CHAPTER 1

INTRODUCTION

"Art and science have their meeting point in method."

Bulwer-Lytton (1803-1873)

1.0 Introduction

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1.1 Typhoid fever

1.1.1 General features

Typhoid fever is still an important health problem in many developing countries. W.H.O. estimates that there are 16.6 million cases of typhoid fever with 600 000 deaths annually. Typhoid fever remains a disease of the developing world, especially in Asia (400 000 deaths annually) and Africa (130 000 deaths annually) (Pang, et al., 1998).

This problem is complicated further by rapid urbanization and the emergence of strains of *Salmonella typhi* that are resistant to previously useful antibiotics (Bhutta et al., 1991; Gupta, 1994) and their wide dissemination throughout Middle East, Northeast Africa, South and Southeast Asia. The clinical manifestations of disease also appear to be changing (Khosla, 1991) with increasing incidence, for example, of neurological manifestations and myocarditis.

1.1.2 History

Because typhoid is a disease with few distinguishing clinical characteristics, it is quite impossible to identify it with confidence in ancient texts. However, by the end of the nineteenth century typhoid had been identified as one of the chief causes of fever in cities throughout the world, especially during war (including The American Civil War, the Boer War, the Spanish-American War), and a cause of small, sporadic epidemics in rural populations.

The first comprehensive account of typhoid and its pathology was written by the French physician Pierre Louis in 1829. Other important early contributors to the understanding of typhoid include Hoffman, who described the ileal lesions of typhoid; Sir William Jenner, who described the differences between typhoid, typhus and relapsing fever in London; and William Budd, who discovered the method of transmission of typhoid.

One of the most interesting stories of typhoid is that of Typhoid Mary, believed to be from Northern Ireland. She was a cook for small, middle-class households. Within weeks of arriving at a new job, typhoid would break out at the household and she would flee, sending health authorities after her. She was identified as the source of at least 53 cases of typhoid fever, with 3 deaths.

1.1.3 Salmonellosis

Organisms from the genus *Salmonella* are capable of causing a large variety of infections in humans, including typhoid (or enteric) fevers, focal systemic infections, septicemias, and gastroenteritis, varying clinically from watery diarrhea to dysentery.

Nontyphoidal salmonellosis usually refers to enteric disease caused by many members of the genus excluding *Salmonella typhi* (*S.typhi*). Convalescent carriage of gastroenteritis strains is usually transient. Patients with *S.typhi* are more likely to become long-term carriers for years or possibly for life, and they serve as reservoirs for the spread of infection.

1.1.4 Chronic carrier status

A chronic *Salmonella* carrier is defined as one who sheds a *Salmonella* species for more than 1 year, as documented by an initial positive culture of a stool sample obtained at least 1 month after resolution of the acute illness and repeated positive cultures for at least 1 year (Corrado et al., 1992). *S. typhi* was the most well known cause of chronic carrier status in the past. Between 1% to 5 % of patients with typhoid fever become chronic carriers. (Wilson & Miles, 1964; McCoy, 1974)

1.1.5 Salmonella

Salmonellae are non-encapsulated gram negative bacilli, motile by means of peritrichous flagellae, expressing two or more forms of H antigens. Generally non-lactose fermenters, this property is used for initial selection in the laboratory. They ferment glucose, resulting in a typical acid butt and alkaline slant on triple sugar iron agar (TSI). They generally produce H_2S, which is detectable as a black reaction product and serves initially to distinguish isolates from *Shigella*, which also give an alkaline/ acid TSI reaction.

There are a very large number of *Salmonella* O and H antigens, allowing the separation of over 2200 different organisms on the basis of the patterns of the O and H antigens. On the basis of major somatic antigens, a limited number of serogroups have been defined, and most human pathogens are members of groups A to D. New *Salmonella* types have been found in various countries every year and have been published in a supplement to the Kaufmann-White Scheme (LeMinor et al, 1974). It is now demonstrated that the genus *Salmonella* contains only one species i.e. *Salmonella enteritica* and this species is divided into 7 subspecies designated I, II, IIIa, IIIb, IV, V and VI (LeMinor and Popoff, 1988). Names were maintained only for subspecies I serovars and must no longer be italicized. Serovars of other subspecies are designated only by their

antigenic formula. A more rational classification divides the genus into 3 species-one scheme includes *S.cholerasuis*, the prototype species; *S.typhi*, the major cause of typhoid (enteric fever) ; and *S.enteritidis*, a catchall designation for all the remaining serotypes, only some of which are pathogenic to humans. Some salmonellae are highly host-adapted to humans (e.g. *S.typhi*, *S.paratyphi A*, and *S. paratyphi B*) while most animal-adapted species cause no human disease.

1.2 Clinical Manifestations

Specific Salmonella serotypes most often produce specific clinical manifestations. These manifestations are often discussed as symptom and sign complexes with a syndrome designation. Though clinically useful, considerable overlap exists between symptom complexes and syndromes, with many patients having characteristics of more than one syndrome. (Mandell, 1995)

1.2.1 Gastroenteritis

Gastroenteritis caused by Salmonella is indistinguishable from that due to other enteric pathogens. Within 48 hours after ingestion of contaminated food or water, nausea, vomiting or diarrhea occurs. Diarrhea may vary in volume and intensity, but is usually without blood or mucus.

Occasionally the diarrhea mimics that of toxigenic *E.coli* or severe cholera and is of large volume and watery, with occasional dysentery with bloody stools and mucus. Fevers, up to 38-39 ^{0}C and abdominal cramping are common, as with chills and nausea. In most patients, if fever is present, it resolves within 48-72 hours.

1.2.2 Enteric Fever

Human typhoid fever and paratyphoid fever is a severe systemic illness characterized by fever and abdominal symptoms. The syndrome of enteric fever is most often caused by *S.typhi*. A similar but less severe syndrome is caused by *S. paratyphi* A, *S.paratyphi* B, and *S.paratyphi* C.

Although the syndrome is classically thought of as an acute illness with fever and abdominal tenderness, these symptoms are non-specific. The clinical features of typhoid and paratyphoid fever are the same, although paratyphoid tends to produce a milder illness. In contrast to patients with an acute abdomen, other gram negative bacteremias, or other catastrophic illnesses, symptoms related to typhoid may be insidious in onset and can be present for weeks.

The incubation period for disease after ingestion of *S.typhi* varies from 5 to 21 days, depending on the inoculum ingested and the health and immune status of the individual. Ingestion of the organism may result in several days of symptoms of diarrhea, although these symptoms usually

resolve before the onset of fever. Diarrhea is more common in certain geographic areas, in AIDS patients, and in children under 1 year of age (Butler et al., 1991). Neuropsychiatric manifestations, including psychosis and confusion, can occur in 5-10 percent of patients with typhoid fever (Stuart et al., 1946; Hoffman et al., 1975; Hoffman et al., 1984).

After appropriate antibiotic therapy, such as beta-lactam antibiotics, chloramphanicol, quinolones, or trimethoprim-sulfamethoxazole, fever usually resolves in 3-5 days (Mandal, 1991). Many of the complications of untreated enteric fever occur in the late stages of the disease in the third or fourth week of infections (Rubin & Weinstein, 1977). These include intestinal hemorrhage, or perforation and other rare focal infections, such as pericarditis, orchitis, and splenic and liver abscess. The severe intestinal complications of typhoid fever are related to hyperplasia of the lymphoid tissue in the ileocaecal area (of the intestine) followed by sloughing, ulceration, and necrosis with subsequent intestinal hemorrhage and perforation. With antimicrobial therapy, these complications occurred in less than 3 percent of patients and occur even less frequently in children (Carmali et al., 1993; Birkhead et al., 1993).

1.3 Laboratory Diagnosis of Typhoid fever

1.3.1 Isolation and Culture

The isolation of *Salmonella* in stool cultures is ideally done using freshly passed stools and not stool swabs. Stool is directly plated onto agar plates. Low selectivity media such as MacConkey and deoxycholate agar and intermediate selective agar such as Salmonella-Shigella (SS) or Hekoten are widely used to screen for Salmonella in primary stool cultures because they also screen for *Shigella* spp. Bismuth sulfite agar is preferred for isolating *S.typhi* and can also be used in the detection of *Salmonella* strains that ferment lactose. Enrichment broths, used to enhance the isolation of low numbers of stool organisms, are highly selective; the most widely used are tetrathionate broth, tetrathionate with brilliant green, and Selenite F broth. (LeMinor, 1984)

After primary isolation, possible *Salmonella* isolates can be tested in commercial identification systems or inoculated into screening media such as Triple Sugar Iron (TSI) and lysine agar in tubes (Le Minor, 1984; Farmer and Kelly, 1991). Lysine agar detects decarboxylation or deamination of lysine and hydrogen sulfide production. Slants of either agar are inoculated by streaking the surface and stabbing with an inoculating needle deep into the agar butt. The reactions are interpreted as slant/butt. Salmonella strains most often are TSI alkaline/acid positive,

gas positive, and H_2S positive. Rarely strains that ferment lactose or sucrose will have an acid/acid slant and be H_2S negative.

Culture of bone marrow aspirate, despite being a highly aggressive and impractical procedure, gives the highest positive rate of isolation (84-86 %) even when specimens are obtained late, or after the patients have been treated with antibiotics. (Tsang and Chau, 1992). Culture of duodenal bile obtained via the string capsule method proves to be a valuable adjunct to blood culture, this method being unaffected by previous antibiotic therapy (Avendano et al., 1986)

1.3.2 Serology

In regions of endemicity, the diagnosis of typhoid is based on the clinical picture in association with the results of Widal's test (Christie, 1987). In this classical technique of Felix and Widal, detection of antibodies is done by detection of 'O' and 'H' antibodies in paired sera drawn two weeks apart. (Widal, 1896) The Widal test, however, is only moderately sensitive (30% of positive cultures have negative Widal tests) and has been reported to cross-react with other Salmonella strains or in cases of cirrhosis. Furthermore, the cut-off titre varies from country to country and from year to year. (Clegg *et al*, 1994)

However, the classical Widal test has been found to be unsatisfactory for serological diagnosis in endemic areas (Levine et al., 1978). This is because the Widal test tends to obscure the serological picture for many reasons, the most important being the presence of

common "shared" antigens by the large numbers of organisms of the *Salmonella* genus and also gram-negative bacteria. Thus, in endemic areas, there is a need to interpret the results of the Widal test based on knowledge of the background titres in the local population (Pang and Puthucheary, 1983).

Various other tests have been developed to detect both *S.typhi* antigen and antibody in serum but they remain unsatisfactory. These include CIE (counterimmunoelectrophoresis) (Gupta and Rao, 1979; Tsang et al., 1981), RIA (radioimmunoassay) to determine anti-LPS antibodies (Tsang et al., 1981), ELISA tests to detect IgG and IgM to LPS and protein antigens (Beasley et al., 1982; Nardiello et al., 1984), IHA (indirect flourescent antibody) towards O, H and Vi antigens and IF (immunoflourescence) for quantitation of Vi antibodies (Doshi and Taylor, 1984) and XIE (counterimmunoelectrophoresis) analysis of serum antibodies (Chau et al., 1984)

Though these assays attempted to be more sensitive, practical, economical and rapid than bacteriological culture, no assay has desirably accomplished this aim (Ivanoff and Levine, 1997).

1.3.3 DNA detection

Techniques based on hybridization with DNA probes and, more recently, the Polymerase Chain Reaction (PCR) have been widely applied to a large variety of human pathogens (Macario and deMacario, 1990; Persing et al., 1993).

Rubin et al. (1985, 1989) reported the development of a DNA probe based on the Vi capsular antigen which was specific for *S.typhi*, but though this probe was highly specific and sensitive, it could not be used to directly detect the presence of the organism due to sensitivity limitations.

Other probes have been developed to detect other genes from other *Salmonella* spp. (e.g. the Hd flagellin gene) but most have had only limited testing on clinical samples (Rubin, 1990). Song et al. (1993) reported the detection of *S.typhi* in blood of patients with typhoid fever by PCR. Some specimens were positive by PCR even in patients suspected to have typhoid fever clinically but who were culture-negative.

Nested PCR has been used in the amplification of the Vi gene from *S. typhi* (Hashimoto et al., 1994). A large number of genes from *Salmonella* spp. have been the target of PCR amplification strategies, but are still of limited usefulness .

1.4 Pathogenesis

Progress towards understanding human typhoid fever has been complicated by the lack of a good animal model, a poorly developed genetic system for *S.typhi*, and a general reluctance to working with *S.typhi*. (Mroczenski-Wildley, 1989). Due to these limitations, most research on typhoid fever has focused on *S. typhimurium* and its pathogenicity in mice. The advantages of this system are that the genetics of *S. typhimurium* are well developed (Niedhart, 1987) and *S.typhimurium*

causes enteric fever in mice, which resembles typhoid fever in humans (Hsu, 1992). Researches using the *S. typhimurium* murine model hope that their results can be extrapolated to *S. typhi* interactions with humans (Groisman et al., 1990; Hsu, 1992).

Following ingestion of a suitable inoculum, *S.typhi* passes the gastric barrier to reach the small bowel. Higher doses of inoculum result in more frequent illness, unless the organisms lack the ability to produce the Vi antigen. Animal studies suggest that *S. typhi* invades the host in the upper small bowel, either by invasion of M cells (Kohbata et al., 1986) or through direct penetration of intestinal cells (Yabuuchi et al., 1986; Yokoyama et al., 1987) resulting in transient and asymptomatic bacteremia. Ultrastructural studies reveal that invading bacteria were highly piliated and flagellated, a characteristic which may play a role in adherence of the organism to the cell surface, as previously reported for other pathogenic bacteria (Yokoyama et al., 1987). The pili appeared to serve as a bridge between the bacteria and the host cell microvilli (Yokoyama et al., 1987). Invading bacteria were surrounded by a host vacuolar membrane where they remained viable and multiplied within the host cell. Jones et al (1994) confimed Kohbata et al.'s (1986) finding that M cells were preferentially and actively invaded by *S. typhimurium* in a murine ligated ileal loop model. Noninvasive mutants of *S.typhimurium* were not internalized by the M cells, suggesting that invasion contributes to this process.

The organisms are ingested by the mononuclear phagocytes, and multiply intracellularly and then invade the liver and spleen. *S.typhi* moves from the liver to the gall bladder and is shed in bile into the intestine, often resulting in intestinal ulceration, which may be fatal if left untreated (Huckstep, 1962; Salyers and Whit, 1994; Owen, 1994).

The invasion locus (***inv*HFGEABC/spaMNOPQRST**) identified in Salmonella which maps to a nearby region on the *S. typhimurium* chromosome (Galan & Curtiss III, 1989; Altemeyer *et al*, 1993; Groisman & Ochman, 1993) encodes for the secretion of antigens are essential for invasion and homologs have been identified in several other pathogenic bacteria (Galan, 1994). This similar invasion locus has also been identified in *S. typhi*, and through site-directed mutagenesis has been shown to be essential for invasion of epithelial cells (Galan & Curtiss, 1991).

Persistent bacteremia initiates the clinical phase of infection. The ability to invade the mononuclear cells and multiply intracellularly determines whether or not this secondary bacteremia occurs. Absence of bactericidal antibodies allows organisms to be phagocytosed in a viable state. Intracellular survival is dependent upon microbial factors that promote resistance to killing and the state of specific T-lymphocyte-activated host cell-mediated immunity. Dose dependence of clinical disease appears to be governed by the balance between bacterial multiplication and acquired host extracellular and intracellular defenses.

When the number of intracellular bacteria surpasses a critical threshold, secondary bacteremia occurs and results in the invasion of the invasion of the gallbladder and Peyer's patches of the intestine. The sustained bacteremia is responsible for the persistent fever of clinical typhoid, while inflammatory responses to the tissue invasion determine the pattern of clinical expression (chloecystitis, intestinal hemorrhage, or perforation). With invasion of the gallbladder and Peyer's patches, bacteria regain entry to the bowel lumen, and by the second week of clinical disease the prevalence of positive stool cultures increases. Seeding of the kidney leads to positive urine cultures but in a much lower percentage of patients. The lipopolysaccharide endotoxin of *S. typhi* may contribute to causing fever, leukopenia, and other systemic symptoms, but the occurrence of such symptoms in patients made tolerant to endotoxin supports a role for other factors, such as cytokines released from infected mononuclear phagocytes, that can mediate inflammation.

1.5 Immune response to *S.typhi*

The first line of defense against enteric pathogens like *Salmonella* is non-specific and relies upon the antibacterial activity of gastric juice and the presence of normal ileal bacterial flora which interferes with the growth of, and epithelial colonization by, bacterial pathogens. Stomach hypoacidity and alterations of the intestinal flora predispose to Salmonella infections (Hook, 1990).

Early work on the immunity to experimental systemic Salmonella infections provided strong evidences that both humoral and cellular immune responses were induced and contributed to protection (Blanden et al., 1966; Collins et al., 1966) .It is still debated how important each arm of the immune response is (Hone & Hackett, 1989; Hsu, 1989; Robbins et al., 1992).

Recent work has confirmed the importance of combined humoral and cellular response in the resistance to oral challenge of innately susceptible BALB/C mice
(Mastroeni et al., 1993). Long term immunity may be related to the persistence of bacterial cells in a L form in the liver of infected mice (Kita et al., 1992). Given the fact that the first contact between the pathogen and the host immune system occurs at the gut mucosa level, infection may be expected to induce a local (secretory) immunity which may later act very early during infection, possibly via direct interference with adherence, invasion and/or multiplication within the mucosa (McGhee and Kiyono, 1993). Secretory IgA (sIgA) antibodies were recently reported to be protective, in a "back-pack" tumor model, in mice orally infected with virulent *S. typhimurium* (Michetti et al., 1992).

Evidence exists for the induction of a human humoral and cellular immune response as a result of contact with *S.typhi* (Germanier, 1984). Circulating serum antibodies are directed against LPS and flagella, whereas the immune response against the VI-Ps is generally very low,

except in chronic typhoid carriers (Tsang et al., 1981; Nardiello et al.,1984 ; Robbins and Robbins, 1984; Edelman and Levine,1986; Sarasombath et al.,1987; Murphy et al.,1987; Losonsky et al.,1987)

In addition, a significant serum antibody response is induced against *S. typhi* protein antigens, including specific outer membrane proteins (Brown & Hormache, 1989; Chau et al., 1984; Ortiz et al., 1989; Aron et al., 1993a,b). The contribution of these serum antibodies to protection is unclear. Furthermore, the induction of a cellular response to *S.typhi* antigens (whole-cell extracts or O-PS) has been demonstrated by different groups in both acute typhoid patients and as a background immunity in residents of endemic regions (Sarasombath et al, 1987; Murphy et al., 1987, 1989; Tagliabue et al., 1989; Bhaskaram et al., 1990). Mucosal immunity has been less well characterized than the systemic immune response. IgM and IgA antibodies against *S.typhi* LPS and crude protein antigen were detected in jejunal secretions typhoid patients, whereas only antibodies of the IgA class were observed in chronic carriers (Chau et al., 1981; Sarasombath et al., 1987).

1.6 Treatment

Although the mortality rate of typhoid fever in the pre-antibiotic era was in excess of 10% (Stuart et al., 1946; Guerrant, 1897), the introduction of chloramphenicol reduced it to less than 2%. Data from

developing countries, however paint a different picture with mortality rates raging from 2-33% form both hospital-based and community-based studies (Rao & Rao, 1959; Kagwa-Nyanzi, 1971; Ellis et al., 1990; Butler et al., 1991; Coovadia et al., 1992).

Usually chloramphenicol, ampicillin and co-trimoxazole constitute the first line antibiotics against *S.typhi*. Until 1950 these antibiotics were effective in patients with acute infection, reducing complications and mortality. However, since 1950 progressive resistance to 1 or 2 of these first line antibiotics has been reported (Rowe et al., 1990; Gupta, 1994) and in 1989 resistance to all three first line antibiotics was noticed in Pakistan, India, China and the Arabian gulf (Mirza & Hart, 1993). Quinolone derivatives and third generation cephalosporins are also effective, particularly ciprofloxacin given orally in a 7-day course of therapy (Wallace et al., 1993). However, the need for increasing doses of ciprofloxacin has been reported from India and resistance to nalidixic acid and ciprofloxacin itself has yet been described in patients returning to the UK from India (Umansankar et al., 1992) Recently the emergence of multidrug resistant strains of *S.typhi* from different parts of the developing world has posed a significant problem, leading to greater morbidity and mortality than previous reports (Bhutta et al., 1991; Mishra et al., 1991). Delayed institution of treatment has been reported to be a major risk factor in typhoid –related mortality (Johnson & Aderle, 1981; Carmali et al., 1993).

The use of steroids in patients with severe typhoid has been recommended by several workers (Hoffman et al., 1984; Rogerson et al., 1991) and is supposed to exert a beneficial effect in critically ill patients with cytokine elevation (Butler et al., 1993).

1.7 Vaccines

In the late 1940s the only typhoid vaccine available to civilian populations was the heat-phenolized whole cell parenteral vaccine (Pfeiffer & Kolle, 1896; Wright & Semple, 1897). This vaccine confers 51-67% protection in controlled field trials sponsored by WHO (Polish Typhoid Committee (1966); Ashcroft et al. 1967; Yugoslav Typhoid Committee (1964). For this reason, and because of the frequent and severe adverse reactions associated with this vaccine, it was rarely used systematically in the control of endemic typhoid fever. Ty21a (Germanier & Furer, 1975; Wahdan et al., 1982; Levine et al. ,1990; Simanjuntak et al., 1991) and non-denatured Vi polysaccharide vaccines can be seen as by-products of the Mexican epidemic of Chloramphenicol-resistant typhoid fever of 1972-1973. The emergence and spread of multiply-resistant strains of *S.typhi* since 1990 have rekindled interest in typhoid vaccines, including the need for further improved vaccines.

The salient features of Ty21a and Vi vaccines, including the level and duration of protection that they conferred in various field trials of efficacy have been previously reviewed in depth (Ivanoff et al., 1994;

Levine, 1994; Levine et al. ,1989a; Levine et al. 1989b). In a large effectiveness trial involving more than 200 000 school children in Santiago, Chile, Ty21a was found to be practical for use in school-based immunization programs (Ferreccio et al., 1989).

While school-based vaccination programs may be appropriate in some areas for routine vaccination programs (Ivanoff et al., 1994), limited attendance at school may make incorporation of typhoid vaccination into the current Expanded Program on Immunization (EPI) schedule most desirable in other areas.

Unfortunately no data are available to show that if either Ty21a or Vi is given to infants would they elicit enduring immunity that would protect years later when the children reach the high-risk school–age years. Although Ty21a has been shown to be immunogenic in toddlers and pre-school children 2-5 years of age, no data have been reported in infants (Cryz et al., 1993). Vi has elicited seroconversions in the majority of toddlers, but titres attained were much lower than those of older children and fell after several months. A second dose of purified Vi does not raise antibody titres over those elicited by a single dose of the vaccine, i.e. immunological memory does not appear to occur. To increase the immunogenicity of Vi by conferring T- cell dependant properties upon the antigen, including the induction of immunologic memory, Szu et al. (1987,1989,1994) have conjugated Vi polysaccharide to various carrier proteins, such as tetanus toxoid.

A recent meta-analysis comparing the efficacy and toxicity of typohid vaccines used in vaccine trials from 1966 to 1996 (Engels et al., 1998) concluded that whole cell vaccines were more effective than the Ty21a and Vi vaccines but were more frequently associated with adverse events

Attenuated *S. typhi* strain CVD 908 was the first engineered *S.typhi* vaccine candidate shown to be highly immunogenic yet well tolerated, thereby generating optimism that it might be possible to develop a single dose, live, oral typhoid vaccine (Tacket et al., 1992; Levine et al., 1997). At a well-tolerated dose, 92% of CVD908 recipients manifested IgG "O" antibody seroconversions and showed evidence of priming of the intestinal immune system (IgA antibody – secreting cells)(Tacket et al., 1992).

Chatfield et al. (1992) found that inactivation of *htrA*, a gene encoding a stress protein that also functions as a serine protease, attenuates wild type *S.typhimurium* in the mouse model. Moreover mice immunized orally with *S.typhimurium* harbouring a deletion mutation in *htrA* were protected against subsequent challenge with a lethal dose of wild-type *S.typhimurium*. Chatfield et al. thereupon introduced a deletion mutation into *htrA* of CVD908, resulting in strain CVD908-*htrA*. Tacket et al. (1992) fed CVD-908-*htrA* as a single dose to three groups of subjects, who tolerated CVD908-*htrA* as well as they did CVD 908.

Curtiss and co-workers (1987) demonstrated that in *Salmonella* the genes *cya* (encoding adenylate cyclase) and *crp* (cyclic AMP receptor

protein) constitute a global regulatory system that affects many genes and operons. They showed that *S.typhimurium* that harbor deletions in *cya* and *crp* are attenuated compared with their wild-type parent, and oral immunization protects mice against challenge with virulent *S.typhimurium*. Curtiss, Kelly and co-workers (1994) constructed vaccine candidate strain c3927, a *cya*, *crp* double mutant of *S.typhi* strain Ty2. In phase I clinical trials, Tackett et al. demonstrated that c3927 was attenuated from wild type but insufficiently attenuated to serve as a live oral vaccine in humans, as occasional subjects developed high fever and typhoid-like symptoms.

Miller, Hohmann and co-investigators constructed 2 candidate *S.typhi* strains harboring deletions in phoP/phoQ (Hohmann et al., 1996a). Strain Ty445, which also harbors a deletion in aroA was only minimally immunogenic. In contrast, strain Ty800, a derivative of Ty2 deleted only in phoP/phoQ, was generally well tolerated and immunogenic when evaluated in a small phase I clinical trial (Hohmann et al., 1996b).

1.8 Heat shock proteins

Ritossa's early observations of puffing of the *Drosophilia* chromosomes in response to elevated temperatures resulted in the quest to characterize the changes produced in organisms during the stress of heat shock. These early observations resulted in identification of a specific set of proteins that were induced after heat shock and conferred protection to the

organism during subsequent heat shock. These proteins were first named the heat shock proteins, but as their induction to a variety of stress signals is now well documented, they are also known synonymously as the stress proteins (Lindquist, 1986).

Heat shock proteins are evolutionarily highly conserved polypeptides with important biological functions in protein biogenesis (Georgopoulos & Welch, 1993; Geeting & Sambrook, 1992). They appear to be produced by prokaryotic and eukaryotic cells to preserve cellular conditions under a wide variety of stress conditions, e.g. temperature (Lindquist & Craig, 1988), oxygen radicals (H_2O_2) (Clerget and Polla, 1990), pH (Wu et al., 1994), cytokines (Gromnowski et al., 1989), viral and bacterial infections (Pelham, 1988; Fayet et al., 1989; Kaufmann, 1990). These conditions increase the synthesis and production of HSPs. These proteins, by themselves or together with other molecules which show biological activities, could interact with host cells involved in the specific and non-specific responses affecting their functions (Polla, 1988; Karlsson-Parra et al., 1990; Kaufmann and Kabelitz, 1991).

The nomenclature of the HSPs has been designed so that the molecular weight of the HSP identifies it as belonging to a particular group, with the known groups ranging in size from the 10-kDa family to the 100 kDa family, so far primarily characterized in eukaryotic cells.

Because of their highly conserved nature, they are also called common antigens and cause significant specificity problems in serological

immunodiagnostic tests because they induce strongly cross-reactive antibodies (Bangsborg et al., 1989).

HSPs function in the intracellular transport to appropriate destinations of folding intermediates, the disassembly of oligomeric structures, & the facilitation of the removal of aggregated and or improperly folded polypeptides, hence the name " molecular chaperones" (Geething & Sambrook, 1992). Hendrick and Hartl (1993) proposed a recent definition - "a molecular chaperone is a protein that binds to and stabilizes an otherwise unstable conformer of another protein, and by controlled binding and release of the substrate protein facilitates its correct fate in vivo, be it folding, oligomeric assembly, transport to another subcellular compartment, or controlled switching between active/inactive conformations".

The most plausible role of HSPs in the stressed cell are the rescue of unfolded or aggregated polypeptides back to an active conformation and the acceleration of the proteolysis of proteins denatured beyond repair.

The sequential interaction of Hsp70 and then Hsp60 with polypeptides in the non-stressed cell, from the time of their synthesis to the final stages of their folding, supports the argument for a direct role for HSPs in protein folding. HSP70 interacts with nascent polypeptide chain while still in the ribosome (Beckman et al., 1990).

Proteins destined for the endoplasmic reticulum (ER) or mitochondria are bound to cytoplasmic Hsp70 in the cytosol and immediately bind

organellar Hsp70s upon emergence into the lumen of the ER or matrix of the mitochondria (Ostermann et al., 1990; Scherer et al., 1990). In the mitochondria, the Hsp70-bound protein is then "passed off" to Hsp60. Experiments with yeast show that Hsp60 is required for the proper folding and assembly of proteins into the multimeric complexes (Cheng et al., 1989).

The idea of a pathway of chaperone action was extended by Hartl (Langer et al., 1992) with an *in vitro* system consisting of the *Escherichia coli* Hsp70 (DnaK), Hsp60 (GroEL), and the additional, unrelated HSPs GrpE, DnaJ, and GroES. The temporal interaction of HSPs in this system closely mimics the pathway delineated for the interaction of HSPs in the import and folding of mitochondrial proteins in vivo. In vitro, Hsp70 (DnaK), DnaJ and Hsp60 interact sequentially with folding polypeptides.

1.8.1 Immune Response to HSPs

HSPs are involved in many basic biological processes and appear to elicit strong cellular and humoral immune responses (Young, 1990; Kaufmann, 1990; DeNagel & Pierce, 1993).

During bacterial infections, the interaction between a bacterial pathogen and its mammalian host exposes both parties to multiple physiological and biological stresses (Murray & Young, 1992).

Bacterial GroELS homologs are known to be immunodominant antigens for B cells and T cells during bacterial infections (Kaufmann & Kabelitz, 1991; Mehra et al., 1992).

T cells recognize a complex of antigenic fragments bound to MHC Class II which is displayed on the surface of antigen presenting cells (APCs). Processing of antigen involves entry into an acidic compartment of an APC, proteolysis, & the subsequent association of peptide fragments with MHC class II molecules (Brodsky et al., 1991; Neefjes et al., 1992; Teyton et al.,1992).

DeNagel and Pierce (1993) suggest that HSPs may potentially function as chaperones in either or both of the 2 phases of this process, i.e. in the assembly process of MHC Class II binding to processed antigen or in the scavenging and transport of processed antigen to MHC Class II.

Very little is now known about the host response against *Salmonella* HSPs. Generally, the majority of the host immune response directed against the bacterial stress proteins is against HSP60. In the *S.typhimurium* model, DnaK and GroEL have been shown to be induced by bacteria which are internalized in macrophages (Buchmeier & Heffron, 1990; Abshire and Neidhart, 1993).

Mice that are immune to virulent *S.typhimurium* infection mount a strong humoral response to GroEL after they are vaccinated with a live attenuated strain (Buchmeier & Heffron, 1990).

An interesting hypothesis has emerged in recent years suggesting that recognition of HSP by γδT cells may have evolved as a first line of defense against pathogenic insult to the host (Murray & Young, 1992) .

Imunological relevance of the *S. typhi* GroEL protein has been demonstrated (Tang et al., 1997). Antibodies specific to this protein were abundantly present in sera of typhoid patients. Other studies also demonstrate that pathogen stress proteins are immunodominant antigens and are targets for the host immune response. Many bacterial HSPs have been shown to be immunogenic proteins, e.g. GroEL of *Salmonella typhimurium* (Buchmeier and Heffron, 1990), GroEL and GroES of *Campylobacter jejuni* (Wu et al., 1994), GroEL and GroES homologs of *Helicobacter pylori* (Perez- Perez et al., 1996).

The fact that HSPs are targets of immune responses in a broad spectrum of infections may be related to the abundance of these proteins under stress conditions, e.g. GroEL is one of the most abundant proteins expressed by Salmonella within infected macrophages (Buchmeier and Heffron, 1990) which are known to be critical antigen-presenting cells in the immune system.

Although the exact role of HSPs in pathogenesis and immunity to microbial infections appears to be complex and incompletely understood, their involvement is well established, particularly with enteric pathogens.

When *htrA*, the gene which encodes a heat shock protein, is deleted in *S. typhi*, it resulted in an attenuated strain, which was then used as a

vaccine candidate for typhoid fever. It has also been shown that a GroEL homolog of *H. pylori* is bound to urease, a recognized virulence factor of this pathogen. Along similar lines, GroEL of *S. typhimurium* is responsible for binding of the bacterium to intestinal mucosa.

Lowrie et al. (1997) recently showed that immunization of mice with genes encoding mycobacterial hsp65 and hsp70 proteins induced strong protective immunity against tuberculosis.

1.9 Epitope Mapping

"Epitope mapping" is usually applied to protein antigens, and is the process of locating the epitope on the surface of the protein or in the protein sequence.

The term epitope mapping has also been used to describe the attempt to determine all major sites on a protein surface that can elicit an antibody response, at the end of which we produce an epitope map of the protein antigen (Atassi, 1984).

Implicit in this view of epitopes is that they are fixed, concrete structures on protein surfaces, which are few in number and are uniquely capable of stimulating the immune system. This kind of epitope map also confuses the important distinction between antigenicity (the ability to recognize a specific antibody) and immunogenicity (the ability to produce antibodies in a given animal species).

It is essential to distinguish between conformational ("discontinuous", "assembled") epitopes, in which amino acids far apart in the protein sequence are brought together by protein folding, and linear ("continuous", "sequential") epitopes, which can be mimicked by simple peptide sequences. Parts of conformational epitopes can be mimicked by peptides, and the term "mimotope" has been coined to describe these peptides. On the other hand, the view that most peptide sequences can produce antibodies that recognize native proteins (Berzofsky, 1985; van Regenmortel, 1989) has been disputed. Given the nature of protein structures, most epitopes on native proteins proteins are likely to be "assembled" (Barlow, 1986).

Consequently, most antibody molecules in polyclonal sera raised against native proteins do not recognise short peptides (van Regenmortel, 1989). If assembled epitopes are found most frequently on native proteins, sequential epitopes are found more often on denatured or partially unfolded proteins.

The definition of epitopes is restricted to studies of proteins of related and known sequence. Even then, the information is limited, and locates the epitope to the neighborhood of the amino acid substitution(s) correlating with the laws of antibody binding (Smith-Gill et al., 1982; Berzofsky et al., 1982).

Monoclonal antibodies raised against the native proteins often fail to bind to any short linear peptide homologous to that of the protein. This

is consistent with the view that a significant proportion of the antibodies generated by the immune response are directed towards assembled or discontinous determinants (Berzofsky et al., 1982; Benjamin et al., 1984).

Monoclonal antibodies work on the operational view that an epitope is defined by an antibody molecule, i.e., if an antibody exists, then whatever it can be shown to recognize in the antigen is the epitope (or part of it). This view has its own problems, notably the fact that monoclonal antibodies often cross-react with sequences or structures other than that of the real antigen. Monoclonal antibodies that bind to proteins on Western blots (after SDS-PAGE) will tend to be against sequential epitopes, whereas those which recognize antigens in liquid-phase immunoassays or in frozen tissue sections are often directed against assembled epitopes.

X-ray crystallography is often recognized as the only method for precise definition of an epitope by identification of all amino acids in contact with the antibody. This technique's contribution to our understanding of epitopes has been outstanding (Saul & Alzari, 1992). This method, however, has its problems. There does not seem to be complete agreement on how close amino acids in the antibody and antigen must be to constitute a "contact". Some residues in the antigen could theoretically be "in contact" with the antibody without contributing significantly to the binding. The method is also restricted by the necessity

of obtaining good crystals of antibody-antigen complexes, and it has usually been applied to conformational epitopes on the surface of soluble proteins.

An alternative to mapping epitopes via the antigen is to employ combinatorial methods, by chemical means, to generate an enormous collection (a library) of all theoretically possible peptides containing 4,5,6 amino acid residues and so on. It is then apparent that complete libraries can only be constructed for relatively short peptides.

Phage-display systems have become popular of late as they enable the production of large libraries (10^9 or more particles / μl) presenting peptides as long as 20 residues or even complete proteins. Epitope mapping is accomplished by reacting the monoclonal antibody with the library, a process called bio-panning. The phages which display a peptide that is homologous to the original epitope are bound by the monoclonal antibody and analyzed. The sequence of the peptide displayed by a particular phage can be determined, and compared with that of the antigen, thus allowing mapping of the epitope (Smith and Scott, 1993).

1.10 The Multipin Peptide Technique

The Multiple peptide synthesis technique permits thorough answers to questions such as where all the linear epitopes in a particular protein are and how long the critical part of each epitope is. It also allows identification of epitopes that are commonly (and rarely) recognized and what the affinities for each epitope are. Further analysis could reveal which amino acid in the epitope are in contact with the antibody/ T cell receptor (TCR) / MHC (major histocompatibility complex) molecule.

Newer methods for the rapid synthesis of large numbers of peptides have facilitated the mapping of antigenic determinants of proteins (Geysen et al., 1984, 1987b). The pin technology developed by Geysen and colleagues is particularly useful for probing the antigenic structure of an entire polypeptide sequence (Geysen et al., 1984, 1987a,b; 1988; Getzoff et al., 1987). Short overlapping peptides, e.g. hexapeptides, covering the sequence of a protein antigen are synthesized onto polyethylene pins by the Merryfield solid phase method (Merryfield, 1963). The pin-bound peptides are then tested for reactivity with antibodies against the protein antigen, using a standard ELISA procedure. Since peptides remain covalently linked to the solid support, bound antibodies can be removed and the peptides re-tested with other antisera.
Of the variety of methods available for multiple peptide synthesis, the Multipin method was the first (Geysen et al., 1984). From its beginning as

a method for testing peptides on the same surface on which they had been synthesized (Smith et al., 1977; Geysen et al., 1984, 1987) it was developed into a method for obtaining solution-phase peptides using a very mild method of cleavage that produced peptides in physiological solution ready for bioassay (Maeji et al., 1990). Further development of the chemistry of grafting, synthesis and cleavage (Bray et al., 1991; Valerio et al., 1993; Maeji et al., 1994) and scaling up of the pin size (Maeji et al., 1995) have allowed a high-quality peptide on the order of 5μmol to be produced from each pin, making it feasible to screen thousands of peptides in assays requiring high peptide concentrations.

Peptides made using this technology can be applied to the search for and understanding of both linear-antibody defined epitopes (B-cell epitopes) (Rodda et al., 1986; Getzoff et al., 1987) and of helper- and cytotoxic T cell epitopes (Mutch et al., 1991; Burrows et al., 1994). For linear B cell epitopes in particular, a decision must be made whether solid-phase or solution-phase peptides are to be used. Use of peptides permanently attached to the solid-phase on which they were synthesized has the advantages of simplicity and sensitivity, but it is subject to uncertainty about the quality of the peptides being used, and to artifact arising from the high density of the peptide and possible interactions with the support matrix (Trifilieff et al., 1991).

The Multipin Peptide method, aided by the availability of molecular sequence information and convenient methods to synthesize libraries of

synthetic peptides, has been used to define immunogenic epitopes within many immunogenic proteins. Among these include B-cell epitopes of *Chlamydia trachomatis* (Yi et al., 1993), the Sta58 major outer membrane protein of *Rickettsia tsutsugamushi* (Lachumanan et al., 1993), a fimbrial protein of *Porphyromonas gingivalis* (Ogawa et al., 1995), the Epstein-Barr replication-activator protein (Cheng et al., 1995), the X protein of Hepatitis B virus (Kumar et al., 1998), the 10K antigen of *Mycobacterium leprae* (Hussain et al., 1999). This method has also proven useful in the identification of epitopes on receptors (Afzalpurkar and Gupta, 1999; Ward et al., 1999) and also in identifying binding sites on enzymes (De Silva et al., 1999).

1.11 Chemistry of Peptide Synthesis

The Multipin Peptide Synthesis System provides a means of applying a constant method applied to "variable" reagents (different amino acids).

The polyethylene gears have been radiation grafted. This provides a reactive surface, on the otherwise inert plastic, consisting of hydroxyl groups (see Figure 1.1). These groups are then esterified with F-moc glycerine to give structure II in Figure 1.1. The gears are then acetylated to "cap" any unreacted hydroxyl groups and so prevent them from taking any further part in the synthesis. The F-moc (9-

fluorenylmethyloxycarbonyl) protecting group is removed and the free amino acid is coupled to F-moc-β-alanine in a peptide bond (see structure III in Figure 1.1). The gears are then acetylated again to cap any unprotected, unreacted amine that may remain.

All amino acids used for the synthesis had their α-amino group protected with the F-moc group and the following side chain protecting groups: *t*- butyl ether (Bu^t) for serine, threonine and tyrosine; t-butyl ester (OBu^t) for aspartic acid and glutamic acid; *t*-butoxycarbonyl for lysine, histidine and tryptophan; 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) for arginine ; and trityl (Trt) for cysteine. The protected amino acids are activated with diidopropylcarbidiimide (DIC)/ hydoxybenzotriazole(HOBT).

Synthesis of peptides is accomplished by repetitive cycles of Fmoc-deprotection, washing and amino acid coupling, adding one amino acid per residue per cycle. After completing the synthesis of the desired peptides, the final F-moc protecting group is removed and the terminal group can be capped by acetylation. Removal of side chain protective groups is effected by trifluoroacetic acid containing one or more scavengers. Scavengers are necessary in order to remove highly reactive *t*-butyl based carbonium ions.

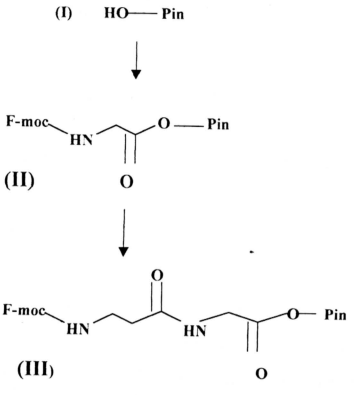

Figure 1.1 Linker assembly

1.12 Objectives of Study

GroEL, the 60-kDa heat shock protein of *Salmonella typhi* has been shown to be immunologically relevant (Tang et al., 1997). In addition, the entire sequence of the GroEL heat shock protein of *S. typhi* has been published (Lindler and Hayes, 1994) and this is an important source of information for this study. The important sites on the *groEL* gene can thus be identified and studied by incorporating knowledge of protein folding, i.e. hydrophobicity and hydrophilicity.

Therefore, the objectives of this study are to:

1. Synthesize short, overlapping peptides covering the identified regions on polyethylene pins using the Multipin Peptide Synthesis technique (Geysen et al., 1987).
2. Screen this GroEL peptide library with culture-confirmed typhoid sera, sera from healthy blood donors and the monoclonal antibody to GroEL.
3. Identify and define the immunogenic epitopes of *S. typhi* GroEL