CLINICAL MICROBIOLOGY REVIEWS, July 1992, p. 328–342 Vol. 5, No. 3
0893-8512/92/030328-15$02.00/0
Copyright © 1992, American Society for Microbiology

Oral Immunization Using Live Attenuated *Salmonella* spp. as Carriers of Foreign Antigens

LUCIA CÁRDENAS AND JOHN D. CLEMENTS*

Department of Microbiology and Immunology, Tulane University School of Medicine, New Orleans, Louisiana 70112

INTRODUCTION 328
IMMUNIZATION AGAINST ENTERIC DISEASES 328
ANTIGEN DELIVERY SYSTEMS FOR ORAL IMMUNIZATION 330
ATTENUATION OF *SALMONELLA* STRAINS 331
galE Mutants 331
aro and *pur* Mutants 331
Cyclic AMP Regulation Mutants 332
Plasmid-Cured Strains 332
Streptomycin-Dependent Mutants 332
Aspartate Semialdehyde Dehydrogenase Mutants 333
phoP and *ompR* Mutants 333
Temperature-Sensitive Mutants 333
ATTENUATED *SALMONELLA* STRAINS AS CARRIERS OF HETEROLOGOUS ANTIGENS 333
Heterologous Antigens of Bacterial Origin 333
Heterologous Antigens of Viral and Eukaryotic Origins 335
OTHER CONSIDERATIONS 336
Stabilization of Antigen Expression in Attenuated *Salmonella* Strains 336
Effect of Multiple Use on Carrier Efficacy 337
CONCLUSIONS 337
REFERENCES 338

INTRODUCTION

Human pathogens that initiate disease following infection of mucosal surfaces represent the single largest cause of morbidity and mortality among the world's populations (2). Diarrheal and respiratory illnesses are among the most common debilitating infectious diseases afflicting people of all ages around the globe. Although extensive research has been conducted to determine the role of cell-mediated immunity and serum antibodies in protection against infectious agents, less is known about the role of mucosal immunity. One major limitation on the development of effective immunoprophylaxis against mucosal pathogens may be the inability to stimulate significant levels of mucosal antibody directed against specific virulence determinants of the pathogens that cause these diarrheal and respiratory diseases.

Parenterally administered vaccines are not effective for eliciting mucosal secretory immunoglobulin A (sIgA) responses and are generally ineffective against organisms that colonize mucosal surfaces and do not invade: hence the relative ineffectiveness of the parenteral cholera vaccine. Orally administered vaccines, especially live attenuated vaccines, have been shown to be effective in inducing specific sIgA responses (17, 34), presumably because antigen is delivered to the T and B lymphocytes of the gut-associated lymphoid tissue (GALT). The primed B cells then migrate to the mesenteric lymph nodes and undergo differentiation. These B cells enter the thoracic duct and then the general circulation and subsequently seed all of the secretory tissues of the body, including the lamina propria of the gut and respiratory tract. IgA is then produced by the mature plasma cells and is transported onto the mucosal surface, where it is available to interact with invading pathogens (17, 31) (Fig. 1). Oral immunization at the level of the gut mucosa can elicit production of secretory antibodies on all mucosal surfaces (17, 34). This greatly enhances the potential of this technique for immunization against infectious diseases.

One recent innovation in this approach has been to use avirulent derivatives of *Salmonella* spp. as carriers for plasmids that code for virulence determinants of heterologous mucosal pathogens. This technique has been examined with a number of antigens and has been shown to be an effective means of stimulating significant levels of specific mucosal sIgA directed against the carrier strain and the heterologous antigen. This review will focus on the background and application of this approach to vaccine delivery.

IMMUNIZATION AGAINST ENTERIC DISEASES

Enteric diseases such as cholera, dysentery, *Escherichia coli*-related diarrheas, typhoid fever, and enterovirus infections are major causes of morbidity and mortality worldwide, especially in developing countries, where sanitation conditions are less than adequate. Several types of vaccines against these enteropathies have been developed and tested over the years, among them killed whole cells, subunits of toxins, and live attenuated bacteria and viruses administered parenterally or orally.

Although widely used, parenterally administered vaccines against these diseases have not proved satisfactory. Results based on well-controlled field trials and human volunteer studies (59, 114) have shown that, with the notable exception

* Corresponding author.

FIG. 1. Generalized pathway for stimulation of specific mucosal immune responses.

of typhoid fever, in which the organisms disseminate systemically, parenterally administered vaccines result only in marginal protection and short-lived immunity. This failure to provide adequate protection is observed despite the development of high titers of circulating antibodies and may be mostly due to an inadequate stimulation of local immunity (53). In addition, parenteral administration of large numbers of killed organisms frequently results in adverse reactions such as local discomfort, redness, swelling, fever, headache, myalgia, and general malaise (1, 21, 73, 145). More severe systemic manifestations such as anaphylactic shock, acute renal disease, appendicitis, and erythema nodosum, although rare, have also been observed (13, 75, 80, 101). Oral killed vaccines against typhoid fever do not generally produce side effects but have been shown to induce only low levels of circulating and secretory antibodies and insignificant levels of protection against challenge (59). In contrast, when examined in a field trial in Bangladesh, the oral vaccine against cholera, which consists of killed whole cells and partially purified cholera toxin B subunit, was shown to provide moderate protection for up to 24 months (22, 23, 137, 138). The protective efficacy of the B-subunit killed whole-cell vaccine was 62% in the first year and 57% in the second year in all age groups for vaccinees receiving three doses. However, by the third year, the protective efficacy had fallen to 17% in all age groups, primarily as a result of the inability to evoke protection in children below the age of 5 years.

Historically, one of the main obstacles to the use of live vaccines was the potential reversion of attenuated strains to virulence. However, in recent years the mechanisms by which a number of pathogens cause infections have been deciphered and manipulation at the molecular level and the application of modern DNA technology have made possible the construction of safe, genetically stable mutants. Mass production, distribution, and administration are easier and less costly for such live attenuated vaccines than for purified component vaccines. This is of great importance since enteric diseases are a leading health problem mainly in countries that cannot afford expensive vaccination programs.

Presently, the only live attenuated enteric bacterial vaccine available for human use is a galactose epimerase-less (*galE*) mutant of *Salmonella typhi* designated Ty21a. This mutant was developed by Germanier and Fürer in 1975 (61) by using the mutagenic alkylating agent nitrosoguanidine to treat the wild-type strain *S. typhi* Ty2. This *galE* mutant also had a 25% reduction of galactokinase and galactose-1-phosphate uridylyltransferase. The mutant was found to be stable; reversion to the parental phenotype has not been demonstrated in vitro or in vivo (10, 11, 61, 62, 88). In the wild-type strain, the galactose needed for the synthesis of smooth lipopolysaccharide (LPS) O antigen is derived from UDP-galactose, which is made by UDP-galactose epimerase from UDP-glucose (Fig. 2). When grown in the absence of galactose, Ty21a has a rough LPS, because it has no source of UDP-galactose. When sufficient galactose is added to the medium, the bacteria have a normal LPS composition but lyse, presumably because of the accumulation of the intermediates galactose-1-phosphate and UDP-galactose to toxic levels. This property, coupled with the fact that Ty21a is also Vi antigen negative, is a factor in the safety of the vaccine strain in vivo (see below). The safety and efficacy of *S. typhi* Ty21a as a human vaccine have been tested in volunteer

FIG. 2. Metabolism of galactose.

studies in the United States (62) and in large, controlled field trials in Egypt (148, 149) and Chile (10, 11, 50, 86, 88). The studies in Egypt demonstrated that oral administration of three doses, each consisting of approximately 5×10^9 bacteria, produced no untoward effects. The overall protection provided by the vaccine, as demonstrated in the Chilean studies, was 65% and the immune status conferred lasted for at least 5 years. This vaccine strain is currently available in several countries (including the United States); it is manufactured by the Swiss Serum and Vaccine Institute and is sold under the trade name of Vivotif.

ANTIGEN DELIVERY SYSTEMS FOR ORAL IMMUNIZATION

The induction of mucosal responses to orally administered antigens usually requires larger quantities of antigen than those used for parenteral immunizations because purified microbial antigens may be degraded by gastric hydrochloric acid or proteolytic enzymes and the amounts that actually get absorbed and are available to evoke an immune response are small (67). To overcome the problem of degradation by gastric acidity, antigen packaging in liposomes or microcapsules has been investigated. Liposomes carrying a *Streptococcus mutans* antigen or *Streptococcus mutans* anti-idiotypic antibodies were shown to potentiate the immune response against the specific antigen (74, 99). A study by Eldridge et al. (47) demonstrated that biodegradable microspheres containing staphylococcal enterotoxin B were efficiently internalized by intestinal specialized adsorptive cells (M cells) and that mice receiving this antigen-carrier preparation showed a rise in specific antibodies.

Delivering soluble antigens with immunopotentiating substances has also been investigated. Muramyl dipeptide, an orally administered adjuvant, has been shown to elevate the salivary IgA responses in rats that received both it and *S. mutans*-derived glucosyl transferase (141). A number of bacterial and viral antigens have been administered in conjunction with cholera toxin or the closely related heat-labile enterotoxin of *E. coli* or their B subunits. Cholera toxin and the heat-labile enterotoxin are potent mucosal adjuvants, and their B subunits bind with high specificity to G_{M1} gangliosides on mucosal epithelial cells (26, 39, 48, 97).

An approach that has been proposed more recently is the use of live attenuated organisms as vectors to deliver specific target antigens to the host (55, 151). The two best-characterized live vector systems are vaccinia virus and attenuated *Salmonella* spp. such as Ty21a. A number of foreign genes have been successfully cloned into vaccinia virus (91). This virus has the capacity to carry up to 25 kb of foreign DNA, which may allow the construction of multivalent vaccines coding for production of several antigens. Zagury et al. (151) and Jones et al. (77) have shown antibody and cell-mediated human immune responses against foreign genes expressed in recombinant vaccinia virus. Despite the potential advantages offered by this viral vaccine vector, a number of problems concerning the safety of this system must be addressed, including the potential for in vivo recombination between poxviruses leading to the creation of strains with increased virulence. Additionally, serious adverse reactions to vaccinia virus, particularly in young children, have been observed (84). This is a point of great concern, because many of the proposed vaccines would be administered to infants and children of preschool age. Moreover, parenteral administration of vaccinia virus does not elicit production of sIgA and, consequently, cannot provide protection at the level of the mucosal surface.

Conceptually, the use of *Salmonella* spp. as carriers for heterologous antigens has a number of advantages in the development of vaccines against bacterial enteric pathogens. Use of a live oral vaccine enhances the repertoire of antigens presented to the mucosal immune system compared with antigens presented in complexes of killed organisms. Killed bacteria prepared for parenteral or oral administration are typically subjected to acetone, formalin, or heat, all of which have the potential to denature relevant immunologic determinants. Moreover, organisms grown in laboratory or industrial-scale fermentors designed to maximize numbers do not necessarily express the same antigens as organisms grown in vivo. Thus, the types and characteristics of antigens presented by live vaccines are more likely to reflect those seen by the host during a natural infection. An additional consideration when any antigen is administered orally is the protection of the antigen from the effects of gastric acidity, pancreatic enzymes, bile salts, and proteases. This is partially overcome by administering the antigen or organism in the presence of bicarbonate to neutralize gastric acidity or by placing antigens into enteric-coated capsules which release their contents in the relatively alkaline environment of the small bowel. Another advantage of live oral vaccines is that once the organisms are past the gastric barrier, antigens expressed by them can be synthesized de novo in the environment in which they naturally occur and are not subjected to stomach acidity.

A *Salmonella* sp. functions as a carrier for stimulation of antigen-specific sIgA because of the natural pathogenesis of the organism and the behavior of appropriately attenuated mutants, which can interact with the lymphoid tissues in the Peyer's patches without causing systemic disease. In the context of the mucosal responsiveness, the immunologically relevant sites are the Peyer's patches, especially for antigen-specific T-cell-dependent B-cell activation (14, 135, 143). Thus, the events up to isotype switching from IgM B cells to IgA B cells occurs in the Peyer's patches. Antigens localized on the epithelial cell surface may contribute to antigen-induced B-cell proliferation in that the class II positive villous epithelial cells may act as antigen-presenting cells for T-cell activation at the secretory site, thereby increasing cytokine production, terminal B-cell differentiation, increased expression of secretory component, and increased external transport of antigen-specific IgA (143). Consequently, live oral vaccines that colonize the proximal small bowel epithelium (78, 90) or killed whole-cell vaccines administered with subunits of toxins that bind to the surface of epithelial cells (22, 138) may not present antigens as efficiently as attenuated *Salmonella* spp. that deliver the antigen directly to the induction site, especially for priming for IgA synthesis. Use of a *Salmonella* sp. as a carrier allows the placement of appropriate antigens directly into the lymphoid follicles, permitting maximum stimulation of relevant lymphoid cells for production of sIgA. This raises the possibility of developing vaccines that can invoke significant levels of mucosal antibodies beyond those achievable by other means.

It has been clearly established that the use of attenuated *Salmonella* strains as live vaccines is a safe and effective means of inducing significant humoral and secretory antibody responses in animal species, including humans (87–89), cattle (130), sheep (103), rabbits (121), fowl (7, 8), and mice (4, 35, 45, 51, 60, 71, 82, 112, 127). This is attributed to the fact that live salmonellae reach the Peyer's patches, where

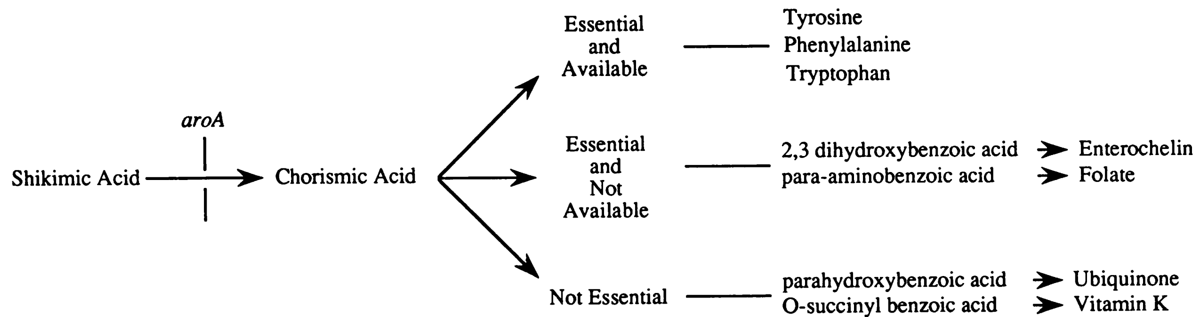

FIG. 3. Aromatic biosynthetic pathway.

they present their array of antigens directly to the T and B lymphocytes of the GALT, therefore priming the mucosal immune system to produce antigen-specific immune responses. This has prompted many investigators to consider and test the use of live attenuated *Salmonella* strains as carriers of genes coding for virulence determinants from other pathogenic microorganisms.

ATTENUATION OF *SALMONELLA* STRAINS

Attenuated *Salmonella* spp. with reduced virulence for their hosts were first described in 1951 (3, 4), when auxotrophic *Salmonella* mutants with exogenous requirements for purines, *p*-aminobenzoic acid, and aspartate were observed to exhibit reduced virulence for mice. Since then, a number of attenuated strains of all *Salmonella* species have been described. Some of the more widely investigated are discussed below.

galE Mutants

Germanier and Fürer noted that *S. typhimurium* and *S. typhi galE* mutants obtained after nitrosoguanidine mutagenesis were avirulent but still immunogenic in mice and humans, respectively. Those authors were the first to propose the use of *S. typhi galE* Ty21a as a vaccine against typhoid fever in humans (60, 61). *S. typhi* Ty21a is phenotypically rough when grown in the absence of galactose but can produce complete LPS and become invasive when exogenous galactose is added to the medium (110). These mutants lyse in the presence of high concentrations of galactose, presumably because of the accumulation of toxic levels of phosphorylated galactose (Fig. 2) (57, 58). It is not known exactly whether the avirulence of these *galE* mutants is due to galactose sensitivity, the inability to synthesize LPS, the fact that they are also unable to synthesize Vi antigen, or unidentified mutations in genes that confer important virulence properties. The importance of galactose sensitivity as a virulence determinant is uncertain. On the one had, *S. typhimurium galE* mutants that were rendered galactose resistant by a second mutation became virulent for mice. However, *S. choleraesuis galE* mutants still retain virulence for mice (111, 112) and *galE via* (Vi antigen-negative) mutants of *S. typhi* Ty2 (strain EX462) retain virulence for humans (72). In the study reported by Hone et al. (72), two of four human volunteers receiving one dose of 7×10^8 *S. typhi* EX462 developed typhoidlike disease with fever and bacteremia. These authors examined the virulence of EX462 only at high doses, so it is not possible to determine the extent of low-dose attenuation. It is clear that the combined *galE* and *via* mutations did not attenuate *S. typhi* Ty2 sufficiently to make it safe for use at high doses in humans. With the cloning and characterization of the *S. typhimurium* galactose operon, *S. typhimurium galE* mutants carrying defined deletions of the *galE* gene have also been constructed. These mutants have been shown to be avirulent and immunogenic in mice (71), in contrast to the results obtained with EX462 in humans.

aro and *pur* Mutants

Mutants with a block in the aromatic pathway have been isolated by transposon mutagenesis (69). By using transposon Tn*10*, which carries the gene that confers resistance to tetracycline, a transposon insertion into the *aroA* gene was generated and subsequently transduced into virulent *S. typhimurium*. Nonreverting *aroA* strains, selected after penicillin enrichment in fusaric acid-containing media, were demonstrated to carry a deletion in the *aroA* gene caused by the imprecise excision of the transposon. The same technique has been used by these (69) and other (129, 130, 134) investigators to obtain other *Salmonella aroA* deletion mutants for use as parenterally administered antisalmonellosis vaccines in calves. The *aroA* gene codes for the synthesis of 3-enolpyruvylshikimate-5-phosphate synthetase, which catalyzes the conversion of phosphoenolpyruvate and shikimate 3-phosphate into 5-enolpyruvoylshikimate-3-phosphate in a pathway that eventually leads to the synthesis of chorismate, a common intermediate compound in the synthesis of aromatic amino acids, *p*-aminobenzoic acid (essential for folate synthesis), 2,3-dihydroxybenzoic acid (needed for production of enterochelin, an iron chelator), *p*-hydroxybenzoic acid (precursor of ubiquinone), and *o*-succinylbenzoic acid (precursor of vitamin K) (Fig. 3). The last two precursors are not essential for bacterial survival. Tryptophan, phenylalanine, and tyrosine are essential for bacterial growth but are available in mammalian cells; thus, their synthesis de novo is not necessary.

p-Aminobenzoic acid and 2,3-dihydroxybenzoic acid are two essential metabolites that are not found in mammalian tissues. Mutants unable to synthesize these metabolites are avirulent, because they cannot replicate in their host cells (24, 118). Several different *aroA* mutants of *S. typhimurium* and *S. dublin* that are immunogenic and exhibit reduced virulence for mice and other animal species have been isolated (46, 64, 69, 82, 103, 122). Other strains harboring mutations affecting the metabolism of aromatic compounds have also been constructed. Mutations in *aroC* or *aroD* or combinations of these mutations are associated with reduced virulence of *S. typhimurium* for mice (42, 45, 100) and of *S. typhi* for humans (140).

Nonreverting, purine-requiring *S. typhi* and *S. typhimu-*

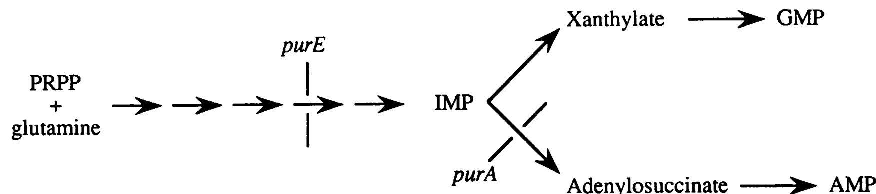

FIG. 4. Purine biosynthetic pathway.

rium mutants with deletions in the *purA* gene have been generated by transposon mutagenesis (96). The *purA* gene codes for adenylosuccinate synthetase, which is the enzyme that catalyzes the first step of the conversion of IMP to AMP in the de novo synthesis of purines (Fig. 4). These mutants were initially constructed to introduce a secondary mutation into *aroA* mutants to make them safer as live oral vaccines. However, when tested in mice, strains harboring either the *purA* alone or a *purA aroA* double mutation were shown to be poorly immunogenic (113, 127). Studies conducted to study the efficacy of these types of mutants as live oral vaccines in humans led to equally disappointing results. Volunteers immunized with *S. typhi* 541TY (*aroA purE*) (Vi positive) or *S. typhi* 543TY (*aroA purE*) (Vi negative) were not colonized by the organisms and did not develop a humoral immune response to the vaccine strains (89). It is clear, then, that some mutations so attenuate the organisms as to make them unsuitable for use in live vaccines.

Cyclic AMP Regulation Mutants

It has been proposed that mutations in the genes coding for the synthesis of cyclic AMP and cyclic AMP receptor protein might render *Salmonella* spp. avirulent, since these proteins form a complex that acts as a positive regulator of transcription for multiple genes that possess catabolite-sensitive promoters (117). Mutants carrying deletions in the *cya* gene (adenylate cyclase), the *crp* gene (cyclic AMP receptor protein), and both the *cya* and *crp* genes have been constructed by using transposon mutagenesis.

Studies on the virulence, stability, persistence, and immunogenicity of these strains demonstrated that a deletion in any of these genes results in reduced virulence. The 50% lethal dose (LD_{50}) for mice was at least 10^4 times the LD_{50} of the wild-type strain. Strains harboring deletions in both genes simultaneously were very stable, whereas strains that had deletions in a single gene, either *cya* or *crp*, reverted with frequencies of 2×10^7 or higher. In contrast to the *aro* or *gal* mutants discussed above, the Δ*cya* Δ*crp* mutants were not able to produce a systemic infection in mice; the numbers of bacteria recovered from the spleens of infected animals were extremely small. Immunization studies with mice demonstrated that the double-deletion mutant (Δ*cya* Δ*crp*) containing the 90-kb virulence associated plasmid (see below) afforded protection against challenge with 10^4 times the LD_{50} of the wild-type strain *S. typhimurium* SR11 (35–37). The Δ*cya* Δ*crp* double-deletion mutation has also been shown to reduce the virulence of *S. typhi* Ty2 for humans (140).

Plasmid-Cured Strains

High-molecular-weight plasmids have been associated with virulence in several nontyphoid *Salmonella* strains: *S. typhimurium* (63, 65, 66, 68, 76, 116), *S. dublin* (18, 93, 142), *S. enteritidis* (105), and *S. gallinarum* (8, 9). It was observed that elimination of the plasmid from these strains resulted in reduced virulence and that in some cases, reintroduction of the plasmid restored complete virulence. For *S. gallinarum*, which causes a dysenterylike syndrome in fowl, the plasmid has been associated with the ability of the organisms to invade via the intestinal mucosal epithelium and to survive and grow in cells of the reticuloendothelial system (9). However, the exact contribution of these plasmids to virulence is uncertain. It has been suggested that, given the reduced virulence traits of these plasmid-cured strains, they may be useful as live attenuated vaccines. Experiments to test this hypothesis have been conducted by several investigators, who have observed that immunization with plasmid-cured strains provides some level of protection against challenge with the virulent organism in chickens and mice (7, 52, 106). Characterization of the plasmid genes and their relationships to other virulence determinants in *Salmonella* spp. is necessary before the plasmid-cured strains can be used as commercial vaccines.

Streptomycin-Dependent Mutants

Streptomycin-dependent *Salmonella* strains described by Reitman and Iverson (120, 121) and Mel et al. (98) were isolated after repeated cultivation of pathogenic *S. typhi* in the presence of streptomycin. The rationale used to obtain these strains is that the organisms can become dependent on the antibiotic without exposure to more severe mutagenic agents that might destroy important protective antigens in the bacterial cell. In host tissues in which streptomycin is not available, the bacteria should not be able to grow and proliferate.

Streptomycin-dependent strains were isolated for use as vaccines against typhoid fever and were tested in both animals (121) and humans (87). It was observed that when mice were simultaneously injected with streptomycin and given the vaccine, the LD_{50} of the attenuated strain was only 10-fold lower than the LD_{50} of the parental strain. Reitman and Iverson noted that parenteral immunization of rabbits with the streptomycin-dependent strain induced humoral antibodies against the O antigen of the vaccine strain (121). Levine et al. (87) tested the efficacy of this vaccine in human volunteers and observed that multiple doses of freshly harvested organisms (10^{11} CFU) did not provoke adverse effects when administered concomitantly with 1 g of streptomycin and 2 g of sodium bicarbonate and that after challenge with the wild-type strain, immunization with streptomycin-dependent *S. typhi* afforded a moderate level of protection (66 to 78% efficacy). In contrast, the same vaccine regimen with lyophilized reconstituted bacteria did not confer on the vaccinees any protection against clinical typhoid fever. Although the reason for this discrepancy is not known, it is obvious that a vaccine strain that loses its

immunogenicity upon lyophilization could not be a good candidate for mass vaccination.

Aspartate Semialdehyde Dehydrogenase Mutants

Diaminopimelic acid is a unique and essential constituent of the rigid layer of the bacterial cell wall. This component is part of a tetrapeptide that cross-links the glycan strands to form peptidoglycan. The enzyme aspartate semialdehyde dehydrogenase catalyzes the conversion of aspartate-4-phosphate to aspartate semialdehyde in a pathway that leads to the synthesis of threonine, methionine, lysine, and diaminopimelic acid (28). *Salmonella* strains with a mutation in the *asd* gene are unable to synthesize normal peptidoglycan and lyse rapidly in the absence of exogenous diaminopimelic acid. These strains are avirulent for mice by all routes of inoculation, but their survival time in the GALT is so limited that their immunogenicity is very reduced (33).

phoP and *ompR* Mutants

Studies on the mechanisms of intracellular survival by *S. typhimurium* have led to the isolation of Tn*10* insertion mutants that are unable to survive within macrophages in vitro and are avirulent for mice in vivo. Molecular and genetic analyses of these mutants indicated that all strains exhibiting the avirulent phenotype had the transposon inserted into the *phoP* gene, which has been hypothesized to be a regulator for the gene that encodes a nonspecific acid phosphatase. These studies indicate that *phoP* may also regulate genes involved in the resistance to antimicrobial factors in the host (51).

Transposon-generated mutations have also led to the isolation of strains lacking one or more outer membrane proteins. Mutations in the *ompC* or *ompF* genes did not affect virulence, whereas strains with mutations in *ompR* failed to kill mice after oral or intravenous inoculation. It has been proposed that *ompR* encodes a positive regulator for the expression of other outer membrane proteins, a tripeptide permease, and other uncharacterized proteins involved in the transport of nutrients and the exclusion of harmful substances from the bacterial cell (41). The immunogenicity of *phoP* and *ompR* strains has yet to be tested.

Temperature-Sensitive Mutants

Temperature-sensitive variants of *S. dansyz* unable to proliferate at 37°C were obtained after nitrosoguanidine mutagenesis. Compared with an LD_{50} of 2×10^2 for the parental strain, doses of 10^7 CFU of the temperature-sensitive mutants were avirulent for mice by the intraperitoneal route, whereas up to 2×10^{10} CFU could be administered orally without causing symptoms of disease. Immunization by either the oral or intraperitoneal route with the temperature-sensitive strains demonstrated that these mutants were able to elicit an immune response capable of protecting the animals upon challenge with the virulent organisms (49). More recently, other temperature-sensitive mutants developed by exposure of virulent strains to UV light have been tested. Most of the mutants obtained by this method reverted to virulence at high frequencies. One strain that did not revert was completely avirulent but exhibited low immunogenicity when tested in a mouse protection assay (115).

It is of interest that the approach of using temperature-sensitive mutants as live vaccines has been tested with other pathogenic organisms. Studies on mutants of *Pseudomonas aeruginosa* that exhibit limited growth at body temperature (36 to 37°C) have indicated that intranasal immunization with these temperature-sensitive mutants enhances the clearance of the virulent organisms from the lungs of challenged animals (102).

ATTENUATED *SALMONELLA* STRAINS AS CARRIERS OF HETEROLOGOUS ANTIGENS

Heterologous Antigens of Bacterial Origin

Formal et al. (55) were the first to report the use of an avirulent *Salmonella* sp. as a carrier for a heterologous antigen. A high-molecular-weight, naturally occurring plasmid of *Shigella sonnei* that codes for a cell surface antigen termed form I was conjugally transferred into the human live vaccine *S. typhi* Ty21a. The recipient strain was shown to contain both the form I plasmid of *Shigella sonnei* and the F′ *lac*(Ts)::Tn*3* plasmid used to mobilize the form I plasmid. Moreover, the transconjugant expressed both the form I antigen and the normal somatic antigens of *S. typhi*. Animal studies demonstrated that mice immunized intraperitoneally or subcutaneously with the recombinant vaccine were protected against challenge with either *S. typhi* or *Shigella sonnei* and that rabbits inoculated intravenously developed high titers of agglutinating antibodies against both the vaccine strain and the foreign antigen. This vaccine has been subsequently tested in human volunteers (11, 144, 146) and has been shown to be safe, to stimulate a significant IgA intestinal response against *Shigella sonnei* LPS, and to afford good but variable protection against bacillary dysentery. The inability to make reproducibly efficacious vaccine lots limits further use of this candidate vaccine.

Clements and El-Morshidy (25) were the first to introduce a recombinant plasmid into an attenuated *Salmonella* strain for use as a vaccine carrier. They constructed a potential live oral vaccine for typhoid fever and the cholera-*E. coli*-related diarrheas by transforming *S. typhi* Ty21a with a recombinant plasmid carrying the gene for the production of the binding subunit of the heat-labile toxin (LT-B) from enterotoxigenic *E. coli*. The *S. typhi* Ty21a derivative, designated SE12, was shown to express LT-B that was recognized by antibody against native LT-B produced in *E. coli*. This recombinant strain caused no diarrhea or other adverse effects in guinea pigs after oral inoculation. When injected intraperitoneally into mice, SE12 induced the production of high levels of antitoxin antibodies that could be specifically boosted with a subsequent injection of the vaccine strain or purified LT-B. To further evaluate the efficacy of this system, Clements et al. (27) introduced a mouse model in which the LT-B-carrying plasmid was transformed into an *S. dublin aroA* strain that, unlike *S. typhi*, is able to produce a transient infection in mice. This transient infection closely resembles enteric fever caused by *S. typhi* in humans. Mice orally immunized with the derivative strain developed progressively increasing secretory and mucosal antibodies to the LPS of the vaccine strain and to LT-B. Significantly, the mucosal antibodies were predominantly of the IgA isotype, and they exerted neutralizing antitoxic activity against cholera toxin and heat-labile enterotoxin (27). Similar studies were subsequently conducted by Maskell et al. (94, 95), who were able to induce serum and mucosal antigen-specific (LT-B) antibodies by immunizing mice with an *S. typhimurium aroA* strain carrying a plasmid containing the LT-B gene from a porcine isolate of enterotoxigenic *E. coli*.

O antigens recognized in *Vibrio cholerae* strains of the O1 serovar belong to two major subclasses: Ogawa and Inaba. High levels of antibodies to the O antigens are present in the serum of convalescent cholera patients. The genes coding for these O antigens were cloned and expressed in *E. coli* (93) and subsequently in an attenuated *Salmonella* sp. to test salmonellae expressing cholera O antigens as a potential vaccine against cholera. A hybrid typhoid-cholera vaccine was constructed by transforming *S. typhi* Ty21a *thyA* with a plasmid containing the genes coding for the biosynthesis of the O antigen of *V. cholerae* O1 serotype Inaba and an intact *thyA* gene to select in favor of plasmid retention. Fourteen human adult volunteers were vaccinated with three doses of 10^{10} viable organisms, and a month later 8 vaccinees and 13 unimmunized controls were challenged with virulent *V. cholerae* O1 El Tor Inaba organisms. The vaccine strain was well tolerated by 10 of 14 volunteers, while the other 4 presented with mild malaise, nausea, and cramps. Serum antibodies against *S. typhi* Ty21a were detected in all vaccinees, while 14% developed a significant rise in antibody to Inaba LPS and 36% had fourfold rises in vibriocidal antibodies. Challenge studies demonstrated that this vaccine conferred only modest protection, since 75% of the vaccinees developed diarrhea, although it was much milder than in the unvaccinated controls. It is not known whether the inability of this candidate vaccine to elicit protective antibodies was due to low expression of the antigen in vivo, low immunogenicity of the antigen when presented on the surface of *S. typhi* (139), or the possibility that Ty21a is a poor carrier of heterologous antigens because of its extensive attenuation (127). Another study with the same vaccine strain was conducted in Australia with 10 adult volunteers. In this trial, the vaccine did not induce adverse reactions in any of the immunized subjects, and, in contrast to results in the report by Tacket et al. (139), 30 to 40% of the vaccinated individuals developed IgG or IgA against Inaba LPS (56).

Enterotoxigenic *E. coli* organisms produce a range of diarrheal diseases in both animals and humans. *E. coli* strains that cause the disease known as scours in swine and other domestic animals express on their surfaces fimbrial antigens (K88) that endow the organisms with the ability to adhere to the mucosal epithelium. Antibodies directed against the K88 fimbrial antigen have been shown to passively protect suckling piglets against diarrhea caused by enterotoxigenic *E. coli* possessing the K88 fimbriae if the sows were previously immunized with K88 preparations (104). To induce relevant secretory antibodies against these adhesins, the gene coding for the fimbrial proteins K88ab was cloned in a plasmid and transformed into an *S. typhimurium galE* mutant. The recombinant strain was shown to agglutinate with anti-K88 antibodies and to express large quantities of K88ab fimbriae as surface proteins. Furthermore, oral immunization of mice with the recombinant vaccine, when followed by intraperitoneal administration of a booster, resulted in high levels of serum and mucosal antibodies against K88ab antigen (132). A similar study was performed by Dougan et al. (44) with an *S. typhimurium aroA* mutant. This strain was transformed with a plasmid carrying the K88 genes from *E. coli*, and the resulting K88-expressing transformant was shown to be avirulent in mice and capable of inducing antibodies against K88, as detected by Western immunoblot. In this study, the immunized mice were strongly protected against virulent *S. typhimurium* on challenge. Hone et al. (7) have used a system of antigen stabilization to clone the K88 gene into the chromosome of a *galE* mutant of *S. typhimurium*. They found that the gene was stably expressed by the attenuated strain and that, after oral immunization of mice, the Peyer's patches were colonized and serum antibodies against K88 developed.

A potential anticaries vaccine using attenuated *Salmonella* strains has been developed by Curtiss and coworkers (31–35, 38, 79, 107). Members of the *Streptococcus mutans* group (including *Streptococcus mutans*, *Streptococcus rattus*, *Streptococcus cricetus*, and *Streptococcus sobrinus*) are the principal etiological agents of dental caries in humans. Studies on the pathogenic mechanisms of *Streptococcus*-induced caries have led to the identification of a surface protein antigen (SpaA) from *Streptococcus mutans* and *Streptococcus sobrinus* that has been implicated in a sucrose-independent reaction used by the organisms to attach to a proteinaceous film coating the tooth surface (30). Also, a glucosyltransferase (GtfA) from *Streptococcus mutans* that is capable of hydrolyzing sucrose and is involved in sucrose-dependent adherence and aggregation of the bacteria to the teeth has been described as an important virulence determinant of these organisms. The *spaA* and *gtfA* genes coding for these proteins have been cloned into plasmids and introduced by transformation into various attenuated *Salmonella* mutants in an effort to construct a vaccine capable of preventing dental caries. These recombinant strains expressed the cloned genes and, on oral immunization of mice, were selectively taken up by the Peyer's patches. Antigen-specific salivary sIgA against both SpaA and GtfA was induced. However, mice immunized with the vaccine strain were not protected against challenge with virulent *Streptococcus mutans*. This may have been a reflection of the low level of antigen expression by the vaccine strains and the low sIgA titers developed by the animals.

The ability of avirulent *Salmonella* strains to evoke a cellular as well as a humoral antibody response against a heterologous antigen was first demonstrated by immunizing mice intravenously with an *S. typhimurium aroA* strain carrying a recombinant plasmid coding for the production of the intracellular protein β-galactosidase. It was observed that after primary immunization and one booster with the vaccine strain, there was a delayed-type hypersensitivity response, as measured by footpad swelling (15, 43), to β-galactosidase.

Shigella flexneri serotype 2a is one of the major etiological agents of shigellosis in the United States. In an effort to develop a vaccine against shigellosis, Baron et al. (6) used bacteriophage Mu-induced transposition to clone the genes specifying the type and group antigens from *Shigella flexneri* 2a into a plasmid. The group antigen that specifies the O repeat unit backbone is encoded by a gene located near the *his* marker at min 44 on the *Shigella* chromosome; the type antigen locus (type 2), which controls the modification of the O repeat unit, is located at min 6. The genetic construct was obtained by using *Shigella flexneri* carrying an F′ *lac*::Mu *cts*62 plasmid grown at the permissive temperature for transposition of Mu. One of the plasmid isolates that carried the *pro* and *his* regions of *Shigella flexneri* 2a was conjugally transferred into a spontaneous *pro his* mutant of *S. typhi* Ty21a. The resulting strain (WR4086) agglutinated with rabbit anti-*Shigella flexneri* 2a serum and with anti-*S. typhi* Ty2 serum. However, in serum adsorption studies, strain WR4086 failed to remove all the *S. typhi* Ty21a O antigens. When examined in a mouse protection test, this strain provided significant protection against intraperitoneal challenge with *Shigella flexneri* 2a but not with the wild-type strain *S. typhi* Ty2. The authors reasoned that this lack of

protection was due to the predominance of *Shigella* O antigens on the bacterial surface; the vaccine strain did not express sufficient *S. typhi* Ty21a somatic antigens to induce protective anti-*S. typhi* antibody levels in the mice.

M protein, an antiphagocytic virulence factor from *Streptococcus pyogenes*, has been cloned and expressed in an *S. typhimurium aroA* attenuated strain. Mice orally immunized with this potential vaccine developed anti-M-specific (type 5) serum IgG, IgA, and IgM as well as specific salivary sIgA. Immune serum was observed to have significant opsonizing activity against virulent *Streptococcus pyogenes*, and vaccinated mice challenged intraperitoneally or intranasally were protected against challenge with *Streptococcus pyogenes* type 5 but not against *Streptococcus pyogenes* type 24 (119). The M protein from *Streptococcus equi*, the causative agent of strangles in horses, has also been cloned into an *S. typhimurium* Δ*cya* Δ*crp* avirulent strain, but thus far no immunogenicity or animal studies have been reported (36).

Stabel et al. (131) have investigated the possibility of constructing a live oral vaccine against brucellosis by cloning a 31-kDa cell surface protein from *Brucella abortus* into an *S. typhimurium* Δ*cya* Δ*crp* strain. The protein was stably expressed by the recombinant strain in vivo and in vitro, and oral immunization of mice led to the development of serum and intestinal antibody responses against both the foreign protein and the *Salmonella* endotoxin. However, the vaccine strain failed to induce a delayed-type hypersensitivity reaction, indicating that the *Brucella* antigen delivered by the live bacteria was not a good inducer of cellular immunity or that suppressor T cells may have been activated by an unknown mechanism.

A novel approach to expressing heterologous antigens on the surface of attenuated *Salmonella* spp. has been recently developed. The gene that codes for *Salmonella* flagellin was cloned into a plasmid, and synthetic oligonucleotides specifying amino acid sequences identified as epitopes of several heterologous antigens were inserted into the flagellin gene. The resulting recombinant plasmids were transformed into flagellin-negative, attenuated *Salmonella* strains. Expression of functional flagella and the foreign epitope was detected by bacterial immobilization in the presence of antipeptide serum. Epitopes from the following antigens have been tested in this system: cholera toxin subunit B (109), hepatitis B surface protein (150), *Streptococcus pyogenes* M protein, human immunodeficiency virus surface glycoprotein gp160, and murine cytomegalovirus immediate-early protein pp80 (92, 133). Analysis of the immunogenicity of the recombinant strains has demonstrated the induction of serum antibodies against the epitopes upon immunization with the recombinant *Salmonella* strains by various routes. Further research to characterize these potential vaccines and study their efficacy is reported to be under way.

Other bacterial antigens that have been expressed in *Salmonella* attenuated mutants include the C fragment of tetanus toxin (136), a lipoprotein from *Treponema pallidum* (136), a membrane T-cell-reactive protein from *Francisella tularensis* (128), and several uncharacterized antigens from *Mycobacterium leprae* (20). Immunogenicity and protection studies in animal models with these recombinant strains are reported to be under investigation.

Heterologous Antigens of Viral and Eukaryotic Origins

The versatility and efficacy of the use of live attenuated *Salmonella* strains as carriers of foreign antigens have prompted investigators to test the feasibility of applying this system in the development of vaccines against viral and eukaryotic pathogens as well.

Several fragments of DNA from human hepatitis B virus and woodchuck hepatitis B virus encoding viral surface or nucleocapsid proteins have been expressed as fusion proteins in *S. dublin aroA*. A pUC8 plasmid derivative carrying the LT-B gene was modified by removing the LT-B translational stop codon and adding a universal polylinker to allow cloning of the viral DNA segments at the 3′ end of the LT-B gene. Various fusion polypeptides were expressed in *E. coli* and *Salmonella* spp. and were detected by immunoblots and enzyme-linked immunosorbent assay with the appropriate anti-virus antibodies. In preliminary immunogenicity studies, mice immunized with the recombinant *Salmonella* strains developed anti-LT-B humoral and cellular responses and a T-cell antiviral priming response when the human hepatitis B virus nucleocapsid was used as the antigen in the fusion protein (125, 126).

Several major neutralizing epitopes of dengue virus are located in the envelope (E) protein. A DNA fragment encoding 76% of the total E protein was cloned into a plasmid and expressed in an *S. typhimurium aroA* strain (29). The plasmid carrying the gene was unstable in vitro, since 50% of the bacteria lost the plasmid after 35 generations of growth under nonselective conditions. In contrast, it was observed that the same plasmid exhibited good stability in vivo, since only 1% of the colonies recovered from spleens of immunized mice had lost the plasmid. Immunization studies with mice showed that the vaccine did not induce any protection against challenge with dengue virus, but, on the contrary, vaccinated animals reacted more severely to the challenge than unvaccinated controls did. It has been observed that different strains of mice show variable ranges of susceptibility to *Salmonella* infection, and, moreover, it has been suggested that infection with *Salmonella* strains may induce some degree of immunosuppression in the animals (68). Another possibility is that the vaccine induced an immune response to the dengue virus E protein, which may have triggered a more severe reaction after challenge.

Herpes simplex virus types 1 and 2 are the cause of several clinical syndromes including acute gingivostomatitis, eczema, genital herpes, and neonatal herpes. Experimental vaccines that use envelope glycoprotein antigens expressed in vaccinia virus and attenuated *Salmonella* strains are being developed. A DNA fragment encoding a terminal domain of herpes simplex virus gB1 was fused to the LT-B gene, and a fusion polypeptide recognized by antibodies against LT-B and herpes simplex virus was expressed in *S. dublin* SL1438 (19). The virion envelope glycoprotein D has been cloned and expressed in *E. coli* under the control of the *lac* promoter. Experiments to express this viral antigen in *Salmonella* spp. and test it in a guinea pig model are currently in progress (12).

Rotaviruses cause most of the diarrheal illness in infants and young children in industrialized nations. This infection is limited to the gastrointestinal tract; therefore, the induction of specific mucosal antibodies plays a major role in preventing the infection. A surface glycoprotein (VP7), which is known to induce neutralizing antibodies, was cloned, and a DNA segment coding for about 86% of the protein was expressed as a fusion product with β-galactosidase in the attenuated *S. typhimurium aroA* SL3261. The plasmid used for this experiment carried the *rop* gene, which decreases the copy number of ColE1-related plasmids, since it was observed that overproduction of the fusion peptide was toxic

for the bacterial cells. After 40 generations in vitro, approximately 90% of the bacteria retained the plasmid without selective pressure. In contrast, only 30% of the bacteria recovered from mice tissues at day 35 postimmunization still retained the plasmid. The vaccinated animals developed circulating antibody titers against *S. typhimurium* and against β-galactosidase but failed to develop antibodies against the viral protein (124).

Sadoff et al. (123) have investigated the use of attenuated *Salmonella* strains as vectors to deliver antigens from *Plasmodium berghei*, the causative agent of malaria in mice, as a model for vaccine development against human malaria. The gene coding for a circumsporozoite (CS) antigen of *P. berghei* was expressed constitutively in *S. typhimurium*. This hybrid strain induced antigen-specific cell-mediated immune responses in mice and, after peroral or subcutaneous immunization, provided protection against sporozoite challenge in the absence of measurable antibody to CS protein. Recently, another group of investigators reported the cloning of a CS gene from *Plasmodium yoelii* into the chromosome of an *S. typhimurium aroA* strain. Mice orally immunized with three doses of the recombinant vaccine were sacrificed 6 weeks after the last immunization, and spleen cells were used as the effector cells in a cytotoxic T-lymphocyte ^{51}Cr release assay. Specific lysis of the target cells labeled with CS antigen was observed with effector cells from mice immunized either with irradiated sporozoites (54% lysis) or with the *Salmonella* vaccine (37% lysis). In contrast, the serum antibody response against CS was negligible, and the mice were not protected against challenge with viable sporozoites. This observation is important, as it constitutes the first report of the generation of cytotoxic T-lymphocyte response to a foreign antigen expressed from the *Salmonella* chromosome (54).

OTHER CONSIDERATIONS

The use of attenuated *Salmonella* strains as carriers of heterologous antigens is a technique of vaccine delivery with significant potential to influence the management of infectious diseases on a large scale not only with vaccines against enteric bacterial pathogens but also with vaccines against a variety of other bacteria, viruses, and parasites. There are, however, a number of unresolved questions that will influence the utility of this approach to vaccine development, including (i) how to stabilize antigen expression in the *Salmonella* carrier and (ii) the effect of multiple use on carrier efficacy. Each of these is addressed below.

Stabilization of Antigen Expression in Attenuated *Salmonella* Strains

Most of the attenuated *Salmonella* strains proposed as candidate vaccines against major human or animal pathogens have been constructed by using plasmids into which the gene(s) encoding the foreign protein has been inserted. The plasmids used for hybrid vaccines should be nonconjugative and nonmobilizable, and although most of the plasmids used have been shown to be highly retained in vitro, problems may arise if hybrid strains carrying recombinant plasmids are administered to humans as vaccines. First, the possibility of plasmid transfer from the hybrid to the human and animal bacterial flora or to environmental bacteria must be considered. Second, the introduction of a recombinant plasmid into the carrier strain usually requires selection for an antibiotic resistance marker on the plasmid. Such markers are very useful in laboratory experiments but are impractical in strains intended for administration to humans or animals, primarily because hybrid strains carrying such selective markers are difficult to maintain in vivo once the selective pressure has been removed. Additionally, it has been observed by several investigators that large amounts of a foreign protein expressed from a multicopy plasmid may be toxic for the bacterial cell, especially when the antigens are of eukaryotic origin (29, 115, 123, 124).

Different strategies for addressing the issue of plasmid instability and avoiding the necessity for a stabilizing antibiotic have been proposed. Nakayama et al. (107) and Curtiss et al. (38) constructed Δ*asd* mutants of *S. typhimurium* and an Asd^+ expression-cloning vector carrying the *spaA* gene of *Streptococcus sobrinus*. Since absence of the recombinant plasmid is lethal for the cells, the antigen was stably expressed in vitro and in vivo. This combination of cloning vector and mutant host eliminates the need for antibiotics to stabilize the cloning vector and may be useful when expression of the antigen from a recombinant plasmid is desirable.

An alternative approach for obtaining stable antigen expression is to integrate the foreign genes into the chromosome of the carrier strain (54, 70, 136). Hone et al. (70) have developed one system whereby heterologous DNA may be recombined into the chromosome of a *Salmonella* sp. This system involves two steps: (i) integration of a *hisOG* deletion mutation into the chromosome and (ii) replacement of the *hisOG* deletion by the complete *hisOGD* region and a segment of heterologous DNA that encodes the antigen of interest. This strategy was used to integrate the genes encoding K88 fimbriae of *E. coli* into the chromosome of a *galE* mutant of *S. typhimurium*. It was observed that after 54 generations of growth in vitro, 90% of the recombinant organisms tested were $K88^+$. However, 18 days after immunization only 73% of the bacteria recovered from the tissues of immunized animals were $K88^+$. The loss of expression of K88 was due either to undetectable point mutations or to a spontaneous deletion of the K88 gene. A similar study was carried out by Strugnell et al. (136), who used the *aroC* gene as the site of integration for the heterologous gene and an *S. typhimurium polA* mutant as the recipient of the recombinant plasmid. Two heterologous antigens were integrated into the *Salmonella* chromosome by this system: the C fragment of tetanus toxin and the *tpd* gene from *T. pallidum*, which encodes a lipoprotein product. Both antigens were expressed, albeit at lower levels than when carried on a plasmid. Results of stability and immunization studies with these recombinant strains are not available.

In a more recent report (54), a chromosomal expression vector was devised by using a modified version of the minitransposon mini-Tn*10* carried on bacteriophage lambda. This vector possesses a transposase gene located outside of the transposable portion of the transposon, which in itself carries a kanamycin resistance gene and an α-*lacZ* fragment under the control of a *lacUV5* promoter. The gene coding for the sporozoite protein from *P. yoelii* (CS) was cloned into this vector fused to the *lacZ* transcription signals. This construct was transformed into *E. coli*, and positive clones were introduced into a restriction-deficient *S. typhimurium*. In the absence of the *lac* repressor, the transposase is expressed and the mini-Tn*10*, along with the CS gene, transposes into the chromosome. The last step in this system involves the transduction of the insertions into an attenuated *Salmonella* vaccine strain by using a P22 lysate. The heterologous protein was expressed by the recombinant strain as detected by immunoblots of whole-cell lysates with an

anti-CS monoclonal antibody. Animal studies demonstrated that *S. typhimurium* recovered from spleens of orally infected mice 3 weeks postimmunization were still expressing the CS protein, although a small deletion in the CS gene was observed in one of the isolates recovered. Although immunization of mice with this vaccine did not confer protection against challenge with viable sporozoites and the antibody responses against CS were negligible, there was a significant anti-CS cytotoxic T-lymphocyte response.

Employing the system developed by Hone et al. (70), Cárdenas and Clements (16) integrated the gene that codes for LT-B into the chromosome of *S. typhimurium galE*. Comparative in vitro and in vivo studies with the strain carrying the gene chromosomally integrated and an isogenic strain carrying the same gene on a multicopy plasmid were conducted. Levels of expression of the foreign antigen were significantly lower when the antigen was expressed from the chromosome than when it was expressed from the plasmid. The in vivo stability of antigen expression in organisms recovered from spleens, livers, and Peyer's patches of orally inoculated mice was determined. By 24 h postinoculation, most of the tissue isolates from the plasmid containing strain had lost the plasmid and the ability to synthesize the antigen in vivo. By contrast, 100% of the cointegrate isolates expressed the antigen in vivo throughout the 21 days of the experiment. Significantly, humoral and mucosal antibody levels against the antigen were greater when the antigen was expressed from the plasmid stabilized by the presence of the antibiotic than from the chromosome. These observations indicate that the most important event for the development of an immune response against a foreign antigen delivered by these vectors may be the initial amount of antigen that primes the GALT and not persistence of the vector in tissues.

There are several points to consider regarding the observed instability of the plasmid expressing the recombinant antigen when the strains are grown in the presence or absence of the stabilizing antibiotic. It should be noted that a number of the studies reported were performed with the genes coding for recombinant antigens on plasmids with a pMB1 origin of replication (147). The relative instability of these plasmids has been widely noted by a number of investigators (29, 54, 107, 113, 123, 124). However, other plasmids derived from different replicons (e.g., ColE1) have also been found to be unstable in these systems. Salas-Vidal et al. (124), for instance, observed a 70% plasmid loss in strains recovered from the tissues of infected animals, even when the *rop* gene was introduced to stabilize the plasmid. It has also been noted that overproduction of many recombinant antigens is toxic to the bacterial host. Whether this plays a role in the observed instability of these constructs is not known. Another consideration is the influence, if any, of the virulence-associated plasmid (63, 65, 66, 76, 116) on the stability of the recombinant plasmids.

Effect of Multiple Use on Carrier Efficacy

A major consideration in the proposed use of a *Salmonella* sp. as a carrier for heterologous antigens is the consequence of repeated use of the carrier. Specifically, if a *Salmonella* strain is used as a carrier for one antigen, will it be an effective vehicle for delivery of other antigens? Does immunologic experience with one carrier (strain) limit the immunologic response to a foreign antigen delivered by the same carrier or a second, immunologically related carrier? Do the serum and mucosal antibody responses differ? Does immunization against typhoid fever limit the use of a *Salmonella* strain as a vaccine carrier? Bao and Clements (5) have examined a number of factors related to these questions, including (i) the nature of the humoral response if two *Salmonella* strains that are serologically identical or serologically different are used, (ii) the temporal relationship of priming and boosting doses on the subsequent immunologic response to the carrier and the foreign antigen, and (iii) differences in serum IgG and mucosal IgA responses between immunologically experienced and immunologically naive animals.

In their findings, immunologic experience with homologous and heterologous *Salmonella* serotype strains potentiated the subsequent antibody response when the same strains were used as vaccine carriers of foreign antigens. This potentiation was positively correlated with the appearance of antibody directed against the LPS of the carrier strain. Both serum and mucosal antibody responses against the foreign antigen increased over time. Serum antibody responses in mice primed with either the homologous or the heterologous serotype strain were not statistically significantly different, whereas mice primed with the homologous serotype strain developed significantly better mucosal antibody responses against the foreign antigen. The findings of Bao and Clements (5) indicated that immunologic experience with a *Salmonella* strain as a vaccine against typhoid fever or as a carrier for one foreign antigen will not preclude its subsequent use as a carrier for antigens of other pathogens. Immunologic experience with one strain does not limit the immunologic response to a foreign antigen delivered by the same strain or a second, immunologically related strain.

CONCLUSIONS

The use of attenuated *Salmonella* strains as vaccine delivery vehicles for heterologous antigens has been extensively studied in a number of animal species and in humans. These mutants are able to establish a limited infection in the host, and during the natural course of this innocuous infection, the bacteria deliver a series of in vivo-synthesized antigens directly to the B and T lymphocytes present in the GALT. The primed B cells migrate to the mesenteric lymph nodes, where they undergo differentiation, enter the general circulation via the thoracic duct, and subsequently populate the gut and other mucosal tissues, where clonal expansion and terminal differentiation into plasma cells occur. It has been demonstrated that immunization with these bivalent vaccines is an effective and safe way to elicit the production of serum and mucosal antibodies against both the *Salmonella* carrier and the foreign antigen. However, certain aspects must be addressed before bivalent attenuated *Salmonella* vaccines can be used for mass immunizations.

This review has focused on the background and application of this approach to vaccine delivery and addresses a number of important aspects of this technique.

(i) Use of avirulent *Salmonella* spp. as carriers for heterologous antigens is safe. This was established not only in the various animal studies cited above but also in the human volunteer studies reported by Tramont et al. (144), Forrest et al. (56), and Tacket et al. (139, 140). The one notable exception may be in the study reported by Cohen et al. (29), in which mice immunized with an *S. typhimurium aroA* strain expressing a fragment of protein E of dengue virus reacted more severely to the challenge than did unvaccinated controls. One possible explanation for these findings is related to an observation made by researchers in a number of

laboratories that immunization of mice with attenuated *Salmonella* mutants can induce protection against challenge with virulent *Salmonella* spp. while concomitantly depressing mitogenic responses to both B-cell and T-cell mitogens (40, 85) or inducing suppression against both *Salmonella* and other antigens (68, 81, 83, 108). The basis for this suppression is not known, and this effect has not been reported to occur following oral immunization. Studies reporting immune suppression as a consequence of immunization with *Salmonella* spp. have employed an intravenous, intraperitoneal, or subcutaneous route of inoculation. These nonphysiologic routes of administration with viable, attenuated bacteria are not appropriate for vaccines intended for human use. Although the mice in the study by Cohen et al. (29) were immunized orally, immunosuppression was not documented in these animals and other factors may have been responsible for those findings.

(ii) Delivery of antigen by this mechanism is effective not only for the development of significant levels of sIgA on various mucosal surfaces but also for the production of significant increases in serum antibody levels and cell-mediated responses against the target antigen. This effect has been demonstrated with a variety of bacterial, viral, and eukaryotic antigens, although not universally so. In the studies by Sadoff et al. (123) and Flynn et al. (54), in which *Plasmodium* antigens were expressed in attenuated *Salmonella* strains, serum antibody responses were not detected even in the presence of demonstrable cell-mediated responses.

(iii) Surface expression or secretion from the bacterial cell is not essential for immunogenicity of a cloned antigen expressed in attenuated *Salmonella* spp. This is clear not only from the studies by Clements et al. with cloned LT-B (25, 27) but also from the studies by Dougan et al. with the K88 fimbrial agent (44), Brown et al. with cloned β-galactosidase (15), and Curtiss and coworkers in the development of a potential anticaries vaccine (31–35, 38, 79, 107). When heterologous antigens have been expressed on the surface of attenuated *Salmonella* strains (92, 109, 133, 150), serum antibodies against the foreign epitopes have been demonstrated.

(iv) Selection of an appropriate mechanism and degree of attenuation for the intended *Salmonella* carrier is a critical consideration. A *Salmonella* sp. functions as a carrier for stimulation of antigen-specific sIgA because of the natural pathogenesis of the organism and the behavior of appropriately attenuated mutants, which can interact with the lymphoid tissues in the Peyer's patches without causing systemic disease. A number of investigators (113, 127) have noted that strains can be overattenuated by inappropriate or multiple mutations and become unsuitable for use as live vaccines. Overattenuation is the most likely reason for the failure of *S. typhi* 541TY (*aroA purE*) (Vi positive) and *S. typhi* 543TY (*aroA purE*) (Vi negative) to colonize and induce a humoral immune response in volunteers (89). Moreover, mutations that attenuate *Salmonella* strains normally pathogenic for mice do not necessarily attenuate to the same degree *Salmonella* strains that are pathogenic for humans (i.e., *galE* mutations in *S. typhimurium*) (72).

(v) The mouse model using appropriately attenuated *Salmonella* mutants is a relevant model system for testing this mechanism of antigen delivery but is not without limitations. It is important to determine the effectiveness of this system of vaccination in an animal model that will permit challenge studies. In general, the lack of relevant animal models has hindered the final assessment of all of the candidate *Salmonella*-based vaccines developed to date. In a number of studies, vaccination and challenge experiments have been performed via intraperitoneal or intravenous injection. Since this route of inoculation deviates from the natural route of infection used by mucosal pathogens, the conclusions concerning efficacy and protection deduced from these kinds of experiments may not apply to natural challenge by the virulent organisms. Furthermore, when the end point of the challenge experiments is death, as in the mouse protection assays, it is not possible to determine the individual contributions of mucosal, serum, and cell-mediated immune responses to animal survival.

(vi) Antigen expression can be stabilized by a variety of mechanisms including use of plasmids that complement metabolic defects on the host strain (38, 107) and integration of the heterologous gene into the host chromosome (16, 54, 70, 136).

(vii) Immunologic experience with a *Salmonella* strain as a vaccine against typhoid fever or as a carrier for one foreign antigen will not preclude its subsequent use as a carrier for antigens of other pathogens. As demonstrated by Bao and Clements (5), immunologic experience with one strain does not limit the immunologic response to a foreign antigen delivered by the same strain or a second, immunologically related strain.

The use of live attenuated *Salmonella* strains as delivery vectors of heterologous antigens to the secretory immune system constitutes a promising approach for the development of new vaccines against a number of diseases. Even though there are unresolved questions about the use of this technique, the results obtained to date are encouraging, and there is great potential for development of safe, effective, affordable vaccines.

REFERENCES

1. **Ashcroft, M. T., J. M. Ritchie, and C. C. Nicholson.** 1964. Controlled field trial in British Guiana school children of heat-killed phenolized and acetone-killed lyophilized vaccines. Am. J. Hyg. **79:**196–206.
2. **ASM News.** 1990. W.H.O. reports decry neglect of world health problems. ASM News **56:**358–359.
3. **Bacon, G. A., T. W. Burrows, and M. Yates.** 1950. The effects of biochemical mutation on the virulence of *Bacterium typhosum*: the virulence of mutants. Br. J. Exp. Pathol. **31:**714–724.
4. **Bacon, G. A., T. A. Burrows, and M. Yates.** 1951. The effects of biochemical mutation on *Bacterium typhosum*: the loss of virulence of certain mutants. Br. J. Exp. Pathol. **32:**85–96.
5. **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.
6. **Baron, L., D. J. Kopecko, S. B. Formal, R. Seid, P. Guerry, and C. Powell.** 1987. Introduction of *Shigella flexneri* 2a type and group antigen genes into oral typhoid vaccine strain *Salmonella typhi* Ty21a. Infect. Immun. **55:**2797–2801.
7. **Barrow, P. A.** 1990. Immunity to experimental fowl typhoid in chickens induced by a virulence-cured derivative of *Salmonella gallinarum*. Infect. Immun. **58:**2283–2288.
8. **Barrow, P. A., and M. A. Lovell.** 1988. The association between a large molecular mass plasmid and virulence in a strain of *Salmonella pullorum*. J. Gen. Microbiol. **134:**2307–2316.
9. **Barrow, P. A., J. M. Simpson, M. A. Lovell, and M. M. Binns.** 1987. Contribution of *Salmonella gallinarum* large plasmid toward virulence in fowl typhoid. Infect. Immun. **55:**388–392.
10. **Black, R., M. M. Levine, C. Ferreccio, M. L. Clements, C. Lanata, J. Rooney, R. Germanier, and the Chilean Typhoid Committee.** 1990. Efficacy of one or two doses of Ty21a *Salmonella typhi* vaccine in enteric-coated capsules in a con-

trolled field trial. Vaccine 8:81–84.

11. **Black, R., M. M. Levine, C. Young, J. Rooney, S. Levine, M. L. Clements, S. O'Donnell, T. Hughes, and R. Germanier.** 1983. Immunogenicity of Ty21a attenuated *Salmonella typhi* given with sodium bicarbonate or in enteric-coated capsules. Dev. Biol. Stand. 53:9–14.
12. **Bowen, J. C., O. Alpar, R. Phillpotts, I. S. Roberts, and M. R. Brown.** 1990. Preliminary studies on infection by attenuated *Salmonella* in guinea pig and on expression of herpes simplex virus. Res. Microbiol. 141:873–877.
13. **Bowers, W. F., and L. Shuppe.** 1942. Acute appendicitis: sequela of typhoid inoculation. Mil. Surg. 90:413.
14. **Brandtzaeg, P.** 1989. Overview of the mucosal immune system. Curr. Top. Microbiol. Immunol. 146:13–25.
15. **Brown, A., C. E. Hormaeche, R. Demarco de Hormaeche, M. Winther, G. Dougan, D. J. Maskell, and B. A. D. Stocker.** 1987. An attenuated *aroA Salmonella typhimurium* vaccine elicits humoral and cellular immunity to cloned β-galactosidase in mice. J. Infect. Dis. 155:86–92.
16. **Cárdenas, L., and J. D. Clements.** Unpublished data.
17. **Cebra, J. J., J. A. Fuhrman, D. A. Lebman, and S. D. London.** 1986. Effective gut mucosal stimulation of IgA-committed B cells by antigen, p. 129–133. *In* F. Brown, R. M. Chanock, and R. A. Lerner (ed.), Vaccines 86: new approaches to immunization. Developing vaccines against parasitic, bacterial, and viral diseases. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. **Chikami, G. K., J. Fierer, and D. G. Guiney.** 1985. Plasmid-mediated virulence in *Salmonella dublin* demonstrated by use of a Tn*5-oriT* construct. Infect. Immun. 50:420–424.
19. **Childress, A. M., and J. D. Clements.** 1988. Construction of a chimeric plasmid expressing glycoprotein B (gB) of herpes simplex virus type 1 and heat-labile enterotoxin (LT-B) of *Escherichia coli*, abstr. S-6, p. 316. Abstr. 88th Annu. Meet. Am. Soc. Microbiol. 1988. American Society for Microbiology, Washington, D.C.
20. **Clark-Curtiss, J. E., J. E. Thole, M. Sathish, B. A. Bosecker, S. Sela, E. F. de Carvalho, and R. E. Esser.** 1990. Protein antigens of *Mycobacterium leprae*. Res. Microbiol. 141:859–871.
21. **Clasener, H. A. L., and B. J. W. Beunders.** 1967. Immunization of man with typhoid and cholera vaccine. Agglutinating antibodies after intracutaneous and subcutaneous injections. J. Hyg. 65:446–449.
22. **Clemens, J. D., D. A. Sack, J. R. Harris, J. Chakraborty, M. R. Khan, B. F. Stanton, B. A. Kay, M. U. Khan, M. Yunus, W. Atkinson, A. Svennerholm, and J. Holmgren.** 1986. Field trial of oral cholera vaccines in Bangladesh. Lancet ii:124–127.
23. **Clemens, J. D., D. A. Sack, J. R. Harris, F. van Loon, J. Chakraborty, F. Ahmed, M. R. Rao, M. R. Khan, M. Yunus, N. Huda, B. F. Stanton, B. A. Kay, S. Walter, R. Eeckels, A.-M. Svennerholm, and J. Holmgren.** 1990. Field trial of oral cholera vaccines in Bangladesh: results from three-year follow-up. Lancet 335:270–272.
24. **Clements, J. D.** 1987. Use of attenuated mutants of *Salmonella* as carriers for delivery of heterologous antigens to the secretory immune system. Pathol. Immunopathol. Res. 6:137–146.
25. **Clements, J. D., and S. El-Morshidy.** 1984. Construction of a potential live oral bivalent vaccine for typhoid fever and cholera-*Escherichia coli*-related diarrheas. Infect. Immun. 46:564–569.
26. **Clements, J. D., N. M. Hartzog, and F. L. Lyon.** 1988. Adjuvant activity of *Escherichia coli* heat-labile enterotoxin and effect on the induction of oral tolerance in mice to unrelated protein antigens. Vaccine 6:269–277.
27. **Clements, J. D., F. L. Lyon, K. L. Lowe, A. L. Farrand, and S. El-Morshidy.** 1986. Oral immunization of mice with attenuated *Salmonella enteritidis* containing a recombinant plasmid which codes for production of the B subunit of heat-labile *Escherichia coli* enterotoxin. Infect. Immun. 53:685–692.
28. **Cohen, G. N., and I. Saint-Girons.** 1987. Biosynthesis of threonine, lysine, and methionine, p. 429–444. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
29. **Cohen, S., C. J. Powell, D. R. Dubois, A. Hartman, P. L. Summers, and K. H. Eckels.** 1990. Expression of the envelope antigen of dengue virus in vaccine strains of *Salmonella*. Res. Microbiol. 141:855–858.
30. **Curtiss, R., III.** 1985. Genetic analysis of *Streptococcus mutans* virulence, p. 253–277. *In* W. Goebel (ed.), Genetic approaches to microbial pathogenicity. Springer-Verlag, New York.
31. **Curtiss, R., III.** 1986. Genetic analysis of *Streptococcus mutans* virulence and prospects for an anticaries vaccine. J. Dent. Res. 65:1034–1045.
32. **Curtiss, R., III, R. M. Goldschmidt, N. B. Fletchall, and S. M. Kelly.** 1988. Avirulent *Salmonella typhimurium* Δ*cya* Δ*crp* oral vaccine strains expressing a streptococcal colonization and virulence antigen. Vaccine 6:155–160.
33. **Curtiss, R., III, R. Goldschmidt, S. Kelly, M. Lyons, S. Michalek, R. Pastian, and S. Stein.** 1987. Recombinant avirulent *Salmonella* for oral immunization to induce mucosal immunity to bacterial pathogens, p. 261–271. *In* H. Kohler and P. T. LoVerde (ed.), Vaccines: new concepts and developments. Proceedings of the 10th International Convocation on Immunology. Longman Scientific and Technical, Harlow, Great Britain.
34. **Curtiss, R., III, R. Goldschmidt, R. Pastian, M. Lyons, S. M. Michalek, and J. Mestecky.** 1986. Cloning virulence determinants from *Streptococcus mutans* and the use of recombinant clones to construct bivalent oral vaccine strains to confer protective immunity against *S. mutans* induced caries, p. 173–180. *In* S. Hamada, S. M. Michalek, H. Kiyono, L. Menaker, and J. R. McGhee (ed.), Molecular microbiology and immunology of *Streptococcus mutans*. Elsevier Science Publishing, Inc., New York.
35. **Curtiss, R., III, and S. M. Kelly.** 1987. *Salmonella typhimurium* deletion mutants lacking adenylate cyclase and cyclic AMP receptor protein are avirulent and immunogenic. Infect. Immun. 55:3035–3043.
36. **Curtiss, R., III, S. M. Kelly, P. A. Gulig, C. R. Gentry-Weeks, and J. E. Galán.** 1988. Avirulent salmonellae expressing virulence antigens from other pathogens for use as orally administered vaccines, p. 311–328. *In* J. Roth (ed.), Virulence mechanisms of bacterial pathogens. American Society for Microbiology, Washington, D.C.
37. **Curtiss, R., III, S. M. Kelly, P. A. Gulig, and K. Nakayama.** 1989. Selective delivery of antigens by recombinant bacteria. Curr. Top. Microbiol. Immunol. 146:35–49.
38. **Curtiss, R., III, K. Nakayama, and S. M. Kelly.** 1989. Recombinant avirulent *Salmonella* vaccine strain with stable maintenance and high level expression of cloned genes in vivo. Immunol. Invest. 18:583–596.
39. **Czerkinsky, C., M. W. Russell, N. Lycke, M. Lindblad, and J. Holmgren.** 1989. Oral administration of a streptococcal antigen coupled to cholera toxin subunit B evokes antibody response in salivary glands and extramucosal tissue. Infect. Immun. 57:1072–1077.
40. **Deschenes, M., M. Guenounou, E. Ronco, F. Vacheron, and C. Nauceil.** 1986. Impairment of in vitro lymphocyte proliferation responses and interleukin-2 production in susceptible (C57BL/6) mice infected with *Salmonella typhimurium*. Immunology 58:225–230.
41. **Dorman, C. J., S. Chatfield, C. F. Higgins, C. Hayward, and G. Dougan.** 1989. Characterization of porin and *ompR* mutants of a virulent strain of *Salmonella typhimurium*: *ompR* mutants are attenuated in vivo. Infect. Immun. 57:2136–2140.
42. **Dougan, G., S. Chatfield, D. Pickard, D. O'Callaghan, and D. Maskell.** 1988. Construction and characterization of *Salmonella* vaccine strains harbouring mutations in two different *aro* genes. J. Infect. Dis. 158:1329–1335.
43. **Dougan, G., D. Maskell, K. Sweeney, D. O'Callaghan, N. Fairweather, A. Brown, and C. E. Hormaeche.** 1987. Live attenuated *Salmonella* vaccines as carriers of heterologous antigens to the immune system, p. 279–282. *In* F. Brown,

R. M. Chanock, and R. A. Lerner (ed.), Vaccines 87. Modern approaches to new vaccines. Prevention of AIDS, and other viral, bacterial, and parasitic diseases. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

44. **Dougan, G., R. Sellwood, D. Maskell, K. Sweeney, F. Y. Liew, J. Beesley, and C. Hormaeche.** 1986. In vivo properties of a cloned K88 adherence antigen determinant. Infect. Immun. **52:**344–347.
45. **Dougan, G., L. Smith, and F. Heffron.** 1988. Live bacterial vaccines and their application as carriers of foreign antigens, p. 271–300. *In* J. L. Bittle (ed.), Advances in veterinary science and comparative medicine. Academic Press, Inc., San Diego, Calif.
46. **Dougan, G., and J. Tite.** 1990. Live bacterial vaccines for delivering antigens to the mammalian immune system. Semin. Virol. **1:**29–37.
47. **Eldridge, J. H., R. M. Gilley, J. K. Staas, Z. Moldoveanu, J. A. Meulbroek, and T. R. Tice.** 1989. Biodegradable microspheres: vaccine delivery system for oral immunization. Curr. Top. Microbiol. Immunol. **146:**59–66.
48. **Elson, C. O., and W. Ealding.** 1984. Generalized systemic and mucosal immunity in mice after mucosal stimulation with cholera toxin. J. Immunol. **132:**2736–2741.
49. **Fahey, K. J., and G. N. Cooper.** 1970. Oral immunization against experimental salmonellosis. I. Development of temperature-sensitive mutant vaccines. Infect. Immun. **1:**263–270.
50. **Ferreccio, C., M. M. Levine, H. Rodriguez, R. Contreras, and the Chilean Typhoid Committee.** 1989. Comparative efficacy of two, three, or four doses of Ty21a live oral typhoid vaccine in enteric-coated capsules: a field trial in an endemic area. J. Infect. Dis. **159:**766–769.
51. **Fields, P. I., E. Groisman, and F. Heffron.** 1989. A *Salmonella* locus that controls the resistance to microbicidal proteins from phagocytic cells. Science **243:**1059–1061.
52. **Fierer, J., L. Chikami, E. Hatlen, E. J. Heffernan, and D. Guiney.** 1988. Active immunization with LD842, a plasmid-cured strain of *S. dublin*, protects mice against group D and group B *Salmonella* infection. J. Infect. Dis. **158:**460–463.
53. **Finkelstein, R.** 1984. Cholera, p. 107–136. *In* R. Germanier (ed.), Bacterial vaccines. Academic Press, Inc., New York.
54. **Flynn, J. L., W. R. Weiss, K. A. Norris, H. S. Seifert, S. Kumar, and M. So.** 1990. Generation of a cytotoxic T-lymphocyte response using *Salmonella* antigen-delivery system. Mol. Microbiol. **4:**2111–2118.
55. **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.
56. **Forrest, B., J. 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.
57. **Fukasawa, T., and H. Nikaido.** 1959. Galactose sensitive mutants of *Salmonella*. Nature (London) **184:**1168–1169.
58. **Fukasawa, T., and H. Nikaido.** 1961. Galactose sensitive mutants of *Salmonella*. II. Bacteriolysis induced by galactose. Biochim. Biophys. Acta **48:**470–483.
59. **Germanier, R.** 1984. Typhoid fever, p. 137–165. *In* R. Germanier (ed.), Bacterial vaccines. Academic Press, Inc., New York.
60. **Germanier, R., and E. Fürer.** 1971. Immunity in experimental salmonellosis. II. Basis for the avirulence and protective capacity of *galE* mutants of *S. typhimurium*. Infect. Immun. **4:**663–673.
61. **Germanier, R., and E. Fürer.** 1975. Isolation and characterization of *galE* mutant Ty21a of *Salmonella typhi*: a candidate strain for a live oral typhoid vaccine. J. Infect. Dis. **131:**553–558.
62. **Gilman, R. H., R. B. Hornick, W. E. Woodward, H. L. DuPont, M. J. Snyder, M. M. Levine, and J. P. Libonati.** 1977. Evaluation of a UDP-glucose-4-epimeraseless mutant of *Salmonella typhi* as a live oral vaccine. J. Infect. Dis. **136:**717–723.
63. **Gulig, P. A., and R. Curtiss III.** 1987. Plasmid-associated virulence of *Salmonella typhimurium*. Infect. Immun. **55:**2891–2901.
64. **Habasha, F. G., B. P. Smith, L. Shwartz, A. Ardans, and M. Reina-Guerra.** 1985. Correlation of macrophage migration-inhibition factor from challenge by exposure in calves vaccinated with *Salmonella typhimurium*. Am. J. Vet. Res. **46:**1415–1421.
65. **Hackett, J., I. K. Kotlarski, V. Mathan, K. Francki, and D. Rowley.** 1985. The colonization of Peyer's patches by a strain of *Salmonella typhimurium* cured of the cryptic plasmid. J. Infect. Dis. **153:**1119–1125.
66. **Hackett, J., P. Wyk, P. Reeves, and V. Mathan.** 1987. Mediation of serum resistance in *Salmonella typhimurium* by an 11 Kd polypeptide encoded by the cryptic plasmid. J. Infect. Dis. **155:**540–549.
67. **Hemmings, W. A.** 1978. Antigen absorption by the gut. University Park Press, Baltimore.
68. **Hoertt, B. E., J. Ou, D. J. Kopecko, L. S. Baron, and R. L. Warren.** 1989. Novel virulence properties of the *Salmonella typhimurium* virulence-associated plasmid: immune suppression and stimulation of splenomegaly. Plasmid **21:**48–58.
69. **Hoiseth, S. K., and B. A. D. Stocker.** 1981. Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. Nature (London) **291:**238–239.
70. **Hone, D., S. Attridge, L. van den Bosch, and J. Hackett.** 1989. A chromosomal integration system for stabilization of heterologous genes in *Salmonella* based vaccine strains. Microb. Pathog. **5:**407–418.
71. **Hone, D., R. Morona, and S. Attridge.** 1987. Construction of defined *galE* mutants of *Salmonella* for use as vaccines. J. Infect. Dis. **156:**167–174.
72. **Hone, D. M., S. R. Attridge, B. Forrest, R. Morona, D. Daniels, J. T. LaBrooy, R. C. A. Bartholomeusz, D. J. C. 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.
73. **Iwarson, S., and P. Larsson.** 1980. Intradermal versus subcutaneous immunization with typhoid vaccine. J. Hyg. **84:**11–15.
74. **Jackson, S., J. Mestecky, N. K. Childers, and S. M. Michalek.** 1990. Liposomes containing anti-idiotypic antibodies: an oral vaccine to induce protective secretory immune responses specific for pathogens of mucosal surfaces. Infect. Immun. **58:**1932–1936.
75. **Joekes, A. M., J. R. Gabriel, and M. J. Goggin.** 1972. Renal disease following prophylactic immunization. Nephron **9:**162–170.
76. **Jones, G. W., D. K. Rabert, D. M. Svinarich, and H. J. Whitfield.** 1982. Association of adhesive, invasive, and virulent phenotypes of *Salmonella typhimurium* with autonomous 60-megadalton plasmids. Infect. Immun. **38:**476–486.
77. **Jones, L., S. Ristow, T. Yilma, and B. Moss.** 1986. Accidental human vaccination with vaccinia virus expressing nucleoprotein gene. Nature (London) **319:**543.
78. **Kaper, J. B., H. Lockman, and M. Baldini.** 1984. Recombinant nontoxigenic *Vibrio cholerae* strains as attenuated cholera vaccine candidates. Nature (London) **308:**655–658.
79. **Katz, J., S. M. Michalek, R. Curtiss III, C. Harmon, G. Richardson, and J. Mestecky.** 1987. Novel oral vaccines: the effectiveness of cloned gene products on inducing secretory immune responses. Adv. Exp. Med. Biol. **216B:** 1771–1747.
80. **Khan, R. J. U.** 1971. Anaphylactoid reaction to typhoid-paratyphoid A and B vaccine. Trop. Geogr. Med. **23:**115–116.
81. **Killar, L. M., and T. K. Eisenstein.** 1984. Differences in delayed-type hypersensitivity responses in various mouse strains in the C3H lineage infected with *Salmonella typhimurium* strain SL3235. J. Immunol. **133:**1190–1196.
82. **Killar, L. M., and T. K. Eisenstein.** 1985. Immunity to *Salmonella typhimurium* infection in C3H/Hej C3H/HeNCr1BR mice: studies with an aromatic-dependent live *Salmonella typhimurium* strain as vaccine. Infect. Immun. **47:**605–612.
83. **Killar, L. M., and T. K. Eisenstein.** 1986. Delayed-type hypersensitivity and immunity to *Salmonella typhimurium*. Infect.

Immun. **52**:504–508.

84. **Lane, J. M., F. L. Ruben, J. M. Neff, and J. D. Millar.** 1969. Complications of small pox vaccinations. National surveillance in the United States. N. Engl. J. Med. **281**:1201–1208.
85. **Lee, J.-C., C. W. Gibson, and T. K. Eisenstein.** 1985. Macrophage mediated mitogenic suppression induced in mice of the C3H lineage by a vaccine strain of *Salmonella typhimurium*. Cell. Immunol. **91**:75–91.
86. **Levine, M. M.** 1990. Modern vaccines: enteric infections. Lancet **335**:958–961.
87. **Levine, M. M., H. L. DuPont, R. B. Hornick, M. J. Snyder, W. Woodward, R. H. Gilman, and J. P. Libonati.** 1976. Attenuated, streptomycin-dependent *Salmonella typhi* oral vaccine: potential deleterious effects of lyophilization. J. Infect. Dis. **133**:424–429.
88. **Levine, M. M., C. Ferreccio, R. E. Black, R. Germanier, and Chilean Typhoid Committee.** 1987. Large-scale field trials of Ty21a live oral typhoid vaccine in enteric coated capsule formation. Lancet **i**:1049–1052.
89. **Levine, M. M., D. Herrington, J. Murphy, J. G. Morris, G. Losonsky, B. Tall, A. Lindberg, S. Svenson, S. Baqar, M. F. Edwards, and B. A. D. Stocker.** 1987. Safety, infectivity, immunogenicity and in vivo stability of two attenuated auxotrophic mutant strains of *Salmonella typhi*, 541Ty and 543Ty, used as oral vaccines in man. J. Clin. Invest. **79**:888–902.
90. **Levine, M. M., J. B. Kaper, D. Herrington, G. Losonsky, C. O. Tacket, J. G. Morris, B. Tall, and S. Cryz.** 1988. Safety, immunogenicity, and efficacy of recombinant live oral cholera vaccines CVD 103 and CVD 103-HgR. Lancet **ii**:467–470.
91. **Mackett, M.** 1990. Vaccinia virus as a vector for delivering foreign antigens. Semin. Virol. **1**:39–47.
92. **Majarian, W. R., S. J. Kasper, and R. N. Brey.** 1989. Expression of heterologous epitopes as recombinant flagella on the surface of attenuated *Salmonella*, p. 277–281. *In* R. A. Lerner (ed.), Vaccines 89: modern approaches to new vaccines including prevention of AIDS. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
93. **Manning, P. A., M. W. Heuzenroeder, J. Yeadon, D. I. Leavesley, P. R. Reeves, and D. Rowley.** 1986. Molecular cloning and expression in *Escherichia coli* K-12 of the O antigens of the Inaba and Ogawa serotypes of the *Vibrio cholerae* O1 lipopolysaccharides and their potential for vaccine development. Infect. Immun. **53**:272–277.
94. **Maskell, D., F. Y. Liew, K. Sweeney, G. Dougan, and C. E. Hormaeche.** 1986. Attenuated *Salmonella typhimurium* as live oral vaccines and carriers for delivering antigens to the secretory immune system, p. 213–217. *In* F. Brown, R. M. Chanock, and R. A. Lerner (ed.), Vaccines 86: new approaches to immunization. Developing vaccines against parasitic, bacterial, and viral diseases. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
95. **Maskell, D., K. Sweeney, D. O'Callaghan, C. E. Hormaeche, F. Y. Liew, and G. Dougan.** 1987. *Salmonella typhimurium aroA* mutants as carriers of the *Escherichia coli* heat-labile enterotoxin B subunit to the murine secretory and systemic immune systems. Microb. Pathog. **2**:211–221.
96. **McFarland, W. C., and B. A. D. Stocker.** 1987. Effect of different purine auxotrophic mutations on mouse virulence of a Vi-positive strain of *Salmonella dublin* and of two strains of *Salmonella typhimurium*. Microb. Pathog. **3**:129–141.
97. **McKenzie, S. J., and J. F. Halsley.** 1984. Cholera toxin B subunit as a carrier to stimulate a mucosal immune response. J. Immunol. **133**:1818–1824.
98. **Mel, D. M., B. L. Arsic, M. L. Radovanovic, R. Kaljarolic, and S. Litvinjenko.** 1974. Safety test in adults and children with live oral typhoid vaccine. Acta Microbiol. Acad. Sci. Hung. **21**: 161–166.
99. **Michalek, S. M., N. K. Childers, J. Katz, F. R. Denys, A. K. Berry, J. H. Elridge, J. R. McGhee, and R. Curtiss III.** 1989. Liposomes as oral adjuvants. Curr. Top. Microbiol. Immunol. **146**:51–58.
100. **Miller, I. A., S. Chatfield, G. Dougan, L. DeSilva, H. Joysey, and C. H. Hormaeche.** 1988. Bacteriophage p22 as a vehicle for transducing gene banks between smooth strains of *Salmonella typhimurium*: use in identifying a role for *aroD* in attenuating virulent *Salmonella* strains. Mol. Gen. Genet. **215**:312–316.
101. **Mittermayer, C. H.** 1976. Lethal complications of typhoid-cholera vaccination (case report and review of literature). Beitr. Pathol. **158**:212–224.
102. **Morris Hooke, A., D. O. Sordelli, M. C. Cerquetti, and J. A. Bellanti.** 1987. Differential growth characteristics and immunogenicity of tight and coasting temperature-sensitive mutants of *Pseudomonas aeruginosa*. Infect. Immun. **55**:99–103.
103. **Mukkur, T. K. S., G. H. McDowell, B. A. D. Stocker, and A. Lascelles.** 1987. Protection against experimental salmonellosis in mice and sheep by immunization with aromatic dependent *Salmonella typhimurium*. J. Med. Microbiol. **24**:11–19.
104. **Nagi, L. K., P. D. Walker, B. S. Bhogal, and T. Mackenzie.** 1978. Evaluation of *Escherichia coli* vaccines against experimental colibacillosis. Res. Vet. Sci. **24**:39–45.
105. **Nakamura, M., S. Sato, T. Ohya, S. Suzuki, and S. Ikeda.** 1985. Possible relationship of a 36-megadalton *Salmonella enteritidis* plasmid to virulence in mice. Infect. Immun. **47**:831–833.
106. **Nakamura, M., S. Sato, T. Ohya, S. Suzuki, S. Ikeda, and T. Koeda.** 1985. Plasmid-cured *Salmonella enteritidis* AL1192 as a candidate for live vaccines. Infect. Immun. **50**:586–587.
107. **Nakayama, K., S. K. Kelly, and R. Curtiss III.** 1988. Construction of an Asd$^+$ expression-cloning vector: stable maintenance and high level expression of cloned genes in a *Salmonella* vaccine strain. Bio/Technology **6**:693–697.
108. **Nauciel, C., E. Ronce, and J.-L. Guenet.** 1988. Genetic control of *Salmonella typhimurium*-induced depression of delayed-type hypersensitivity to sheep erythrocytes in mice. Infect. Immun. **56**:310–313.
109. **Newton, S. M. C., C. O. Jacob, and B. A. D. Stocker.** 1989. Immune response to cholera toxin epitope inserted into *Salmonella* flagellin. Science **244**:70–72.
110. **Nikaido, H.** 1961. Galactose-sensitive mutants of *Salmonella*. I. Metabolism of galactose. Biochim. Biophys. Acta **48**:460–469.
111. **Nnalue, N. A., and B. A. D. Stocker.** 1986. Some *galE* mutants of *Salmonella choleraesuis* retain virulence. Infect. Immun. **54**:635–640.
112. **Nnalue, N. A., and B. A. D. Stocker.** 1987. Test of the virulence and live-vaccine efficacy of auxotrophic and *galE* derivatives of *S. choleraesuis*. Infect. Immun. **55**:955–962.
113. **O'Callaghan, D., D. Maskell, F. Y. Liew, C. S. F. Easmon, and G. Dougan.** 1988. Characterization of aromatic- and purine-dependent *Salmonella typhimurium*: attenuation, persistence, and ability to induce protective immunity in BALB/c mice. Infect. Immun. **56**:419–423.
114. **Ogra, P. L., Y. Chiba, K. R. Beutner, and A. Morag.** 1976. Vaccination by non-parenteral routes: characterization of immune response. Dev. Biol. Stand. **33**:19–26.
115. **Ohta, M., N. Kido, Y. Fujii, Y. Arakawa, T. Komatsu, and N. Kato.** 1987. Temperature-sensitive growth mutants as live vaccines against experimental murine salmonellosis. Microbiol. Immunol. **31**:1259–1265.
116. **Pardon, P., M. Y. Popoff, C. Coynault, J. Marly, and I. Miras.** 1986. Virulence associated plasmids in *Salmonella typhimurium* in experimental murine infection. Ann. Inst. Pasteur Microbiol. **137B**:47–60.
117. **Pastan, I., and A. Adhya.** 1976. Cyclic adenosine 5′-monophosphate in *Escherichia coli*. Bacteriol. Rev. **40**:527–551.
118. **Pittard, A. J.** 1987. Biosynthesis of the aromatic amino acids, p. 368–394. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
119. **Poirier, T. P., M. A. Kehoe, and E. H. Beachey.** 1988. Protective immunity evoked by oral administration of attenuated *aroA Salmonella typhimurium* expressing cloned streptococcal M protein. J. Exp. Med. **168**:25–32.
120. **Reitman, M.** 1967. Infectivity and antigenicity of streptomycin dependent *Salmonella typhosa*. J. Infect. Dis. **117**:101–107.

121. **Reitman, M., and W. P. Iverson.** 1953/1954. The immunizing properties of dihydrostreptomycin-dependent *Salmonella typhosa*. Antibiot. Annu. **1953/1954:**604–608.
122. **Robertsson, J. A., A. A. Lindberg, S. Hoiseth, and B. A. D. Stocker.** 1983. *Salmonella typhimurium* infection in calves: protection and survival of virulent challenge bacteria after immunization with live or inactivated vaccines. Infect. Immun. **41:**742–750.
123. **Sadoff, J. C., W. R. Ballou, L. S. Baron, W. R. Majarian, R. N. Brey, W. T. Hockmeyer, J. F. Young, S. J. Cryz, J. Ou, G. H. Lowell, and J. D. Chulay.** 1988. Oral *Salmonella typhimurium* vaccine expressing circumsporozoite protein protects against malaria. Science **240:**336–340.
124. **Salas-Vidal, M., M. Plebañski, S. Castro, G. Perales, E. Mata, S. Lopez, and C. F. Arias.** 1990. Synthesis of the surface glycoprotein of rotavirus SA11 in the *aroA* strain of *Salmonella typhimurium* SL3261. Res. Microbiol. **141:**883–886.
125. **Schödel, F., and H. Will.** 1989. Construction of a plasmid for expression of foreign epitopes as fusion proteins with subunit B of *Escherichia coli* heat-labile enterotoxin. Infect. Immun. **57:**1347–1350.
126. **Schödel, F., and H. Will.** 1990. Expression of hepatitis B virus antigens in attenuated *Salmonella* for oral immunization. Res. Microbiol. **141:**831–837.
127. **Sigwart, D. F., B. A. D. Stocker, and J. D. Clements.** 1989. Effect of *purA* mutation on the efficacy of *Salmonella* live vaccine vectors. Infect. Immun. **57:**1858–1861.
128. **Sjöstedt, A., G. Sandström, and A. Tärnvick.** 1990. Immunization of mice with an attenuated *Salmonella typhimurium* strain expressing a membrane protein of *Francisella tularensis*. A model for identification of bacterial determinants relevant to the host defense against tularemia. Res. Microbiol. **141:**887–891.
129. **Smith, B. P., M. Reina-Guerra, S. K. Hoiseth, B. A. D. Stocker, F. Habasha, E. Johnson, and F. Merritt.** 1984. Aromatic-dependent *Salmonella typhimurium* as modified live vaccines for calves. Am. J. Vet. Res. **45:**59–66.
130. **Smith, B. P., M. Reina-Guerra, B. A. D. Stocker, S. K. Hoiseth, and E. Johnson.** 1984. Aromatic-dependent *Salmonella dublin* as a parenteral modified live vaccine for calves. Am. J. Vet. Res. **45:**2231–2235.
131. **Stabel, T. J., J. E. Mayfield, L. B. Tabatabai, and M. J. Wannemuehler.** 1990. Oral immunization of mice with attenuated *Salmonella typhimurium* containing a recombinant plasmid which codes for production of a 31-kilodalton protein of *Brucella abortus*. Infect. Immun. **58:**2048–2055.
132. **Stevenson, G., and P. Manning.** 1985. Galactose epimeraseless (*galE*) mutant G30 of *Salmonella typhimurium* is a good potential live oral vaccine carrier for fimbrial antigens. FEMS Microbiol. Lett. **28:**317–321.
133. **Stocker, B. A. D.** 1990. Aromatic-dependent *Salmonella* as live vaccine presenters of foreign epitopes as inserts in flagellin. Res. Microbiol. **141:**787–796.
134. **Stocker, B. A. D., S. K. Hoiseth, and B. P. Smith.** 1983. Aromatic-dependent *Salmonella* species as live vaccines in mice and calves. Dev. Biol. Stand. **53:**47–54.
135. **Strober, W., and D. Jacobs.** 1985. Cellular differentiation, migration, and function in the mucosal immune system, p. 1–30. *In* J. I. Gallin and A. S. Fauci (ed.), Advances in host defense mechanisms, vol. 4. Mucosal immunity. Raven Press, New York.
136. **Strugnell, R. A., D. Maskell, N. Fairweather, D. Pickard, A. Cockayne, C. Penn, and G. Dougan.** 1990. Stable expression of foreign antigens from the chromosome of *Salmonella typhimurium* vaccine strains. Gene **88:**57–63.
137. **Svennerholm, A.-M., and J. Holmgren.** 1986. Oral combined B subunit-whole cell cholera vaccine, p. 33–43. *In* J. Holmgren, A. Lindberg, and R. Mölby (ed.), Development of vaccines and drugs against diarrhea. 11th Nobel Conference. Studentlitteratur, Stockholm.
138. **Svennerholm, A.-M., M. Jertborn, L. Gothefors, A. M. Karim, D. A. Sack, and J. Holmgren.** 1984. Local and systemic antibody responses and immunological memory in humans after cholera disease and after immunization with a combined B subunit-whole cell vaccine. J. Infect. Dis. **149:**884–893.
139. **Tacket, C. O., B. Forrest, R. Morona, S. R. Attridge, J. LaBrooy, B. D. Tall, M. Reimann, D. Rowley, and 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.
140. **Tacket, C. O., D. M. Hone, R. Curtiss III, S. M. Kelly, G. Losonsky, L. Guers, A. M. Harris, R. Edelman, and M. M. Levine.** 1992. Comparison of the safety and immunogenicity of Δ*aroC* Δ*aroD* and Δcya Δcrp *Salmonella typhi* strains in adult volunteers. Infect. Immun. **60:**536–541.
141. **Taubman, M. A., J. L. Ebersole, D. J. Smith, and W. Stack.** 1983. Adjuvants for secretory immune response. Ann. N.Y. Acad. Sci. **409:**637–648.
142. **Terakado, N., T. Sekizaki, K. Hashimoto, and S. Naitoh.** 1983. Correlation between the presence of a 50-megadalton plasmid in *Salmonella dublin* and virulence for mice. Infect. Immun. **41:**443–444.
143. **Tomasi, T. B., and A. G. Plaut.** 1985. Cellular differentiation, migration, and function in the mucosal immune system, p. 31–61. *In* J. I. Gallin and A. S. Fauci (ed.), Advances in host defense mechanisms, vol. 4. Mucosal immunity. Raven Press, New York.
144. **Tramont, E. C., R. Chung, S. Berman, D. Keren, C. Kapfer, and S. B. Formal.** 1984. Safety and antigenicity of typhoid-*Shigella sonnei* vaccine (strain 5076-1C). J. Infect. Dis. **149:**133–136.
145. **Tuft, L., E. M. Yagle, and S. Rogers.** 1932. Comparative study of the antibody response after various methods of administration of mixed typhoid vaccine. J. Infect. Dis. **50:**98–110.
146. **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.
147. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19:**259–268.
148. **Wahdan, M. H., C. Serie, Y. Cerisier, S. Sallam, and R. Germanier.** 1982. A controlled field trial of live *Salmonella typhi* strain Ty21a oral vaccine against typhoid: 3 year results. J. Infect. Dis. **145:**292–296.
149. **Wahdan, M. H., C. Serie, R. Germanier, A. Lackany, Y. Cerisier, N. Guerin, S. Sallam, P. Geoffroy, A. S. el Tantawi, and P. Guesry.** 1980. A controlled field trial of live oral typhoid vaccine Ty21a. Bull. W.H.O. **58:**469–474.
150. **Wu, J. Y., S. Newton, A. Judd, B. A. D. Stocker, and W. S. Robinson.** 1989. Expression of immunogenic epitopes of hepatitis B surface antigen with hybrid flagellin proteins by a vaccine strain of *Salmonella*. Proc. Natl. Acad. Sci. USA **86:**4726–4730.
151. **Zagury, D., J. Bernard, R. Cheynier, I. Desportes, R. Leonard, M. Fouchard, B. Reveil, D. Ittele, Z. Lurhuma, K. Mbayo, J. Ware, J. Salaun, B. Goussard, L. Dechazal, A. Burny, P. Nara, and R. C. Gallo.** 1988. A group specific anamnestic immune reaction against HIV-1 induced by a candidate vaccine against AIDS. Nature (London) **332:**728–731.