Discussion
The effectiveness of vaccines for prevention and control of human and animal infectious diseases is well recognized. However, the mechanisms of protective immunity are uncertain, the identity of the protective antigen(s) has not been fully elucidated, and the immunological correlates of protection conferred by vaccination are undefined. Consequently the requirements for a good vaccine are unclear, selection of vaccine strains is empirical, the reasons for the success and failure of a vaccine are unknown, and the protective capacity of a vaccine can only be ascertained by challenge or natural exposure to the infection (Hormaeche et al., 1996). These are serious obstacles to rational vaccine design. The concept of live vector vaccines, whereby a well tolerated, highly immunogenic vaccine strain delivers foreign antigens to the mammalian immune system and stimulates relevant protective immune responses to a pathogen distinct from the live vector, has been popularized in recent years (Formal et al., 1981; Clements et al., 1986; Fairweather et al., 1990; Langermann et al., 1994). Attenuated *Salmonella* (Curtiss et al., 1989; Gonzalez et al., 1994; Khan et al., 1994) are particularly promising among bacterial live vectors because of their ability to immunize via mucosal surfaces and their capacity to stimulate a broad array of immune responses including secretory IgA mucosal antibodies (Clements et al., 1986), serum antibodies (Gonzalez et al., 1994), and various cell mediated responses (Gonzalez et al., 1994; Sztein et al., 1994). In view of these facts there is a continuing need for improved *Salmonella* vaccines (Robbins, 1990). *Salmonella typhimurium* live vector constructs administered orally to mice have established the feasibility of eliciting immune responses to foreign antigens by mucosal immunization (Clements et al., 1986; Curtiss et al., 1989; Khan et al., 1994; Chatfield et al., 1992).

The main objective of the work documented here was to ascertain whether mucosal immunization with attenuated *Salmonella* live vectors could result in stimulating high titers of IgG serum antibodies against cholera toxin B epitope CTP1. To accomplish this goal, two routes of immunization were explored. In addition to this it was also decided to study comparatively the antibody response conferred by CTP1 in chimeric flagellin administered either as bacterial vaccine or as DNA vaccine. It has been noted that DNA vaccines apart from other advantages, is adaptable for pediatric vaccine formulation (Hassett et al., 2000).
IV.1. Cholera: An Ancient Scourge

It appears that disease will always be part of the human existence. Nonetheless, it is worthwhile to pause and consider the role and importance of vaccines in our individual and collective public health. Barely one year after Koch isolated the causative agent of cholera (Koch, R., 1884), Ferran in 1885 reported the first candidate cholera vaccine (Levine and Kaper, 1993). More than one hundred years have elapsed between the first (live, parenteral) cholera vaccine that entered clinical trials in 1885 and the field trials of two oral inactivated cholera vaccines undertaken in Bangladesh in the mid-1980s. This disease has a long history; descriptions of it can be found in ancient Sanskrit writings dating back at least two millennia. Since 1871, seven cholera pandemics have occurred. Despite 11 decades of research, efforts to develop effective cholera vaccines have produced few successes (Mekalanos and Sadoff, 1994). The limited success of these vaccines is attributed to their inability to induce a local intestinal or "mucosal" immune response. Such mucosal immunity appears to be a critical feature of natural convalescence from cholera, a highly immunogenic process that provides long-lasting protection.

Cholera and other diarrheal diseases are relatively easy to treat by intravenous and oral rehydration therapies. However, in the grip of explosive epidemics, medical facilities can be overwhelmed and significant mortality can result. Cholera can have a devastating economic impact on the countries, that is measured not only in treatment costs but also in the deleterious effect that this disease has on food exportation and tourism. Although provision of safer water sources and sewage treatment is in no doubt the best way to control cholera, estimates by World Health Organization (WHO) indicate that this goal would cost Latin America alone tens of billions of dollars.

Cholera has been a great educator in the public health arena and a marvelous catalyst for scientific discovery. We hope to reap the benefits of what we have learned from cholera and _V. cholerae_ can be applied to effective immunization. In addition, continued study on the properties that enable _V. cholerae_ to be such a potent immunogen may help to clarify the general physiology of mucosal immunity.
In the present study an attempt has been made to understand whether the mucosal immunization with attenuated Salmonella live vectors could result in stimulating high titers of IgG serum antibodies against cholera toxin B epitope CTP1.

IV.2. Selection of Immunodominant Epitope of Cholera Toxin B

The elucidation of protein antigenic structures is presently a difficult, uncertain, and time-consuming task. Many methods for visualizing immunogenic epitopes have been developed based on locating hydrophilic regions in a protein (Hopp and Woods, 1981; Kyte and Doolittle, 1982), since it was argued that antigenic determinants are surface located and often contained charged and polar residues (Hopp and Woods, 1981). These methods are very useful to obtain a rough estimate of potentially antigenic regions. Previous investigations have demonstrated that antigenic determinants are surface features of proteins and indicate that they are frequently found on regions of a molecule that have an unusually high degree of exposure to solvent—i.e. regions which project into the medium (Atassi, 1975; Reichlin, 1975). This together with the fact that charged, hydrophilic amino acid side chains are common features of antigenic determinants led us to find out potential antigenic regions of cholera toxin B, which is nonpathogenic subunit of the holotoxin. The secondary structure prediction of cholera toxin B and Salmonella flagellin was made using Chou & Fasman program. Cholera toxin was also scanned to localize its antigenic regions using a computer based algorithm 'Predict7' (Rmenes, et al., 1988).

Jacob et al., (1983) synthesized six peptides 8-20, 30-42, 50-64, 69-85, 75-85 and 83-97 of cholera toxin B. Immunoprecipitation experiments showed that antibodies against peptides 8-20, 50-64 and 83-97 showed the highest cross reactivity with the intact toxin. In the present study the epitope CTP1 (8-20), with slight modifications, of cholera toxin B was chosen based on our study of secondary structure prediction and the findings of Jacob et al., (1983).

IV.3. Construction of Chimeric Flagellin

Most bacteria of the genus Salmonella are motile by means of flagella, which are organelles divided into three regions (DePamphilis and Adler, 1971). The extracellular helical filament of each flagellum is a polymer of a single protein, termed flagellin.
(Astbury et al., 1955), specified by either a fliC (phase-1) or a fliB (phase-2) gene (Iino et al., 1988). Flagellins appear to have a general structure consisting of extremely conserved ends progressing inwardly with diminishing homology towards a central hypervariable region (Joys, 1988). B cell epitopes have been detected in this extremely variable region (Joys and Schodel, 1991) and the large number of flagellar antigens found in *Salmonella* is thought to result from such variety. These properties have led to the suggestion (Newton et al., 1989) that flagella might be useful in vaccine development if part of the hypervariable regions could be substituted with medically important epitopes. Attempts at this substitution have used a unique restriction site in the structural gene for the *i* antigen flagellin of *Salmonella typhimurium* (Newton et al., 1990) and substitution of a 48 bp region between two EcoRV restriction sites in the gene of the *d* flagellin of *Salmonella muenchen* (Newton et al., 1989; 1990). To date, such attempts have been partially successful with a variety of B cell epitope (He et al., 1994) and two T cell epitopes (Verma et al., 1995a,b) and the concept appears worthy of further examination.

In the present study, we employed a similar system using *Salmonella* chimeric flagellin by inserting, in frame, an epitope (CTP1) of cholera toxin B into the EcoRV site of the hypervariable region IV of *Salmonella* flagellin.

**IV.4. Construction of DNA Vaccine Vector**

Construction of chimeric flagellin containing heterologous inserted sequences, their expression by Gram-negative bacteria and the use of these recombinant stains have been reported by several groups (Maskell et al., 1987; Leclerc et al., 1989; Newton et al., 1989; Agterberg et al., 1990). This is the first report to our knowledge where chimeric flagellin was used as a DNA vaccine. Immunization by plasmid DNA by a variety of routes has been shown to elicit immune responses that are protective in a variety of experimental models (Boyle et al., 1996; Donnelly et al., 1994; Xiang et al., 1995; Zarozinsky et al., 1995). This novel approach to vaccination is attractive as it offers several desirable features. First, the DNA is not infectious, it does not replicate and it encodes only the protein of interest. Second, the DNA is stable, and it can be made inexpensively in large quantities at high levels of purity. Third, plasmid DNA does not contain a heterologous protein component, as compared to recombinant vaccinia virus
vaccines, to which the host may respond impairing the desired responses to a booster vaccination. Fourth, DNA vaccines can successfully induce both cell-mediated and humoral immunity and finally antigen expression persists after DNA vaccination, promoting the induction of long lived memory immune cells. In order to compare the vaccine potential of chimeric flagellin it was decided to deliver chimeric flagellin as DNA vaccine through intramuscular (i.m.) and subcutaneous (s.c.) routes. Chimeric flagellin (CTP1 bearing) was cloned into a mammalian expression vector pCI-neo, which carries the human cytomegalovirus (CMV) immediate early enhancer/promoter region to promote constitutive expression of cloned DNA inserts in mammalian cells. It will be important to deliver entire B subunit of cholera toxin for a comparison with the chimeric flagellin in terms of attained antibody titer.

IV.5. Expression of Chimeric Flagellin

We hypothesized that chimeric flagellin were exported to bacterial surface in order to facilitate their direct interaction with the host immune system and that bacterial replication and expression of an epitope containing recombinant protein within the gut when given orally would result in generating mucosal immune response against "passenger" antigen. As all or most of the antigenic activity of some proteins reside in short amino acid sequences, constituting linear epitopes, the inclusion of a foreign oligonucleotide epitope in a protein made by a live-vaccine strain might be expected to stimulate production of antibody with affinity for the peptide epitope, and perhaps also for the protein from which it came. To test these ideas we inserted the cholera toxin B epitope CTP1 into the central hypervariable domain of Salmonella flagellin. The chimeric flagellin expressed by recombinant Salmonella strains contain a 14 amino acid CTP1 epitope. This epitope was inserted in a region of flagellin sequence that specifies the flagellar serotype and thus is considered to be "hypervariable" and relatively tolerant of amino acid additions, deletions and substitutions. For this reason and because the epitope is relatively small with the approximately 500 amino acid length of a native flagellin, the resulting chimera were expected to form functional flagella. Flagella normally propel bacteria along chemotactic gradients, a function that can be assessed using standard motility assay that is conducted with semisolid agar. Motility assay, western blotting and dot blotting ascertained expression of chimeric flagellin.
IV.6. Conformational Changes in Flagellin

CD spectra of the recombinant flagellin show a reduction in the α-helical content of the protein vis-à-vis the native protein. With increasing insert size, there seems to be a reduction in motility conferred on bacteria. Reduced mobility observed could be caused by either a reduction in the ability of the flagellin monomers to polymerise or an interference in polymers switching from the left handed to right handed supercoil. Monomeric and dimeric flagellin have reduced helicity as the terminal regions- α-helical coiled coils in polymeric flagellin- are disordered in solution (Kostyukova et al., 1988; Vondervistz et al., 1989).

Experimental evidence points to the central region of flagellin not being essential for filament formation (Kuwajima, 1988). The polymeric filament is energetically favourable, and extreme physiological conditions of heat, acid or pH are necessary for depolymerisation into monomeric form. The immune response against the cholera toxin B CTP1 seen from the recombinant flagellin is suggestive of this epitope being displayed from polymeric filament on the surface. Mobility by bacterial flagellar coiling has been hypothesized (Asakura, 1970) and a model proposed based on experimental findings (Yamashita et al., 1998). The reversible spiral twisting of the flagellar propellar arises from combinations of interconvertible left and right tilted arrays of identical flagellin subunits in 11 near-axial protofilament strands of different length in the flagellum. This switch operates at the quarternary level without significant change in tertiary structure, and the actual molecular displacement is consistent with the idea that it may involve alternate alpha-helical interlocking patterns observed in coiled-coil (Casper, 1998). The loss in helicity in the recombinant flagellin is probably due to the reduction of helicity in the central region, due to cholera toxin B epitope CTP1 insert. This perturbation in secondary structure may affect the switching at the quarternary coil, resulting in reduced mobility.

IV.7. Immunogenicity of Chimeric Flagellin

The oligonucleotide used in this investigation comprised 14 codons specifying the cholera toxin B epitope CTP1. The insert thus replaced the 48-bp fragment deleted from the flagellin gene in plasmid pLS408, the target for the insertion. The resulting
recombinant flagellin gene of plasmid pLS408 caused production of flagellin, which was assembled into functional flagella, as shown by motility of flagellin deficient *E. coli* given this plasmid. Expression of recombinant flagellin in flagellin-deficient *Salmonella dublin* SL5928, SL1438 and *S. typhimurium* SL3235 strain was ascertained by protein immunoblotting. By contrast, in the experiments of Wu et al., (1991), in which the inserts used specified hepatitis B surface protein epitopes, three of the six tested plasmids with in-frame inserts in the flagellin gene did not confer motility on the *Salmonella dublin* live vaccine strain. However, the three plasmids that did not confer motility did cause production of chimeric flagellin, as shown by immunoblotting and by the flagellin specific and epitope specific antibodies by animals given the live vaccine. Current evidence on specificity of protection conferred by live *Salmonella* vaccines is conflicting. As shown in Table: 1.2., a broad spectrum of antigens have been successfully expressed in attenuated strains of *Salmonella*. Heterologous genes have been introduced either on plasmids or integrated into the chromosome, the latter being the method of choice as it provides a higher degree of stability.

In our study, we have demonstrated high levels of serum antibodies against the cloned heterologous antigen after the oral and intraperitoneal (i.p.) immunization. This system was used to investigate whether epitope of the nontoxic B subunit of cholera toxin can make use of *Salmonella* flagellin for display. The flagellar system of antigen delivery would seem to have several advantages. In particular the epitope inserted in chimeric flagellins are present in multiple copies as part of the flagellar filament, and the foreign epitope they contain appears to be surface accessible. Because each flagellar filament consists of several thousand flagellin subunits and each bacterium carries three to ten flagella, an individual bacterium could carry as many as 100,000 copies of an individual epitope. This hypothesis is proved in our experiments when CTP1 (cholera toxin B epitope) could result in as much as 60% of the titer attained by positive control, despite the fact that the epitope represents less than 3% of the flagellin molecule. When chimeric flagellin was used as DNA vaccine the antibody titer goes down by almost half, suggesting that chimeric flagellin offers better processing and presentation of foreign epitope, when given as live attenuated bacterial vaccine. Another plausible explanation could be that as a consequence of bacterial proliferation in the gut, continued production of flagella provide substantial quantities of chimeric flagellin for antigen processing and presentation. DNA vaccine will also hold promise in situations
where the recipient rapidly clears the carrier molecule because of high titers of carrier
specific antibodies already in circulation due to prior exposure to the carrier.

IV.8. Splenomegaly in Mice

Inoculation of *Salmonella* via intraperitoneal (i.p.) route resulted in profound
splenomegaly. Splenomegaly is a measure of the inflammatory response associated with
the infection. There seems to be no correlation between splenomegaly and immune
response because mice immunized via oral route showed higher antibody titer in
comparison to i.p. route. It has been reported in literature that splenomegaly happens
because of accumulation of neutrophils, macrophages and precursor macrophages (Al-
Ramadi et al., 1991). In the splenic sections observed under microscope we found out
some dark particulate matter of bacterial dimension near lymphatic nodules, suggesting
that *Salmonella* might have disseminated to the spleen thereby inducing splenomegaly.
We are aware of one such study in which the authors found out that depending on the
species and host, *Salmonella* may disseminate to the spleen, the liver, and regional
lymph nodes, take residence in macrophages, and thereby induce serum antibody and
cellular immune responses (Michalek et al., 1995). However, these are qualitative
observations to find out more subtle changes happening in the tissue it will be necessary
to study them quantitatively in terms of structural details and expression of bacterial and
host marker molecules.