THE EFFECTIVE DELIVERY OF A BIVALENT VACCINE AGAINST DIARRHOEAL DISEASE

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Thesis for the Degree of Doctor of Medicine of the University of Adelaide
Submitted March 1990

To my parents, Leo and Glenice Forrest

"I'm an idealist: I don't know where I'm going but I'm on my way."

Carl Sandburg

The object of all vaccination processes is, first, to achieve a degree of immunity which shall be equal or greater to that which accrues to a patient who undergoes and recovers from an actual attack of the disease; and, secondly, to achieve that immunity without any risk to life or health.

Almroth .E. Wright, 1897 [1]

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ABSTRACT

The work comprising this thesis, describes a methodology for the detailed evaluation of the processes involved in the assessment of recombinant orally administrable vaccines against mucosal pathogens, in this instance, a bivalent vaccine against diarrhoeal disease. For such an evaluation to provide a meaningful insight into the worth of a particular vaccine candidate, a human model for the assessment of local intestinal immunity to an attenuated bacteria had to be established. This model, utilizing the attenuated live oral typhoid vaccine, *Salmonella typhi* Ty21a, permitted the development of a new, novel immunoassay that allowed the correlative determination of a specific intestinal immune response using peripheral blood lymphocytes. Combining this new assay, with improved assays of local and serum specific antibody, it was possible to provide a detailed picture of the kinetics of the human immune response to intestinally presented live attenuated bacterial vaccines - permitting the definition of the optimal time for sampling of fluids and secretions in the assessment of live bacterial vaccines. In addition the effect of different manufacturing processes, vaccination routes and regimens on the resulting ability of this strain to induce an intestinal immune response was also assessed. This work formed the basis upon which several new hybrid typhoid/cholera attenuated live orally administrable bacterial vaccines, based upon *Salmonella typhi* Ty21a, could be assessed in volunteer subjects. This work culminated in the protective efficacy against clinical cholera of one of these strains, designated EX645, being assessed in human volunteer subjects at the Center for Vaccine Development in Baltimore, USA.

STATEMENT

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference has been made in the text.

I hereby consent to this thesis being made available loan and for photocopying within the constraints of the applicable State and Commonwealth laws, if accepted for the award of the degree.

Bruce D. Forrest

London, January 1990

ACKNOWLEDGEMENTS

I wish to acknowledge:

Dr JT LaBrooy, Professor DC Shearman and Professor D Rowley for their supervision and support throughout the period during which this work was performed;

Enterovax Limited for providing the financial support for the studies comprising this thesis;

Enterovax Scientific Group comprising (in addition to the aforementioned) Mr. L Beyer, Dr G Boehm, Dr SR Attridge, Dr R Morona, Dr J Hackett and Mr. J Hodges, for the provision of vaccine strains, doses, and comradeship;

Dr R Pridham, Dr CE Dearlove, Mrs. W Ferguson and Ms. C Danz for their assistance at various times in the recruitment and management of the volunteers;

Miss L Mundy, Miss PD Dixon, and Mrs. D Pyle for their expert laboratory and technical assistance;

Professor MM Levine, Dr CO Tacket, Mr. B Tall, Mr. CR Young and Sylvia O'Donnell at the Center for Vaccine Development, University of Maryland, Baltimore, USA for permitting my involvement in, and assisting me with the conduct of the Phase 2 cholera challenge study of the final candidate vaccine strain; and

the volunteers, both in Adelaide and Baltimore, far too many to be acknowledged individually, but without whom none of this work could have been performed.

1.1 MECHANISMS OF IMMUNITY IN THE HUMAN INTESTINE

It is considered that the most effective control of infectious diarrhoeal diseases can be best achieved through the adequate supply of potable water, sanitation and health education. However, it is the lack of available funds and the presence of other competing priorities that appears likely to prevent this goal from being achieved in the regions of the world where it is most needed.[2]

Vaccination provides a complimentary approach to effective intervention against diarrhoeal disease. An orally administered live attenuated vaccine against an enteric pathogen is believed to provide a convenient and effective means of vaccinating against diarrhoeal disease; the success of this approach with the live attenuated (Sabin) poliovirus vaccines indicates that this approach may be significantly beneficial to a region at risk of disease, especially when utilized in cooperation with the other aforementioned interventions.[2]

Any attempt to discuss the effective delivery of an orally administered live attenuated bacterial vaccine, must first take into consideration the processes involved in the generation of an antigen-specific immune response at the intestinal mucosal surface, since it is the mucosal membranes of the intestinal tract which represent a massive area of direct environmental exposure, and as a result it is these surfaces that have the largest accumulation of lymphoid tissue.

1.2

HISTORICAL INTRODUCTION

The concept of local immunity, that is, immunity of the mucosal surface at which the antigen was presented is not a recent concept. Indeed, Witebsky[3] has suggested that it was first demonstrated by Mithridates VI Eupator (132-63 BC) the King of Pontus (Northern Turkey) who, to protect himself against being assassinated by the ingestion of poison, developed a "universal antidote" from the blood of ducks that had been fed on poisonous weeds, which he drank daily. After losing a battle with the Romans he attempted to kill himself with a poison which he carried in a vial that had been extracted from certain plants. While this poison successfully killed two of his daughters, the poison from the same vial was ineffective against him.

Louis Pasteur, working with Roux and Chamberland, probably deserves credit for being the first in the modern era to demonstrate the effect of local immunity with his report of the successful protection of chickens against chicken cholera by the addition of attenuated *Bacillus avisepticum* to chicken feed in 1880. This type of experiment was not as successful when it was attempted to orally vaccinate sheep against anthrax by administering attenuated *Bacillus anthracis* spores.[4]

By the middle of the 1920's there had been a gradual accumulation of evidence for the existence of local immunity. In 1924, Frederick Gay in a good review of the early history of local immunity,[4] recognized that the likely outcome of a bacterial infection at any given site was dependent upon the interplay between bacterial virulence characteristics and local host resistance factors. He defined local immunity as *"an acquired, increased protection of some part or tissue superior to that existent elsewhere in the body. This local immunity moreover follows the local administration of the particular*

microorganism against the which protection is evidenced". He added to this definition *"a locally superior mechanism for the disposal of the particular microorganism rather than a local mobilization of antibodies generally present in the body"*. Pondering the difficulties associated with demonstrating the existence of a local immune response that would support his definition, he proposed that *"local immunity may be proved by the local presence of antibodies before their appearance elsewhere in the body or by their presence in greater concentration"*. In general terms, these concepts still hold true 65 years later.

For local immunization to be both safe and effective it was necessary that methods of attenuating microorganisms be developed. It was the ability to develop processes of attenuation that was the cornerstone of Louis Pasteur's work. Pasteur's four methods of attenuating organisms were: aging in the presence of oxygen (chicken cholera, 1880); prolonged culture at higher than normal temperatures (anthrax, 1881); passage through different host species (swine erysipelas, 1883); and drying (rabies, 1885).[5]

It was Jaime Ferran y Clua (1852-1929) who held the distinction of being the first investigator to immunize humans against a bacterial disease, with his highly successful administration of broths of live *Vibrio cholerae* organisms subcutaneously to 30 000 Spaniards in 1885 during a cholera epidemic, a full eight years before the work of Waldemar Haffkine. In addition, Ferran was also the first to orally immunize humans against a bacterial disease, also cholera, using attenuated live bacteria.[5] In a paragraph quoted by Bornside:

"The vibrio isolated from dejecta ... becomes rapidly and spontaneously attenuated when it is cultured in nutrient broth. Thus, the third culture in a series can be drunk in small quantities with impunity, and inoculated subcutaneously without danger at a dose of two cc. With a number of my vaccines I follow a very

simple and convenient procedure to sustain and reinforce this immunity. This consists of drinking from time to time a specified number of drops of culture of the comma bacillus. Long experience has demonstrated to me that this procedure is also as inoffensive to those who submit to it as it is to those with whom they live. In view of these results, I do not hesitate to say that the most practical way to confer immunity to an entire population rapidly would consist of infecting their drinking water with large quantities of attenuated culture of the comma bacillus. This practice would suddenly produce in all the inhabitants one or two diarrhoeal stools followed by a slight reaction and a fleeting lassitude, symptoms which we obtain in drinking our cultures. For this method to be harmless it is not necessary that one be vaccinated previously by hypodermic injection.".[6] Perhaps these comments represent the earliest concepts of the principles of mass oral vaccination.

During the late 19th Century it had become increasingly apparent to many European investigators that the oral route of vaccination appeared likely to provide a better degree of protective immunity against enterically presented bacterial infections than the parenteral route. The problem that they encountered was that there was not available any measure of local intestinal immunity that correlated with protection. Subsequently, several investigators attempted to determine methods of quantifying the magnitude and significance of the local immune response. Both Klemperer[7] and Metchnikoff[8] were able to induce and measure agglutinating antibodies (agglutinins) in serum against *V. cholerae* when administered by the by oral route. However, the role and significance of serum antibody following local immunization was considered doubtful by Gay, Besredka and Calmette, who, in Gay's words,[4] believed that *"It is particularly true that a general reaction as evidenced by serum antibodies is no indication of a superior local production, for example in the intestine, if we admit that this exists"*. In addition he emphasized,

"Although the antibody reaction has been definitely stated, little accrues from these experiments to our knowledge of local protection although in some instances experimental infection by various routes was and indicates that at least some grade of general immunity can be produced against these bacteria as well". By using the term "general immunity" it is apparent that he is referring to serum antibody (agglutinins).

Besredka, through his studies with Metchnikoff, was able to show that serum antibodies (agglutinins) did not necessarily indicate protective immunity. They reported that chimpanzees subcutaneously vaccinated with a killed typhoid vaccine, then infected by the oral route with virulent *S. typhi* bacteria contracted typhoid fever; where guinea pigs vaccinated subcutaneously and challenged intraperitoneally with *S. typhi* were protected.[9] Nicolle in 1922 reported the first successful controlled human challenge study with a enteric bacterial pathogen.[10] In his study, two volunteers took a killed *Shigella dysenteriae* vaccine preparation on three consecutive days. The vaccine had been prepared by heat inactivating the cultures at between 72°C-75°C. On Day 17 and 20 after the commencement of vaccination these two subjects together with two unvaccinated controls were challenged orally with $1x10^9$ live, virulent *Sh. dysenteriae*. Both control subjects became ill with clinical dysentery, whereas the two vaccinated subjects remained well.

These early experimental studies demonstrated that the oral route represented an effective means of protecting against enteric disease through the mechanism of local immunity. A finding further confirmed by the extensive use of oral typhoid, cholera and dysentery vaccines throughout Europe and South America during the 1920's with great success.[9]

However all this work was empirical, with no understanding of the mechanisms involved in the conferring upon an individual a degree of protection through immunization utilizing the oral route.

1.3

THE ROLE OF IgA IN MUCOSAL IMMUNITY

It is generally considered that the intestinal secretory IgA system is quantitatively the most important humoral immune system in the body.[11-13] It has been repeatedly demonstrated that after infection or oral vaccination with attenuated organisms, the immune response generated at the intestinal mucosal surface is mainly of the IgA antibody class, and always of the secretory type.[14-16] Other studies have confirmed that a similar situation occurs after local immunization at other mucosal surfaces, such as the genital tract [17] or the upper respiratory tract.[18,19]

Secretory IgA, the predominant immunoglobulin in the human gastrointestinal tract exists in a dimeric form, and not in the monomeric form of the bone marrow-derived serum IgA.[20,21] Serum IgA irrespective of its form, be it monomeric or polymeric, does not significantly contribute to the total IgA in secretions.[22] Secretory IgA has been found to have several roles in the maintenance of mucosal defences, through its ability to neutralize toxins and viruses, and its ability to block bacterial adherence to surface epithelium.[23] In fact, secretory IgA *in vitro* is able to specifically inhibit bacterial adherence and colonization of cultured respiratory epithelial cells.[24] One possible reason for the effectiveness of secretory IgA is its retention within the mucous layer over the epithelial cells after its secretion into the intestinal lumen, achieved by interacting with cysteine residues contained in the mucins within the glycocalyx. It is this relative juxtaposition to the

intestinal epithelial cell which is responsible for its efficient interaction with intestinal antigens. These antibodies are strongly adherent to the intestinal surface and can only be removed with difficulty.[25]

However, accurately determining the level of intestinal secretory IgA antibody has proven to be fraught with difficulties. The reported dissociation of serum and local intestinal IgA responses to an orally administered antigen in humans rendered serum IgA determination as an indicator of local IgA inadequate,[26-28] as originally encountered by the 19th Century investigators and further described by Besredka.[9] One significant contribution to this situation has been the inadequacy of assays used in the determination of intestinal specific IgA antibody,[28,29] as well as the difficulties encountered due to proteolysis of the specific IgA immunoglobulin that occurs at various levels of the gut[13] - despite a variety of measures that have been used to minimize this event.[30-32] This is one of the major reasons for most investigators continuing to utilize serum IgA antibody levels as an indicator of a specific IgA local antibody response, while acknowledging its purported inadequacies.

What appears to be certain is that following clinical infection with an enteric pathogen, *Salmonella* gastroenteritis for example, it has been observed in adults that a marked specific antibody response most evident at two to three weeks following the onset of diarrhoea, with persisting elevated levels of specific IgA antibody in intestinal fluid specimens obtained up to six weeks after the onset of diarrhoea occurs.[28] In some cases these elevated IgA levels were still significantly elevated above normal control levels one year after the illness however they were usually associated with asymptomatic intestinal carriage of the pathogen.[16] The importance of this prolonged duration of specific antibody response in protection is unclear, although the

observation that the long term excreters of the pathogenic *Salmonella* did not have any evidence of symptomatic infection suggests that the specific IgA antibody may have been providing a degree of protection.[16] It should be mentioned that the need for a persisting specific IgA intestinal antibody in conferring protection may not be an absolute requirement. Subjects who were challenged with homologous organisms three years after recovery from experimental *V. cholerae* infection were found to be significantly protected against disease in the absence of detectable specific IgA anti-cholera antibodies,[33] although this may represent an inadequacy in the sampling or assaying as discussed previously. Therefore, it appears that although the humoral immune response may not remain persistently elevated above baseline following oral vaccination, there may still have been priming of the immune system with the generation of immunological memory, which would facilitate a rapid response to a later challenge.[34-36]

The human immune response to enteric pathogens has been variously investigated in the past,[16,28,37-39] with some of this information having been obtained with pathogenic organisms such as *V. cholerae*.[40-43] However, the opportunity to intensively examine the kinetics of the humoral intestinal and serum IgA responses concurrently has not been previously afforded using modern techniques.

The establishment of the importance of local IgA production in the provision of protection against enteric disease is unquestioned, however it is often the varying nature and duration of that protection that is disputed.

Despite 100 years or more of systematic investigation into local immunity and its role in protection, and in the 30 years since IgA was described in mucosal secretions, there is still a paucity of available techniques of accurately and reliably determining the levels of intestinal

specific IgA antibody that have enabled its relationship to protection to be unequivocally elucidated.

1.4

THE STRUCTURAL BASIS FOR LOCAL INTESTINAL IMMUNITY

1.4.1 *Distribution of IgA Producing Cells*

Animal studies have indicated that following oral immunization, a preponderance of specific IgA plasma cells were observable in the intestinal lamina propria,[44] with the plasma cells containing antigen-specific IgA being found in close contact with the epithelial cells, which in turn contained demonstrable antigen-specific IgA antibody concentrated in their apical ends.[45]

In the human, it has been discovered that IgA has two subclasses, IgA_1 and IgA_2 - the distinction being based largely upon antigenic differences, and differences of amino acid sequence and carbohydrate composition.[46,47] In adult peripheral blood it has been shown that there is a preponderance of IgA_1 producing B lymphocytes (mean 80%) compared to IgA_2 producing cells (mean 20%),[48] whereas in external secretions IgA_1 constitutes only 50% to 74% of the total IgA.[49,50] The distribution of the IgA subclasses throughout human tissues appears as in Table 1.1.

As can be seen from Table 1.1, in the gastrointestinal tract it is the IgA_1 subclass which predominates in the upper small intestine, and the IgA_2 subclass is the predominant immunoglobulin in the colon.[46,50,51] It is particularly advantageous that the IgA_2 production is maximal in the colon, mammary glands and in saliva, since these secretory tissues produce

antibodies to mucosal surfaces that have a particularly heavy bacterial load, and IgA_2 is resistant to several bacterial proteases that can cleave IgA_1.[51-53]

TABLE 1.1

DISTRIBUTION OF IgA SUBCLASS PRODUCING CELLS IN HUMANS

	IgA_1	IgA_2
Nasal mucosa	96%	14%
Lacrimal glands	52.5%-81%	19%-47.5%
Salivary glands	53%-66%	34%-47%
Mammary glands	63%	37%
Stomach	84%	16%
Duodenum/Jejunum	56.2%-77%	23%-43.8%
Ileum	55%	45%
Colon	33%-38.6%	61.4%-67%
Rectum	45%	55%
Spleen	78.6%-91%	9%-21.4%
Peripheral nodes	73.4%-94%	6%-26.6%

(adapted from Crago SS *et. al.* 1984, and Kett K *et. al.* 1986; references 50,51)

The evidence is clear that IgA is the predominant immunoglobulin at the intestinal mucosal surface, and that it appears to have a significant role in the protection against enteric disease.

The following text, attempts to detail the mechanism involved in the production of this specific IgA antibody in response to an antigenic stimulus.

1.4.2 *Role of Peyer's Patches in Local Intestinal Immunity*

Broadly speaking, for an immune response to be produced at a mucosal surface against a specific antigen, that antigen must in some manner

access the lymphoid tissue and stimulate the appropriate lymphocytes, a process which subsequently results in the production of an antigen-specific immune response.

The Peyer's patches are in this context the receptor of an antigen locally presented at a small intestinal mucosal surface. In 1677, J.C. Peyer described these structures present in the wall of the terminal ileum which he originally thought were in some way involved in the digestive process, since they appeared to exuded fluid when pressed. Since that time our understanding of the role that these structures, known as Peyer's patches, have in local mucosal immunity has increased substantially. However, most of this knowledge has only been acquired in the past 25 years.

Peyer's patches form the bulk of the structured and organized lymphoid tissue of mammals. A careful study of the fetal intestine indicated that as early as 11 weeks of gestation clusters of CD4 expressing cells were present in the fetal ileum.[54] It was concluded that since these cells were CD3 negative that they probably represented macrophages. It was from 14 weeks gestation that aggregates of lymphoid tissue were apparent which contained CD4 and CD8 T lymphocytes. By 16 weeks it was found that both B and T lymphocytes were present. From 19 weeks of gestational age, fetal lymphoid follicles were found with B lymphocytes surrounded by T lymphocytes. The B lymphocytes were found to be expressing surface IgM, IgG, C3b- and C3d-receptors. Evidence of antigen-independent B cell proliferation was demonstrated by the expression of the CD23 surface antigen. Follicular dendritic cells were also present at this time. These fetal small intestinal lymphoid follicles present by the fifth gestational month are Peyer's patches.[55]

In 1965, Cornes performed the first extensive study of the number and distribution of human Peyer's patches and observed a mean of 59 well-defined Peyer's patches identifiable at the 24th gestational week, these patches containing five or more lymphoid follicles.[56] He also made the observation that in humans, unlike other animals, both the number and size of the Peyer's patches increased throughout the second half of fetal life, and continued to do so for the first ten years of post-natal life at least. The number of Peyer's patches containing five or more lymphoid follicles peaked in the 12-14 year age group with 239 being identified. During aging a decrease in both the number and size of Peyer's patches was noted, from a mean of over 200 patches containing five or more follicles in persons under 20 years to a mean of 100 in the 70-95 year age group. Large patches exceeding 4 cm in length tended to appear after puberty and remained identifiable despite aging. There was no observable alteration in the distribution of Peyer's patches throughout the small intestine, patches being found from the Ampulla of Vater in the duodenum (rarely between the Ampulla and the pyloris) through to the terminal ileum. On most occasions, though not always, the bulk of these patches were found in the ileum - on occasions some subjects were found to have the bulk located in the jejunum.[57]

Peyer's patches have been found to be generally oval in shape and were usually found on the antimesenteric wall of the small intestine, especially in the ileum and distal two-thirds of the jejunum, however they can be situated randomly around the intestinal wall, especially in the duodenum and proximal one-third of the jejunum.[56] Light microscopic examination has shown that each patch was only ever one follicle thick, irrespective of the number of follicles present in the patch, and this preserved

the intimate relationship between the follicle and the overlying intestinal epithelium.[58] It is this apparent relationship between the intestinal epithelium and the underlying follicles of Peyer's patches that requires further discussion.

1.4.3 *The Function of Intestinal Epithelial M Cells*

The follicles comprising Peyer's patches are covered with a dome-like epithelium, the ultrastructure of which is similar to that found over the bronchus associated lymphoid tissue (BALT).[59] High resolution, high magnification scanning electron micrographs of this epithelium demonstrated the existence of a different epithelial cell type, which was named the microfold or M cell.[58] This epithelium was found to comprise a reticulum formed by ultra-fine processes of the M cells, which appeared to allow lymphoid cells to closely approach the gut lumen without being lost into the lumen. Owen and Jones [58] concluded from their ultrastructural study of these M cells that due to the very close proximity of the inner and outer plasma membranes and the absence of the terminal web, that these cells were involved in some form of transport activity. They also observed that the lymphoid cells lying within the M cell processes appeared, due to their prominent Golgi apparatus and rough endoplasmic reticulum, to be actively stimulated.

A similar epithelial cell structure had been observed by other investigators in some animal intestines. In one electron microscopic study the transmission of India ink and ferritin that had been placed into the lumen of a ligated mouse intestine into the underlying Peyer's patch was observed to occur throughout the apical vesicles of the follicular epithelial cells.[60]

It is now apparent that these intestinal M cells clearly play a role in the selective uptake of specific antigens and microorganisms from the small intestinal lumen, transporting them into the underlying lymphoid tissue.[61] Various investigators have observed the specific uptake and transport of certain bacteria,[61-65] while others have demonstrated the specific affinity that specific viral agents, such as certain strains of reovirus, have for the small intestinal M cell in mice, and the subsequent selective uptake by these cells of this virus permitting viral access to the intestinal lymphoid follicles,[66,67] and still others have shown that inert particulate antigens such as latex microspheres can also be selectively taken up and transported into the underlying Peyer's patch lymphoid tissue and beyond.[68]

Macrophages have also been demonstrated to be in close association with the epithelial M cell and with the lymphocyte clusters of the Peyer's patches, and have been found to insert processes between the M cells, enabling them to phagocytose intraluminal organisms such as *Salmonella*.[69,70]

1.5

LOCAL IgA SYNTHESIS IN THE SMALL INTESTINE

It is known that Peyer's patches selectively sample luminal contents but with little or no antibody synthesis occurring within these tissues[71-73] despite the knowledge that the gut-associated lymphoid tissue (GALT) (Peyer's patches form the major part) possesses nonadherent, nonphagocytosing and highly Ia-positive antigen-presenting cells.[74-76] It is in this respect that the Peyer's patches lymphoid tissue differs significantly from peripheral lymphoid tissue. The exact reasons for this remain unclear.

An important step in the pathogenesis of many microbial diseases is the successful penetration of the protective mucosal epithelium of the

respiratory or gastrointestinal tracts. Many organisms are able to breach the epithelial barrier through their selective uptake by the epithelial M cell,[62,77,78] others such as *S. typhimurium*[79] and *S. typhi*[80] have also been shown to penetrate and disrupt the mucosa directly. The successful uptake of such an antigen into the underlying lymphoid aggregate, such as the Peyer's patches of the small intestine, has been shown to result in the stimulation of IgA-secreting precursor plasma cells. Peyer's patches appear to contain large numbers of cells predisposed to the synthesis of IgA in mucosal tissues.[81,82] Upon stimulation these precursor cells appear to migrate into the blood circulation, via the mesenteric lymph nodes and the lymphatics (thoracic duct).[83] During this migration these cells undergo maturation and eventually seed to the lamina propria of a variety of mucosal surfaces where they secrete specific IgA.[84,85] It has been determined that not all the migrating lymphocytes that derived from the GALT necessarily return to the intestinal mucosa.[86] Certainly the bulk of these cells (70%) have been observed to return, but many in fact are found to seed to other mucosal surfaces, such as respiratory tract and genital tracts.[86]

While it has been noted that GALT derived B lymphocytes have a predisposition to return to mucosal lymphoid tissues and may be found within 30 minutes of transfer, in the subcapsular sinus of the mesenteric lymph nodes,[87] and that Peyer's patch-derived lymphocytes, precommitted to IgA synthesis, were capable of recognizing and extravasating in the intestine before they are sufficiently differentiated to remain there as IgA plasma cells,[87] it is worth noting that the Peyer's patches do not exclusively contain B lymphoblast precursors for IgA,[88] and the organ selectivity described for IgA has also been shown for IgG lymphoblasts, implying a mucosa-seeking B lymphocyte population not exclusive to the IgA class.[86]

The reasons for this migration and homing pattern have still not been satisfactorily elucidated (the process of antigen uptake, lymphocyte migration and mucosal IgA production is summarized in Figure 1.1),[89] although it is apparent that the process is independent of secretory component and antigen presence.[12] A chemotactic substance, however, may have been identified in breast milk that could be responsible for migration of B lymphocytes to that secretory site.[12]

1.6 SYNTHESIS AND SECRETION OF SECRETORY IgA

Once the mature plasma cell has located itself at the lamina propria, it begins synthesizing and secreting secretory IgA.

IgA is like most other immunoglobulin classes in that it is made up of four-chain-monomer subunits with two antigen combining sites oriented with two-fold rotational symmetry. To enable it to function as a secretory antibody, IgA has several distinctive features, two are detailed below:

(a) the ability of the IgA monomer subunits to polymerize, due to a special C-terminal extension containing an extra cysteine, enabling cross-linking.[90] J chain (a 15 000 M_r peptide) initiates the polymerization and becomes incorporated into the molecule.[91] The polymer formation is believed to be essential for the binding to the mucosal transport receptor, increases antigen avidity, and directly increases the resistance of IgA to proteolytic digestion.[92]

(b) Whereas the immunoglobulin hinge is usually regarded as a labile site for proteolysis, the particular amino acid composition of the IgA hinge renders it relatively unsuitable as a substrate for many proteolytic enzymes.[90]

The plasma cells secrete IgA in a dimeric or polymeric form into the subepithelial space. However, since the epithelial cells of the intestinal mucosa form a tight barrier between the site of IgA synthesis and the intestinal lumen, a specific transport mechanism has evolved to move individual polymeric IgA molecules through the epithelial cell to the surface.

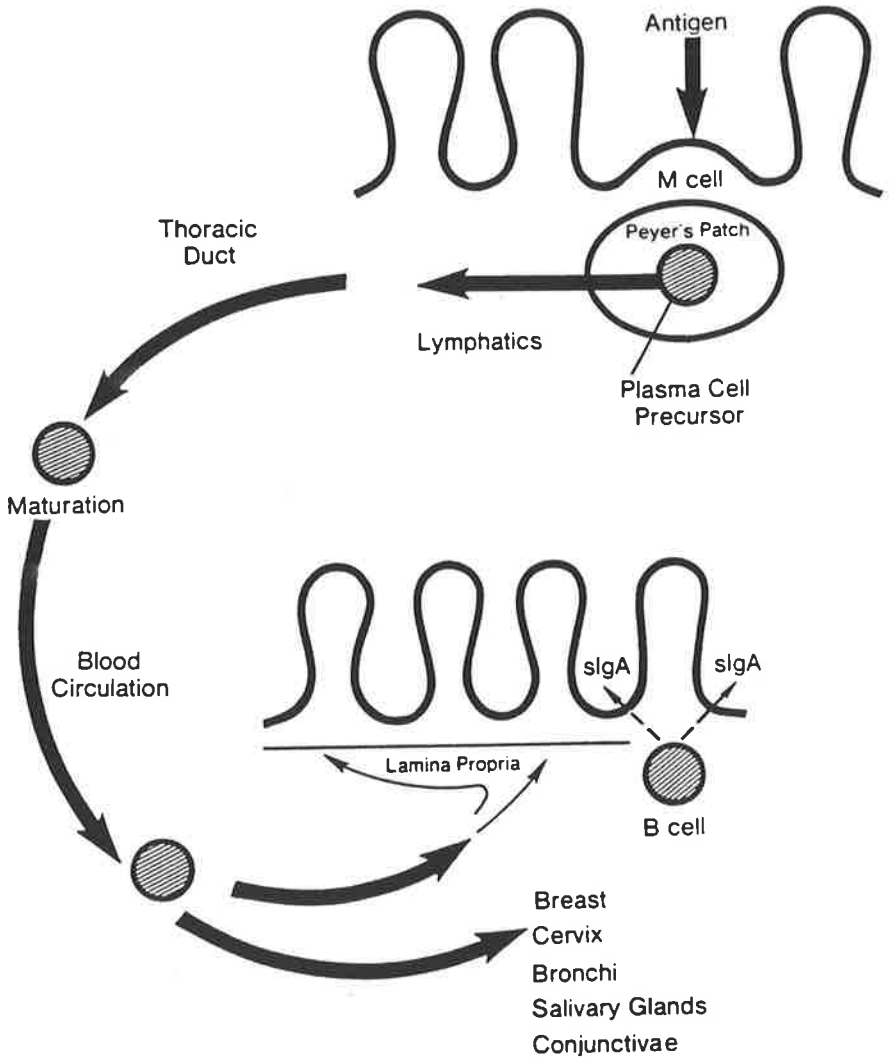
In humans the major transport process involves the diffusion of the IgA molecule from the site of synthesis to the columnar epithelial cells, where it complexes to a 70 000 M_r polypeptide called Secretory Component (SC) which is synthesized only by these cells. Immunohistochemistry has failed to identify the presence of SC within the plasma cells synthesizing IgA heavy chain. Part of the activity was localized to the basolateral membrane which faced the plasma cells, implying that SC-IgA coupling was an early event in the transportation process. The secretory form of IgA ($(IgA)_2$-SC) is subsequently transported in vesicles across the epithelial cells and released into the intestinal epithelium by exocytosis.[90,93]

1.7 APPLICATION OF THE MUCOSAL IgA RESPONSE

It was the evolution of the concept of a common mucosal immune system through our understanding of migratory IgA plasma cell precursors and the production of a mucosal immune response, that permitted an explanation for the detection of secretory antibody against orally administered antigens at mucosae other than that of the gastrointestinal tract, such as in colostrum, tears and saliva.[94-96] Through this concept, the possibility of immunizing more than one mucosal surface, perhaps using a polyvalent oral vaccine may become an eventuality.

FIGURE 1.1

MATURATION CYCLE OF PEYER'S PATCH PRECURSOR PLASMA CELLS FOLLOWING LOCAL MUCOSAL ANTIGENIC STIMULATION



2.1

TYPHOID FEVER: AN INTRODUCTION

2.1.1 *Definition*

Typhoid fever is an acute systemic disease resulting from infection with the bacterium, *Salmonella typhi.* It is a disease that is unique to humans with no other animal naturally acquiring the illness after the oral ingestion of the organisms.[97]

2.1.2 *Aetiological Agent*

The causative organism of typhoid fever, *Salmonella typhi*, is a non-gas producing member of the Kauffmann and White *Salmonella* serological group D.[98] *S. typhi* is motile and non-capsulate, and on culture resembles other enterobacteria except that following incubation on MacConkey or DCA medium it forms non-lactose-fermenting colonies.[98] Antigenically, it elaborates the 9 and 12 O-polysaccharide (O-antigen) components of lipopolysaccharide (LPS), with most strains also producing the Vi antigen, a polygalacturonic acid polymer.[99] It does not demonstrate the flagellar phase variation of other *Salmonellae*, producing flagellar (H-d) antigen only in phase I. Bacteriophage typing of the Vi-positive strains enables epidemiological surveillance of outbreaks of disease and assist with source identification.[100]

2.1.3 *Epidemiology*

The incidence of typhoid fever has declined throughout the developed world since 1900, so that now the annual incidence in western Europe, Japan and the United States of America (USA) has reportedly

declined to be about 0.24-3.7 cases per 100 000 population, and to about 4.3-14.5 cases per 100 000 in southern Europe.[101] In the USA the annual incidence of typhoid fever has remained steady at 0.2 cases per 100 000 since 1966.[101,102]

Unfortunately accurate surveillance data is not available from developing countries, however it has been crudely estimated that there are approximately 12.5 million new cases of typhoid fever in the world (excluding China) each year. This represents an annual incidence of 365 cases per 100 000 of the world's total population (excluding China), and 540 cases per 100 000 (0.5%) of the population of the developing countries. In some areas of Indonesia studies have reported incidences exceeding 1000/100 000 per year, and that typhoid fever was among the five common causes of death.[101,103] These figures indicate that typhoid fever remains a significant cause of morbidity in underdeveloped regions of the world.

Typhoid fever remains essentially a young person's disease with the majority of reported cases occurring in persons under 20 years of age.[104] In Chile, where typhoid fever has quite marked seasonality, the peak incidence occurs in children between the ages of eight and 13 years [105] but is also found in 2.0% of children under the age of 2 years presenting with fever.[106] In Nigeria, it was found that 42% of all cases of typhoid fever occurred in children under 10 years of age, with 14.3% occurring in children under 2 years.[107] This disease pattern was first reported from British Guiana where it was found that the typhoid incidence in children aged between three and 10 years living on sugar estates was 65/1249 (5204/100000 or approximately 5%); not a single case was diagnosed in 1695 adults aged 20 to 50 years living on the same estates and so apparently exposed to the same degree of risk of infection.[108]

Most cases of typhoid fever in developed countries appear to be acquired overseas and subsequently imported. In the USA the proportion of cases that are imported appears to be increasing annually, in 1984 62% of cases being imported.[102]

Mortality from typhoid fever in the USA has remained quite low with a case-fatality rate of 1.3%. This rate has been <0.5% in adult cases under 40 years of age, progressing to 29% in persons 80 years or older. In developing countries assessment of any changes that may be occurring in the clinical severity of typhoid fever are unreliable. In general terms it appears that there was a reduction in case-fatality rates following the introduction of chloramphenicol therapy to 1%-12% from 10%-32% in the pre-antibiotic era.[109,110] The case-fatality rates of some developing countries have been reported to continue to be between 9%-32% implying that the situation has not significantly improved.[111,112]

It has been described by Gangarosa[113] that developing countries reporting increased typhoid severity share these characteristics:

(1) rapidly increasing populations,

(2) rapidly increasing urbanization,

(3) inadequate processing of human wastes,

(4) decreasing water supply per capita,

(5) intimate contact between humans, food, and heavily contaminated water supplies,

(6) overburdened health care delivery systems.

Most of these points have been supported by an epidemiological survey of typhoid fever in Santiago, Chile.[114]

Chronic carriers also represent a means of transmission of typhoid fever. It has been reported that approximately 2-4% of treated patients become chronic carriers. Usually carriers are faecal excreters of the organism for long periods of time, however urinary excreters are occasionally identified, most commonly in the presence of *Schistosoma haematobium* infection. *Clonorchis sinensis* and other schistosomal infections, as well as chronic chololithiasis, also predispose to the carrier state.[115]

In Santiago, Chile, which has the combination of one of the highest prevalences of chololithiasis in the world and endemic typhoid fever, it has been determined that the crude chronic *S. typhi* carrier rate was 694 carriers per 100 000 population.[116] Since chronic carriers can excrete up to 10^{11} live organisms per gramme of faeces[97] (typically 10^6-10^9 organisms[104]), it is easy to see how they make a substantial contribution to the transmission of the disease.

2.1.4 *Infecting Dose*

Infective Dose for Symptomatic Infection

The infective dose of *S. typhi* Vi^+ (Quailes strain) that resulted in the development of symptomatic typhoid fever in previously unexposed North American adult volunteers is detailed in Table 2.1.[97] The challenge organisms were prepared and administered while the organisms were in logarithmic-growth phase, suspended in 30 ml of milk and administered by gargling and swallowing. No special precautions were taken to protect the organisms against the potentially sterilizing effects of gastric acid. From this table it can be determined that the dose required to infect 50% of adult

human subjects (ID_{50}) without neutralizing gastric acid was 10^7 viable organisms.

TABLE 2.1

INFECTIVE DOSE FOR CLINICAL INFECTION WITH *Salmonella typhi* IN HUMANS

Challenge Dose*	Clinical Illness Rate	Incubation Period+
Vi+ Strain		
10^9	95%	5 (3-32)
10^8	89%	
10^7	50%	7.5 (4-56)
10^5	32%	9 (6-33)
10^3	0%	
Vi- Strain		
10^7	26%	

* - Number of viable organisms administered in milk without pretreatment of the subject with a sodium hydrogen carbonate solution.
+ - Incubation period in days following ingestion of challenge dose, with range observed.

(adapted from Hornick RB *et al* 1970; reference 97)

The contributory effect of the presence of the Vi antigen on challenge strains of *S. typhi* is also evident from Table 2.1. It was demonstrated that the disease rates were significantly higher in individuals who ingested Vi antigen-containing strains, $p<0.05$. It also confirms that Vi$^-$ strains are capable of inducing clinical infection and symptomatic disease.

Asymptomatic Infection

It has been shown that of 64 human subjects who ingested at least 10^8 viable *S. typhi* (Quaile's strain), seven failed to develop clinical typhoid fever.[117] In two of these totally asymptomatic persons, receiving $1x10^8$ and $1x10^9$ live organisms respectively, it was possible to isolate the infecting strain from their blood and faeces.

Asymptomatic typhoid infection potentially provides major public health problems with respect to the effective control and epidemiology of the disease.

2.1.5 *Clinical Description*

Depending on the size of the infecting dose, the incubation period of typhoid fever varies from 3 to 60 days but averages about 10-14 days.[97,118] The length of the incubation period appears to be inversely proportional to the size of the inoculum.[97]

However, in volunteer studies conducted in the USA, where prisoner-volunteers aged 18 to 50 years ingested 10^5 viable organisms of *S. typhi* Quailes strain, and where the incubation period of the infection could be accurately determined (being defined as the first day the subject's temperature reached or exceeded 37.8°C), the frequency-distribution of the number of cases against the incubation period was quite obviously trimodal. The overall attack rate was 35%, with most subject's having an incubation period of 7 days. The other peaks were at 21 and 28 days. It has been suggested that an infecting dose of a specific size and virulence is able to produce an attack rate with two or three modes in the case-onset pattern because groups within the exposed population may differ genetically in their susceptibility to infection.[119]

Following the onset of clinical infection in a typical, untreated patient, the illness usually lasts about 4 weeks. Typhoid fever normally has an insidious onset, but follows an irregular course characterized by complaints of a severe headache, malaise, fever, lethargy, anorexia, and abdominal pain and tenderness.[104] In adults constipation occurs more commonly than diarrhoea.[118] Diarrhoea occurs in half of infected children, constipation occurring much less frequently.[104]

During the first week of illness the patient may experience fever, severe headache, constipation and lethargy. In the ensuing week, the fever becomes unremitting, with fatigue, anorexia, cough and diarrhoea increasing in severity. The patient is often severely obtunded, mental depression, delirium and stupor have all been reported to occur at this stage. Abdominal tenderness is usually present, and in children the spleen frequently is enlarged.[104,107] A macular or maculopapular rash (rose spots) is evident on the lower chest and abdomen in up to 80% of patients, each crop of lesions lasting 2-3 days. In adults there is commonly the paradoxical relationship of fever and a low pulse rate.[118] This observation is much less common in children.[104]

The total duration in patients receiving only supportive care is thirty to forty days, with resolution and full recovery without complications usually occurring.[120] However, the patient may progress to develop an illness with complications, rather than to recovery.

2.1.6 *Complications*

It is probably quite difficult to define an uncomplicated attack of typhoid fever. In one study of clinical typhoid fever, one or more complications occurred in 103 of 530 patients receiving 585 courses of

treatment.[121] The commoner complications were: "relapse" which occurred in 5%-15% of untreated cases (10.8% Rowland,[121] 8.8% McCrae [122]) and to up to 18.3% of chloramphenicol treated patients;[120] intestinal haemorrhage occurred in 2.9%,[121] 7%,[122] 21%,[123] and 13.8%;[120] intestinal perforation in 1.5% (in all but one case the lesion was solitary and all were located within 30 cm of the end of the ileum,[121] 1.9%,[123] 3.1%,[122] and 5.1%;[120] myocarditis in 1.7% which was associated with a 78% case-fatality rate;[121] meningitis in 0.75%; endocarditis and arthritis 0.19%;[121] and jaundice in 1.1%.[121]

Perforation of the ileum in children has been reported to be a rare occurrence, with no cases being reported at all in one study but with a sharply increasing incidence being noted with increasing age.[107]

2.1.7 *Pathology*

Orally ingested *S. typhi* has been demonstrated in human volunteers to induce a diffuse enteritis of the upper small intestine even in the absence of any lymphoid follicles during the incubation period. This enteritis clearly affected the epithelial lining of the villi, crypt glands, and the tunica propria and was observed during the incubation period as well as during the fully developed disease. The enteritis was shown to be completely reversible in that there was no evidence of any mucosal lesions in convalescent subjects.[80]

Typically, after the incubation period the ingested organisms, which have already invaded and colonized the Peyer's patches of the small intestine, enter the bloodstream, probably via the lymphatics. The resulting bacteraemia is the cause of the progressive fever. During the first week of the fever it is possible to isolate the organisms from blood cultures, but as the disease progresses the developing immunity results in the clearance of the organisms from the blood. From this stage onwards the organisms persist in

the liver and biliary tract, re-entering the small intestine in large numbers from the bile. It is during this stage they can be often be isolated from faecal specimens.

Reinfection of the small intestinal lymphoid tissue by organisms in the bile leads to the development of the marked Peyer's patches lesions of the distal ileum, which after initially showing inflammation, undergo necrosis and ulceration from about the tenth day of the illness. It is from these ulcers that the severe haemorrhage or perforation of the small intestine that can be associated with typhoid fever occurs.[124]

2.2

IMMUNITY TO TYPHOID FEVER

2.2.1 *Major Antigenic Determinants of Salmonellae*

1. Lipopolysaccharide (endotoxin)

The lipopolysaccharide (LPS), also referred to as endotoxin, has two main components: an hydrophobic lipid component (Lipid A); and an hydrophilic polysaccharide region.

(a) Lipid A

The Lipid A comprises a glucosaminyl-ß(1-6)-glucosamine backbone which has been substituted with six or seven saturated fatty acid residues, and forms part of the bacterial cell outer membrane. The Lipid A is well conserved between the Enterobacteria, displaying only intergenus microheterogeneity (essentially only minor backbone substitutions) and as a result there is extensive intergenus antigenic cross-reactivity of Lipid A.

(b) Polysaccharide Core

The oligosaccharide core of *Salmonella* is actually a pentasaccharide. The composition and structure of the core is virtually identical within the *Salmonella* species, though differing significantly in structure from other Enterobacteria. Despite there being specific differences between the *Salmonella* pentasaccharide core and that of other Enterobacteria, a degree of antigenic cross-reactivity is apparent with some strains of *Escherichia coli*, for example *E. coli* O111, and *E. coli* O8. Despite the *Shigella* oligosaccharide having an identical sugar composition, the structure is sufficiently different for there to be virtually no antigenic cross-reactivity.

It appears that the terminal N-acetylglucosamine-α(1-2)-D-glucose sugar residue of the oligosaccharide core confers the *Salmonella* specificity. In *Salmonella* strains with a full complement of O polysaccharide (O antigen) sidechains ("smooth" or "S" strains), the immunodominant structures of the core are incompletely expressed.[125]

(c) Polysaccharide O Antigen

The polysaccharide O antigen of the *Salmonella* species, is a heat-stable somatic antigen which comprises four saccharide moieties in a single unit which is repeated between eight and 30 times; 22 times in *S. typhi*. The O antigen may be an homopolysaccharide or an heteropolysaccharide.[125]

The O antigen is covalently linked to the core oligosaccharide via the glucose II residue within the core through a 1,4 linkage. This in turn is linked to through a 1,2 linkage to the galactose I residue.[126]

Some of the O antigens can be induced by bacteriophage, for example 1, 14 and 27, so these factors are not a consistent finding in any particular serotype. It is the O antigens that form the basis for the

Kauffmann-White Serological Classification of the *Salmonellae* and are responsible for the pathogenicity of the organisms.[98]

Antibodies raised against the O antigen are able to confer protection on an animal against challenge with an organism expressing the homologous O antigen type.[127]

2. H antigens

The flagellar or "H" antigen comprises a peritrichous flagella, $1x10^{-5}$ mm wide, and is composed of flagellin (a member of the keratin-myosin-epiderm-fibrinogen group) of M_r 40 000.

A wide range of flagellar types have been identified and these have all been allocated to specific antigenic groups. The amino acid composition of the flagellae of any one antigenic group is constant. Many *Salmonella* isolates express flagellae of more than one antigenic type.

The flagellae of *Salmonella* are known to undergo "Phase Variation". This phase variation was originally described by Bruce White as "a saltative oscillation of the labile (H) antigen complex between two serologically contrasting states".[128] There are two phases described: the specific phase, where the strain can be agglutinated only by homologous antiserum; and the non-specific phase where the strain can be agglutinated by homologous and heterologous antisera. In any one culture of a particular *Salmonella* strain, it is possible to find organisms with specific phase flagella production only, as well as finding some producing flagella in the non-specific phase. However, some strains are monophasic, such as *S. typhi* (H-d), producing only a single flagellar phase, while others, such as *S. typhimurium* (H-i:1,2) are diphasic.

3. Capsular Vi antigen

Some strains of *Salmonella* produce a capsular polysaccharide antigen in addition to the LPS, an antigen that is equivalent to the K antigens of

E. coli. This polysaccharide antigen, known as the Vi antigen, is an N-acetyl-D-galacturonic acid polymer with a M_r of $2x10^5$-$2x10^6$. In addition, it has been recently reported that the native molecule also contains O-acetyl groups.[129]

The Vi antigen can be found to be produced by some strains of *S. typhi*, *S. dublin*, *S. paratyphi* C as well as by *Citrobacter freundii*. The Vi antigen of *S. paratyphi* C is not identical to that of the others, as it contains proteinaceous material.

The virulence of certain *Salmonella* strains was originally attributed to the presence or absence of Vi antigen, its absence believed to attenuate virulent organisms. This certainly appeared to be the case with the human virulence of *S. typhi*, as it has been shown that Vi^+ strains have a significantly lower ID_{50} than Vi^- strains (Table 2.1).[97]

In its purified form this antigen has been shown to be strongly immunogenic in humans.[130]

4. Pili or Fimbrial antigens

Fimbriae are filamentous protein appendages, 0.01 x 0.3-1.0 μm, approximately 100-250/cell, and they are only visible using electron microscopy. They are found on most Enterobacteria members, and in the *Salmonella* species, seven putative types have been described. They have also been found to be subject to phase variation, the bacterium oscillating between a fimbriate and non-fimbriate form depending upon the culture conditions.[98]

The most numerous type, the Type 1 fimbriae, have been demonstrated to allow the bacterial cell to bind to the mucous membranes of the mammalian intestinal wall.[98]

2.2.2 *Protective Immunity*

Reinfection with typhoid fever is said to be a rare occurrence. A study in a British Air Force camp in Egypt did provide evidence that reinfection with resulting disease can occur, but the subsequent attack rate of 20% indicated that natural disease confers only moderate immunity.[131] In another study, where twenty-two previously challenged American volunteers were re-challenged two to twelve months later with a dose of 105 same Vi-phage type organisms, the attack rate observed was 23% compared with 30% among the 34 controls ($p>0.05$). This group of volunteers, who were susceptible prior to the first challenge, appeared to acquire partial immunity following natural disease, however no correlation between resistance to reinfection and anti-O, -H, or -Vi antigen antibody levels was demonstrated.[132] It is evident that immunity is not always conferred by an attack of typhoid fever.

One explanation for these high reinfection rates was that the immunity to typhoid fever may be Vi-phage type specific.[131] However, there is no definite evidence for that apart from Marmion's own observations, and a recent case report.[133] It should also be kept in mind that in DuPont's volunteer studies the re-challenge occurred using the same Vi-phage type organisms.[132]

2.3

IMMUNITY TO NATURALLY ACQUIRED INFECTION

2.3.1 *Humoral Immunity*

The development of specific antibodies in human typhoid fever is well established.[134]

The lipopolysaccharide (LPS) antigens, especially the O polysaccharide side-chains, of *Salmonella* can be regarded as the major antigenic determinants of the organisms.[135] Early investigations into the humoral immune response to *S. typhi* O-antigens, utilized the more primitive assays of bacterial agglutination or indirect agglutination using antigen coated red blood cells for antigen-specific antibody determination. With these primitive immunological tools, initial reports indicated that there was an anti-O antigen antibody response following clinical typhoid fever, and it was believed that this was in the IgM immunoglobulin class.[136,137] More recent studies have demonstrated that the intestinal immune response to the *S. typhi* O-antigen following an acute episode of typhoid fever mainly occurred in the IgA and IgM immunoglobulin classes,[138-140] but in chronic carriers it was only of the IgA class with a near complete absence of *S. typhi* O-antigen-specific IgM antibodies.[138,141] In serum, the anti-*S. typhi* O antigen response is measurable in the IgA, IgG and IgM immunoglobulin classes,[15,142] however it has been shown that there is no correlation between the serum antibody response and that measured in the fluid obtained from the jejunum.[15] In one report, significant salivary anti-*S. typhi* O antigen IgA antibody responses were measurable in the saliva of some convalescent patients.[139]

The human antibody response to the O antigens of *S. typhi* are not necessarily protective.[143] It has been reported that convalescent human volunteers who have substantial anti-*S. typhi* O-antigen antibodies can still be susceptible to reinfection with the subsequent development of clinical disease.[132] Additionally, volunteers who were made tolerant to *S. typhi* lipopolysaccharide by its repeated intravenous administration and as a result had developed high anti-O antigen antibody titres, developed unameliorated clinical disease following oral infection with *S. typhi*.[144] Relapses are also known to occur in convalescent patients in the presence of high anti-O antigen antibody responses.[145,146]

Recently there have been a few studies investigating the human humoral response to various *S. typhi* protein antigen preparations in clinical typhoid fever. Using Barber's protein antigen preparation (BP) (veronal extraction and trichloroacetic acid precipitation of protein from Vi-negative *S. typhi* O901), one group reported that the intestinal IgA and IgM anti-BP antibody response was significantly elevated above that of normal controls at one to three months after clinical illness.[147]

In a more detailed study lasting over two years, it has been shown that a wide range and magnitude of immune responses were found in patients with typhoid fever, these responses varying considerably in duration. It was reported that after the onset of clinical illness, the IgG, IgM and anti-*S. typhi* O antigen and anti-H (flagellar) antigen agglutinins of serum persisted for 2 years, 16 weeks, 16 weeks, and 36 weeks respectively. However, the specific anti-BP antigen secretory IgA response of the intestine was still measurable at 48 weeks.[140]

This confirmed earlier reports that in typhoid fever, the anti-protein antibody responses in serum and intestinal fluid can be elevated to at least the same extent as anti-O antigen antibody responses.[15,138]

2.3.2 *Cell Mediated Immunity*

The investigation of the development of cellular immune response following infection with *S. typhi* are even more poorly documented than the humoral response. One of the major problems encountered in assessing the published data in this area, is the widely disparate antigen preparations used in attempts to measure the cell mediated immune (CMI) response.

In one study performed in Sri Lanka, where the CMI response to an antigen preparation comprising the supernatant from a centrifuged culture of *S. typhi* that had been subjected to repeated freeze-thawing was measured using the lymphocyte migration inhibition assay (LMIA), it was shown that typhoid patients and sewerage workers both demonstrated significantly higher CMI responses than normal "unexposed" controls.[148] This has been reported earlier by others using an ultrasonic lysate of *S. typhi*.[142] Thevanesam and associates[148] were also able to demonstrate significantly higher serum antibody responses directed against all somatic antigens in the patient/sewerage worker groups than in controls. They were also unable to demonstrate a difference in the CMI and humoral responses between adult and child patients, nor were they able to find any correlation between the development of a CMI response and the occurrence of complications. However it has been reported by others that patients with a detectable CMI response as determined using the LMIA had a significantly lower rate of complications and relapses,[149,150] a feature frequently reported by others.[146] In addition, they confirmed the finding of others that antibiotic therapy did

not impair the development of the CMI and humoral immune responses.[151,152]

Other investigators have attempted to define the kinetics of the development of the CMI response in patients with typhoid fever.[152] The LMIA was used to detect a CMI response using an ultrasonic lysate of *S. typhi* as the antigen preparation. In this study it was further demonstrated that more adult typhoid patients who presented in their first week of clinical illness had a detectable CMI response than control patients (67% vs 39%). In their patients, the number of CMI responders increased during the illness, from 23.5% in the first week, to 77.4% in the second week and 81.2% in the third or later weeks. This pattern of response was later confirmed in other studies.[139] Chloramphenicol did not appear to impair the development of a positive CMI response. More importantly, it was demonstrated that typhoid fever complications occurred significantly more frequently in patients who failed to demonstrate a detectable CMI response. It was therefore concluded that uncomplicated clinical recovery from typhoid fever was dependent upon the development of a CMI response, and that this was independent of antibodies directed against somatic antigens or antibiotic therapy.[139,152] It has been shown that this CMI response may still be measurable up to one year after recovery using a similar LMIA.[139]

More recent studies have shown that the lymphocyte replication assay using whole cell particulate antigen is a more sensitive measure of prior contact with *S. typhi* than conventional serologic or cellular assays.[153] However, it has been acknowledged that a major problem in using any measure of CMI in endemic regions is the difficulty in locating a negative control population, that is, a group of individuals without any previous environmental exposure to the organism, since it has been found that most

residents in an endemic area have evidence of exposure to *S. typhi* without any history of clinical disease,[153] and as a result specific statistical methods have had to be utilized to determine a meaningful difference.[154]

In summary, infection with *S. typhi* induces a serum and local intestinal immune response that while mainly directed against the immunodominant O polysaccharides of the LPS, also has a component directed against protein antigens. However, these antibody responses appear to arise independently of the CMI response and have not been directly correlated with recovery from disease, nor protection against subsequent exposure to an infecting dose of the organism. CMI appears to play a role in the recovery from infection, however its role in protection against disease also remains unclear.

For the rational development of a suitable vaccine against an enteric pathogen, the nature of the immune response that is stimulated from natural infection with the organism, whether that immune response is directed against specific antigens and the manner in which it relates to protective immunity need to be fully investigated and clarified. In typhoid fever, this has not been adequately investigated. As a result the development and evaluation of new candidate vaccines has far outpaced the attainment of knowledge regarding the fundamental aspects of protective immunity.

2.4

PARENTERAL TYPHOID VACCINATION

2.4.1 An Historical Perspective

In 1895, Waldemar Haffkine, the man who introduced mass parenteral cholera vaccination to the world, suggested that the approach used by him for cholera may also be applied to typhoid fever. In 1896, Wright first reported the effective use of a parenterally-administered heat-inactivated typhoid vaccine in humans.[155] Two months later Pfeiffer and Kolle reported their experiences using an identically prepared killed typhoid vaccine.[156] There is some dispute as to which group should be awarded priority for the discovery, and the reader is referred to a paper by Groschel and Hornick for a factual consideration.[157]

Wright's reported experience with his typhoid vaccine which was administered into the flank of the subject,[1] showed that it was immunogenic (using bacterial agglutination to determine a specific immune response) and safe. Immunogenicity of the vaccine was determined by comparing its agglutinating ability with that of sera obtained from recently convalescent typhoid patients, on the assumption that these patients had a substantial degree of protection conferred upon them by the disease. Since the agglutinating ability of the sera from the vaccinated subjects was similar to that of the patients, it was inferred that the vaccine would provide protection against typhoid fever.

The vaccine was associated with a considerable number of adverse reactions, which he divided into local and constitutional symptoms. Local symptoms were common and found to be associated with the size of the dose used. These mainly comprised local tenderness and erythema, but were also associated with a marked, albeit transient, lymphadenitis. The commoner

constitutional symptom reported was a high fever seen in all cases. Others included faintness, nausea, vomiting, and anorexia.

In this study, one subject volunteered to be challenged orally with a single inoculum of live *S. typhi* following his parenteral vaccination. This subject failed to develop clinical illness, and it was concluded that the vaccine afforded some protection against infection with typhoid. Although uncontrolled, this study was the first to investigate the protective efficacy of a typhoid vaccine against an oral challenge with virulent organisms.

In 1898-9 Wright,[158] as a member of the Plague Commission, travelled to India, where he proceeded to vaccinate 4000 young soldiers recently arrived from Britain against typhoid fever. It appears that only one dose was administered, and the results based on reports from regimental medical officers varied from good to inconclusive. It was necessary to prepare batches of the vaccine during his visit to India, and Wright was particularly concerned with the sterility of the resulting preparations. So much so, in fact, that at each break in the journey, he re-sterilized his entire stocks by repeatedly heating the batches to 60°C before they were administered. It is doubtful whether such a preparation retained any significant immunogenicity.

This uncontrolled field trial represented the first using a typhoid vaccine.

Wright collaborated with Leishman to perform another field trial in British soldiers in South Africa during the Boer War in 1899. In this study 100 000 soldiers were vaccinated.[159] It was hoped that this study would definitively demonstrate the protective efficacy of this new typhoid vaccine in the field, especially considering the very high incidence of typhoid fever and its associated mortality rate. Unfortunately this study failed to provide

this proof due to a host of factors, such as poor documentation, noncompletion of the vaccine schedule, doubtful diagnoses and inadequate discrimination between typhoid and paratyphoid fevers.

After Wright, Pfeiffer and Kolle almost simultaneously reported the protective efficacy of parenterally administered killed typhoid organisms in humans, this form of vaccination became a widespread practice.

In 1912 Leishman described what he believed was the optimal typhoid vaccine and detailed the laboratory preparation of it,[160] however, despite this attempt to standardize the preparation and formulation of typhoid vaccine, none of the early field trials were subject to any adequate control procedures, hence the wide variation in the reported efficacies.[161]

In 1946, one epidemiological study strongly suggested that parenteral typhoid vaccination was efficacious in providing protection against disease.[162] In this study approximately 360 young women (including 181 military personnel) who were resident in a hotel for business women in Cleveland, Ohio, USA, were involved in a localized typhoid fever epidemic. Most of the civilian women had not been vaccinated against typhoid fever, in contrast to the military personnel. The vehicle of infection was presumed to be contaminated orange juice, however this was not definitively established. There were 17 cases of clinical typhoid fever in the 140 non-immunized women, and one case in the 211 immunized women (Chi-squared=21.2 d.f.=1, p=0.0000036, my calculations). Therefore it was inferred from these figures that parenteral vaccination against typhoid fever was able to confer substantial protection upon the recipient.

However until 1954, there had not been any properly controlled field trials to actually determine whether this form of vaccination really did provide any significant protection against disease, and if it did, for what

period of time. Major field trials, some sponsored by the World Health Organization (WHO), using various preparations of killed typhoid vaccines for parenteral administration were conducted between 1954 and 1967 in several countries in an attempt to provide answers for these questions. These field studies were prompted by a growing dissatisfaction with the then currently available typhoid vaccines, supported by the increasing number of reports of cases of clinical typhoid in persons receiving the killed typhoid vaccine.[131,163-166]

In addition, these field studies provided the first real opportunity to correlate the antigenicity of a variety of typhoid vaccine preparations as measured by the demonstration of specific antibodies in humans, with their protective efficacy against infection in the field.

2.4.2 Field Evaluation of Parenterally Administered Killed Typhoid Vaccines

Yugoslavia 1954/1960

The Yugoslav field trial area comprised the town and district of Osijek, which was known to have a persistently high incidence of typhoid fever.[167] The township and surrounding villages had a total population of 108 352 inhabitants in 1954. From 1946 to 1954 the annual incidence of typhoid fever varied from 151.9 to 365 cases per 100 000 population, averaging 305/100 000. The typhoid incidence was much higher in the town compared to the villages and this was attributed to the poorer standard of water supply. There was very little seasonal variation in incidence. In the five year period prior to the study, 70% of cases were in persons under 25 years of age.

A serological survey of nearly 200 randomly selected volunteers aged from 5 to 50 years, indicated previous exposure to typhoid, with 42% having H antibodies, 26.3% with O antibodies, and 12.5% with Vi antibodies.

In 1954, the study only used volunteers aged 5 to 50 years, and in 1955 from 2 to 50 years, as a result of the recognition that the incidence of typhoid in children was much greater than previously determined, due to the milder nature of their symptoms.

Two preparations of killed typhoid vaccines were prepared for evaluation in this study. The organism used was *S. typhi* Ty2 that was either treated with alcohol according to the method of Felix,[168] or inactivated by heating at 56°C for one hour after which 0.5% phenol was added as a preservative. Each typhoid batch contained $3x10^9$/ml total organisms. Adult males received two doses of $2.25x10^9$ organisms three to five weeks apart, females received $1.8x10^9$ organisms and children between 2 and 12 years received $4.5x10^8$ organisms using the same schedule. An inactivated control vaccine was prepared from *Shigella flexneri* II and contained $1.5x10^9$/ml.

A total of 35 508 people, randomly divided into three groups, participated in the field study. This represented 33% of the total population of the area, or 50% of the 5-50 year age group.

A diagnosis of proven typhoid fever was only considered if confirmed by a positive blood culture. This stringent definition was used due to the high anti-typhoid serological positivity found in the population.

The population receiving the heat-killed-phenolized vaccine were significantly protected against typhoid fever ($p<0.001$), and that this protection was observed to last for at least three years. This indicated a protective efficacy of 70%, with much more marked protection being evident in the 5-14 year age group. No better protection was provided by the booster

dose given 12 months later. This preparation also provided significantly better protection than the alcohol-inactivated vaccine preparation ($p<0.001$). Interestingly the alcohol-inactivated preparation was also shown to stimulate better anti-Vi antibodies, whereas the heat-killed-phenolized preparation stimulated a significantly better elevation in anti-H antibodies in serum. The serum anti-O antibody response was significantly better with the heat-killed-phenolized preparation, but this difference was not evident after 5 months.

Yugoslavia 1960/1962

The initial field studies performed by the Yugoslav Typhoid Commission in the Osijek district and described above, conclusively demonstrated the effectiveness of freshly prepared liquid phase heat-phenolized-killed typhoid vaccines in the protection against disease in the field. However the laboratory potency tests that were carried out on the prepared batches were inconclusive, and as a result could not be correlated with the observations in the field. Therefore the WHO sponsored further controlled field trials using lyophilized heat-phenol-inactivated typhoid vaccine and lyophilized acetone-dried and -inactivated typhoid vaccine that had proven effective in some animal tests.[169]

The areas chosen for the field trials were two old Yugoslav towns, Pristina (population in 1961 of 38 891) and Bitola (population in 1960 of 46 947).[170] It had been determined that the annual incidence of typhoid fever in both towns had been quite high in the previous fifteen years. The average incidence in Bitola had been about 200/100 000, and in Pristina more than 300/100 000, in both towns it was highest in the 5-25 year age group. Once again previous and/or continuing exposure to typhoid fever was indicated

by the high levels of O, H and Vi antibodies identified in a serological survey of a sample the trial population.

In this study, as for the following ones to be described in British Guiana, Poland and the USSR, the vaccines were prepared by the Walter Reed Army Institute of Research, Washington DC, USA. The acetone-inactivated typhoid vaccine was labelled "K", the heat-phenol-inactivated typhoid vaccine was labelled "L" and the tetanus toxoid control vaccine was labelled "M". Adult doses (over 14 years of age) comprised two doses four weeks apart of $1x10^9$ organisms and for children under 14 years the same schedule was used however the doses were only $0.5x10^9$.

In Bitola, 32 492 persons received the first dose with 94% of these attending for the second dose and so completing the vaccination programme. This represented 65% of the town's population. In Pristina, 16 087 persons had the first dose, with 94% returning for the second dose, representing 39% of the town's population.

The vaccinated populations were divided into three groups of approximately equal numbers. Allocation to each vaccine group K, L or M was according to a strict randomization protocol.

Following vaccination all three vaccine groups were followed up for 30 months. A diagnosis of typhoid fever was only recorded following the isolation of *S. typhi* by blood culture.

Two years after the vaccination, there was a severe epidemic outbreak of typhoid fever in Pristina. In the unvaccinated population the incidence was 260/100 000.

Refer to Table 2.2 for protective efficacy of vaccines in Pristina.

TABLE 2.2

RESULTS OF PARENTERAL KILLED TYPHOID VACCINE FIELD TRIALS, PRISTINA, YUGOSLAVIA 1964

Vaccine Preparation	Typhoid Incidence*	Protective Efficacy
K	320	79%
L	730	51%
Control	1490	0%

K - Acetone-inactivated vaccine
L - Heat-phenol-inactivated vaccine
C - Unvaccinated population
* - Incidence rates expressed as number of confirmed cases/100 000 population

(Adapted from Yugoslav Typhoid Commission 1964; reference 170)

Overall, in both Bitola and Pristina, it was found that both the acetone-inactivated and heat-phenol-inactivated typhoid vaccines provided significant ($p<0.01$) protection. The acetone-inactivated preparation however was demonstrated to provide significantly better protection ($p<0.05$) than the heat-phenol-inactivated typhoid vaccine. Both vaccines were shown to be effective for at least two years, especially considering the heavy exposure to *S. typhi* in the water supply experienced by the vaccinated population in Pristina.

The differences in protection afforded by either the acetone-inactivated or the heat-phenol-inactivated typhoid vaccines appeared to be due to the failure of the heat-phenol-inactivated typhoid vaccine to provide any significant protection in adults, whereas it did provide significant ($p<0.01$) protection in the under 15 years age group.

This field study confirmed the usefulness of parenteral typhoid immunization as a public health tool. It also indicated that the lyophilized

acetone-inactivated typhoid vaccine provided far better protection against typhoid in all age groups than the heat-phenol-inactivated typhoid vaccine.

British Guiana 1960/1967

The British Guiana field trial area comprised school children aged 6-15 years attending schools in the coastal area of Berbice and Demerara; the city of Georgetown was excluded.[171,172] At the time, typhoid fever was endemic with annual notification rate being about 100/100 000 population. In 1960, 72 000 school children 6-15 years of age were randomly allocated to three groups.

The acetone-inactivated and heat-phenol-inactivated typhoid vaccine doses used in this study were exactly the same as those prepared for the 1960 Yugoslavian field trials in Pristina and Bitola, as was the control tetanus toxoid vaccine doses. The vaccine doses were administered on a strictly randomized basis to the three student groups, the doses and dose schedule being the same as for the Pristina/Bitola trial.

The study population was followed up for seven years. As for the Yugoslav study, a diagnosis of typhoid fever was confirmed if a positive blood culture for *S. typhi* was obtained, but also if a typical clinical history was obtained together with the isolation of *S. typhi* from faeces.

Over the period of the follow-up, both typhoid vaccine types provided substantial protection against disease, however the acetone-inactivated typhoid vaccine over the first 3 years had a protective efficacy of 94.4% and over the entire seven years of 89.5% providing substantially better protection than the heat-phenol-inactivated preparation, which after 3 years had a protective efficacy of 76.4% and after seven years of 67.4%, the differences reported as being highly significant.[172]

Despite the lower attack rates of typhoid fever in the control group compared to those in the Yugoslavian study, the results that were obtained from that trial largely supported those of the Yugoslavian study.

Poland 1961/64

The Polish typhoid vaccine field trial area comprised five provinces - Kielce, Lodz, Poznan, Warsaw and Wroclaw.[173,174] The incidence of typhoid fever in this area between 1953 and 1960 was reported as varying from 2.0 to 3.8 cases per 100 000 population. The field trial area had been subject to mass typhoid immunization programmes using a modified Grasset's vaccine until 1958.

The vaccines that were used in the study are described below:

Vaccine N: formol-killed phenol-preserved *S. typhi* Ty2,

Vaccine P: acetone-killed and -dried *S. typhi* Ty2,

Vaccine S: *S. typhi* Ty2 lipopolysaccharide prepared by Westphal's method and adsorbed on aluminium hydroxide,

Vaccine T: *S. typhi* Ty2 lysate prepared by the Grasset-Slopek method,[175]

Vaccine K: the acetone-inactivated *S. typhi* Ty2 typhoid vaccine used in British Guiana and Yugoslavia, prepared and supplied by the Walter Reed Army Institute of Research.

Vaccine O: tetanus toxoid.

The populations aged 5-14 years and 15-60 years were divided into 4 groups. Of the child population, 370 920 attended for the first vaccine dose, 89.4% of these attended for the second dose and so completed the schedule.

Of the adult population, 472 718 attended for the first dose, 76% attended for the second dose.

Each of the four 5-14 year groups received one of either vaccines K, T, N or O. Each of the 15-60 year age groups received one of either vaccines N, O, P or S.

All cases of typhoid fever in the areas were recorded for two years. Diagnoses of typhoid fever based on clinical manifestations only were detailed separately from those made on blood, urine or faecal cultures.

Over three years surveillance, Vaccine N provided highly significant ($p<0.001$) protection against typhoid in all age groups, having a protective efficacy of 87% in the first year, declining to 66% by the end of the third year post-vaccination. In children its protective efficacy was not statistically different from the proven Vaccine K (acetone-inactivated).

In the 5-14 year age groups immunized twice with vaccines K, N or T, the incidence of typhoid fever was significantly lower than in the control group (1.8/100 000 vs. 18.5/100 000).

There was no statistical difference between the incidences of typhoid fever in the control group and those receiving the typhoid lipopolysaccharide, indicating that the Westphal LPS preparation alone failed to provide protection in the field. This preparation also failed to stimulate a measurable serum immune response to H, O or Vi antigens in human subjects, whereas the N and K vaccine preparations stimulated maximal anti-H antibodies. However these other preparations provided only marginal improvement in anti-O antibody responses compared to the Grasset preparation, which itself provided only limited protection against disease and this only in the first year after vaccination. In the second year the

protective efficacy of the Grasset preparation had declined to 35% and by the third year it was found to provide no protection.

This study confirmed that acetone-inactivated typhoid vaccine preparations provided the best protection against typhoid in the field for at least three years, and for the first time indicated that the anti-H antigen antibody response could be linked to protective efficacy.

In effect, it was subsequently concluded from studying the immunogenicity data from a number of studies and sources, that the anti-H antibody response was a good predictor of the protective efficacy of a typhoid vaccine against naturally acquired infection.[169,176-179]

USSR 1958/62

Between 1958 and 1962 a series of field trials using a variety of parenterally administrable typhoid vaccines were conducted in the USSR, and it was only the last commencing in 1962 which was conducted in association with the World Health Organization.[180]

For these studies, 182 491 subjects were recruited, each was to receive either one or two doses of either:

(a) polyvalent chemical vaccine (the LPS preparations of *S. typhi*, *S. paratyphi* A, *Sh. flexneri*, *Sh. sonnei* and *Clostridium tetani* adsorbed onto calcium phosphate); or

(b) bivalent typhoid/paratyphoid B chemical vaccine (similar to the polyvalent vaccine preparation); or

(c) bivalent alcohol-inactivated typhoid/paratyphoid B whole-cell vaccine ($1x10^9$ killed organisms/ml); or

(d) monovalent alcohol-inactivated typhoid vaccine ($1x10^9$ killed organisms/ml); or

(e) monovalent heat-phenol-inactivated typhoid vaccine ($1x10^9$ killed organisms/ml).

In total, 45 938 persons received one of the chemical vaccine formulations, 45 187 received the monovalent heat-phenol-inactivated whole-cell typhoid vaccine, 45 594 received the alcohol-inactivated whole-cell monovalent typhoid vaccine, and 45 772 received the tetanus toxoid control vaccine; all vaccines being administered subcutaneously.

There was no significant differences observed between the different vaccine preparations with respect to adverse reactions.

The polyvalent chemical vaccine was found to have an overall protective efficacy of 72% ($p=0.0002$) when compared to the tetanus toxoid control vaccine over a ten month observation period (incidence in vaccinated 19/100 000, control 68/100 000), however neither this preparation when administered at reduced doses or the bivalent typhoid/paratyphoid B vaccine was capable of inducing protective immunity in children aged 13-16 years (protective efficacy 33.0%, not significant) and 7-12 years (protective efficacy 11.0% not significant) respectively.

In a comparative study evaluating the protective efficacies of the monovalent heat- and acetone-inactivated preparations and a monovalent typhoid chemical (adsorbed LPS preparation) vaccine, it was demonstrated that all three preparations conferred significant protection upon the respective recipients over a ten month observation period, with the chemical vaccine having a protective efficacy of 59% (incidence 61/100 000, $p=0.00001$), the heat-inactivated vaccine 81% (incidence 29/100 000, $p<0.00001$), and the acetone-inactivated vaccine 66% (incidence 50/100 000, $p<0.00001$), versus the tetanus toxoid control population (incidence 149/100 000).

The results of these studies can be seen to be quite different from the trend observed in the aforementioned studies. Here, the Russian studies suggested that the heat-phenol-inactivated preparation of the typhoid vaccine conferred a significantly greater degree of protection than the acetone-inactivated preparation - a result in direct opposition to the extensive studies performed subsequently. Also it was shown that extracted LPS adsorbed to a carrier compound was also able to provide a significant degree of protection, a finding not supported by the Polish studies. I believe that the only practical explanation for these observed discrepancies would lie in the methods of inactivation and preparation, or in the population used. One additional point which was evident through this study, was that the vaccine preparations tended to provide a greater degree of protection upon adult subjects than in children, perhaps indicating a boosting effect of naturally acquired immunity by these various vaccine preparations.

Baltimore, USA 1965

Both types of the commonly used heat-phenol-inactivated and acetone-inactivated monovalent parenterally administered killed typhoid vaccines evaluated in all the aforementioned field studies where typhoid fever was endemic, were also evaluated for there protective efficacy in previously unexposed North American adult volunteers in experimental challenge studies performed at the University of Maryland, USA.[161] Some differences noted in the physical characteristics of the two vaccine preparations included that acetone-inactivated typhoid organisms (K vaccine) retained much of the Vi antigen whereas the heat-phenol-inactivated preparation had much of its Vi antigen destroyed. In addition, a

purified preparation of Vi antigen was also included in these studies for evaluation.

When the volunteers who had been immunized with differing preparations were challenged with an ID_{50} dose (10^7) of viable virulent *S. typhi* no evidence of protection was observed in comparison to with the unvaccinated controls. At an ID_{25} challenge dose (10^5), a significant degree of protection was conferred upon the subjects who received the killed whole cell vaccines; K vaccine protective efficacy of 65.4%, Chi-squared=4.56 p=0.033; L vaccine protective efficacy of 73.5%, Chi-squared=6.64 p=0.010 (my calculations). The purified Vi antigen vaccine preparation was found not to provide any significant protection at any challenge dose, at an ID_{25} challenge dose the protective efficacy was calculated to be 34.5%, Chi-squared=0.263 p=0.61 (my calculations).[97,161,181]

These studies demonstrated that at low challenge inocula, as would be expected in water-borne disease, the parenteral whole cell vaccines were able to provide a significant degree of protection in previously unexposed adult subjects. However, at larger challenge inocula, as would be expected in food-borne disease through a carrier, no protection was provided by any of the parenterally administered preparations.

Bangkok, Thailand 1986

Recently a study conducted in Thailand was able to show the impact of mass typhoid vaccination on incidence rates in an epidemic.[182] In 1977 all Thai schoolchildren aged from 7 to 12 years received a single dose of a locally produced heat-phenol-inactivated typhoid vaccine containing $2.5x10^8$ organisms, annually for six consecutive years. In total, more than 5 million children were vaccinated from 1977 to 1985.

This national program proved extremely effective. In a survey of four major Bangkok hospitals, the isolation rate of *S. typhi* from all blood cultures was reduced from 4.6% in 1976 to only 0.3% in 1985. The case ration of *S. typhi* to *S. paratyphi* A infection from 4.1:1 to 0.9:1. The *S. paratyphi* A isolation rate used as a control did not significantly decline during the period of study, indicating that the vaccination programme was responsible for the decline in *S. typhi* isolation.

2.5

THE ROLE OF FLAGELLA IN THE PROTECTIVE EFFICACY OF PARENTERAL IMMUNIZATION AGAINST TYPHOID FEVER

The other major difference between the acetone-inactivated typhoid vaccine used in these studies and other vaccine preparations derived from *S. typhi* Ty2, was the inability of the other preparations to induce anti-H antibody responses following vaccination, either due to the absolute absence of H antigen or its presence in denatured or markedly diminished quantities.

In order to evaluate the role of flagella (or H antigen) in the protection of vaccinated individuals against natural typhoid infection, a controlled field trial of a lyophilized acetone-inactivated *non-motile* mutant of *S. typhi* Ty2 was performed in Egypt in 1972-74.[183] The vaccine strain used was able to synthesize complete O and Vi antigens but was devoid of flagella so unable to stimulate anti-H antibodies.

The field trial area comprised newly admitted schoolchildren aged 6-7 years, none of which had been vaccinated against typhoid or tetanus previously. Two vaccine doses were given subcutaneously three weeks apart such that the total dose contained 1.2×10^9 organisms. tetanus toxoid was used as the control vaccination. In all 42 080 children were vaccinated.

A definite diagnosis of typhoid fever was made only in children presenting with clinical symptoms consistent with typhoid and upon investigation returned blood, urine or faecal cultures positive for *S. typhi*, or whose Widal tests were positive.

Of the 11 071 schoolchildren vaccinated with the H-negative typhoid vaccine, the incidence of typhoid fever was 161/100 000, compared to an incidence of 209/100 000 in the control population of schoolchildren who received the tetanus toxoid instead. The difference in the attack rates was not statistically significant.

In a group of 55 student nurses receiving this vaccine to examine the serological responses to this vaccine, it was found that the O and Vi antigen responses were comparable to those reported using an H-positive lyophilized acetone-inactivated typhoid vaccine,[178] however no anti-H antibody could be detected in the serum of these students.

In summary, the experience of this study, combined with the observations in British Guiana, Yugoslavia, Poland and the USSR suggested that high levels of antibodies directed against the H antigen following parenteral vaccination possibly correlated with protection against naturally acquired typhoid fever.

2.6

PURIFIED Vi ANTIGEN VACCINE

The role of Vi antigen, the capsular polysaccharide of *S. typhi*, in protection against typhoid fever remains controversial. The protective effect of a purified Vi antigen vaccine in human volunteer subjects was originally demonstrated to be quite poor, not achieving statistical significance.[143] However, it was subsequently reported that this poor immunogenicity may have been attributable to the methods employed in the preparation of the original Vi antigen vaccine.[99] A new preparation obtained using cetyl bromide extraction which resulted in the preservation of the N-acetyl and O-acetyl linkages of the purified Vi antigen was administered to adult volunteers and was shown to be safe and immunogenic in adult volunteers, although associated with significant local and systemic adverse reactions almost identical to those observed following vaccination with the currently available parenterally administered killed whole cell vaccines.[130]

South Africa 1986

In a randomized double-blind controlled field trial performed in South Africa,[184] a total of 11 384 children were immunized with either a single intramuscular dose of 0.025 mg of purified Vi antigen or the 0.050 mg dose of the combined meningococcal type A and C polysaccharide vaccine; 11 691 other children acted as unvaccinated controls. During the subsequent 21 months post-vaccination observation the Vi antigen vaccine was demonstrated to have a protective efficacy against blood-culture positive typhoid fever of 60% when compared to the meningococcal polysaccharide vaccine, and 77.4% when compared to the unvaccinated control children.

Nepal 1986

In this field evaluation of the purified Vi antigen vaccine preparation, 6 907 Nepalese subjects aged between 5 and 44 years and living in five villages west of Kathmandu each received either a single intramuscular (deltoid region) injection of 0.025 mg of purified Vi antigen (3 457 subjects) or the pneumococcal polysaccharide vaccine (23 valence) (3 450 subjects).[185] There were some subjects vaccinated who did not belong to the target group, 165 children under 5 years but over two years, and 304 adults over 44 years of age.

All subjects were followed up regularly during the subsequent 17 months after vaccination, and the diagnosis of typhoid fever was made bacteriologically or clinically in suspected cases.

The incidence of blood-culture confirmed typhoid fever in the Vi antigen vaccinated subjects was 260/100 000 and 926/100 000 in the control group (protective efficacy 72%, p=0.004); the rate of clinically suspected cases without blood culture confirmation was 145/100 000 in the Vi antigen group and 725/100 000 in the control group (protective efficacy 80%, p=0.0003). The overall protective efficacy was 75%, and this difference in the attack rates was statistically significant (p=0.00001).

These studies confirmed that the Vi antigen is immunogenic in humans and that immunity against this antigen contributes to protective immunity. Therefore it is apparent that any future typhoid vaccine development must result in a vaccine which contains the Vi antigen.

2.7 Parenteral Vaccination Summary

From the wealth of field studies and experimental challenge studies, it could be concluded that parenteral vaccination:

(1) was more effective against low dose challenge inocula than against high dose inocula (water-borne versus food-borne disease)

(2) was more effective in endemic regions with very high incidence rates

(3) was equally effective in an endemic area with one vaccine dose as with two doses

(4) with purified Vi antigen preparation prepared using modern extraction procedure was as effective as using whole killed organisms

(5) possibly required the ability to stimulate anti-H (flagellar) antibody responses to confer maximal protection.

(6) was associated with severe and possibly unacceptable adverse reactions.

2.8 ORAL VACCINATION

2.8.1 An Historical Introduction

The use of oral vaccines against typhoid fever, was a very popular and widespread practice during the period between the First and Second World Wars. It was Besredka's oral vaccine preparations that essentially were responsible for this, due to their outstanding success in controlling episodic outbreaks of clinical infection.

However, Besredka was not the first to advocate or attempt to vaccinate humans orally against typhoid fever. The first attempt to immunize humans against typhoid fever using the oral route was performed

by James Carroll, the Director of Laboratories at the Army Medical School, Washington DC with assistance by Edward Vedder and Harry Gilchrist, in 1904. This particular study represented the initial effort to develop an oral vaccine for typhoid using killed organisms and was first fully described by Tigertt.[186]

The need for an effective vaccine against typhoid fever was obvious to the United States Army where the peacetime typhoid fever rate was reported to be 514/100 000 in 1904.

In the proposed study, for which it was eventually determined to use soldier-volunteers, the candidate heat-killed typhoid vaccine was prepared by inoculating a flask of nutrient broth with *S. typhi* Dorset strain isolated from a fatal case six years previously and maintained in culture. After six days of incubation, the one litre culture of *S. typhi* was killed by heating at 56°C for one hour. The flask was subsequently incubated for a further seven days at 36°C to produce autolysis of the dead bacteria. Sterility was confirmed by plating one millilitre lots of the flask contents. The vaccine was kept on ice until required.

The vaccine was initially tested in rabbits and guinea pigs, being administered orally using a stomach tube, with increasing weekly doses up to 25 ml in rabbits and 10 ml in guinea pigs being used. Weekly bleeding confirmed that all physiological and haematological parameters remained within the normal ranges. Agglutinating antibody titres of 1:1200-1:2000 could be demonstrated after the fourth or fifth dose, indicating an intestinal immune response was being generated. The vaccine had therefore been proven to be safe and immunogenic in animals.

Due to these results the vaccine was orally administered to the three investigators and 11 soldier-volunteers. Carroll was the only subject

definitely known to have had typhoid fever in the past. The vaccination schedule was that each subject would receive three doses of the vaccine, each dose comprising 30 ml of the vaccine suspension to be given orally 10 days apart. No effort was made to neutralize the potentially deleterious effects of gastric acid on the vaccine. Most of the subjects received only one dose in practice, although Vedder and Gilchrist did receive second doses.

Following the first dose seven of the 13 subjects had undoubted attacks of typhoid fever, a further three had febrile illnesses that may have been mild attacks of typhoid fever. Carroll the only participant with known past exposure to typhoid, remained well throughout the study. One blood culture (from Vedder) was known to be positive for *S. typhi* and two others had positive Widal reactions. The incubation period was shown to vary from six to sixteen days.

The study was performed during the summer when the maximum number of typhoid cases was expected. However the Washington Barracks Post and General Hospital only recorded 11 cases of typhoid fever for the year 1904, seven of them were the volunteers in the vaccine study!

Subsequently the vaccine preparation was replated and found to have contained 2-3 live organisms per millilitre, indicating that each subject received 60 to 90 live typhoid organisms per dose. It appears that the flask had been inadequately heated, and that while the outer temperature was 56°C, the interior temperature may only have reached 52°C, permitting some organisms to survive.

This study was important as it was the first demonstration of anti-typhoid antibodies in any animal following oral administration of a typhoid vaccine, it proved that *S. typhi* obeyed Koch's postulates, and that an infecting inoculum for man need be only a small number of viable

organisms, perhaps more in keeping with natural acquisition, unlike the large doses needed to induce disease as shown by Hornick *et al.*.[97]

Soon after this pioneering experiment, Wright reported his human studies using an orally administered killed typhoid vaccine.[187] In his study, seven individuals ingested heat-killed cultures of *S. typhi*, after which three of whom developed marked increases in the level of their serum bactericidal antibodies. The other four involved in the study were reported to have had a decrease in their post-vaccination serum bactericidal antibodies.

By the time of Garbat in 1928,[188] the use of the parenteral route for immunization against typhoid fever had become well established. There were few doubts that this means of protecting against the disease had a major impact on the incidence of typhoid between the Spanish-American War of 1898 and the First World War, even taking into account the steadily improving sanitary conditions. There were however two observations that had not been fully addressed by 1928. It appeared that parenteral typhoid vaccine using killed organisms did not confer absolute protection against typhoid upon the recipient. In addition, the use of typhoid vaccination had not been accepted by the general community, mainly due to the severe adverse reactions that were frequently observed in following vaccination.

Besredka's concept of local immunity being the major protective mechanism against an orally ingested pathogen led to the development of several killed oral vaccines. Besredka acknowledged the importance of intestinal mucous as a protective barrier against microorganisms, and believed that an oral killed typhoid vaccine would be unable to induce an adequate local immune response due to its inability to penetrate the overlying mucus layer to make contact with the intestinal epithelium, or in his words, "*The vaccine glides along the intestinal canal and ends by being expelled,*

without having come into contact with the receptive cells". Therefore, he considered it necessary to clear away the intestinal mucous with the prior administration of ox-bile.[9] This form of vaccination became known as Besredka's bilivaccination.

2.8.2 Besredka's Oral Typhoid Vaccine

This preparation was the most widely used oral vaccine against typhoid. The description is based upon that of Garbat.[188]

Composition

Appearance and Administration

The vaccine came in the form of small tablets. After fasting, one tablet containing ox bile was swallowed, then 15 minutes later a tablet containing the killed typhoid vaccine organisms was swallowed. After vaccination, no food was permitted for one hour. This procedure was repeated on the next two successive days.

Duration of immunity

It was stated that the duration of protection provided was no less that afforded by the parenteral vaccination, therefore annual re-vaccination was recommended with immediate re-vaccination in the case of an epidemic.

Contraindications

There were no absolute or relative contraindications to the administration of this typhoid vaccine preparation. It was claimed that this vaccine could be safely administered to anyone with the following pre-existing conditions; cardiac, hepatic, tuberculous, syphilitic patients; pregnant, lactating or menstruating women; old age or infancy.

Reactions

Due to the vaccine's inability to penetrate into the recipient, no adverse reactions were to be expected. In fact it was found that only about 4% of recipients demonstrated unspecified minor reactions.

Refractory condition

Garbat stated that the immunity induced by oral vaccination was much more rapidly acquired than that afforded by parenteral vaccination. However, he did caution that some individuals were refractory to either form of vaccination. That is, they failed to produce measurable serum agglutinins following vaccination.

It was Vaillant who was probably the first to apply large-scale oral vaccination using Besredka's bilivaccine.[189] In 1921, he vaccinated six villages in the Pas-de-Calais region where a massive typhoid epidemic existed. The vaccine was administered solely on the grounds of disease control, so as a result, the study had inadequate control populations. The reported figures suggested that the vaccine had no effect on the disease incidence in the vaccinated subjects compared to those who failed to be vaccinated, for whatever reason. This conclusion was probably unfair in view of the poor study design.

Lodz, Poland 1923/1927

One of the earliest field studies using the bilivaccine, was performed in Lodz, in Poland, by Starzynski between 1923 and 1925 during which 60 000 vaccinations were administered, followed by a two year post-vaccination observation period extending from the end of 1925 to 1927.[190] It is difficult to gauge the effects of the vaccine when the overall figures are examined, however, if only the households where not all inhabitants were

vaccinated, therefore having a control population exposed to a similar risk of infection as the vaccinated inhabitants, then we find this represents a total population of 28 166. Of this population, 3 051 did not receive the vaccine, and 47 cases of clinical typhoid fever were recorded in that group. The vaccinated group comprised 25 115 subjects, and only 2 cases of clinical typhoid fever were recorded. If these figures are re-analyzed, it implies that there was a vaccine protective efficacy of 99.5% - certainly an outstanding result.

Starzynski drew the conclusion that vaccination using the oral route was a powerful weapon against epidemics of typhoid fever.

The success of Starzynski was quickly followed by that of Gauthier in Greece.[191] The bilivaccine was administered to a small Greek town where 200 cases of typhoid fever had been recorded in two months. The vaccine had been administered to more than 1 000 inhabitants, one dose on three consecutive days. Only one further case was recorded following vaccination. The epidemic was completely halted in two larger villages when the entire community took the oral vaccine.

Besredka reported a massive vaccination campaign in Brazil during 1926-1927 in response to a typhoid fever epidemic.[9] This vaccination programme potentially provided the opportunity to evaluate the protective efficacies of both the parenteral typhoid vaccine preparation and the oral typhoid bilivaccine since both were used to attempt to control the outbreak. While Besredka claimed that significant protection was conferred by the oral vaccine in comparison to the parenteral vaccine, reference to the original records clearly makes it apparent that it is impossible to determine exactly the numbers vaccinated, what they were vaccinated with and upon how many occasions, and actual disease rates.

Besredka was in little doubt about the benefits of oral vaccination, *"Vaccination practiced by the mouth works, we now know, in the area of the intestinal parietes; it takes place without general reaction, without the production of [serum] antibodies, and is established in a very short time".*[9]

By 1928, Garbat had been estimated that 525 000 people had been orally immunized with Besredka's oral bilivaccine, with a substantial degree of success.[188]

South Africa 1929

In 1929, Cluver described a major comparative field trial conducted in the Union of South Africa.[192] The field trial area comprised a gold mine in the Germiston area near Johannesburg. Contract labour was used in all mines in South Africa, with very few workers remaining at the same mine after the completion of their 12 month contract. As a result typhoid fever epidemics occurred on a regular 12 month cycle. Initial studies using a locally produced preparation of Besredka's typhoid bilivaccine in 1922/23, confirmed the usefulness of this form of vaccination in the prophylaxis of typhoid fever at this mine. Therefore from 1924 all new recruits to the mine received the oral vaccine. Following the introduction of oral typhoid vaccine the annual incidence of typhoid fever in the mineworkers at the declined from 2 330/100 000 in 1923, to 1 040, 1 010, 520, 400 and 470/100 000 in the succeeding five years. This decrease in the presence of continued unsatisfactory sanitary conditions was considerable.

The control group represented workers of a nearby mine which was identical in conditions to the trial mine except that they were not orally or parenterally vaccinated routinely. The differences between the typhoid fever incidences are detailed in Table 2.3. This study confirmed that the orally

administered typhoid bilivaccine prepared as described by Besredka, was an effective means of controlling typhoid fever.

TABLE 2.3

EFFECTIVENESS OF KILLED ORAL TYPHOID VACCINE IN SOUTH AFRICAN GOLD MINES, 1929

Year	Vaccine	Cases	Total Population	Incidence	Protective Efficacy
1927	A	13	3 929	398	71.4%
	B	127	10 978	1 157	
1928	A	16	3 373	474	51.2%
	B	115	11 826	972	

A - Population receiving oral bilivaccine
B - Unvaccinated control population

(adapted from Cluver E 1929; reference 192)

Throughout the 1920's and 1930's numerous inadequately designed studies were conducted in western Europe using Besredka's orally administered bilivaccine, with a whole spectrum of success rates being reported from augmenting disease through to 100% protection. many of these are summarized in the review by Dolman.[193] In addition several from Romania were reported with variable successes.[194]

2.8.3 Specific Immune Response in Humans Following Oral Vaccination with Killed Typhoid Vaccine

Besredka's persistent inability to measure any serum antibody responses in subjects who had ingested his vaccine preparation, had erroneously led him to conclude that antibodies played no role in protection against enteric disease.[195]

Until 1929, the role of specific antibody following oral vaccination in the protection against enteric bacterial disease was doubtful. Very few investigators had been able to measure anything that enabled them to predict the likelihood of protection in a subject following oral vaccination. Most of the evidence for the effectiveness of oral immunization against typhoid fever had been based largely upon empirical clinical data.

One particularly thorough study performed in 1929,[196] involved the vaccination of 93 university students with the triple heat-killed bacterin vaccine (heat-killed $1x10^9$ *S. typhi*, $7x10^6$ *S. paratyphi* A and $7x10^6$ *S. paratyphi* B respectively) administered either as 1 ml in a glass of cold water one-half hour before breakfast on three consecutive mornings, or with two capsules alcohol-extract of ox-bile with the first dose only.

Table 2.4 presents the specific serum anti-typhoid antibody response (serum agglutinins), in the vaccinated subjects. The response data has been divided according the subjects' previous parenteral vaccination status, and whether they received the oral vaccine with or without the ox-bile capsules.

From this data it was evident that an anti-typhoid serum antibody response could be consistently measured following oral vaccination with heat-killed typhoid organisms irrespective of whether the first dose of the schedule was administered with ox-bile or not. However, the real difference provided by the ox-bile was in the magnitude of the serum titres achieved following vaccination. Concentrating only on the responses to *S. typhi* Hopkins strain, of those immunized with prior treatment with ox-bile, 75% of those previously parenterally vaccinated were found to have agglutinin titres exceeding 1:400, while 57.3% of previously unvaccinated subjects had agglutinin titres exceeding 1:100 with 19.1% reaching 1:400 or more. In the vaccination group not receiving pre-treatment with ox-bile, 51.6% of the

previously vaccinated group had titres exceeding 1:100, with 41% exceeding 1:200, while 24.7% of the previously unvaccinated group had titres exceeding 1:100. In this study, the upper limit of dilution for the normal population was considered to be 1:50. In the group receiving the ox-bile pretreatment, none of the subjects previously parenterally vaccinated had titres below the background following oral vaccination, while only 11.5% of those not previously parenterally vaccinated had post-oral vaccination titres less that 1:50. However, 21% of the previously vaccinated and 27.8% of the previously unvaccinated group who received the oral vaccine without ox-bile pretreatment had post-oral vaccination titres less than 1:50.

While statistically, ox-bile made no difference to the immune response despite an obvious trend that suggested it did, neither did previous parenteral vaccination. This study confirmed that following oral vaccination with killed *S. typhi* organisms a specific serum antibody response was evident.

When this study was repeated using gelatin capsules containing $4x10^9$ *S. typhi* Rawling's strain, $2.8x10^7$ *S. paratyphi* A and $2.8x10^7$ *S. paratyphi* B, similar pattern of response was identified to that found in the above study in the groups not receiving pretreatment with the ox-bile capsules.[197]

The bilivaccine preparation of Besredka was being extensively used in South Africa in the late 1920's, especially in the gold mining settlements. One South African group of investigators were able to further characterize the serum anti-typhoid agglutinin response following oral vaccination with the typhoid bilivaccine.[198] Using *S. typhi* H901, a flagellated strain, and *S. typhi* 0901, a flagella-less mutant of the H901 strain, they were able to determine that the serum agglutinin response was directed against the O antigen., with no anti-H (flagellar) response detectable at all.

In another South African study comparing the specific antibody responses to three different typhoid vaccine preparations:[199]

Group I - a parenteral typhoid vaccine comprising heat-phenol-inactivated organisms of the *S. typhi* Watson and Ty2 strains, two doses administered subcutaneously 14 days apart;

Group II - a formalin-inactivated preparation of the mixed bacterial culture, vacuum dried over calcium chloride, compressed into sugar-coated tablets containing $4x10^{10}$ organisms, and administered on three consecutive mornings following the administration of a chocolate-coated bile-tablet on the previous nights; and

Group III - a parenterally administrable endotoxoid vaccine preparation, prepared according to the method of Grasset,[175] and two doses were administered subcutaneously 14 days apart.

The serological responses are detailed in Table 2.5.

In this study it was demonstrated that both the typhoid endotoxoid (Group III) and heat-phenol-inactivated whole cell typhoid (Group I) vaccine preparations administered subcutaneously produced anti-H and anti O antigen agglutinins to a high titre. The oral vaccine (Group I) however, stimulated measurable antibodies in only a few subjects and only then to a low titre.

Despite the positive findings of Hoffstadt *et al.*[196,197] and Dennis *et al.*[200] of high O antibody responses to oral typhoid vaccine in the majority of recipients, the findings of Lewin *et al.*[199] supported by those of Crimm *et al.*[201] suggested that the either the response to the different oral preparations was dependent upon the means of preparation or the dose schedule, or was variable due to inadequate or differing methods employed in the measurement of those responses.

The conclusions drawn from this intensive pre-Second World War period study of oral typhoid vaccination, was that it appeared worthwhile because it had fewer adverse reactions, the antibody titres (when detectable) persisted for up to six months,[201] and was as efficacious in protection as subcutaneously administered killed mixed typhoid vaccine.

After a period of interruption where attention again focussed upon the parenterally administrable killed typhoid vaccines, it was not until the late 1960's early 1970's when attention once again swung back to the oral route of vaccination following the unimpressive results of the extensive field and experimental studies of the various parenterally administered vaccines.

TABLE 2.4

SERUM AGGLUTININ ANTIBODY RESPONSE FOLLOWING ORAL KILLED TYPHOID VACCINATION

	Administered with Bile		Administered without Bile		
Bacteria	**Vaccinated+**	**Unvaccinated***	**Vaccinated+**	**Unvaccinated***	**Controls**
S. typhi					
Hopkins	73.7%	88.8%	91.6%	100%	100%
Patton	57.8%	88.8%	91.6%	100%	100%
S. paratyphi					
A	73.7%	58.4%	91.6%	84.6%	100%
B	52.5%	68.9%	83.3%	57.4%	80%
All	42.1%	22.2%	83.3%	57.4%	80%
None	26.3%	11.1%	8.4%	0%	0%
Total Subjects	19	36	12	26	5

\+ - Subjects known to have been previously parenterally vaccinated with killed typhoid vaccine

* - Subjects known not to have been previously parenterally vaccinated with killed typhoid vaccine

(adapted from Hofstadt RE *et. al.* 1929; reference 196)

TABLE 2.5

SERUM AGGLUTININ ANTI-H AND ANTI-O ANTIBODY TITRES FOLLOWING TYPHOID VACCINATION

Vaccine	Pre-vaccination Day 0		Post-vaccination Day 14		Post-vaccination Day 28	
	H	O	H	O	H	O
Bacteria (s.c)	1	18	1790	451	1494	394
Endotoxin (s.c.)	6	30	2470	350	1133	550
Bacteria (oral)	8	18	16	41	22	36

All values represent the arithmetic mean of the reciprocal of the dilution at a defined end-point

s.c. - administered subcutaneously

oral - orally administered in tablet form

Bacteria - *S. typhi* Ty2

Endotoxin - extracted lipopolysaccharide

(adapted from Lewin W *et. al.* 1937; reference 199)

2.8.4 Field Studies of Modern Oral Killed Typhoid Vaccine Preparations

Delhi 1968/69

A double-blind randomized controlled field trial of an orally administrable killed typhoid vaccine was conducted in Delhi during 1968/69.[202] The field trial area comprised children under 17 years of age resident in the Geeta Colony of Delhi on the Jamuna River. Sanitation was poor, water being supplied from shallow tube wells, and faecal waste removal by bucket latrines and open drains.

It was estimated from hospital records that the incidence of typhoid fever in Delhi was 500-700/100 000 population/per annum. The vaccine used was supplied by the World Health Organization and comprised $1x10^{11}$ acetone-inactivated *S. typhi* Ty2 in tablet form.

Each child received either one tablet daily on three alternate days, or on three consecutive days. In total, 6 591 children received the vaccine, with 92% receiving all three doses; 6 783 children received the placebo tablets, with 92% receiving all three tablets. The trial area was placed under strict surveillance for 12 months from the completion of the vaccination. A diagnosis of typhoid fever was confirmed only following a positive blood culture or a faecal culture positive for *S. typhi* Ty2 in a clinically diagnosed case.

Using these criteria for diagnosis, the incidence of typhoid fever in the vaccinated subjects was 1 540/100 000, and in the control group 1 390/100 000. This difference was not statistically significantly. Furthermore six-monthly analysis of the data indicated that the killed vaccine at no stage provided any significant protection.

This study indicated that the orally administered killed typhoid vaccine given to an at risk population using this dosage schedule was not effective against naturally acquired *S. typhi* infection.

Delhi 1970/71

The failure of the first Indian field trial was considered to be possibly due to an inadequate dose. Therefore this second study was effectively a repeat of the 1968/9 study, except that the vaccine organism used was *S. typhi* Ty58 and the dose had been increased to $3x10^{11}$ killed organisms.[203] The field trial area comprised a residential area nearby the one used previously, which shared exactly the same conditions. Similar numbers of children were used as previously described (vaccine group 6 136, control group 6 428).

The incidence of typhoid fever in the vaccinated group was found to be 760/100 000 and in the control group 840/100 000 during the 12 month surveillance period. This difference was not significant. Three monthly analysis again failed to demonstrate even short term protection in the vaccinated group when compared to the controls.

This study confirmed the findings of the earlier one in that orally administered killed typhoid organisms were unable to provide protection against disease, even when very large doses were administered.

Delhi 1974/75

The third, and as it became, final controlled field study was performed by these investigators using a new preparation of an oral killed typhoid vaccine.[204,205] On this occasion the vaccine tablets comprised $4.0x10^{11}$ acetone-inactivated *S. typhi* Ty2 and were supplied by

Behringwerke, West Germany. The field trial area used was the district extending from Laxmi Nagar to Chander Nagar, Delhi - an area near where the previous two studies were performed. As in the previous studies, only schoolchildren between 6 and 17 years were included in the study (7 312 received all three doses of the vaccine, 7 292 received all three doses of the placebo).

During the first seven months of surveillance, the incidence of typhoid fever in the vaccinated group was 900/100 000, and 740/100 000 in the control group. This difference was again not statistically significant.

The conclusion of the investigators after three unsuccessful studies, was that killed typhoid organisms even in large doses were unable to induce any protective immunity in humans, and that a live attenuated vaccine strain was probably required.

Santiago, Chile 1973/75

In a WHO sponsored field trial undertaken in the Province of Santiago, Chile from 1973 to 1975, the effectiveness of two different preparations of killed orally administrable typhoid vaccines was evaluated in a placebo-controlled randomized double-blind field trial.[206]

One vaccine preparation was manufactured by the SSW Laboratory of the German Democratic Republic (Vaccine GDR), the other was prepared by Behringwerke Laboratories of the German Federal Republic (Vaccine GFR). The antigenic composition of both vaccines differed significantly, as did the method of preparation. The GFR vaccine comprised $5x10^{11}$ acetone-inactivated *S. typhi* Ty2 organisms in capsules, whereas the GDR vaccine preparation comprised $1.8x10^{11}$ formalin-inactivated *S. typhi* organisms and $1.2x10^{11}$ formalin-inactivated *S. paratyphi* B organisms in a combined tablet.

Three doses of each formulation was administered each day on three consecutive days to the subjects. The GFR vaccine was administered to 37 566 schoolchildren aged 10-19 years, and 38 137 schoolchildren of the same age received the placebo, from the western district of Santiago. The GDR vaccine was administered to 37 517 children of the same age-group as in the GFR study with 35 464 others being the placebo controls. This part of the study was done in the northern district of Santiago. During the 18 month post-vaccination observation period, the GDR vaccine had a protective efficacy of 24.7% (disease incidence rates: GDR vaccinated 125.3/100 000; control 166.4/100 1000); the GFR vaccine had a protective efficacy of 42% (disease incidence rates: GFR vaccination 53.2/100 000; control 91.8/100 000). These protective efficacies were not statistically significant. These results were significantly lower than those obtained using the GDR vaccine and a control preparation in an earlier study in Santiago and San Fernando, Chile, where a protective efficacy of 75-80% during the first year was reported.[207]

Baltimore, USA 1969

Two groups of North American adult male volunteers were orally vaccinated with two different killed typhoid vaccines: "Typhoral" (Behringwerke, Marburg-Lahn, West Germany) an enteric-coated tablet containing 0.33×10^{11} acetone-inactivated *S. typhi* Ty58 organisms with equal numbers of *S. paratyphi* A and B organisms; or "Taboral" (Swiss Serum Vaccine Institute, Berne, Switzerland) a monovalent Keratine coated tablet preparation containing 1×10^{11} acetone-inactivated *S. typhi* Ty2 organisms and 20% sorbitol.[132] The subjects were vaccinated and subsequently challenged with 10^5 viable virulent *S. typhi* Quailes strain along with 34

control subjects. The vaccination schedules and protective efficacies are detailed in Table 2.6.

TABLE 2.6

PROTECTIVE EFFICACY OF KILLED ORAL TYPHOID VACCINE TABLETS IN AMERICAN VOLUNTEER SUBJECTS

Group	Number of Subjects	Incidence of Typhoid	Protective Efficacy
A	35	14 (40%)	6.7%
Control	28	12 (43%)	
B	21	8 (38%)	29.7%
Control	24	13 (54%)	

A - six daily tablets of "Taboral"
B - twelve daily tablets of "Taboral"

(adapted from DuPont HL *et. al.* 1971; reference 132)

In addition the serum antibody responses to the vaccines when administered according to their recommended schedules was examined. Only 3% of "Typhoral" recipients demonstrated a anti-H (flagellar) response, where 12% and 17% had four-fold or greater rises in anti-O and anti-Vi antibody. Those subjects receiving the 12 sequential doses of "Taboral" (twice the recommended dose) demonstrated a more frequent response (anti-H 24%, anti-O 17%, anti-Vi 48%).

Despite the promising results of Vladoianu and associates in the mid-1960's with their oral vaccination of children with killed mixed typhoid/paratyphoid vaccines and their demonstration of serum antibody responses,[208] subsequent studies in the field and in volunteers demonstrated that these responses did not appear to be a useful or reliable indicator of

potential protection, and of no value as a predictive index of overall vaccine efficacy.

Modern killed typhoid vaccines when evaluated in properly controlled field and experimental studies, consistently failed to provide any useful degree of protection irrespective of whether the recipients were in an endemic area or had no possible environmental exposure.

It was this outcome that directed research into the possible development of live attenuated typhoid vaccines, work that was spurred on by the success of Mel and his attenuated shigella vaccine.[209]

2.9
ATTENUATED LIVE ORALLY ADMINISTERED TYPHOID VACCINES

The basis for the concept of the use of attenuated live orally administrable *S. typhi* organisms as vaccines against typhoid fever, relies on the belief that the ideal vaccine is one that retains the protective antigens of the pathogenic bacterial cell, and mimics the behaviour of the pathogenic form but to a far lesser extent, that is retains the ability to penetrate intestinal mucus, adhere to the intestinal epithelium, penetrate the epithelium, colonize and proliferate in the epithelial layer.

The older methods of attenuation such as those of Pasteur (Section 1.1) were regarded as being unsuitable for the modern era since their safety was questionable - there was no understanding of the mechanisms of attenuation in those processes, and so no guarantees of inability to regain virulence.

The first method of rational vaccine development proposed was the creation of auxotrophic strains of the pathogen. This was first proposed in 1948 by Iverson and Waksman with respect to the possible use of

streptomycin-dependence as a means of developing a rationally attenuated vaccine.[210]

2.9.1 Streptomycin-Dependent Live Typhoid Vaccine

In 1967, the first report of a study on the immunogenicity and antigenicity of a streptomycin-dependent (Sm-D) *S. typhi* in animals was published.[211] This strain, known as *S. typhi* 27V, exhibited several desirable traits as a viable vaccine: stability, antigenicity, lack of degradation and safety in animals.

Subsequently another Sm-D *S. typhi*, a Vi-negative variant *S. typhi* 20SD candidate vaccine was derived from the streptomycin-sensitive parent of the *S. typhi* 27V strain, *S. typhi* 19V. This vaccine strain was Vi-negative because of the belief at the time that Vi antigen played none or had only a slight role in protective immunity in typhoid fever.[212]

This vaccine organism, *S. typhi* 20VSD, was orally administered to chimpanzees in the following schedule:

(a) 5 control animals received no vaccine;

(b) 5 animals received 4 doses, each dose three days apart;

(c) 4 animals received 4 doses, each dose three days apart and administered with a concurrent dose of streptomycin.

Individual vaccine doses comprised between $3.6x10^{10}$ to $8.2x10^{10}$ viable vaccine organisms. Ten days after immunization the chimpanzees were challenged with $2.6x10^{10}$ virulent *S. typhi* Ty2 orally. All animals in the first two groups developed bacteriological confirmation of *S. typhi* infection, and only in the last group did one animal remain well without evidence of infection.

The failure of this vaccine to provide adequate protection against virulent challenge was attributed to the very high challenge dose and/or inadequate number of doses.

In 1964, the Sm-D *S. typhi* 27V vaccine strain had been fed to adult volunteers in doses of 10^8 or 10^9 viable bacteria with or without streptomycin. Some of these volunteers were challenged with virulent *S. typhi* and not protected against disease.[213] However, subsequent studies demonstrating the effectiveness of the Sm-D *Shigella* vaccines using greater doses (>10^{10} viable organisms) and pretreating vaccine recipients with oral sodium hydrogen carbonate solution,[214] resulted in the Sm-D *S. typhi* 27V strain to be re-evaluated.

In the re-evaluation study, 30 men who had received six weekly doses of between $3x10^{10}$ and $1x10^{11}$ live *S. typhi* 27V organisms with or without sodium hydrogen carbonate pretreatment, and with or without oral streptomycin, were challenged along with 26 unvaccinated control subjects.

The overall protective efficacy of this vaccine preparation was reported to be 66.7%. No breakdown of the differing groups and their respective protection was provided.

When the serum anti-typhoid antibody responses were evaluated post-vaccination, 9% of subjects were reported as having four-fold or greater rise in serum anti-O antigen antibody responses, 54% having a similar rise in anti-H antibody responses and 12% in Vi haemagglutination responses. The investigators conclusion was the same as that drawn by other investigators using the killed preparations 40 years earlier, in that the humoral antibody response was not predictable after oral vaccination.[132]

In a series of subsequent studies using this strain to further evaluate its protective efficacy,[215] the protective efficacy of this strain was consistently

demonstrated to be 66%-78%. However the lyophilized preparation of the vaccine strain, only provided a protective efficacy of 29% which was not significant. More subjects receiving the fresh preparation of the *S. typhi* 27V vaccine strain demonstrated an anti-H antibody response than those receiving the lyophilized preparation (58% vs. 33%). No correlation was found to exist between the serum anti-Vi and anti-O antibody responses and protection. Similarly, no difference in the attack rate was observed between the vaccinees with (21%) and those without (26%) anti-H antibody at the time of challenge.

A similar vaccine candidate prepared as a one-step mutant from the pathogenic strain *S. typhi* Ty2, was found to be safe in children and adults at doses of $6x10^{10}$ to $1.8x10^{11}$ live organisms, but was not evaluated for protective efficacy.[216]

2.9.2 Attenuated Typhoid Vaccine by Intergeneric Mating

Another approach was the development of an attenuated live candidate typhoid vaccine through the intergeneric mating of a pathogenic *S. typhi* strain and the non-pathogenic *E. coli* K-12 organism.[217] The resulting undefined hybrid mutant strain was attenuated in animal studies as well as proving to be attenuated in human subjects when evaluated up to doses of $6.6x10^{10}$ viable organisms. Once again poor serum antibody responses were detected, but on this occasion, coproantibody measurements were made in an effort to determine the presence of local specific antibody, implying a fair degree of local specific antibody production. Good anti-typhoid coproantibody responses were evident in most subjects. In this study, seven out of the 17 vaccinated subjects were challenged with $1.7x10^5$ virulent *S. typhi* Ty 1554 (Walter strain). None of the challenged subjects developed

clinical or bacteriological evidence of typhoid fever. Unfortunately this study was uncontrolled, so there is no way of accurately determining the protective efficacy of this particular vaccine strain. In addition, the lack of a defined attenuating marker most likely precludes this organism or any others developed using this approach from being a seriously considered candidate vaccine, there being no guarantees concerning its unlikelihood of reversion to a virulent strain.

2.10 *Salmonella typhi* TY21a

2.10.1 *Development and Protective Efficacy*

In 1975, the development of *S. typhi* Ty21a, a potential new candidate live oral typhoid vaccine organism, was described.[218] This candidate vaccine strain was developed through chemical and physical mutagenesis of *S. typhi* Ty2 while grown in the presence of galactose. It was described as differing from pathogenic *S. typhi* Ty2 by being defective in the enzyme uridine diphospho-(UDP)-galactose-4-epimerase (hence described as being a *galE* mutant), as well as having sharply reduced galactokinase and galactose-1-phosphate uridyltransferase activity (only 20% of that of the parent *S. typhi* Ty2 strain).

GalE mutants of *S. typhi* are characterized by their inability to synthesize complete cell wall LPS due to the complete block of UDP-galactose-4-epimerase. When grown in the absence of galactose, no galactose can be detected in the cell wall LPS, however in the presence of exogenous galactose *in vivo*, it is claimed that a complete smooth-type LPS is produced. While this alleged *in vivo* smooth-type LPS production ensures that the

immunogenicity of this strain remains substantially better than other rough-type LPS mutants, the *galE* defect also ensures that sufficient galactose is accumulated in the cytoplasm to induce bacteriolysis.

S. typhi Ty21a has been demonstrated to be substantially less virulent in mice than the parent *S. typhi* Ty2 strain as well as providing excellent protection against challenge with virulent organisms.

2.10.2 *Experimental Human Evaluation of* S. typhi *Ty21a*

Preliminary human studies performed on a total of 173 human volunteers in Baltimore, USA, who received orally administered doses up to $5x10^{10}$ live organisms demonstrated the strain's safety, stability and relative absence of adverse reactions.[219-221]

The protective efficacy of *S. typhi* Ty21a as a live oral vaccine against typhoid in human volunteers was reported in 1976.[220] Subjects vaccinated with five to eight doses of $3\text{-}10x10^{10}$ live *S. typhi* Ty21a which had been grown in the presence of exogenous galactose (producing a smooth-type LPS) and subsequently challenged with a dose of 10^5 live pathogenic *S. typhi* Quailes strain that caused clinical typhoid fever in 53% of unimmunized controls, were observed to have conferred upon them 87% protection. There was a clinical attack rate of 50% in challenged volunteers who received live *S. typhi* Ty21a grown in the absence of exogenous galactose (producing rough-type LPS), demonstrating the failure of the rough LPS-bearing strain to provide significant protection against disease (Table 2.7).[220,221] The anti-O antigen antibody response was deemed to be low, with very few subjects achieving a four-fold or greater rise in antibody titre, however this response was greater in those subjects receiving "smooth" vaccine organisms, than in those receiving "rough" vaccine organisms. This study suggested that the

serum anti-H antibody response was not a useful predictor of protection, since there was no difference in the post-vaccination anti-H antibody responses, being present in 31% and 33% of subjects ingesting smooth and rough vaccine organisms respectively, in spite of the significant differences in protection afforded by the two different vaccine preparations.

TABLE 2.7

PROTECTIVE EFFICACY OF "SMOOTH" AND "ROUGH" *Salmonella typhi* TY21a IN AMERICAN VOLUNTEER SUBJECTS

Group	Number of Subjects	Typhoid Fever Rate
"Smooth"		
Vaccinated	28	7%*
Controls	43	53%*
"Rough"		
Vaccinated	27	19%+
Controls	21	38%+

* - Protective Efficacy 87%, p=0.0002
+ - Protective Efficacy 50%, not significant

(adapted from Gilman R *et. al.* 1977; reference 220)

2.10.3 *Field Evaluation of* S. typhi *Ty21a*

Alexandria, Egypt 1978/81

In 1978 a controlled field trial was performed using *S. typhi* Ty21a in Alexandria, Egypt.[222] A total of 32 388 school-children aged 6-7 years were included in the study: 16 486 receiving the oral vaccine; 15 902 received an oral placebo using the same schedule as for the vaccinees; and there were 25 628 non-vaccinated controls.

The lyophilized vaccine doses comprised $2.7x10^9$ live *S. typhi* Ty21a per dose after reconstitution in 20-30 ml of diluent. A total of three doses were given, each dose was two days apart. The doses were only given after the child had chewed a sodium hydrogen carbonate tablet to neutralize gastric acidity.

In the ensuing year, not one confirmed or probable case of typhoid fever was identified in the vaccinated group, while seven confirmed cases and 13 probable cases were identified in the placebo group (incidence 125.7/100 000). This difference was significant. In the non-vaccinated group the incidence of confirmed or probable cases was 132.7/100 000.

This demonstrated that *S. typhi* Ty21a was a safe and effective vaccine against typhoid fever, providing protection for at least one year in the field.

In 1982 the results of a further three years of observation was reported. The attack rate of typhoid fever in the 16 486 vaccinated children was 0.2 cases/10 000 children/year, compared to 5 cases/10 000 children/year in the placebo control group of 15 902 and in the non-immunized group of 25 628. This represents a vaccine protection rate of 96% over a three year period following vaccination.[223]

"Vivotif", Switzerland 1981/83

While a lyophilized form of the vaccine given as a reconstituted liquid has been demonstrated to provide substantial protection against typhoid fever in recipients, such a formulation is neither convenient nor practical for commercial manufacture for mass immunization programmes. A lyophilized gelatin-capsule form of *S. typhi* Ty21a became available in Switzerland in 1981, manufactured by the Swiss Serum Institute, Berne, and

marketed under the trade name "Vivotif". One study involving Swiss travellers who had taken either the three doses of the *S. typhi* Ty21a preparation, or the three doses of the oral killed *S. typhi* Ty2 vaccine "Taboral", reported a typhoid incidence of 1.1/10 000 doses of *S. typhi* Ty21a sold, which was not significantly different from the incidence of 0.9/10 000 doses sold of the proven ineffective killed *S. typhi* Ty2 vaccine. On examination of *S. typhi* Ty21a capsules bought off the shelf in 1983, it was observed that they contained only 6-10x10^8 viable bacteria, which dropped by a further factor of ten following one week of storage at room temperature.[224] It appeared that these travellers were receiving substantially less viable *S. typhi* Ty21a organisms than had been used to demonstrate protection in the previously mentioned studies. This particular preparation was withdrawn from sale in 1984, and replaced with an enteric-coated lyophilized formulation.

Santiago, Chile 1982/83

In 1982/83 a comprehensive field study was conducted in Santiago, Chile, to evaluate the protective efficacy of one or two doses of 10^9 viable *S. typhi* Ty21a contained within enteric-coated capsules.[225] In a randomized double-blind study, 91 954 schoolchildren were orally vaccinated on two occasions, 1 week apart with: two vaccine doses; one vaccine dose and one placebo; or two placebos. An additional 45 743 children did not participate in the study. Intensive post-vaccination surveillance identified 260 cases of bacteriologically-confirmed typhoid fever; the annual incidence rate in the placebo and unvaccinated groups was determined to be in excess of 200/100 000. Over the first nine months post-vaccination the vaccine efficacy for the two dose schedule was 67% and for the single dose was 41%. No

protective efficacy was found after nine months. A subsequent study, using two doses of the enteric-coated preparation, given one week apart, showed a protective efficacy of 59% over a two-year period, whereas a single dose only provided 29% protection over the same period.

These studies demonstrated the practicality of using an enteric-coated formulation in the field, despite the dose schedule being inadequate.

Santiago, Chile 1984/87 (Study A)

The likely usefulness of *S. typhi* Ty21a as a public health tool was evaluated in Santiago, Chile between 1984 and 1987. Two studies were performed in parallel in different administrative regions of the city.

This first study was a larger and more complete study than that described above, conducted again in Santiago, between 1984 and 1987, using both the gelatin-capsule form of the vaccine as well as the formulation in which gelatin capsules were enteric-coated with hydroxyprophymethyl-cellulosephthalate.[226] In this study 109 594 schoolchildren 6-21 years of age received all three doses of the vaccine or the placebo and were placed under surveillance for three years. A vaccine efficacy of 67% was observed in the group receiving three doses of the enteric-coated vaccine capsules, each dose two days apart, as against an efficacy of 19% using the gelatin capsules with the same schedule or 49% with enteric-coated capsules and 21 days between doses. Of two dosage schedules used, either three doses, each dose two days apart, or three doses with 21 days between doses, the former provided significantly better protection against typhoid regardless of the formulation. The gelatin capsules failed to provide any better protection than placebo, whereas the enteric-coated preparation using either schedule provided significant protection against typhoid.

Therefore the enteric-coated formulation was able to provide at best 67% protection against typhoid fever over a three year period. This is markedly better than the two dose schedule, but is obviously not as good as the 96% protection observed in the Alexandria field study of 1978, however, this current formulation is a substantially more practical form of delivery.

Santiago, Chile 1984/87 (Study B)

The selection of the three doses, each dose two days apart, as the vaccination schedule had been initially made as a direct result of the success of that schedule in Alexandria, Egypt, and so permitted the comparison of the protective efficacies of the two studies.

In two different regions of Santiago from those used in the field trials described above, a concurrent study was performed to evaluate the relative effectiveness of differing dose regimens on the protective efficacy of *S. typhi* Ty21a against typhoid fever.[227] This study was uncontrolled, there being no placebo group involved apparently as a result of a direct request by the WHO Ethical Review Committee.

Schoolchildren selected as for Study A, received the enteric-coated capsule formulation as above, either as two doses, three doses or four doses, each dose being administered two days apart. The results are detailed in Table 2.8.

From this study it can be determined that as the number of vaccine doses increased so did the degree of protection afforded by the vaccine.

TABLE 2.8

DOSE RESPONSE PATTERN OF PROTECTIVE EFFICACY OF *Salmonella typhi* TY21a IN AN ENDEMIC AREA

	Time after Vaccination (Years)			
Doses Administered	**1**	**2**	**3**	**Total**
2				
Cases	37	53*	37#	123
Rate/10^5	49.5	79.6	55.6	184.6
3				
Cases	26+	48	30	104
Rate/10^5	40.1	74.1	46.3	160.5
4				
Cases	12+	24*	20#	56
Rate/10^5	20.5	41.1	34.2	95.8

* - p=0.008
\+ - p=0.07
\# - p=0.1

(adapted from Ferreccio C *et. al.* 1989; reference 227)

These two extensive field studies have convincingly demonstrated the if long-term protection is necessary in an endemic area of high prevalence, then a four or possibly more dose vaccination regimen should be employed, however, if short-term incomplete protection will suffice, for example in an epidemic situation, then it may be possible to use only the two dose regimen.[227] *S. typhi* Ty21a has proven itself to be a highly effective, flexible public health tool in the prevention of typhoid fever, however, it has the inconvenience of requiring multiple doses to achieve high degrees of protection.

Plaju, Indonesia 1987

In Plaju, Indonesia, 22 000 persons aged 3-44 years received either the vaccine or a placebo administered weekly for three weeks.[103] The vaccine was prepared as either enteric-coated capsules as used in the Chilean field studies, or as a liquid preparation consisting of lyophilized organisms and buffer salts which was reconstituted in water immediately before use, with each vaccine dose comprising $1\text{-}4 \times 10^9$ viable vaccine organisms.

The incidence of blood-culture proven typhoid fever in this region was particularly high in children being distributed as follows:

(a) 3-6 years	1 600/100 000
(b) 7-9 years	1 400/100 000
(c) 10-14 years	1 300/100 000

The overall rate for the 3-44 year age span was 900/100 000; substantially higher than that encountered in Santiago, Chile.

During the subsequent 24 months of post-vaccination surveillance, the overall efficacy against blood culture proven typhoid fever was 55% for the liquid preparation and 41% for the capsule formulation; both vaccine

preparation appearing to be more efficacious in adults than in children. The results are detailed in Table 2.9.

TABLE 2.9

EFFICACY OF *Salmonella typhi* TY21a IN PLAJU, INDONESIA

Age of Vaccinees[a]	Incidence of Typhoid Fever[b]	Vaccine Formulation	Vaccine Efficacy (%)[c]
3-14 years	1 403	liquid capsule	53 36
15-44 years	382	liquid capsule	63 60
All ages	896	liquid capsule	55 41

a - Each vaccine dose comprised 1-4x10^9 viable vaccine organisms, three doses administered one week apart.
b - Cases of typhoid fever/10^5 persons per years in placebo recipients.
c - 24 months of observation, all significances $p>0.05$.

(adapted from World Health Organization Diarrhoeal Disease Control Programme 1989; reference 486)

This result was considered disappointing since in a preliminary in schoolchildren in Santiago, Chile, the liquid preparation had a protective efficacy of 80% compared with that of only 45% for the enteric-coated capsules ($0<0.001$).

No satisfactory reason was given for the greater vaccine efficacy of the liquid preparation observed in Chile as compared with Indonesia, however, the possibility that vaccine efficacy may be greater where the incidence of typhoid fever is lower was considered as being relevant.

2.10.4 Immunity to Salmonella typhi *Ty21a*

As discussed in Section 2.3, local humoral immunity appears to be important in providing protection against typhoid fever. The live oral

typhoid vaccine organism, *S. typhi* Ty21a, has satisfactorily demonstrated its ability to stimulate a consistent and long-lasting specific intestinal immune response following oral administration. This response was observed in several volunteers who received three doses of the gelatin capsule formulation (containing a minimum of 10^9 viable organisms/dose) and had jejunal fluid samples collected before vaccination and again three weeks and 1 year post-vaccination. A striking immune response was evident at three weeks, which was still present, though slightly reduced, one year after vaccination.[228] In addition, this group observed with fresh vaccine doses, that as the vaccine dose was increased ten-fold, both the number and the magnitude of the intestinal immune responses increased. This study, though dealing with small numbers of subjects, also examined the immunizing potential of an inoculum of 10^8 organisms directly placed into the jejunum on two occasions, 14 days apart. It was observed that there was no detectable anti-typhoid intestinal antibody response evident. Despite this possibly not being the optimal dose schedule, it was suggestive that the minimum dose of *S. typhi* Ty21a required to induce an intestinal immune response in previously unexposed subjects was of the order of 10^8-10^9 organisms. This would be in keeping with the observation made earlier that gelatin capsules containing 10^8 viable organisms failed to confer protection.[224]

S. typhi Ty21a has also been observed to stimulate a significant cell mediated immune response in volunteers ingesting 10^9 freshly prepared vaccine organisms, as determined by the Lymphocyte Migration Inhibition Assay.[229] However, this group was unable to detect an anti-typhoid LPS antibody response in serum or faeces following vaccination, where others have claimed to be able to identify quite a strong faecal anti-typhoid LPS antibody response following oral vaccination with either the "Vivotif" gelatin

capsules or with the enteric-coated preparation which were both purported to contain the same number of viable organisms, 10^9.[230,231] In a study comparing the CMI responses, as determined with a highly sensitive lymphocyte replication assay using whole killed *S. typhi* Ty2 as the antigen, it was shown that 100% of North American volunteer subjects who received the enteric-coated preparation of *S. typhi* Ty21a using the three dose schedule had a detectable CMI response following vaccination, compared to 92% of typhoid fever patients from Santiago, Chile.[153] The specificity of the assay was shown to be good, with 0% of North American subjects with no known previous exposure to *S. typhi* through disease or previous vaccination having a detectable response. Further more, there was no cross-reaction detected against killed *S. enteritidis* or *S. thompson*, when used as antigens.

One study, which evaluated CMI using an assay of antibody-dependent cell cytotoxicity, was able to clearly distinguish differing cellular responses in subjects depending upon whether they were orally vaccinated with the enteric-coated capsules or received a traditional TAB parenterally administered vaccine (mixed *S. typhi*, *S. paratyphi* A and *S. paratyphi* B heat-phenol-inactivated organisms).[231] The subjects receiving the oral typhoid vaccine had distinct cellular responses directed against *S. typhi*, *S. paratyphi* A and *S. paratyphi* B, but not against the antigenically unrelated *S. paratyphi* C. None of the subjects parenterally vaccinated with the TAB vaccine had CMI levels as determined using this assay above the natural background against any of the three organisms used above for the vaccination.

Since cell mediated immunity (CMI) has been suggested to be as important in providing protection against typhoid fever as humoral immunity (Section 2.3.2), the ability of *S. typhi* Ty21a to stimulate a strong

CMI response provides a further mechanism for its efficacy as an anti-typhoid vaccine.

2.11 AUXOTROPHIC ATTENUATED TYPHOID VACCINES

Although *S. typhi* Ty21a has been shown to be a useful public health tool in the control of typhoid fever in an endemic region, there are several aspects of it that does not make it the ideal oral typhoid vaccine. These factors include:

(a) it has been demonstrated that the *galE* enzyme deletion is not an attenuating marker of some *Salmonella* strains including those of *S. cholerasuis* in mice,[230] and more importantly *S. typhi* for humans,[233] so the actual mechanism of attenuation of *S. typhi* Ty21a remains unknown;

(b) that *S. typhi* Ty21a affords very little protection when administered as only a single dose;[226]

(c) the method of development of *S. typhi* Ty21a by treating virulent *S. typhi* Ty2 with N-methyl-N'-nitro-N-nitrosoguanidine appears to have resulted in a vaccine strain with multiple undefined genetic lesions in addition to the *galE* enzyme defect.[218,234]

In view of the above problems, investigators have set about attempting the development of attenuated *S. typhi* strains carrying defined gene deletions in biochemical pathways,[235] as putative candidate vaccine strains. In the early 1950's, it was observed by some investigators that certain strains of *S. typhi* which had sustained one of three biochemical mutations were highly attenuated in the highly artificial mouse typhoid model:[234] the requirement for a purine, such as adenine or adenosine; the requirement for *p*-amino-benzoic acid (pAB); or the requirement for aspartic acid.

The aromatic-dependent *S. typhimurium* strains have been demonstrated to be attenuated in mice and calves,[237,238] as have the purine-dependent strains alone.[239]

A double mutant *S. typhimurium* strain, containing non-reverting *purA* and *aroA* gene deletions (requiring the exogenous addition of a purine derivative and pAB respectively to enable growth) was orally administered to mice and shown to be safe, immunogenic and protective against virulent challenge.[240] The attenuation of this organism was a result of the absence of appreciable quantities of the required compounds in mammalian tissue.[234]

In a human volunteer study performed in the USA,[234] 33 subjects ingested *purA aroA* mutants of either a Vi-negative or Vi-positive *S. typhi* strain in single doses of either 10^8, 10^9 or 10^{10} viable organisms. Four other subjects ingested two doses of $2x10^9$ viable vaccine organisms four days apart. No adverse reactions were observed as a result of orally ingesting the vaccine organism. The humoral immune response to *S. typhi* O, H, Vi and lysate antigens in both serum and intestinal fluid was poor. However, in most vaccinated subjects cell-mediated immune responses were demonstrable. In 69% of subjects overall, or 89% of recipients of doses exceeding 10^9 viable organisms, CMI responses were detectable to *S. typhi* particulate or purified O polysaccharide antigens in lymphocyte replication assays, but not to antigens of other *Salmonella* or *E. coli*.

It was concluded from this study that despite there being a measure of immunity conferred upon the subjects who were vaccinated, the likelihood of this being protective was considered to be low in view of the absence of humoral immune responses, this possibly being due to the over-attenuation of the vaccine strain by the double mutation.

Subsequent studies in mice using *S. typhimurium* with *purA* alone or *purA aroA* double deletions, demonstrated their ineffectiveness in protection against virulent challenge, and so their ineffectiveness as orally administrable vaccines.[240]

As a result of the above findings, investigators have concentrated on developing potential candidate live oral typhoid vaccines by the construction of *S. typhi* strains harbouring gene deletions involving two separate genes in the aromatic pathway only.[241] Equivalent *S. typhimurium* strains carrying these gene deletions have been shown to be attenuated and protective against challenge with virulent *S. typhimurium* organisms in mice. Therefore, an *aroA aroC* mutant of *S. typhi* Ty2 has been constructed and will be administered to volunteer human subjects in the near future.

An additional benefit of these aromatic-mutant strains of *Salmonella* may also be as potential carriers of defined protective antigens from foreign organisms, possibly providing a single multivalent attenuated live orally administered vaccine (Section 8.1).[242]

3

CHOLERA

3.1 *Introduction*

3.1.1 *Definition*

Cholera is an acute enteric illness resulting from small intestinal infection with *Vibrio cholerae* O1. It is a disease that like typhoid fever, is unique to humans with no other animal naturally acquiring the illness after oral ingestion of the organisms.[243] Cholera is characterized by the sudden onset of frequently life-threatening diarrhoea associated with massive fluid and electrolyte losses, together with its tendency for epidemic occurrence.

3.1.2 *Aetiological Agent*

Vibrio cholerae, first described by Robert Koch in 1884, is a Gram-negative, motile, curved bacillus with a single polar flagellum (the heat labile H or flagellar antigen). It is non-sporing, non-capsulated, facultative anaerobe, and which ferments glucose without gas production. *Vibrio cholerae* can be distinguished from other non-cholera vibrios on the basis of a heat stable somatic (O) antigen, O1 which is specific to the classical and El Tor biotypes of *V. cholerae*. The two pathogenic biotypes can be subdivided into three serotypes according to which combinations of the three fractions A, B and C of the O-antigen are present - Ogawa (AB), Inaba (AC) and Hikojima (ABC). A rare fourth serotype containing only the A determinant also exists.[244,245]

3.2

EPIDEMIOLOGY

3.2.1 *An Historical Review*

The review of the epidemiology of cholera is best presented by the inclusion of an historical perspective.

Cholera possesses the unique ability to intermittently spread rapidly throughout the world, resulting in major pandemics. It has demonstrated this ability on at least seven occasions during the nineteenth and twentieth centuries, and there is still no apparent diminishing of the current seventh cholera pandemic. Prior to 1817, however, there were no reports of the spread of cholera from the Indian subcontinent.[246] Historically, the earliest probable report of clinical cholera is believed to have been documented during the reign of Ti-song De-tsen in Tibet (AD 802-845) which graphically described an epidemic purging illness.[244] In Macnamara's classic treatise on the history of cholera,[248] he discovered further evidence of the existence of cholera in the Indian subcontinent since time in memorium, including the reports of a temple at Gujrat in western India containing a monolith dating from the time of Alexander the Great bearing an inscription plainly referring to cholera. However it was only from the time of the Portuguese explorer Vasco de Gama's arrival in the Gangetic delta in 1498 that the first accurately documented reports of cholera were compiled. Over 60 documented references to cholera were believed to have been made by independent authorities between 1503 and 1817, with at least 10 of these referring to the epidemic spread of the disease.[249] However, despite the spread of cholera in epidemic form throughout the Indian subcontinent, and possibly into China, Pollitzer[250] expounds the general consensus of opinion that the disease did

not exist in Europe before 1817; the evidence suggesting that the diseases described by historical figures such as Hippocrates (Greece) and Sydenham (1679-82, London) were not cholera.

First Pandemic 1817-1823

In 1817, cholera broke its subcontinent shackles and went on to cause the first cholera pandemic.[250] The first six pandemics all originated in the Gangetic Delta, in what is now Bangladesh, and the causative organisms were of the classical *V. cholerae* O1 biotype. This particular outbreak appeared to have been an especially aggressive form of the disease, and by 1820 had spread throughout India, becoming localized there in 1821. The regions affected by the 1817-1823 pandemic were: India 1817; Ceylon, Burma, Mauritius 1819; Thailand, Philippines, Indonesia, Malacca, Singapore, Penang, China 1820; Arabia, Basra, Zanzibar 1821; Japan, Iran, Tbilsi and Astrakhan (USSR), Syria, Peking (Beijing) 1822. By 1823, cholera had disappeared just as rapidly from nearly all those places outside of the Indian subcontinent, not without first inflicting massive mortality in these regions.

Second Pandemic 1829-1847

The second pandemic is worthy of some discussion being significant because it truly represented a pandemic. Cholera commenced its march out from the Gangetic Delta in 1827 across India and into Afghanistan in 1829.[250] From there it spread northwesterly, invading Russia, and then in 1831 it commenced its European campaign with Poland. Due to the Russian-Polish war and the infection of the troops involved, the disease spread into Austria with the invasion by the Polish troops. In 1831 Germany and England both reported the presence of the disease in epidemic form, followed in 1832 by

Ireland, Belgium and France. In 1834 cholera reached North America, commencing in New York and Philadelphia and rampaging across the continent to the west coast, and as far south as Mexico City. In 1835 an outbreak occurred in Guyana in South America, which was followed by a devastating occurrence in Nicaragua in 1837. North Africa and once again Zanzibar were involved in the disease spread during 1835-7.

By now it was apparent that cholera had established itself as a disease capable of rapid spread with devastating consequences throughout the world and one that was able to defeat most quarantine measures adopted at the time. The periods of the subsequent pandemics are detailed in Table 3.1.

TABLE 3.1

CHOLERA PANDEMICS

Pandemic Number	Period
1	1817-1823
2	1829-1849
3	1853-1859
4	1863-1875
5	1881-1896
6	1899-1923
7	1961-

(adapted from Pollitzer R 1959; reference 250)

In 1905, a new biotype of *V. cholerae* was described, *V. cholerae* O1 El Tor, in Sinai. In 1937, in the new focus of Sulawesi, Indonesia, this biotype initiated the seventh pandemic which has become the longest and most widespread pandemic in history.[251]

The seventh pandemic

The seventh pandemic of cholera commenced with the spread from Sulawesi of the El Tor biotype of *V. cholerae* in 1961. Since that time there has been a continual increase in the numbers of countries affected, so that now more countries than ever before are reporting the presence of cholera. The El Tor biotype has effectively replaced the classical biotype as the major causative agent of cholera, however occasional isolated outbreaks of cholera due to classical biotype strains have occurred, such as the reports from India in 1978 and 1979 and from Bangladesh in 1979. The El Tor biotype appears to have a greater tendency to become endemic than classical *V. cholerae* O1 strains, in addition to having a higher infection-to-case ratio.[252]

It should be remembered when reading the following incidence and prevalence figures, that outside of the Gangetic Delta and parts of Indonesia, cholera is an epidemic disease and that outbreaks in a region in one year can have completely resolved by the following year.

In 1963, two years into the current pandemic, 14 countries reported the presence of cholera, representing 59 564 notified cases. All of these notifications came from Asia; the bulk occurring in India, Bangladesh, Philippines, Burma and Thailand, with far smaller numbers of cases being reported from Indonesia, South Korea, Malaysia and Singapore.

Europe reported its first cases in this pandemic in 1965, from the USSR.

In 1970, cholera returned to Africa, but this time spread throughout North Africa and Sub-saharan Africa, with large numbers of cases being initially reported from Malawi, Ghana, Guinea, and Ethiopia.

In 1982, 37 countries notified the WHO of the presence of cholera, 12 less than the previous year, 1981, representing 54 856 reported cases of

cholera. This was less than one-third of the peak reported cases of 178 111 in 1971. In 1982, 16 African countries reported cholera (37 427 of 54 856 or representing 68% of total reported cases) with most case-reports coming from Somalia, Zaire, Tanzania, Kenya, Mozambique, Ghana and Zambia (94.5% of total African case-reports).

Cholera has failed to make large inroads into Europe during this pandemic, with only six large outbreaks occurring: USSR 1965 and 1970 (570 and 720 reported cases respectively); Italy 1973 (278 reported cases); Portugal 1974-1975 (2 467 and 1 066 reported cases respectively); Spain 1979 (267 reported cases). In Europe in 1982, only Spain reported one locally acquired case of cholera, although Finland did report 18 imported cases.

In the Oceania region, only the Trust Territory of the Pacific Islands reported a large outbreak during 1982, with 2 214 reported cases. Kiribati in 1977 and 1978 had a size-able outbreak with 1 307 and 494 reported cases respectively, but did not report any cases in 1982.

In 1982, most of the Asian reported cholera cases derived from Indonesia with 8 183. At that time India was the only other significant reporter of cholera cases with 4 656, followed by Iran, Malaysia, and Thailand. Interestingly, Bangladesh did not report any cases of cholera during 1982, nor for that matter for the previous three years.

The global situation since 1981 is presented in Table 3.2. WHO notifications for the year ending 30 April 1988 recorded 48 507 cases of cholera worldwide, indicating little change from the previous year (46 473 cases).[253] These reports came from 34 countries, compared with 36 the previous year, with no further spread of the pandemic into new countries reported. In Africa 17 countries reported cholera (the same as in 1986), however a considerable reduction in the number of cases was observed, from

40 626 in 1986 to 30 929 in 1987. this reduction appeared to be due to a reduction in cases in West Africa, and the termination of the epidemic in Somalia. Despite this, there were significant increases in the number of reported cases in Guinea-Bissau and Senegal, and a large outbreak in Angola of 16 222 cases associated with 1 460 deaths for the period 8 April to 31 December 1987.

Ten countries in Asia reported cholera cases in 1987, compared to 11 in 1986. However, there was a significant increase in the numbers of notified cases, increasing from 5 774 in 1986 to 17 558 in 1987. Most countries reported increases, especially India, Iran, Malaysia, and a large outbreak of 6 353 cases occurred in Thailand.

In Europe, only Spain reported two indigenously acquired cases of cholera, all other notified cases were imported. The USA also reported four indigenously acquired cases of cholera in 1987.

The breakdown of cholera notified cases in 1987 according to individual country are represented in Table 3.3

A large outbreak of cholera occurring between 26 June and 7 October 1988, was notified from the Southern Xinjiang region of China. In this outbreak 3 961 cases occurred with 55 deaths (a case-fatality rate of 1.39%). This epidemic was caused by *V. cholerae* El Tor biotype Ogawa. The epidemic was rapidly brought under control and only sporadic cases were occurring in October.[254]

Recently, the incidence of clinical cholera in endemic areas has been reported to vary between 100-2 000/100 000.

TABLE 3.2

GLOBAL CHOLERA SITUATION, 1981-1987

Year	1981	1982	1983	1984	1985	1986	1987
Number of Countries Reporting Cholera	42	37	33	35	36	36	34
Number of New Countries Infected	1	1	-	-	-	1	-
Number of Cases	36 840	54 856	64 061	28 893	40 510	46 473	48 507

(Adapted from World Health Organization Weekly Epidemiological Record 1988; reference 253)

TABLE 3.3

MAJOR CHOLERA NOTIFICATIONS TO WORLD HEALTH ORGANIZATION, 1987

Country	Cases Notified
Angola	16 222
India	9 375*
Thailand	6 353#
Senegal	2 757+
Guinea-Bissau	2 443
Tanzania	1 892
Mauretania	1 578
Algeria	1 507
Nigeria	1 298
Zaire	1 150+
Indonesia	659
Malaysia	584
Burundi	523
Iran	295
Viet Nam	188*
Others	1 683
Total	**48 507**

* - figures incomplete
+ - confirmed cases only
# - certified cholera-free, April 1989 (reference 487)

(adapted from World Health Organization Weekly Epidemiological Record 1988; reference 253)

In an endemic area, cholera is a young person's disease. Previous findings from Bangladesh, have determined that children under 10 years of age represented 36% of the total population, 98% of the cholera carriers and 74% of the cholera cases.[255]

3.2.2 *Transmission of cholera*

John Snow

It was John Snow who first thoroughly investigated the mechanism of transmission of cholera in his classic treatise "*On the mode of communication of cholera*" of 1855.[256] Snow, through his investigations determined that cholera was a disease transmitted from person to person by the faecal oral route:

"Diseases which are communicated from person to person are caused by some material which passes from the sick to the healthy, and which has the property of increasing and multiplying in the systems of the persons it attacks...As cholera commences with an affection of the alimentary canal, and as we have seen that the blood is not under the influence of any poison in the early stages of this disease, it follows that the morbid material producing cholera must be introduced into the alimentary canal - must, in fact, be swallowed accidentally, for persons would not take it intentionally; and the increase in the morbid material, or cholera poison, must take place in the interior of the stomach and bowels. It would seem that the cholera poison, when reproduced in sufficient quantity, acts as an irritant on the surface of the stomach and intestines, or, what is still more probable, it withdraws fluid from the blood circulating in the capillaries, by a power analogous to that by which the epithelial cells of the various organs abstract the different secretions in the healthy body. For the morbid matter of cholera having the property of reproducing its own kind, must necessarily have some sort of structure, most likely that of a cell. It is no objection to this view that the structure of the cholera poison cannot be recognized by the microscope, for the matter of smallpox and of chancre can only be recognized by their effects, and not by their physical properties."

In that paragraph, Snow clearly expressed his views on the transmission of the disease and its site and mechanism of action. In addition, Snow went further to delineate situations in which transmission occurred, indicating that he also firmly believed that cholera was not simply a waterborne disease. Through this treatise, it is apparent that Snow viewed cholera as being transmitted through the faecal-oral route, with this transmission being direct, foodborne or waterborne. He also related the transmission of the disease to low socioeconomic status and the accompanying poor standards of hygiene.

The removal of the pump handle of the Broad Street Pump (now known as Broadwick Street, London W1), is where Snow's popular historical reputation lies. However, the Soho cholera outbreak had all but ceased by the time of the pump-handle removal, and it was Snow's detailed investigation of the cholera outbreak in the Southwark, Lambeth and Vauxhall areas of South London and his relation of that outbreak to the water companies supplying the particular households affected that proved conclusively his theories of the waterborne nature of the disease and established his reputation as an epidemiologist.[257]

Snow proposed a strategy for the control of cholera requiring:

(1) good drainage (sewerage)

(2) good water supply

(3) improved housing and less crowding

(4) public wash-houses

(5) education in "personal and domestic cleanliness"

(6) quarantining of sick persons on incoming vessels.

Robert Koch and Max von Pettenkofer

Snow's belief that a cell might be responsible for the disease, was supported by the discovery in 1884 by Robert Koch of the cholera comma-bacillus in the faeces of infected individuals. The importance of this discovery was disputed and openly ridiculed by members of the scientific community at the time, in particular by the influential Max von Pettenkofer. In 1892 von Pettenkofer and his disciple Rudolf Emmerich, neutralized their gastric acid with a sodium hydrogen carbonate solution, then subsequently ingested 10^9 viable comma-bacilli of Koch. von Pettenkofer developed mild diarrhoea, while Emmerich developed much more severe diarrhoea. Despite this convincing evidence of the ability of Koch's comma-bacillus to induce a cholera like illness, von Pettenkofer refused to believe that it was the sole cause of the disease.[258]

3.3

MODERN EPIDEMIOLOGY OF ENDEMIC CHOLERA

3.3.1 *Seasonality*

Between 1963 and 1970, cholera in Bangladesh was observed to occur mainly during one season, November to January, as sporadic and scattered localized outbreaks, ranging in size from single cases to 20 or more.[259,260] Different areas were affected at differing times during the season: Dakha being affected during November-December, Matlab during January-February, and Calcutta (in India) during May-June.[261] Since 1970, the pattern has shifted, so that by 1982 the number of cholera cases were peaking during September-October in all three of the above locations,[257] the reasons for these shifts being unknown.

3.3.2 *Infective Dose for Symptomatic Infection*

The infective dose of *V. cholerae* that resulted in the development of clinical disease in normal healthy North American male volunteer subjects, was found to be quite high: 10^8 viable classical strain organisms in water produced diarrhoea in 50% of subjects (ID_{50}), with 10^{11} viable such organisms producing a cholera-like illness.[262] The prior administration of 2 grammes of sodium hydrogen carbonate in solution reduced the ID_{50} to 10^4 viable organisms to induce diarrhoea, and 10^8 for a cholera-like diarrhoea. Diarrhoea was not induced in these subjects with infecting doses of less than 10^8 viable classical strain organisms administered without sodium hydrogen carbonate, nor with less than 10^3 such organisms with prior treatment of the subject with sodium hydrogen carbonate.[260] However, when 10^3 viable *V. cholera* El Tor Inaba strain organisms were administered to volunteer subjects following gastric acid neutralization with sodium hydrogen carbonate, it was reported that 67% of these subjects had symptomatic infection.[252,264]

The requirement for such large infectious doses to induce clinical disease, tends to suggest that foodborne transmission of the organism is the more likely mechanism of spread than water transmission. Foodborne transmission has been reported to be especially associated with high infectious doses owing to the potential for growth prior to ingestion. In support of this, it has been noted that *V. cholerae* concentrations in natural waters are usually very low. In addition, *V. cholerae* has not been found to multiply in natural waters, in fact they have been shown to be more susceptible than other bacterial pathogens.[262] One study examining heavily polluted surface waters in Bangladeshi villages during the cholera season, found that more than 70% of samples contained less than 5 *V. cholerae*/ml with no sample containing more than 500 *V. cholerae*/ml.[266]

Despite this evidence, several studies have put forward strong claims for the near exclusive waterborne transmission of cholera,[267-269] while the evidence reported by others support non-waterborne transmission.[266,270] One study intending to support the concept of waterborne mechanisms of infection, actually found that the prevalence of infection in 1 078 boatmen using a heavily polluted canal over a three-month period was only 0.46%.[271] Yet in one Australian study,[272] five cases of clinical cholera resulting from infection with *V. cholerae* El Tor Inaba strain were reported between 1977 and 1984, seemingly acquired from the riverine environment in the state of Queensland. In four of these five case-reports the river concentration of *V. cholerae* could be ascertained, and was found to vary between 3 and 75 organisms/l.

In volunteer studies performed in the USA,[264] subjects were divided into three groups and administered 10^6 viable *V. cholerae* El Tor Inaba organisms with either 300 ml of water, 2 grammes of sodium hydrogen carbonate or a standard meal comprising fish, rise custard and skim milk. None of the subjects who ingested the pathogenic organisms in water developed clinical or bacteriological evidence of infection, 90% of the subjects who ingested the organisms with sodium hydrogen carbonate developed symptomatic and had bacteriological evidence of infection, and 100% of the subjects who ingested the organisms with food developed symptomatic cholera with bacteriological evidence of infection to the same degree of magnitude as those who ingested the organisms with sodium hydrogen carbonate. This provided further support for the non-waterborne concept of cholera transmission.

Whatever the mechanism of transmission involved, the infectious dose of *V. cholerae* required to induce clinical infection can be quite low, 10^2-

10^3 viable organisms as detailed above, a level which could be found in heavily contaminated surface waters, although the overwhelming weight of the evidence from endemic areas suggests that in the field non-waterborne transmission appears more likely.

Evidence exists for extensive person to person spread of cholera through the analysis of family contacts of a single case. Following the identification of a single index case of clinical cholera, evidence of infection was found between 13% and 34% of contacts if the primary case was caused by a classical strain of *V. cholerae*,[259,260,273-275] and between 18% and 39% of contacts if the primary case was due to infection with an El Tor strain of *V. cholerae*.[259,276,277] One extensive review of the published data concerning infection of family contacts of an index case,[257] indicated that after exclusion of infections occurring within 2 days after the reporting of the index case since they may represent co-primaries with the index case, 35-80% of infected family contacts were detected more than two days after the identification of the index case.

Therefore it appears likely that person to person transmission of *V. cholerae* infection plays an important role in the spread of disease.

3.3.3 *Cholera Carriers*

In an endemic area, the point prevalence of *V. cholerae* excretion among the healthy population has been reported to be usually less than 1%.[257] However, quite a range has been observed in the prevalence of asymptomatic excretion of viable *V. cholerae*, ranging from 0.03%,[278] 1.3%,[279] to 8.5%.[280] Despite these reports, one study suggested that asymptomatic infection was far more common than this,[281] with 21% of children aged 1-9

years in one village during the peak cholera season excreting live *V. cholerae*, and 18% excreting the organisms in the cholera season the following year.

The median duration of excretion of *V. cholerae* by asymptomatic carriers is between one and eight days with a reported range between 5 and 43 days,[257] the excretion duration being slightly longer for El Tor strains.[278]

3.4 CLINICAL DESCRIPTION

3.4.1 *Incubation Period*

In volunteer studies performed in the USA, the mean incubation period following the ingestion of 10^6 viable classical *V. cholerae* Inaba strain organisms, following the pre-treatment of the subject by the ingestion of a sodium hydrogen carbonate solution to neutralize gastric acid, which was consistently associated with the onset of diarrhoea varied from 18 to 36.5 hours, and appeared to be independent of the size of the inoculum.[264] In 1857 Greisinger made the observation that the incubation period for naturally acquired cholera could be as short as 12-24 hours,[250] however there were also many instances when the incubation period had to have been 2-4 days. Since then it has been generally believed that the incubation period for cholera was variable, being as short as a few hours ranging up to three days; anything longer being exceptional.[250]

3.4.2. *Clinical Manifestations*

The clinical manifestations of cholera cover the entire spectrum from asymptomatic infection (as described above) to severe diarrhoea and death. In many instances, attacks are mild and easily mistaken for other less serious

causes of gastroenteritis. In a typical adult case, the diarrhoea is characterized by precipitous onset of diarrhoea - initially the stools are formed but progressive stools become liquid and clear, with occasional specks of mucus but no blood. Vomiting may accompany the early stages of diarrhoea and may contribute to the dehydration.

The clinical signs of cholera relate to the severe dehydration which results form the infection and are graphically depicted in Figure 3.1: diminished skin turgor, sunken eyeballs, depressed fontanelle in infants, dry mucous membranes, cold extremities, thready pulse and systolic hypotension. Fluid loss is maximal in the first 24 hours. Provided adequate rehydration occurs, the patient survives, with the diarrhoea declining over the second 24 hours of the illness, with complete remission within 1-6 days.[282]

In children, symptoms such as fever, lethargy, seizures, altered consciousness and hypoglycaemia are more commonly seen, the latter two being important risk factors for death.

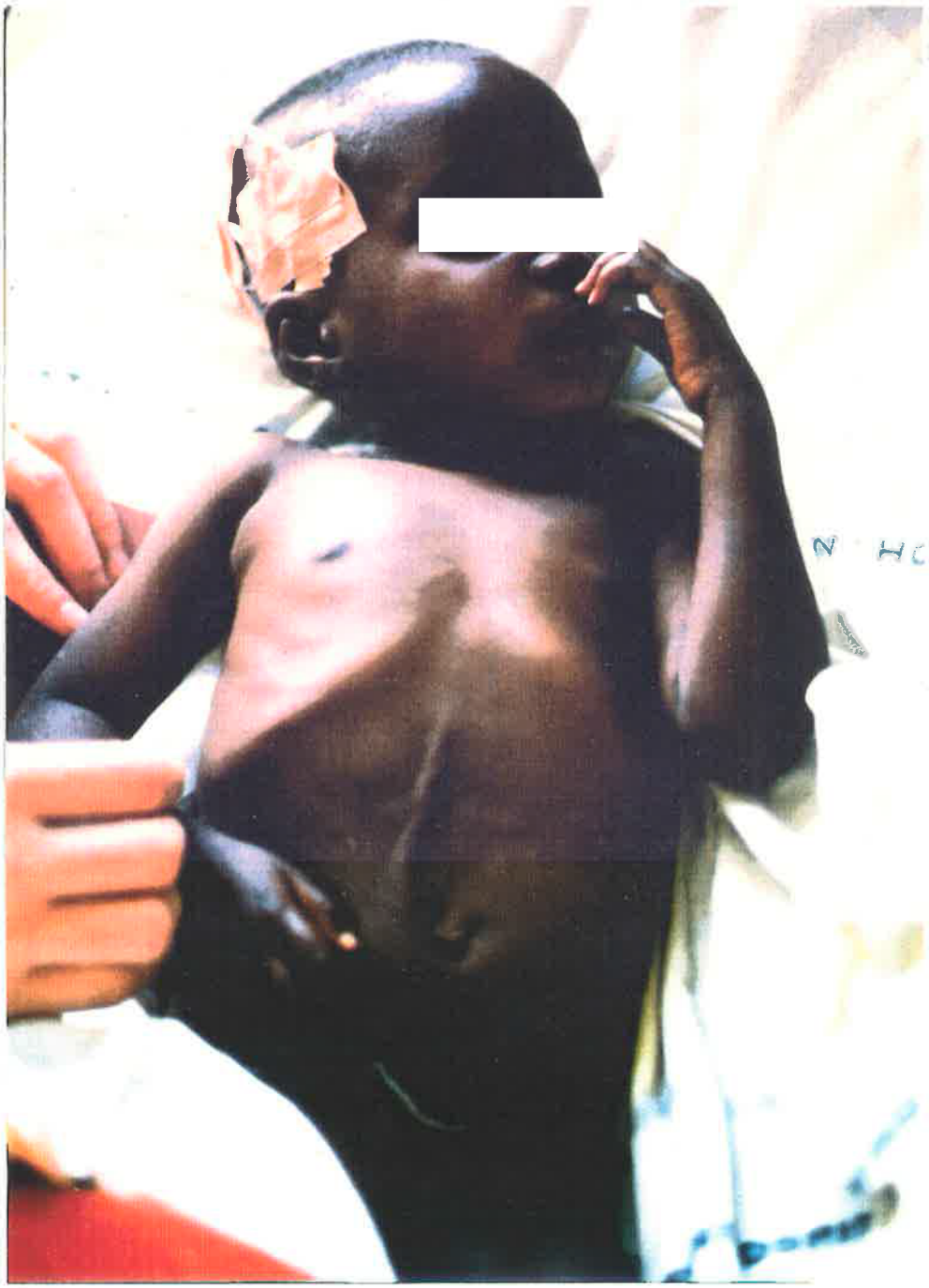
Adequate fluid and electrolyte replacement is the mainstay of effective treatment of symptomatic cholera, being first demonstrated in the early 1830's.[283,284]

3.4.3 *Complications*

Just as the clinical features of cholera relate to the severe dehydration, it is the fluid and electrolyte loss that results in the complications that may accompany an attack of cholera; these may include hypovolaemic shock, metabolic acidosis; hypokalaemia; death.

FIGURE 3.1

CLINICAL FEATURES OF CHOLERA



Photographed at Mashoko Christian Hospital, Mashoko Mission, Mashoko, Bikita, Zimbabwe, February 1984

3.5

PATHOGENESIS

3.5.1 *Adherence*

Following ingestion of a sufficiently large inoculum of viable pathogenic *V. cholerae* O1, and its successful passage through the stomach, the development of symptomatic illness depends upon the ability of the *V. cholerae* to colonize the small intestine.

The first step in the colonization of the small intestine was believed to be the primary adherence of the organisms to the microvilli of the intestinal absorptive epithelial cells. However a recent electron microscopic study has discovered that pathogenic *V. cholerae* O1 organisms also adhered strongly to the intestinal mucus coat, and that this binding was more marked than to the epithelial surface of the villi. From this study it was apparent that the intestinal mucus also can act as a primary binding site for *V. cholerae*. The actual site and mechanism of attachment to the mucus has not yet been elucidated but these investigators believed that it may be to a L-fucose sugar residue in the mucin or glycocalyx, and that the haemolysin appeared to play a significant role.[285]

Furthermore, recent studies have identified specific cell-surface pili or fimbria which also appear to be required for effective adherence and colonization (Section 5.6.1).

3.5.2 *Cholera toxin*

Once adherence has occurred, *V. cholerae* multiplies, and produces a heat-labile enterotoxin. In 1959, De in Calcutta in 1959,[286] convincingly demonstrated that pathogenic strains of *V. cholerae* produced an enterotoxin,

by demonstrating that a cell-free culture filtrate could stimulate fluid secretion in the rabbit intestinal-loop model. Since then, the cholera toxin has been purified and its mechanism of action elucidated at the molecular level.

Cholera toxin is a protein with a molecular mass of 84 000, comprising two immunologically different regions designated "A" and "B". The B region (previously known as choleragenoid) has an approximate Mr of 56 000, and is composed of five non-covalently associated subunits each of about Mr of 11 500 arranged in a circular fashion.[252] The B subunit encircles the A region, which comprises two peptides, A_1 (M_r 20 500) and A_2 (M_r 7 500) linked by a disulphide bond.[287]

The B subunit is responsible for the binding of the holotoxin to host-cell membrane receptors that contain the glycolipid, the GM_1 ganglioside. At present, the Fishman model provides an adequate explanation for the mechanism of action of cholera toxin that results in the secretory diarrhoea.[288] According to this model, the first step in the pathogenesis of diarrhoea is the irreversible binding of the toxin to the cell outer membrane through the interaction of the B subunit and the GM_1 ganglioside. This permits the active component of the toxin, the A_1 subunit to penetrate the cell where it acts enzymatically to stimulate the enzyme adenylate cyclase. It achieves this by cleaving nicotinamide adenosine dinucleotide (NAD) and transferring adenosine 5'-diphosphate (ADP)-ribose to the guanosine 5'-triphosphate (GTP)-binding protein associated with adenylate cyclase. This ADP-ribosylation of the GTP-binding protein prevents the breakdown of GTP to guanosine 5'-diphosphate (GDP), effectively locking adenylate cyclase in its active state. This results in the production of excessive levels of cyclic-adenosine monophosphate (cAMP) which results in the stimulation of

the secretion of water and electrolytes from the intestinal crypt cells and inhibits the absorption by the villous cells - the cholera stool.[252,289]

3.6 IMMUNITY TO CHOLERA

3.6.1 *Major Antigenic Determinants of* Vibrio cholerae

1. Lipopolysaccharide

It appears that the structure of the lipopolysaccharide of *V. cholerae* may be fundamentally different from that of *Salmonella* species described in Section 2.2.1. The sugar components of the Inaba and Ogawa serotypes include glucose, heptose (L-glycero-D-manno-heptose), mannose, glucosamine, glycerol and ethanolamine.[290] In addition fructose, an unusual component of Gram-negative LPS, has been reported to be an important constituent, while galactose appears to be absent.[291,292] While having a Lipid A region which comprises 30% of the constitution, *V. cholerae* LPS lacks a KDO region, a core component found in most other Gram-negative bacteria.[293]

Using the infant mouse model, it has been demonstrated that antibodies raised in rabbits against the O1 antigen of *V. cholerae* were able to confer protection on the animals against challenge with pathogenic *V. cholerae* O1.[127,294]

3. Pili or Fimbrial antigens

Recently a fimbrial antigen of *V. cholerae* has been described that appears to play a major role in the ability of pathogenic strains of *V. cholerae* to adhere to the intestinal mucosae.[295] This pilus, designated the toxin co-regulated pilus (TCP) and the cholera toxin are regulated by the *toxR* gene

complex. The importance of this pilus in the pathogenesis of clinical cholera has been evaluated in human subjects at the Center for Vaccine Development of the University of Maryland, Baltimore, USA. Volunteer subjects were orally challenged with a classical *V. cholerae* Ogawa strain O395-N1, others with a mutant of the O395 strain (designated JJM43) in the *ctxA* gene rendering it unable to produce the cholera toxin A subunit, and a third strain (designated TCP2) carrying defined deletions in the *toxR* and *tcpA* genes.

Subjects receiving the O395-N1 strain reported abdominal cramps, malaise diarrhoea, anorexia and generated a good immune response indicating active colonization. Those receiving the strain TCP2 remained asymptomatic, with no evidence being found that the strain had colonized, nor had it generated an immune response. Recipients of strain JJM43 remained asymptomatic, although there was some evidence of poor colonization and the strain did stimulate an immune response.

This study indicates the importance of the TCP in the ability of the pathogenic *V. cholerae* strains to induce clinical disease. A large number of non-pathogenic environmental isolates of *V. cholerae* have been probed for the TCP gene without success while all pathogenic isolates carry the gene.

3.6.2 *Protective Immunity*

It was Robert Koch, who in 1884, published his observations that convalescent anti-cholera protective immunity was only of short duration, since a person suffering an attack of cholera during one epidemic could have another attack during another outbreak, but that his person was not attacked twice during the same epidemic, despite the fact that recovery meant that the patient returned home and was again exposed to the disease.[296]

It is now generally believed that clinical cholera appears to invoke a significant degree of protection upon convalescent patients. In endemic areas cholera incidence is maximal in children aged 2-4 years, decreasing in older age groups despite continuing exposure to the organism.[274,297] In addition, in such areas it has been demonstrated that primary infection confers solid protection against second attacks for several years.[298] Clinical infection in North American volunteer subjects evoked solid protection that lasted for at least 10 weeks against rechallenge with homologous or heterologous serotype,[299,300] and which was still evident one year later against homologous challenge[33] or against heterologous or homologous challenge up to three years later.[300,301]

3.6.3 *Antibacterial Humoral Immunity*

Local intestinal immunity to cholera is probably now generally accepted as being important in the prevention of clinical disease. As mentioned previously, there are numerous technical difficulties encountered in obtaining satisfactory specimens of intestinal fluid for the accurate determination of a specific antibody response. Early studies using guinea pigs suggested that they could be protected against challenge with pathogenic *V. cholerae* by coproantibody (faecal antibody) induced by previous infection.[26] This led to the discovery that acutely ill cholera patients also produced coproantibody against cholera.[37] Early estimations of the IgA antibody content in the stools of 25 acute cholera patients supported the view that the coproantibody being measured was being locally produced in the intestine and was not a transudate from serum, the serum levels of IgA being no lower than convalescent levels.[41,302] Further studies were able to determine that much of the coproantibody IgA was in fact the polymeric

secretory form. However, there was a significant proportion of monomeric IgA and smaller IgA fragments present, possibly representing the enzymatic breakdown products of the secretory form.[41] When specific anti-cholera antibody levels were examined in the jejunal fluid obtained by the intestinal intubation of patients, it was observed that during the early stage of infection the predominant intestinal immunoglobulin was IgG initially,[42] but by seven days after recovery the predominant immunoglobulin had become IgA,[42] which peaked two to four weeks post-convalescence.[40] In one detailed study the increase in specific anti-cholera IgA was a mean 30-fold in the small intestinal secretions after cholera, with no concomitant rise in the total IgA; this is in marked contrast to a mean fold rise in specific serum IgA of only two-fold,[40] further supporting the importance of specific local antibody production in clinical cholera. In that study, it was also determined that the intestinal IgG and IgM responses appeared to be due to a transudate from serum.

A study which examined the specific immune responses against cholera in jejunal fluid obtained by the technique of intestinal intubation from bacteriologically confirmed cholera convalescent patients in Thailand,[303] demonstrated 100% of patients had anti-cholera LPS specific IgA antibodies predominating in the intestinal fluid, with much lower levels of LPS-specific IgM and no IgG detectable. These levels were observed to increase during the convalescent period. Therefore, it is apparent that elevated anti-cholera LPS IgA antibodies occur in the gut following clinical cholera. Similar responses have also been detected following the oral administration of a combined killed whole-cell/B toxin subunit.[304] This vaccine, both with and without the cholera toxin B subunit has been subsequently shown to be protective in challenged human volunteers.[305]

Serum antibodies directed against cholera antigens can be found following cholera infection, but since *V. cholerae* is non-invasive - remaining confined to the lumen and mucosal epithelium of the small intestine - these circulating antibodies probably play a minor role in protective immunity, and that it is most likely that secretory IgA antibodies produced locally in the small intestine provide the main protective role.[306] In an extensive study using one of the "modern" methods of vibriocidal antibody determination, significant ten-fold rises in antibody were found in 70 of 76 cholera patients infected with *V. cholerae* Inaba, and in only 2 of 30 non-cholera patients. In addition, 69 of these 76 patients and 15 of the 30 non-cholera patients were found to have at least four-fold vibriocidal antibody rises. The antibody response was observed to be uniform in cholera patients, and was independent of previous vaccination and antibiotic therapy,[307] a finding further supported by the very low vibriocidal antibody titres that was found in USA Army servicemen, all of whom had been exposed to cholera antigen through parenteral vaccination.[308] The findings of Mosley,[309,310] who demonstrated that each two-fold rise in vibriocidal antibody titre was associated with a two-fold reduction in susceptibility to cholera in the field, while others have suggested a more prominent role for vibriocidal antibody determination as a measure of a specific immune responses following naturally acquired infection.[40,42] The recent clinical studies with the whole-cell killed cholera vaccine (without the B subunit of the cholera toxin) which stimulated geometric serum antibody titres of as little as two-fold,[311] and yet provided 56% protection in recipients,[305] suggest that the ability of convalescent or post-vaccination serum to kill live *V. cholerae in vitro* may be a useful indicator of protective immunity.

The vibriocidal antibody estimation, however, is not against a defined antigen, rather against the whole live organism - so the specific role of any one antigen in protection against disease can not be accurately determined by this method, although it has been reported that the specific anti-*V. cholerae* O antigen IgA antibody response accounts for the bulk, but not all, of the *in vitro* vibriocidal antibody response.[243,312]

The specific outer membrane proteins of *V. cholerae* have been implicated in contributing to the generation of a protective immune response in experimental animal infection[313,314] and following clinical infection.[315] In a study involving a large group of previously unexposed adult volunteer subjects who were challenged with a variety of strains of *V. cholerae* and developed clinical illness, 50% of the 79 symptomatic subjects challenged with the El Tor biotype and 54% of those challenged with the classical biotype had significant serum IgG rises against an outer membrane protein (OMP) preparation. Significant rises were identified against the OMP preparation of both the homologous and heterologous strains.[315] A particular protein antigen of M_r 45 000 to 48 000 found within both bio- and serotypes may represent the immunodominant protein antigen.[316] Local antibody responses the OMP in humans have indicated that the specific secretory IgA response to be directed against components of the OMP of M_r 25 000 or less. In addition, it was observed that the post-challenge jejunal IgA antibody response specifically recognized two antigens not found on the heterologous challenge strain, and since protective immunity is greater for the homologous challenge strain than the heterologous strain, it was believed that these two antigens may have an important, although yet undefined role, in the generation of a protective immune response.[317]

3.6.4. *Anti-cholera Toxin Humoral Immunity*

As described in Section 3.5.2, cholera toxin can be produced by both biotypes and serotypes of *V. cholerae* O1. In a major survey performed on the sera obtained from convalescent bacteriologically confirmed cholera patients in East Pakistan (Bangladesh) in 1967, nine-fold or greater rises in the cholera toxin-neutralization titre were observed in 73% of the 111 cholera patients, with 89% having three-fold or greater rises, while bacteriologically-negative patient admissions all had very low anti-cholera toxin titres.[318] Other studies have confirmed this type of pattern of response, demonstrating in acute and convalescent patients that serum antitoxin antibody responses peaked on Day 15 following the onset of diarrhoea with a geometric mean fold rise of nine-fold, before declining quite slowly, especially when compared to the rapid fall in the vibriocidal antibody titre observed in this study (peaking on Day 15 and declining to be near pre-vaccination levels by Day 45).[43] In experimental animal studies, it has been demonstrated that anti-cholera toxin antibodies were able to protect against symptomatic infection.[319-324] As a direct result of much of this work a stable purified cholera toxoid was prepared,[325] which was subsequently used in a large scale field trial in Bangladesh in 1974.[326] In this trial, parenterally administered glutaraldehyde-treated cholera toxoid was found to elicit high titres of serum anti-cholera toxin antibodies, but was observed to only offer very short-term protection in one vaccinated group only.

Studies using adult volunteer subjects were performed to assess the importance of local and systemic anti-cholera toxin immunity in protection against clinical cholera.[299,300] In one report,[300] two groups of volunteers received three monthly doses of either 2 mg or 8 mg of purified glutaraldehyde-treated cholera toxoid enterally via an intestinal tube in

order to attempt to stimulate local intestinal immunity. A serum antitoxin antibody response was determined in 6/10 subjects and 7/9 subjects respectively. Upon challenge with 10^6 pathogenic *V. cholerae* organisms, there was no significant difference from the control groups in attack rate, stool volume or incubation period. In another study, the same toxoid preparation was administered as 2 mg doses enterally using an intestinal tube on three occasions 28 days apart to one group of volunteer subjects, while in the other group the primary vaccination was administered subcutaneously as 0.200 mg doses, then the subsequent two doses administered enterically as in the former group.[299] Specific anti-cholera toxin antibodies were measured in the serum of all seven subjects receiving the toxoid preparation parenterally, but in only six of ten subjects receiving the toxoid enterally. Intestinal specific antitoxin antibodies could not be detected by the particular assay system utilized by these investigators. Upon challenge 25 weeks after the primary vaccination (17 weeks after the last dose) with 10^6 live pathogenic *V. cholerae* the attack rates of the two groups were observed to be identical to that of a a control group, as was the severity of the clinical illness, stool volume, and incubation period.

When these results were added together with the observation that subjects previously challenged with *V. cholerae* when rechallenged were protected against clinical disease and had a significant and substantial reduction in the number of pathogenic organisms that could be isolated from their jejunum, it was concluded that local antibacterial immunity played a more significant role in providing protection against reinfection than did antitoxin immunity.[327]

One explanation for the failure of the toxoid preparation to induce any degree of protective immunity may have been attributable to the method

employed to prepare it, however it may genuinely have no role in the protective immune response.

Other studies had suggested that the B subunit component of the cholera toxin may have been more suitable as an immunogen. Although less imunogenic than the holotoxin,[328] anti-B subunit antibodies have been demonstrated to have greater cholera-toxin neutralizing capacity than the anti-A subunit antibodies.[329]

The immunogenicity of the purified B subunit of cholera toxin was evaluated in volunteer female subjects, 24 women in the mature lactating phase (2-10 months post-natal).[330] For this study the 24 lactating women were randomly allocated to four experimental groups:

(a) Group 1 received a 0.500 mg dose of the cholera toxin B subunit preparation as their primary vaccination orally, followed 25 days later by a second 0.500 mg oral dose and a third oral dose of 0.500 mg 15 months later;

(b) Group 2 received a 0.150 mg intramuscular dose of the B subunit preparation, followed 25 days later by a second 0.150 mg intramuscular dose and 15 months later by a third dose of the subunit preparation this time as a 0.500 mg orally administered dose;

(c) Group 3 received the same 0.500 mg primary oral vaccination as Group 1, followed by a second dose, intramuscularly of 0.150 mg 25 days later, and finally a third dose of 0.500 mg, orally, 15 months later;

(d) Group 4 received a 0.150 mg intramuscular dose as for Group 2, followed by a second dose of 0.500 mg, orally, and a third dose of 0.500 mg orally 15 months later.

Following the oral primary vaccination with the B subunit preparation, 8/11 subjects exhibited a four-fold or greater rise in intestinal antitoxin IgA specific antibody, and after the single intramuscular primary

vaccination 7/12 subjects had a four-fold or greater rise in intestinal antitoxin specific IgA. The magnitude of these responses were similar, with geometric mean fold rises of nine-fold and eight-fold respectively. A significant local IgA antitoxin response was also detectable, however this was only 10% of that observed in the IgA class. It was observed that 86% of the antitoxin specific intestinal IgA antibody obtained by intestinal lavage from the orally vaccinated subjects was of the secretory type, and only 71% from the intramuscularly vaccinated subjects was.

The second oral dose following a primary vaccination with either the oral or intramuscular route resulted in a responses similar in frequency and magnitude to those resulting from the primary vaccination. a second dose administered intramuscularly resulted in greater frequency and magnitude of responses than the primary vaccination, irrespective of the route used for that vaccination.

Significantly elevated specific antitoxin IgA antibody responses were found in only 27% of subjects four weeks after either of the two intramuscular injections, but was observed in 57% of subjects four weeks after the initial or second oral administration. However, these responses were relatively short-lived, as fifteen months later only 1/16 had levels of specific antitoxin intestinal IgA antibody significantly elevated above their pre-vaccination baseline levels. The third and final oral vaccination of the B subunit preparation resulted in four-fold or greater rises in intestinal specific antitoxin IgA antibody in 8/15 subjects, the magnitude and duration of which did not differ significantly from that observed following the first oral vaccination.

Significantly elevated antitoxin specific serum antibodies were observed in 11/12 lactating women following the primary oral vaccination

and this was mainly in the IgA and IgG antibody classes, with a similar number of responders following the intramuscular vaccination but of a greater magnitude. A second vaccination by either route did not result in significantly higher IgA, IgG or IgM titres than those induced following the primary vaccination - except where intramuscular vaccination followed a primary oral vaccination.

This study appeared to confirm the observation that antitoxin immunity was of only a short duration and required repeated doses/exposure to maintain a significant level of specific intestinal or serum antibodies. This was supported by the observation that each recurrent orally administered dose of the B subunit preparation, failed to stimulate a local antibody response of greater magnitude and duration than that observed following the primary vaccination, although it was purported that the pattern of response was comparable to that observed in cholera convalescents in Bangladesh.[304]

3.7

CHOLERA VACCINATION: AN HISTORICAL PERSPECTIVE

3.7.1 *Protective Efficacy of Parenterally Administered Killed Cholera Vaccines*

Jaime Ferran: Spain 1885

It is only appropriate that this review be commenced with Jaime Ferran and his demonstration in 1885, just one year following Koch's description of the agent responsible for cholera, *V. cholerae*, of the protective efficacy of parenterally administered live whole cell cholera vaccine in humans. During 1884/1885, there had been sporadic outbreaks of cholera in

Spain, but their impact had been rather limited. However, the epidemic of 1885 was Spain's most devastating with 338 685 cases and 119 620 deaths (mortality rate 35.3%) in a population of only 16 000 000.[331] In October 1884, after his return from investigating the cholera epidemic in Southern France, Ferran investigated the pathogenicity of *V. cholerae* in guinea pigs observing that animals which survived injection of virulent organisms subsequently were able to resist further injections of much greater numbers of virulent organisms. He proceeded to conduct a Phase 1 clinical trial of a parenterally administrable live cholera vaccine, using doses comprising 0.5 ml of pure culture, using himself, friends other physicians and students. Adverse reactions were frequently observed including chills, fever, headache and local pain and erythema. These symptoms diminished by 24 hours and had usually disappeared by 48 hours.[5] In March 1885, the cholera epidemic commenced in Jativa in Spain, and in from April 27 he and a small group of physicians and assistants, proceeded to vaccinated 30 000 members of the community. Ferran was systematic and thorough, with all vaccine recipients receiving a numbered certificate which corresponded with his register entry and also contained a disclaimer.

In early 1886, Ferran was the first to report achieving the same effect in guinea pigs that was observed previously, except by using killed whole vibrios.[332]

However, Ferran was subjected to the rigorous investigation of several international commissions of inquiry into the methods and success of his vaccine preparation. Regrettably, his great work was repudiated and even considered dangerous, primarily because he would not release the method of attenuation nor the actual vaccine strain for analysis in other investigators laboratories.

The data obtained from his field trial was statistically analyzed by Bornside,[5] who was able to show that in Ferran's town of Alcira, 4 957 persons were not inoculated at any stage and the cholera attack rate in this group was 82 900/100 000 and was associated with a mortality rate of 51.8%. There were two vaccinated groups: one of 2 220 persons vaccinated once only, where the attack rate was 1 580/100 000 (protective efficacy of 80.8% and had an associated mortality rate of 22.9% (both highly statistically significant compared to the unvaccinated group); and another group of 8 830 who had been revaccinated, where the attack rate was only 6 200/100 000 (protective efficacy of 92.5%), with an associated mortality rate of 16.4% (these figures too, are statistically significant compared to the unvaccinated group).

If all 30 491 subjects in east Spain who received one dose of Ferran's vaccine were compared to the district population which was not vaccinated, the attack rate in the vaccinated group was 1 270/100 000 (26.9% mortality) compared to the unvaccinated population attack rate of 7 700/100 000 (43.6% mortality rate) giving an overall vaccine protective efficacy of 83.5%. In retrospect, Ferran's vaccine was quite successful, but his steadfast refusal to fully co-operate with the international commissions left him out of favour, and when cholera returned to Spain in 1890, vaccination was not used.

Waldemar Haffkine 1895

As a direct result of Ferran's refusal to comply with the requests of the various international commissions investigating his successful anti-cholera vaccine, for most of this century, it was Waldemar Haffkine who has been credited with the priority for the development of cholera vaccination in humans.[6] Haffkine, a Russian dissident Jew who was an active member of

the political group responsible for the assassination of Tsar Alexander II, and who had studied under Metchnikoff, fled Russia in 1888 to Switzerland following his numerous arrests and brief terms of imprisonment for his political activities. Eventually, in 1889 he joined the Pasteur Institute in Paris. During his early years at the Pasteur Institute, he was able to induce protection against cholera in several laboratory animals. So, on 18 July 1892, he and three others were inoculated into the flank with, firstly, an attenuated live *V. cholerae* candidate vaccine attenuated by the growth of the broth cultures at 39°C in the presence of oxygen, and then six days later with an enhanced live vaccine whose pathogenicity had been increased through the serial passage through a variety of animal species.

All subjects experienced significant adverse reactions as a result of this vaccination process including fever (38.6°C), local pain, tenderness and erythema. There were no significant systemic symptoms recorded.[333] From 1894-1896, Haffkine refined the two-inoculation live anti-cholera vaccination method, vaccinating over 72 000 volunteer subjects randomly throughout India. He was able to determine that in Calcutta, his vaccination programme conferred significant protection on the recipients, compared to non-vaccinated subjects in the same household, commencing on Day 5 post-vaccination and remaining significant four the subsequent 14 months. By 1896, he had determined that the primary, attenuated vaccine was unnecessary for protection, and altered his schedule to a single vaccination with the enhanced vaccine preparation.

However, much of his efforts were hampered by the restrictions placed upon him by the authorities resulting in no accurate determination of the protective efficacy of the vaccine in other regions could be calculated.

From 1896-1899 he conducted the first properly controlled field trials using his cholera vaccine, in the Cachar tea plantations. In this study, 5 778 persons were vaccinated, and 6 549 acted as controls. In the ensuing cholera epidemic, 198 cases (3.0% incidence) of cholera occurred in the control group associated with 124 deaths (mortality rate of 62.6%), whereas only 27 cases (0.5% incidence) occurred in the vaccinated group but was associated with 14 deaths (mortality rate of 51.9%). The protective efficacy of Haffkine's vaccine was 83.3% which was statistically significant. Vaccination had no effect on the mortality rate associated with cholera.[334]

Despite Ferran leading the way, it was Haffkine who definitively demonstrated the efficacy of vaccination against cholera in a well-controlled field trial.

3.7.2 *Review of the Field Trial Evaluation of Parenteral Cholera Vaccines*

As with the parenteral typhoid vaccination, despite the success of the approaches of Ferran and Haffkine late in the 19th century, there was no adequately controlled study into the usefulness of killed parenterally administered cholera vaccines until, once again carefully planned WHO sponsored field trials were performed the 1960's.

East Pakistan (Bangladesh) 1963

This field trial conducted by the Pakistan-SEATO Cholera Research Laboratory in Dhaka represented the first randomized double-blind controlled trial of a standardized killed parenterally administrable cholera vaccine in humans.[335]

The field trial area comprised 23 villages in the densely populated Matlab Thana region of the Comilla district of East Pakistan, about 40 miles

from Dhaka. This region was selected due to the high population density (>590 people/km^2) and because of the persistent high prevalence of cholera documented in the region.

The vaccine used was a commercial preparation containing a mixture of Inaba (strain 35A3) and Ogawa (strain 41) serotypes which had been phenol-inactivated (0.5%) and divided into 0.5 ml doses containing $8x10^9$ organisms/ml. The control vaccine contained $1x10^9$ heat-phenol-inactivated *S. typhi* and $7.5x10^8$ *S. paratyphi* A and *S. paratyphi* B respectively. The vaccine was administered as single doses delivered by jet injectors of 0.5 ml for subjects over 12 years of age, 0.25 ml for subjects 2-12 years, and 0.1 ml for subjects under 2 years of age.

The side-effects observed were apparently sufficiently severe to result in the dose to those over 12 years to be reduced to 0.4 ml after 7 027 vaccinations because they impaired adult participation in the study.

The diagnosis of cholera was confirmed by an acute onset episode of diarrhoea associated with the bacteriological isolation of the organism from faeces.

In total, 6 956 subjects received the parenterally administered phenol-inactivated cholera vaccine, and 7 102 received the combined *S. typhi/S. paratyphi* A & B vaccine; the total of 14 059 subjects representing 50.9% of the regional at risk population. At the end of the first year, the incidence of bacteriologically confirmed cholera in the cholera vaccinated group was 170/100 000, and in the typhoid/paratyphoid vaccinated group 610/100 000. This difference was statistically significant ($p<0.001$) and corresponded with a vaccine protective efficacy of 71.5%.

This study demonstrated that this particular cholera vaccine preparation, which was known to have unusually high mouse potency, was

able to induce a significant degree of protection on members of an endemic community after a single dose.

The Philippines 1964

The first WHO sponsored field trial of parenterally administrable killed cholera vaccines, was performed in the Philippines in 1964.[336] The study was a randomized double-blind controlled trial that adhered to the principles of strictly controlled field trials.[337] The Philippino field trial area comprised the Negros Occidental province, an area of 7 740 km^2 containing more than 1.3 million people. The overall cholera incidence was expected to be 50/100 000 population.

The vaccines used in this study were: a cholera vaccine prepared as for the East Pakistan study above using the same strains; a cholera El Tor fluid vaccine prepared from lyophilized cultures of *V. cholerae* El Tor Ogawa strain 1418 and Inaba strain 6973; a cholera oil-adjuvant vaccine prepared by H. Ogonuki of the Chiba Serum Institute, Japan using the Inaba strain 35A3 and Ogawa strain 41; and a monovalent typhoid vaccine as a control. The oil adjuvant used was a 9:1 mixture of a highly pure liquid petroleum mineral oil and an emulsifying agent (mannide mono-oleate).

Each vaccine dose comprised a concentration of $8x10^9$ heat-phenol-inactivated organisms/ml. The classical cholera and cholera El Tor vaccines' doses comprised 0.25 ml for subjects under 4 years of age, 0.5 ml for subjects 5-9 years, and 1.0 ml for subjects over 10 years. The cholera oil-adjuvant vaccine was administered in doses of 0.05 ml, 0.10 ml and 0.20 ml to the above age groups respectively. All vaccinations were administered as a single subcutaneous injection in the upper arm to all subjects. In total 584 000 subjects were vaccinated, 146 000 in each vaccine group.

Several adverse reactions observed at an overall frequency of 301/100 000 following vaccination including erythema, swelling, pain, induration, fever and malaise. The erythema and induration were more pronounced with the oil-adjuvant vaccine. The cholera, cholera El Tor and control typhoid vaccines accounted for only 0.8%, 1.7%, and 1.4% of the more severe reactions observed, whereas the oil-adjuvant vaccine accounted for 96.1% including a large number of subjects who developed a hard mass (119/100 000), ulcer (81/100 000) or sterile abscess (99/100 000) at the site of injection.

In assessing vaccine protective efficacy, only bacteriologically confirmed cases of cholera were recorded. Following vaccination, it was observed that:

(a) the cholera vaccine had a protective efficacy of 48% during the first four months only, failing to provide any protection after that time;

(b) the cholera El Tor vaccine had a protective efficacy of 55% during the first four months, falling to 26% during the next three months, and then also failing to provide any protection after seven months;

(c) and the oil-adjuvant vaccine provided 66% protection over the first seven months, still providing 50% protection after ten months.

Furthermore, the clinical course of the disease was independent of vaccination.[338]

The conclusion was that the cholera vaccination with heat-phenol-inactivated vaccine was able to provide a limited short-term degree of protection upon the recipients, and that the addition of the oil-adjuvant enhanced the magnitude and the duration of the protection but at the expense of frequent and often severe adverse reactions.

The Philippines 1966/67

A follow-up randomized double-blind controlled trial was organized in the Negros Occidental province of the Philippines to evaluate the degree and duration of protection afforded by one or two doses of the cholera El Tor vaccine described above (Section 6.2.2).[339,340] In total, 359 600 subjects received two doses of the vaccines preparations, the vaccine dose schedules and the protective efficacy associated with those schedules are detailed in Table 3.4.

The best protection afforded after six months was 58%, conferred by the single subcutaneous dose of 1.6×10^{10} heat-phenol-inactivated organisms. While this effect was statistically significant, it was not significantly greater than that afforded by one or two doses of 8×10^9 heat-phenol-inactivated organisms (protective efficacies of 53% and 55% respectively).

TABLE 3.4

RESULTS OF PHILIPPINES' FIELD STUDY 1966-67

Group	Number Vaccinated	Cholera Cases	Incidence ($/10^5$)
A	89 100	19	21.3
B	89 350	18	20.1
C	90 250	17	18.8
D	90 900	41	45.1
Total	359 600	95	26.4

A - Single subcutaneous dose of killed cholera El Tor vaccine (8×10^9 organisms)
B - Two subcutaneous doses of killed cholera El Tor vaccine ($2 \times 8 \times 10^9$ organisms)
C - Single subcutaneous dose of cholera El Tor vaccine (1.6×10^{10} organisms)
D - Control population (two subcutaneous doses of killed monovalent typhoid vaccine)

East Pakistan (Bangladesh) 1966/1967

A cohort of 40 000 children aged 0-14 years was divided into four groups, a control group and three vaccine groups.[341]

Vaccination occurred annually for three years prior to the cholera season, and follow-up continued for an additional two years. The vaccines used were a tetanus-diphtheria toxoid vaccine and a phenol-inactivated bivalent cholera vaccine comprising $4x10^9$ killed Ogawa strain 41 and Inaba strain 35A3 serotype organisms respectively (total dose $8x10^9$). All vaccines were administered subcutaneously as 0.5 ml vaccine doses.

From this study it was apparent that there was increasing protection with repeated revaccination, which appeared to be at a maximum following three doses. The observed protective efficacies were: one dose 43%; two doses 64%; three doses 81%; four doses 76%. The duration of protection was more sustained following the four dose schedule with 50% and 39% protection being afforded one and two years after the completion of the vaccination schedule respectively. There was no significant protection afforded in the two or less dose schedules two years after the last dose.

East Pakistan (Bangladesh) 1968/69

The 1968/69 cholera field trial involved a similar population to that described in Section 6.2.4, 45 000 children aged 0-14 years in the Matlab district.[342] The vaccines used in this study were: monovalent classical *V. cholerae* Ogawa strain 41 and a monovalent classical *V. cholerae* Inaba strain 35A3 both formalin-inactivated and each vaccine preparation comprised $8x10^9$ killed organisms/ml. These two whole cell vaccines were administered as a single subcutaneous dose of 0.5 ml annually for two years. A purified

V. cholerae El Tor Inaba strain V86 LPS antigen preparation was also used, each subject receiving a single dose of 0.100 mg.

During the ensuing cholera epidemic, which was almost exclusively due to infection with the Inaba serotype, it was observed that two annual injections of the monovalent whole cell Inaba vaccine afforded the greatest protection averaging 84% over the three years of follow-up; the single injection of the purified Inaba antigen preparation gave significantly less protection of only 51%; two annual injections of the whole-cell Ogawa vaccine failed to protect children under the age of 5 years, but conferred 48% protection against Inaba cholera on those 5-14 years old. It was observed that despite the Ogawa vaccine producing very high anti-Inaba serum antibody titres in young children, it failed to protect them. The cross-protection seen in the older children was attributed to boosting of naturally acquired immunity in this population group.

The Philippines 1970/71

Another extensive field trial of 223 566 subjects was conducted in the province of Negros Occidental of the Philippines during 1970/71.[343] This study was conducted to evaluate the protective efficacies of four monovalent cholera vaccines: a monovalent El Tor Inaba vaccine containing $8x10^9$ heat-phenol-inactivated *V. cholerae* El Tor Inaba strain 8273 and strain 6973 organisms/ml; a monovalent El Tor Ogawa vaccine containing $8x10^9$ heat-phenol-inactivated *V. cholerae* El Tor Ogawa strain 299 and strain 1418 organisms/ml; a lyophilized monovalent classical Ogawa vaccine containing $8x10^9$ formalin-inactivated classical *V. cholerae* Ogawa strain 41 organisms/ml; and a lyophilized monovalent classical Inaba vaccine containing $8x10^9$ formalin-inactivated classical *V. cholerae* Inaba strain 35A3

organisms/ml. A heat-phenol-inactivated monovalent typhoid vaccine preparation containing $1x10^9$ organisms was used as a control vaccine.

In the subsequent seven month follow-up period, all four types of the cholera vaccines used offered significant degrees of protection against the *V. cholerae* El Tor Ogawa strain causing the epidemic in the field trial region, with protective efficacies varying from 58% to 71%. The Ogawa vaccine preparations afforded slightly better protection than the Inaba vaccines, although this difference was not statistically significant. In addition, no significant difference could be demonstrated with respect to the biotype of the vaccine preparation used.

In the 1968-69 East Pakistan (Bangladesh) vaccine study,[342] it had been shown that the classical Inaba monovalent cholera vaccine protected only against the homologous classical Inaba infection, with the classical Ogawa vaccine conferring no protection against the classical Inaba infection. Since the classical Ogawa vaccine induced good titres of heterologous vibriocidal antibody, but failed to protect against classical Inaba infection, it had been concluded that immunity related to the Inaba type-specific C antigen played a greater role in human protection than immunity related to the common A antigen.

This Philippines vaccine study demonstrated that the reverse was not true, in that vaccination with Inaba vaccine afforded protection against classical Ogawa infection. Despite Ogawa strains having a small proportion of the C antigenic component, anti-C antigen antisera had been reported to not be protective against experimental Ogawa infection.[343] The combined results of these two field studies suggested that immunity against Inaba infection was dependent only upon the presence of anti-C antigen

component antibodies, and that immunity against Ogawa infection was dependent upon either anti-A antigen component (the "common antigen") or the specific anti-B antigen component. This would then provide a satisfactory explanation for the ability of Inaba organisms to protect against Ogawa infection, but not vice versa.

Surabaya, Indonesia 1973/75

The extensive investigations of the protective efficacies of parenterally administered inactivated whole-cell and purified component cholera vaccines as detailed above, all indicated that the protective efficacies of these types of cholera vaccines were approximately 50% with the duration of protection lasting little more than six months. As a result, some investigators were moved to argue that parenteral cholera vaccination was neither of benefit to the recipient in protecting them against the disease nor was economical or cost-effective.[341,342] In fact it was was further suggested that it was more cost-efficient to treat cholera patients with oral rehydration therapy than to vaccinate them against cholera.[341]

The poor immunogenicity was the main problem with these vaccines despite the several methods of preparation that had been utilized. The one previous attempt to use an adjuvant to enhance the immune response to a parenterally administered cholera vaccine preparation had resulted in severe adverse reactions precluding it from widespread human use.[336]

The purpose of this Indonesian field trial was to evaluate a new preparation of the whole-cell killed cholera vaccine incorporating another adjuvant, and to compare its protective efficacy with that of the former vaccine preparations.[344]

Surabaya, the field trial area, was the capital of the East Java province with a population of 1 500 000 in 1971 and a population density of 19 000/km^2. Between 1970 and 1972 the cholera incidence was 74, 23 and 60/100 000, with the cholera season occurring from May to July (the dry season).

The vaccine preparations used were; a "conventional" plain whole-cell killed cholera bivalent vaccine containing a total of $1.6x10^{10}$ killed classical *V. cholerae* Inaba strain 35A3 and Ogawa strain 41 organisms ($8x10^9$ of each)/ml and prepared by inactivation with 0.02% Thiomersol and heating to 56°C for one hour; an aluminium hydroxide-adsorbed vaccine prepared by adding 1.6 mg/ml of aluminium hydroxide to the plain vaccine detailed above;[345] and a control vaccine preparation comprising aluminium phosphate-adsorbed tetanus toxoid. All vaccines were administered as a single subcutaneous dose. The numbers of subjects in the three groups were 156 300, 155 600, and 158 500 respectively.

Throughout the fourteen month follow-up the predominant cholera vibrio was *V. cholerae* El Tor Inaba. In the vaccinees aged 1-4 years the protective efficacy of the aluminium hydroxide-adsorbed cholera vaccine was 88% during the first six months falling to 50% after 14 months, whereas in the same age group the plain vaccine had a protective efficacy of only 53% after the first six months, and failed to provide a significant degree of protection after that. In those subjects over five years of age, both vaccine preparations afforded protective efficacies of 50%-60% throughout the fourteen month period of observation. The aluminium hydroxide-adsorbed cholera vaccine was associated with an increase in the number and severity of the side-effects usually reported following the parenteral administration of

a plain cholera vaccine,[336] but this increase was not significantly different from the plain vaccine used in this study.

Once again, this study has demonstrated that the maximal protective effect of parenterally administrable killed cholera vaccines was observed in young children, under five years of age.

Calcutta, India 1975/77

The results of the aforementioned field trial using an aluminium hydroxide-adsorbed inactivated cholera vaccine, were repeated in a similarly constructed field trial performed in eastern Calcutta during the period 1975 to 1977.[346]

The vaccine preparations used in this study were: a plain bivalent cholera vaccine prepared identically to that used in the Surabayo, Indonesia field trials and containing the same numbers and strains, was aluminium phosphate-adsorbed by the addition of 3 mg/ml of aluminium phosphate to the plain vaccine preparation. The same aluminium phosphate-adsorbed tetanus toxoid vaccine was used as the control vaccine.

The protective efficacy of this cholera vaccine formulation in children under five years was shown to be a statistically significant 100% for the first six months, 88.9% for twelve months and 91.7% for eighteen months. No protection was conferred upon these recipients after eighteen months. In those over five years of age, the protective efficacy of the vaccine during the first six months was 51.9% falling to 35.7% after eighteen months. However, there was still a protective efficacy of 255 at the end of two years, suggesting that in vaccinating individuals over the age of five years, a boosting of naturally acquired immunity occurs, something which has not yet developed

in those subjects under five years of age. This finding was also implied in an earlier study.[342]

3.8 ANALYSIS OF THE HUMAN IMMUNE RESPONSE TO PARENTERALLY ADMINISTERED KILLED CHOLERA VACCINE

3.8.1 *Induction of Specific Intestinal Antibody*

There has only been one study investigating the ability of parenterally administered inactivated whole-cell cholera vaccines to induce an anti-cholera specific intestinal antibody response.[347] In this particular study a large number of volunteer subjects were drawn from the local population of Calcutta, an area where cholera was endemic at that time, although none of the subjects used had any previous history of cholera in the previous six months and all returned a stool culture negative for *V. cholerae*.. Various cholera vaccine preparations were compared including some orally administered ones, some of these will be considered later. One group received the monovalent inactivated *V. cholerae* Inaba whole-cell vaccine containing $8x10^9$ organisms subcutaneously. Intestinal fluid from the duodenojejunal flexure was collected from all volunteers using a technique of intestinal intubation pre-vaccination (Day 0), and on Days 14 and 28 post-vaccination. Specific anti-cholera antibody production in the intestinal fluid of these volunteers was determined using an infant mouse model and an opsonization test with the mouse peritoneum assay. By either method, a significant rise in intestinal specific anti-cholera antibodies following parenteral immunization could not be determined at any of the collection times, although 15% of subjects did elaborate an elevated pre-vaccination

anti-cholera specific antibody level, probably reflecting asymptomatic environmental exposure.

The poor protective efficacies of this type of cholera vaccine, even when utilized in endemic areas where the administration of any preparation by any route could conceivably be regarded as boosting any pre-existing naturally acquired immunity,[347] and the absence of any significant increases in cholera-specific intestinal antibody, further supported the view that the stimulation of local antibody may be more important in protecting against cholera infection.

3.8.2 *Induction of Specific Serum Antibody*

Despite evidence existing that suggested that the measurement of serum cholera-specific antibody was not the optimal method of assessing protective immunity against reinfection, collection of serum was the easiest procedure that could be adequately performed in the field. The serum vibriocidal assay described in Section 3.6.3, had been developed to permit the field determination of a specific immune response following clinical infection, and rises in the serum vibriocidal antibody titre had been demonstrated to indicate a reduction in the incidence of cholera in a specific area.[274,310]

As a result of that promising survey, serum vibriocidal antibody titres were determined in several studies following parenteral vaccination with the inactivated cholera vaccines,[348-350] to assess the predictive value of this assay in assessing the likely protective efficacy of any particular cholera vaccine.

In a study involving volunteer subjects from a non-endemic area designed to assess ability of several different cholera vaccine preparations to

stimulate a serum cholera-specific antibody (vibriocidal) response,[351] it was observed that:

(a) two subcutaneous doses administered two weeks apart stimulated vibriocidal responses greater than those of a single doses, or two doses four weeks apart;

(b) the second dose, administered on the fourth week, resulted in virtually no boost in the serum vibriocidal response, except for a few subjects who failed to have a detectable response following the first dose;

(c) no dosage regimen significantly affected the pattern of decline of vibriocidal antibody, following the primary immunization;

(d) revaccination at six months resulted in vibriocidal titres lower than those following the primary vaccination.

It was also observed that those subjects with elevated baseline vibriocidal titres, although most without having had any previous exposure to *V. cholerae*, had the greater vibriocidal responses following the initial vaccination. This finding was supported in another study examining the serum vibriocidal antibody response to revaccination of a closed community in an endemic area subject to repeated annual vaccination.[352] In this study significant increases in serum vibriocidal antibody were observed, especially in the 2-14 year age group with geometric mean rises of 646% who had quite low pre-vaccination titres, but with much lower rises of only 251% in those subjects over 15 years with elevated baseline titres. It was concluded from that study that revaccination against cholera using a parenterally administered inactivated whole-cell cholera vaccine appeared to be only useful as a booster for subjects having low vibriocidal titres.

In addition, in an endemic area the absence of any effect on the primary serum vibriocidal response with repeated vaccinations has been also

observed.[341] The previous findings that serum vibriocidal antibody titres may be a useful predictor of protective immunity following naturally acquired infection did not hold completely in the case of parenteral vaccination.[274,310,353] Although in some instances the serologically predicted level of protective immunity approximated the observed protection, the levels of vibriocidal antibody titres could not be directly related to levels of protection.[341]

It appears that serum antibodies may only be a measure of the extent of prior cholera exposure and therefore be indicative of herd immunity but not individual resistance to disease. Predictive immunity in an individual cannot be determined from the presence of specific serum antibody.[350]

It has also been demonstrated that the parenterally administered inactivated whole-cell cholera vaccines failed to stimulate any serum anti-cholera toxin immunity,[341,349,352] considered to be also important in the protective immune response.

3.9 ORAL VACCINATION AGAINST CHOLERA

3.9.1 *Inactivated Whole Cell Vaccines*

As was the case with orally administered typhoid vaccines, the use of the oral route for cholera vaccination has waxed and waned in popularity during the last 100 years. Ferran, as detailed in Section 1.1, was the first to advocate the use of live attenuated oral cholera vaccines, and demonstrated their protective value in humans.[6]

Sawtschenko and Sabolotny 1896

Although it was Klemperer in 1892 who claimed that he was able to protect guinea pigs against oral infection with *V. cholerae* by the administration of small doses of viable organisms into their stomachs,[7] it was Sawtschenko and Sabolotny who in 1893, orally vaccinated themselves and one other human subject with numerous doses of agar-grown heat-inactivated *V. cholerae*, were able to demonstrate that their serum obtained 25 days after vaccination was completed, was able to protect guinea pigs against virulent challenge.[253]

Furthermore, these two investigators, following a further oral dose of the vaccine preparation, ingested 0.1 ml of a 24 hour culture of *V. cholerae* O1, neither of them showed any clinical signs of cholera - despite both subjects excreting viable *V. cholerae* up to three days post-challenge. This study represents the first in humans showing the protective effect of large multiple orally administered doses of killed whole cells.

Metchnikoff 1896

It was Metchnikoff in 1894 who was able to demonstrate with his infant rabbit model that parenteral vaccination did not protect them against developing symptomatic infection upon oral challenge with pathogenic organisms.[354] However, during the subsequent years, parenteral cholera vaccination was applied extensively in humans in large field trials, with apparent success (Section 3.7). In 1910, Metchnikoff with Choukevitch,[355] repeated their studies in the infant rabbit. Three groups of infant rabbits were involved in this study. One group of infant rabbits received two subcutaneous doses of the killed cholera vaccine, commencing on the third day after birth, and the second dose seven days later. This group and a

control group were orally challenged with live pathogenic *V. cholerae* on the 17th day after birth, that is seven days after the last subcutaneous injection of the vaccinated group. In the vaccinated group 14/19 rabbits died from cholera, while only 6/12 control rabbit died. The third group, which was also vaccinated subcutaneously using the same schedule as detailed above, were completely protected against an intraperitoneal challenge with live pathogenic *V. cholerae* organisms.

Metchnikoff (and Besredka[9]) both concluded that this repeated study demonstrated that parenteral vaccination was ineffective against clinical cholera.

Once again, it was Besredka's bilivaccine formulation that became the most widely used oral cholera vaccine between the two World Wars.

Zabolotny advocated the use of mass oral vaccination using a killed cholera vaccine preparation,[356] as a result of his preliminary studies demonstrating that doses of $1x10^9$ to $1x10^{10}$ induced significant rises in serum anti-cholera agglutinating and vibriocidal titres. Other investigators using three orally administered daily doses of Besredka's bilivaccine containing $5x10^{10}$ heat-inactivated *V. cholerae* organisms were able to demonstrate significant rises in serum agglutins in all recipients by Day 19,[357] which persisted for as long as nine months, although diminishing to half the maximal titre after the first four months.[358]

Pondicherry, India 1925/26

The efficacy of this type of vaccine preparation, the cholera bilivaccine, was demonstrated in several extensive field trials conducted during the 1920's. The first major demonstration was during an outbreak in Pondicherry in India during 1925/1926.[9] At the commencement of the

epidemic 5 200 subjects were vaccinated orally using the same vaccination schedule as for the typhoid bilivaccine preparation (Section 2.8.3). There were no recorded cases of cholera in the vaccinated group of subjects, while there were 1 039 cases in the unvaccinated population. This spectacular success was similar top that encountered with typhoid vaccination using the bilivaccine formulation.

Madras, India 1925/27

In this large-scale field trial conducted in 360 villages in the Cauvery River region, two cholera vaccine formulation were compared to a control population.[359] The vaccines used were the standard heat-inactivated subcutaneously administered cholera vaccine ($8x10^9$ killed organisms/dose), and the orally administered cholera bilivaccine preparation of Besredka ($7x10^{10}$ killed organisms/dose). In total, the parenteral vaccine was administered exclusively in 236 villages, the oral vaccine exclusively in 52 villages, and both in 72 villages.

The results are best summarized as follows:

(a) In the villages receiving only the oral bilivaccine there were 18 cases of cholera in the 4 982 vaccinated subjects (incidence 0.36%), whereas there were 222 cases in the 11 004 unvaccinated control population (incidence 2.02%);

(b) In the villages receiving the parenteral vaccine only there were 31 cases of cholera in the 8 485 vaccinated subjects (incidence 0.37%), compared to 489 cases in the 29 254 unvaccinated control subjects (incidence 1.67%);

(c) In the villages where both formulations were used there were 15 cases of cholera in the 3 085 orally vaccinated subjects (incidence 0.49%), six

cases in the 1 448 subcutaneously vaccinated subjects (incidence 0.41%) and 160 cases in the 7 664 unvaccinated control subjects (incidence 2.2%).

This study confirmed that the oral route of vaccination using the cholera bilivaccine was as efficacious as the subcutaneous route using killed whole organisms but without the frequency or severity of adverse reactions. Overall, the number of cases of cholera in the vaccinated groups irrespective of the method of vaccination were 5.6 times less than in the unvaccinated control population.

Unlike oral typhoid vaccination, very little work was undertaken to ascertain the mechanism of protective immunity induced through the oral route, although most commentators did agree that the disease was toxin mediated and the effect probably local,[360] necessitating local immune mechanisms.

3.9.2 *Cholera Protein Extract Vaccine*

In 1975, Dodin and Wiart of the Institute Pasteur, Paris, described the isolation and purification of an antigenic component from *V. cholerae* lysate.[361] This antigen was characterized and found to be a 16 amino acid peptide of approximately M_r 57 500. This antigen appeared to be serotype specific, that is, a similar protein could be extracted from both the Ogawa and Inaba serotypes, there being only some cross-specificity demonstrated by hyperimmune sera raised in rabbits, protective in mice against pathogenic *V. cholerae* challenge and capable of inducing an agglutinating and vibriocidal response in the serum of rabbits, donkeys and humans greater than that induced by the heat-inactivated subcutaneously administered cholera vaccine.[362]

In human volunteer subjects, 50 mg of this preparation orally administered induced serum agglutinins and vibriocidal antibodies that persisted for as long as that induced by 0.100 mg of this preparation administered subcutaneously, four months.[362,363]

Malemba-NKulu, Zaire 1983

The efficacy of the Institute Pasteur oral cholera vaccine preparation was evaluated in a controlled field trial performed in the Malemba-NKulu district of Shaba province in Zaire in 1983.[364,365] This region had been involved in annual outbreaks of cholera since 1978, an outbreak occurred seven months after the vaccine was administered in this study. There were 216 cases of cholera in the 18 377 unvaccinated control subjects (incidence 1.17%), 57 cases in the 6 249 subjects who received the commercial heat-inactivated subcutaneously-administered cholera vaccine (incidence 0.91%), and only 6 cases in 12 014 subjects who received the orally administered purified cholera protein extract vaccine (incidence 0.05%). These differences were found to be statistically significant, and indicated that the protective efficacy of this vaccine preparation was 97.9% compared with 22.4%, that being the protective efficacy of the commercial parenterally administered cholera vaccine in this population.

3.9.3 *Immunogenicity of Cholera Protein Extract Oral Vaccine*

This vaccine preparation, which has recently been described as comprising a lipopolysaccharide-outer membrane protein complex released by intact *V. cholerae* as particles into culture medium,[366] was prepared from two strains of *V. cholerae* (El Tor Ogawa strain HK1 and El Tor Inaba strain Ekoko) separated from the culture medium, and lyophilized and placed into

enteric-coated granules. Each vaccine dose consisted of 215 mg of these enteric-coated granules containing 0.5 mg of complexes from each strain. Eighteen adult subjects received either two or three doses of the vaccine seven or fourteen days apart. Significant secretory IgA antibody responses against the complex were detectable in the jejunal fluid of vaccinated subjects post-vaccination, with no correlation being found between the salivary and intestinal antibody responses. Serum vibriocidal antibodies were also detectable in 8/18 (against Inaba strains) and 9/18 (against Ogawa strains) and were reported to be of the same magnitude as those observed following immunization with the orally administered B subunit whole-cell cholera vaccine (Section 7.3),[311] that is, approximately two-fold increases.

3.9.4 Vibrio cholerae *L forms, 1971*

A *V. cholerae* Ogawa L-form lysate, filtered through sintered glass and heat-inactivated was shown to be immunogenic when orally administered as a 10 ml dose to six human volunteer subjects following their ingestion of two tablets of aluminium hydroxide gel. Each dose was administered twice a week to a total of five doses.[367]

This vaccine preparation was shown to induce a rise in specific coproantibody from a baseline titre of 1:10 to a peak of 1:640, before declining after three to four weeks to 1:160. Similarly there was a gradual rise in indirect haemagglutinating antibodies which peaked later and substantially higher than the coproantibody titres. This was supported by a substantial increase in the vibriocidal antibody. All antibody responses were measurable against both homologous and heterologous serotypes of either biotype.

The protective efficacy of this rather crude vaccine preparation has never been published if it was, in fact, ever assessed.

3.10

CHOLERA TOXIN B SUBUNIT WHOLE-CELL CHOLERA VACCINE

The combined cholera toxin B subunit whole-cell cholera vaccine was developed as a result of the synergistic amplification effect that concurrent administration had on the resulting immune response in the intestine of rabbits, which was associated with a greater degree of protection against challenge with pathogenic organisms than which was achieved with the individual components separately administered.[321] The intestinal immune response in humans to two doses of 10^{11} phenol-inactivated classical Ogawa and Inaba *V. cholerae* organisms orally administered 14 days apart had been investigated by Ganguly *et al.*[347] and, in their assay systems, demonstrated to be poorly immunogenic, with only 9/21 volunteer subjects having detectable levels of anti-cholera antibodies in their intestinal fluid during at any time the first month following vaccination. A similar situation was found to exist for the adsorbed oral cholera toxoid vaccine and with the purified B subunit of cholera toxin alone (Section 5.6.4), although orally administered cholera toxin B subunit has been shown to stimulate good intestinal antitoxin antibodies when orally administered to volunteer subjects in endemic areas.[368]

Bangladesh 1981

A combined purified B subunit whole-cell cholera vaccine (referred to from here as B-WCV) was first administered to a group of Bangladeshi volunteer subjects in 1982.[304] The B-WCV comprised 0.5 mg or 2.5 mg of B

subunit in 1.0 ml of 0.85% sodium chloride solution and containing 10^{10} of heat-thiomersol-inactivated classical *V. cholerae* Ogawa and Inaba organisms respectively. The vaccine was administered as two oral doses 28 days apart, each dose preceded by 100 ml of 0.1 mol/l sodium hydrogen carbonate solution.

In this population, it was observed that a 2.5 mg dose of purified B subunit was as effective as clinical cholera in inducing a local intestinal secretory IgA antitoxin response in frequency and in magnitude. The 0.5 mg dose was found to be inadequate as a stimulant of local intestinal immunity when compared to the 2.5 mg dose. The second oral dose of B subunit induced antitoxin responses that were more frequent and of greater magnitude than those induced by an initial 0.5 mg dose. In those subjects who received the 2.5 mg oral primary dose, their response to the 0.5 mg second dose was identical to that of convalescent cholera patients. The B-WCV was only able to induce irregular increases in intestinal specific anti-cholera LPS IgA antibody responses of significantly lower magnitude than those observed following clinical cholera where significant intestinal anti-LPS antibody responses evident in 8/9 patients. After two doses the responses in the vaccinated subjects were better with an increase from 8/14 to 12/13 responders (a two-fold or greater rise in specific intestinal anti-LPS antibodies).

A marked difference was observed between the antibody responses of serum and the intestine which was attributed to the fact that the antibody in the intestine was of local origin. This was supported by the observation that the serum IgG response to cholera toxin was lower after disease and vaccination than following parenteral immunization, yet the specific IgA

response in the intestine was substantially greater after disease and oral vaccination than parenteral vaccination.

Sweden 1983

Since it was possible that the good immune responses to the vaccine were a result of boosting pre-existing immunity from previous disease or environmental exposure, 27 Swedish volunteer subjects with no previous history of exposure to cholera were orally vaccinated with B-WCV.[369] Subjects received either 0.5 mg or 2.5 mg of purified B subunit with the 10^{10} heat-thiomersol-inactivated classical Ogawa and Inaba strain organisms as their primary vaccination, and 0.5 mg B subunit with the inactivated organisms as a booster vaccination. In this group of volunteers subjects, a single oral immunization with the 0.5 mg B subunit dose combined vaccine induced a significant (two-fold or greater) intestinal antitoxin IgA response in 4/6 (67%) vaccinated subjects, which was not enhanced by the additional dose. This response was independent of the dose of B subunit administered, as was the serum antitoxin response (four-fold or greater response). Furthermore, a single administration appeared to be as efficient as two doses in the production of antitoxin immunity.

Once again, single or repeated oral vaccination with the B-WCV resulted in the near absence of anti-LPS antibodies, and only modest vibriocidal antibody responses being significant (four-fold or greater response) in only 7/27 subjects following a single dose, and 9/27 after two doses.

This study confirmed that the responses to the vaccine in Swedish volunteers were identical in magnitude of final specific antibody titre as those observed in the Bangladeshi subjects, although the Swedish pre-

vaccination antitoxin titres were ten-fold lower than those of the Bangladeshis.

Baltimore USA, 1985

The protective efficacy of one particular preparation of this type of vaccine was assessed in North American volunteer subjects at the Center for Vaccine Development, Baltimore, USA.[305] The whole-cell preparation used in this study comprised $5x10^{10}$ each of heat-inactivated classical *V. cholerae* Inaba strain Cairo 48 and Ogawa strain Cairo 50 organisms, and $1x10^{11}$ formalin-inactivated *V. cholerae* El Tor Inaba strain Phil 6973 organisms all suspended in 8 ml of phosphate-buffered saline. When required, the purified B-subunit was administered as a dose of 5 mg.

The B-WCV was orally administered to nineteen subjects while another fourteen ingested the WCV without the B subunit. Of the B-WCV vaccine group, four-fold or greater rises in anti-cholera LPS IgA antibody titres were observed in the jejunal fluid of 53% of vaccinated subjects, and in the serum of 68%. Antitoxin antibody responses of the same magnitude were observed in serum of 100% of B-WCV subjects but in only 74% were jejunal secretory IgA antitoxin responses detectable. Serum vibriocidal antibody responses were significantly elevated in 89% of vaccinated subjects.

In the group receiving the whole-cell vaccine without B subunit, specific anti-cholera LPS jejunal secretory IgA responses were not detected in any subject, yet serum IgA anti-LPS responses being elevated in 57% of subjects. The serum vibriocidal antibody response was significantly elevated in 71% of vaccinated subjects; of course no antitoxin antibody response was detected in the either the serum or jejunal fluid of any volunteer.

Both groups failed to produce a measurable anti-OMP antibody response in jejunal fluid, yet such a response was detected in the serum of 58% of B-WCV subjects and in 57% of WCV recipients only.

Following oral challenge with $2x10^6$ viable pathogenic *V. cholerae* El Tor Inaba organisms the protective efficacy of the combined B-WCV vaccine was 64% against a dose that induced clinical illness in all control subjects (p=0.01), and the WCV without B subunit was 56% against a dose that induced clinical illness in 75% of control subjects (p=0.11). There was no significant difference between the protective efficacies of the two vaccine preparations, with or without the B subunit.

Matlab, Bangladesh 1985

As a result of the aforementioned clinical studies investigating the immunogenicity and protective efficacy of the B-WCV and the WCV alone in volunteer subjects, a large-scale randomized double-blind placebo-controlled field trial was undertaken in the Matlab area in Bangladesh during 1985.[370]

The trial population comprised 63 498 children aged 2-15 years, and women over 15 years. The whole-cell component of the vaccines used comprised $1x10^{11}$ total killed organisms made up of $2.5x10^{10}$ of each of heat-inactivated classical *V. cholerae* Inaba strain Cairo 48, heat-inactivated classical *V. cholerae* Ogawa strain Cairo 50, formalin-inactivated *V. cholerae* El Tor Inaba strain Phil 6973, and formalin-inactivated classical *V. cholerae* Ogawa strain Cairo 50. The B-WCV also comprised 1 mg of purified cholera toxin B subunit. An *E. coli* K12 strain prepared to the same optical density as the WCV was used as a placebo.

The vaccines were administered as single doses once a week for three weeks, although there were some variations on this schedule in a small number of participants.

The incidence of cholera and the protective efficacies (PE) of the respective vaccine groups versus the placebo group during the following six months post-vaccination surveillance, are presented in Table 3.5.

TABLE 3.5

EFFICACY OF THE ORAL KILLED WHOLE-CELL/B SUBUNIT COMBINATION CHOLERA VACCINE AND THE ORAL KILLED WHOLE-CELL VACCINE ALONE (DHAKA, BANGLADESH)

Vaccinated Subjects and Follow-up Period	Protective Efficacy (%) WCV	B-WCV
All vaccinated subjects		
Year 1	53	62
Year 2	57	57
Year 3	43	17
All three years	52	50
Protection by biotype*		
Classical	60	58
El Tor	40	39
Protection by age*		
< 6 years old	23	26
> 6 years old	68	63

WCV - Killed whole-cell vaccine only.
B-WCV - Killed whole-cell vaccine with cholera toxin B subunit.
Three doses of the vaccines or placebos were given at one month intervals.
None of the levels of protection for the WCV or B-WCV were significantly different ($p>0.05$).
* - Protection over three year period.

(adapted from World Health Organization Diarrhoeal Disease Control Programme 1989, reference 371)

This study demonstrated that the combined B subunit killed whole-cell cholera vaccine was able to induce significantly greater protective

immunity in the residents of an endemic area than the orally administered killed whole-cell cholera vaccine alone, and that this protective immunity remained significantly elevated above that of the WCV alone for at least six months. During the subsequent three years of observation vaccine efficacy gradually declined, so that by the end of the third year of post-vaccination surveillance the protective efficacy of the WCV alone was 43% and on the B-WCV only 17% (Table 3.5).[371]

Interestingly, this B-WCV appeared to provide the same degree of protection against clinical cholera in both children and adults.

3.11

LIVE ATTENUATED *V. cholerae* VACCINES

The guidelines for one approach to the development of a live attenuated cholera vaccine were outlined in 1971 by Howard.[372] His proposals concerning the development of a prototype live oral cholera vaccine, suggested that the ideal vaccine should continue to act antigenically for a prolonged period, something which could only be achieved with a particular kind of live orally administrable organism - an attenuated cholera bacterium. He developed candidate vaccine strains apparently deficient in the A subunit of cholera toxin, but essentially unchanged in all other respects, using chemical mutagenesis.

This approach was intended to result in a strain which not only produced local antibacterial immunity but also antitoxin immunity.

All the evidence acquired over the past thirty years indicate that cholera is a toxin-mediated disease, that antitoxin immunity is an important component of the protective immune response although antibacterial immunity may be of greater importance in establishing protection against

disease. Therefore, one possible approach to the development of an attenuated live cholera vaccine, was the development of a non-toxigenic *V. cholerae*, which should be able to colonize the small intestine as would the pathogenic strains, but due to its inability to produce cholera toxin, would be unable to induce disease.

3.11.1 V. cholerae *El Tor Ogawa strain EW-6, Calcutta 1968*

An environmentally isolated inherently non-toxigenic *V. cholerae* El Tor Ogawa strain EW-6, which had been confirmed as nonpathogenic in rabbits, was evaluated in a human study for safety, toxicity and immunogenicity in Calcutta in 1968, to assess the likelihood of it being a potential live oral cholera vaccine.[373]

The immunogenicity of this attenuated oral vaccine strain was also compared to that of an orally administered killed whole-cell vaccine in humans.

Thirty-five adult subjects aged between 22 and 58 years participated in the study; 25 of which received the live attenuated orally administered *V. cholerae* strain EW-6 and ten ingested killed strain EW-6 organisms. The 25 subjects were randomly allocated to four groups receiving two doses of the live vaccine administered seven days apart of $8x10^9$ followed by $4x10^{10}$ viable organisms. The first group of four subjects represented a pilot study receiving three doses sequentially of $2x10^9$, $8x10^9$, $4x10^{10}$ viable organisms seven days apart. All doses were administered following neutralization of gastric acid by aluminium hydroxide gel.

Following vaccination with the live strain EW-6 organisms, viable vibrios could only be isolated from the faeces of four subjects, between 24 and 48 hours following the administration of either the first or second

vaccine dose. The vaccine was well tolerated by all recipients with no adverse reactions reported.

Significant rises in the serum vibriocidal antibody response was evident in 22/25 subjects following vaccination with the live EW-6 strain organisms, this being detectable within ten days of vaccination ($p<0.01$) and remaining significantly elevated over the subsequent six months ($p=0.05$). Similarly, a coproantibody response was observed in all but one of the vaccinated subjects, evident from 8 days post-vaccination and persisting for up to 12 weeks, with only a slight decrease in magnitude. No elevation of serum vibriocidal antibody was detected in those subjects receiving the killed strain EW-6 organisms using the same dose regimen and schedule.

This study confirmed that non-toxigenic strains of *V. cholerae* El Tor were capable of colonizing the intestine of volunteer subjects in endemic areas for *V. cholerae* infection, resulting in the generation of both local and systemic immune responses.

Subsequently this candidate vaccine strain was evaluated for its immunogenicity and protective efficacy in previously unexposed volunteer subjects in the USA.

3.11.2 V. cholerae *El Tor Ogawa strains C14-S5 & EW-6, Baltimore, 1974*

These two candidate vaccine strains were evaluated in volunteer subjects at Baltimore in the USA, to assess their immunogenicity when orally administered as a variety of doses, and their likely protective efficacy against challenge with pathogenic *V. cholerae*.[374]

The *V. cholerae* El Tor Ogawa strain C14-S5 was an attenuated streptomycin-resistant dwarf vibrio, while the Ogawa strain EW-6 was a naturally (undefined) attenuated isolate from a water source in Calcutta,

previously demonstrated to be safe in both animals and humans.[368] In only one subject, who received 10^9 viable organisms of the strain C14-S5 were the vaccine organisms found tp be excreted and then only within 24 hours of ingesting the dose. Of the 32 subjects who participated overall, in only two were four-fold or greater rises in homologous serum vibriocidal titres demonstrated post-vaccination, and in one of these a significant heterologous vibriocidal antibody response was also demonstrated. One subject also had a serum antitoxin antibody response. When the five subjects who ingested the C14-S5 vaccine candidate at doses of 10^9 viable organisms were challenged along with eight unvaccinated controls with 10^4 live virulent *V. cholerae* Ogawa strain 395, 80% of the vaccinated subjects and 50% of the control subjects recorded an attack of clinical cholera, there being no difference in incubation times, stool grades or volumes nor duration of illness.

The absence of any evidence of colonization the EW-6 strain was considered surprising in view of the significantly elevated vibriocidal antibody titres reported previously to occur following the ingestion of this strain by humans.[373] However, in that earlier study conducted in Calcutta, it was be noted that 23 of the 35 subjects used had a past history of clinical cholera, therefore it was considered that previous exposure to either the vaccine or to other pathogenic strains may have resulted in an anamnestic response after exposure to the vaccine strain.[374]

3.11.3 V. cholerae *El Tor Ogawa strains 1196-78 and 1074-78*

South America has been spared the scourge of cholera since the middle part of the 19th Century, the reasons for this have not been satisfactorily elucidated. In 1978, Brazilian microbiologists isolated and identified *V. cholerae* O1 El Tor Ogawa in the sewerage water of Sao Paulo,

Brazil.[375] However, no outbreak of clinical cholera was ever identified, nor were these strains isolated from any patient presenting with diarrhoea. Both of these strains have been shown to lack the *tox* genes responsible for cholera toxin production.[376]

These strains were investigated and found to not produce cholera holotoxin or have A subunit activity.[377] The immunogenicity and pathogenicity of these environmental non-toxigenic strains were evaluated in previously unexposed volunteer subjects. Three groups of volunteer subjects were used, two groups orally ingested the 1196-78 strain at doses of 10^6 or 10^8 viable organisms, and the remaining group orally ingested the 1074-78 strain at a dose of 106 viable organisms. In no group did any subject develop a diarrhoeal illness. The stool cultures of the 1074-78 strain subjects were negative for challenge organisms, while 8/13 recipients of the 1196-78 excreted viable vibrios, independently of the magnitude of the challenge dose. Serum antitoxin antibody responses were not detected in any subject, and significantly elevated serum vibriocidal antibody responses were only recorded in 2/7 recipients of the 1074-78 strain, and 4/13 of the 1196-78 strain recipients (again irrespective of size of ingested dose).

One month later, four of the subjects who ingested the 1196-78 strain and had been noted to have positive stool cultures following that ingestion, along with six unvaccinated control subjects were challenged with 10^6 viable pathogenic *V. cholerae* El Tor Ogawa strain E7946 organisms. All subjects in both groups developed clinical cholera, there being no difference in the incubation period, number of stools or magnitude of stool volume. In addition all subjects faecally excreted the challenge strain, and there were no differences in the resulting serum antitoxin and vibriocidal antibody responses.

These studies indicated that nontoxigenic strains of *V. cholerae* are attenuated in humans, however that for such strains to be effective as vaccines they should avidly colonize the small intestine as has been demonstrated for all pathogenic strains.

3.11.4 V. cholerae *El Tor Ogawa Texas Star-SR*

Naturally acquired antitoxin immunity has been shown to have a significant role in protection against reinfection with *V. cholerae*. This has been utilized in the development of the orally administered B subunit whole-cell cholera vaccine described in Section 7.3. However, in that particular vaccine preparation, the duration of protection attributable to the antitoxin immunity was no more than four months, after which the antibacterial immunity provided the bulk of the protective immune response. The success of the B subunit whole-cell cholera vaccine in the field was still only marginal with rather average protective efficacies, certainly no better than a single subcutaneous dose of the commercial heat-inactivated cholera vaccine.

Since the specific bacterial antigens that have a major role in the conferring protective immunity have not been fully elucidated, and since cholera is a toxin-mediated disease, one approach to vaccine development has been to construct a toxin-less mutant of a *V. cholerae* O1 strain as a possible candidate oral vaccine. This approach provided the potential benefit of developing an attenuated vaccine strain which retained all the bacterial antigenic components, and was unable to induce clinical disease as a result of its inability to produce cholera toxin. The first of these toxinless mutants was the candidate vaccine strain *V. cholerae* El Tor Ogawa Texas Star-SR (from now on referred to as Texas Star-SR) which was derived through the nitrosoguanidine treatment of *V. cholerae* El Tor Ogawa strain 3083 resulting

in live mutant strain which elaborated the nontoxic immunogenic B subunit oligomer of cholera toxin, but was incapable of making the full cholera toxin, due to its inability to produce the A subunit.[378]

This strain was orally administered to a total of 68 volunteer subjects at the Center for Vaccine Development (CVD), Baltimore USA,[379] in doses varying from 10^5 to $5x10^{10}$ viable organisms with sodium hydrogen carbonate. Sixteen subjects (24%) were recorded as having experienced loose stools which appeared to have been independent of the size or frequency of the vaccine dose. Sixty-three vaccinated subjects (93%) were observed to have significant rises (four-fold or greater) in serum vibriocidal antibody, only 20 (29%) had significant rises in serum antitoxin antibody, while paired intestinal fluids from 41 subjects showed significant rises in jejunal secretory IgA against lipopolysaccharide (29%), Ogawa OMP (29%) and toxin antigens (12%).

Four to six weeks after vaccination, eight vaccinated subjects and four control subjects were challenged with 10^6 of the presumably pathogenic parent *V. cholerae* El Tor Ogawa strain 3083. Neither control nor vaccinated subjects developed clinical cholera, although all of the control subjects provided evidence of colonization with excretion of the challenge strain and significant rises in serum antitoxin and vibriocidal antibody titres. Since this was the dose that induced clinical illness in 70%-100% of subjects when challenged with all the other pathogenic strains in use at the CVD, it was concluded that the parent strain 3083 was of naturally diminished virulence despite its production of cholera holotoxin.

Subsequently, another group of seven vaccinated subjects who had received only a single dose of $5x10^{10}$ viable vaccine organisms eight weeks previously together with ten unvaccinated control subjects were orally

challenged with 10^6 homologous *V. cholerae* El Tor Ogawa strain E7946 organisms. Diarrhoea occurred in 7/10 control subjects and in 2/7 vaccinated subjects (protective efficacy of 59.2%, p=0.15). While significant protection was not afforded by this single dose, the stool volume was significantly reduced in the vaccinated group of subjects compared with the control group ($p<0.01$), and faecal excretion of challenge organisms was also reduced.

Eighteen subjects who received two doses of the vaccine strain (either 10^9 or $2x10^{10}$ viable organisms) seven weeks earlier and fifteen unvaccinated control subjects were challenged orally with 10^6 pathogenic heterologous *V. cholerae* El Tor Inaba strain N16961. Diarrhoea occurred in 11/15 control subjects (73%) but in only 5/18 previously vaccinated subjects (28%) (protective efficacy of 62.1%, $p<0.02$). The vaccine failures were not restricted according to dose, as they were identified in both dosage groups. Once again it was observed that the stool volume of the vaccinated group of subjects was significantly less than that of the control subjects ($p<0.01$), with another 100 fold reduction in the quantity of challenge organisms detectable in faeces.

No evidence of reversion to a toxigenic form was observed in any of 503 isolates from the faeces of vaccinated subjects screened.

Texas Star-SR induced a mild infection in subjects ingesting it which was associated with mild diarrhoea in 24% of recipients, induced significant vibriocidal antibody responses which however were significantly less than those found in convalescent patients, and induced significant antitoxin responses in only a few vaccinated subjects. The failure to detect good jejunal antibody responses was attributed to the collection of jejunal fluid on Day 8 post-vaccination, which may not be the optimal time for such sampling, in

addition the intestinal fluid was also heat-treated at 56°C for one hour prior to lyophilization - a technique which has been claimed to significantly reduce the quantity of secretory IgA present in intestinal fluid.[32]

Texas Star-SR represented a significant advance over the use of oral killed vaccine organisms and ill-defined attenuated environmental vibrios. However there were some acknowledged disadvantages using this particular candidate vaccine:

(a) the precise genetic lesion responsible for the inability to produce the A subunit of cholera toxin was not defined, allowing the theoretical possibility of reversion in the host or the environment;

(b) as was observed with the live oral typhoid vaccine *S. typhi* Ty21a, nitrosoguanidine frequently induces other mutations in the genome which may not be immediately evident;

(c) the reasons for the slow growth of Texas Star-SR and its failure to induce immunity comparable to virulent vibrios were not apparent.

Texas Star-SR, did represent an important step forward in cholera vaccine development and represented the prototype for a new approach to cholera vaccine development.

3.11.5 *Recombinant Non-toxigenic Mutant* V. cholerae *Strains*

The Texas Star-SR cholera strain, while not an entirely satisfactory vaccine candidate and one for a variety of reasons unsuitable for evaluation in the field, did provide one new direction in cholera vaccine development. The next prototype vaccine strains were a recombinant mutants of *V. cholerae* in which the genes encoding either the A or B subunits or both had defined deletions rendering them inactive. The first candidate, a *V. cholerae* El Tor Inaba strain N16961 in which genes encoding both the A and B subunits of

cholera toxin were deleted using site-directed mutagenesis with the insertion of a mercury-resistance gene. This putative vaccine strain was designated JBK 70.[380,381] Strains constructed through this approach are incapable of becoming toxigenic. In the initial evaluation of this strain in volunteer subjects at the Center for Vaccine Development, Baltimore, USA,[382] a total of 14 subjects ingested single doses ranging from 10^6 to 10^{10} viable organisms. Mild to moderated diarrhoea was observed in 1/4 (mean stool volume 543 ml), 2/5 (mean stool volume 1 180 ml) and 4/5 (mean stool volume 802 ml) subjects respectively. The stool volume ranged from 235 ml to 1 878 ml, and abdominal cramps were reported by all subjects with diarrhoea; one of which had rice-water stools. Other adverse reactions including anorexia, fever and vomiting were also recorded by recipients. Significant four-fold or greater rises in serum vibriocidal antibody titre were observed in all subjects, the magnitude of such, being as high as that observed in subjects challenged with pathogenic *V. cholerae*, and was far greater than that recorded in subjects following ingestion of the B subunit whole-cell cholera vaccine. The serum response to specific bacterial components such as purified LPS, TCP pili and OMP were less frequently detected and were significantly lower in magnitude than the vibriocidal titre. There was not a serum antitoxin antibody response in any of the vaccine recipients. In only some subjects was a specific intestinal secretory IgA response detectable, this absence of a consistent response was attributed to sampling before the optimal time. One month after vaccination, both the vaccinated subjects and a control group of unvaccinated volunteer subjects were orally challenged with 10^6 pathogenic *V. cholerae* El Tor Inaba strain N16961 in sodium hydrogen carbonate. Following challenge, diarrhoea was observed in 7/8 control subjects and in only 1/10 vaccinated subjects (protective efficacy of 88.6%, $p<0.003$),

indicating that this toxin-negative strain was capable of inducing highly significant protection. However, the frequency and magnitude of the adverse effects accompanying vaccination prohibited this vaccine strain from being seriously evaluated any further.[383]

Other *V. cholerae* strains have since been developed as either newly isolated recombinants, such as the classical *V. cholerae* Ogawa strain O395 in which the only the gene encoding for the A subunit of cholera toxin has been deleted (designated CVD 101),[381] as well as some being derivatives of both CVD 101 and JBK 70.

Several important points that have been derived from this work:

(a) Deletion of the structural genes for cholera holotoxin result in a strain incapable of inducing the massive purging diarrhoea of cholera gravis. These strains however are not completely attenuated and even at low doses of 10^4 live organisms are still able to induce mild diarrhoea in more that 50% of recipients;

(b) A single oral dose of an attenuated *V. cholerae* strain has been demonstrated to be capable of colonizing the small intestine and inducing vibriocidal antibody responses comparable to that induced by pathogenic strains, and, depending upon the vaccine strain used, strong serum antitoxin responses;

(c) Protection against cholera does not require antitoxin immunity as demonstrated by the strong protective immunity shown by subjects receiving the toxin-less mutants of *V. cholerae* and subsequently orally challenged with pathogenic organisms, protection which was equal to that conferred by pathogenic organisms (90%),[301] and significantly greater than that observed following the B subunit whole cell cholera vaccine (64%),[305] and from ingested killed whole cells alone (56%);[305]

(d) The mild diarrhoea observed even in cholera holotoxin-less mutants when administered to volunteers subjects may have been due to the presence of some other metabolic product, such as the haemolysin,[384,385] or the Shiga-like toxin (verotoxin).[386]

3.11.6 *Live Oral Cholera Vaccines CVD 103 and CVD 103HgR*

The attenuated live orally administrable candidate cholera vaccine strain designated CVD 103, was derived from classical *V. cholerae* Inaba strain 569B and was developed through recombinant techniques resulting in an organism that could only produce the non-toxigenic immunogenic cholera toxin B subunit.[382] The problems with diarrhoea encountered with the earlier strains developed through this technique were circumvented through the selection of the classical 569B strain which does not elaborate Shiga-like toxin, nor does it have a functional gene for production of El Tor haemolysin/cytotoxin. The strain CVD 103-HgR was prepared by the insertion of a gene encoding for mercury-resistance into the haemolysin locus, permitting the vaccine strain to be differentiated.

Both these vaccine candidates were evaluated in cohorts of 5-18 volunteer subjects at the Center for Vaccine Development, Baltimore USA. Each cohort received a single oral dose of either CVD 103 or CVD 103-HgR. In those subjects receiving 10^2 or 10^3 viable CVD 103 organisms, no adverse reactions were reported, however, only 33% of vaccinated subjects had four-fold or greater rises in serum vibriocidal antibody, with a geometric mean peak titre of only 1:120, and only 17% had significant rises in serum antitoxin responses.

In those subjects receiving an oral dose of 10^6 viable CVD 103 organisms, 17% had diarrhoea exceeding 400 ml (but less than 1 000 ml),

however 100% had significant serum vibriocidal (geometric mean titre 1:2873) and antitoxin responses; a dose of 2-3x10^8 viable organisms resulted in no further increase in the magnitude of the responses observed with 10^6 organisms, and 11% of subjects had diarrhoea with a stool volume exceeding 400 ml but less than 1 000 ml.

These responses were identical to those induced in volunteer subjects challenged with 10^6 viable classical *V. cholerae* Inaba strain 569B organisms, except the numbers and volume of diarrhoetic stools were substantially more in the clinical cholera subjects.

The vaccine strain CVD 103-HgR was administered as either a freshly harvested preparation or as a lyophilized and reconstituted preparation to a group of previously unexposed volunteer subjects. Only 1/25 subjects experienced a single loose stool of more than 400 ml volume, 24/25 had significant serum vibriocidal antibody responses (geometric mean tire 1:1810) and 22/25 had significantly elevated antitoxin responses. There were no significant differences between the responses observed between CVD 103 and CVD 103-HgR.

The groups vaccinated with the CVD 103 organisms were divided into three equal groups and each group was subsequently challenged with 10^6 viable pathogenic organisms of either classical *V. cholerae* Inaba strain 569B or classical *V. cholerae* Ogawa strain O395 or *V. cholerae* El Tor Inaba strain N16961 along with the relevant unvaccinated control subjects. The recipients of CVD 103-HgR were challenged with 10^6 viable pathogenic *V. cholerae* El Tor Inaba N16961 organisms only.

The protective efficacy of the CVD 103 strain was 87% ($p<0.003$), 82% ($p<0.001$) and 67% ($p<0.01$) respectively. The CVD 103-HgR was observed to have a protective efficacy of 62% ($p=0.06$).

These investigations of these new candidate vaccine strains have demonstrated the practicality of this approach to oral vaccination against cholera. The B-WCV, reviewed in Section 3.10, was effective as a vaccine but the requirement for large multiple doses of killed organisms ($1x10^{11}$) and the need to use large quantities of purified cholera toxin B subunit make this an expensive approach to vaccination. The single dose of these live vaccine strains which provided a sound degree of protection against clinical cholera (more so against the classical rather than El Tor biotype) using a relatively low dose of organisms suggests this is a more cost-effective approach and that these particular candidate strains should be further evaluated in the field.

The protective efficacies of a variety of putatative vaccine preparations are summarized in Table 3.6.

TABLE 3.6

PROTECTIVE EFFICACY OF VARIOUS CHOLERA VACCINE PREPARATIONS

Vaccine Preparation	Diarrhoea Attack Rate		Protective Efficacy (%)
	Controls	Vaccinees	
Pathogenic classical *V. cholerae*	71/85	0/37	100
Pathogenic El Tor *V. cholerae*	32/37	2/22	90
Parenteral killed whole cell vaccine	47/58	2/13	81
Oral killed *V. cholerae*	47/58	5/16	62
Oral glutaraldehyde-treated toxoid:			
- 3 x 2 mg doses	3/6	3/6	0
- 3 x 8 mg doses	8/8	6/8	25
- with alcohol-killed *V. cholerae*	4/6	2/9	67
V. cholerae Texas Star-SR	18/25	7/25	61
Oral killed whole vibrio/B subunit vaccine	7/7	4/11	64

(adapted from Kaper JB 1985; reference 488)

...one cannot be too conservative when evaluating any new prophylactic or therapeutic measure, especially so when there exists a tried out method which meets fairly successfully the particular indication.

A.L. Garbat [188]

4

DEVELOPMENT OF AN ORAL HYBRID TYPHOID/CHOLERA VACCINE

4.1 *Recombinant DNA Vaccine Development*

In the absence of alternative control measures, vaccination has proven to be an effective approach to infectious disease control. The traditional approach to vaccine development, as described in detail in the previous sections, has been to utilize killed or attenuated whole microorganisms or toxoids, however for both cholera and typhoid this method of vaccine production has been shown to be useful but not entirely effective.

Factors which affect the potential usefulness of a particular approach to vaccine development relate to the manner in which the immune system recognizes and responds to the pathogenic microorganism. Different organisms, even different antigens on any one organism, are often recognized differently by the immune system. In the accurate design of a suitable vaccine for any one disease, these differences must be taken into consideration.

Therefore, the objectives for the rational design of an effective vaccine must include the ability:

(a) to block those processes essential for the establishment of infection;

(b) to neutralize those substances, produced by the infective agent, that mediate the pathological effects;

(c) to enhance the specific immune defences of the host.

The advent of recombinant DNA technology has provided a mechanism for the rational design of vaccines. The major difficulty encountered in adopting this approach to vaccine development is the identification of specific important virulence factors of the relevant pathogen. When a protective antigen has been identified the possibility of a designer vaccine can be explored.

In the development of an attenuated live combined orally administrable hybrid typhoid/cholera vaccine one of many approaches offered by recombinant DNA technology was applied for the development of the candidate vaccines against the two infectious diseases. The following section attempts to explain the essential principles involved in the particular approach which resulted in the candidate typhoid/cholera hybrid vaccines that form the basis of this thesis. It is by no means a full account of the molecular biology and genetic engineering utilized, such detail being beyond the scope of this thesis.

4.2 *Genetic Engineering as Applied to Vaccine Development*

A wide range of recombinant DNA techniques exist which permit the placement and maintenance of genetic material into cells, be it bacteria, viruses or even mammalian cells. These techniques, often referred to as "cloning", require the identification of the appropriate gene, isolation, mobilization and insertion of the gene into the recipient cell (often achieved by the use of a plasmid or by chromosomal integration), and achieving expression of the gene-product. If the product only is to be used as the vaccine, then a subsequent purification step is required.

The basic assumptions of rational vaccine design stem from advances in our knowledge of molecular biology. If a molecule can be identified as a

protective antigen then the gene coding for that molecule can be identified. The nucleic acid sequence comprising this gene can then be removed from the pathogen, attached to a suitable vector DNA to form a recombinant and introduced into suitable host cells, most usually *E. coli* or a yeast. The transformed cells containing the recombinant molecules can subsequently used in several ways that may contribute to vaccine development. Though the process can be described simply, the theoretical and technical problems should not be underestimated.[387,388] The type of host-vector system chosen depends on the nature of the experiment and the use of the product. Important considerations include the ability to express the product, the yield of recombinant DNA produced, ease of growth and suitability for large scale production.

The realization of the potential of these techniques has reinforced the expectation that infectious diseases can be controlled by means of safe and specific vaccines.

Attenuated bacterial vaccine organisms have the advantage that they can act as carriers of defined protective antigens of other organisms. Therefore a bacterial strain developed as an attenuated vaccine against a particular disease can be used as a delivery system for a range of protective antigens relevant to an unrelated disease. The use of attenuated bacteria to carry and express the genes of heterologous organisms is gradually increasing in importance. Essentially this development is focussed on the use of the oral route to vaccinate against the specifically targeted organisms. As a result, it is mainly attenuated enteric bacterial pathogens that have been used as vectors to deliver the foreign antigen(s) to the immune system (Table 4.1). Attention has been drawn recently to the use of attenuated *S. typhi* strains as vectors,[235,389] due to the strong intestinal and systemic immune responses

that are frequently initiated following infection with the pathogenic parent strain. This includes marked cell-mediated immune responses; orally administered *Salmonella* strains carrying defined antigens having also been used to protect animals against diseases where the cell-mediated immune response is considered to be more important, such as malaria.[390] Since the oral route of vaccination offers considerable advantages over other delivery routes, such as ease of administration and reduction in side effects, this approach will continue to be further developed.

Other approaches to vaccine development which have been used with varying degrees of success include the development of specifically attenuated bacterial vaccines (Table 4.2), where a pathogenic organism is rendered avirulent losing all potential to cause disease, however retaining its immunogenicity and ability to grow. Attenuated live whole organism vaccines are possibly best suited for protecting against pathogens which access the body through the mucosal surfaces, and so much of the development has focussed on enteric and respiratory pathogens, where strong local immune responses have been shown to be responsible for long term protection against reinfection. As described in Section 2.10, this approach has been used to develop the live oral typhoid vaccine *S. typhi* Ty21a and the promising monovalent attenuated orally administrable single dose cholera vaccine (Section 3.11.5).[382]

Purified component vaccines have also been successfully utilized as an approach to vaccine development. The ability of host cells to express recombinant genes and produce the "foreign" protective antigen can be used as the basis of an industrial process. Fermentation technology can, therefore, be used for the production large amounts of antigen, which, after suitable purification can form the basis of a vaccine (Table 4.3). The most successful

products of this approach to vaccination have been the synthetic hepatitis B surface antigen vaccine and the oral killed whole-cell/purified B subunit cholera vaccine described in Section 3.10.

Vaccines can be assembled in a biological vector, such as yeast (as for the hepatitis B vaccine) or can be synthesized using peptide synthesizers (the falciparum malaria peptide vaccine [391] is the best example). Often it is necessary for the smaller peptide vaccines to be linked to another carrier peptide or protein (the CSP malaria peptide is linked to tetanus toxoid [391]) or to be delivered in one of the newer delivery systems, liposomes or as immunostimulatory complexes (ISCOMS). This approach is the antithesis of the attenuated whole organism one, as it often requires that not just the major antigen be determined, but that the amino acid sequence responsible for the immunogenicity of the whole antigen be accurately determined. It is therefore possible to create vaccines containing only B or T lymphocyte epitopes. These are "designer" vaccines that use the whole range of technology to produce a discriminating product, and represent the peak of current technology.

TABLE 8.1

ANTIGENS EXPRESSED IN BACTERIAL VECTORS

Origin	Antigen	Vector	Trialed in Animals	Trialed in Humans	Reference
Shigella flexneri 2a	LPS O antigen	*Escherichia coli*	Yes	Yes	392
Sh. sonnei	Form I antigen	*Salmonella typhi* Ty21a	Yes	Yes	393,394
Sh. flexneri 2a	LPS O antigen	*S. typhi* Ty21a	No	No	395
Vibrio cholerae Inaba	LPS O antigen	*E. coli*	Yes	No	127
V. cholerae Inaba	LPS O antigen	*S. typhi* Ty21a	Yes	Yes	396
Escherichia coli	CFA-I fimbria	*S. typhi* Ty21a	Yes	No	397
E. coli	B-LT	*S. typhi* Ty21a	Yes	No	398
E. coli	B-LT	*S. enteritidis*	Yes	No	399
E. coli	B-LT	*S. typhimurium* AroA	Yes	No	242
E. coli	K88ab fimbria	*S. typhimurium*	Yes	No	401
Campylobacter pylori	66 kDa peptide	*E. coli*	Yes	No	400
Plasmodium berghei	CSP	*S. typhimurium*	Yes	No	390
Streptococcus sobrinus	SpaA	*S. typhimurium*	Yes	No	402
Mycobacterium leprae	protein antigens	*E. coli*	No	No	403
Hepatitis B virus	surface antigen	*E. coli*	No	No	404

SpaA - Streptococcal colonization factor
CT - Cholera toxin; LT - Heat-labile toxin
A - A subunit of either CT or LT (e.g CT A); B - B subunit of either CT or LT (e.g. B-LT)
CSP - Circumsporozoite protein LPS - Lipopolysaccharide

TABLE 8.2

ATTENUATED BACTERIAL VACCINE STRAINS

Parent Organism	Vaccine strain attenuating factor	Trialed in Animals	Trialed in Humans	Reference
Salmonella typhi	UDP-galactose-4-epimeraseless (*galE*)	Yes	Yes	218
S. typhi	AroA PurA	Yes	Yes	234
S. typhi	AroA AroC	Yes	No	240,241
V. cholerae El Tor Inaba	Cholera toxin (CT) A^-B^- (toxinless)	Yes	Yes	381
V. cholerae El Tor Inaba	CT A^-B^-, deleted haemolysin (HA^-)	Yes	Yes	381
V. cholerae 569B Inaba	CT A^-B^-, HA^-, no Shiga-like toxin	Yes	Yes	382
V. cholerae Ogawa 395	CT A^-B^+, (A subunit deletion only)	Yes	Yes	381
V. cholerae Ogawa 395	CT A^-B^+, (overproducer of Tcp pili)	Yes	No	295
Shigella flexneri Y	AroD	Yes	No	405

SpaA - Streptococcal colonization factor
CT - Cholera toxin; LT - Heat-labile toxin
A - A subunit of either CT or LT (e.g CT A); B - B subunit of either CT or LT (e.g. B-LT)
CSP - Circumsporozoite protein LPS - Lipopolysaccharide

TABLE 8.3

SYNTHETIC PEPTIDE VACCINES

Origin	Antigen	Trialed in Animals	Trialed in Primates	Reference
Poliovirus 1	Structural protein VP1	Yes	No	406
P. falciparum	$(NANP)_3$ of CSP	Yes	Yes	391,407
P. falciparum	Pfs25, asexual	No	No	410
P. falciparum	$R32tet_{32}$ CSP	Yes	Yes	411
P. vivax	CSP	Yes	No	412
P. berghei	$(DPPPPNPN)_2D$ of CSP	Yes	No	408
Melanoma	GM2	Yes	Yes	409
Hepatitis B	surface antigen	Yes	Yes	413,414
Liposomes				
P. falciparum	$(NPNA)_3$	Yes	No	415
V. cholerae	CT	Yes	No	415

SpaA - Streptococcal colonization factor
CT - Cholera toxin; LT - Heat-labile toxin
A - A subunit of either CT or LT (e.g CT A); B - B subunit of either CT or LT (e.g. B-LT)
CSP - Circumsporozoite protein LPS - Lipopolysaccharide

5

SELECTION AND ASSESSMENT OF VOLUNTEERS

5.1 *Phases of Experimental Drug Evaluation in Humans*

Most drug testing performed by pharmaceutical companies in the world, follows the pattern laid down by the Food and Drug Administration of the USA (FDA).[416] Vaccines are considered to be experimental new drugs for this purpose. Before any new vaccine can be administered to humans in the USA it must first satisfy the FDA by providing details of the results of laboratory and animal research.

Clinical drug or vaccine trials are normally carried out in three or four phases:[416,417]

Phase 1 trials, "Investigation of New Chemicals", are primarily concerned with assessing the initial introduction of new drug into humans with respect to safety of the candidate vaccine organism and normally involves the administration of the vaccine to between 20 and 100 subjects. These trials are also interested in examining the excretion of the organism, transfer of the inserted foreign DNA to other microorganisms where applicable, and most importantly it provides details of the immunogenicity of the vaccine strain. Phase 1 trials can also be used to optimize the dose-response as assessed by the maximal dose that will provide the maximal immune response without impairing the safety of the vaccine.

Phase 2 trials, "Limited Clinical Trials", are concerned with assessing the protective efficacy of the particular candidate vaccine organism against the relevant disease, assuming that it has been shown to be safe, immunogenic, and that the dose has been optimized. In ordinary drug evaluation, this would entail the use of only several hundred patients with

the particular disorder that is to be controlled or treated, however with vaccines, it is preferable to administer the vaccine to a much smaller group of normal subjects, then challenge these subjects along with an unvaccinated control group with the particular organism that the vaccine is meant to protect against. This is certainly the ideal, and is by no means possible with a wide range of infectious agents. However, with most enteric organisms it is possible to challenge normal subjects with the virulent organism in the knowledge that the subject, while certainly experiencing a degree of discomfort, in a closely monitored facility is most unlikely to suffer from any post-study effects. This is the situation with cholera - the onset of disease is quite obvious, and the treatment is simple rehydration and electrolyte replacement and the discriminate use of specific antibiotics to render the subject non-infectious.

Since it is practically impossible to recruit large numbers of normal subjects that can be safely observed during such a challenge study, often only small groups of subjects are used. However, if a sufficiently large challenge inoculum of virulent organisms is used, for example one that will induce clinical illness in 95% of normal control subjects, then any reduction in the numbers of clinically ill vaccinated subjects tends to become statistically significant.

Phase 3 trials, "Broad-scale Clinical Trials" are in effect field studies of the candidate vaccine in regions with a high incidence of naturally acquired infection. Large scale studies involving tens and hundreds of thousands subjects have been performed in recent years with candidate cholera[370] and typhoid vaccines.[226,418] These trials need to last several years, at least 1-4 years, to permit adequate follow-up of the population and to carefully document the incidence of the disease in the vaccinated population

in comparison to the unvaccinated or placebo-controlled population to accurately determine the protective efficacy of the vaccine in the population in which it will be required. This also provides the opportunity to assess differing preparations and formulations, allows comparison of dose regimens, and can frequently reveal rare or unusual adverse reactions. These studies are usually performed as randomized double-blind controlled trials in order to gain the maximal amount of statistically valid data.

Phase 4 trials, "Postmarketing Surveillance", is exactly what it says it is, a continuing evaluation of the vaccine's efficacy after it has been approved for general distribution or commercial marketing.

Unfortunately such an organization as the US FDA does not really exist in Australia. From the experience I gained through my association with Enterovax Limited, the University of Adelaide's biotechnology company for this project, it became apparent that if a group wished to evaluate in humans a vaccine in which the genetic material from another organism had been inserted (as in Phase 1 studies), then approval was required from the Recombinant DNA Monitoring Committee (RDMC) of the Government of the Commonwealth of Australia. If the putative vaccine was developed through the removal of genetic material in an attempt to attenuated a virulent pathogen, then it was left to locally formed institutional ethical committees comprising non-expert members to determine whether it was ethical to administer such a preparation to unnamed human subjects. On the occasion of my first exposure to this mechanism, I considered the process manifestly inadequate - especially considering that one of the putative vaccine strains developed was in fact demonstrated to be virulent in human subjects.[233] In the United Kingdom, a local investigator can administer any drug or preparation to a human subject on an experimental basis provided

the local ethical committee has agreed to its use and the subject's name has been forwarded to the Department of Health.

Neither of these two latter mechanisms can possibly be considered to be satisfactory in view of the ever widening use of recombinant DNA technology in the development of putative vaccines. There is a definite need for a centralized expert committee to be formed in order to assess all proposed human use of genetically manipulated microorganisms.

5.2 *Volunteer Recruitment*

Any vaccine evaluation programme that uses volunteer subjects must follow a set of guiding ethical principles, whether they be adapted from existing guidelines for the evaluation of pharmaceuticals in humans (as set out in the code of Good Clinical Practice by the USA Food and Drug Administration (FDA)) or be set down as regulations from local ethical committees or Government agencies (for example, those established by the Royal College of Physicians [419]). The studies comprising this work for this thesis were guided by a hybrid of the FDA Code of Good Clinical Practice and guidelines established by the Australian Government's National Health and Medical Research Council, the Human Ethics Committee of the Royal Adelaide Hospital, the Committee on the Ethics of Human Experimentation of the University of Adelaide, and in accordance with the Declaration of Helsinki (1975). In addition, one's own personal beliefs can also come into consideration.

Subjects for the studies in this thesis were healthy adult volunteers, who were recruited through advertisement or by direct approach. A newspaper advertisement was only used on two occasions seeking subjects. One was a tactfully worded small advertisement in the city's morning daily

paper which resulted in 32 enquiries, 21 of which agreed to participate in the studies following detailed discussion of the particular study and what was involved with through this participation. The other advertisement was a large, full-page, advertisement placed in the local student campus newspaper of our University. This was placed at a time when large numbers of new students were enrolling for the first time, and many older students were re-enrolling in their courses. This advertisement attracted no enquiries, despite the paper having a circulation time of one week.

Many of our early recruitments were obtained from friends of friends, members of colleagues' families, or through word of mouth from people who had participated in a previous study. The use of the student employment office of our own University was not productive, mainly through difficulties experienced in obtaining the necessary co-operation of the student union administrators. Eventually, we were able to come to an arrangement with the administrators of the student employment office of the Flinders University of South Australia, whereby they would leave a standard note on the noticeboard advising interested persons that we were seeking volunteers for a variety of studies. Any interested persons could collect copies of the detailed information sheets for a variety of studies that were being performed from the administrators of the local student employment office, and contact a research nurse in the Department of Medicine at the Royal Adelaide Hospital for further information, to obtain answers to any specific questions, or to arrange an appointment to see me for an interview. All intending subjects attended a clinical interview to determine their suitability for inclusion in the studies or if not considered suitable for that study, to examine their suitability for another.

All subjects were advised very early in the selection process that they would be financially compensated for their involvement, to cover costs incurred through their participation in the study for such items as travel expenses and any discomfort or inconvenience suffered. The sum offered varied considerably depending upon the sort of commitment required for a particular study, the number and extent of the investigative procedures involved and the size of the time commitment. This sum was selected as being equal to A$10 per hour as suggested by the NH&MRC, and could not be in any way considered an inducement to participate. Students who were required to remain within the biological containment unit were offered the sum of A$56 per day or part thereof, which approximated the sum of the average Australian weekly male wage at that time.

5.3 *Subject Selection Criteria*

Subjects were selected using the following criteria:

1) Male or female adults in good health between the ages of 18 and 60 years were accepted, provided that if the volunteer was female and was of reproductive age:

a) she was not, nor had any reason to believe that she was pregnant,

b) that if she was sexually active that she and/or her partner were using an effective form of contraception,

c) that she consented to a serum ß-HCG estimation prior to acceptance into the study.

2) Prior exposure to typhoid or cholera either through disease or immunization could be accurately determined.

3) On history and/or physical examination there was no abnormality that would be a contraindication to the receipt of a live vaccine, or for undergoing any of the required procedures. Special emphasis was placed on current or past gastrointestinal or respiratory symptoms or disease, including past episodes of diarrhoea.

For the studies where parenteral immunization was a requirement, persons with epilepsy, previous severe head injury or other neurological illness, and/or any past severe reaction to parenteral vaccination were not accepted.

4) Results from biochemical and haematological screening tests performed on samples of blood obtained prior to acceptance into the study were within the normal parameters for age and sex.

5) serum obtained on the same occasion as 4) above was negative for Hepatitis B surface antigen, and antibodies against Human Immunodeficiency Virus (HIV/AIDS) could not be identified.

6) Students enrolled in any subject taught or examined by the Departments of Medicine or Microbiology and Immunology of the University of Adelaide were precluded from the studies.

7) That all volunteers gave their free and written informed consent to participate in the studies after they had been interviewed and had read the appropriate volunteer information sheet, and understood that they were free to withdraw from the study at any time with prejudice.

All studies using human volunteer subjects detailed in this thesis had prior approval from the Human Ethics Committee of the Royal Adelaide Hospital and Committee on the Ethics of Human Experimentation of the University of Adelaide, and were performed in strict accordance with the Declaration of Helsinki (1975).

6

ESTABLISHMENT OF A BIOLOGICAL CONTAINMENT FACILITY FOR THE CLINICAL ASSESSMENT OF LIVE RECOMBINANT VACCINE ORGANISMS

6.1 *Necessity for Biological Containment*

The use of recombinant DNA technology in vaccine development, to alter old or to construct new microorganisms must raise concerns in the thinking individual. Everyone must be concerned about the potential risks or hazards of genetic manipulation, no matter how they are perceived, be they real or only theoretical, for this technology has certain consequences for all of us. Investigators in the field have from time to time expressed concern about the need for adequate containment measures for many types of experiments involving genetic manipulation.[420-423] As can be seen in Section 8, there has been a recent explosion in the development of putative vaccines using the approaches offered by recombinant DNA technology.

In the development of our vaccine strains it was necessary for them to have incorporated into the parent strain and the plasmid, a selection system to permit only the vaccine to grow in specific conditions, and to enable the laboratory identification of plasmid-bearing organisms in environmental samples. This was initially achieved through the use of antibiotic resistance marker genes.

In view of the problems that could be encountered through the transfer of plasmid-borne DNA from recombinant bacterial vaccine strains to environmental microorganisms, especially the possible transfer of antibiotic resistance, the RDMC recommended that the initial human studies with any new recombinant vaccine organism should be performed in a suitably approved biological containment facility that met the standards for a

restricted C3 category laboratory. Once, a particular organism had been shown to be safe, and stable, with no transfer of the recombinant genes to the subjects' normal intestinal flora, then additional clinical studies using that strain could be performed on an outpatient basis.

6.2 *Biological Containment Facility*

Our initial studies requiring biological containment were performed in a small facility located on the grounds of the Royal Adelaide Hospital's Hampstead Centre located off Hampstead Road, Northfield. The facility was the refurbished front ground floor section of Nurses' Home B. Access was restricted through the front of the building through the use of an intercom system. It comprised three double bedrooms, a food preparation area, eating area, a nurses bedroom, a television/reading room, a large recreation room, a security laboratory for the primary processing of faecal samples, bathroom and separate toilet facilities. The problem of ensuring that viable faecally excreted vaccine organisms did not access the general public sewerage system, was solved through the use of portable flush toilets ("Portapotty" brand), each individually labelled with a subject's name, and containing a concentrated formalin solution in the lower 5 litre capacity reservoir. We had been assured that formalin was sufficient to kill all viable vaccine organisms used, in laboratory based experiments. Spare toilets were available should they have been required. Upon the completion of the study all toilet reservoirs were sampled, and these samples were analyzed for the presence of viable organisms with the plasmid carrying the recombinant DNA.

All subjects were permitted to wear their own street clothes, and to bring with them essential items of personal grooming, as well as a limited number of personal recreational items. Upon the completion of the trial,

underclothes, as well as these other items were all disinfected before returning to the community environment. No visitors were permitted under any circumstances, and no unauthorized personnel were permitted to enter the facility.

Three members of nursing staff were rotated through eight hour shifts to provide a continuous cover for observation of the subjects, and to assist with the organization of recreational activities. Medical staff (the author) visited the facility twice daily to monitor the study and to record any adverse reactions and to settle any personal disputes that may have arisen through keeping six unrelated individuals in close proximity for a week or more. In addition, the member of the medical staff was on 24 hour call through a long-distance paging system.

All meals were provided by the hospital cafeteria, and subjects were also provided with a well-stocked pantry to allow the preparation of breakfasts, snacks and light meals.

The final Phase 1 containment study utilizing the vaccine strain EX645 was performed at a new larger facility on the same site, but permitting up to 15 volunteer subjects in individual study/bedrooms. This facility was originally constructed as a wing for a tuberculosis hospital and eventually became a rehabilitation ward for neurosurgical patients. As a result of the modifications required for its latter purpose, this ward had a large amount of recreational and general living space, as well as considerably more toilet and bathing facilities. Apart from these improvements, the general daily functioning of the unit followed the guidelines established with the initial facility.

7

DETAILS OF THE METHODS USED FOR THE DETERMINATION OF A SPECIFIC HUMORAL IMMUNE RESPONSE IN ADULT SUBJECTS

7.1 *Collection and Processing of Samples*

7.1.1 *Intestinal (Jejunal) Fluid*

In studies requiring the analysis of intestinal fluid, such fluid was obtained from the upper jejunum using a technique of intestinal intubation.

This procedure required each volunteer to have fasted for at least eight hours prior to the procedure. A 10 ml solution of a local anaesthetic gargle (2% amethocaine with benzoic acid as a preservative, prepared by the Royal Adelaide Hospital Pharmacy) in water (1:1 v/v) was administered, and swallowed.

After 1 to 2 minutes the intestinal tube was passed using the oral route. The intestinal tube used was a polyvinyl plastic ANPRO AN 20 Andersen tungsten-weighted sump tube (H.W. Andersen Products, Oyster Bay, New York, USA) that had been cut to 1.50 metre length and which had fitted on the proximal end a three way tap with Luer adapter.

Correct positioning of the tube *in vivo* was confirmed using fluoroscopy. This method was examined by the hospital Scientific Officer and approved by the Human Ethics Committee of the Royal Adelaide Hospital. The total radiation dose received by any individual subject for a completed study was substantially less than 5.0 mSv, in accordance with the regulations of the National Health and Medical Research Council. A log was maintained of exposure times and settings, and periodically reviewed by the

Scientific Officer of the Royal Adelaide Hospital. Subjects were not permitted to participate in more than one study.

Intestinal fluid samples with a pH >6.5 (as determined using Merck Special Indicator paper, pH 4.0-7.0) were collected and kept on ice, until 25 to 30 ml had been collected from each volunteer. The samples were then centrifuged at 4000 xg at 4°C for 30 minutes and the supernatant stored at -70°C until required. Intestinal fluid was not heat-inactivated by heating to 56°C for 1 hour, as performed by some investigators, since convincing evidence shows that this is in fact detrimental to the concentration of secretory IgA present in intestinal fluid.[32] It has been recommended that a cocktail of intestinal protease inhibitors be used,[32] however others have shown that this is also an inadequate measure.[424] The technique as described above has been proven to be quite effective in determining specific secretory IgA levels in intestinal fluid collected using the aforementioned technique of intestinal lavage.[16,28]

7.1.2 *Serum*

Whole blood (usually 20 ml) collected by venipuncture (usually from an antecubital vein) was allowed to clot at room temperature for two hours, before being kept overnight at 4°C. The serum was subsequently pipetted and stored at -20°C until required.

7.1.3 *Saliva*

When required, saliva was collected from one of the parotid glands by placing a Curby cap over the buccal opening of the appropriate parotid duct, and stimulating saliva production by placing small amounts of citric acid ("AnalaR", BDH Chemicals Aust. Pty. Ltd. Cat. No. 10081) on the tongue

of the volunteer, until 5 to 7 ml of saliva had been collected. The collected saliva was centrifuged at 4000 x*g* for 30 minutes at 4°C and the supernatant stored at -70°C until required.

7.1.4 *Peripheral Blood Lymphocytes*

Peripheral blood lymphocytes (PBL) were collected for the determination of antigen- and class-specific antibodies produced by circulating antibody secreting cells, a full description of this procedure is detailed in Section 12.

7.2

DESCRIPTION OF ENZYME-LINKED IMMUNOSORBENT ASSAYS FOR THE QUANTIFICATION OF ANTIGEN-SPECIFIC ANTIBODIES IN SERUM, INTESTINAL FLUID AND SALIVA

The principle of the enzyme-linked immunosorbent assay (ELISA) was first described in 1973 by Engvall and Perlmann.[425] The ELISA is a simple and sensitive method for the quantitative determination of antibodies in a sample, be it serum or secretions. Briefly, the wells of 96-well polyvinyl or polystyrene microtitre plates are coated with the antigen for which you are attempting to determine antibodies against. This coating is performed by simple adsorption to the plastic, however there are available more sophisticated methods of covalently binding proteins to the plastic. After coating the wells the plates are thoroughly washed to remove any excess unbound antigen. It is then necessary to fill any spaces on the wells not covered by the coating antigen with an inert "blocking" agent, such as casein, bovine serum albumin (BSA), glycine or gelatin.[426] This is necessary since because immunoglobulins are simply proteins they would non-specifically

bind to vacant areas on the wells in addition to binding to the coating antigen, resulting in an increase in the background optical "noise". This would result in a loss of assay sensitivity. After the blocking step, the samples are added to the wells and the plate incubated for a predetermined length of time at a suitable temperature. It is during this incubation step that antigen-specific antibodies of all isotypes will bind to the coating antigen.

The plate is subsequently thoroughly washed to remove any excess unbound or loosely bound antibody from the wells. Despite not necessarily being antigen-specific, any residual unbound antibody or loosely bound antibody would still bind the enzyme-conjugated secondary antibody in the next step, resulting in a loss of specificity of the assay. Those non-specific antibodies which have bound the secondary antibody, may be washed out at the next washing step. By non-specifically binding and then being removed, there may be a reduction in the amount of specific antibody-antigen complexes detected by the labelled secondary antibody, further reducing the sensitivity of the assay. If wells are inadequately washed at the next washing step, then these poorly or unbound antibodies will also contribute to the background noise.

After the thorough washing, an enzyme-labelled secondary antibody is added. These antibodies can be either polyclonal or monoclonal, and are directed against a specific immunoglobulin isotype. For example an alkaline-phosphatase conjugated goat IgG anti-human IgA antiserum is one made by immunizing goats with human IgA, purifying the serum sample so that only the goat IgG fraction remains then further purification so that only goat IgG antibodies directed against human IgA remains. Subsequently one or two molecules of alkaline-phosphatase are covalently bound (conjugated) to this purified goat antiserum. The resulting antiserum contains enzyme-labelled

antibodies that are highly specific for human IgA. Upon incubation in the wells, this antiserum will specifically bind only to any human IgA that may be present, be it a specific antibody-antigen complex, or non-specifically bound or residual human IgA.

After the appropriate predetermined dilution of this antiserum is added to the wells, the plates are further incubated then washed.

A solution containing the appropriate substrate for the conjugated enzyme is added, and the optical density of the colour change that occurs as a result of further incubation, is measured using a spectrophotometer, an ELISA reader. The degree of the colour change is directly proportional to the amount of specific antibody bound to the coating antigen. The most common substrate used in the alkaline phosphatase enzyme system is a 1 mg/ml solution of *p*-nitrophenyl phosphate in a 10% diethanolamine solution that has been supplemented with magnesium chloride. The enzyme cleaves the phosphate off, leaving the bright yellow compound *p*-nitrophenyl.

In the studies described in this thesis, antigen-specific antibodies were quantified using an indirect "sandwich" enzyme-linked immunosorbent assay (ELISA)[426] detailed below, that had been previously described with some modifications.[14,228]

For anti-lipopolysaccharide antibody determination, 96-well polyvinyl microtitre plates (either round-bottomed "Costar" Data Packaging Corp. Cambridge, Mass. USA cat. no. 2595 or "highly-activated" flat-bottomed "Titertek" Flow Laboratories Holland) were coated with 0.005 mg/ml solution of the relevant lipopolysaccharide that had been linked to methylated bovine serum albumin,[427] in carbonate-bicarbonate coating buffer pH 9.6 (0.100 ml/well), overnight at 4°C.

The plates were shaken and then 0.100 ml of a 0.05% bovine serum albumin (BSA) in phosphate buffered saline solution (BSA-PBS) was added to each of the wells and left for 45 minutes at room temperature before washing in PBS-"Tween 20" (Sigma Chemical Co., St. Louis, Missouri, USA cat. no. P 1379).

After this washing, 0.200 ml of either 1:10 serum dilutions in 0.05% BSA-PBS or 1:2 jejunal fluid or 1:1 saliva dilutions in 0.05% BSA-PBS were added in duplicate wells of the first row of the plates. Two or three fold dilutions were then performed down the rows in 0.05% BSA-PBS. The plates were incubated for 16 hours at 4°C in an humid atmosphere, before being washed with PBS-"Tween 20".

For the determination of human antibodies, alkaline-phosphatase conjugated goat antihuman IgA, IgG or IgM heavy chain specific antisera ("KPL", Kirkegaard and Perry Laboratories, Gaithersburg, Maryland USA) were used.

The correct dilution of the enzyme-conjugated antisera had been previously determined and each new batch used was compared to the previous one to ensure consistency of performance.

After the second washing step 0.100 ml the appropriate enzyme-conjugated secondary antibody diluted in 0.05% BSA-PBS was added to each of the wells. The plates were left to incubate at 37°C for four hours, after which time they were shaken and washed as above.

The substrate used was a 1 mg/ml solution of *p*-nitrophenyl phosphate (Sigma Phosphatase Substrate No. 104-105) in a 10% diethanolamine buffer, 0.100 ml of which was added to all of the wells. The plates were then incubated at 37°C for two hours.

Following this incubation all plates were read using a "Titertek" ELISA reader Model 310C (Flow Laboratories) at 405 nm.

All plates were initially blanked using antigen-coated wells, containing residual, non-specifically bound, enzyme-conjugated secondary antibody and the substrate solution.

In each assay, serum obtained from an individual with a known high titre directed against the relevant antigen was included as a positive control, and serum obtained from an individual with a known low titre against the antigen was included as a negative control.

7.3

THE SINGLE RADIAL IMMUNODIFFUSION ASSAY FOR THE DETERMINATION OF TOTAL CLASS-SPECIFIC IMMUNOGLOBULIN CONTENT

The single radial immunodiffusion assay as previously described by Mancini *et al.*[428] was used to quantify the total class-specific immunoglobulin levels in the samples of intestinal fluid and saliva.

This assay is a modification of the older gel immunoprecipitation assay. The principle of the assay is quite simple in that immunoglobulin placed in a well cut in agarose gel will diffuse out into the gel and will react with any specific antiserum dissolved in the gel forming a precipitation line. In the method of Mancini *et al.*[428] a precipitation ring is formed around the well, the square of the diameter of this ring being directly proportional to the immunoglobulin concentration. Any sample's immunoglobulin concentration can be determined by comparison to the diameters of precipitation rings formed by standards of known concentration on the same plate.

The method entailed a 200 mm x 200 mm glass plate being carefully washed with alcohol and dry wiped with silicon chloroform. A clearly labelled 100 mm x 100 mm glass plate was supported by each corner 1.5 mm above the large base plate. A 2% agarose solution was heated using a microwave oven until the solution became clear. For each plate, 7 ml of this solution was mixed with 7 ml of a 0.1 M sodium barbitone solution resulting in a final concentration of 1% agarose. To this solution was added either a rabbit antihuman IgG (gamma-chain specific) antiserum (Behringwerke AG, Marburg West Germany, Behring Cat. No. ORCM 14/15) (final concentration 0.026 mg/ml), or a rabbit antihuman IgM (mu-chain specific) antiserum (Behring Cat. No. ORCK 14/15) (final concentration 0.050 mg/ml), or a rabbit antihuman IgA (alpha-chain specific) antiserum (Behring Cat. No. ORCI 14/15) (final concentration 0.022 mg/ml).

The heated solution was then pipetted between the two glass plates and was allowed to set. Once set, the excess agarose was cut from the edges, and sixty-four circular 0.003 ml wells were cut in the plate.

Intestinal fluid or saliva samples were used neat, 0.003 ml of each were added in duplicate to the wells of the appropriate plate. IgG and IgM standard curves were constructed using 0.003 ml samples in duplicate of the appropriate dilutions of a Standard Human Serum (Behring Cat. No. ORDT 06/07). The standard curve for IgA, was constructed using secretory IgA in the form of human colostrum of predetermined IgA and secretory IgA content.

After the addition of the standards and samples, the plates were stored in a humid atmosphere at 4°C for 72 hours, and were then washed four times in 0.9% saline, each wash lasting 12 hours. Following this washing step the plates were thoroughly rinsed with distilled water to remove any

salt residue then dried in a 56°C oven until the agarose was hard. The plates were then stained using a 0.1% w/v solution of Coomassie Blue dissolved in a water:acetic acid:methanol (50:7:50) solution.

Excess stain was removed by rinsing the plates with a double distilled water:acetic acid:methanol (85:7:5) solution.

7.4

VACCINE ADMINISTRATION

7.4.1 *Standard Method of Oral Vaccination*

Unless otherwise indicated in the text, all vaccines intended for oral use were administered using this "*Standard Method of Vaccination*".

All intending vaccinees were required to have fasted for at least eight hours prior to their presentation for vaccination.

They were all required to attend for vaccination between 0830 and 1030 in the mornings. Upon arrival they all drank 50 ml of a 2% solution of sodium hydrogen carbonate in distilled water, five minutes later they swallowed the vaccine which comprised the vaccine organisms suspended in 30 ml of 0.9% saline. Immediately after taking the vaccine, they were all required to drink a further 60 ml of distilled water.

All vaccinees were required to fast for a further hour after vaccination.

Three doses of the vaccine were usually given according to either Schedule A. (a single dose on each of days 0, 2 and 4) or Schedule B. (a single dose on each of days 0, 2 and 5).

7.4.2 *Parenteral Vaccination*

The commercial killed typhoid vaccine ("CSL", Commonwealth Serum Laboratories, Melbourne, Australia) was administered to volunteers by subcutaneous injection through the skin overlying the left deltoid muscle, of 0.5 ml as per manufacturer's instructions.

The same method of administration was used for the commercial killed cholera vaccine (CSL).

Depending upon the stated aims of the particular study being performed, the number of vaccinations, their times and the duration between doses varied.

7.4.3 *Standard Method of Rectal Vaccination*

In those volunteers who were receiving the vaccine through the rectal route, the following procedure was used:

(a) Volunteers were required to attend for vaccination as soon as practicable after they had opened their bowels on the days they were required to attend for vaccination.

(b) Upon attendance, lyophilized doses of *S. typhi* Ty21a were freshly reconstituted in 10 ml of 0.9% saline. The volunteers were required to remove their lower garments and to lie in the left lateral position with their knees bent towards the chest. A rectal catheter ("Rectal Catheter FG22", AHS Australia, Cat. No. 86408) was inserted 10-15 cm from the anus into the rectum. The catheter had been lubricated with lanolin (F.H. Faulding and Co. Thebarton, South Australia) to assist with entry. The 10 ml suspension of vaccine organisms was poured into a 60 ml catheter syringe which was attached to the external opening of the rectal catheter, the plunger reinserted into the syringe and the vaccine injected into the rectum. A Spencer-Wells

clamp was used to occlude the catheter to prevent spillage of the vaccine, and 25 ml of 0.9% saline was poured into the syringe. After the plunger was inserted and the clamp removed, the saline was injected into the rectum in order to wash out the catheter. The catheter was clamped near the anus and removed without spillage occurring. This method of administration was quick and without discomfort for the volunteer. All volunteers were required to hold the vaccine for at least six hours after inoculation or preferably until they opened their bowels as usual the following day.

8

DEVELOPMENT OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY FOR THE QUANTIFICATION OF *in vitro* ANTIGEN-SPECIFIC ANTIBODY PRODUCTION BY PERIPHERAL BLOOD LYMPHOCYTES

Due to delays and problems associated with the development of the hybrid cholera/typhoid vaccine strains, it was considered practical to investigate the basic pattern of the human humoral immune response and factors that may affect it using the standard parent strain *S. typhi Ty21a.*

The necessity to accurately determine a primary intestinal immune response to orally administered enteric vaccines demanded the development of a sensitive, reliable and rapid alternative to the existing methods.

Briefly (to summarize the description in Section 1.2), bacterial infections of the gastrointestinal tract, as well as oral vaccines, normally produce a secretory immune response (usually of the IgA isotype) in the intestine which may be accompanied by a more widespread response with specific secretory IgA detectable in the external secretions of other distant mucosal surfaces.[94,95] This pattern of response has been reported to occur without the corresponding appearance of detectable specific antibodies in serum.[15,95,429] However, serum antibody determination has been of limited value, as significant changes in specific antibody titres are not always present following infection.[15,28,96] When a serum response has been measurable it has not necessarily reflected the intestinal immune response. This made, and continues to make, the assessment of the effectiveness of administered orally enteric vaccines in stimulating local immunity difficult. The most reliable method for the determination of the intestinal immune response has been intestinal intubation with sampling of the intestinal fluid.[15,28] This technique is both time consuming and inconvenient for the subject involved, and

variable due to a variety of uncontrolled factors. These include the methodological difficulties relating to enzymatic digestion, timing of sampling,[30] and the non-homogeneous distribution of immunoglobulin in collected samples. The use of other body fluids, such as serum and saliva, as indirect measures of intestinal immunity has met with mixed success.[96,369] Thus, to measure a local intestinal immune response in volunteers vaccinated orally, repeated intestinal intubations have been necessary.

The development of the haemolytic plaque-forming assay was the initial effort to overcome some of the problems associated with the determination of an intestinal immune response to an orally administered antigen. This assay and its and subsequently improved successors were developed for the quantification of the circulating immunoglobulin producing lymphocytes and proved useful as an indirect measure of an immune response at difficult to access mucosal surfaces.[430-432] The haemolytic plaque assays were a convenient and accurate technique for evaluating the numbers of activated B lymphocytes producing class-specific immunoglobulin.[423] As a result of this, the ELISPOT and the ELISA-plaque forming assays were developed.[434-437]

Both assays have been applied in two independent trials of oral vaccines.[435,438] The information obtained from these applications demonstrated the kinetics of the numbers of antigen-specific lymphocytes that reached the peripheral blood following oral vaccination, with the peak number of antibody producing cells reported to occur between days 6 and 10 inclusive.

Following this background work, a new enzyme-linked immunosorbent assay (ELISA) which determined the amount of specific

antibody of any class secreted by circulating PBL following oral vaccination with a live vaccine organism was developed.

For this assay, peripheral blood lymphocytes were obtained by diluting heparinized venous blood 1:1 v/v with Dulbecco's Buffered Salts Solution (Dulbecco's BSS) then centrifuging at 800 xg for 25 minutes at room temperature on Ficoll-Paque (Pharmacia, Uppsala, Sweden).[434] The mononuclear cells at the plasma/ficoll interface were pipetted into a V-bottomed universal container (Bunzl, later Disposable Products, Adelaide, South Australia) and made up to 25 ml with Dulbecco's BSS. These isolated cells were washed a further three times by centrifugation at 135 xg for 10 minutes at room temperature with resuspension in 25 ml of fresh Dulbecco's BSS after each centrifugation.

After the final wash the cells were resuspended to a concentration of 10^7 mononuclear cells/ml in RPMI 1640 culture medium (Flow Laboratories Australasia, North Ryde, NSW, Australia) supplemented with 10% heat inactivated fetal calf serum, 2 mmol/l L-glutamine and mixed antibiotics. The cells were then allowed to stand at room temperature, being added to the assay plates within two hours of final resuspension.

For this assay, 96-well polyvinyl microtitre plates were coated with the relevant antigen and blocked as described in Section 7.2.

After the plates were blocked and washed as described in Section 7.2, 10^6 (0.100 ml) peripheral blood lymphocytes (PBL) in supplemented RPMI 1640 culture medium were added to replicate wells of the plates. The plates were incubated for 16 hours at 37°C in a 5% CO_2 atmosphere, before washing out the cells.

Addition of the alkaline-phosphatase conjugated goat antihuman IgA, IgG or IgM heavy chain specific antiserum (Kirkegaard and Perry Laboratories) and subsequent washing were as described in Section 7.2.

The substrate used was a 1 mg/ml solution of *p*-nitrophenyl phosphate in diethanolamine buffer, 0.100 ml of which was added to all of the wells. The plates were then incubated at 37°C for four hours.

Following this incubation all plates were read using a Titertek Elisa reader Model 310C (Flow Laboratories) at 405 nm.

All plates were initially blanked using wells coated with the relevant antigen, containing residual, non-specific bound enzyme-conjugated secondary antibody and the substrate solution.

In this initial study, the specific IgA antibody response detected in the PBL of three of the twelve volunteers exceeded the upper limit of detection of this ELISA reader (>2.00 absorbance units). For the purpose of obtaining meaningful statistical analysis of the data, in these cases a value was determined by pipetting the replicate wells and reading them using a conventional spectrophotometer. In subsequent studies this was not performed, and the value representing the upper limit of detection (2.00 absorbance units) was used instead. All results were expressed in ELISA absorbance units/10^6 PBL.

In each assay, high titre serum obtained from a convalescent typhoid patient was included as a positive control, and PBL suspensions obtained from at least three unimmunized normal volunteers on the same occasion as the vaccinees, were included as negative controls.

Development of PBL-ELISA

Subjects and Methods

Twelve healthy adults (two women and ten men, 19-45 years of age) volunteered to participate in this study. None of these volunteers had any past contact with typhoid fever nor had they been previously immunized against typhoid.

The vaccine organism used was *S. typhi* Ty21a. Each vaccine dose comprised 10^{11} freshly harvested live organisms suspended in 10 ml of 0.9% sodium chloride solution. The vaccine was supplied by Dr G. Boehm of Enterovax Limited, Adelaide, South Australia.

Vaccination of the subjects was performed in accordance with the standard method of vaccination as detailed in Section 7.4.1, intestinal fluid and serum were collected as described in Section 8.1.

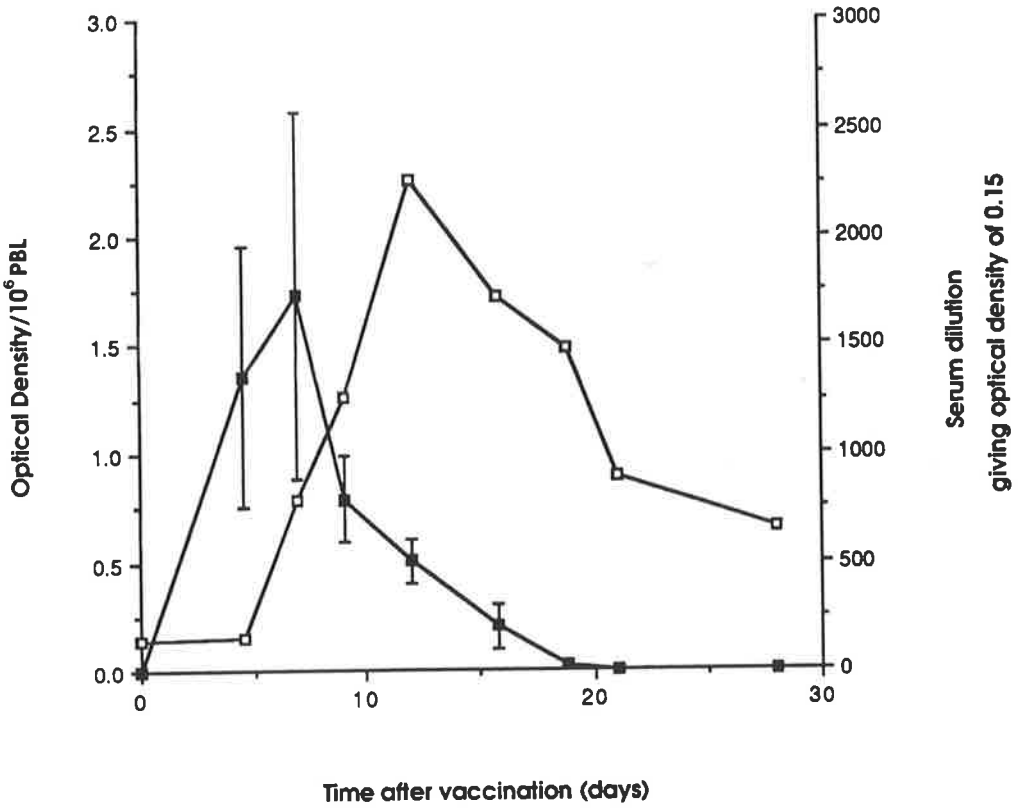
Results

Specific PBL Responses

In all twelve of the volunteers who ingested the *S. typhi* Ty21a, an anti-typhoid LPS IgA antibody response (optical density >0.100 units/106 PBL) was detected in their sampled PBL, with the mean optical density peak of 1.73 units (95% CI 0.88 to 2.57 units) occurring at a mean of 6.2 days (95% CI 5.5 to 6.8 days) after the first dose of the vaccine (Figure 8.1). In all but one volunteer, there was a response measurable as early as four days. IgA antibodies to typhoid LPS could not be detected by this method before vaccination nor later than 18 days after vaccination. In no assay was any typhoid specific antibody detectable in any of the negative controls' PBL (Table 8.1).

FIGURE 8.1

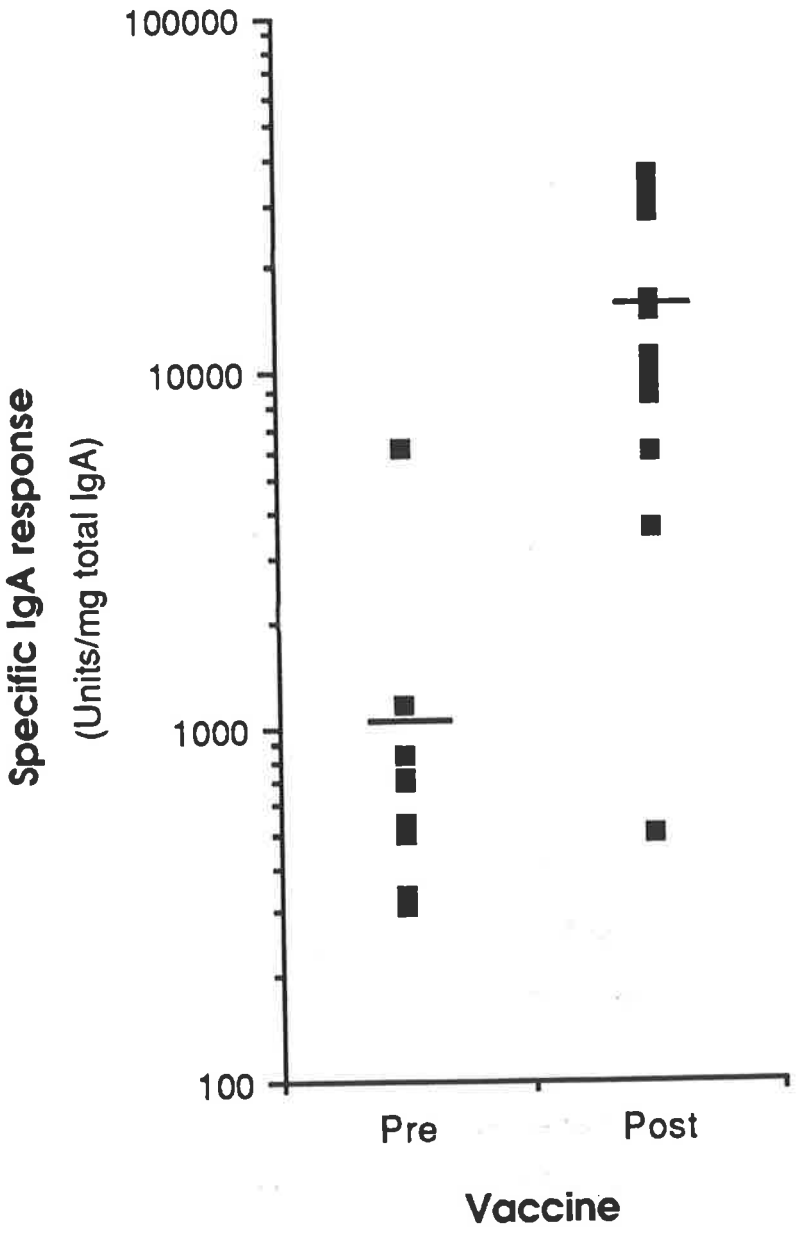
TIME COURSE RELATIONSHIP BETWEEN IgA PRODUCTION BY PERIPHERAL BLOOD LYMPHOCYTES AND IgA RESPONSE IN SERUM



PBL response (■) is the arithmetic mean optical density (absorbance units) ± 95% confidence intervals
Serum response (□) is the geometric mean of the serum dilution giving an optical density of 0.15 absorbance units

FIGURE 8.2

SPECIFIC INTESTINAL IgA RESPONSE IN VOLUNTEERS FOLLOWING ORAL VACCINATION WITH *Salmonella typhi* Ty21a



Line (-) indicates arithmetic mean value.

TABLE 8.1

TIMING OF PBL AND SERUM ANTIBODY PEAKS

	Days*	Peak+
IgA		
PBL	6.3 (5.5-6.8)	1.728 (0.882-2.574)
Serum	13.2 (10.7-15.7)	2310 (809-6580)
IgG		
PBL	6.8 (5.7-8.0)	0.707 (0.278-1.136)
Serum	17.1 (13.6-20.6)	3344 (1240-8990)
IgM		
PBL	7.8 (6.1-9.4)	0.646 (0.359-0.933)
Serum	16.8 (14.2-19.4)	2431 (1320-4480)

* - arithmetic mean
+ - PBL values expressed as arithmeic mean absorbance values, serum values expressed as the geometric mean peak titres
Numbers in parentheses refer to 95% CI.

The organism specificity of the antibody response was demonstrated by the failure to measure either an anti-cholera or an anti *E. coli* antibody response in any of the PBL assayed in wells coated with either *V. cholerae* 569B LPS or *E. coli* O55:B5 LPS.

A similar chronology of response was demonstrated in IgM (11/12 responders on day 7) and IgG (10/12 responders) antibody classes but of a lesser magnitude.

Incubation of the PBL in a 0.075 mg/ml solution of cycloheximide resulted in the observation of a 65-94% reduction in IgA, 75-88% reduction in

IgG, and 68-86% reduction in IgM production, indicating that the PBL were the source of the specific antibody production.

Specific Serum Responses

Typhoid specific serum IgA antibodies were measurable in 10/12 volunteers (geometric mean fold rise >4.0); pre-vaccine geometric mean titre 137 units, 95% C.I. 105 to 180 units, post-vaccine geometric mean titre 2900 units, 95% C.I. 1100 to 7900 units; using Student's paired t test after ln(x) transformation of titres, $t=7.634$ d.f.=11 $p<0.001$. The mean peak serum IgA response occurred at 13.2 days, 95% C.I. 10.7 to 15.7 days (Table 8.1).

Specific Intestinal Responses

After correction of intestinal antibody titres for total class-specific immunoglobulin, 9 of the 12 volunteers had a four-fold or greater rise in typhoid LPS specific IgA antibody response; pre-vaccine mean 1020 units, 95% C.I. 0 to 2050 units, post-vaccine mean 14800 units, 95% C.I. 7500 to 22100 units; using Student's paired t test, $t=4.029$ $p=0.002$ (Figure 8.2).

There was a significant relationship (as determined using Wilcoxon Rank Sum Test) between the magnitude of the PBL IgA antibody response and the fold rise in specific IgA in both intestinal fluid ($p=0.0024$) and in serum ($p=0.00098$).

Specific IgA antibody production was detected in the PBL obtained from all vaccinated volunteers, however neither a serum nor an intestinal immune response was always detected.

Conclusion

This section described a new enzyme-linked immunosorbent assay (ELISA) which allows the determination of the amount of specific antibody of any class secreted by circulating PBL following oral vaccination with a live vaccine organism. Czerkinsky *et al.*[434] in their description of the ELISPOT assay also referred to the possible conversion of that assay to an ELISA. This present assay differs in that the PBL were incubated in antigen-coated round-bottomed polyvinyl microtitre plates which as a result dispensed with the need to pipette solutions from petri-dishes and does not require the addition of beads to the culture medium. In comparison to the ELISPOT or ELISA-plaque assays, it is far more convenient, since time is not spent laboriously counting spots in agarose, and it can be used to quantify the specific antibody production by the PBL.

The necessity of having to pipette the replicate wells of three of the vaccinated subject's PBLs in this part of the study, because the specific IgA antibody response by their PBL exceeded the detectability of the ELISA reader, could have been avoided by reading the plates after a shorter substrate incubation time or by using a lower concentration of PBL.

The result has been the establishment of a highly sensitive, specific ELISA which enables the rapid assessment of the immunogenicity of candidate orally administrable vaccines.

9

THE HUMORAL IMMUNE RESPONSE TO *Salmonella typhi* Ty21a

9.1 *The Kinetics of the Specific Humoral Immune Response*

Introduction

The continued development of effective vaccines against enteric bacterial infections requires a thorough understanding of the local and systemic immune responses to a locally presented bacterial pathogen. The meaningful evaluation of potential candidate vaccines against pathogens such as *S. typhi* or *V. cholerae* rely heavily upon measuring the resulting immune response at an optimal time point.

As detailed in Section 1.2, the human immune response to enteric pathogens has been variously investigated in the past,[16,28,37-39] with some of this information having been obtained with pathogenic organisms such as *V. cholerae*.[40,42,43] However, the opportunity to intensively examine the kinetics of the humoral intestinal, serum and peripheral blood lymphocyte immune responses concurrently has not been previously afforded using modern techniques.

While the humoral immune response to *S. typhi* Ty21a in humans has been studied in the past,[15,153,220,221,228-231] there has not been a detailed examination of the time sequence of the serum and intestinal immune responses. In order to conduct our further studies examining the humoral immune response of candidate oral hybrid typhoid/cholera vaccines based on *S. typhi* Ty21a,[396,440] it was essential that the optimal time point for sampling serum or jejunal fluid be determined. Therefore there was an

obligation to plot the time course of the human humoral immune response to *S. typhi* Ty21a more carefully.

In this study the generation of the humoral immune response over the first few weeks in human subjects following their ingestion of *S. typhi* Ty21a was examined in detail. From this, it was hoped to obtain an accurate representation of this response which would permit more accurate evaluation of the immunogenicity of some of the more recently developed live enteric bacterial vaccines.

Subjects and Methods

Nineteen healthy adults (three women and sixteen men, 18-35 years of age) agreed to participate in this study. None of the volunteers had any previous exposure to typhoid either through vaccination or disease, nor did any of the subjects had any history or current symptoms of gastrointestinal tract disease.

All volunteers received *S. typhi* Ty21a. Each lyophilized vaccine dose comprised $1.7x10^{11}$ viable organisms after reconstitution with 0.9% (w/v) saline. The vaccine was supplied by Enterovax Limited, Adelaide, South Australia.

The subjects were allocated randomly to three study groups, A, B and C:

Group A subjects received three doses of the vaccine given on days 0, 2 and 5, and underwent the procedure of intestinal intubation on four occasions, once prior to vaccination then again on three subsequent occasions on Days 7, 14 and 21.

Group B were vaccinated as for Group A, but underwent intestinal intubation on Days 15, 29 and 43.

Group C received two doses of the vaccine, one dose on each of Days 0 and 21.

Groups A and B comprised the short schedule of vaccination, Group C had the long schedule.

Statistics

Serum antibody responses are represented as the reciprocal of the dilution that gave an optical density of 0.15 ELISA absorption units and are expressed as units of antibody. These and the adjusted intestinal antibody titres were ln(x) transformed prior to their graphical presentation and statistical analysis. Differences between the means of two groups for any given time point, or between two time points of any given group were determined using Student's t-test. The t value and significance levels are cited only. When the differences between the means of all three groups were assessed for any given time point, the one-way analysis of variance (ANOVA) was employed, with only the F value and significance level cited in the text.

The PBL responses were analyzed using the two-tailed Wilcoxon Rank Sum Test for independent samples.

Results

Pattern of Intestinal Specific Immune Response

The mean baseline pre-vaccination intestinal anti-typhoid antibody levels between all three groups were not significantly different ($F=1.328$, $p=0.293$).

Following vaccination, there was a significant anti-typhoid IgA antibody response evident in both groups A and B by Day 14 ($t=4.406$,

p=0.014) and Day 15 (t=6.832, p=0.00048) respectively (Figure 9.1). There was a stepwise increase in antibody titre from the baseline to the Day 14 peak, evident as early as Day 7 in Group A (t=2.823, p=0.030), with a further significant rise occurring from Day 7 to 14 (t=3.051, p=0.023). No difference was evident between the antibody levels of days 14 and 21 (t=0.011, p=0.99).

This antibody response continued to remain significantly elevated above baseline levels on Day 43 in Group B (t=2.592, p=0.041), despite a significant decline in titre from Day 15 (t=8.468, p=0.00015).

We have previously shown that a consistent specific intestinal antibody response can only be demonstrated in the IgA class.[14] In this study the same situation prevailed, with only specific IgG and IgM responses determined in exceptional responders, therefore those results are not presented here.

Pattern of Specific Serum Immune Responses

Serum IgA Response

As observed with the intestinal fluid samples, the mean baseline pre-vaccination serum specific IgA responses did not differ between the three groups (F=0.477, p=0.629).

Following the commencement of vaccination, an increase in the specific antibody titre was observed (Figure 9.2). This rise reaching significance as early as Day 7 in Group A (t=4.966, p=0.00254). The increase continued as a series of significant rises over the previous sample from Day 4 to 7 (t=2.483, p=0.048), and Day 7 to 11 (t=3.348, p=0.016), the peak response occurred in the Days 11 to 14 period, with no difference between these titres (t=2.378, p=0.055). Following this peak response a decline in the specific antibody titre occurred, with significant falls evident between days 15 and 19

in Group B (t=4.081, p=0.0065) and Days 17 and 21 in Group A (t=2.788, p=0.032). Despite this rapid decline from Day 15, the specific serum IgA antibody titre remained significantly elevated above the pre-vaccine baseline on Day 43 (t=3.900, p=0.023).

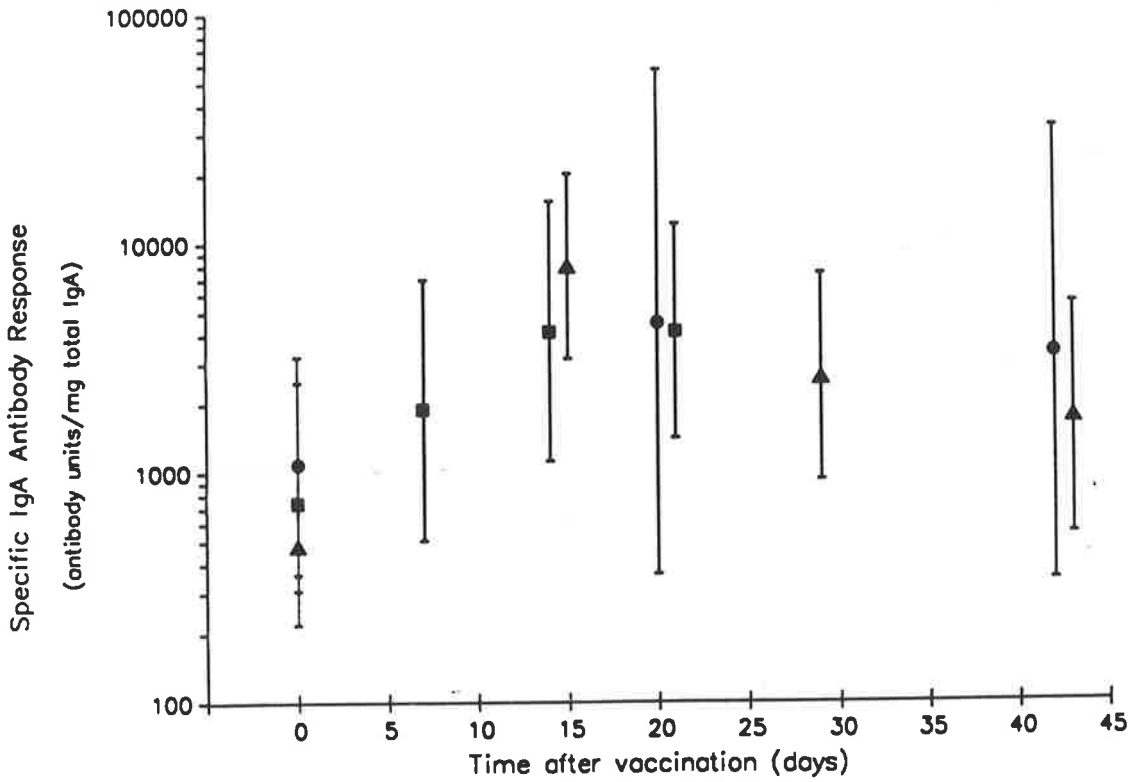
It is apparent from this study that the serum specific antibody response parallels that of the intestine following oral vaccination.

Just as was observed with three doses, a single dose of *S. typhi* Ty21a resulted in a significant increase in the specific IgA antibody titre by Day 7 (t=3.359, p=0.028) (Figure 9.2). Similarly, the mean Day 14 response was no different from that observed in Groups A and B (F=0.472, p=0.632). There was a subsequent decline in titre until the second dose was administered on Day 21. There was no significant difference between the Day 21 responses of Groups A and C, and the Day 19 response of Group B (F=0.431, p=0.658). Following the second dose, there was an apparent increase in antibody titre peaking on Day 35 - although this increase from Day 21 was not statistically significant (t=1.029, p=0.362). However, this second dose had no long term benefit as a significant decline in antibody titre was still evident from Day 21 to 42 (t=2.828, p=0.047), although remaining significantly elevated above the baseline pre-vaccination titre (t=3.886, p=0.018).

The pattern of the serum response following the divided single doses closely resembles that following the short three dose course. However the presence of significant serum IgA antibody response in the absence of a similar intestinal IgA response further demonstrates the dissociation between local antibody production and serum antibodies.

FIGURE 9.1

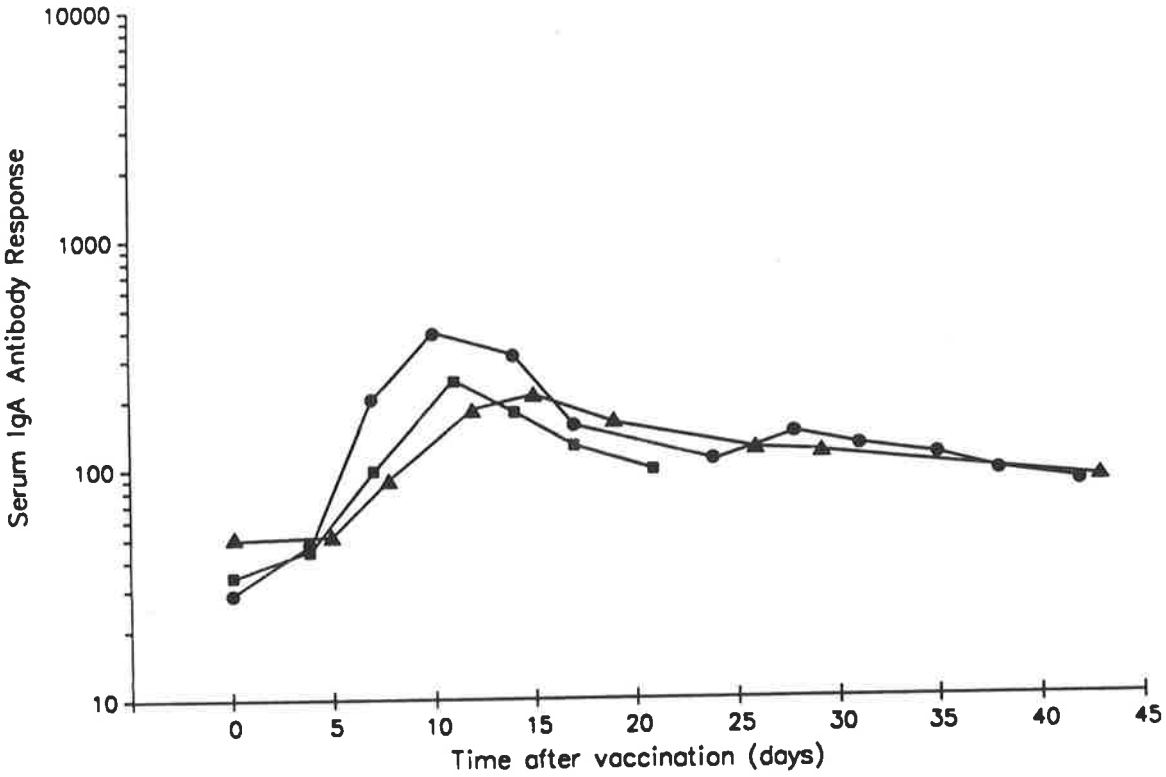
SPECIFIC INTESTINAL IgA RESPONSES FOLLOWING ORAL IMMUNIZATION WITH *Salmonella typhi* Ty21a



All responses given as geometric mean titre ± 95% CI and adjusted for total IgA.
Group A response (■) - three doses two days apart
Group B response (▲) - three doses two days apart
Group C response (●) - two doses 21 days apart

FIGURE 9.2

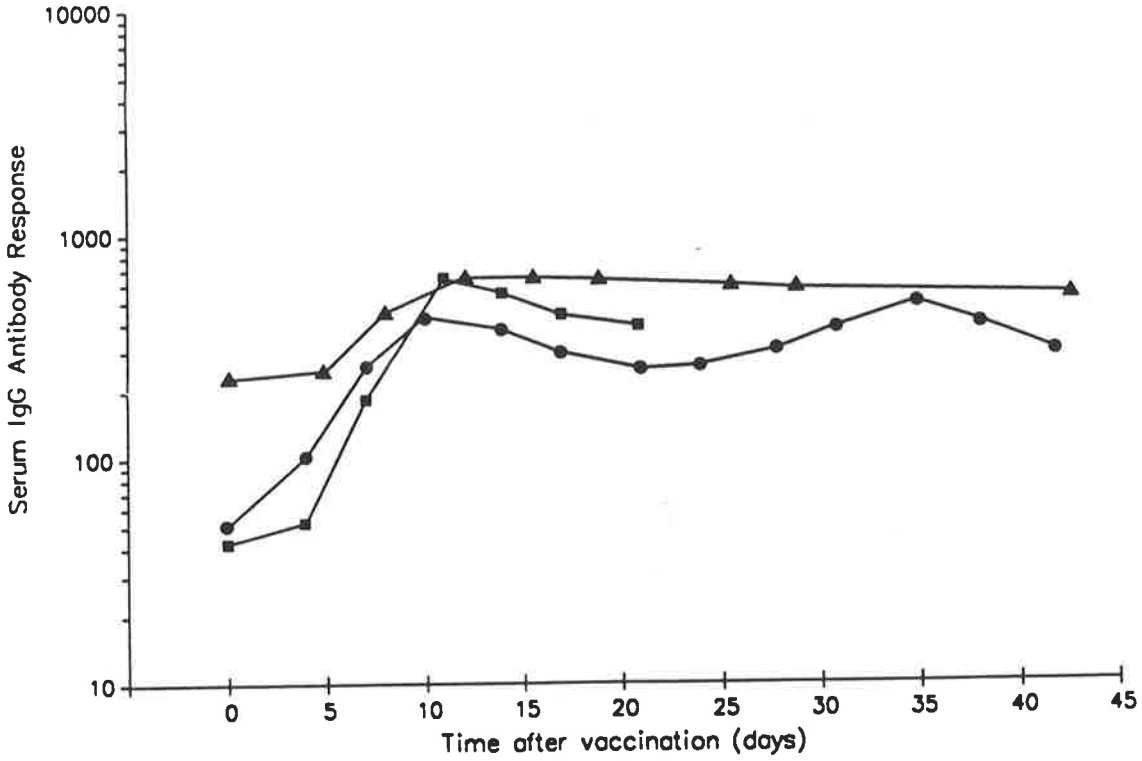
SPECIFIC SERIAL SERUM IgA RESPONSES



Serum antibody titre is the reciprocal of the dilution giving an OD of 0.15 units.
All responses given as geometric mean titre ± 95% CI
Group A response (■) - three doses two days apart
Group B response (▲) - three doses two days apart
Group C response (●) - two doses 21 days apart

FIGURE 9.3

SPECIFIC SERIAL SERUM IgG RESPONSES



Serum antibody titre is the reciprocal of the dilution giving an OD of 0.15 units.
All responses given as geometric mean titre ± 95% CI
Group A response (■) - three doses two days apart
Group B response (▲) - three doses two days apart
Group C response (●) - two doses 21 days apart

Serum IgG Response

In this instance, the baseline pre-vaccination titres were not comparable. The difference was due to the significantly lower titres observed in Group A. There was no difference between the baseline values of Groups B and C (t=1.987, p=0.074). Despite the low initial pre-vaccination titres, the Group A response followed the pattern of the Group B and C responses (Figure 9.3).

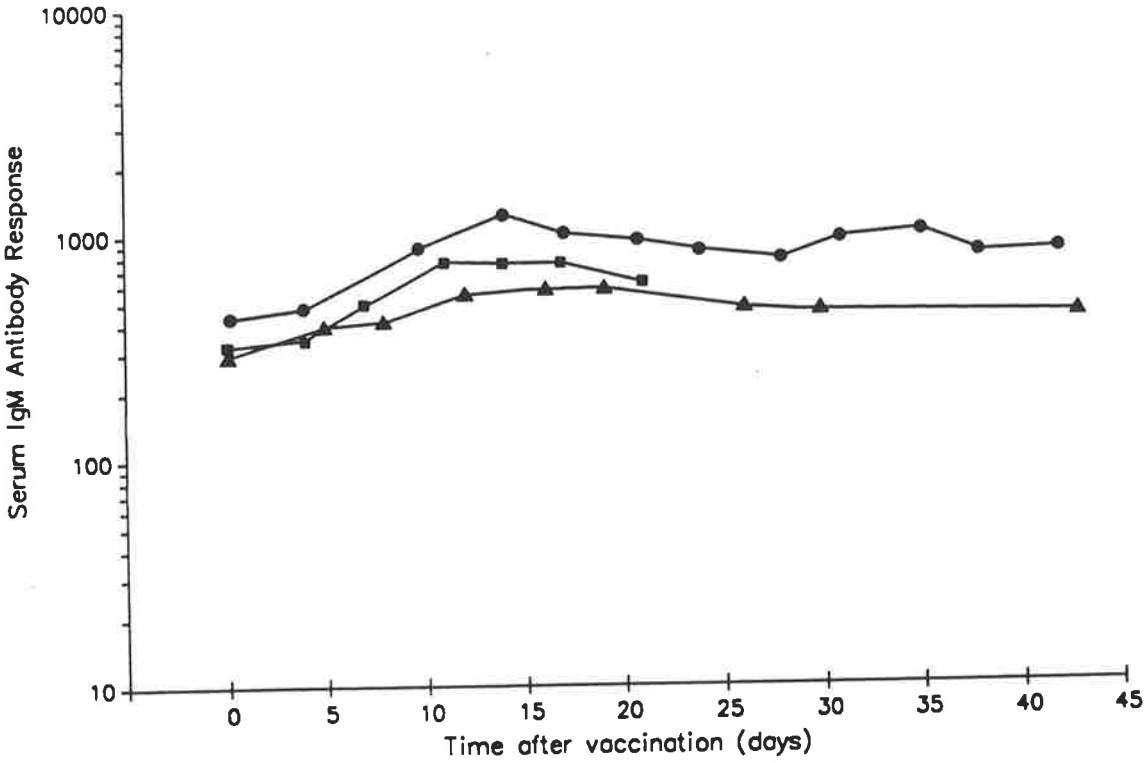
Following vaccination, there was a rise in the serum IgG antibody response which became statistically significant on Day 8 in Group B (t=3.043, p=0.023). Such a response was not evident on Day 7 of Group A (t=2.252, p=0.065). The IgG response appeared to peak around Day 15 (t=3.129, p=0.020). It did not decline from Day 15 to Day 43 (t=1.734, p=0.134) and remained significantly elevated above baseline pre-vaccination levels (t=3.142, p=0.014).

Following the first single dose the specific serum IgG antibody titre gradually rose to peak on Day 14 (t=4.662, p=0.0096). There was a rapid decline in titre which on Day 21 was significantly lower than on Day 14 (t=4.458, p=0.011), but still significantly elevated above the pre-vaccination baseline (t=4.353, p=0.012). From the second dose on Day 21 there was a significant rise to Day 31 (t=3.963, p=0.017) in specific IgG antibody. This second peak response was only short-lived, with there being a significant decline from Day 35 to Day 42 (t=2.949, p=0.042), which still remained significantly elevated above baseline (t=4.353, p=0.012).

There was no significant difference between the Day 21 responses of Groups A and C, and the Day 19 response of Group B (F=0.420, p=0.664), nor was there any difference between the Day 43 response of Group B and the Day 42 response of Group C (t=0.842, p=0.420).

FIGURE 9.4

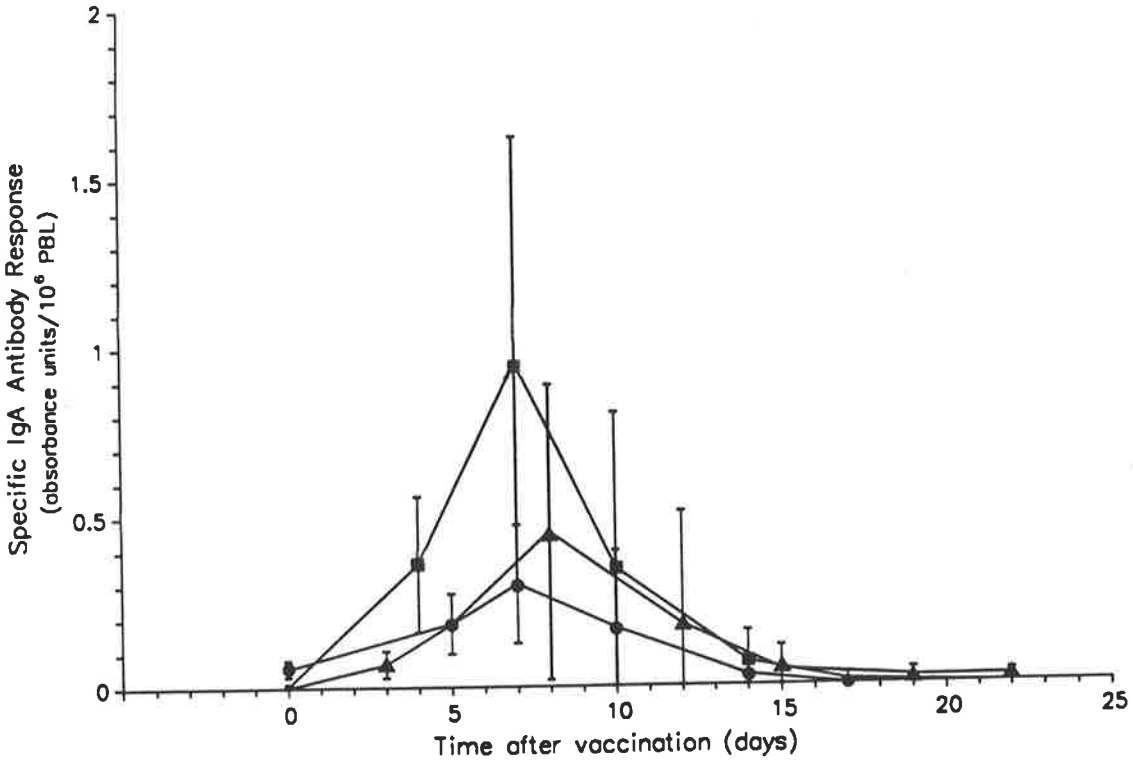
SPECIFIC SERIAL SERUM IgM RESPONSES



Serum antibody titre is the reciprocal of the dilution giving an OD of 0.15 units.
All responses given as geometric mean titre ± 95% CI
Group A response (■) - three doses two days apart
Group B response (▲) - three doses two days apart
Group C response (●) - two doses 21 days apart

FIGURE 9.5

SPECIFIC ANTI-TYPHOID IgA RESPONSES IN PERIPHERAL BLOOD LYMPHOCYTES



All responses given as arithmetic mean titre ± 95% CI
Group A response (■) - three doses two days apart
Group B response (▲) - three doses two days apart
Group C response (●) - two doses 21 days apart

Serum IgM Response

The mean baseline pre-vaccination specific serum IgM responses did not differ between the three groups (F=0.299, p=0.746).

Following vaccination there was a significant rise in the serum IgM response evident on Day 7 in Group A (t=2.543, p=0.044), peaking on Day 15 for Group B (t=3.024, p=0.023) and Day 17 for Group A (4.295, p=0.0051), and declining to become not significantly different from the pre-vaccination baseline on Day 43 (t=1.843, p=0.115) (Figure 9.4).

It was observed in this study that the serum IgM response to one dose of the vaccine was more rapid than after three doses, being significantly elevated on Day 7 (t=3.513, p=0.025), peaking on Day 10 (t=4.11, p=0.015) and declining significantly from Day 14 to Day 42 (t=4.028, p=0.016) (Figure 9.4). The serum IgM response on Day 38 was not significantly different from the pre-vaccination baseline level (t=2.289, p=0.084).

The second dose does not seem to have any significant effect on this pattern of response, in that there was no difference between the mean Day 21 responses of Groups A and C, and the mean Day 19 response of Group B (F=0.811, p=0.462). Furthermore, there was not difference between the Day 43 level of Group B and the Day 42 level of Group C (t=1.148, p=0.278).

Pattern of Specific Peripheral Blood Lymphocyte Response

In all subjects receiving the short schedule of vaccination, an anti-typhoid LPS IgA antibody response (optical density >0.100 units/10^6 PBL) was detected in their sampled PBL (Group A Day 7 mean optical density peak of 0.952 units, 95% CI 0.283 to 1.620 units; Group B Day 8 mean optical density peak 0.454 units, 95% CI 0.020 to 0.888 units). The pattern of the

response was the same as that described in previously[14] peaking around Day 7 and declining to be not detectable after Day 17 (Figure 9.5).

Following the first single dose a response was identified in all subjects (Group C Day 7 mean optical density peak 0.302 units, 95% CI 0.129 to 0.475 units) with a pattern of response identical to that of three doses, except of a lesser magnitude. Following the second single dose on Day 21, no specific antibody response was observed in the subjects' PBL at any stage or in any antibody class over the subsequent 21 days.

Anti-typhoid IgM and IgG responses were evident but variable in Groups A and B with not all subjects having such responses. In Group C, no IgM response was identified in any subjects' PBL.

Conclusion

The main conclusion from this study was that in the evaluation of a candidate vaccine against an enteric bacterial pathogen, the optimal time for obtaining intestinal fluid samples for determination of a specific local antibody response was during the period commencing with Day 14 and concluding with Day 21 post-vaccination. In addition, a single time-point determination of specific serum antibody was not a reliable indicator of a specific local immune response to an orally administered vaccine.

9.2 *Effect of Dose Regimen and Formulation on the Humoral Immune Response*

Introduction

The continued development of effective vaccines against enteric bacterial infections requires a thorough understanding of the local and systemic immune responses to a locally presented bacterial pathogen and the

factors which may affect this. The meaningful evaluation of potential candidate vaccines against pathogens such as typhoid fever rely heavily upon measuring the resulting immune responses at an optimal point. The orally administrable typhoid vaccine, *S. typhi* Ty21a, represents one potential intervention against an enteric bacterial disease. However, this one vaccine has been demonstrated to provide quite variable degrees of protection against typhoid fever when administered to very different populations in several quite different formulations and dose schedules.[103,220,222,223,225-227,441] In one instance a particular formulation failed to provide any better protection than the parenterally administered killed typhoid vaccine,[226] although it did have negligible adverse reactions in comparison.

With any newly developed drug it is necessary to investigate the dose response patterns and to evaluate the efficacy of a particular delivery system or formulation in providing the optimal dose of the drug to the desired site. Live oral bacterial vaccines are no different from other pharmaceuticals in this respect; specific measures that need to be undertaken to maximize their bioavailability are lyophilization of the bacteria to ensure their survival during prolonged storage and the development of suitable measures for the protection of the organisms from the lethal effects of gastric acid.[442]

The information obtained from this study possibly provided one explanation for the variation in protective efficacy seen in the previous studies, and enable more effective production and delivery of attenuated bacterial vaccines to the intestine, and to attempt to provide a further understanding of the potential effects on the humoral immune response of differing dose regimens and formulations of the attenuated live vaccine organism *S. typhi* Ty21a.

Having earlier defined the optimal timepoints for obtaining samples of serum and intestinal fluid following oral vaccination (Section 9), it was hoped that additional information would be provided by this study, which would contibute to the more effective production and delivery of attenuated bacterial vaccines to the intestine, and to attempt to provide a further understanding of the potential effects on the humoral immune response of differing dose regimens and formulations of the attenuated live vaccine organism, *S. typhi* Ty21a.

Subjects and Methods

Fifty-three healthy adults (15 women and 38 men, 18-40 years of age) participated in this study. None of the volunteers had any previous exposure to typhoid fever, and only one subject (a member of Group A) had been vaccinated against typhoid (with the parenterally administered heat-phenol-inactivated typhoid vaccine) in the past 7 years. None of the subjects had any history or current symptoms of gastrointestinal tract disease.

The subjects were allocated randomly to nine study groups, called A to J. Each group was vaccinated by means of vaccine organism, route, dose, formulation and schedule appropriate to their respective group (Table 9.1).

All vaccine doses were supplied by Enterovax Limited, Adelaide South Australia, except: formalin-killed doses for Group B were supplied by Dr J. Hackett of the University of Adelaide's Department of Microbiology and Immunology; the enteric-coated capsules ("Typh-Vax (Oral)") used as the vaccine doses for Group H, were manufactured by the Swiss Serum and Vaccine Institute, Berne, Switzerland, with these doses being obtained from the Australian distributor, the Commonwealth Serum Laboratories, Parkville, Victoria, with careful attention being paid to maintaining the cold

chain; and the monovalent heat-killed typhoid vaccine doses used in Group C were also obtained from the Commonwealth Serum Laboratories.

One batch of fermenter-grown lyophilized "smooth" *S. typhi* Ty21a vaccine doses was used in this study. The total number of organisms by direct microscopy in the batch were $5.2x10^{11}$, with $1.7x10^{11}$ (33%) viable by colony counts. However, it appears that whenever *S. typhi* Ty21a is viable-counted directly from fermentation broths or agar plates, the viable count represents 65% of the total (microscope) count. If this observation is taken into consideration, then the percentage of initially viable cells actually surviving lyophilization is approximately 50%. One explanation for this discrepancy is through the possible existence of viable but non-culturable bacterial cells (Beyer L. unpublished observation).

The vaccine doses that comprised "rough" *S. typhi* Ty21a (Group J) were prepared by growing the organisms in the absence of exogenous galactose. This resulted in the organisms being unable to assemble the O-antigen polysaccharide side-chains of the lipopolysaccharide (LPS). All "rough" strains failed to slide agglutinate with anti-9, 12 O-antigen typing sera, whereas all "smooth" strains agglutinated.

Notes on Collection of Samples

Intestinal fluid and serum were obtained as detailed in Section 11.1. Where possible, intestinal fluid samples were obtained through the technique of intestinal intubation on Day 14 or 15 after the commencement of vaccination. In one group (Group E) it was necessary to obtain the samples on Day 21 instead. It is not believed that this delayed collection would have any adverse effect on the relevance of the results or the comparisons made with other groups, since it was demonstrated in Section 13 that there was not

a significant difference between Day 14 and Day 21 levels of intestinal specific antibody. The intestinal fluid samples from the group receiving the subcutaneous injection of killed typhoid vaccine (Group C) were obtained fourteen days following the second dose of the vaccine, that is 26 days after the commencement of vaccination.

Statistics

Serum and intestinal antibody responses are represented as the reciprocal of the dilution that gave an optical density of 0.15 ELISA absorbance units and are expressed as units of antibody. This absorbance was chosen as it represented the upper limit of the 95% confidence intervals (CI) above background levels. The intestinal fluid specific antibody units were adjusted for total class-specific immunoglobulin content and were expressed as units of specific antibody/mg of total class specific immunoglobulin. Sera and intestinal antibody responses were ln(x) transformed prior to their statistical analysis. Differences between the means of the pre- and post-vaccination antibody titres within any one group were determined using Student's t-test. Comparisons of the fold rises in specific antibody titres and the PBL responses where available, between groups, were analyzed using the two-tailed Wilcoxon Rank Sum Test (WRST) for independent samples.

Intra-group analysis of the significance of PBL responses was determined using the non-parametric Sign Rank Test for paired data.

Any differences between the means of the baseline samples for each group was examined using The one-way analysis of variance (ANOVA), with only the F value and significance level being reported.

TABLE 9.1

DOSE REGIMEN AND FORMULATION OF *Salmonella typhi* Ty21a

Group	Organism Description	Formulation	Number of Organisms Total	Number of Organisms Viable (% Total)	Number of Subjects	Dose Schedule
A	smooth Ty21a	live, freshly-harvested suspension	$1.8x10^{11}$	$1.4x10^{11}$ (78%)	6	A
B	smooth Ty21a	formalin-killed suspension	$1.3x10^{11}$	(0%)	4	A
C	smooth Ty2	heat killed, phenol preserved	$5.0x10^{8}$	(0%)	6	D
D	smooth Ty21a +	lyophilized and reconstituted	$5.2x10^{11}$	$1.7x10^{11}$ (33%)	7	A
E	smooth Ty21a +	lyophilized and reconstituted	$5.2x10^{11}$	$1.7x10^{11}$ (33%)	5	B
F	smooth Ty21a +	lyophilized and reconstituted	$5.2x10^{10}$	$1.7x10^{10}$ (33%)	7	A
G	smooth Ty21a +	lyophilized and reconstituted	$5.2x10^{9}$	$1.7x10^{9}$ (33)	7	A
H	smooth Ty21a	enteric-coated capsule, "Typh-Vax (Oral)"	$5x10^{10}$	$1.1x10^{9}$ (2.2%)	6	C
J	rough Ty21a	live, freshly-harvested suspension	$1.8x10^{11}$	$1.3x10^{11}$ (72%)	5	A

Smooth - grown in presence of exogenous galactose; Rough - grown in absence of exogenous galactose; + - all doses used from same batch

Dose Schedules:

A - three orally administered doses, each dose on alternate days. Pre-treatment with sodium bicarbonate solution.

B - single orally administered dose only. Pre-treatment with sodium bicarbonate solution.

C - three orally administered doses, each dose on alternate days. No pre-treatment.

D - two subcutaneously injected doses 14 days apart. No pre-treatment.

Results

Intestinal IgA Immune Responses

The mean baseline pre-vaccination intestinal anti-typhoid antibody levels between all nine groups were not significantly different (F=1.07, p=0.403) confirming that the subjects were drawn from the same population.

For comparison of the differences in the change in the intestinal specific antibody levels following the various doses, regimens and formulations refer to Figure 9.6. The individual specific intestinal IgA responses expressed as fold-rises post-vaccination, are depicted in Figure 9.7, with the group values and geometric mean fold rises presented in Table 9.2.

As was evident in the antibody kinetics study detailed above, lyophilization of *S. typhi* Ty21a did not appear to adversely affect its ability to generate a significant local intestinal antibody response (Group D), with 6/7 subjects having a four-fold or greater rise in specific intestinal IgA (Figure 9.7). The overall response was not significantly different from that induced with the same dose of the freshly harvested preparation (Group A) (Table 9.2) with 6/6 subjects having a similar level of response, but was significantly greater than that induced by a similar dose of killed organisms (Group B) (1/4 responders). There was both a greater proportion of responders and a more consistent level of intestinal antibody responses to the larger viable vaccine (10^{11}) doses, whether freshly-harvested or lyophilized, than with any other preparation (Figure 9.7).

A single dose of *S. typhi* Ty21a failed to generate a satisfactory response with only 2/5 subjects responding with a four-fold or greater rise in specific intestinal antibody (Group E), and an overall geometric mean fold rise of only a quarter of that produced by the three doses of 10^{11} viable organisms (Table 9.2). However this response was only slightly better than

that induced by three doses of 10^{10} viable organisms (Group F) where there were only 2/7 satisfactory responders (Figure 9.7).

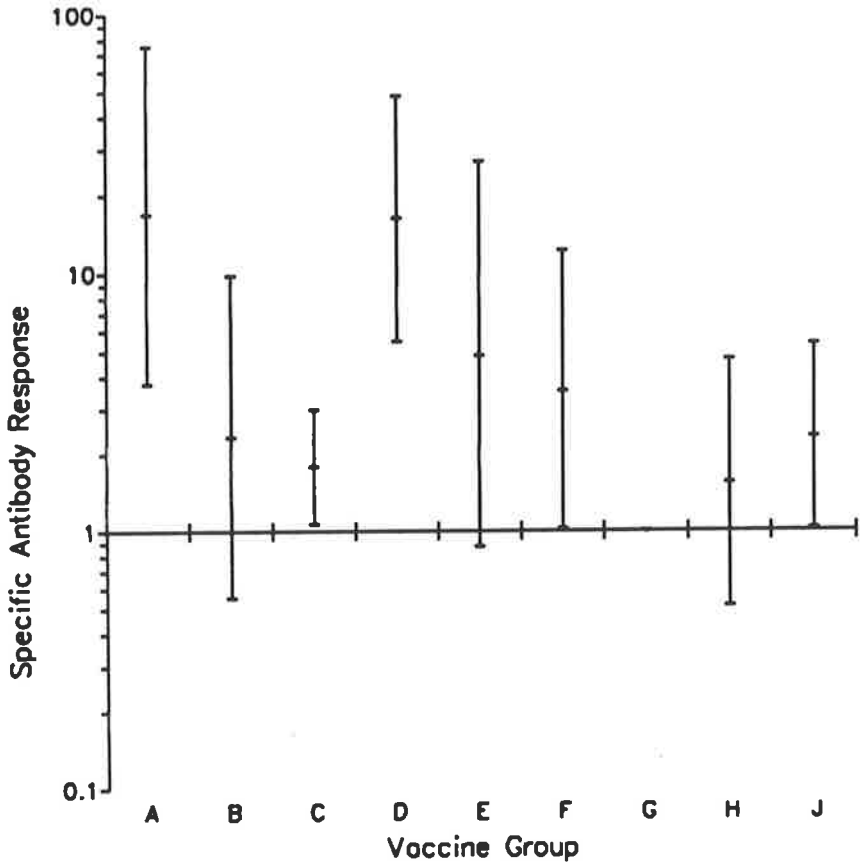
An intestinal IgA immune response was not measurable with our assay system in any of the subjects receiving three doses of 10^9 live organisms (Group G), irrespective of the presence of $3.5x10^9$ killed organisms per dose. This situation, previously reported, is the presumed fringe region of efficacy for *S. typhi* Ty21a,[228] as doses less than this have been reported not to confer protection in any but exceptional cases.[224]

The commercial enteric coated formulation (Group H) failed to produce a consistent local intestinal anti-typhoid antibody response with only 1/5 subjects from whom intestinal fluid could be obtained having a four-fold or greater specific IgA antibody response. The response that was stimulated was not significantly different from that induced by the oral killed vaccine doses (Group B). This latter association was not unexpected, since nearly all the organisms comprising the enteric coated capsules were killed (approximately $5x10^{10}$, or 97.8% of total organisms) which is comparable to the number of organisms comprising the killed doses (10^{11}) used in Group B. The 10^9 live organisms present in these doses did not appear able to produce a local antibody response detectable in our assay system as observed in the responses found in Group G.

When the subjects in Group J received the three doses of 10^{11} freshly harvested "rough" doses a significant intestinal anti-typhoid LPS antibody response was detected in only 1/5 subjects. As a result, the overall response was much also less than that observed with a similar course of smooth organisms, but was not significantly different from any other dose regimen or formulation (Figure 9.7).

FIGURE 9.6

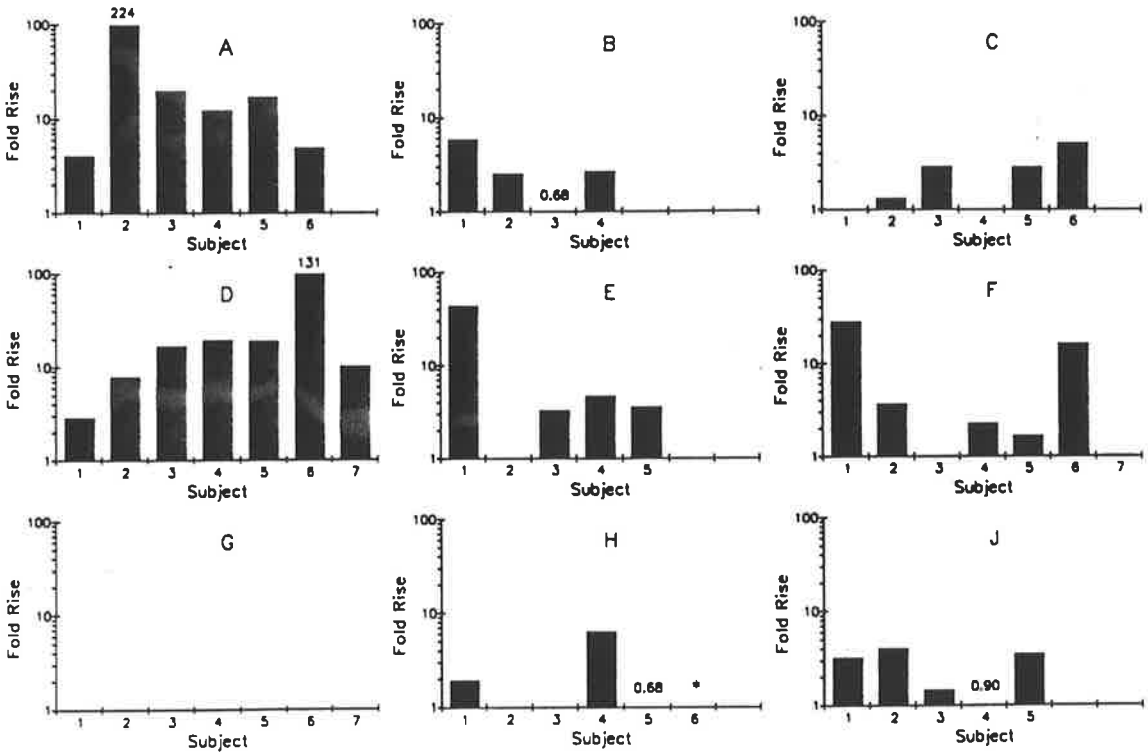
COMPARATIVE FOLD-RISE SPECIFIC JEJUNAL IgA ANTIBODY RESPONSES



All responses represented as the geometric mean fold-rise ± 95% CI

FIGURE 9.7

INDIVIDUAL SPECIFIC JEJUNAL IgA ANTI-TYPHOID LPS ANTIBODY RESPONSES BY VACCINATION GROUP



All columns represent the fold rise increase in Day 14 post-vaccination intestinal anti-typhoid IgA antibody response with respect to Day 0 (pre-vaccination) for an individual subject.
Refer to Table 9.1 for details of vaccine doses, regimens and formulations.
Columns with fold rise increases above 100 or below 1 are labelled with the actual response.
Group B responses have not been included for ease of presentation, that schedule being represented by Group A.
All responses depicted by the solid columns are the post-primary vaccination responses, shaded columns represent the post-secondary immunization in comparison with the pre-vaccination titre.
* intestinal fluid could not be collected from this subject.

TABLE 9.2

SPECIFIC JEJUNAL ANTI-TYPHOID IgA ANTIBODY RESPONSE

Vaccine Group	Vaccination Pre	Vaccination Post	Fold Rise	Significance (p value)
A	580	9740	16.8	0.0047
	(330-1020)	(2700-35140)		
B	690	1600	2.32	0.159
	(260-1830)	(247-10400)		
C	609	1078	1.77	0.037
	(579-641)	(527-2200)		
D	518	7940	16.3	0.0011
	(327-822)	(3135-20100)		
E	938	4545	4.84	0.063
	(343-2570)	(361-57200)		
F	929	3280	3.53	0.048
	(284-3030)	(542-19800)		
G	Specific Antibody Titre Below Limit of Detection			
H	371	573	1.54	0.34
	(137-1000)	(306-1070)		
J	427	988	2.32	0.047
	(189-961)	(527-1850)		

Jejunal antibody titre is the reciprocal of the dilution giving an O.D. of 0.15 units, adjusted for total IgA.
All responses given as geometric mean titres with values in parentheses indicating 95% CI

Similarly, parenteral immunization with a commercial killed typhoid vaccine, resulted in only 1/6 subjects just achieving a four-fold or greater rise in specific intestinal IgA antibody.

Serum Antibody Responses

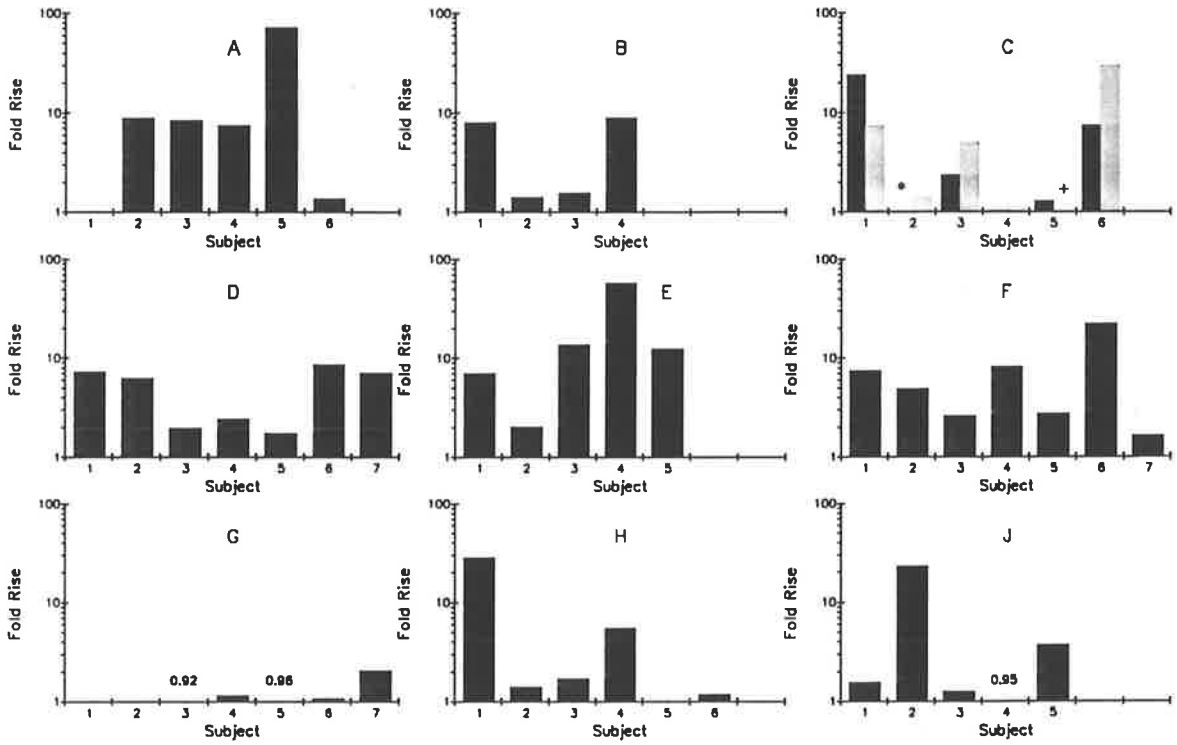
As can be deduced by comparison of the individual intestinal specific IgA responses in Figure 9.8 and the individual serum specific IgA responses in Figure 9.9, this study reconfirmed the previously reported observations that the magnitude of specific antibody responses in serum samples obtained at a single time point correlate poorly with the magnitude of local intestinal specific antibody responses,[14] although it is broadly apparent that those subjects with the largest intestinal antibody responses were more likely to have the largest serum IgA responses.

In addition, we observed that for any one subject group, a change in the geometric mean fold rise of any one particular antibody class was reflected in the others (Figure 9.8). The collated results for each subject group are presented in Table 9.3.

It is worth noting that there were no significant differences between the two groups (Group A and Group B) in the geometric mean fold rises in specific serum IgA (WRST, $p>0.50$), IgG (WRST, $p=0.35$) or IgM (WRST, $p=0.26$). In Group C, the serum anti-typhoid IgA and IgG antibody responses were not significantly elevated after either one or two doses (Table 9.3), however there was a significantly elevated IgM response following the first dose. The second dose had no effect on the serum antibody response detectable after the primary dose in any serum antibody class (Table 9.3) (Figure 9.8).

FIGURE 9.8

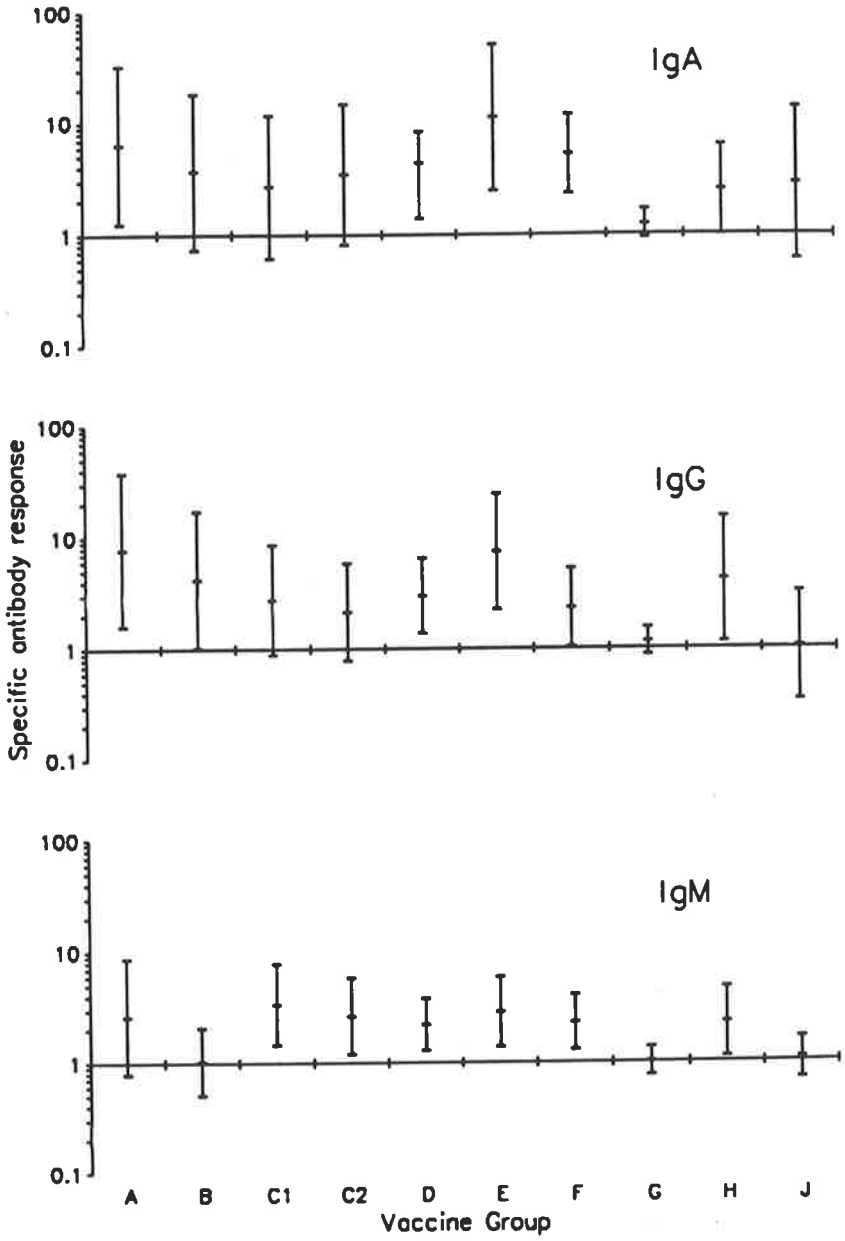
INDIVIDUAL SPECIFIC SERUM IgA ANTI-TYPHOID LPS ANTIBODY RESPONSES BY VACCINATION GROUP



All columns represent the fold rise increase in Day 14 post-vaccination intestinal anti-typhoid IgA antibody response with respect to Day 0 (pre-vaccination) for an individual subject.
Refer to Table 9.1 for details of vaccine doses, regimens and formulations.
Group B responses have not been included for ease of presentation, that schedule being represented by Group A.
Columns with fold rise increases above 100 or below 1 are labelled with the actual response.
All responses depicted by the solid columns are the post-primary vaccination responses, shaded columns represent the post-secondary immunization in comparison with the pre-vaccination titre.
* indicates a post-primary vaccination fold rise of 0.57.
+ indicates a post-secondary vaccination fold rise of 0.86.

TABLE 9.9

COMPARATIVE FOLD-RISE SPECIFIC SERUM IgA ANTIBODY RESPONSES



All responses represented as the geometric mean fold-rise ± 95% CI
C1 geometric mean fold-rise represents the Day 12 post (1)-vaccination titre/pre-vaccination titre
C2 geometric mean fold-rise represents the Day 26 post (2)-vaccination titre/pre-vaccination titre

TABLE 9.3

SPECIFIC SERUM ANTI-TYPHOID IgA ANTIBODY RESPONSE

Vaccine Group		IgA	IgG	IgM
A	Pre	46.3 (18.3-117)	266 (115-614)	375 (159-882)
	Post	291 (33.0-2570)	2060 (424-10000)	929 (205-4220)
Significance		0.033	0.021	0.11
B	Pre	26.8 (11.2-64.2)	103 (42.6-251)	207 (60.3-707)
	Post	97.3 (23.2-407)	742 (251-2190)	213 (34.3-1320)
Significance		0.084	0.048	0.90
C	Pre	114 (39.8-329)	209 (64.1-680)	692 (372-1290)
	Post (1)	304 (121-763)	575 (257-1290)	2350 (853-6480)
	Post (2)	393 (158-979)	447 (283-708)	1840 (912-3700)
Significance*		0.15/0.079	0.070/0.11	0.014/0.025
D	Pre	49.4 (18.6-131)	226 (85.1-598)	272 (79.3-935)
	Post	210 (90.8-485)	674 (172-2640)	606 (245-1500)
Significance		0.0013	0.014	0.011
E	Pre	28.5 (7.95-102)	71.2 (6.19-818)	428 (377-486)
	Post	313 (122-803)	283 (152-526)	1230 (574-2620)
Significance		0.012	0.18	0.016

Serum antibody titre is the reciprocal of the dilution giving an OD of 0.15 units.
All responses given as geometric mean titres + 95% CI
* - For Group C two significance values are provided. The first is the Day 12 Post (1)-vaccination vs. Pre-vaccination, the second is the Day 26 Post (2)-vaccination vs. Pre-vaccination.

TABLE 9.3 (CONTINUED)

SPECIFIC SERUM ANTI-TYPHOID IgA ANTIBODY RESPONSE

	Vaccine Group	IgA	IgG	IgM
F	Pre	38.3 (16.4-91.7)	146 (62.6-343)	198 (68.4-575)
	Post	198 (48.1-817)	340 (129-899)	401 (123-1310)
Significance		0.0027	0.042	0.031
G	Pre	38.3 (15.8-93.0)	164 (78.1-344)	127 (51.6-315)
	Post	43.8 (18.6-103)	190 (75.9-477)	129 (53.9-310)
Significance		0.26	0.23	0.91
H	Pre	78.0 (27.8-219)	102 (29.0-359)	249 (117-532)
	Post	220 (78.4-619)	425 (245-737)	612 (293-1280)
Significance		0.11	0.036	0.016
J	Pre	43.5 (18.3-104)	60.8 (27.0-137)	450 (285-711)
	Post	122 (20.5-731)	63.6 (14.8-273)	482 (346-670)
Significance		0.15	0.92	0.68

Serum antibody titre is the reciprocal of the dilution giving an OD of 0.15 units.
All responses given as geometric mean titres + 95% CI
* - For Group C two significance values are provided. The first is the Day 12 Post (1)-vaccination vs. Pre-vaccination, the second is the Day 26 Post (2)-vaccination vs. Pre-vaccination.

Significant serum IgA anti-typhoid antibody responses were evident after both the single oral dose of 10^{11} (Group E) (t=4.42, p=0.012) and the three oral doses of 10^{10} (Group F) (t=4.91, p=0.0027). These serum IgA anti-typhoid responses were not significantly different from those induced by any other dose, route or formulation used (Figures 9.8, 9.9), except for the three oral doses of 10^9 live organisms (Group G). This latter dose regimen failed to stimulate a significant anti-typhoid response in any serum antibody class (Table 9.3), and any response that was generated was consistently lower than that for any other dose, route or formulation used.

PBL Specific IgA Antibody Responses

The pattern of responses observed in the intestinal fluid detailed above, were confirmed with the determination of specific IgA antibody release *in vitro* by the subjects' PBL. The live freshly harvested vaccine organisms (Group A) were found to stimulate a significantly greater PBL response than rough fresh organisms of the same dose (Group J) (WRST, p=0.029), than a single dose of 10^{11} (Group E) (WRST, p=0.016), enteric-coated formulation (Group H) (WRST, p=0.0095), and three doses of 10^9 live organisms (Group G) (WRST, p=0.0061). There was no difference in the response compared to lyophilized organisms of the same dose and schedule (Group D) (WRST, p=0.12) or to three doses of 10^{10} organisms (Group F) (WRST, p>0.50). Although the magnitude of responses did vary between groups, there was very little variation in the numbers of responders between groups, with 100% of subjects in Groups A, D, E, F, J responding, and with only 4/6 subjects in Group H responding. There were no responders in Group G who received the three doses of 10^9 live organisms, supporting the intestinal and serum findings (Figure 9.10).

Following their primary parenteral vaccination only 2/6 subjects in Group C were noted to have a low magnitude PBL IgA response occurring by Day 7 post-vaccination.

Unfortunately, it was not possible to perform the PBL-ELISA on the PBL of subjects in Group B who received the three doses of 10^{11} killed organisms.

Conclusion

This section described a detailed evaluation of a variety of dose regimens and formulation of typhoid vaccines examining the duration and magnitude of the resulting specific immune response.

Several conclusions could be drawn from this study:

(a) *S. typhi* Ty21a does not appear capable of obtaining sufficient exogenous galactose *in vivo* to synthesize the O polysaccharide of LPS as reflected in the consistently poor intestinal, PBL and sera antibody responses following immunization with rough *S. typhi* Ty21a (Group J).

(b) *S. typhi* Ty21a retained approximately 50% viability after lyophilization, and demonstrated no impairment in its ability to generate significant local antibody responses. The responses observed were identical to those produced with freshly prepared doses containing 10^{11} viable organisms.

(c) The presence of the killed vaccine organisms in the doses used in this study appeared unlikely to have contributed significantly to the immune responses observed following vaccination with the lyophilized doses, since the immune responses following the administration of 10^{11} formalin-killed organisms (Group B) were not significant.

(d) Three sequential doses of 10^9 live *S. typhi* Ty21a failed to stimulate a measurable immune response in the intestine of previously unexposed subjects. It is unlikely that this reflects any limitations of the assay for intestinal specific antibody, as a specific antibody response was also not identifiable at this dose using subjects' PBL. This is suggestive that the meagre response observed with the enteric-coated formulation is more likely attributable to the $5x10^{10}$ killed organisms present than to the $1.1x10^9$ viable ones. However even this response was substantially less than that of three doses of 10^{11} or 10^{10} live organisms in the presence of a similar number of killed vaccine organisms.

(e) A single oral dose of 10^{11} live *S. typhi* Ty21a generated widely disparate responses within that vaccination group (Group E), as represented by the broad range covered by the 95% confidence intervals (CI). This observation was initially noted previously (Section 13) where it was attributed to individual variation in response. From this observation it appears that to achieve the maximal recruitment of responders to *S. typhi* Ty21a several sequential doses comprising 10^{11} live organisms are required.

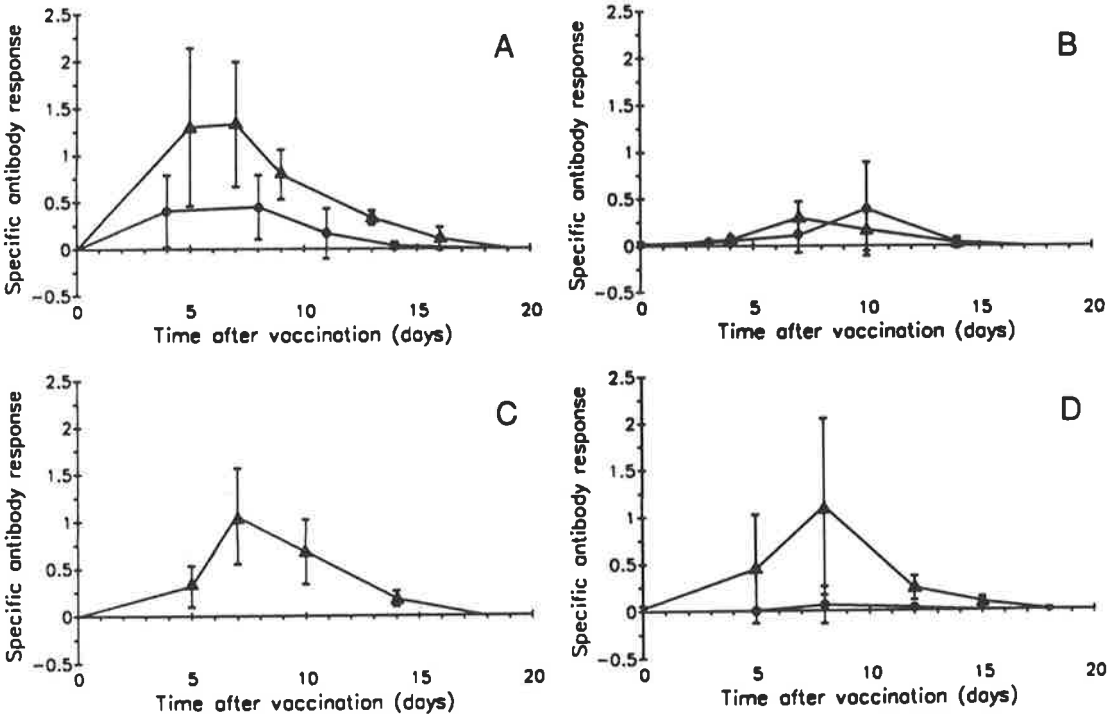
(f) This study supported the previous findings of the inadequacy of single-point determinations of antigen-specific serum antibody, irrespective of class (Section 13) for the accurate determination of an intestinal antibody response. However, sequential determination of a groups' response within an antibody class has been determined to be of most value (Section 13). If a point determination of serum antibody response is the only method available for the evaluation of the immunogenicity of a particular preparation, then this study suggests that the magnitude of the serum IgG response may provide the most consistent and sensitive predictor, as significant antibody responses were evident in this class when absent in or not significant in

others (for example: Group B, t=3.23, p=0.048; Group H, t=2.85, p=0.036) (Table 9.3), although the magnitude of the response may not correlate with that of the intestine.

(g) The PBL-ELISA was confirmed as a highly sensitive indicator of the primary intestinal exposure of an individual to an enteric bacterial organism. However due to its high degree of sensitivity, it was not a particularly useful discriminatory measure of the probable effectiveness of a particular vaccine formulation. It does however remain the most rapid way of determining an immune response in an individual following primary oral vaccination.

FIGURE 9.10

SPECIFIC *in vitro* PBL IgA ANTIBODY RESPONSES



All responses represented as the arithmetic mean absorbance at 405 nm ± 95% CI.

A -	▲	Group A mean responses	●	Group J mean responses
B -	▲	Group E mean responses	●	Group H mean responses
C -	▲	Group D mean responses		
D -	▲	Group F mean responses	●	Group G mean responses

10

SPECIFICITY OF THE IgA ANTI-LIPOPOLYSACCHARIDE ANTIBODY RESPONSE

Introduction

The local humoral response has been shown to be one of the major determinants of protection against enteric bacterial disease. Since the O-polysaccharide (O antigen) of the lipopolysaccharide (LPS) is the major immunodominant surface structure of the members of the Enterobacteriaceae, it is not surprising that following enteric infection with one of these organisms, most of the specific antibody is directed against the O antigen.[443] However, while some of the specific antibody may also be directed against the oligosaccharide constituting the core LPS structure,[444] the O antigen may block access to the core LPS.[445] While there is a some overlap of O antigens between the organisms comprising the different genera, with there being identical or very similar O antigens on occasions, the O antigens are usually specific for a particular species serotype.[446] Therefore it may also possible that specific antibodies directed against one O antigen type may provide cross-protection against bacteria with similar O antigen types of different species.[447]

The antigenic specificity of the core LPS structures is more diverse, with a reduction in genera or family specificity as antibodies are directed to lower portions of the core structure,[448,449] before reaching the Lipid A component, which only expresses microheterogeneity between species.[450,451] It has been shown that specific monoclonal antibodies directed against the oligosaccharide components of the core LPS may provide cross-protection

against other species members,[452,453] however others have reported that this is by no means certain.[454]

The attenuated live orally administrable typhoid vaccine organism, *S. typhi* Ty21a, being derived from the pathogenic typhoid organism *S. typhi* Ty2,[218] shares the same 9, 12 O antigens with it and many other members of the Kauffmann-White Serological Groups A, B, D_1 and D_2 *Salmonella*.[455]

The purpose of this study was to further characterize the antigenic specificity of the human jejunal and serum humoral antibody response to *S. typhi* Ty21a, and to review its potential protective efficacy against other *Salmonella* members as well as other members of the genus Enterobacteria.

Materials and Methods

For this study samples of serum and intestinal fluid which had been obtained from six human subjects orally immunized in a previous study with the live attenuated orally administered typhoid vaccine organism *S. typhi* Ty21a according to the standard method of vaccination (Section 7.4.1) were used.[14]

15.2.1 *Selection of lipopolysaccharide preparations*

The different LPS preparations used in this study are detailed in Table 10.1. The choice of LPS was mainly on our perceived needs and commercial availability. All LPS preparations were obtained from Sigma Chemical Co., St Louis, USA.

TABLE 10.1

LIPOPOLYSACCHARIDE PREPAPRATIONS USED IN ASSESSING SPECIFICITY OF HUMORAL IMMUNE RESPONSE

Souce Organism	Sigma* Catalogue Number	Method of Preparation
Escherichia coli Serotype O111:B4	L 2630	A
Escherichia coli Strain EH 100 (Ra mutant LPS)	L 9641	B
Salmonella enteritidis Serotype O9,O12	L 6011	A
S. minnesota Serotype O21	L 6261	A
S. minnesota Strain Re595 (Re mutant LPS)	L 9764	B
S. typhi Strain Ty2 Serotype O9,O12	L 6386	A
S. typhimurium Serotype O2,O4,O5,O12	L 6511	A
S. typhimurium Strain TV 119 (Ra mutant LPS)	L 6016	B
Shigella flexneri 1a	L 4393	A
Vibrio cholerae Serotype Inaba 569B	L 0385	

* - Sigma Chemical Company, St. Louis, Missouri, USA.

A - Phenol extracted
B - Chloroform-phenol-petroleum ether extracted

Ra mutant LPS comprises only Lipid A, KDO, and core oligosaccharide.
Re mutant LPS comprises only Lipid A and KDO.

The LPS from a small range of *Salmonella* serovars was selected. The *S. enteritidis* and *S. typhi* serovars both share the 9, 12 O antigens; *S. typhimurium* has the 4, 5, 12 O antigens (sharing the O 12 with *S. typhi*). *S. minnesota* only has the 21 O antigen, which is not shared by the other *Salmonella* LPS preparations used. For the examination of cross-reacting core LPS antibody production, LPS obtained from a Ra mutant strain of

S. typhimurium was used, and for anti-Lipid A antibodies the LPS from a Re mutant strain of *S. minnesota* was used. Ra mutants are incapable of producing the O antigen, so only have the core LPS present, and are known as "rough" strains. Re mutants are incapable of producing the O polysaccharide or the core oligosaccharide of LPS, only having the Lipid A and 2-keto-3-deoxy-D-manno-octonoate (KDO) present. This selection of LPS preparations would enable us in a limited manner to examine the IgA antibody specificity to O antigens within the same *Salmonella* serotype group (Group D_1), between other groups (Group B and 21), as well as assaying for the presence of anti-core LPS and Lipid A antibodies.

The remaining four LPS preparations were chosen so as to screen for cross-reactivity with members of other species. *E. coli* O111 is a common isolate from human faeces, the O antigen not having any similarities to the selected *Salmonella* LPS preparations to the best of our knowledge; the *E. coli* Ra mutant was chosen to screen for cross-reacting core LPS structures, providing any anti-core LPS antibodies were produced; *Sh. flexneri* 1a since shigella members are the closest phylogenetically to *Salmonella*;[125] and classical *V. cholerae* Inaba 569B, being quite distinct from *Salmonella* species.

Assays for Quantifying Specific Antibody

Quantification of the specific IgA antibody response in serum and intestinal fluid was determined using the ELISA described previously (Section 7.1).

Statistics

Sera and jejunal antibody responses were ln(x) transformed prior to their statistical analysis. Differences between the means of jejunal and sera

pre- and post-vaccination antibody titres within any one group were determined using Student's *t*-test for paired data. Comparisons of fold rises in specific antibody titres between groups, were analyzed using the two-tailed Wilcoxon Rank Sum Test (WRST) for independent samples.

Results

Antigenic Specificity of Jejunal Specific IgA Response

As has been reported previously,[14] the oral vaccination with *S. typhi* Ty21a stimulated the production of a significant jejunal (t=6.82, p=0.0024) (Table 10.2).

The intestinal and serum IgA responses were maximal against the *S. typhi* and *S. enteritidis* serovars LPS indicating that the antibody response was mainly directed against the 9, 12 O antigens (Table 10.2). There was no difference between the geometric mean fold rises of these responses (WRST, both serum and jejunum p>0.50).

In addition to the anti-typhoid LPS antibody responses, significant jejunal fluid IgA antibody responses were recorded post-vaccination against *S. enteritidis* LPS (t=7.64, p=0.0016), *S. typhimurium* LPS (t=5.41, p=0.0057), the Ra mutant of *S. typhimurium* LPS (t=2.84, p=0.047), and the *S. minnesota* LPS (t=3.32, p=0.029). There were no specific antibodies detectable against the Lipid A component as represented by the remaining *Salmonella* mutant LPS, prepared from the Re mutant of *S. minnesota* (Table 10.2), this was not surprising, since it is well established that antibodies do not generally react with LPS-bound Lipid A.[125]

The geometric mean fold rises in specific jejunal IgA reflects the similarities in the specificity of the antibody response (Figure 10.1). There was no significant difference between the response to *S. typhi*, *S. enteritidis*

and *S. typhimurium* LPS preparations (F=0.431, p=0.660); this difference retaining significance when the *S. minnesota* response was included (F=2.62, p=0.087). The responses as measured by the geometric mean fold rises against the LPS of the Re mutant of *S. minnesota* and the other non-*Salmonella* organisms were significantly less than the response against the *S. typhi* LPS (WRST, vs. *S. minnesota* Re LPS p=0.0079; vs. *E. coli* O111 LPS p=0.016; vs. *Sh. flexneri* 1a p=0.016; vs. *V. cholerae* Inaba LPS p=0.0079) (Figure 10.1). Interestingly, there was some evidence for cross-reactivity of the IgA antibody response with the particular *E. coli* core LPS used in this study reflected in the absence of a significant difference in the jejunal specific antibody response between the *S. typhi* LPS and the *E. coli* Ra mutant LPS (WRST, p=0.095), confirming similarities in the lower core structure LPS components.

These findings support the premise that most jejunal specific antibody produced is mainly directed against the O antigens, and that there are also produced specific IgA antibodies that cross-react with the *Salmonella* core oligosaccharide but not the Lipid A component. Apart from a suggestion of a response to the *E. coli* core LPS used in this study, there was no convincing evidence of cross-reaction with any of the other LPS types of other non-*Salmonella* members of the Enterobacteriaceae.

Antigenic Specificity of the Serum IgA Response

The serum anti-typhoid LPS IgA antibody response following the oral vaccination with *S. typhi* Ty21a was similar to that noted previously (Section 8), supporting the jejunal fluid finding above.

The patterns of serum antibody anti-LPS specificity differed only slightly from that observed in the jejunum. Significant responses were

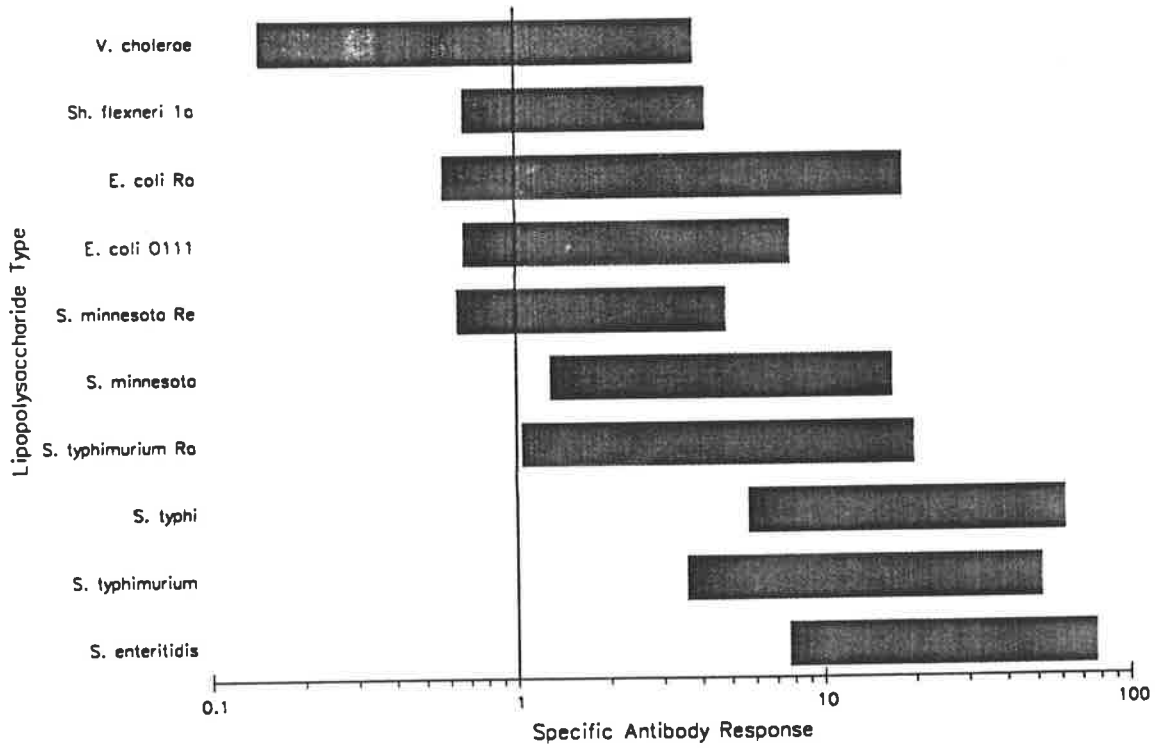
observed against *S. typhi* (t=6.84, p=0.0010), *S. enteritidis* (t=5.03, p=0.0040), *S. typhimurium* (t=4.25, p=0.0081) LPS preparations. In serum, an overall significant response to the *S. minnesota* LPS was not detectable (t=2.23, p=0.076) (Table 10.3), however there was not a significant difference between the geometric mean fold rises in IgA against these four LPS preparations (F=1.43, p=0.263) (Figure 10.2). Also the serum IgA antibody response also cross-reacted strongly with the *Salmonella typhimurium* Ra mutant core LPS preparation (t=3.31, p=0.021), supporting the specific jejunal anti-core oligosaccharide response observed. There were no other significant specificities observed, except it is difficult to explain the observation of a significant cross-reaction with the *E. coli* O111 LPS in serum in the absence of a similar reaction to the *E. coli* core LPS and the absence of a jejunal response (Table 10.3).

There were some additional differences observed in the magnitudes of the serum cross-reactivities as reflected in the geometric mean fold rises. There were no significant differences between the anti-typhoid LPS response and that against *S. enteritidis* LPS (WRST, p>0.50), *S. typhimurium* (WRST, p=0.394) and *S. minnesota* LPS (WRST, p=0.093). The *S. typhi* LPS serum IgA response was significantly elevated above that of all the remaining other LPS types, including that of the *Salmonella* core oligosaccharide (the *S. typhimurium* Ra mutant) (Figure 10.2), possibly confirming the either the low levels present or poorer immunogenicity.

Suffice to say, the relevance and reliability of serum antibody responses as indicators of local mucosal immunity or antibody response patterns, has been shown to be of questionable value.[14,16,28]

FIGURE 10.1

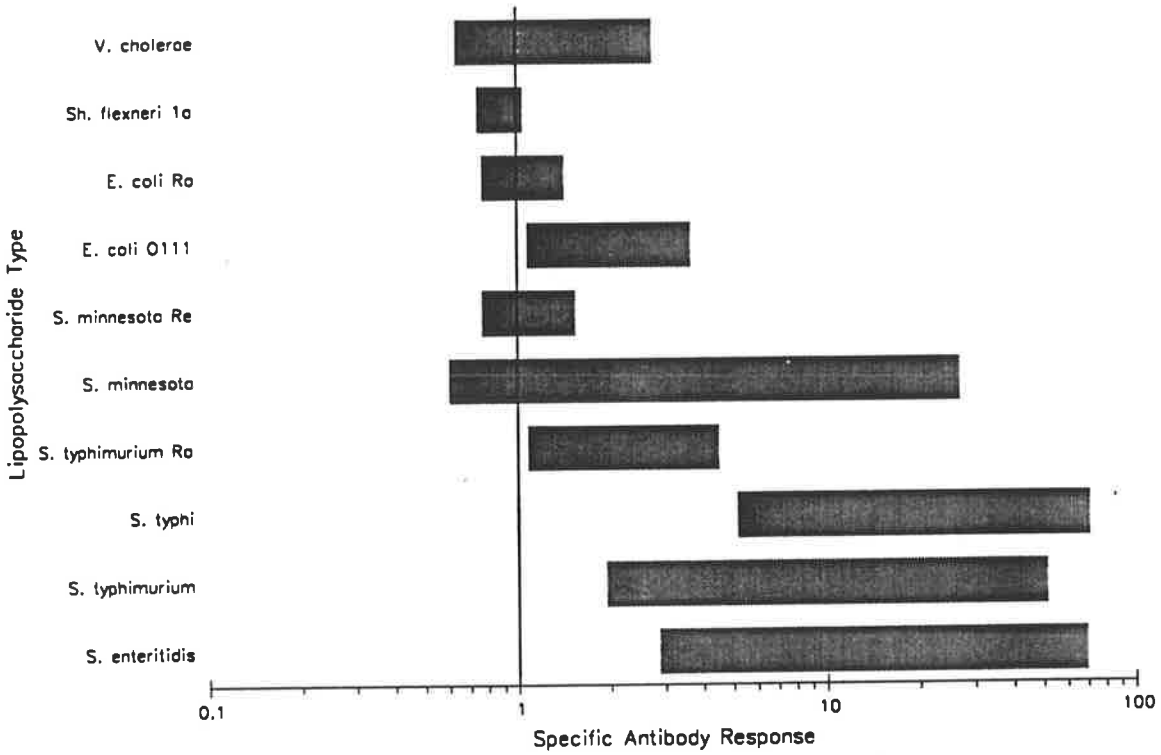
SPECIFICITY OF THE JEJUNAL ANTI-LIPOPOLYSACCHARIDE ANTIBODY RESPONSE



Hatched bars represent the range covered by the 95% confidence intervals about unshown geometric mean antibody responses. Ranges including a geometic mean fold rise of 1.00, are not statistically significant.

FIGURE 10.2

SPECIFICITY OF THE SERUM ANTI-LIPOPOLYSACCHARIDE ANTIBODY RESPONSE



Hatched bars represent the range covered by the 95% confidence intervals about unshown geometric mean antibody responses. Ranges including a geometic mean fold rise of 1.00, are not statistically significant.

TABLE 10.2

LIPOPOLYSACCHARIDE SPECIFICITY OF JEJUNAL IgA ANTIBODY RESPONSE

Organism	Vaccination		Fold Rise	t-statistic	Significance (p value)
	Pre	Post			
S. enteritidis	1010 (180-5690)	24500 (6280-95700)	24.2	7.64	0.0016
S. typhi	1010 (180-5690	18900 (5100-70000)	18.7	6.82	0.0024
S. typhimurium	1010 (180-5690)	13700 (2400-78100)	13.5	5.41	0.0057
S. typhimurium (Ra mutant)	1160 (237-5710)	5240 (1340-20600)	4.51	2.84	0.047
S. minnesota	1600 (442-5820)	7450 (1470-37900)	4.66	3.32	0.029
S. minnesota (Re mutant)	1010 (180-5690)	1780 (520-6050)	1.76	1.54	0.20
E. coli (O111)	1190 (256-5530)	2730 (619-12030)	2.29	1.92	0.13
E. coli (Ra mutant)	1260 (290-5540)	4090 (525-31900)	3.25	1.89	0.13
Sh. flexneri Ia	3624 (1092-12030)	6080 (1650-22400)	1.68	1.57	0.19
V. cholerae	1010 (180-5690)	752 (498-1140)	0.75	0.74	0.64

Antibody titre is the reciprocal of the dilution giving an optical density of 0.15 units, adjusted for total IgA. All responses given as geometric mean titres + 95% CI. Fold rises are expressed as the geometric mean fold rise in specific antibody titre The significance value was determined using Student's paired t-test after ln(x) transformation of data

TABLE 10.3

LIPOPOLYSACCHARIDE SPECIFICITY OF SERUM IgA ANTIBODY RESPONSE

Organism	Vaccination		Fold Rise	t-statistic	Significance (p value)
	Pre	Post			
S. enteritidis	49.2 (21.0-115)	689 (105-4520)	14.0	5.03	0.0040
S. typhi	33.5 (15.6-72.0)	18900 (120-3380)	19.0	6.84	0.0010
S. typhimurium	59.5 (20.2-175)	591 (91.6-3810)	9.93	4.25	0.0081
S. typhimurium (Ra mutant)	37.3 (11.0-126)	81.6 (19.2-347)	2.19	3.13	0.021
S. minnesota	91.5 (22.8-366)	366 (124-1080)	4.00	2.23	0.076
S. minnesota (Re mutant)	21.4 (7.40-61.7)	23.2 (8.28-64.7)	1.08	0.705	0.51
E. coli (O111)	52.6 (16.5-167)	104 (20.1-539)	1.98	3.38	0.020
E. coli (Ra mutant)	165 (60.1-454)	294 (78.5-1100)	1.78	2.09	0.091
Sh. flexneri Ia	425 (119-1520)	374 (98.0-1430)	0.880	2.30	0.070
V. cholerae	17.2 (6.43-45.8)	22.7 (5.98-86.4)	1.32	1.16	0.30

Antibody titre is the reciprocal of the dilution giving an optical density of 0.15 units, adjusted for total IgA. All responses given as geometric mean titres + 95% CI. Fold rises are expressed as the geometric mean fold rise in specific antibody titre The significance value was determined using Student's paired t-test after ln(x) transformation of data

Conclusion

The investigation into the LPS antigenic component specificity detailed in this section, demonstrated that:

(a) while antibodies against the core oligosaccharide of LPS were evident following oral vaccination with *S. typhi* Ty21a in this study, they were present in only low amounts;

(b) that there was significant cross-specificity of the IgA responses in serum and intestinal fluid among *Salmonella* species members sharing antigenic components, especially the 12 O antigen.

11

SPECIFIC IMMUNE RESPONSE IN HUMANS FOLLOWING RECTAL DELIVERY OF LIVE TYPHOID VACCINE

Introduction

As touched upon in Section 7.4.1, gastric acid provides a formidable barrier against pathogenic micro-organisms, especially enteric bacteria. The beneficial protective effect of gastric acid in the human has been repeatedly observed and documented, as has the greater risk of enteric infection in patients with achlorhydria - a physiologic absence of gastric acid.[262,442,456-460]

This barrier, however, is also highly effective in killing orally administered attenuated live bacterial vaccines and altering the antigenicity of ingested killed bacterial vaccines, unless special regard is paid to neutralizing gastric acid prior to their ingestion.[214,461] One approach to overcoming this problem, has been to use enteric coated preparations. This approach has been shown to be useful in the case of the effective live orally administered typhoid vaccine, *S. typhi* Ty21a (Section 2.10).[226] This formulation has been demonstrated to be practical and easily administered to older children and adults. Liquid preparations, while ensuring greater vaccine organism viability, are not particularly useful in developing countries, and enteric-coated capsules are not easily administered to infants or small children - the groups at greatest risk of enteric bacterial disease.[462]

The oral route of vaccination relies on the stimulation of the gut-associated lymphoid tissue and the subsequent local production of specific antibody for its effectiveness. The emerging concept of the common mucosal immune system suggested that it may be possible to immunize distant

mucosal surfaces by the local application of antigen to another mucosal surface (Section 1).[84]

The rectal route is a well established avenue of effective delivery of pharmaceuticals due to its large absorptive area, the absence of the requirement for special methods of neutralizing effects of gastric acid, and as a means of reducing local adverse reactions.[463] The colon has also been previously shown to play a role in the generation of mucosal and systemic immune responses to a locally presented microbial agent.[464]

The rectal mucosa has been shown to be more densely populated with lymphoid follicles ($25/cm^2$) than any other part of the colon.[465] These follicles are covered with a dome-like epithelium, the ultrastructure of which is similar to that of the M cells found over the Peyer's patches of the small intestine,[466] and over the bronchus associated lymphoid tissue (BALT).[59] M cells clearly play a role in the selective uptake of specific antigens and microorganisms from the small intestinal lumen, transporting them into the underlying lymphoid tissue.[61-65] It is therefore possible that these similar cells found in the rectal mucosa play a like role.

In this study the ability of the live attenuated typhoid vaccine strain *S. typhi* Ty21a to access the immune system following atraumatic rectal inoculation and produce a systemic immune response, inferring that the rectum may represent a route for microorganisms to enter the immune system was investigated.

Subjects and Methods

Seven healthy adults (two women and five men, 20-42 years of age) agreed to participate in this study. Two volunteers had prior exposure to typhoid through parenteral vaccination five and six years previously

respectively. None of the other volunteers had any previous exposure to typhoid either through vaccination or disease.

None of the subjects had any history of colitis or bowel disturbance but sigmoidoscopy was not carried to confirm the normality of the rectal mucosa.

Volunteers were encouraged to attend for vaccination in the morning as soon as possible after defaecation, and were requested to refrain from further defaecation for at least six hours after vaccination.

Calculation of Antibody Responses

Serum, intestinal and salivary specific antibody responses are presented as the reciprocal of the dilution that gave an optical density of 0.15 ELISA absorbance units and are expressed as units of antibody. This absorbance was chosen as it represented the upper limit of the 95% confidence intervals (CI) above background levels. The intestinal fluid and salivary specific antibody units were further adjusted for total class-specific immunoglobulin content and were expressed as units of specific antibody/mg of total class specific immunoglobulin.

All serum samples were diluted 1:10 for assaying, therefore the lower limit of detection for the serum assay is 10 units of antibody activity as defined above.

Results

In all seven of the volunteers who received *S. typhi* Ty21a rectally, an anti-typhoid LPS IgA antibody response (optical density >0.100 units/10^6 PBL) was detected in their sampled PBL, with the mean optical density peak of 0.565 units (95% C.I. 0.023 to 1.11 units) occurring at a mean of 8.7 days

(95% C.I. 6.4 to 11.0 days) after the first dose of the vaccine (Figure 11.1). This response was significantly different from their pre-vaccination PBL IgA antibody response (p=0.009, Wilcoxon Rank Sign Test). In one volunteer there was a response measurable as early as five days post-vaccination. IgA antibodies to typhoid LPS could not be detected by this method before vaccination nor later than 16 days after vaccination. In no assay was any typhoid specific antibody detectable in any of the negative control's PBL.

Anti-typhoid IgM responses were detected in 6/7 subjects post-vaccination, with IgG responses in only 3/7 subjects (Table 11.1).

Significant typhoid specific serum IgA responses were determined; pre-vaccine geometric mean titre 118 units, 95% C.I. 79.0 to 175 units, post-vaccine geometric mean titre 382 units, 95% C.I. 153 to 952 units; using Student's paired t test after ln(x) transformation of titres, t=3.728 d.f.=6 p=0.010. Four-fold or greater anti-typhoid serum IgA responses were measurable in 3/7 volunteers. The mean peak serum IgA response occurred at 13.40. days, 95% C.I. 9.5 to 17.3 days (Figure 11.1).

The magnitude of the serum anti-typhoid antibody response was observed to be greatest in the IgG class (Table 11.1).

After correction of intestinal antibody titres for total class-specific immunoglobulin, a two-fold or greater typhoid LPS specific IgA antibody response was observed in 3/7 volunteers. Overall, there was a significant anti-typhoid antibody response determined in intestinal fluid; pre-vaccine geometric mean 418 units, 95% C.I. 366 to 477 units, post-vaccine geometric mean 668 units, 95% C.I. 422 to 1059 units; using Student's paired t test after ln(x) transformation of titres, t=2.749 p=0.033 (Figure 11.2).

TABLE 11.1

PEAK PBL AND SERUM SPECIFIC ANTIBODY RESPONSES FOLLOWING RECTAL VACCINATION WITH *Salmonella typhi* TY21a

	IgA	IgG	IgM
PBL			
Peak (Days)	8.7 (6.4-11.0)	8.1 (6.2-10.0)	8.7 (6.4-11.0)
Mean Peak OD	0.565 (0.023-1.11)	0.211 (0.000-0.484)	0.215 (0.061-0.369)
Significance+	p=0.009	p=0.108	p=0.014
SERUM			
Peak (Days)	13.4 (9.5-17.3)	17.4 (13.6-21.1)	17.6 (14.0-21.1)
Mean Pre-vaccination Titre*	118 (79.0-175)	161 (55.4-470)	507 (325-791)
Mean Post-vaccination Titre*	382 (153-952)	649 (336-1250)	908 (599-1380)
Significance++	p=0.010	p=0.004	p=0.029

Numbers in parentheses refer to 95% CI.
* - Geometric mean.
OD - optical density.
+ - level of significance determined using Wilcoxon rank sum test.
++ - level of significance determined using Student's *t* test following ln(x) transformation of data.

Similarly a significant IgA anti-typhoid LPS salivary antibody response was measured; pre-vaccine geometric mean 202 units, 95% C.I. 165 to 248 units, post-vaccine geometric mean 297 units, 95% C.I. 228 to 386 units; using Student's paired t test after ln(x) transformation of titres after correction for total class-specific immunoglobulin, t=2.573 d.f.=5, p=0.050 (Figure 11.2).

Conclusion

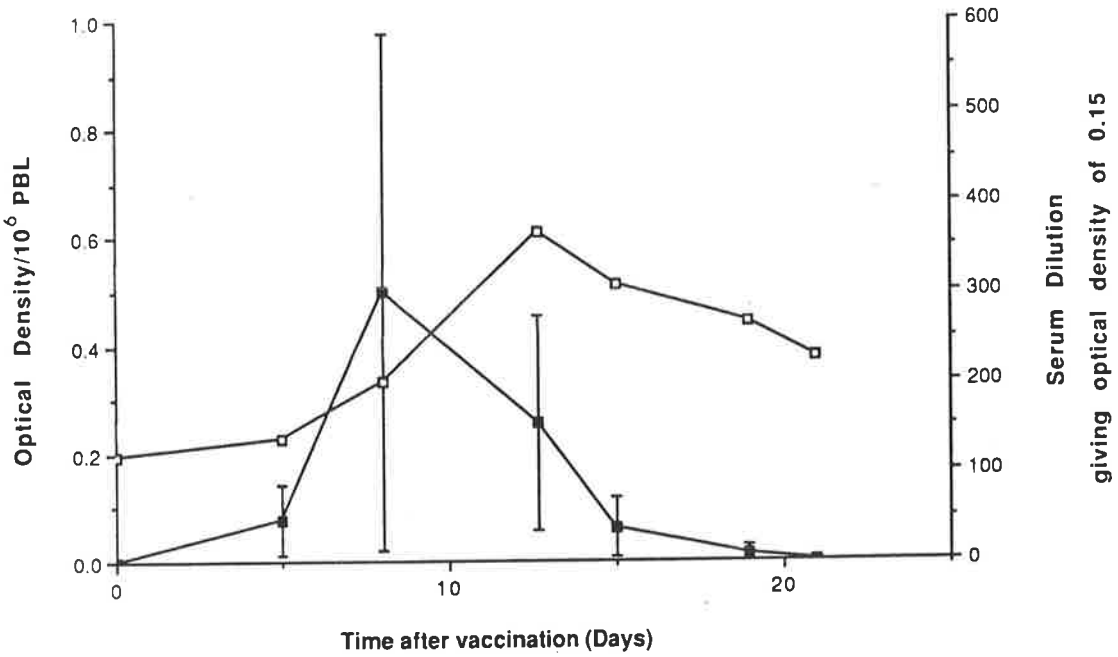
In the study described in this section, a significant anti-typhoid IgA antibody response could be identified in all subjects' post-vaccination peripheral blood lymphocytes, however the magnitude of this response was only one-third of that observed following oral vaccination with the same dose. Unlike Ogra and Karzon,[464] it was possible to demonstrate distant mucosal anti-typhoid IgA responses in jejunal fluid and saliva. While these post-vaccination responses were statistically significant, they were substantially lower than previous findings using the oral route. However, it was also found the that the anti-typhoid antibody response in the IgG immunoglobulin class, as measured by the mean fold rise, to be greater following rectal vaccination.

It has been demonstrated previously that following the rectal administration of a single dose of 100 ml of a 40% barium sulphate solution, some of this fluid could be detected in the upper descending colon as far as the splenic flexure, or even the ascending colon.[467] It is possible that some of the vaccine dose did enter the descending colon following administration, however with the much smaller volume used in this study, it is likely that this amount would be of little consequence.

This study conclusively demonstrates that it is possible for microbial agents to access the immune system via the rectal mucosa in the absence of pre-existing trauma. These results confirm the role of the rectal mucosa in the generation of distant mucosal and systemic immunity to a locally presented microbial agent providing additional evidence in support of the existence of the common mucosal immune system in humans.

FIGURE 11.1

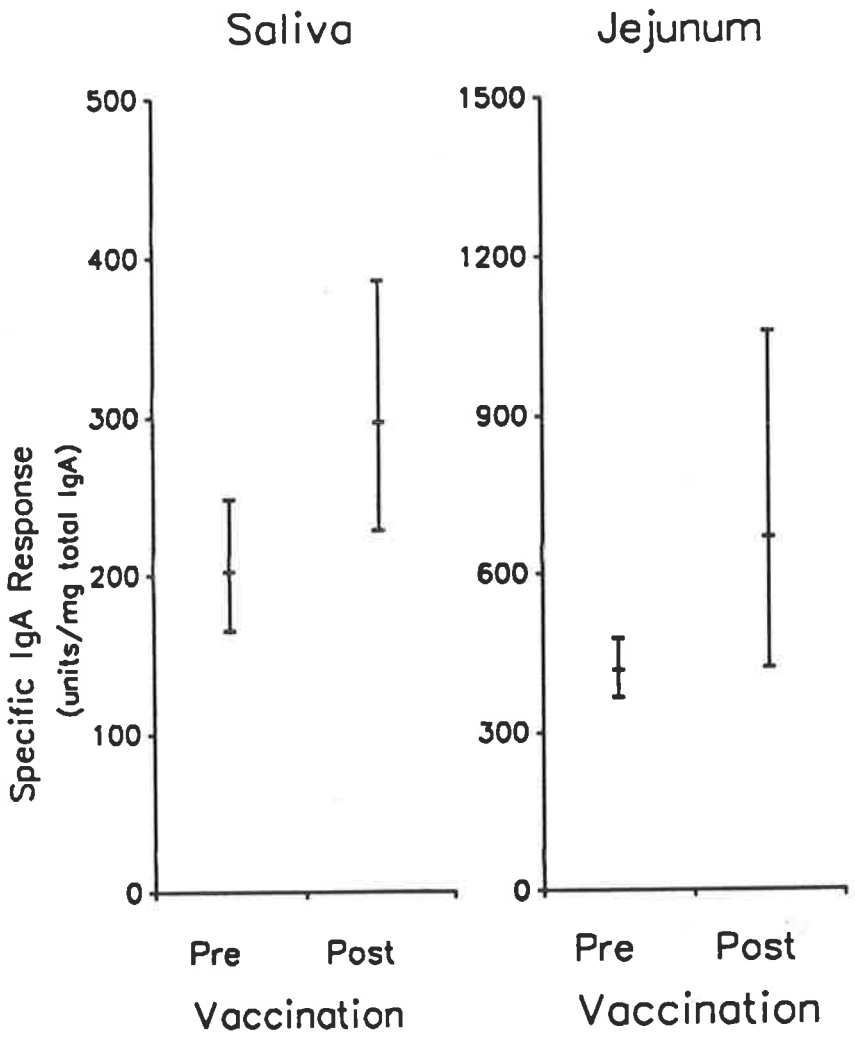
TIME COURSE RELATIONSHIP BETWEEN IgA PRODUCTION BY PERIPHERAL BLOOD LYMPHOCYTES AND IgA RESPONSE IN SERUM



PBL response (■) is the arithmetic mean optical density (absorbance units) ± 95% confidence intervals
Serum response (□) is the geometric mean of the serum dilution giving an optical density of 0.15 absorbance units.

FIGURE 11.2

SPECIFIC JEJUNAL AND SALIVARY IgA RESPONSES FOLLOWING RECTAL VACCINATION WITH *Salmonella typhi* Ty21a



Graphs represent geometric mean titres ± 95% confidence intervals, following adjustment for total IgA content.

12

CONSTRUCTION OF A HYBRID *Salmonella typhi* TY21a-*Vibrio cholerae* ORAL VACCINE

12.1 *Introduction*

The failure of the parenteral heat-killed vaccine to effectively provide any long-term protection against clinical cholera,[468] has lead to a search for effective replacements. Since cholera stimulates poor systemic immunity but excellent protective local intestinal immunity (Section 3.6.2),[300] recent research has focussed upon development of improved vaccines suitable for oral administration. approach to the development of an oral cholera vaccine described here, involved the cloning of protective antigens from *V. cholerae* into *S. typhi* Ty21a. The rationale for this approach are that:

(a) experimental studies were performed that demonstrated the feasibility of bivalent oral vaccines; for example, immunization of mice with a *S. typhimurium/E. coli* hybrid resulted in protection against *S. typhimurium* challenge as well as stimulating a good anti-*E. coli* O8 lipopolysaccharide IgA antibody response.[62] This indicated that the hybrid strain not only retained its status as a vaccine against *S. typhimurium* but also stimulated a specific antibody response against expressed foreign antigens. This was supported by studies in other laboratories which confirmed that attenuated *S. typhimurium* strains could present cloned determinants to the humoral and cellular immune systems.[469]

(b) *S. typhi* Ty21a is a safe, effective live orally administrable vaccine which has shown itself able to successfully act as a vector for foreign putative protective antigens (Section 4.2);

(c) The correlation of anti-cholera LPS antibodies in the field with protection against disease,[470] and the finding that anti-LPS anti-serum is protective in the infant mouse model[471] and in rabbits,[294,472] together support a major role for anti-LPS antibodies in providing protection against cholera. In support it has been demonstrated that anti-LPS antibody contributes significantly to the serum vibriocidal antibody response (in fact the entire vibriocidal antibody response can be removed by absorption of the serum with purified LPS),[369,473] which has been shown to correlate with protection (Section 3.6.3); and

(c) as extensively discussed above, locally produced specific antibody is the most effective mechanism of providing protection against enteric pathogens.[23,474,475]

For these reasons, the localization and subsequent transfer of the genes encoding the O antigen polysaccharide of the cholera LPS into potential carrier organisms was initiated. The genes required for the biosynthesis of the O antigens of the Inaba and Ogawa serotypes of *V. cholerae* were initially transferred into *E. coli* K-12. The anti-Inaba O antigen antiserum raised in rabbits using this *E. coli*/Inaba hybrid was demonstrated to provide the same degree of protection against classical *V. cholerae* Inaba 569B challenge in the baby mouse protection test as the antiserum raised using the parent cholera strain.[127] Subsequent work involved the transfer of these cloned genes for O antigen biosynthesis into *S. typhi* Ty21a.

The hybrid typhoid/cholera vaccines described in this thesis were jointly developed by investigators at the biotechnology company Enterovax Limited and at the Department of Microbiology and Immunology at the University of Adelaide.

Description of the genetic manipulative processes which resulted in the various hybrid strains have been published previously.[127,476] It is necessary and important that a brief description of these processes and their products is provided here in order to understand the evolution and relationship between the different strains that will be described subsequently. The end-product of these processes was a candidate vaccine strain based on the safe, attenuated live oral typhoid vaccine *S. typhi* Ty21a into which the genes encoding for the O antigen of the LPS of *V. cholerae* Inaba strain 569B had been inserted by means of a plasmid.

S. typhi Ty21a as already discussed in some detail (Section 2.10) is an attenuated *S. typhi* strain containing numerous undefined genetic mutations, one of which the *galE* deletion permits the quantity of the O antigen polysaccharide component of the LPS to be varied according to the exogenous galactose concentration. From this strain, a spontaneous rifampicin-resistant mutant was isolated by growing *S. typhi* Ty21a on rifampicin-containing medium. The resulting mutant was designated EX759. A new strain designated V487, was derived from EX759 through the transfer by conjugation of a plasmid which encoded tetracycline resistance and carried genes encoding for the *V. cholerae* Inaba O antigen. This strain, V487, has been evaluated in human volunteer subjects (Section 12.2.1). As detailed in Section 12.2.1, this strain was not immunogenic in human volunteer subjects and this was attributed to the inadequacy of the *S. typhi* core oligosaccharide of the LPS to serve as a suitable substrate for the polymerization of *V. cholerae* O antigen polysaccharide.

The next strain constructed, designated EX210, in which the chromosomal *rfa* region of V487, which encodes the core oligosaccharide, was replaced with the homologous region from *E. coli* K12 by conjugation.

This was performed as it was believed that the good expression of *V. cholerae*-like LPS in the *E. coli* K12 and the poor or absent expression in *S. typhi* Ty21a was due to *V. cholerae* O antigen polymerizing onto the *E. coli* core oligosaccharide and not onto the *S. typhi* Ty21a core oligosaccharide which appeared to be unsuitable. Strain EX210 was found to produce *V. cholerae* O antigen polysaccharide in the absence of exogenous galactose and both *V. cholerae* and *S. typhi* O antigen polysaccharide in the presence of exogenous galactose.

EX210 was considered unsuitable as a vaccine due to the presence of plasmid-borne tetracycline resistance and chloramphenicol resistance which had been transferred from the *E. coli* K12 with the *rfa* region DNA. Therefore, the antibiotic resistances were removed by removing the existing plasmid (resulting in a strain designated EX233) and excising the chloramphenicol resistance (strain EX256). Into EX256 a non-reverting mutation was introduced rendering the strain with a thymine-dependence ($ThyA^-$), an auxotrophy, designated EX259.

A newly constructed plasmid, which carried the genes for classical *V. cholerae* Inaba strain 569B O antigen polysaccharide as well as the genes for thymine-independence ($ThyA^+$), was inserted into EX259, resulting in a new strain designated EX645.

The strain EX645 was to become the first candidate attenuated live orally administrable hybrid typhoid/cholera vaccine to have its protective efficacy evaluated in humans.

12.2 *Prototype Candidate Hybrid Typhoid/Cholera Vaccines*

12.2.1 *V487: Phase 1 study*

Subjects and Methods

This study was the very first involving the administration of a live attenuated hybrid typhoid/cholera vaccine to humans and so was required the subjects to be detained within the containment facility established in the Nurses' Home B at the Hampstead Centre. Six healthy adult volunteers aged between 20 and 23 years, consented to participate in this study. Only one of the subjects had previous exposure to cholera and/or typhoid through being immunized against both five years previously.

The vaccine doses were supplied by Dr J. Hackett of the Department of Microbiology and Immunology of the University of Adelaide. Being the first study using this type of organism, the initial dose administered was 10^{10} viable organisms, ten-fold lower that which was known to be both safe and immunogenic of *S. typhi* Ty21a, with subsequent doses comprising 10^{11}. The vaccine organisms were grown in brain-heart infusion broth (BHIB) in the presence of 0.001% galactose, a concentration known to result in the maximal production of *S. typhi* O polysaccharide.

The vaccine doses and schedules are detailed in Table 12.1.

At the time of this study the standard method of vaccination had not been established. Therefore, the administration of these vaccine doses followed a different method. All subjects were required to fast overnight, and then to ingest 50 ml of a sodium hydrogen carbonate solution. Five minutes later, the vaccine doses were administered in 30 ml of skim milk, which was followed by a 50 ml glass of distilled water.

TABLE 12.1

VACCINE DOSES AND SCHEDULES FOR V487 AND EX210 CLINICAL STUDIES

	Vaccination Days	Dose*
V487	0	8.8×10^{9}
	2	1.1×10^{11}
	4	1.2×10^{11}
EX210 (Group A)	0	1.2×10^{11}
	2	0.9×10^{11}
	3	1.1×10^{11}
EX210 (Group B)	0	2.1×10^{11}
	2	1.3×10^{11}
	4	1.2×10^{11}
EX210 (Group C)	0	1.1×10^{11}
	2	1.2×10^{11}
	3	1.1×10^{11}
EX210 (Group D)	0	2.2×10^{11}
	2	1.65×10^{11}
	4	1.7×10^{11}
	24	1.75×10^{11}
EX210 (Group E)	0	1.75×10^{10}
	2	2.45×10^{10}
	4	1.98×10^{10}
	26	1.4×10^{11}
EX210 (Group F)	0	1.98×10^{11}
	2	1.4×10^{11}
	4	1.35×10^{11}
	24	3.5×10^{11}
	26	3.85×10^{11}
	28	3.9×10^{11}
EX210 (Group G)	0	1.23×10^{11}
	21	1.33×10^{11}
EX363 (Group A)	0	1.7×10^{11}
	2	1.6×10^{11}
	4	1.9×10^{11}
EX363 (Group B)	0	1.4×10^{10}
	2	1.3×10^{10}
	4	1.7×10^{10}

* - Number of viable organisms comprising vaccine dose.

The subjects were not permitted to eat for a further one hour after oral vaccination. All subjects were required to remain within the containment facility until 24 hours after the last faecal specimen to return a negative culture following the final vaccine dose.

The specific immune response in intestinal fluid and serum was determined as previously described (Section 7.2) using an ELISA, except that the coating antigens were heat-phenol extracted alkali-treated *S. typhi* Ty21a LPS and classical *V. cholerae* Inaba 569B LPS neither being methylated-BSA linked.

Results

The vaccine appeared well tolerated in this small group of subjects with no frequent adverse effects being observed.

Anti-typhoid LPS immune response

Significant typhoid specific serum IgA responses were determined as presented in Table 12.2. Four-fold or greater rises in serum IgA typhoid-specific antibodies were measurable in 4/6 subjects respectively.

Four-fold or greater rises in intestinal IgA anti-typhoid LPS antibodies were detected in 5/6 subjects. Overall, there was a significant intestinal anti-typhoid IgA antibody response (Table 12.2).

Anti-cholera LPS immune response

A four-fold or greater rise in intestinal IgA anti-cholera LPS antibodies was not detected in any subject; in fact there was no detectable specific intestinal anti-cholera IgA antibody response in the intestinal fluid obtained from any subject.

This was supported by the absence of any detectable anti-cholera LPS IgA antibody response in the serum from any subject.

Conclusion

Despite V487 producing cholera O antigen (J. Hackett, personal communication), this was not in a form that was presentable to the immune system. It was believed that this was due to the inadequacy of the *Salmonella* core oligosaccharide to act as a suitable anchor upon which the cholera O antigen could polymerize.

Subsequent development resulted in the candidate vaccine strain EX210, in which some of the technical problems associated with V487, were supposedly overcome.

12.2.2 *EX210: Phase 1 Studies - Group A*

Introduction

The development of the new candidate vaccine strain, EX210, promised a significantly better result than that achieved with V487. The laboratory evaluation of this strain indicated that it produced significant amounts of *V. cholerae* O antigen that was polymerized on the *E. coli* K12 core oligosaccharide which had replaced the *Salmonella* one present in V487.

Since this strain represented a new construction, the human evaluation of the safety and immunogenicity of this strain was required to be performed in the biological containment facility at the Nurses' Home B of the Hampstead Centre as described in Section 10 and for V487.

Subjects and Methods

Six healthy adult subjects (four male and two female) aged between 18 and 23 years agreed to participate in this study. All conditions of containment were as for the V487 study (Section 12.2.1).

The vaccine strain, EX210, was grown in the BHIB with the addition of 0.001% galactose, resulting in maximal production of S. typhi O antigen. The production of V. cholerae O antigen being independent of the presence or absence of galactose. The vaccine was administered according to the dose schedule in Table 12.1, and as detailed in Section 12.2.1 for strain V487.

Results

This new candidate vaccine appeared well tolerated in this small group of subjects with no frequent adverse effects being observed.

Anti-typhoid LPS immune response

Significant typhoid specific serum IgA responses were determined and are presented in Table 12.2. Four-fold or greater rises in serum IgA typhoid-specific antibodies were measurable in 4/6 subjects respectively.

Four-fold or greater rises in intestinal IgA anti-typhoid LPS antibodies were detected in 4/6 subjects. Overall, there was a significant intestinal anti-typhoid IgA antibody response (Table 12.2).

Anti-cholera LPS immune response

A four-fold or greater rise in intestinal IgA anti-cholera LPS antibodies was not detected in any subject (Table 12.2).

This was supported by only one subject having a four-fold or greater rise in serum specific anti-cholera O antigen IgA antibody response (Table 12.2).

Conclusions

These results were yet a further disappointment, especially in view of the laboratory data implying the abundance of *V. cholerae* O antigen produced by this strain. Following this study, further laboratory work performed by Dr S.R. Attridge, suggested that when the vaccine strain was grown in the presence of an increasing concentration of galactose, there was an increasing amount of *S. typhi* O antigen production as determined by haemagglutination inhibition assays and a concurrent reduction in the amount of *V. cholerae* O antigen present. However, additional evidence provided from Dr R. Morona showed that when EX210 was grown in the presence of sufficient galactose to ensure maximal *S. typhi* O antigen production, upon disruption of the cell and performance of gel electrophoresis, both *V. cholerae* O antigen and *S. typhi* O antigen were present polymerized to the *E. coli* core oligosaccharide.

These results together with the human experimental results implied that there was a masking phenomenon occurring, such that the long O polysaccharide chains of *S. typhi* were preventing access to the much shorter *V. cholerae* O antigens by the human immune system.

TABLE 12.2

V487 AND EX210 RESULTS OF PRIMARY INVESTIGATION OF IMMUNOGENICITY

	JEJUNUM*		SERUM+	
	TYPHOID	CHOLERA	TYPHOID	CHOLERA
V487				
Pre-vaccination	862 (337-2200)	#	483 (113-2055)	#
Post-vaccination	9020 (5174-15730)	#	2091 (632-6912)	#
Fold Rise	10.5		4.33	
Significance	0.0036		0.018	
EX210 (Group A)				
Pre-vaccination	928 (363-2373)	193 (119-314)	529 (226-1235)	104 (34.8-309)
Post-vaccination	6554 (3068-14000)	187 (77.6-449)	2669 (1110-6417)	154 (36.6-649)
Fold Rise	7.06	0.969	5.05	1.48
Significance	0.0072	0.91	0.014	0.30

* - Reciprocal of geometric mean titres following correction for total IgA; + Reciprocal of geometric mean titres

\# - All values below limit of detection

** - Analysis of geometric mean fold rises between these two groups using Student's t-test for independent samples, t=0.71, p=0.50 indicating no significant difference

Fold rises expressed as geometric mean fold rises. Significance values expressed as p value only following ln(x) transformation of data and analysis by Student's paired t-test

TABLE 12.2 (CONTINUED)

V487 AND EX210 RESULTS OF PRIMARY INVESTIGATION OF IMMUNOGENICITY

	JEJUNUM*		SERUM+	
	TYPHOID	CHOLERA	TYPHOID	CHOLERA
EX210 (Group B)				
Pre-vaccination	1576 (435-5700)	1835 (722-4665)	702 (326-1510)	198 (38.7-1017)
Post-vaccination	9851 (5091-19060)	4312 (1550-11990)	3325 (1280-8660)	470 (91.6-2406)
Fold Rise	6.25	2.35**	4.74	2.37
Significance	0.035	0.079	0.037	0.15
EX210 (Group C)				
Pre-vaccination	1716 (699-4210)	268 (153-467)	64.9 (35.4-119)	56.5 (30.2-105)
Post-vaccination	7170 (4829-10650)	471 (278-798)	608 (238-1553)	104 (37.6-289)
Fold Rise	4.18	1.76	9.37	1.84
Significance	0.0025	0.049**	0.0017	0.11

* - Reciprocal of geometric mean titres following correction for total IgA; + Reciprocal of geometric mean titres

\# - All values below limit of detection

** - Analysis of geometric mean fold rises between these two groups using Student's t-test for independent samples, t=0.71, p=0.50 indicating no significant difference

Fold rises expressed as geometric mean fold rises. Significance values expressed as p value only following ln(x) transformation of data and analysis by Student's paired t-test

12.2.3 *EX210: Phase 1 Studies - Groups B and C*

Introduction

To further evaluate the possible presence of a masking phenomenon, two further human studies were conducted at the biological containment facility.

In the first of these studies (Group B) EX210 was grown in the complete absence of exogenous galactose and administered to five healthy adult subjects (four males and one female) aged 18 to 25; and in the second study (Group C) EX210 was grown in the presence of 0.0001% exogenous galactose which had been determined by Dr S.R. Attridge to result in the production in immunologically equal quantities the *S. typhi* and *V. cholerae* O antigens.

All vaccine doses were administered according to the schedules in Table 12.1, and followed the same protocol as for V487 vaccination, except that for Group C the skim milk was replaced by an equal volume of 0.9% saline.

Results

Anti-typhoid LPS immune response

Significant typhoid specific serum IgA responses were determined in subjects for Groups B and C of the study and are presented in Table 12.2. Four-fold or greater rises in serum IgA typhoid-specific antibodies were measurable in 3/5 Group B subjects and 5/6 Group C subjects respectively.

Four-fold or greater rises in intestinal IgA anti-typhoid LPS antibodies were detected in 3/5 Group B subjects and 4/6 Group C subjects respectively. Overall, there were significant intestinal anti-typhoid IgA

antibody responses determined in both Groups B and C of the study (Table 12.2).

Anti-cholera LPS immune response

A four-fold or greater rise in intestinal IgA anti-cholera LPS antibodies was detected in 1/5 Group B subjects and not in any of the Group C subjects (Table 12.2).

This was supported by rises of a similar magnitude in specific anti-cholera O antigen IgA serum antibody responses in 1/5 Group B subjects and 1/6 Group C subjects (Table 12.2).

However, as it can be seen in Table 12.2, the overall intestinal specific anti-cholera O antigen IgA antibody response was statistically significant in Group C and not in Group B. The anti-cholera O antigen IgA antibody responses in serum of both Groups failed to achieve statistical significance.

In addition, despite the overall anti-cholera O antigen intestinal antibody response being significantly different in Group C subjects compared with those of Group B, there was no significant difference in the geometric mean fold rises in this specific antibody (Table 12.2).

Conclusion

These two additional studies supported the premise that the long *S. typhi* O polysaccharide side-chains were inhibitory to the ability of the human immune system to recognize the presence of the *V. cholerae* O antigen, and that by reducing the quantity of the *S. typhi* O antigen by growing the organism in a lower concentration of exogenous galactose, the shorter *V. cholerae* O polysaccharide side-chains were unmasked or exposed.

12.2.4 *EX210: Dose Schedule Evaluation*

Introduction

The above studies had indicated that there was really very little difference in the antigenicity of the strain EX210 in humans whether it was grown "rough" and administered in skim milk, or grown in the presence of 0.0001% galactose and administered in saline. The three dose schedule had been shown to be effective in stimulation a specific immune response against both the O antigens. However, there needed to be an investigation into the optimal dose regimen and schedule that would result in the stimulation of the maximal amount of local intestinal specific anti-cholera antibody.

Subjects and Methods

The number of subjects involved in these four studies, together with their vaccination and sampling schedules are detailed in Table 12.1.

These studies were performed on an out-patient basis, the safety and environmentally secure nature of the vaccine strain organism EX210 having been determined in the studies previously.

All doses of EX210 were grown in the absence of exogenous galactose and so were designated as "rough" and supplied by Dr R. Morona of Enterovax Limited. The vaccination protocol followed that established in the V487 study described in Section 12.2.1, in which the freshly harvested vaccine doses were administered in a specified volume of skim milk. It was decided that this approach would be more acceptable for the subjects in view of the unpalatability of the vaccine organisms in saline. The disadvantage associated with using this protocol possibly was the presence of galactose in the milk and the effect that may have had on the *S. typhi* O antigen

production. However the previous study described in Section 12.2.3 indicated that there was no difference in the magnitude of the fold rise against cholera O antigen in subjects receiving either form of the vaccine. In addition to this it was impossible to control for the presence of accessible galactose in the intestinal tract of the subjects and the effect that may have had.

Results

Anti-typhoid LPS immune response

The specific anti-typhoid LPS IgA antibody responses in the vaccinated subjects are detailed in Table 12.3. It can be seen that the subjects in Group E who received three doses of 10^{10} vaccine organisms failed to generate an intestinal specific antibody response, and a further single oral dose of 10^{11} live organisms three weeks later did not result in any stimulation of the response. Similarly, a single dose of 10^{11} live vaccine organisms just failed to generate a significant intestinal specific antibody response (Group G), and this was not improved by a subsequent single oral dose of 10^{11} live organisms three weeks later. All subjects in both Groups D and F who ingested three doses of 10^{11} live vaccine organisms produced a significant anti-typhoid intestinal immune response. In both of these groups the second course of either a single oral dose or three oral doses of 10^{11} live vaccine organisms failed to significantly improve the antityphoid antibody response, although in the latter group the specific anti-typhoid antibody response did approach the same magnitude as that observed following three doses of 10^{11} smooth *S. typhi* Ty21a only, all the other vaccine groups' anti-typhoid response being substantially lower.

Anti-cholera LPS immune response

Only the subjects comprising Group F generated an anti-cholera LPS intestinal IgA antibody response following the completion of their vaccination course. As with all the other groups, Group F also failed to generate a significant intestinal anti-cholera antibody response following their primary vaccination course, however, unlike the other groups which maintained this absence of response, Group F subjects did generate a significant intestinal anti-cholera IgA antibody response following their three dose secondary vaccination schedule (Table 12.3).

As is apparent in Table 12.3, the specific anti-typhoid and anti-cholera IgA serum antibody responses are singularly unremarkable, with few significant rises in titre evident.

Conclusions

This series of studies was performed in order to optimize the dose and schedule of vaccination with the oral candidate vaccine EX210 in human subjects.

These studies demonstrated that EX210 was of low immunogenicity, and that the best vaccination schedule that resulted in a significant intestinal immune response against both typhoid and cholera was one involving frequent large multiple doses. Such a schedule is totally impractical, and as a result further development of EX210 was terminated. Despite the overall failure of EX210 to be a successful immunogen against cholera, it demonstrated that the process that resulted in its development could be made to work.

Therefore, a new direction of development was instituted using EX210 as the progenitor strain.

TABLE 12.3

EX210: INVESTIGATION OF EFFECT OF DOSE REGIMEN ON IMMUNOGENICITY

	JEJUNUM*		SERUM+	
	TYPHOID	CHOLERA	TYPHOID	CHOLERA
EX210 (Group D)				
Pre-vaccination	1092 (178-6720)	252 (91.1-698)	220 (108-451)	100 (35.1-287)
Post (1)-vaccination	4460 (623-31900)	355 (158-800)	529 (206-1362)	120 (80.1-179)
Significance (1)	0.012	0.40	0.0069	0.51
Post (2)-vaccination	5020 (622-40550)	577 (198-1677)	299 (136-655)	132 (89.4-193)
Significance (2)	0.85	0.41	0.011	0.59
Significance (3)	0.13	0.21	0.082	0.47

All values expressed as the geometric mean titre with values in parentheses beneath indicating the 95% CI.

* Reciprocal of geometric mean titres following correction for total IgA

\+ Reciprocal of geometric mean titres

Numbers in parentheses following post-vaccination indicate whether the value was obtained from the first post-vaccination sample (Post (1)) before the second vaccine course, or the post-vaccination sample obtained following the second vaccination course (Post (2)).

Significance values expressed as p value only following ln(x) transformation of data and analysis by Student's paired t-test. The number in the parenthesis represents (1) = Post (1)/Pre-vaccination; (2) = Post (2)/Pre-vaccination; (3) = Post (2)/Pre-vaccination.

TABLE 12.3 (CONTINUED)

EX210: INVESTIGATION OF EFFECT OF DOSE REGIMEN ON IMMUNOGENICITY

	JEJUNUM*		SERUM+	
	TYPHOID	CHOLERA	TYPHOID	CHOLERA
EX210 (Group E)				
Pre-vaccination	627 (177-2220)	363 (172-768)	122 (26.4-560)	69.6 (16.3-297)
Post (1)-vaccination	1318 (230-7550)	382 (208-700)	307 (126-750)	103 (58.5-183)
Significance (1)	0.28	0.74	0.23	0.31
Post (2)-vaccination	1285 (313-5266)	412 (204-834)	221 (74.7-654)	106 (135-1150)
Significance (2)	0.88	0.51	0.64	0.95
Significance (3)	0.23	0.17	0.40	0.47

All values expressed as the geometric mean titre with values in parentheses beneath indicating the 95% CI.

* - Reciprocal of geometric mean titres following correction for total IgA

\+ - Reciprocal of geometric mean titres

Numbers in parentheses following post-vaccination indicate whether the value was obtained from the first post-vaccination sample (Post (1)) before the second vaccine course, or the post-vaccination sample obtained following the second vaccination course (Post (2)).

Significance values expressed as p value only following ln(x) transformation of data and analysis by Student's paired t-test. The number in the parenthesis represents (1) = Post (1)/Pre-vaccination; (2) = Post (2)/Pre-vaccination; (3) = Post (2)/Pre-vaccination.

TABLE 12.3 (CONTINUED)

EX210: INVESTIGATION OF EFFECT OF DOSE REGIMEN ON IMMUNOGENICITY

	JEJUNUM*		SERUM+	
	TYPHOID	CHOLERA	TYPHOID	CHOLERA
EX210 (Group F)				
Pre-vaccination	358 (147-872)	248 (179-343)	263 (108-638)	127 (27.1-600)
Post (1)-vaccination	6321 (4295-9305)	271 (185-395)	501 (333-753)	191 (84.4-434)
Significance (1)	0.0024	0.49	0.055	0.44
Post (2)-vaccination	12010 (5274-27340)	311 (241-402)	292 (147-583)	393 (135-1150)
Significance (2)	0.16	0.43	0.10	0.038
Significance (3)	0.00063	0.034	0.67	0.026

All values expressed as the geometric mean titre with values in parentheses beneath indicating the 95% CI.

* - Reciprocal of geometric mean titres following correction for total IgA

\+ - Reciprocal of geometric mean titres

Numbers in parentheses following post-vaccination indicate whether the value was obtained from the first post-vaccination sample (Post (1)) before the second vaccine course, or the post-vaccination sample obtained following the second vaccination course (Post (2)).

Significance values expressed as p value only following ln(x) transformation of data and analysis by Student's paired t-test. The number in the parenthesis represents (1) = Post (1)/Pre-vaccination; (2) = Post (2)/Pre-vaccination; (3) = Post (2)/Pre-vaccination.

TABLE 12.3 (CONTINUED)

EX210: INVESTIGATION OF EFFECT OF DOSE REGIMEN ON IMMUNOGENICITY

	JEJUNUM*		SERUM+	
	TYPHOID	CHOLERA	TYPHOID	CHOLERA
EX210 (Group G)				
Pre-vaccination	1018 (210-4930)	312 (150-648)	122 (30.9-486)	169 (44.3-642)
Post (1)-vaccination	1950 (411-9250)	504 (134-1890)	251 (53.5-1180)	137 (22.4-833)
Significance (1)	0.053	0.31	0.11	0.46
Post (2)-vaccination	1822 (314-10580)	447 (123-1624)	187 (45.1-773)	129 (23.0-723)
Significance (2)	0.85	0.59	0.18	0.52
Significance (3)	0.11	0.46	0.084	0.34

All values expressed as the geometric mean titre with values in parentheses beneath indicating the 95% CI.

* - Reciprocal of geometric mean titres following correction for total IgA

\+ - Reciprocal of geometric mean titres

Numbers in parentheses following post-vaccination indicate whether the value was obtained from the first post-vaccination sample (Post (1)) before the second vaccine course, or the post-vaccination sample obtained following the second vaccination course (Post (2)).

Significance values expressed as p value only following ln(x) transformation of data and analysis by Student's paired t-test. The number in the parenthesis represents (1) = Post (1)/Pre-vaccination; (2) = Post (2)/Pre-vaccination; (3) = Post (2)/Pre-vaccination.

12.2.5 *EX363: Phase 1 Study*

Introduction

Two new similar candidate vaccine strains resulted from the development of EX210 - EX363 and EX645. The process of development is described in Section 12.1. Both strains utilized the thymine selection system detailed in Section 12.1. The first strain, EX363, carrying the cloned DNA for the O antigen of a classical *V. cholerae* Ogawa strain; and the other, EX645, carrying the DNA for the O antigen of classical *V. cholerae* Inaba strain 569B - in both cases it was plasmid-borne.

Since the development of EX363 was further advanced than EX645 at the time, it was decided that EX363 should be assessed in humans.

After an initial safety and toxicity study using volunteer subjects kept in the biological containment facility, but from whom no immunological data was obtained, a subsequent out-patient study was performed to evaluate the immunogenicity of this new vaccine candidate, EX363.

Subjects and Methods

In total two studies were performed evaluating the immunogenicity of this vaccine strain. The first involving seven volunteer subjects (Group A), and the second (Group B) using six subjects.

The vaccination doses and schedules including the sampling times, are detailed in Table 12.1. All subjects were vaccinated according to the standard method of vaccination detailed in Section 7.4.1.

Intestinal fluid and sera samples were obtained as detailed Sections 7.1.1 and 7.1.2 in accordance with the schedule in Table 12.1. These fluids were assayed using the ELISA system described in Section 7.2, except that

the specific cholera antibody responses were determined using a phenol extracted LPS preparation obtained from the classical *V. cholerae* Ogawa strain RA411, which was linked to methylated BSA.

Results

Anti-typhoid LPS immune response

Significant typhoid specific serum IgA responses were determined in subjects for Groups A and B of the study and are presented in Table 12.4. Four-fold or greater rises in serum IgA typhoid-specific antibodies were measurable in 3/7 Group A subjects and 1/6 Group B subjects respectively.

Four-fold or greater rises in intestinal IgA anti-typhoid LPS antibodies were detected in 4/7 Group A subjects and 1/6 Group B subjects respectively. Overall, there was a significant intestinal anti-typhoid IgA antibody response only determined in Group A and not in Group B of the study (Table 12.4). In neither group was a significant anti-typhoid IgA antibody response detectable.

Anti-cholera LPS immune response

A four-fold or greater rise in intestinal IgA anti-cholera LPS antibodies was detected in 2/7 Group A subjects and in only 1/6 of the Group B subjects (Table 12.4).

This was supported by four-fold or greater rises in specific anti-cholera O antigen IgA serum antibody responses in 2/7 Group A subjects and 1/6 Group B subjects.

However, as it can be seen in Table 12.4, that the overall intestinal specific anti-cholera O antigen IgA antibody response was not statistically

significant in either group, and that the anti-cholera O antigen IgA antibody responses in serum of both groups also failed to achieve statistical significance.

In addition to the poor immunogenicity, the three oral dose schedule of 10^{11} viable organisms containing the plasmid was associated with an unacceptable high incidence of nausea (4/7), vomiting (3/7) and diarrhoea (more than three loose bowel actions during the day following the first dose) (2/7). One reason for this high rate of adverse reactions could be attributable to the fact that to achieve a dose of 10^{11} live organisms carrying the plasmid, the total dose of organisms (including killed and viable not containing plasmid) was 1.7×10^{12}. This very high dose when used with *S. typhi* Ty21a was associated with a similar incidence of adverse reactions (author's unpublished observations). The poor yield of vaccine organisms carrying the plasmid can be ascribed to the inefficiency of the ThyA selection system incorporated in EX363 and EX645 in comparison to the antibiotic selection system used in EX210.

At the lower dose of 10^{10} viable organisms with plasmid, no adverse reactions were reported by the subjects.

Conclusions

EX363 was considered unsuitable as a candidate vaccine strain due to the unacceptably high rate of adverse reactions associated with doses that are sufficient to generate an albeit poor immune response, and the absence of any indication of immunogenicity at a dose that was free from adverse reactions. In addition, in the absence of a commercial source of a standardized preparation of purified *V. cholerae* Ogawa LPS that was subject to quality control, with the resulting necessity to prepare batches of Ogawa

LPS in the laboratory which may have contained contaminants of a varying degree, rendered further evaluation of this strain unpopular.

TABLE 12.4

EX363: RESULTS OF PRIMARY INVESTIGATION OF IMMUNOGENICITY

	JEJUNUM*		SERUM+	
	TYPHOID	CHOLERA	TYPHOID	CHOLERA
EX363 (Group A)				
Pre-vaccination	337 (211-538)	322 (221-471	305 (127-734)	200 (77.2-520)
Post-vaccination	2664 (699-10150)	880 (268-2890)	717 (286-1795)	255 ((108-603)
Fold Rise	7.91	2.73	2.35	1.28
Significance	0.0089	0.073	0.083	0.46
EX363 (Group B)				
Pre-vaccination	693 (335-1430)	354 (254-494)	150 (56.0-403)	23.8 (8.60-65.9)
Post-vaccination	2578 (380-17480)	677 (152-3030)	210 (70.4-625)	33.5 (6.97-161)
Fold Rise	3.72	2.73	1.40	1.41
Significance	0.12	0.27	0.064	0.35

All values expressed as the geometric mean titre with values in parentheses beneath representing the 95% CI.

* - Reciprocal of geometric mean titres following correction for total IgA

\+ - Reciprocal of geometric mean titres

\# - All values below limit of detection

Significance values expressed as p value following ln(x) transformation of data and analysis by Student's paired t-test

13

EX645: PHASE 1 STUDY

Introduction

I have chosen to dedicate a complete section to EX645 because it was the final candidate vaccine strain organism whose development I was involved in.

EX645 could be considered the "sister" strain to EX363, except that it carried the genes on a plasmid for *V. cholerae* Inaba strain 569B O antigen. This enabled the immunological evaluation to be performed reliably and accurately, with standardized purified antigen.

Since this strain was subsequently evaluated in a cholera challenge study, I have decided to make this section more detailed than the previous sections dealing with the earlier candidate strains.

13.1 *Freshly harvested EX645*

Subjects and Vaccine Preparation

Ten healthy adults (two women and eight men, 19-23 years of age) volunteered to participate in this study. Only one of the volunteers had any past contact with typhoid fever or cholera; she had been parenterally immunized against both 12 years previously.

The vaccine doses were fermenter grown in minimal salts medium containing 0.0001% supplemental galactose, and in the absence of thymine so as to apply selection pressure for the retention of the plasmid. Each vaccine dose was freshly harvested and suspended in 10 ml of 0.9% (w/v) sodium chloride solution, and contained a mean total number of organisms of

$9.3x10^{10}$; of these $5.8x10^{10}$ (63%) were viable and 92% of the viable organisms carried the plasmid. All doses slide agglutinated positive for *S. typhi* H-d (flagellar) antigen and 9, 12 O polysaccharide antigens as well as for *V. cholerae* Inaba LPS. The amount of *S. typhi* O antigen (33%) and *V. cholerae* O antigen (63%) expressed as a percentage of the parent organisms were quantified by haemagglutination inhibition assay,[231] performed by Dr S.R. Attridge.

Assays for anti-cholera and anti-typhoid bactericidal antibody were performed by Dr SR Attridge of Enterovax Limited, as previously described,[233] on the same serum samples as assayed by ELISA. Endpoints were obtained by interpolation using a graph of residual viability of indicator organisms versus serum dilution, the titre being the reciprocal serum dilution resulting in 50% killing.

Statistics

After ln(x) transformation of the data, Student's t-test was used to assess the significance of any change in antigen-specific serum antibody and bactericidal titres following vaccination. Wilcoxon's Rank Sign Test was used to assess the significance of any change in the absorbances of the PBL-ELISAs observed following vaccination.

Results

Tolerance of Vaccine

The vaccine was well tolerated by all recipients, with no adverse reactions being reported by any of the vaccinees during the period of the study.

TABLE 13.1

HUMAN IMMUNE RESPONSE TO ORAL TYPHOID/CHOLERA HYBRID VACCINE

	PBL+			Serum*		
	IgA	IgG	IgM	IgA	IgG	IgM
Typhoid						
Pre-vaccination	0.071 (0.058-0.083)	0.001 (0.000-0.003)	0.018 (0.007-0.029)	97.2 (79.4-349)	225 (145-349)	576 (406-818)
Post-vaccination	1.03++ (0.510-1.55)	0.213 (0.005-0.421)	0.905 (0.342-1.47)	911 (699-1190)	784 (349-1760)	2110 (1250-3580)
Fold Rise	14.8	213	50.3	9.37	3.48	3.67
Significance	0.002	0.009	0.003	<0.001	0.008	<0.001
Cholera						
Pre-vaccination	0.055 (0.044-0.066)	0.016 (0.007-0.025)	0.035 (0.019-0.051)	41.0 (22.4-75.2)	91.5 (54.8-153)	581 (383-880)
Post-vaccination	0.265 (0.062-0.469)	0.164 (0.000-0.431)	0.207 (0.000-0.438)	132 (40.8-423)	241 (73.3-792)	1103 (464-2624)
Fold Rise	4.82	10.3	5.91	3.22	2.64	1.90
Significance	0.002	0.193	0.003	0.022	0.081	0.104

+ - arithmetic mean absorbance values, p values determined by Wilcoxon Signed Rank Test

++ - absorbance values for three subjects' PBL exceeded the upper limit of detection and so were recorded as 2.00 absorbance units

* - geometric mean peak titres, p values determined by Student's t-test following ln(x) transformation of data. Values in parentheses represent 95% CI.

Anti-typhoid LPS immune response

An anti-typhoid IgA antibody response was detected using the post-vaccination PBL obtained from all ten volunteers who ingested the *S. typhi* Ty21a/*V. cholerae* Inaba vaccine (EX645). IgG responses (7/10 responders) and IgM responses (10/10 responders) were also observed.

Significant serum responses in all antibody classes were observed (Table 13.1), with four fold or greater rises in serum IgA, IgG and IgM typhoid-specific antibodies measurable in 7/10, 4/10 and 3/10 individuals respectively. Similarly four fold or greater rises in typhoid bactericidal titres were evident in 8/10 vaccinated subjects, with all ten demonstrating at least a two-fold rise.

Anti-cholera LPS immune response

A post-vaccination anti-cholera Inaba O antigen IgA antibody response was detected in 8/10 vaccinees using the PBL-ELISA, with an IgM response identified in 6/10 vaccinees, and an IgG response in 3/10. The IgA and IgM responses were highly significant, but the IgG response was not, despite a mean fold-rise of 10.3 (only three vaccinees responded) (see Table 13.1).

Anti-cholera Inaba O antigen-specific serum IgA antibody responses were observed, with 4/10 vaccinees (4/8 PBL IgA responders) having a four fold or greater rise. A similar rise was observed in the IgG class anti-Inaba antibodies in 3/10 vaccinees, although only 1/10 had an IgM response of that magnitude.

Evidence of cross-reactivity to the heterologous serotype was obtained by the demonstration of rises in the anti-Ogawa LPS IgA antibody produced by PBL (8/10 vaccinees; 7/8 Inaba responders), and in serum

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Anti-cholera LPS immune response

A post-vaccination anti-cholera Inaba O antigen IgA antibody response was detected in 8/10 vaccinees using the PBL-ELISA, with an IgM response identified in 6/10 vaccinees, and an IgG response in 3/10. The IgA and IgM responses were highly significant, but the IgG response was not, despite a mean fold-rise of 10.3 (only three vaccinees responded) (see Table 13.1).

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Evidence of cross-reactivity to the heterologous serotype was obtained by the demonstration of rises in the anti-Ogawa LPS IgA antibody produced by PBL (8/10 vaccinees; 7/8 Inaba responders), and in serum

(4/10 vaccinees with the large anti-Inaba responses also having four-fold or greater anti-Ogawa IgA antibody rises).

Serum anti-cholera Inaba bactericidal antibody rises of four-fold or greater magnitude were observed in 5/10 subjects post-vaccination (pre-vaccine geometric mean titre 72.2, 95% CI 21.5-242; post-vaccine geometric mean titre 296, 95% CI 52.1-1683; Student's paired t test following ln(x) transformation of data, p=0.015). However, only 2/10 vaccinees (2/5 Inaba LPS responders) recorded a four-fold or greater rise in the anti-Ogawa LPS vibriocidal response (Table 13.2).

TABLE 13.2

SERUM BACTERICIDAL RESPONSES TO FRESHLY HARVESTED EX645

	Typhoid	569B*	O17+
Pre-vaccine**	297 (154-573)	72.2 (21.5-242)	1220 (462-3210)
Post-vaccine**	2900 (1250-6710)	296 (52.1-1680)	2670 (1030-6940)
Fold Rise@	9.76	4.10	2.19
Significance#	p<0.001	p=0.015	p=0.073

* - *Vibrio cholerae* Inaba 569B
+ - *Vibrio cholerae* Ogawa O17
** - geometric mean peak titres
@ - geometric mean fold rise
# - significance values determined using Student's paired t-test following ln(x) transformation of data.
Values in parentheses indicate 95% confidence intervals.

Conclusion

This study demonstrated that EX645 was both well tolerated and immunogenic in human subjects. The magnitude of the resulting immune response compared favourably with observed levels in other studies, like those of Mosley,[274,309] who demonstrated that a two-fold rise in vibriocidal

antibody titre was associated with a two-fold reduction in susceptibility to cholera in the field, and the clinical studies with the whole-cell killed cholera vaccine (without the B subunit of the cholera toxin) which stimulated geometric serum antibody titres of as little as two-fold,[311] and yet provided 56% protection in recipients.[305] These reports indicated that the results obtained so far with this vaccine strain EX645 suggested that it would have a similar efficacy in the prevention of cholera.

The further attraction of EX645 as a vaccine was the likelihood that it will provide protection against typhoid in addition to that against cholera, and possibly against other non-typhoid *Salmonella* infection, though again this would require demonstration in the field.

13.2 *Lyophilized EX645*

Despite the many advantages of using a freshly harvested liquid vaccine which retains excellent viability and plasmid retention, economic and practical considerations demand a more convenient and reliable preparation for administration to high risk populations in the field. The preparation of large batches of lyophilized vaccine doses provides a convenient means of vaccine delivery, and ensures that there is some degree of quality control of the doses. This provides for some consistency in and control over variables such as dosage size, organism viability, plasmid retention, lipopolysaccharide production by the organism and maintenance of immunogenicity.

Therefore, the successful lyophilization of vaccine organisms represents the first step in the preparation of a commercial formulation of the candidate vaccine suitable for protection studies.

In addition, this study provided the first opportunity to evaluate this particular candidate vaccine strain's ability to induce an intestinal immune response. The thorough nature of the data provided here reflects its use, it was obtained for the purpose of inclusion into an IND application to the FDA in the USA.

Subjects and Vaccine Preparation

Nine healthy adults (three women and six men, 21-42 years of age) volunteered to participate in this pilot study. Only one of the subjects had any past contact with typhoid fever or cholera; he had been parenterally immunized against both three years previously.

The vaccine doses were fermenter grown in the same defined medium as for the freshly harvested doses in Section 13.1. The vaccine doses were subsequently lyophilized and these lyophilized vaccine doses were stored at -20°C until required for administration. The vaccination process required that the vaccine doses be warmed to room temperature for 15 minutes, before being resuspended in 10 ml of 0.9% (w/v) sodium chloride solution. Each reconstituted lyophilized vaccine dose comprised a mean total number of organisms of 5.0×10^{11}; of these 5.6×10^{10} (11%) were viable and 50% of the viable organisms carried the plasmid. All doses slide agglutinated positive for *S. typhi* H-d (flagellar) antigen and 9, 12 O antigens as well as for *V. cholerae* Inaba LPS. The amount of *S. typhi* O antigen (12.5%) and *V. cholerae* O-antigen (50%) expressed as a percentage of the parent organisms were quantified by Dr S.R. Attridge using a haemagglutination inhibition assay.[233]

In one subject despite successful intestinal intubation, it was not possible to collect intestinal fluid on either occasion.

Faecal samples were collected from all volunteers on days 0 to 9 of the study inclusive. Diluted 20 g/100 ml faecal suspensions were prepared by homogenization of 2 grammes of faeces in 10 ml sterile 0.9% (w/v) saline; 1 ml of the homogenate was added to 9 ml of Oxoid Nutrient Broth containing 0.050 mg/ml thymine and 0.100 mg/ml rifampicin for enrichment. The broth was incubated for 18 hours at 37°C with aeration. The broth was then sampled using a 0.010 ml loop and directly plated onto selection agar. These plates were incubated for 24 hours at 37°C. Dr R. Morona of Enterovax Limited was responsible for the processing of the faecal samples.

Results

Tolerance of Vaccine

Following vaccination, some adverse effects were reported by 7/9 subjects. These are recorded in Table 13.3. All adverse reactions reported occurred within twelve hours of receiving a vaccine dose, except in two instances. In these two subjects the reported adverse reactions occurred more than 60 hours after the last vaccine dose. In addition, these two subjects had symptoms of a concurrent viral upper respiratory tract infection. Generally, the vaccine was well tolerated by the recipients.

The nausea and abdominal discomfort observed in some subjects after taking a vaccine dose could be due to the size of the vaccine doses, each of which had a high total cell count. A situation that was observed with the EX363 human evaluation (Section 12.2.5).

TABLE 13.3

ADVERSE REACTIONS REPORTED IN HUMANS FOLLOWING ORAL ADMINISTRATION OF LYOPHILIZED EX654

Clinical Complaint	Incidence
Abdominal Discomfort	5/30 (17%)
Headache	3/30 (10%)
Nausea	3/30 (10%)
Vomiting*	1/30 (3.3%)
Diarrhoea+	1/30 (3.3%)

Incidence is number of reports of a specific adverse reaction/total number of doses administered.
* - single episode occurring 60 hours after last vaccine dose.
+ - single loose bowel action occurring 60 hours after last vaccine dose.

Anti-typhoid LPS immune response

An anti-typhoid IgA antibody response was detected using the post-vaccination PBL obtained from 9/9 subjects who ingested EX645 ($p=0.004$ using Wilcoxon's Rank Sign Test). IgG responses (9/9 responders) and IgM responses (6/9 responders) were also observed.

Significant typhoid specific serum IgA responses were determined (Table 13.4) with four-fold or greater rises in serum IgA, IgG and IgM typhoid-specific antibodies measurable in 9/9, 8/9 and 5/9 individuals respectively. Typhoid bactericidal antibody responses were also significantly elevated ($p=0.011$), with four fold or greater rises in antibody titres evident in 4/8 vaccinees, with 6/8 demonstrating at least a two-fold rise (Table 13.5).

A four-fold or greater rise in intestinal IgA anti-typhoid LPS antibodies was detected in 6/8 subjects. Overall, the intestinal anti-typhoid IgA antibody response was significant (Table 13.4).

TABLE 13.4

EX645: RESULTS OF INVESTIGATION INTO IMMUNOGENICITY OF LYOPHILIZED DOSES

	JEJUNUM*		SERUM+	
	TYPHOID	CHOLERA	TYPHOID	CHOLERA
EX645				
Pre-vaccination	1078 (358-3250)	431 (161-1150)	43.3 (24.4-76.7)	32.2 (16.5-63.0)
Post-vaccination	8409 (4840-14600)	830 (345-1990)	626 (258-1520)	75.8 (29.1-198)
Fold Rise	7.80	1.93	14.5	2.35
Significance	0.0010	0.12	<0.001	0.046

All values expressed as the geometric mean titre with values in parentheses beneath representing the 95% CI.

* - Reciprocal of geometric mean titres following correction for total IgA

\+ - Reciprocal of geometric mean titres

Fold rises expressed as geometric mean fold rises. Significance values expressed as p value only following ln(x) transformation of data and analysis by Student's paired t-test.

TABLE 13.5

SERUM BACTERICIDAL RESPONSES TO EX645

	Typhoid	Cholera*
Pre-vaccine**	271 (94.0-783)	45.5 (6.14-337)
Post-vaccine**	2059 (1022-4149)	203 (30.7-1340)
Fold Rise@	7.60	4.46
Significance#	p=0.011	p=0.011

* - *Vibrio cholerae* Inaba 569B
** - geometric mean peak titres
@ - geometric mean fold rises
# - p values determined using Student's paired *t*-test following ln(x) transformation of data.
Values in parentheses indicate 95% confidence intervals.

Anti-cholera LPS immune response

An anti-cholera Inaba O-antigen IgA antibody response in the PBL-ELISA was identified in 5/9 subjects post-vaccination, an IgM response in 6/9, and an IgG response in 4/9. The IgA (p=0.009), IgM (p=0.025) and IgG (p=0.034) responses were significant.

Significant serum anti-cholera IgA responses were also identified (Table 13.4) with a four-fold or greater rise in serum IgA anti-cholera antibodies was measurable in only 2/9 subjects. A similar rise was observed in the IgG class anti-cholera antibodies in 5/9 subjects.

A four-fold or greater rise in serum bactericidal antibody against *V. cholerae* Inaba LPS was observed in 3/8 subjects (Table 13.5).

Similarly, a four-fold or greater rise in intestinal IgA anti-cholera LPS antibodies was detected in 2/8 subjects. However, the overall intestinal anti-cholera IgA antibody response was not significantly elevated; pre-vaccine geometric mean 431 units, 95% C.I. 161 to 1149 units, post-vaccine geometric

mean 830 units, 95% C.I. 345 to 1993 units; using Student's paired t test after ln(x) transformation of titres, t=1.772 d.f.=7 p=0.120 (Figure 13.1).

Faecal Excretion of EX645

EX645 was detected on at least one occasion in the faeces of all subjects following vaccination.

In only two subjects was it possible to isolate the vaccine strain from faecal specimens obtained more than 48 hours after the last dose of the vaccine, and in both of these subjects, EX645 could not be detected in faeces more than 72 hours after the final dose.

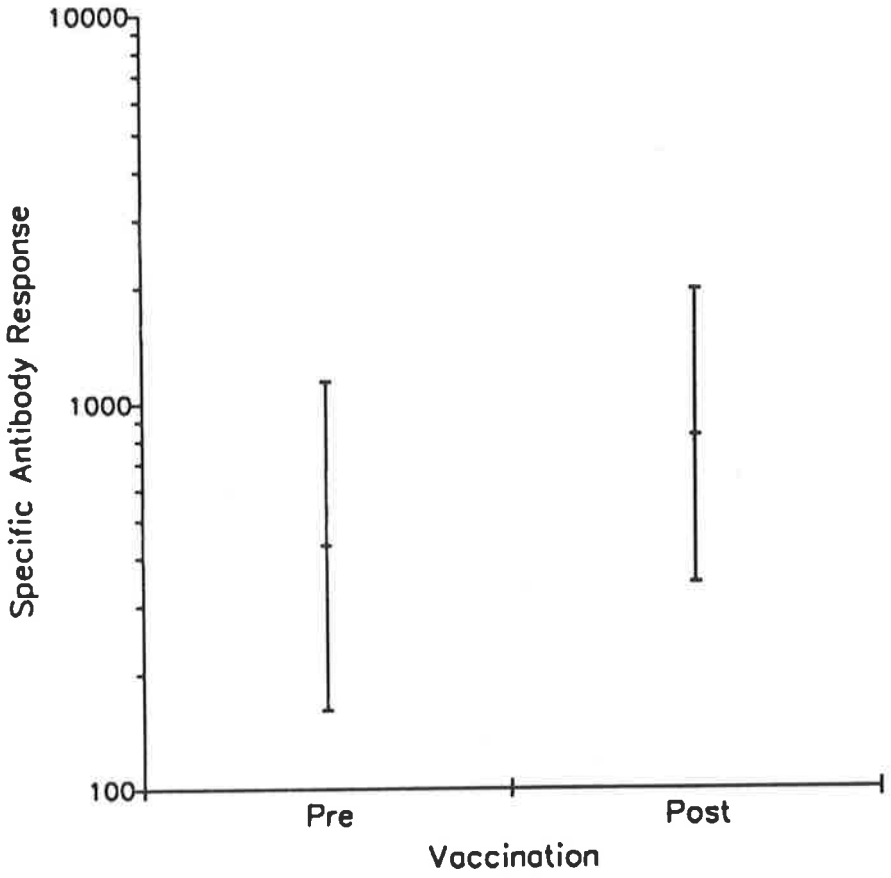
In all subjects, peak faecal excretion of EX645 occurred within the 24 hours following a vaccine dose, and there was no detectable transfer of the cloned DNA to indigenous microorganisms.

Conclusion

In conclusion this study demonstrated that, despite the viability of the vaccine strain EX645 and its plasmid retention being considerably adversely affected by the process of lyophilization, it retained its ability to stimulate significant anti-typhoid and anti-cholera immune responses in humans. It was this batch of vaccine doses that were used in the subsequent protective efficacy evaluation study.

FIGURE 13.1

SPECIFIC ANTI-CHOLERA INTESTINAL IgA RESPONSE FOLLOWING VACCINATION WITH LYOPHILIZED EX645



Graph represents geometric mean titres ± 95% confidence intervals following adjustment for total IgA content.

13.3 *Specific Immune Response Following Oral Vaccination With Killed Whole-Cell Vaccine*

Introduction

In order to have some indication of the likely protective efficacy against cholera challenge of the EX645 candidate oral typhoid/cholera vaccine, the intestinal, serum and PBL specific immune responses were determined using the killed oral whole-cell vaccine only (Section 3.10), a vaccine known to have conferred protection against cholera challenge in 56% of recipients.[305]

Six healthy adult subjects ingested three doses of the killed oral whole-cell vaccine (Supplied by the Institute Merieux, Lyon, France), according to the standard method of oral vaccination (as described in Section 7.4.1). Blood was obtained every three-four days for serum and PBL, jejunal fluid was obtained pre-vaccination and again 20 Days post-vaccination (Sections 7.1.1, 7.1.2 & 7.1.3).

Specific anti-cholera Inaba LPS antibody responses were determined using previously detailed methods (Section 7.2 & 7.3).

Results

Intestinal Immune Responses

A four-fold or greater rise in intestinal IgA anti-cholera Inaba LPS antibodies was not detected in any of the six subjects receiving the WCV, nor was such a response detectable directed against the *V. cholerae* Ogawa RA411 LPS. However, the post-vaccination mean jejunal specific anti-cholera Inaba LPS IgA antibody titre in this group was significantly elevated above the pre-

vaccination mean level, whereas the anti-cholera Ogawa LPS post-vaccination mean antibody titre was not significantly greater than the pre-vaccination level (Table 13.6, Figure 13.2).

TABLE 13.6

ORAL KILLED WHOLE-CELL VACCINE: IMMUNOGENICITY SUMMARY

	JEJUNUM*	
	INABA	OGAWA
Pre-vaccination (Day 0)	724 (288-1820)	393 (218-709)
Post-vaccination (Day 20)	1062 (419-2700)	514 (356-741)
Fold Rise	1.47	1.31
Significance	0.0046	0.28

* - Reciprocal of geometric mean titres following correction for total class-specific immunoglobulin
Fold rises expressed as geometric mean fold rises
Significance values expressed as p value only following ln(x) transformation of data and analysis by Student's paired *t*-test for jejunal fluid

Serum Immune Responses

The low magnitude intestinal immune responses were supported by the failure to identify four-fold or greater rises in specific anti-cholera Inaba LPS IgA serum antibody responses in any of the subjects. The serum immune response directed against the *V. cholerae* Ogawa LPS was not investigated. The mean post-vaccination anti-cholera Inaba LPS serum IgA antibody titres did achieve statistically significance as early as Day 5 post-vaccination (geometric mean titre 56.0, 95% CI 27.6-113; t=2.73, p=0.041) peaking on Day 9 (geometric mean titre 73.1, 95% CI 22.7-236; t=2.77, p=0.040) but declining after this to return to near pre-vaccination baseline levels. The serum specific

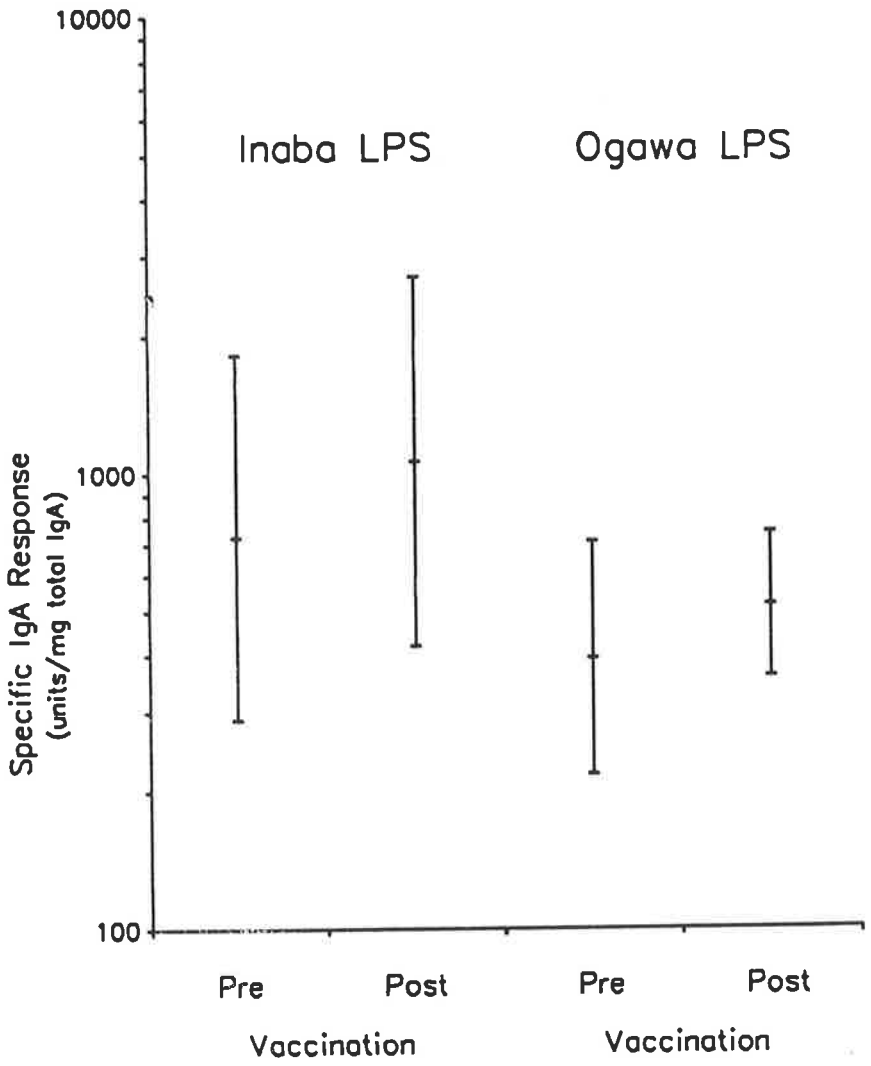
anti-cholera Inaba LPS IgG titres also increased significantly post-vaccination however also without any subject achieving four-fold or greater rises, peaking on Day 12 (geometric mean titre 112, 95% CI 66.5-118; t=5.10, p=0.0038) declining significantly from day 15 (Day 15 vs Day 19 titres t=3.25, p=0.023) however the Day 19 titre post-vaccination remained significantly elevated above the pre-vaccination baseline (Day 19 geometric mean titre 64.5, 95% CI 38.7-108; t=2.97, p=0.031).

Conclusion

Although the killed whole-cell vaccine preparation had an overall marginally better immune responses than the lyophilized vaccine candidate EX645, the differences were not sufficiently large to indicated that EX645 would behave in a largely similar manner with respect to protective efficacy. It should be noted that EX645 did really perform any better than the WCV in the ability to induce good levels of specific antibody, reflecting the low levels of available cholera O antigen produced by the hybrid strain.

FIGURE 13.2

SPECIFIC ANTI-CHOLERA INTESTINAL IgA RESPONSE FOLLOWING ORAL VACCINATION WITH KILLED WHOLE CELL CHOLERA VACCINE



Graph represents geometric mean titres ± 95% confidence intervals following adjustment for total IgA content.

14

PROTECTIVE EFFICACY OF EX645

Introduction

The evaluation of the protective efficacy of the candidate live oral hybrid typhoid/cholera vaccine strain EX645 was performed at the Center for Vaccine Development, which is located within the University of Maryland School of Medicine in Baltimore, Maryland, USA. Since 1952 this institution has been developing human models of infectious diseases of relevance to clinical medicine. Commencing under Professor T.E. Woodward with the initial experimental human typhoid fever studies in 1952, until the present where under the authority of Professor M.M. Levine, the Center has developed human models for the examination of the pathogenesis and immunology of numerous enteric diseases and malaria.

With the massive expensive that is incurred with the study of vaccine efficacy in the field, the Center for Vaccine Development (CVD) provides the ideal opportunity to accurately evaluate the protective efficacy of a candidate vaccine against diarrhoeal disease in a strictly controlled environment. It is also the only biological containment facility in the world that currently offers this service on a regular basis.

Under the requirements of the IND granted by the FDA of the USA, it was necessary to repeat the Phase 1 studies at least once with the airfreighted lyophilized vaccine doses, and so this was built into the challenge study protocol producing a sequential two-part investigation.

Subjects and Methods

The clinical protocol for this study was approved by the University of Maryland Human Volunteers Research Committee. Fourteen healthy adult volunteers agreed to participate in the study and after the necessary health screening were admitted to the Isolation Ward of the CVD at the University of Maryland Hospital.

The lyophilized vaccine organisms had been pre-packaged in single dose vials, and prior to administration were reconstituted in 30 ml of 0.9% saline. Each reconstituted dose contained $1.0x10^{10}$ viable vaccine organisms containing the plasmid. vaccination occurred according to the standard method of vaccination as previously detailed. Following vaccination all subjects were maintained within the facility under strict observation for nine days.

Intestinal fluid was collected on Days 0, 7 and 14, using a slightly modified procedure to that described in Section 7.1.1. The subjects swallowed an 2.5 mm internal diameter weighted polyvinyl tube and this remained *in situ* until 70 ml of fluid had been collected or for 36 hours duration whichever was sooner. Fluoroscopy was not approved for use to permit accurate siting of the tube in the small intestine, however all samples were tested for pH and only those of pH>6.0 were collected. The intestinal fluid was subsequently handled as described in Section 7.1.1, that is, it was not heat-inactivated but simply aliquoted and stored at -70°C until required for assaying. Whole blood for PBL and serum was also collected on Days 0, 7 and 14.

Specific anti-typhoid and anti-cholera LPS antibody responses in serum and intestinal fluid were determined using the ELISA described in

Section 7.2, and antigen-specific antibodies produced by circulating PBL *in vitro* were determined using the PBL-ELISA as described in Section 8.

Eight of the 14 vaccinated subjects agreed to return to be evaluate the vaccine's protective efficacy against an oral challenge with pathogenic *V. cholerae*. Four weeks after vaccination, these eight subjects together with 13 previously unexposed control subjects were admitted to the Isolation Ward. The challenge dose schedule, the subjects from both groups to swallow 120 ml of a 1.3% sodium hydrogen carbonate solution, then one minute later ingested $1.1x10^6$ pathogenic *V. cholerae* El Tor Inaba strain N19691 organisms suspended in 30 ml of the 1.3% sodium hydrogen carbonate solution.

The subjects were kept on the ward for eight days following challenge, being observed, examined once daily, and if experiencing diarrhoea being treated with oral glucose-electrolyte solution to maintain hydration.[477] All subjects received tetracycline 500 mg six hourly for five days to eradicate the challenge organism from their stools prior to discharge.

All stools after vaccination and challenge were collected and recorded for weight, consistency and description. A rectal swab was obtained from any subject not able to provide a daily faecal specimen. All stools were graded according to the scale in Table 14.1.

TABLE 14.1

STOOL CONSISTENCY GRADES

Stool Grade	Stool Description
1	Formed
2	Soft, formed
3	Thick liquid
4	Opaque, watery
5	Rice water

TABLE 14.2

SERUM IMMUNE RESPONSE IN NORTH AMERICAN SUBJECTS FOLLOWING ORAL VACCINATION WITH EX645

Subjects who returned for challenge only

Serum IgA Responses

	S. typhi Ty2			*V. cholerae* Inaba 569B		
Subject Code	Day 0	Day 7	Day 14	Day 0	Day 7	Day 14
11001-4	148	2420	3110	92.4	288	175
11001-5	27.1	299	219	14.8	10	10
11001-9	26.2	188	178	10	10	10
11001-10	260	1450	2010	24.4	47.0	52.9
11001-12	17.0	84.0	47.2	10	21.7	71.0
11001-13	10	85.0	227	10	10	10.6
11001-14	72.0	873	1074	29.2	49.8	41.2
11001-17	45.0	157	90	11.0	10	10

All values expressed as the reciprocal of the dilution of serum that gave an optical density of 0.15 by ELISA for specific antibody activity.

TABLE 14.3

SERUM IMMUNE RESPONSE IN NORTH AMERICAN SUBJECTS FOLLOWING ORAL VACCINATION WITH EX645

Subjects who returned for challenge only

Serum IgG Responses

	S.typhi Ty2			*V. cholerae* Inaba 569B		
Subject Code	Day 0	Day 7	Day 14	Day 0	Day 7	Day 14
11001-4	88.0	2385	5750	10	10	10
11001-5	14.6	70.0	167	10	10	10
11001-9	77.8	254	382	33.9	23.5	25.0
11001-10	82.4	330	630	10	10	10
11001-12	84.0	370	670	21.7	20.8	89.5
11001-13	10	795	848	10	10	10
11001-14	59.8	177	401	48.9	78.6	85.5
11001-17	15.0	283	686	10	10	10

All values expressed as the reciprocal of the dilution of serum that gave an optical density of 0.15 by ELISA for specific antibody activity.

TABLE 14.4

SERUM IMMUNE RESPONSE IN NORTH AMERICAN SUBJECTS FOLLOWING ORAL VACCINATION WITH EX645

Subjects who returned for challenge only

Serum IgM Responses

	S.typhi Ty2			*V. cholerae* Inaba 569B		
Subject Code	Day 0	Day 7	Day 14	Day 0	Day 7	Day 14
11001-4	65.8	88.9	84.6	61.5	81.6	67.8
11001-5	62.4	606	595	62.0	83.9	208
11001-9	45.3	182	87.7	100	85.0	72.5
11001-10	179	219	263	268	307	348
11001-12	47.2	257	749	71.0	92.0	350
11001-13	346	350	393	395	425	526
11001-14	385	696	828	537	470	380
11001-17	170	272	269	310	260	363

TABLE 14.5

PERIPHERAL BLOOD LYMPHOCYTE IMMUNE RESPONSE IN NORTH AMERICAN SUBJECTS FOLLOWING ORAL VACCINATION WITH EX645

Subjects who returned for challenge only

PBL IgA Responses

	S.typhi Ty2		*V. cholerae* Inaba 569B	
Subject Code	Day 0	Day 7	Day 0	Day 7
11001-4	0.033	>2.00	0.049	0.081
11001-5	0.023	1.863	0.022	0.056
11001-9	0.000	0.202	0.008	0.000
11001-10	0.009	>2.00	0.043	0.171
11001-12	0.045	1.905	0.000	0.048
11001-13	0.000	0.323	0.008	0.076
11001-14	0.025	0.846	0.017	0.017
11001-17	0.007	0.411	0.002	0.031

PBL IgG Responses

	S.typhi Ty2		*V. cholerae* Inaba 569B	
Subject Code	Day 0	Day 7	Day 0	Day 7
11001-4	0.030	1.598	0.000	0.000
11001-5	0.043	0.209	0.020	0.008
11001-9	0.018	0.118	0.000	0.068
11001-10	0.025	0.202	0.000	0.003
11001-12	0.005	0.291	0.000	0.056
11001-13	0.008	0.367	0.000	0.000
11001-14	0.030	0.073	0.001	0.000
11001-17	0.007	0.138	0.000	0.000

All values expressed as the reciprocal of the dilution of serum that gave an optical density of 0.15 by ELISA for specific antibody activity.

TABLE 14.5 (CONTINUED)

PERIPHERAL BLOOD LYMPHOCYTE IMMUNE RESPONSE IN NORTH AMERICAN SUBJECTS FOLLOWING ORAL VACCINATION WITH EX645

Subjects who returned for challenge only

PBL IgM Responses

	S.typhi Ty2		*V. cholerae* Inaba 569B	
Subject Code	Day 0	Day 7	Day 0	Day 7
11001-4	0.068	0.211	0.004	0.159
11001-5	0.050	1.014	0.061	0.088
11001-9	0.076	0.272	0.042	0.095
11001-10	0.023	0.329	0.074	0.168
11001-12	0.052	0.423	0.068	0.084
11001-13	0.049	0.363	0.027	0.042
11001-14	0.093	0.470	0.075	0.075
11001-17	0.079	0.676	0.071	0.092

All values expressed as the reciprocal of the dilution of serum that gave an optical density of 0.15 by ELISA for specific antibody activity.

Results

The anti-typhoid O antigen and anti-cholera Inaba LPS specific IgA, IgG and IgM serum antibody responses for each vaccinated subject are presented in Tables 14.2, 14.3 and 14.4 respectively.

Anti-typhoid LPS Immune Response

An anti-typhoid IgA antibody response was detected using the Day 7 post-vaccination PBL obtained on from all eight subjects who ingested EX645 ($p=0.0078$ using Wilcoxon's Rank Sign Test). IgG responses (7/8 responders) and IgM responses (8/8 responders) were also observed (Table 14.5 and 14.6).

TABLE 14.6

EX645: IMMUNOGENICITY SUMMARY
PERIPHERAL BLOOD LYMPHOCYTE RESPONSES*

	TYPHOID	CHOLERA
IgA		
Pre (Day 0)	0.018 (0.004-0.032)	0.019 (0.004-0.034)
Post (Day 7)	1.194 (0.507-1.881)	0.060 (0.016-0.104)
Significance	0.0078	0.031
IgG		
Pre (Day 0)	0.021 (0.010-0.032)	0.003 (0.000-0.009)
Post (Day 7)	0.375 (0.000-0.796)	0.017 (0.000-0.041)
Significance	0.0078	0.44
IgM		
Pre (Day 0)	0.061 (0.043-0.079)	0.053 (0.031-0.075)
Post (Day 7)	0.470 (0.251-0.689)	0.100 (0.065-0.135)
Significance	0.0078	0.016

* - arithmetic mean of absorbances at 405 nm
Significance values expressed as p value only following Wilcoxon Rank Sign Test

Significant anti-typhoid specific serum IgA responses were identified (Table 14.7) with four-fold or greater rises in serum IgA, IgG and IgM typhoid-specific antibodies measurable in 6/8, 8/8 and 2/8 individuals respectively (Tables 14.2, 14.3 and 14.4).

A four-fold or greater rise in intestinal IgA anti-typhoid LPS antibodies was detected in 7/8 subjects (Table 14.8). Overall, the intestinal anti-typhoid IgA antibody response was significant (p=0.00047) (Table 14.9).

TABLE 14.7

EX645: IMMUNOGENICITY SUMMARY

	SERUM*	
IgA	TYPHOID	CHOLERA
Pre (Day 0)	44.4 (17.7-111)	17.9 (9.30-34.6)
Post (Day 14)	338 (96.1-1190)	27.1 (10.5-69.4)
Fold Rise	7.61	1.51
Significance	0.00029	0.16
IgG		
Pre (Day 0)	39.7 (34.5-45.7)	15.7 (9.05-27.1)
Post (Day 14)	660 (283-1540)	19.3 (8.46-44.0)
Fold Rise	16.6	1.23
Significance	0.00022	0.31
IgM		
Pre (Day 0)	117 (56.4-244)	162 (76.3-344)
Post (Day 14)	306 (145-644)	234 (121-452)
Fold Rise	2.62	1.44
Significance	0.029	0.18

* - Reciprocal of geometric mean titres.
Fold rises expressed as geometric mean fold rises.
Significance values expressed as p value only following ln(x) transformation of data and analysis by Student's paired *t*-test for sera.

TABLE 14.8

INTESTINAL IMMUNE RESPONSE IN NORTH AMERICAN SUBJECTS FOLLOWING ORAL VACCINATION WITH EX645

Subjects who returned for challenge only

Jejunal IgA Responses

	S.typhi Ty2		*V. cholerae* Inaba 569B	
Subject Code	Day 0	Day 14	Day 0	Day 14
11001-4	195	11060	195	195
11001-5	125	11900	125	8955
11001-9	248	7836	247	209
11001-10	267	24420	42.7	1134
11001-12	457	4405	457	706
11001-13	NS	NS	NS	NS
11001-14	581	8798	150	156
11001-17	66.7	221	65	66.3

All values expressed as the reciprocal of the dilution of serum that gave an optical density of 0.15 by ELISA for specific antibody activity.

TABLE 14.9

EX645: IMMUNOGENICITY SUMMARY

	JEJUNUM*	
	TYPHOID	CHOLERA
Pre (Day 0)	224 (337-2200)	142 (68.1-297)
Post (Day 14)	5781 (1406-23760)	383 (69.8-2096)
Fold Rise	25.8	2.70
Significance	0.00047	0.23

* - Reciprocal of geometric mean titres following correction for total class-specific immunoglobulin.
Fold rises expressed as geometric mean fold rises.
Significance values expressed as p value only following ln(x) transformation of data and analysis by Student's paired *t*-test for jejunal fluid.

TABLE 14.10

CLINICAL AND BACTERIOLOGICAL RESPONSES OF VACCINATED AND CONTROL SUBJECTS FOLLOWING CHALLENGE WITH *V. cholerae* EL TOR INABA N16961

Subjects	Diarrhoeal Stool Volume (ml)	Number of Diarrhoeal Stools	Incubation Period (hours)	Peak *V. cholerae* Excretion ($x10^7$)
VACCINEES				
11001-4	956	7	17.0	10
11001-5	497	2	25.5	1.5
11001-9	2 053	9	20.0	7.5
11001-10	784	7	53.0	0.065
11001-12	0	0	-	0.002
11001-13	0	0	-	0.00027
11001-14	312	5	16.0	11
11001-17	602	6	22.0	5
MEAN	**867***	**6**	**25.6**	**3.5+**
CONTROLS				
11002-1	2 054	10	31.5	12
11002-2	1 168	8	22.0	10
11002-3	889	8	22.0	7.5
11002-6	4 399	17	16.5	15
11002-9	1 819	13	13.5	30
11002-12	1 597	6	16.0	0.5
11002-15	4 619	12	17.5	0.8
11002-17	3 405	11	21.0	10
11002-19	906	8	32.0	10
11002-20	622	3	27.5	1
11002-21	3 294	20	22.0	10
11002-22	2 634	13	5.5	10
11002-23	6 434	22	20.0	13
MEAN	**2 603***	**11.6**	**20.5**	**6.2+**

* - p=0.0013, Wilcoxon Rank Sum Test; + - p=0.053, Wilcoxon Rank Sum Test

Anti-cholera LPS Immune Response

An anti-cholera Inaba O-antigen IgA antibody response in the PBL-ELISA was identified in 4/8 subjects post-vaccination, an IgM response in 5/8, and an IgG response in 2/8. The IgA (p=0.031) and IgM (p=0.016) responses achieved statistical significance, the IgG (p=0.44) responses failed to do so (Table 14.6).

Significant serum anti-cholera IgA responses were also identified (Table 14.9) with a four-fold or greater rise in serum IgA anti-cholera antibodies was measurable in only 1/8 subjects. A similar rise was observed in the IgG class anti-cholera antibodies in only 1/8 subjects (the same subject recording the rise in IgA class), with the same subject being the only one of eight recording such a rise in specific IgM antibodies (Tables 14.2, 14.3 and 14.4).

A four-fold or greater rise in serum bactericidal antibody against *V. cholerae* Inaba LPS was observed in 2/8 subjects.

A similar four-fold or greater rise in intestinal IgA anti-cholera LPS antibodies was detected in 2/7 sampled subjects. However, the overall intestinal anti-cholera IgA antibody response was not significantly elevated (Table 14.9) (p=0.23).

Results of Cholera Challenge

All thirteen previously unexposed control subjects (100%) and 6/8 (75%) of the vaccinated subjects developed diarrhoea following their simultaneous challenge with 1.1×10^9 viable pathogenic *V. cholerae* Inaba strain N16961. The protective efficacy of the vaccine strain EX645 was determined to be 25%, which was not found to be statistically significant (p=0.13, Fisher's exact test, 2-tailed). Of the two protected vaccinated

subjects, one had was the subject who recorded the four-fold or greater serum anti-cholera LPS responses noted earlier. The protection of the other subject could not have been predicted from the data presented above. However, both protected vaccinated subjects were those observed to have the significantly elevated serum vibriocidal antibody responses as determined by the CVD.

Despite the failure to confer a significant degree of complete protection against challenge with pathogenic *V. cholerae* organisms, the vaccine did significantly reduce the mean stool volume of the vaccinated subjects with respect to the control subjects ($p=0.0013$) (Table 14.10), with maximal protection being afforded against moderate (>2.0 litres) to moderately-severe (>3.0 litres) diarrhoea. There was no statistical difference in the incubation periods between the control or vaccinated subjects, nor was there a statistically significant reduction in the peak number of challenge organisms faecally excreted ($p=0.053$) (Table 14.10).

This study also provided the opportunity to investigate the PBL-ELISA as a predictor of protective immunity. However, the ELISA absorbances following oral vaccination with the candidate vaccine strain EX645, although significantly increased over the pre-vaccine levels, were very low. The two greatest responders in the anti-cholera LPS IgA and IgM antibody classes both developed clinical illness following challenge.

With the anti-cholera responses following vaccination being so low, and the vaccine failing to provide any degree of protection, the levels of PBL specific anti-cholera antibodies in the previously unexposed control subjects was examined following challenge with pathogenic organisms in order to

obtain some indication of the levels that might have to be achieved by subsequent vaccine candidates.

Table 14.11 presents the pre- and post-challenge PBL IgA anti-cholera Inaba LPS antibody responses, with the diarrhoeal stool volume.

TABLE 14.11 SPECIFIC IgA PERIPHERAL BLOOD LYMPHOCYTE RESPONSE IN PREVIOUSLY UNEXPOSED SUBJECTS CHALLENGED WITH PATHOGENIC *V. cholerae*

Subject	Pre-Challenge	Post-Challenge	Diarrhoeal Stool Volume (ml)
11002-1	0.000	0.007	2 054
11002-2	0.000	0.001	1 168
11002-3	0.033	0.121	889
11002-6	0.015	0.161	4 399
11002-9	0.011	0.132	1 819
11002-15	0.009	0.367	4 619
11002-17	0.002	0.146	3 405
11002-19	0.000	0.057	906
11002-20	0.012	0.072	622
11002-21	0.024	0.101	3 294
11002-22	0.044	0.153	2 634
11002-23	0.019	0.017	6 434
Mean (95% CI)	0.013* (0.004-0.022)	0.111* (0.048-0.174)	

* p=0.00053, Student's t-test

It can be seen from this table, that only 9/12 (75%) sampled subjects with clinical cholera had IgA anti-cholera PBL responses by Day 7 post-challenge that exceeded the upper limit of the 95% confidence intervals of

their pre-challenge PBL, and in only 7/12 (58.3%) did the magnitude of the post-challenge response exceed 0.100 ELISA absorbance units. The post-challenge mean specific IgA PBL response was particularly low, barely 10% of the anti-typhoid LPS IgA PBL response seen with the EX645 vaccine doses. The increased response was highly statistically significant, and also was substantially greater than that observed following vaccination with EX645.

In addition there was no obvious correlation between the magnitude of the post-challenge specific IgA PBL antibody levels (as expressed in ELISA absorbance units) and the severity of the clinical attack of cholera as determined by the diarrhoeal stool volume ($r=0.32$, $t=1.05$, $p=0.32$).

Conclusion

It was apparent from this clinical evaluation of the protective efficacy of the candidate hybrid live oral typhoid/cholera vaccine, EX645, that this strain was unable to confer a significant degree of protection upon recipients of three oral doses of this formulation. The vaccine did appear to stimulate a low level of specific anti-cholera immunity reflected in the difference in the mean diarrhoetic stool volumes between the vaccinated and unvaccinated control groups following virulent challenge. Apart from the serum vibriocidal assay, all other assays of specific immunity failed to provide a predictive measure of likely protective efficacy.

The failure of the vaccine to protect against clinical cholera most likely reflects the low cholera O antigen load presented to the small intestine as supported by the very low levels of cholera O antigen specific antibody detected.

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DISCUSSION

Vaccine development is not inexpensive; one study determining that the cost of development of a single vaccine to a marketable stage required an overall investment of US$30-50 million.[478] The requirement for such a large financial investment into what could be considered a high-risk enterprise with no likelihood of a short-term financial return upon that investment, demands that when a suitable candidate vaccine is constructed, that it can be accurately and meaningfully evaluated. The ability to accurately determine the specific immune response in human subjects has the major role in determining the continued development of a particular candidate vaccine.

The studies comprising this thesis form three components each dealing with one major area of vaccine development. The first such area concerned the aforementioned issue of the optimal timing, methods and nature of samples obtained for the determination of a specific immune response to one orally administered vaccine organism. The second area dealt with the very important issues of dosage, formulation and route of administration; once a suitable vaccine organism has been developed each of these issues must be considered, initially individually, and finally as a whole, in order that the best possible vaccine preparation can be delivered to the community that requires it, in the safest, most convenient and cost-efficient form. The final area focussed upon the product of one particular vaccine development programme which utilized one particular area of recombinant DNA technology to construct a hybrid vaccine that theoretically should have provided a reasonable degree of protection against at least two major enteric bacterial diseases, typhoid and cholera, and possibly against several others in addition.

In attempting to assess the immunogenicity of any enteric bacterial candidate vaccine, the nature of the samples obtained and the optimal timing of that sampling is often critical. The traditional approach to the evaluation of a specific immune response, has been to utilize serum samples. Serum has been preferred since it is relatively easy to acquire reasonable amounts from a vaccinated subject without causing any substantial degree of discomfort or distress, unlike the invasive processes required to obtain samples of intestinal (jejunal) fluid. However, since the humoral immune response in some individuals can decline rapidly after reaching an early peak, it was necessary to examine in detail the human humoral immune response to the successful oral typhoid vaccine *S. typhi* Ty21a, so as to determined an accurate picture of the pattern of the human immune response, enabling assessment of the immune response to occur at the time of maximal response. This vaccine strain was used since it represented the only widely used orally administrable live attenuated bacterial vaccine against an enteric disease currently available, together with the possibility that it or other attenuated *S. typhi* strains may be used as carriers of cloned heterologous protective antigens. In addition, this was the vector strain which was to be used in the development of a bivalent hybrid typhoid/cholera oral vaccine.

Following the oral vaccination of volunteer subjects with the live *S. typhi* Ty21a vaccine organisms, it was noted that the pattern of the serum and intestinal responses differed only slightly irrespective of whether a short course of three doses was used with two days between doses, or a single dose was administered, although the numbers of four-fold or greater responders were reduced. A second single dose three weeks after a first single dose, except for a brief peak in the serum IgG, did not significantly alter the pattern of the response from that of the three dose schedule at a

similar time point. The only difference observed in the immune response was in the range covered by the 95% confidence intervals, indicating the presence of low or non-responders. From these observations it was concluded that following a single dose of *S. typhi* Ty21a some individuals responded significantly better than others, who did not respond at all. One explanation for this was that the effect of three doses may simply have been to recruit more responders, by giving additional opportunities for subjects who did not respond to the earlier dose (or doses) to produce a primary response. This may increase the vaccine's "take rate", without having a significant impact on the magnitude of the resulting response. Interestingly it was a multiple dose schedule which provided the maximal degree of protection against virulent challenge in subjects previously unexposed to *S. typhi* through vaccination, disease or the environment,[220] as well as in residents of endemic regions.[227]

However, it is unlikely that the magnitude of the primary humoral immune response is simply the most reliable indicator of potential efficacy. As referred to in Section 1.1, clinical *Salmonella* gastroenteritis has been shown to result in an marked specific antibody response that peaks around two to three weeks after the onset of diarrhoea, and in the intestine can persist for up to six weeks or more - or even as long as one year with the persistant colonization of the pathogenic strain.[28] The primary pattern of specific antibody production was very similar to that observed using the attenuated typhoid vaccine in this study. An earlier study which examined the intestinal immune response to *S. typhi* Ty21a one year after receiving three doses of 10^9 viable organisms in gelatin capsules two days apart, demonstrated persistent elevated levels of specific antibody in two of three vaccinated subjects.[228] The importance of a prolonged duration of specific antibody response in protection is unclear, although it has been

demonstrated that the same gelatin capsule formulation which resulted in this elevated detectable specific antibody response in volunteers, conferred 96% protection in a field study.[222,223]

However, the relationship between the persistance of specific intestinal antibody and likely protection against reinfection has not been a consistent finding. Subjects rechallenged with homologous pathogenic *V. cholerae* three years after an experimentally induced attack of clinical cholera were protected against cliunical disease in the absence of detectable local intestinal antibodies.[33,301] Priming of the immune system with the generation of immunological memory, facilitating a rapid anamnestic response to a later challenge,[34-36] appears equally important in considering the likely protective efficacy of a particular candidate vaccine organism.

It could then be anticipated that in the evaluation of a candidate vaccine against an enteric bacterial pathogen, both the magnitude of the primary local antibody response during the Day 14 to Day 21 post-vaccination period, and the duration of persistence of that local antibody response, and the duration of any immunological memory should be considered in estimating its likely protective value.

That *S. typhi* Ty21a is an effective vaccine in conferring a substantial degree of protection against typhoid fever in endemic areas cannot be denied, although the mechanism for this protection has not been clearly determined. This vaccine strain grown in conditions for optimal LPS O antigen expression and administered in 5-8 doses of $3x10^{10}$-10^{11} freshly-harvested live organisms three to four days apart over four weeks, soon after harvesting, has been shown to have a protective efficacy of 87% against virulent challenge in previously unexposed American volunteers.[220] The same strain grown in the absence of exogenous galactose such that it did not

possess LPS O antigen was administered according to a similar regimen to previously unexposed volunteers, failed to confer a significant degree of protection upon its recipients. It was observed that there was no significant difference in the serum immune responses to the flagellar (H) antigen, the production of which being independent of the the presence or absence of galactose, between the recipients of both the "smooth" and "rough" preparations - with 51% and 60% responders accordingly.[220] This may imply a similar colonization rate for both bacterial phenotypes. Hence it would appear reasonable to ascribe anti-LPS antibodies a definite role in protection against disease.

However the factors that precluded this effective formulation from widespread use include the fact that freshly harvested doses are totally impractical for use in the field, with maintenance of the quality control in minimizing batch variation as well as maintaining an effective cold chain being major cost factors together with the necessity for multiple doses to confer maximal protection.

The cost of commercially producing the very large doses of 10^{11} live *S. typhi* Ty21a organisms is prohibitive, especially for fresh doses grown in brain-heart infusion broth, since the medium is expensive and the yield poor (approximately $2x10^9$ colony forming units (cfu)/ml in fermenters). Overnight static cultures of *S. typhi* Ty21a (grown in either brain heart infusion broth or in semi-defined media) yield only $5x10^8$ cfu/ml, therefore one dose represents approximately 200 ml of media. Fermenter grown cultures using a semi-defined medium have better yields ($1x10^{10}$ cfu/ml), however one dose of 10^{11} *S. typhi* Ty21a still requires approximately 10 ml of culture medium (Beyer L. unpublished observations).

While *S. typhi* Ty21a may well be markedly attenuated, it still is capable of stimulating a far greater intestinal specific IgA antibody response than an equivalent oral dose of killed organisms.

However the evidence obtained from the studies detailed in Section 9.2 support the proposition that *S. typhi* Ty21a is not able to obtain sufficient exogenous galactose in vivo to synthesize a complete O polysaccharide, a finding supported by the retention of virulence by a *S. typhi* oral vaccine candidate strain which was able to synthesize complete O polysaccharide as well as accumulate sufficient galactose to spontaneously osmotically lyse, *in vitro*.[233] This finding is reflected in the consistently poor anti-O polysaccharide immune response observed in the intestinal fluid, sera and PBL of subjects receiving *S. typhi* Ty21a organisms grown in the complete absence of exogenous galactose and so are devoid of O polysaccharide when administered (rough strains). So it appears that for *S. typhi* Ty21a to retain its efficacy as an oral vaccine it must be grown *in vitro* in the presence of a limited amount of exogenous galactose and administered as a smooth preparation. This observation may also be important, especially in regards to the use of *galE* mutants of *S. typhi* as carriers of heterologous protective antigens. This was implied with the EX210 hybrid *S. typhi* Ty21a/*V. cholerae* oral vaccine organism, where the regulation of the exogenous galactose concentration during *in vitro* growth permitted optimal amounts of both *S. typhi* and *V. cholerae* O-antigen expression to occur (Sections 12.2.2, 12.2.3, 12.2.4).[396]

The effective delivery of a live oral vaccine in the field requires that it be available for distribution in a convenient and stable form. The first step in achieving this is to lyophilize the vaccine doses. The possibility that the process of lyophilization may adversely affect the immunogenicity and

protective efficacy of a live bacterial vaccine has been raised following the significant reduction in the ability of an attenuated streptomycin-dependent *S. typhi* typhoid vaccine to stimulate a significant local immune response, or to subsequently confer protection on subjects orally vaccinated with it, when compared to an equivalent freshly harvested doses.[215] In the study reported in Section 9.2, it was observed that following lyophilization, *S. typhi* Ty21a, although only retaining approximately 50% viability after lyophilization, demonstrated no impairment in its ability to generate significant intestinal antibody responses. There was no identifiable difference in the pattern or magnitude of the response produced compared with that of the freshly prepared doses containing 10^{11} viable organisms. In addition, it appeared unlikely that the presence of any killed vaccine organisms may have significantly contributed to the immune responses observed following vaccination with the lyophilized doses, as the immune responses observed following the administration of 10^{11} formalin-killed organisms did not achieve statistical significance. The retention of the immunogenicity of *S. typhi* Ty21a after lyophilization may reflect a retention in protective efficacy, and others have suggested that this may be highly dependent on the nature of the lyophilization support medium.[479,480,481]

Studies conducted in endemic typhoid fever areas have demonstrated the conferring of adequate protection on the vaccinated population with doses as little as 10^9 live organisms.[222,223,226] However there have been reports that this dose may be of questionable value in travellers from non-epidemic regions,[224] although in this instance a different formulation was used. It was observed in the studies reported in this thesis, that three sequential doses of 10^9 live *S. typhi* Ty21a failed to stimulate a measurable local intestinal immune response in previously unexposed

volunteer subjects. This confirmed other reports of the failure of this dose to effectively stimulate a local antibody response in individuals from non-endemic regions.[228,229] It is considered unlikely that this inability to detect a specific immune response at this dose reflects any limitations of the assay for intestinal specific antibody, as a specific antibody response was also not identifiable at this dose using subjects' PBL, a far more sensitive measure of immunogenicity. This suggests that the meagre immune response observed with the commercially available enteric-coated formulation is most likely attributable to the $5x10^{10}$ killed organisms present than to the $1.1x10^{9}$ viable ones. If local antibody production is used as an indicator of vaccine organism colonization of the gut lymphoid tissue, by combining the evidence that frequent doses of 10^{11} live organisms were only capable of providing 87% protection in previously unexposed subjects,[220] and the work reported here, it could be implied that the minimum effective dose of *S. typhi* Ty21a for residents of non-endemic areas may be more in the order of 10^{10} live vaccine organisms, than the currently available 10^{9} live organisms (with the accompanying $6x10^{10}$ killed ones). Therefore any candidate vaccine using *S. typhi* Ty21a as a vector for heterologous protective antigens would require a minimum dose in the order of 10^{10} viable vaccine organisms.

The intestinal antibody response as determined using this human model system did appear to provide a useful baseline for assessing any future oral typhoid vaccine preparation. This is supported by the fact that *S. typhi* Ty21a administered orally as either killed vaccine, or as vaccine doses of 10^{9} live organisms, or as the enteric coated capsules containing a mixture of 10^{9} live and $5x10^{10}$ killed organisms did not stimulate any greater intestinal IgA specific antibody responses than the commercial heat-phenol-inactivated parenterally administered vaccine.

Although the enteric coated preparation of *S. typhi* Ty21a ("Typh-vax (Oral)") has been shown to be as effective and to have far fewer adverse reactions than the single dose of the heat-phenol-inactivated parenterally administered vaccine,[226] in requiring three doses to be as effective it could be argued that it becomes far less convenient or practical in the field, than the heat-phenol-inactivated typhoid vaccine which can confer 70% protection following a single vaccination dose,[172] or the single dose purified Vi antigen vaccine,[185] despite the frequently accompanying adverse reactions, some of which on occasions can be quite severe. In fact the annual administration of a single dose of the heat-phenol-inactivated vaccine has been shown to be highly effective in reducing typhoid fever incidence rates in children in Bangkok, Thailand.[182]

While the studies in this thesis confirm the inadequacy of single-point determinations of antigen-specific serum antibody, irrespective of class,[14] for the accurate determination of an intestinal antibody response and while the sequential determination of a groups' response within an antibody class has been determined to be of most value, it is often the least practicable. If a point determination of serum antibody response is the only method available for the evaluation of the immunogenicity of a particular preparation, then this study suggests that the magnitude of the serum IgG response may provide the most consistent and sensitive indicator, as significant antibody responses were evident in this class when absent in or not significant in others (for example: Group B, $t=3.23$, $p=0.048$; Group H, $t=2.85$, $p=0.036$) (Table 3), although the magnitude of the response may not correlate with that of the intestine. This finding has been recently supported in field trials, where the serum IgG anti-LPS O antigen antibody responses were observed to roughly correlate with vaccine efficacy.[482]

These studies of the humoral immune response to typhoid vaccines, suggest that the measurement of the humoral immune response to an enteric vaccine may well provide useful information with respect to its protective efficacy, and that previous failures to detect a specific humoral immune response may simply reflect factors such as inadequate dosage, sub-optimal timing or techniques of sampling, inadequate processing of samples or insensitive assay systems.[28,30,32]

In underdeveloped regions, diarrhoeal disease caused by bacterial or viral pathogens has been reported to occur most frequently in children less than two years of age.[461] One potential realistic intervention is vaccination. It is now generally accepted that the optimal method of immunization against enteric pathogens is through the stimulation of a local mucosal (intestinal) immune response by using the oral route of administration. It is the concept of a common mucosal immune system that provides an explanation for the detection of secretory antibody against orally administered antigens at mucosae other than that of the gastrointestinal tract, such as in colostrum, tears and saliva.[94-96] Not only does this system permit the generation of a distant immune response to mucosally presented antigens but it also permits microbial agents to access the systemic immune system and be circulated.

As mentioned previously, the oral route is associated with several difficulties: such as the problem of neutralizing the sterilizing effects of gastric acid; and the virtually unconsidered problem of administration of oral vaccine formulations to infants or very young children who have the most to benefit from vaccination. One approach used for the administration of drugs and other pharmaceuticals to this age group has been through the utilization of the rectal route.

The colon has been previously shown to play a role in the generation of mucosal and systemic immune responses to a locally presented microbial agent.[464] Ogra and Karzon were able to demonstrate in children with transverse colostomies that following the colonic administration of live poliovirus (Sabin) vaccine a systemic immune response was evident, especially noticeable in serum where the response was observed to be mainly in the IgG antibody class. They were able to demonstrate a weak IgA response locally in the proximal colonic segment and a better response in the distal colonic segment. This is not surprising in view of the large numbers of lymphoglandular complexes each with overlying M cells present. However, following the administration of the Sabin vaccine into the distal colonic segment they were unable to demonstrate a distant mucosal secretory immune response.[464]

In previous studies the magnitude and kinetics of the immune response to orally administered live *S. typhi* Ty21a has been determined (Sections 8, 9.1 and 9.2).[14,228] Significant anti-typhoid IgA antibody responses could be identified in all subjects' post-vaccination peripheral blood lymphocytes but of a diminished magnitude in comparison to using the same dose by the oral route. In addition distant mucosal anti-typhoid IgA responses in jejunal fluid and saliva were also demonstrated, and, like that which was observed in the PBL, the magnitude of the jejunal response was of a much diminished magnitude. Interestingly the salivary antibody response achieved statistical significance, something which in my studies was as uncommon finding when utilizing the oral route. However considering the low numbers of subjects used, and the borderline nature of that response, its real relevance could only be determined using much greater numbers of subjects.

This study has other wider implications with respect to the access of the human immune system by microorganisms utilizing the rectal route, for example the HIV-1 virus family, confirming that trauma is not an absolute prerequisite for the access of the immune system through the rectum. Most importantly, this study indicated that the rectal route may provide a satisfactory alternative for the administration of live attenuated enteric bacterial vaccines other than the oral route for infants and very young children, as it is possible to generate a significant specific small intestinal immune response using this route.

The establishment of the human model for the evaluation of enteric bacterial vaccines and the optimizing of the sampling of relevant fluids and secretions, permitted the accurate evaluation of candidate vaccine strains intended to provide bivalent protection against typhoid and cholera.

However, while the vaccine development programme was deliberately attempting to construct a bivalent vaccine, it may be possible that *S. typhi* Ty21a alone could confer protection upon an individual against more than one infectious cause of enteric disease. Since all *Salmonellae* appear to be members of a single species, and in many instances share common antigenic components as a result, it is possible that immunization with *S. typhi* Ty21a may provide a degree of protection against other serious common conditions caused by other *Salmonellae*. The human specific antibody response to a range of bacterial enteric pathogens has been examined in the past,[16,28,38,39] with the consistent finding that specific antibody affords effective protection against invasive and non-invasive pathogenic bacteria.[40,42,43] In a variety of animal experiments, it has been determined that specific antibody directed against the O polysaccharide component of LPS is sufficient alone, to provide substantial protection

against homologous challenge.[127,483] However, it has also been determined that specific antibodies directed against the oligosaccharide of the core LPS are also capable of providing protection against homologous and heterologous challenge in some, but not all instances.[452,453] *S. typhi* Ty21a is a *galE* mutant of the "wild type" *S. typhi* Ty2 - the aetiological agent of typhoid fever. Although in typhoid fever, cell mediated immunity may make a significant contribution to providing protection, specific local antibody does seem to be important in the provision of protection against reinfection with an homologous enteric pathogen, this may be especially true in enteric disease caused by non-invasive organisms. In convalescent subjects in which high levels of specific antibody could be measured in intestinal fluid following experimental cholera infection, an illness where it is considered that little intestinal mucosal invasion if any occurs by the pathogen, were afforded solid protection against rechallenge six weeks later.[301] Human infection with non-typhoid *Salmonellae* usually results in a non-invasive gastroenteritis in immunocompetent individuals, with a septicaemic illness being uncommon. Therefore it would appear likely that cross-reacting O antigen antibodies stimulated by oral vaccination with *S. typhi* Ty21a, may provide a degree of protection against other members of the species sharing common or similar O antigens, such as *S. enteritidis*, *S. dublin* and *S. typhimurium*, a finding supported by recent animal experiments.[447] The findings of this investigation confirmed that most jejunal specific antibody produced is directed against the O polysaccharide antigens. However, antibodies against the core oligosaccharide of LPS but not the Lipid A component, were also evident following oral vaccination with *S. typhi* Ty21a in this study, but were present in only low amounts. While anti-core LPS antibodies have been demonstrated to stimulate a degree of protective

immunity in some animal models of infection,[453] their role in protection against human enteric infection is dubious. In one study where human subjects received three large doses of *S. typhi* Ty21a grown in the absence of exogenous galactose and therefore not elaborating any O antigen (a "rough" organism), were not protected against challenge with virulent *S. typhi* organisms.[220] Apart from a suggestion of a response to the *E. coli* core LPS used in this study, there was no convincing evidence of cross-reaction with any of the other LPS types of other non-*Salmonella* members of the Enterobacteriaceae.

Significant cross-specificity of the IgA response with the LPS preparations of other Kauffmann-White serological Groups B and D_1 organisms following oral immunization with *S. typhi* Ty21a was observed. Since salmonellosis accounts for more than 80% of the worldwide incidence of enteric infection and that the responsible organisms are the usually of the *S. typhimurium* and *S. enteritidis* serovars,[484] one unexpected benefit of extensive vaccination campaigns with *S. typhi* Ty21a may be the overall reduction in clinical illness resulting from infection with these organisms.

As described in Section 3.2, cholera remains a problem of major public health importance in developing countries, with more countries than ever before recording its presence. Yet, clinical cholera evokes good protective immunity that has been shown to confer resistance to rechallenge for at least three years,[301] and serum antibodies directed against cholera antigens can be found following cholera infection; but since *V. cholerae* is non-invasive - remaining confined to the lumen and mucosal epithelium of the small intestine - these circulating antibodies probably play a minor role in protective immunity.[306] It is now generally accepted that local intestinal immunity to cholera is important in preventing clinical disease. Significantly

elevated anti-cholera LPS IgA antibodies have been demonstrated in the gut following clinical cholera, and similar responses have been detected following the oral administration of a combined killed whole-cell/B toxin subunit.[304,330] This vaccine, both with and without the cholera toxin B subunit has been subsequently shown to be protective in challenged human volunteers.[305]

S. typhi Ty21a has been examined as a potential vector for the carriage of "protective" antigens of other enteric pathogens.[393,397,398] This thesis describes for the first time the effectiveness of *S. typhi* Ty21a as a carrier of a defined protective antigen for *V. cholerae*, the O polysaccharide antigen. This candidate vaccine demonstrated its ability to stimulate significant immune responses to both typhoid and cholera O antigens, whether measured by *in vitro* release of antibody by circulating PBL, or by ELISA or bactericidal assays of serum and jejunal specific antibody. The specific anti-cholera antibody response by PBL is indicative of antigen uptake into the Peyer's patches, and it has been shown that antigen-specific PBL antibody response can correlate strongly with the intestinal immune response following oral vaccination.[14]

However, as detailed earlier, freshly harvested preparations are unsuitable for field evaluation. In addition, since the immunological expression of the cholera O antigen on the *S. typhi* Ty21a strain is heavily dependent upon the growth conditions, it was necessary to only use in the Phase 2 (challenge) study a stable form of the vaccine of known immunogenicity, rather than risk slight alterations in production in the USA that may dramatically have altered the immunogenicity of the vaccine strain. However, it was obvious that the viability of the hybrid vaccine strain, EX645, was markedly adversely affected by the lyophilization process. In the

last Phase 1 study (Section 13.1) the number of viable vaccine organisms upon reconstitution was found to be only 11%, in comparison to the 63% viability found in the fresh vaccine preparation used in Section 13.2.[396] Plasmid retention was also quite low, only 50% of the viable reconstituted organisms retained the plasmid, where in the fresh doses 92% retention was obtained. As a result the number of viable vaccine organisms in the reconstituted lyophilized vaccine doses that were capable of producing cholera O-antigen was less than 10% of that found in the freshly harvested doses (lyophilized 5.5%, fresh 58%).

Despite this handicap, EX645 again demonstrated its ability to stimulate significant immune responses against both typhoid and cholera O antigens. In fact, it appeared that the reduced viability had at worst only a minimal effect on the anti-typhoid immune responses, while the anti-cholera responses in peripheral blood lymphocytes, and serum still achieved statistical significance.

Disappointingly, this study failed to demonstrate a consistent anti-cholera IgA antibody response in intestinal fluid. However, the assays for specific antibody were only able to detect an intestinal antibody response in 75% of the serum or PBL anti-typhoid responders following vaccination with EX645. This implied that either the technique of intestinal intubation may not provide satisfactory samples for assaying, hence proving to be an unreliable measure of an intestinal immune response, or confirms the previous observation that the PBL-ELISA may not provide an indication of the degree off local immunity but indicates the uptake of an orally presented antigen into the Peyer's patches. In addition, it appeared that this preparation was unlikely to provide a degree of protection any better than that of afforded by the orally administered killed whole-cell vaccine preparation, which was

previously shown to confer 56% protection on subjects against virulent challenge.[302] Like the killed WCV, EX645 failed to induce sgnificant intestinal specific anti-cholera LPS antibody responses, and its serum responses were lower in magnitude than those induced by the WCV. Therefore it could have ben predicted that EX645 may in fact have conferred a lower degree of protective immuntiy on recipients than the killed WCV.

Therefore the Phase 2 (challenge) study was performed with the bulk of the evidence suggesting that the candidate vaccine strain EX645 would provide a degree of protection at best comparable to that afforded by the killed whole-cell cholera vaccine without the B subunit of cholera toxin, about 50%-60%.

The final laboratory investigation involving EX645 before the assessment of its protective efficacy, was the examination of the pattern of faecal excretion which needed to be determined to assess the potential for the strain to be an environmental hazard. This needed to be evaluated prior to release of this candidate vaccine strain. It was conclusively shown that while all recipients did excrete viable vaccine organisms in their faeces, isolation rates rapidly declined over the 24 hour period following a vaccine dose. In only two subjects was it still possible to detect EX645 in their faecal specimens, and these isolates were at only very low levels, in fact, on the limit of detection. From this study, it is evident that EX645 does not represent a significant environmental hazard.

Therefore, the failure of EX645 to confer a significant degree of protection against clinical cholera on previously unexposed volunteer subjects, was predictable from the Phase 1 studies' data.

Some of the reasons for the failure of the vaccine strain EX645 in the protective efficacy evaluation studies have already been alluded to:

(a) poor production of immunologically accessible *V. cholerae* Inaba O antigen;

(b) poor plasmid retention in freshly prepared vaccine organisms despite growth in selective media; and

(c) poor plasmid retention and viability following lyophilization.

There certainly was O antigen available to stimulate specific humoral immune responses but due to the low quantities, multiple large doses were required to induce significant levels specific of local and serum antibodies (Section 12.2.5).

Despite this failure of protection against clinical illness, the vaccine did have an observable effect, as measured by the reduction in diarrhoetic stool frequency and volume in the vaccinated subjects in comparison to that of the unvaccinated control subjects. However, this effect could not in any way be regarded as a substantial success, although it did indicate that this approach to vaccine development may be possible with strain improvement through further developments in recombinant DNA and fermentation/lyophilization technologies.

The major contribution made by the work in this thesis was the description of the development of a particularly sensitive ELISA technique which permits the rapid determination of a specific immune response directed against an orally administered antigen. This assay, referred to as the PBL-ELISA was confirmed as a highly sensitive, correlative indicator of an individual's primary intestinal exposure to an enteric bacterial organism. However, this assay may have proved to be too sensitive, as it was found not

to be a particularly useful discriminatory measure of the probable effectiveness of a particular vaccine formulation when differing *S. typhi* Ty21a doses and preparations were evaluated using it, although it certainly is techincally far less denmanding upon the subject than intestinal intubation. As a predictive measure of protective immunity against *V. cholerae* O1 infections, it was also a disappointment, in that following infection with one *V. cholerae* O1 strain that induced diarrhoea in 100% of challenged subjects, only 75% developed detectable specific PBL in their peripheral blood that secreted *in vitro* specific anti-cholera LPS IgA antibodies. This failure could be attributable to either to inappropriate timing of the sampling of whole blood for the PBL (the study was restricted to a single post-challenge sample obtained upon Day 6 post-challenge), or that although *V. cholerae* O1 is taken up into the Peyer's patches by epithelial M cells of the small intestine,[65] possibly inadequate numbers of viable organisms do so to stimulate a response detectable using this assay system. These possibilities are not mutually exclusive, the former is supported by the variation in the peak PBL response observed in Sections 8, 9.1 and 9.2, and the latter through the understanding of the pathogenesis of clinical cholera - a toxin-mediated disease with only limited numbers of the cholera bacterium actually adhering to the small intestinal epithelial cell surface hence reducing the opportunities for transport to the underlying Peyer's patch. The latter point in itself, though, is insufficient to explain the observation, since enough cholera bacteria must be taken up into the Peyer's patch to result in the normally good level of intestinal (jejunal) and serum specific immune responses observed, suggesting that the single day timing of the PBL sampling was inadequate.

Therefore the assertion that the PBL-ELISA is a highly sensitive indicator of antigen uptake into Peyer's patches, than as an indicator of likely protective immunity could be supported by this interpretation of the data, however this may only represent the biased view represented through a single method of data analysis, and that other factors such as the duration of detectable PBL response in addition to its magnitude may also be important in the determination of the relationship between the specific *in vitro* PBL ELISA responses and the likely protective efficacay of a particular vaccine preparation.

The interpretation of the data from this type of immunassay, utilizing isolated PBL, must continue if it is to have a meaningful role in the evaluation of candidate oral bacterial vaccines against enteric disease.

Antityphoid inoculation has also, in common with all measures designed to protect the public health, been bitterly opposed by agitators who, when faced by a fancied infringement of personal rights, are blind to all calm inspection of evidence.

Frederick P. Gay [485]

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APPENDIX

IDENTIFICATION OF AN INTESTINAL IMMUNE RESPONSE USING PERIPHERAL BLOOD LYMPHOCYTES

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Summary The intestinal immune response of volunteers given the oral vaccine *Salmonella typhi* Ty21a was assessed with a new immunoassay which measures specific antibody secretion by peripheral blood lymphocytes (PBL). Peak IgA, IgG, and IgM production by PBL occurred on day 7 after the start of vaccination. Peak antibody secretion by PBL occurred several days earlier than the serum antibody peak. All volunteers showed a specific PBL antibody response, but serum or intestinal immune responses, when demonstrable, were highly variable. Peak PBL IgA antibody response correlated with degree of rise in IgA antibody in serum ($p = 0{\cdot}00098$) and the intestinal fluid ($p = 0{\cdot}0024$). The assay is a useful means of measuring humoral immune response at a mucosal surface after local administration of antigen.

Introduction

THE effectiveness of orally administered enteric vaccines is difficult to assess because serum responses do not necessarily reflect intestinal immune responses. Thus, to measure immune response to oral vaccines repeated intestinal intubations have been necessary.[1-5]

Bacterial infections of the gastrointestinal tract and oral vaccines normally produce a secretory immune response (usually IgA) in the intestine.[2,3] This response may be accompanied by the secretion of specific IgA by other distant mucosal surfaces,[6] but so far corresponding specific antibodies have not been detected in serum.[2,4,7] Here we describe a simple assay for measuring the immunological priming of the intestinal immune system.

Subjects and Methods

12 healthy volunteers (2 women and 10 men, 19–45 years of age) gave written informed consent for this study, which conformed to the ethical standards of the Human Ethics Committee of the Royal Adelaide Hospital and with the Helsinki Declaration of 1975. None of the volunteers had had contact with typhoid fever or been immunised against the disease.

The organism used was *Salmonella typhi* Ty21a. Each vaccine dose contained 10^{11} freshly harvested live organisms suspended in 10 ml of 0·9% sodium chloride solution. The vaccine was supplied by Dr G. Boehm, Enterovax Research Pty Ltd, Adelaide, South Australia.

After an 8 h fast, all volunteers drank 50 ml of a 2% sodium bicarbonate solution. After 5 min they took the vaccine in 40 ml of 0·9% saline, followed by 100 ml of distilled water. The three doses were taken on days 0, 2, and 4.

30 ml samples of blood were collected before and every 3–4 days after vaccination for up to 42 days. Intestinal aspirates were obtained from the upper jejunum with an 'ANPRO AN 20 Andersen' tungsten weighted sump tube (H. W. Andersen Products, Oyster Bay, New York, USA) 1 week before vaccination and again on day 21. Correct positioning of the tube was confirmed by fluoroscopy.

Intestinal fluid samples with a pH > 6·8 were collected and kept on ice until 25–30 ml had been collected from each volunteer. The samples were then centrifuged at 4000 *g* at 4°C and stored at −70°C until required.

Lymphocytes were obtained by centrifugation of heparinised venous blood on 'Ficoll-Paque' (Pharmacia, Uppsala, Sweden).[8] Isolated cells were washed thrice in Dulbecco's buffered salts solution and resuspended, to a concentration of 10^7 PBL/ml, in RPMI 1640 culture medium (Flow Laboratories, North Ryde, New South Wales) supplemented with 10% heat inactivated fetal calf serum, 2 mmol/l L-glutamine, and antibiotics.

Measurement of Antibody Production by PBL

An enzyme-linked immunosorbent assay (ELISA) was developed for quantifying the amount of specific antibody produced by circulating peripheral blood lymphocytes (PBL) during incubation in vitro. For this assay, 96-well polyvinyl microtitre plates (Costar, Data Packaging Corp, Cambridge, Massachusetts, catalogue no 2595) were coated with 5 µg/ml solution of *S typhi* Ty2 LPS (Sigma catalogue no L 6386) that had been linked to methylated bovine serum albumin (BSA),[9] in carbonate-bicarbonate coating buffer pH 9·6 (100 µl/well), overnight at 4°C. 100 µl of a 0·05% BSA in phosphate buffered saline (PBS) solution was added to each of the wells and left for 45 min at room temperature before being washed in PBS-'Tween 20' (Sigma Chemical Co, St Louis, Missouri, catalogue no P–1379). 10^6 PBL (100 µl) in supplemented RPMI 1640 culture medium were added to replicate wells of the plates. The plates were incubated for 16 h at 37°C in a humid atmosphere, before the cells were washed out. Addition of the alkaline-phosphatase conjugated goat antihuman IgA, IgG, or IgM heavy chain specific antiserum (Kirkegaard and Perry Laboratories, Gaithersburg, Maryland) and subsequent washing were as described elsewhere.[1] The substrate used was a 1 mg/ml solution of *p*-nitrophenyl phosphate (Sigma phosphatase substrate no 104–105) in diethanolamine buffer, 100 µl of which was added to all of the wells. The plates were then incubated at 37°C for 4 h. Following this incubation all plates were read with a 'Titertek' ELISA reader model 310C (Flow Laboratories) at 405 nm. All plates were blanked on antigen-coated wells containing residual, non-specifically-bound enzyme-conjugated secondary antibody and the substrate solution.

The specific IgA antibody response by the PBL of 3 of the volunteers exceeded the upper limit of detection of the ELISA reader (> 2·00 absorbance units). In these cases the contents of the replicate wells were pipetted and read with a conventional spectrophotometer.

In each assay, high-titre serum obtained from a convalescent typhoid patient was included as a positive control, and PBL

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TIMING AND MAGNITUDE OF PEAK PBL AND SERUM SPECIFIC ANTIBODY RESPONSES AFTER ORAL VACCINATION WITH SALMONELLA TYPHI TY21A

—	IgA	IgG	IgM
PBL			
Peak (days)	6·2 (5·5–6·8)	6·8 (5·7–8·0)	7·8 (6·1–9·4)
Mean peak OD	1 73 (0·88–2·57)	0·71 (0·28–1 14)	0·65 (0·36–0·93)
Serum			
Peak (days)	13·2 (10 7–15·7)	17·1 (13·6–20·6)	16·8 (14 2–19·4)
Mean peak titre*	2900 (1100–7900)	3350 (1250–9000)	2400 (1300–4500)
Mean pre titre*	140 (105–180)	380 (190–780)	600 (380–940)

Numbers in parentheses refer to 95% CI.
*Geometric mean.
OD = optical density.

suspensions obtained from at least 3 non-immunised normal volunteers on the same occasion as the vaccinees were included as negative controls.

Measurement of Antibody Concentrations in Intestinal Fluid and Serum

Specific antibody was measured in intestinal fluid and serum with an ELISA described by Bartholomeusz et al[1] except that the antigen used to coat the wells of the plates was the same as described in the PBL assay above.

A single radial immunodiffusion method[10] was used to determine the total class-specific immunoglobulin content of intestinal fluid.

The specificity of the antibody responses detected were determined by applying the described assays to the samples, except that the plates had been either coated with *Vibrio cholera* 569B LPS (Sigma catalogue no L 5262) or *Escherichia coli* O55:B5 LPS (Difco, Detroit, Michigan, catalogue no 3120-25-0) that had been linked to methylated BSA.[9]

Results

In all 12 volunteers who ingested the *Salmonella typhi* Ty21a, the PBL showed an anti-typhoid LPS IgA antibody response (optical density >0·100 units/10^6 PBL), with the mean optical density peak of 1·73 units (95% CI 0·88–2·57 units) occurring at a mean of 6·2 days (95% CI 5·5–6·8 days) after the first dose of the vaccine (table, fig 1). In all but 1 volunteer, the response was measurable by 4 days. IgA antibodies to typhoid LPS were not detected before

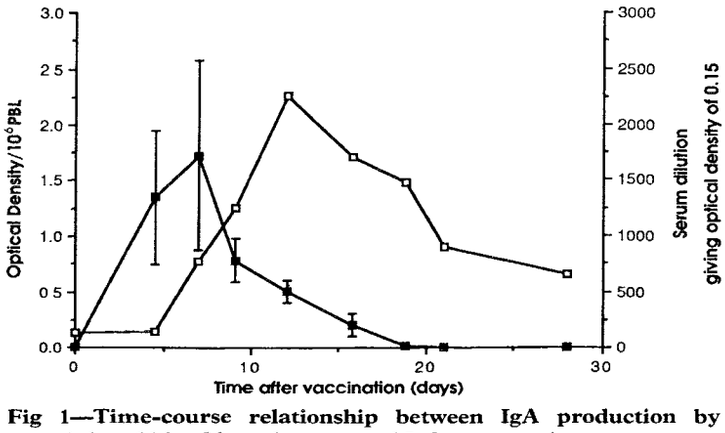

Fig 1—Time-course relationship between IgA production by peripheral blood lymphocytes and IgA response in serum.

PBL response (■) given as arithmetic mean optical density (absorbance unit) + 95% CI.

Serum response (□) given as geometric mean of the serum dilution giving an optical density of 0·15 absorbance units.

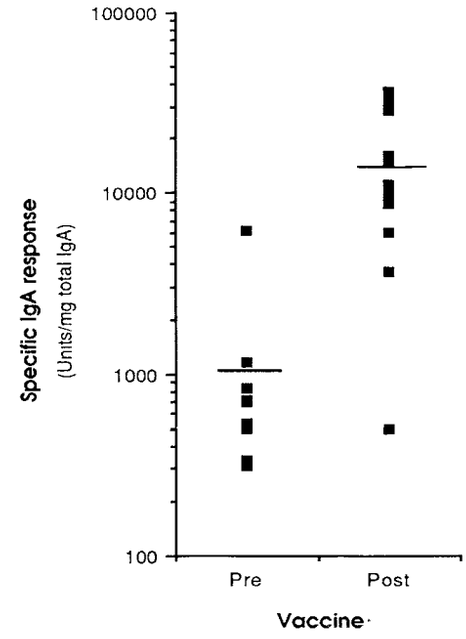

Fig 2—Specific intestinal IgA response in volunteers after oral vaccination with *Salmonella typhi* Ty21a.

Horizontal bar indicates arithmetic mean value.

vaccination or later than 18 days after vaccination. Typhoid specific antibody was not detected in any of the negative controls.

The organism specificity of the antibody response was demonstrated by the absence of an antibody response to either cholera or *E coli* in any of the PBL assayed in wells coated with either *Vibrio cholerae* 569B LPS or *E coli* O55:B5 LPS.

IgM (11/12 responders) and IgG (10/12 responders) antibody classes were also detected on day 7, but the responses were smaller than those for IgA.

Incubation of the PBL in a 75 μg/ml solution of cycloheximide reduced the production of IgA by 65–94%, IgG by 75–88%, and IgM by 68–86%; these observations indicate that the PBL were the source of the specific antibody.

Typhoid-specific serum IgA antibodies were measurable in 10/12 volunteers and they showed a greater than fourfold rise in geometric mean titre: pre-vaccination geometric mean titre 137 units (95% CI 105–180), post-vaccination 2900 units (95% CI 1100–7900 units) (by Student's paired *t* test after ln(x) transformation of titres, $t = 7{\cdot}634$, $df = 11$, $p < 0{\cdot}001$). The mean peak serum IgA response occurred at 13·2 days (95% CI 10·7–15·7).

After correction of intestinal antibody titres for total class-specific immunoglobulin, 9 of the 12 volunteers had a fourfold or greater rise in typhoid LPS specific IgA antibody response (fig 2): pre-vaccination mean 1020 units (95% CI 0–2050), post-vaccination mean 14 800 units (95% CI 7500–22 100) (by Student's paired *t* test, $t = 4{\cdot}029$ $p = 0{\cdot}002$).

There was a significant relation (as determined using Wilcoxon rank sum test) between the magnitude of the PBL IgA antibody response and the degree of rise in specific IgA in both intestinal fluid ($p = 0{\cdot}0024$) and serum ($p = 0{\cdot}00098$).

Specific IgA antibody production was detected in the PBL obtained from all vaccinated volunteers but serum or intestinal immune responses were not always detected.

Discussion

The most reliable method of determining intestinal immune response is by examination of the intestinal fluid,[2,3] but intestinal intubation is time-consuming and inconvenient for the patient, and the results are variable because of uncontrollable factors.[3] The use of other body fluids, such as serum and saliva, for assessing intestinal immunity indirectly has not been a universal success. Despite claims by some, serum antibody determination has been of limited value because significant changes in specific antibody titres do not always follow infection.[2,3,11]

To overcome some of the problems associated with the determination of an intestinal immune response to an orally administered antigen, some investigators re-examined haemolytic plaque-forming assays, which can be used to quantify the circulating immunoglobulin-producing lymphocytes and are useful as an indirect means of measuring immune response at mucosal surfaces that are difficult to gain access to.[12-14] As a result of this, the 'Elispot' assay[15,16] and the ELISA plaque-forming assay[17,18] were developed. Application of these two assays in two independent trials of oral vaccines[16-19] showed that the numbers of antigen-specific lymphocytes that entered the peripheral blood after oral vaccination reached a peak between days 6 and 10 inclusive.

This paper describes a new enzyme-linked immunosorbent assay (ELISA) which determines the amount of specific antibody of any class secreted by circulating PBL after oral vaccination with a live vaccine. In their description of the Elispot assay Czerkinsky et al[15] also referred to its possible modification to an ELISA. Our assay is more convenient than the Elispot or ELISA-plaque assays because the PBL are incubated in antigen-coated round-bottomed polyvinyl microtitre plates, so there is no need to pipette solutions from petri-dishes and to add beads to the culture medium; there is also no need for laborious counting of spots in agarose. The necessity of having to pipette solutions from the replicate wells of 3 of the vaccinees in this study whose specific IgA antibody response by their PBL exceeded the detectability of the ELISA reader could have been avoided by reading the plates sooner or by using a lower concentration of PBL.

Salmonella typhi Ty21a is ideal as a model for the examination of the intestinal immune response since its infection is self-limiting. An intestinal immune response has been demonstrated in volunteers receiving this organism in three doses of 10^{11} live organisms.[1] In this study, volunteers receiving three such doses of the vaccine gave a peak PBL antibody response between days 4 and 7 after the start of vaccination. This was significantly earlier than the mean peak serum response. All the volunteers in whom an intestinal or serum antibody response could be measured also demonstrated specific antibody secretion by their PBL in vitro.

IgA proved to be more reliable than IgG or IgM as a measure of the immune response since the PBL from all vaccinees produced a substantial specific IgA response, whereas not all of the vaccinees had a detectable IgG or IgM response. Also, IgG and IgM responses were weaker than the IgA responses.

The assay described in this paper should prove useful for the rapid assessment of immunological priming of the intestine of individuals receiving oral vaccines. It may be of most use in field studies where the impracticality of intestinal tubes precludes their use. It may also be of use in examining the intestinal immune response to other orally ingested antigens of clinical significance.

I thank Dr J. T. LaBrooy, Prof D. J. C. Shearman, and Prof D. Rowley for their supervision and their guidance in the preparation of this manuscript; Mrs W. Ferguson and Dr R. Pridham for their assistance with the intestinal intubations; and Miss L. Mundy for technical assistance. Enterovax Research Pty Ltd, Adelaide, South Australia funded the study. B. F. is the recipient of an Enterovax Postgraduate Research Scholarship.

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"A pawnbroker has only three balls to deal with, and they are static; but a professor of medicine has to juggle with four—practice, teaching, research, and administration—and they are constantly changing. In addition, a professorial chair seems to act as some kind of magnet for national commitments of various kinds. Academic theory dictates that in the interests of academic mobility every effort should be made to attract a candidate from the ends of the earth, preferably bringing with him a new discipline. Almost always in these hard times such a theory is overset by practicality, since few university departments can now afford a complete change of emphasis and the consequent replacement of equipment . . .

Leaving aside any consideration of personality, I actually do believe that a professor appointed from within the same hospital has got certain advantages to set against those which might attend the bringing in of new blood. Having worked in the same hospital for some years, he knows which of his colleagues are both helpful and appropriately knowledgeable; and, just as important, he knows the minority who are deficient in either one of those attributes. To pursue what may be a rationalisation a little further, it may be possible for an internally appointed professor to accept national commitments somewhat earlier than would be wise for someone who had to learn the ways of an unfamiliar hospital, and also build up a department virtually from scratch."—SIR DOUGLAS BLACK. Recollections and Reflections. Published by British Medical Journal, 1987: 9.