139.
25. **Cohen, M. B., R. A. Giannella, J. Bean, D. N. Taylor, S. Parker, A. Hoeper, S. Wowk, J. Hawkins, S. K. Kochi, G. Schiff, and K. P. Killeen.** 2002. Randomized, controlled human challenge study of the safety, immunogenicity, and protective efficacy of a single dose of Peru-15, a live attenuated oral cholera vaccine. Infect. Immun. **70:**1965–1970.
26. **Coster, T. S., C. W. Hoge, L. L. VanDeVerg, A. B. Hartman, E. V. Oaks, M. M. Venkatesan, D. Cohen, G. Robin, A. Fontaine-Thompson, P. J. Sansonetti, and T. L. Hale.** 1999. Vaccination against shigellosis with attenuated *Shigella flexneri* 2a strain SC602. Infect. Immun. **67:**3437–3443.
27. **Covone, M. G., M. Brocchi, E. Palla, W. Dias da Silveira, R. Rappuoli, and C. L. Galeotti.** 1998. Levels of expression and immunogenicity of attenuated *Salmonella enterica* serovar Typhimurium strains expressing *Escherichia coli* mutant heat-labile enterotoxin. Infect. Immun. **66:**224–231.
28. **Coynault, C., V. Robbe-Saule, and F. Norel.** 1996. Virulence and vaccine potential of *Salmonella typhimurium* mutants deficient in the expression of the RpoS (sigma S) regulon. Mol. Microbiol. **22:**149–160.
29. **Curtiss, R., III.** 2002. Bacterial infectious disease control by vaccine development. J. Clin. Investig. **110:**1061–1066.
30. **Curtiss, R., III, K. Nakayama, and S. M. Kelly.** 1989. Recombinant avirulent *Salmonella* vaccine strains with stable maintenance and high level expression of cloned genes in vivo. Immunol. Investig. **18:**583–596.
31. **Dietrich, G., S. Spreng, D. Favre, J. F. Viret, and C. A. Guzman.** 2003. Live attenuated bacteria as vectors to deliver plasmid DNA vaccines. Curr. Opin. Mol. Ther. **5:**10–19.
32. **DiPetrillo, M. D., T. Tibbetts, H. Kleanthous, K. P. Killeen, and E. L. Hohmann.** 1999. Safety and immunogenicity of *phoP*/*phoQ*-deleted *Salmonella typhi* expressing *Helicobacter pylori* urease in adult volunteers. Vaccine **18:**449–459.
33. **Dreher, D., M. Kok, L. Cochand, S. G. Kiama, P. Gehr, J. C. Pechere, and L. P. Nicod.** 2001. Genetic background of attenuated *Salmonella typhimurium* has profound influence on infection and cytokine patterns in human dendritic cells. J. Leukoc. Biol. **69:**583–589.
34. **Duc le, H., H. A. Hong, and S. M. Cutting.** 2003. Germination of the spore in the gastrointestinal tract provides a novel route for heterologous antigen delivery. Vaccine **21:**4215–4224.
35. **Duc le, H., H. A. Hong, N. Fairweather, E. Ricca, and S. M. Cutting.** 2003. Bacterial spores as vaccine vehicles. Infect. Immun. **71:**2810–2818.
36. **Dunstan, S. J., C. P. Simmons, and R. A. Strugnell.** 1998. Comparison of the abilities of different attenuated *Salmonella typhimurium* strains to elicit humoral immune responses against a heterologous antigen. Infect. Immun. **66:**732–740.
37. **Edelman, R., K. Palmer, K. G. Russ, H. P. Secrest, J. A. Becker, S. A. Bodison, J. G. Perry, A. R. Sills, A. G. Barbour, C. J. Luke, M. S. Hanson, C. K. Stover, J. E. Burlein, G. P. Bansal, E. M. Connor, and S. Koenig.** 1999. Safety and immunogenicity of recombinant Bacille Calmette-Guerin (rBCG) expressing Borrelia burgdorferi outer surface protein A (OspA) lipoprotein in adult volunteers: a candidate Lyme disease vaccine. Vaccine **17:**904–914.
38. **Eko, F. O., T. Schukovskaya, E. Y. Lotzmanova, V. V. Firstova, N. V. Emalyanova, S. N. Klueva, A. L. Kravtzov, L. F. Livanova, V. V. Kutyrev, J. U. Igietseme, and W. Lubitz.** 2003. Evaluation of the protective efficacy of *Vibrio cholerae* ghost (VCG) candidate vaccines in rabbits. Vaccine **21:**3663–3674.
39. **Evans, D. T., L. M. Chen, J. Gillis, K. C. Lin, B. Harty, G. P. Mazzara, R. O. Donis, K. G. Mansfield, J. D. Lifson, R. C. Desrosiers, J. E. Galan, and R. P. Johnson.** 2003. Mucosal priming of simian immunodeficiency virus-specific cytotoxic T-lymphocyte responses in rhesus macaques by the *Salmonella* type III secretion antigen delivery system. J. Virol. **77:**2400–2409.
40. **Fairweather, N. F., S. N. Chatfield, A. J. Makoff, R. A. Strugnell, J. Bester, D. J. Maskell, and G. Dougan.** 1990. Oral vaccination of mice against tetanus by use of a live attenuated *Salmonella* carrier. Infect. Immun. **58:**1323–1326.
41. **Favre, D., S. J. Cryz, Jr., and J. F. Viret.** 1996. Development of *Shigella sonnei* live oral vaccines based on defined rfbInaba deletion mutants of *Vibrio cholerae* expressing the *Shigella* serotype D O polysaccharide. Infect. Immun. **64:**576–584.
42. **Formal, S. B., L. S. Baron, D. J. Kopecko, O. Washington, C. Powell, and C. A. Life.** 1981. Construction of a potential bivalent vaccine strain: introduction of *Shigella sonnei* form I antigen genes into the *galE Salmonella typhi* Ty21a typhoid vaccine strain. Infect. Immun. **34:**746–750.
43. **Forrest, B. D., J. T. LaBrooy, S. R. Attridge, G. Boehm, L. Beyer, R. Morona, D. J. Shearman, and D. Rowley.** 1989. Immunogenicity of a candidate live oral typhoid/cholera hybrid vaccine in humans. J. Infect. Dis. **159:**145–146.
44. **Fouts, T. R., R. G. Tuskan, S. Chada, D. M. Hone, and G. K. Lewis.** 1995. Construction and immunogenicity of *Salmonella typhimurium* vaccine vectors that express HIV-1 gp120. Vaccine **13:**1697–1705.
45. **Francisco, J. A., R. Campbell, B. L. Iverson, and G. Georgiou.** 1993. Production and fluorescence-activated cell sorting of *Escherichia coli* expressing a functional antibody fragment on the external surface. Proc. Natl. Acad. Sci. USA **90:**10444–10448.
46. **Francisco, J. A., C. F. Earhart, and G. Georgiou.** 1992. Transport and anchoring of beta-lactamase to the external surface of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **89:**2713–2717.
47. **Francisco, J. A., C. Stathopoulos, R. A. Warren, D. G. Kilburn, and G. Georgiou.** 1993. Specific adhesion and hydrolysis of cellulose by intact *Escherichia coli* expressing surface anchored cellulase or cellulose binding domains. Bio/Technology **11:**491–495.
48. **Galen, J. E., and M. M. Levine.** 2001. Can a "flawless" live vector vaccine strain be engineered? Trends Microbiol. **9:**372–376.
49. **Galen, J. E., J. Nair, J. Y. Wang, S. S. Wasserman, M. K. Tanner, M. B. Sztein, and M. M. Levine.** 1999. Optimization of plasmid maintenance in the attenuated live vector vaccine strain *Salmonella typhi* CVD 908-*htrA*. Infect. Immun. **67:**6424–6433.
50. **Gentschev, I., G. Dietrich, and W. Goebel.** 2002. The *Escherichia coli* alpha-hemolysin secretion system and its use in vaccine development. Trends Microbiol. **10:**39–45.
51. **Gentschev, I., I. Glaser, W. Goebel, D. J. McKeever, A. Musoke, and V. T. Heussler.** 1998. Delivery of the p67 sporozoite antigen of *Theileria parva* by using recombinant *Salmonella dublin*: secretion of the product enhances specific antibody responses in cattle. Infect. Immun. **66:**2060–2064.
52. **Gerdes, K., J. S. Jacobsen, and T. Franch.** 1997. Plasmid stabilization by post-segregational killing. Genet. Eng. **19:**49–61.
53. **Gonzalez, C., D. Hone, F. R. Noriega, C. O. Tacket, J. R. Davis, G. Losonsky, J. P. Nataro, S. Hoffman, A. Malik, E. Nardin, et al.** 1994. *Salmonella typhi* vaccine strain CVD 908 expressing the circumsporozoite protein of *Plasmodium falciparum*: strain construction and safety and immunogenicity in humans. J. Infect. Dis. **169:**927–931.
54. **Grangette, C., H. Muller-Alouf, M. Geoffroy, D. Goudercourt, M. Turneer, and A. Mercenier.** 2002. Protection against tetanus toxin after intragastric administration of two recombinant lactic acid bacteria: impact of strain viability and in vivo persistence. Vaccine **20:**3304–3309.
55. **Gundel, I., G. Weidinger, V. ter Meulen, J. Heesemann, H. Russmann, and S. Niewiesk.** 2003. Oral immunization with recombinant *Yersinia enterocolitica* expressing a measles virus CD4 T cell epitope protects against measles virus-induced encephalitis. J. Gen. Virol. **84:**775–779.
56. **Hantman, M. J., E. L. Hohmann, C. G. Murphy, D. M. Knipe, and S. I. Miller.** 1999. Antigen delivery systems: development of recombinant live vaccines using viral or bacterial vectors, p. 779–791. *In* P. L. Ogra, J. Mestecky, M. E. Lamm, J. Bienenstock, W. Strober, and J. McGhee (ed.), Mucosal immunology, 2nd ed. Academic Press, Inc., New York, N.Y.
57. **Hayes, L. J., J. W. Conlan, J. S. Everson, M. E. Ward, and I. N. Clarke.** 1991. *Chlamydia trachomatis* major outer membrane protein epitopes expressed as fusions with LamB in an attenuated *aroA* strain of *Salmonella typhimurium*; their application as potential immunogens. J. Gen. Microbiol. **137**(Pt. 7)**:**1557–1564.
58. **Herrington, D. A., L. Van de Verg, S. B. Formal, T. L. Hale, B. D. Tall, S. J. Cryz, E. C. Tramont, and M. M. Levine.** 1990. Studies in volunteers to evaluate candidate *Shigella* vaccines: further experience with a bivalent *Salmonella typhi*-*Shigella sonnei* vaccine and protection conferred by previous *Shigella sonnei* disease. Vaccine **8:**353–357.
59. **Hess, J., I. Gentschev, D. Miko, M. Welzel, C. Ladel, W. Goebel, and S. H. Kaufmann.** 1996. Superior efficacy of secreted over somatic antigen display in recombinant *Salmonella* vaccine induced protection against listeriosis. Proc. Natl. Acad. Sci. USA **93:**1458–1463.
60. **Hindle, Z., S. N. Chatfield, J. Phillimore, M. Bentley, J. Johnson, C. A. Cosgrove, M. Ghaem-Maghami, A. Sexton, M. Khan, F. R. Brennan, P. Everest, T. Wu, D. Pickard, D. W. Holden, G. Dougan, G. E. Griffin, D. House, J. D. Santangelo, S. A. Khan, J. E. Shea, R. G. Feldman, and D. J. Lewis.** 2002. Characterization of *Salmonella enterica* derivatives harboring defined *aroC* and *Salmonella* pathogenicity island 2 type III secretion system (*ssaV*) mutations by immunization of healthy volunteers. Infect. Immun. **70:**3457–3467.
61. **Hohmann, E. L., C. A. Oletta, K. P. Killeen, and S. I. Miller.** 1996. *phoP*/*phoQ*-deleted *Salmonella typhi* (Ty800) is a safe and immunogenic single-dose typhoid fever vaccine in volunteers. J. Infect. Dis. **173:**1408–1414.
62. **Hohmann, E. L., C. A. Oletta, and S. I. Miller.** 1996. Evaluation of a *phoP*/*phoQ*-deleted, *aroA*-deleted live oral *Salmonella typhi* vaccine strain in human volunteers. Vaccine **14:**19–24.
63. **Hoiseth, S. K., and B. A. Stocker.** 1981. Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. Nature **291:**238–239.
64. **Hone, D. M., S. R. Attridge, B. Forrest, R. Morona, D. Daniels, J. T. LaBrooy, R. C. Bartholomeusz, D. J. Shearman, and J. Hackett.** 1988. A *galE* via (Vi antigen-negative) mutant of *Salmonella typhi* Ty2 retains virulence in humans. Infect. Immun. **56:**1326–1333.
65. **Hone, D. M., C. O. Tacket, A. M. Harris, B. Kay, G. Losonsky, and M. M. Levine.** 1992. Evaluation in volunteers of a candidate live oral attenuated *Salmonella typhi* vector vaccine. J. Clin. Investig. **90:**412–420.
66. **Jagusztyn-Krynicka, E. K., J. E. Clark-Curtiss, and R. Curtiss III.** 1993. *Escherichia coli* heat-labile toxin subunit B fusions with *Streptococcus sob-*

rinus antigens expressed by *Salmonella typhimurium* oral vaccine strains: importance of the linker for antigenicity and biological activities of the hybrid proteins. Infect. Immun. **61:**1004–1015.
67. **Janssen, R., and J. Tommassen.** 1994. PhoE protein as a carrier for foreign epitopes. Int. Rev. Immunol. **11:**113–121.
68. **Johansson, E. L., L. Wassen, J. Holmgren, M. Jertborn, and A. Rudin.** 2001. Nasal and vaginal vaccinations have differential effects on antibody responses in vaginal and cervical secretions in humans. Infect. Immun. **69:**7481–7486.
69. **John, M., T. I. Crean, S. B. Calderwood, and E. T. Ryan.** 2000. In vitro and in vivo analyses of constitutive and in vivo-induced promoters in attenuated vaccine and vector strains of *Vibrio cholerae*. Infect. Immun. **68:**1171–1175.
70. **Kang, H. Y., and R. Curtiss III.** 2003. Immune responses dependent on antigen location in recombinant attenuated *Salmonella typhimurium* vaccines following oral immunization. FEMS Immunol. Med. Microbiol. **37:** 99–104.
71. **Kaufmann, S. H., and J. Hess.** 1999. Impact of intracellular location of and antigen display by intracellular bacteria: implications for vaccine development. Immunol. Lett. **65:**81–84.
72. **Kirschmann, D. A., P. A. De Ciechi, C. P. Bono, M. L. Zacheis, B. D. Schwartz, and S. L. Woulfe.** 1995. Utilization of soluble fusion proteins for induction of T-cell proliferation. Cell. Immunol. **160:**193–198.
73. **Kochi, S. K., K. P. Killeen, and U. S. Ryan.** 2003. Advances in the development of bacterial vector technology. Expert. Rev. Vaccines **2:**31–43.
74. **Kohler, J. J., L. B. Pathangey, S. R. Gillespie, and T. A. Brown.** 2000. Effect of preexisting immunity to *Salmonella* on the immune response to recombinant *Salmonella enterica* serovar Typhimurium expressing a *Porphyromonas gingivalis* hemagglutinin. Infect. Immun. **68:**3116–3120.
75. **Koprowski, H., 2nd, M. M. Levine, R. J. Anderson, G. Losonsky, M. Pizza, and E. M. Barry.** 2000. Attenuated *Shigella flexneri* 2a vaccine strain CVD 1204 expressing colonization factor antigen I and mutant heat-labile enterotoxin of enterotoxigenic *Escherichia coli*. Infect. Immun. **68:**4884–4892.
76. **Kotloff, K. L., D. A. Herrington, T. L. Hale, J. W. Newland, L. Van De Verg, J. P. Cogan, P. J. Snoy, J. C. Sadoff, S. B. Formal, and M. M. Levine.** 1992. Safety, immunogenicity, and efficacy in monkeys and humans of invasive *Escherichia coli* K-12 hybrid vaccine candidates expressing *Shigella flexneri* 2a somatic antigen. Infect. Immun. **60:**2218–2224.
77. **Kotloff, K. L., G. A. Losonsky, J. P. Nataro, S. S. Wasserman, T. L. Hale, D. N. Taylor, J. W. Newland, J. C. Sadoff, S. B. Formal, and M. M. Levine.** 1995. Evaluation of the safety, immunogenicity, and efficacy in healthy adults of four doses of live oral hybrid *Escherichia coli-Shigella flexneri* 2a vaccine strain EcSf2a-2. Vaccine **13:**495–502.
78. **Kotloff, K. L., F. Noriega, G. A. Losonsky, M. B. Sztein, S. S. Wasserman, J. P. Nataro, and M. M. Levine.** 1996. Safety, immunogenicity, and transmissibility in humans of CVD 1203, a live oral *Shigella flexneri* 2a vaccine candidate attenuated by deletions in *aroA* and *virG*. Infect. Immun. **64:** 4542–4548.
79. **Kotloff, K. L., F. R. Noriega, T. Samandari, M. B. Sztein, G. A. Losonsky, J. P. Nataro, W. D. Picking, E. M. Barry, and M. M. Levine.** 2000. *Shigella flexneri* 2a strain CVD 1207, with specific deletions in *virG*, *sen*, *set*, and *guaBA*, is highly attenuated in humans. Infect. Immun. **68:**1034–1039.
80. **Kotloff, K. L., J. P. Winickoff, B. Ivanoff, J. D. Clemens, D. L. Swerdlow, P. J. Sansonetti, G. K. Adak, and M. M. Levine.** 1999. Global burden of *Shigella* infections: implications for vaccine development and implementation of control strategies. Bull. W. H. O. **77:**651–666.
81. **Kramer, U., K. Rizos, H. Apfel, I. B. Autenrieth, and C. T. Lattemann.** 2003. Autodisplay: development of an efficacious system for surface display of antigenic determinants in *Salmonella* vaccine strains. Infect. Immun. **71:**1944–1952.
82. **Kutteh, W. H., A. Kantele, Z. Moldoveanu, P. A. Crowley-Nowick, and J. Mestecky.** 2001. Induction of specific immune responses in the genital tract of women after oral or rectal immunization and rectal boosting with *Salmonella typhi* Ty21a vaccine. J. Reprod. Immunol. **52:**61–75.
83. **Lecuit, M., S. Vandormael-Pournin, J. Lefort, M. Huerre, P. Gounon, C. Dupuy, C. Babinet, and P. Cossart.** 2001. A transgenic model for listeriosis: role of internalin in crossing the intestinal barrier. Science **292:**1722–1725.
84. **Lee, J. S., K. S. Shin, J. G. Pan, and C. J. Kim.** 2000. Surface-displayed viral antigens on *Salmonella* carrier vaccine. Nat. Biotechnol. **18:**645–648.
85. **Lee, S. F.** 2003. Oral colonization and immune responses to *Streptococcus gordonii*: potential use as a vector to induce antibodies against respiratory pathogens. Curr. Opin. Infect. Dis. **16:**231–235.
86. **Levine, M. M.** 1999. Typhoid fever vaccines, p. 781–814. *In* S. A. Plotkin and W. A. Orenstein (ed.), Vaccinesz, 3rd ed. The W. B. Saunders Company, Philadelphia, Pa.
87. **Lundin, B. S., C. Johansson, and A. M. Svennerholm.** 2002. Oral immunization with a *Salmonella enterica* serovar Typhi vaccine induces specific circulating mucosa-homing $CD4^+$ and $CD8^+$ T cells in humans. Infect. Immun. **70:**5622–5627.
88. **Mastroeni, P.** 2002. Immunity to systemic *Salmonella* infections. Curr. Mol. Med. **2:**393–406.
89. **Mata, M., and Y. Paterson.** 1999. Th1 T-cell responses to HIV-1 Gag protein delivered by a *Listeria monocytogenes* vaccine are similar to those induced by endogenous listerial antigens. J. Immunol. **163:**1449–1456.
90. **McDermott, P. J., P. Gowland, and P. C. Gowland.** 1993. Adaptation of *Escherichia coli* growth rates to the presence of pBR322. Lett. Appl. Microbiol. **17:**139–143.
91. **McGhee, J. R., J. Mestecky, M. T. Dertzbaugh, J. H. Eldridge, M. Hirasawa, and H. Kiyono.** 1992. The mucosal immune system: from fundamental concepts to vaccine development. Vaccine **10:**75–88.
92. **Miller, S. I.** 1991. PhoP/PhoQ: macrophage-specific modulators of *Salmonella* virulence? Mol. Microbiol. **5:**2073–2078.
93. **Mutsch, M., W. Zhou, P. Rhodes, M. Bopp, R. T. Chen, T. Linder, C. Spyr, and R. Steffen.** 2004. Use of the inactivated intranasal influenza vaccine and the risk of Bell's palsy in Switzerland. N. Engl. J. Med. **350:**896–903.
94. **Nardelli-Haefliger, D., J. P. Kraehenbuhl, R. Curtiss III, F. Schodel, A. Potts, S. Kelly, and P. De Grandi.** 1996. Oral and rectal immunization of adult female volunteers with a recombinant attenuated *Salmonella typhi* vaccine strain. Infect. Immun. **64:**5219–5224.
95. **Nardelli-Haefliger, D., R. B. Roden, J. Benyacoub, R. Sahli, J. P. Kraehenbuhl, J. T. Schiller, P. Lachat, A. Potts, and P. De Grandi.** 1997. Human papillomavirus type 16 virus-like particles expressed in attenuated *Salmonella typhimurium* elicit mucosal and systemic neutralizing antibodies in mice. Infect. Immun. **65:**3328–3336.
96. **Newton, S. M., C. O. Jacob, and B. A. Stocker.** 1989. Immune response to cholera toxin epitope inserted in *Salmonella* flagellin. Science **244:**70–72.
97. **Noriega, F. R., G. Losonsky, C. Lauderbaugh, F. M. Liao, J. Y. Wang, and M. M. Levine.** 1996. Engineered Δ*guaB-A* Δ*virG Shigella flexneri* 2a strain CVD 1205: construction, safety, immunogenicity, and potential efficacy as a mucosal vaccine. Infect. Immun. **64:**3055–3061.
98. **Noriega, F. R., G. Losonsky, J. Y. Wang, S. B. Formal, and M. M. Levine.** 1996. Further characterization of Δ*aroA* Δ*virG Shigella flexneri* 2a strain CVD 1203 as a mucosal *Shigella* vaccine and as a live-vector vaccine for delivering antigens of enterotoxigenic *Escherichia coli*. Infect. Immun. **64:** 23–27.
99. **Noriega, F. R., J. Y. Wang, G. Losonsky, D. R. Maneval, D. M. Hone, and M. M. Levine.** 1994. Construction and characterization of attenuated Δ*aroA* Δ*virG Shigella flexneri* 2a strain CVD 1203, a prototype live oral vaccine. Infect. Immun. **62:**5168–5172.
100. **Nouaille, S., L. A. Ribeiro, A. Miyoshi, D. Pontes, Y. Le Loir, S. C. Oliveira, P. Langella, and V. Azevedo.** 2003. Heterologous protein production and delivery systems for *Lactococcus lactis*. Genet. Mol. Res. **2:**102–111.
101. **Orr, N., J. E. Galen, and M. M. Levine.** 2001. Novel use of anaerobically induced promoter, dmsA, for controlled expression of fragment C of tetanus toxin in live attenuated *Salmonella enterica* serovar Typhi strain CVD 908-htrA. Vaccine **19:**1694–1700.
102. **Pasetti, M. F., E. M. Barry, G. Losonsky, M. Singh, S. M. Medina-Moreno, J. M. Polo, J. Ulmer, H. Robinson, M. B. Sztein, and M. M. Levine.** 2003. Attenuated *Salmonella enterica* serovar Typhi and *Shigella flexneri* 2a strains mucosally deliver DNA vaccines encoding measles virus hemagglutinin, inducing specific immune responses and protection in cotton rats. J. Virol. **77:**5209–5217.
103. **Pasetti, M. F., R. Salerno-Goncalves, and M. B. Sztein.** 2002. *Salmonella enterica* serovar Typhi live vector vaccines delivered intranasally elicit regional and systemic specific $CD8^+$ major histocompatibility class I-restricted cytotoxic T lymphocytes. Infect. Immun. **70:**4009–4018.
104. **Pecota, D. C., C. S. Kim, K. Wu, K. Gerdes, and T. K. Wood.** 1997. Combining the *hok*/*sok*, *parDE*, and *pnd* postsegregational killer loci to enhance plasmid stability. Appl. Environ. Microbiol. **63:**1917–1924.
105. **Peters, C., X. Peng, D. Douven, Z. K. Pan, and Y. Paterson.** 2003. The induction of HIV Gag-specific $CD8^+$ T cells in the spleen and gut-associated lymphoid tissue by parenteral or mucosal immunization with recombinant *Listeria monocytogenes* HIV Gag. J. Immunol. **170:**5176–5187.
106. **Qadri, F., T. R. Bhuiyan, K. K. Dutta, R. Raqib, M. S. Alam, N. H. Alam, A. M. Svennerholm, and M. M. Mathan.** 2004. Acute dehydrating disease caused by *Vibrio cholerae* serogroups O1 and O139 induce increases in innate cells and inflammatory mediators at the mucosal surface of the gut. Gut **53:**62–69.
107. **Rayevskaya, M. V., and F. R. Frankel.** 2001. Systemic immunity and mucosal immunity are induced against human immunodeficiency virus Gag protein in mice by a new hyperattenuated strain of *Listeria monocytogenes*. J. Virol. **75:**2786–2791.
108. **Rees, J. H., S. E. Soudain, N. A. Gregson, and R. A. Hughes.** 1995. *Campylobacter jejuni* infection and Guillain-Barre syndrome. N. Engl. J. Med. **333:**1374–1379.
109. **Richie, E. E., N. H. Punjabi, Y. Y. Sidharta, K. K. Peetosutan, M. M. Sukandar, S. S. Wasserman, M. M. Lesmana, F. F. Wangsasaputra, S. S. Pandam, M. M. Levine, P. P. O'Hanley, S. J. Cryz, and C. H. Simanjuntak.** 2000. Efficacy trial of single-dose live oral cholera vaccine CVD 103-HgR in North Jakarta, Indonesia, a cholera-endemic area. Vaccine **18:**2399–2410.
110. **Rizos, K., C. T. Lattemann, D. Bumann, T. F. Meyer, and T. Aebischer.** 2003. Autodisplay: efficacious surface exposure of antigenic UreA fragments from *Helicobacter pylori* in *Salmonella* vaccine strains. Infect. Immun. **71:**6320–6328.

111. **Robbins, J. B., R. Schneerson, and S. C. Szu.** 1995. Perspective: hypothesis: serum IgG antibody is sufficient to confer protection against infectious diseases by inactivating the inoculum. J. Infect. Dis. **171:**1387–1398.
112. **Roberts, M., A. Bacon, J. Li, and S. Chatfield.** 1999. Prior immunity to homologous and heterologous *Salmonella* serotypes suppresses local and systemic anti-fragment C antibody responses and protection from tetanus toxin in mice immunized with *Salmonella* strains expressing fragment C. Infect. Immun. **67:**3810–3815.
113. **Ruiz-Perez, F., R. Leon-Kempis, A. Santiago-Machuca, G. Ortega-Pierres, E. Barry, M. Levine, and C. Gonzalez-Bonilla.** 2002. Expression of the *Plasmodium falciparum* immunodominant epitope (NANP)(4) on the surface of *Salmonella enterica* using the autotransporter MisL. Infect. Immun. **70:**3611–3620.
114. **Russell, M. W., M. H. Martin, H. Y. Wu, S. K. Hollingshead, Z. Moldoveanu, and J. Mestecky.** 2000. Strategies of immunization against mucosal infections. Vaccine **19**(Suppl. 1)**:**S122–S127.
115. **Russmann, H., U. Gerdemann, E. I. Igwe, K. Panthel, J. Heesemann, S. Garbom, H. Wolf-Watz, and G. Geginat.** 2003. Attenuated *Yersinia* pseudotuberculosis carrier vaccine for simultaneous antigen-specific CD4 and CD8 T-cell induction. Infect. Immun. **71:**3463–3472.
116. **Russmann, H., H. Shams, F. Poblete, Y. Fu, J. E. Galan, and R. O. Donis.** 1998. Delivery of epitopes by the *Salmonella* type III secretion system for vaccine development. Science **281:**565–568.
117. **Ryan, E. T., J. R. Butterton, R. N. Smith, P. A. Carroll, T. I. Crean, and S. B. Calderwood.** 1997. Protective immunity against *Clostridium difficile* toxin A induced by oral immunization with a live, attenuated *Vibrio cholerae* vector strain. Infect. Immun. **65:**2941–2949.
118. **Ryan, E. T., J. R. Butterton, T. Zhang, M. A. Baker, S. L. Stanley, Jr., and S. B. Calderwood.** 1997. Oral immunization with attenuated vaccine strains of *Vibrio cholerae* expressing a dodecapeptide repeat of the serine-rich *Entamoeba histolytica* protein fused to the cholera toxin B subunit induces systemic and mucosal antiamebic and anti-*V. cholerae* antibody responses in mice. Infect. Immun. **65:**3118–3125.
119. **Ryan, E. T., and S. B. Calderwood.** 2000. Cholera vaccines. Clin. Infect. Dis. **31:**561–565.
120. **Ryan, E. T., T. I. Crean, M. John, J. R. Butterton, J. D. Clements, and S. B. Calderwood.** 1999. In vivo expression and immunoadjuvancy of a mutant of heat-labile enterotoxin of *Escherichia coli* in vaccine and vector strains of *Vibrio cholerae*. Infect. Immun. **67:**1694–1701.
121. **Ryan, E. T., T. I. Crean, S. K. Kochi, M. John, A. A. Luciano, K. P. Killeen, K. E. Klose, and S. B. Calderwood.** 2000. Development of a Δ*glnA* balanced lethal plasmid system for expression of heterologous antigens by attenuated vaccine vector strains of *Vibrio cholerae*. Infect. Immun. **68:**221–226.
122. **Salerno-Goncalves, R., M. F. Pasetti, and M. B. Sztein.** 2002. Characterization of $CD8^+$ effector T cell responses in volunteers immunized with *Salmonella enterica* serovar Typhi strain Ty21a typhoid vaccine. J. Immunol. **169:**2196–2203.
123. **Salerno-Goncalves, R., T. L. Wyant, M. F. Pasetti, M. Fernandez-Vina, C. O. Tacket, M. M. Levine, and M. B. Sztein.** 2003. Concomitant induction of $CD4^+$ and $CD8^+$ T cell responses in volunteers immunized with *Salmonella enterica* serovar Typhi strain CVD 908-*htrA*. J. Immunol. **170:**2734–2741.
124. **Schafer, R., D. A. Portnoy, S. A. Brassell, and Y. Paterson.** 1992. Induction of a cellular immune response to a foreign antigen by a recombinant *Listeria monocytogenes* vaccine. J. Immunol. **149:**53–59.
125. **Scheifele, D. W., B. Duval, M. L. Russell, R. Warrington, G. DeSerres, D. M. Skowronski, M. Dionne, J. Kellner, D. Davies, and J. MacDonald.** 2003. Ocular and respiratory symptoms attributable to inactivated split influenza vaccine: evidence from a controlled trial involving adults. Clin. Infect. Dis. **36:**850–857.
126. **Silva, A. J., A. Mohan, and J. A. Benitez.** 2003. Cholera vaccine candidate 638: intranasal immunogenicity and expression of a foreign antigen from the pulmonary pathogen *Coccidioides immitis*. Vaccine **21:**4715–4721.
127. **Soussi, N., H. Saklani-Jusforgues, J. H. Colle, G. Milon, N. Glaichenhaus, and P. L. Goossens.** 2002. Effect of intragastric and intraperitoneal immunization with attenuated and wild-type LACK-expressing *Listeria monocytogenes* on control of murine *Leishmania major* infection. Vaccine **20:**2702–2712.
128. **Spreng, S., G. Dietrich, W. Goebel, and I. Gentschev.** 2003. Protection against murine listeriosis by oral vaccination with recombinant *Salmonella* expressing protective listerial epitopes within a surface-exposed loop of the TolC-protein. Vaccine **21:**746–752.
129. **Stevenson, A., and M. Roberts.** 2003. Use of *Bordetella bronchiseptica* and *Bordetella pertussis* as live vaccines and vectors for heterologous antigens. FEMS Immunol. Med. Microbiol. **37:**121–128.
130. **Tacket, C. O., B. Forrest, R. Morona, S. R. Attridge, J. LaBrooy, B. D. Tall, M. Reymann, D. Rowley, and M. M. Levine.** 1990. Safety, immunogenicity, and efficacy against cholera challenge in humans of a typhoid-cholera hybrid vaccine derived from *Salmonella typhi* Ty21a. Infect. Immun. **58:**1620–1627.
131. **Tacket, C. O., J. Galen, M. B. Sztein, G. Losonsky, T. L. Wyant, J. Nataro, S. S. Wasserman, R. Edelman, S. Chatfield, G. Dougan, and M. M. Levine.** 2000. Safety and immune responses to attenuated *Salmonella enterica* serovar Typhi oral live vector vaccines expressing tetanus toxin fragment C. Clin. Immunol. **97:**146–153.
132. **Tacket, C. O., S. M. Kelly, F. Schodel, G. Losonsky, J. P. Nataro, R. Edelman, M. M. Levine, and R. Curtiss III.** 1997. Safety and immunogenicity in humans of an attenuated *Salmonella typhi* vaccine vector strain expressing plasmid-encoded hepatitis B antigens stabilized by the Asd-balanced lethal vector system. Infect. Immun. **65:**3381–3385.
133. **Tacket, C. O., M. B. Sztein, G. A. Losonsky, S. S. Wasserman, J. P. Nataro, R. Edelman, D. Pickard, G. Dougan, S. N. Chatfield, and M. M. Levine.** 1997. Safety of live oral *Salmonella typhi* vaccine strains with deletions in *htrA* and *aroC aroD* and immune response in humans. Infect. Immun. **65:**452–456.
134. **Valentine, P. J., B. P. Devore, and F. Heffron.** 1998. Identification of three highly attenuated *Salmonella typhimurium* mutants that are more immunogenic and protective in mice than a prototypical *aroA* mutant. Infect. Immun. **66:**3378–3383.
135. **Valle, E., T. Ledon, B. Cedre, J. Campos, T. Valmaseda, B. Rodriguez, L. Garcia, K. Marrero, J. Benitez, S. Rodriguez, and R. Fando.** 2000. Construction and characterization of a nonproliferative El Tor cholera vaccine candidate derived from strain 638. Infect. Immun. **68:**6411–6418.
136. **Van de Verg, L., D. A. Herrington, J. R. Murphy, S. S. Wasserman, S. B. Formal, and M. M. Levine.** 1990. Specific immunoglobulin A-secreting cells in peripheral blood of humans following oral immunization with a bivalent *Salmonella typhi-Shigella sonnei* vaccine or infection by pathogenic *S. sonnei*. Infect. Immun. **58:**2002–2004.
137. **VanCott, J. L., S. N. Chatfield, M. Roberts, D. M. Hone, E. L. Hohmann, D. W. Pascual, M. Yamamoto, H. Kiyono, and J. R. McGhee.** 1998. Regulation of host immune responses by modification of *Salmonella* virulence genes. Nat. Med. **4:**1247–1252.
138. **Vindurampulle, C. J., and S. R. Attridge.** 2003. Impact of vector priming on the immunogenicity of recombinant *Salmonella* vaccines. Infect. Immun. **71:**287–297.
139. **Vindurampulle, C. J., and S. R. Attridge.** 2003. Vector priming reduces the immunogenicity of salmonella-based vaccines in *nramp1*$^{+/+}$ mice. Infect. Immun. **71:**2258–2261.
140. **Viret, J. F., D. Favre, B. Wegmuller, C. Herzog, J. U. Que, S. J. Cryz, Jr., and A. B. Lang.** 1999. Mucosal and systemic immune responses in humans after primary and booster immunizations with orally administered invasive and noninvasive live attenuated bacteria. Infect. Immun. **67:**3680–3685.
141. **Whittle, B. L., and N. K. Verma.** 1997. The immune response to a B-cell epitope delivered by *Salmonella* is enhanced by prior immunological experience. Vaccine **15:**1737–1740.
142. **Wick, M. J., C. V. Harding, N. J. Twesten, S. J. Normark, and J. D. Pfeifer.** 1995. The *phoP* locus influences processing and presentation of *Salmonella typhimurium* antigens by activated macrophages. Mol. Microbiol. **16:**465–476.
143. **Wu, K., D. Jahng, and T. K. Wood.** 1994. Temperature and growth rate effects on the hok/sok killer locus for enhanced plasmid stability. Biotechnol. Prog. **10:**621–629.
144. **Yu, D. T.** 2003. Definition and diagnosis of undifferentiated spondyloarthropathy, Reiter's syndrome, and reactive arthritis. UpToDate 2003. [Online.]

Editor: J. B. Kaper