CONSTRUCTION AND CHARACTERISATION OF *Escherichia coli* HEAT-LABILE TOXIN B-SUBUNIT FUSION PROTEINS

A thesis by

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ABBREVIATIONS

APC	- antigen presenting cell
ASC	- antibody secreting cell
BALT	- bronchus-associated lymphoid tissue
BALU	- bronchus-associated lymphoid unit
BSA	- bovine serum albumin
CFA	- complete Freund's adjuvant
CT	- cholera toxin
CT-B	- cholera toxin B-subunit
EDTA	- ethylene diaminotetra-acetic acid
ELISA	- enzyme-linked immunosorbent assay
GALT	- gut-associated lymphoid tissue
G_{M1}	- monosialoganglioside
HRV2	- human rhinovirus type 2
Ig	- immunoglobulin
LT	- *Escherichia coli* heat-labile enterotoxin
LT-B	- *Escherichia coli* heat-labile enterotoxin B-subunit
µg	- microgram
µl	- microlitre
mg	- milligram
ml	- millilitre
mM	- millimolar
P.69	- P.69 pertactin, a membrane-associated protein from *B. pertussis*
p110	- peptide representing residues 147-161 from influenza A virus nucleoprotein
p114	- peptide representing residues 364-382 from influenza A virus nucleoprotein

p167	- peptide representing residues 270-283 from influenza A virus nucleoprotein
PBS	- phosphate-buffered saline (pH7.2)
PEG	- polyethylene glycol 6000
SD	- standard deviation
SDS	- sodium dodecyl sulphate
Tris	- Tris(hydroxymethyl)-methylamine
VP2	- virion protein 2, the major capsid protein of HRV2
v/v	- volume per volume
w/v	- weight per volume
‡	- novel junction

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DECLARATION

All of the work described in this thesis is my own, except where stated otherwise. Some of the data contained within this thesis have been published in the following paper: "Intranasal immunization using the B subunit of the *Escherichia coli* heat-labile toxin fused to an epitope of the *Bordetella pertussis* P.69 antigen", Lipscombe *et al.*, (1991). Molecular Microbiology, 5, 1,385-1,392.

DEDICATION

This thesis is dedicated to my parents - thanks for everything.

ABSTRACT

A plasmid vector was constructed which allowed for the in-frame insertion of peptide-encoding sequences at the 3' terminal of the *Escherichia coli* (*E. coli*) heat-labile enterotoxin B-subunit (LT-B) structural gene. Several synthetic oligonucleotides, encoding various B and T cell epitopes from other proteins, were ligated into this vector. The sequence across the junctions of these novel plasmid constructs was determined and found to be as predicted. The chimeric fusion proteins expressed by these constructs were characterised *in vitro* by SDS-PAGE, Western blotting and G_{M1}-linked ELISA. All the fusion proteins were shown to behave like native LT-B in that they were transported to the periplasmic space when expressed in *E. coli*. In addition they formed pentamers which dissociated into their constituent monomers upon boiling. Furthermore, the pentameric forms were found to retain G_{M1}-binding properties as determined by G_{M1}-linked ELISA.

Some of these plasmids, expressing LT-B fusion proteins containing T cell epitopes, were transferred into an aromatic-dependent attenuated strain of *Salmonella typhimurium* SL1344, and these strains were used to inoculate mice. A weak serum antibody response to one of these epitopes was demonstrated. However, a consistent *in vitro* T cell response to these epitopes could not be detected.

Another of the fusion proteins, termed LT-B69, was partially purified by ion-exchange chromatography and used to inoculate mice intranasally. Mice immunised in this way developed serum antibodies against LT-B and P.69 (an important *Bordetella pertussis* antigen). Additionally, LT-B-specific and P.69-specific antibody secreting cells could be detected in their lungs. There was some evidence to suggest that these mice were slightly protected against colonisation by *B. pertussis* after an aerosol challenge with live organisms, compared to the levels of colonisation in a control group.

CHAPTER 1

Introduction

1.1 Some introductory comments on the mucosal immune system

The immune system can mount both systemic and local responses. There are many significant differences in the type of stimulus required to provoke the two types of immune response and in the nature of the immunity generated. In simple terms, the systemic response gives rise to serum antibody and effector T cells which mediate a wide range of processes: for example, opsonisation of bacteria, clearance of viral infections and transplant rejection. Such responses can be efficiently stimulated in experimental animals by the administration of µg amounts of antigen parenterally (e.g. intraperitoneally, intramuscularly) if strong adjuvants are used (such as complete Freund's adjuvant), leading to long-lasting immunological memory. Vaccination of humans usually requires multiple inoculations because powerful adjuvants like CFA cannot be used as they cause unacceptable side effects (e.g. granuloma formation).

In contrast, mucosal responses are difficult to induce and are often of short duration. Mucosal immunity is mediated in large part by secretory IgA (sIgA), mainly produced locally by plasma cells adjacent to the mucosal epithelium, although in rats significant amounts of the sIgA present at mucosal surfaces are derived from serum directly or indirectly via bile (Orlans *et al.*, 1976, Halsey *et al.*, 1980). In man, the derivation of sIgA from bile is thought to be less important because human hepatocytes do not express secretory component, an essential element in the secretion of IgA (described in detail later). Delacroix (1985) estimated that human bile contributes about 1mg/kg/day to gut IgA, whereas locally secreted IgA exceeds 30mg/kg/day. It has been calculated that IgA synthesis is responsible for 2/3 of total human immunoglobulin production (Mestecky & McGhee, 1987).

There are numerous sites of IgA secretion - the gut, lungs, mammary, salivary and lacrymal

glands. It is worthy of note that in the human small intestine there are about 10^{10} immunoglobulin producing cells per metre, which is comparable to the 2.5×10^{10} estimated to be present in bone marrow, spleen and lymph nodes collectively (Brandtzaeg *et al.*, 1987). It has been demonstrated that there is considerable circulation of immunocompetent cells between the various mucosal sites. For example, Mestecky *et al.*, (1978) showed that after oral immunisation of mice with killed *Streptococcus mutans*, specific sIgA in salivary and lacrymal secretions could be detected without an elevation in serum antibody titres. Similarly, McDermott and Bienenstock (1979) found evidence that murine B plasma cell precursors migrated into intestinal, respiratory and genital tissues after stimulation by antigen. These and other findings led to the postulation of the existence of a "common mucosal immune system" (Bienenstock & Befus, 1980). Further evidence supporting this concept was provided by DeSousa (1981), who demonstrated that mouse lymphocytes derived from mucosal tissues, when transferred to a syngeneic animal, have a tendency to return to the tissue of origin. If the common mucosal immune system is a reality then it raises the exciting possibility of oral immunisation being able to protect against infection at distal mucosal surfaces. Such vaccines would possess a number of intrinsic advantages over more conventional vaccines which are outlined below.

1.2 Advantages of vaccines designed to stimulate mucosal immunity

The majority of pathogens of man (and other animals) be they bacterial, viral or protozoal, cause disease by initially colonising or entering via mucosal surfaces. Thus an extremely important advantage of any vaccine which can elicit a protective mucosal immune response is that it may prevent infection *per se*. This is very significant for a number of reasons. In some acute viral infections, such as the common cold, there is a rapid onset of symptoms which are experienced before any secondary systemic response can be mounted. In addition, such local mucosal immunity would prevent the establishment of a carrier population. This would eliminate reservoirs of infection. Also, a mucosal immunisation strategy, by reducing or eliminating the

carrier population, would increase the likelihood of achieving "herd immunity", inhibiting the development of an outbreak of disease into an epidemic.

Equally important is the observation that, for many diseases, mucosal immunity is simply more relevant for protection than systemic immunity. A good illustration of this phenomenon is cholera. There are estimated to be nearly 8 million cases of cholera world wide each year, resulting in 124,000 deaths (Black, 1986). The disease is caused by infection with *Vibrio cholerae*, an organism which in man is essentially non-invasive. Parenteral inoculation with whole-cell cholera vaccines generates high titres of anti-bacterial serum antibodies in man (Svennerholm & Holmgren, 1986*a*). However, protective levels in the gut are maintained for only a short time. The parenteral vaccine is not very effective at protecting people travelling to areas where cholera is endemic (Behrens, 1991). In contrast, certain killed *V. cholerae* whole cell/subunit combination vaccines given orally provide solid protection for a comparatively long period (Svennerholm & Holmgren, 1986*a*). Yet another important advantage is that as stated earlier, if the concept of common mucosal immune system is correct, one oral inoculation might be able to provide protective immunity at all mucosal surfaces.

There are also a number of very practical advantages which accompany the stimulation of mucosal immunity. Principal among these is that the oral or intranasal routes of inoculation, which best elicit mucosal responses, obviate the need for injections performed by medically-trained personnel. Such an approach also avoids the risk of transmission of blood-borne agents, such as hepatitis B virus or HIV. The take-up rate for this sort of vaccine would be higher than for a corresponding vaccine given parenterally, as many people dislike injections. In addition, although there is little experience of oral or intranasal immunisation, it seems probable that these routes of inoculation would be safer than conventional routes (lipopolysaccharide, for instance, is comparatively harmless when given orally but can be fatal if given intravenously).

Finally, there is some evidence that the ability to mount secretory immune responses arises in human infants in advance of the capacity to mount systemic responses (McGhee & Mestecky, 1990). Thus it may be possible to protect very young children against diarrhoeal

diseases (which are a major cause of infant mortality in less-developed countries) by the use of a vaccine which induces secretory responses. In the light of these advantages it is important to understand as fully as possible the immune mechanisms operating at mucosal surfaces. These are outlined below.

1.3 The structure and function of the mucosal immune system

1.3.1 Historical background

The importance of the mucosal immune system was first demonstrated as long ago as 1891, when Paul Ehrlich showed that oral immunisation with ricin could protect rabbits against the necrotic effects caused when ricin was introduced into the conjunctival sac (Mestecky & McGhee, 1989). Research in 1922 by Vaillant, which compared the efficacy of oral versus subcutaneous inoculation with a killed *Salmonella typhi* vaccine, showed that superior protection resulted from the oral route of immunisation (Gay, 1924), underlining the significance of local immunity. Subsequently, strenuous efforts have been made to obtain an oral vaccine against a number of pathogens, most notably *V. cholerae*, *S. typhi*, *Shigella dysenteriae*, *Streptococcus mutans* and polio virus.

With regard to the intranasal route of vaccination, experiments by Bull and McKee (1929) proved that intranasal immunisation of rabbits with killed *Streptococcus pneumoniae* was protective against lethal pneumococcal infection. These workers could not detect serum antibody in protected animals. However, Walsh & Cannon, (1938) showed that intranasal immunisation resulted primarily in the local production of antibody. Later work by Tomasi and others (reviewed by Brandtzaeg, 1984) identified this locally-produced antibody as IgA and that most of it was present as secretory IgA (sIgA) (Rossen *et al.*, 1966 *a,b*).

1.3.2 The structure of sIgA

The structure of sIgA is well-known and well-documented (Waldman & Ganguly, 1974, Tomasi, 1989). It consists of the familiar arrangement of two light and two heavy chains of the α isotype joined by disulphide bridges. In man it is usually present as a dimer (but can exist as higher order polymers, especially in mice), the two IgA molecules held together by a J ("joining") chain. This is a 15kDa polypeptide which is also present in pentameric IgM. Dimeric IgA is found both in secretions and in serum. However sIgA possesses an additional element, an 80kDa glycoprotein called secretory component (SC).

1.3.3. Processes in the secretion of IgA

The necessary steps in the secretion of IgA at mucosal surfaces represent one of the better-understood processes operating in mucosal immunity (reviewed by Brandtzaeg, 1985). Secretory component is synthesised by mucosal epithelial cells on the rough endoplasmic reticulum as a 95kDa polypeptide. It is core-glycosylated to a M_r of 105kDa and is transported to the Golgi cisternae. There, complex sugars are added and terminal glycosylation occurs. The glycoprotein is transported to the basolateral cell membrane where phosphorylation of a serine residue takes place to give the final 120kDa receptor molecule. J chain/IgA dimers produced by neighbouring plasma cells bind non-covalently to SC (which serves as an epithelial cell membrane receptor for the antibody molecule) and enter the epithelial cell in endocytic vesicles. These vesicles transcytose to the luminal membrane, with which they fuse. The transmembrane domain of SC is cleaved, releasing mature sIgA. At some stage, between the initial interaction between SC and IgA and the final secretion from the epithelial cell, two disulphide bridges form covalent links between SC and the C_H2 domain of one of the α heavy chains.

1.3.4 Properties of sIgA

The association of polymeric IgA with secretory component endows sIgA with certain properties which enhance its efficiency. For instance, SC is thought to reinforce the natural resistance of IgA to proteolysis. Clearly this is relevant for antibodies operating in the respiratory and gastro-intestinal tracts, which are milieux rich in proteolytic enzymes (McGhee & Mestecky, 1990).

Perhaps a more significant structural feature is the dimerism of sIgA, which is reflected in several of the biological properties of the molecule. For example, sIgA has been shown to be highly effective at neutralising viruses (e.g. Taylor & Dimmock, 1985) and its multiple antigen-binding sites allow it to agglutinate and inhibit the adsorption of bacteria very effectively. Magnusson & Stjernström (1982) demonstrated that in this respect sIgA is considerably more efficient than IgG.

There is much evidence to show that, at least in the gut, sIgA inhibits the absorption of soluble antigens in an antigen-specific manner (André *et al.*, 1974). This phenomenon is termed "immune exclusion". The binding of sIgA to antigen does not result in the activation of complement. Indeed, it has been shown that IgA can inhibit complement-dependent reactions mediated by antibodies of different classes (Russell-Jones *et al.*, 1980, 1981). Thus, by combining with antigen and inhibiting its absorption, sIgA prevents the antigen being bound by, for example, serum IgG, which might result in potentially harmful immune complexes, such as found in IgA-deficient patients (Cunningham-Rundles *et al.*, 1978). Alternatively, antigen might be bound by IgG at the mucosal surface with concomitant activation of complement, leading to local inflammation and loss of integrity of the mucosal epithelium, resulting in increased absorption of "bystander" antigens with the possibility of inappropriate immune responses. Thus an important role of sIgA is thought to be the down-regulation of other, potentially pathological, immune-effector responses to environmental antigens.

1.3.5 The initiation of a secretory IgA response

The main events which are necessary for the initiation of a secretory IgA response at mucosal surfaces are reasonably well-understood and have been reviewed recently (McGhee & Mestecky, 1990), but the details, especially those concerning cellular interactions and the regulation of the response, remain poorly defined.

Immunocompetent cells can be found in various locations throughout the gastro-intestinal and respiratory tracts (GIT and RT respectively). In particular, they are concentrated in gut-associated lymphoid tissue (GALT) and bronchus-associated lymphoid tissue (BALT). These are defined structures found throughout the length of the GIT and RT.

Initially, antigen crosses the mucosal epithelium. In the gut this is thought to be mainly via the thin dome-shaped layer of epithelial tissue (the follicle-associated epithelium, "FAE") which overlies specialised lymph nodes termed Peyer's patches (named after the Swiss scientist Johan Conrad Peyer, who first described them in 1677). Although enterocytes are also capable of macromolecular absorption, the mucus layer is thinner above the FAE, thus the microfold cells ("M" cells) which constitute the FAE are more accessible to antigen via pinocytosis. Furthermore beneath the FAE, within the Peyer's patch, is lymphoid tissue rich in B and T lymphocytes, arranged in discrete zones. However, the M cells do not express MHC class II antigens. Therefore, although they may or may not process antigen, they are presumably incapable of presenting antigen to T helper cells. This function is probably performed by the "professional" antigen-presenting cells (APC), such as macrophages and dendritic cells, which are well-represented in Peyer's patches. Thus, it is hypothesised that antigen becomes associated with MHC class II^{+ve} APC and is processed and presented to T helper cells. These antigen-stimulated T cells provide various regulatory signals to B cells, resulting in B cell differentiation (which commits the B lymphoblasts to being IgA^{+ve}). Antigen- and cytokine-stimulated plasma cell precursors migrate from the lymphoid tissue via the lymphatic system and enter the circulation through the thoracic duct. They then target to a variety of secretory mucosal tissues where they undergo terminal differentiation into sIgA-secreting plasma cells.

This re-distribution of immunocompetent cells is discussed in greater detail in the next section.

The processes operating in the respiratory tract are not so well-documented. Nevertheless, primary stimulation of B cells is also thought to occur in specialised structures, such as the tonsils and the bronchus-associated lymphoid tissue (BALT). The BALT exists as aggregates of immunocompetent cells and accessory cells. These structures (for which some authors have proposed the term "Bronchus-associated lymphoid units" or BALU; Sminia *et al.*, 1989) are analogous to the Peyer's patches in the gut. They are randomly distributed along the length of the bronchial tree but are often found around bifurcations in the bronchi. Their number appears to differ from species to species. In adult rats, for example, between 30 and 50 have been found to exist (Piesch, 1982). As with Peyer's patches, BALU comprise both B and T cell areas. Histochemical studies conducted on rat BALT follicles have found this lymphoid cell component to consist of ~60% B cells and 40% T cells. Of the latter, there are roughly twice as many T cells with the helper phenotype as with the cytotoxic/suppressor phenotype (van der Brugge-Gamelkoorn & Sminia, 1985). Also present are many non-lymphoid cells: fibroblasts, macrophages, interdigitating cells and follicular dendritic cells.

Despite these similarities between BALU and Peyer's patches, various important distinctions can be drawn between BALT and GALT. For instance, as stated above, T cells constitute 40% of the lymphocytes present in rat BALT follicles. This contrasts with the situation in rat Peyer's patches, where only 15% of lymphocytes are T cells (Jeurissen *et al.*, 1984). Moreover, other studies, again conducted on rats, have demonstrated that most of the intraepithelial T cells in the respiratory tract possess the T helper phenotype (van der Brugge-Gamelkoorn, 1986). Similarly, T cells in the epithelium lining the human nose are found to be mainly T helper cells (Winther *et al.*, 1987), whilst in the gut 80% of intraepithelial T cells bear the cytotoxic/supressor phenotypic markers (Mowat, 1990). This might be a reflection of the generally immunosuppressive processes occuring along the length of the gut, which presumably are necessary to maintain oral tolerance. It may well be that, in the light of this finding and the presence of the acid barrier in the stomach (together with the digestive enzymes in the gut), the respiratory tract represents a more attractive route of inoculation than

does oral delivery.

Another difference between BALT and GALT concerns the uptake of antigen: whereas antigen crosses the intestinal epithelium and enters Peyer's patches via M cells, there are no M cells as such in the epithelium lining the respiratory tract. Nevertheless, there are non-ciliated epithelial cells which are active in antigen-sampling. In contrast to M cells, which tend to form a layer above adjacent lymphocytes, the BALT epithelial antigen-sampling cells usually extend from the bronchial lumen right down to the basement membrane. There is some debate as to their relevance. It has been demonstrated that exogenous material can be ingested by the entire respiratory tract (Richardson *et al.*, 1976). In addition, the great majority of antigen in the lung is taken up by alveolar macrophages (Hocking & Golde, 1979). However, the local uptake of even small amounts of antigen by non-ciliated antigen-sampling cells in BALT aggregates is probably significant because of the intimate association between these cells and infiltrating lymphocytes and accessory cells. As with M cells, it is unclear if these specialised epithelial cells simply take up and transport antigen or whether they also process and present antigen. Certainly they can be induced to express Ia molecules upon antigen challenge, at least in rats (Gregson *et al.*, 1979, Simecka *et al.*, 1988). Interestingly, it has been proven that rat intestinal epithelial cells (other than M cells) can also express MHC class II antigens and present antigen to T cells in vitro, resulting in T cell proliferation (Bland & Warren, 1986). However, it has not yet been shown whether such epithelial cells can present antigen *in vivo*. Also, it should be remembered that Ia antigen has been found to have a role in intracellular transport systems and is not necessarily an indicator of immunological processes (Unanue & Allen, 1986).

1.3.6 Lymphocyte traffic

According to Bjerke and Brandtzaeg (1986), once stimulated by antigen and cytokines most B cells immediately migrate from the mucosa-associated lymphoid tissue, via the lymphatic and vascular systems, to various secretory tissues such as the gut, respiratory tract, mammary and

salivary glands. It is thought that various endothelial surface receptors are at least partly responsible for the specificity of the homing of these lymphocytes (Streeter *et al.*, 1988). Apparently different recognition mechanisms operate in the GALT and BALT (Brandtzaeg, 1988), thus it would seem reasonable to assume that different subsets of B cells, with different surface markers, would target with different specificities. That such migration of B cells occurs is beyond question. There are numerous examples of local immunisation resulting in secretion of antigen-specific IgA at distal mucosal sites (reviewed by Mestecky, 1987). The evidence for T cell migration is less abundant. However, there have been reports indicating that, in mice and rats, T cells traffic in a similar manner to B cells (Guy-Grand *et al.*, 1978, Mattingly & Waksman, 1978, respectively).

The exact molecular basis for the specific targeting of IgA-precursor cells to mucosal tissues has not been fully determined although it is clear that there are a number of vascular endothelial receptors involved (Streeter *et al.*, 1988). Considerable interest was generated when it was reported that virtually all $CD3^{+ve}/CD8^{+ve}$ intraepithelial lymphocytes (IEL) found in the mouse intestine were of the γδ type T cell receptor (TcR), (Bonneville *et al.*, 1988, Goodman & Lefrançois, 1988), whereas $CD3^{+ve}/CD8^{+ve}$ lymphocytes present in non-mucosal lymphoid organs and blood far more commonly possess a TcR of the classical αβ type. Naturally it was speculated that this difference in T cell receptor structure could be the molecular basis for specific targeting of IEL to the intestinal epithelium. However, subsequent experiments demonstrated the existence of two populations of intestinal IEL in mice (reviewed by Viney *et al.*, 1990). One was $\gamma\delta^{+ve}$ and was thymus-independent in its ontogeny (they could be found in athymic mice). The other was α/β^{+ve} and thymus-dependent. The two populations were present in roughly equal numbers in normal mice. This discrepancy with earlier results is explained at least in part by the finding that in gnotobiotic or very young animals there are very few IEL and those that are present are mostly $\gamma\delta^{+ve}$, whilst in older mice there are far more IEL of which many more are α/β^{+ve}. Thus the ratio of α/β to γδ cells among the intestinal IEL population depends on the degree of antigen stimulation of the gut and the age of the mouse. It would seem therefore, that if the γδ TcR is part of a molecular homing signal it can

only be operable at an early stage of development. A similar degree of uncertainty exists concerning the situation in the mouse lung, where estimates of the $\gamma\delta^{+ve}$ T cell population vary from 2-20% (Abraham *et al.*, 1990, and Augustin *et al.*, 1989 respectively).

As stated earlier, the majority of antigens given orally (such as food and inert environmental antigens) will result in the suppression of subsequent systemic responses to the same antigen given parenterally. This phenemenon is known as "oral tolerance". It can occur in adults and is independent of the mechanisms responsible for the generation of self-tolerance in neonates. However, this mechanism does not operate for all antigens to which mucosal surfaces are exposed. For instance, replicating antigens such as viruses and bacteria can induce secretory and serum antibody responses. Among inert (i.e. non-replicating) antigens, cholera toxin (CT) and its non-toxic, binding B-subunit (CT-B) have been known for some time to act as potent immunogens when fed to rats (Pierce & Gowans, 1975). Subsequently, several studies have shown that CT, CT-B and their analogous *E. coli* counterparts heat-labile toxin (LT) and its non-toxic, binding B-subunit (LT-B), can generate strong secretory and serum antibody responses (Elson & Ealding, 1984*a*, Chen & Strober, 1990, Clements, 1990, Maskell *et al.*, 1987). To understand why CT, LT and their B-subunits act in this way it is necessary to describe their structure and properties.

1.4 The properties of CT and LT

CT is elaborated by wild-type *V. cholerae* and is largely, but not exclusively, responsible for the symptoms of diarrhoea that can follow infection of humans by *V. cholerae* (Levine *et al.*, 1984). There are many strains of *E. coli* which can also cause diarrhoea, in both man and animals. Among the virulence factors of some of these strains are one or more enterotoxins. The best characterised of these are the heat-stable and heat-labile toxins (ST and LT respectively). Two types of ST have been identified, STa and STb (Burgess *et al.*, 1978). These are both relatively small peptides whose relative molecular mass has been determined at between 2kDa and

5kDa. Their small size partly explains their heat-stability and also their very poor immunogenicity, which has hindered attempts to develop a vaccine. In contrast, LT is highly immunogenic. LT is very similar, but not identical, to CT in terms of structure and biological activities. LT consists of one A-subunit of 28kDa, non-covalently linked to five identical B-subunit monomers. Each monomer has a M, of ~ 11.5kDa, thus the LT-B pentamer is 56kDa whilst the LT holotoxin is 84kDa in size. This is an arrangement very close to that found in CT (Gill *et al.*, 1981). It should be stated that the LT-B structural genes in LT-producing strains of *E. coli* isolated from different sources exhibit small but consistent sequence differences (Leong *et al.*, 1985). This is reflected in different amino acid compositions. Thus heat-labile toxin B-subunit produced by strains isolated from pigs, referred to as pLT-B, differs by 6 amino acids from the equivalent protein synthesised by strains isolated from humans (hLT-B). Two of these different amino acid residues are in the leader sequence and so are not represented in the mature protein. The other different residues are at positions 4, 13, 46 and 102, as represented in the mature protein after cleavage of the signal sequence (Leong *et al.*, 1985). These subtle differences between pLT-B and hLT-B can be distinguished serologically (Honda *et al.*, 1981 and Svennerholm & Holmgren, 1986*b*). Quite why these apparently rigorous strain differences exist is puzzling, as the enterotoxin structural genes are plasmid-borne in both porcine and human isolates. In addition, these plasmids have been shown to be transmissible (Smith & Halls, 1968). It may be that such exchange does occur, but only infrequently, and that simply insufficient isolates have been studied.

1.5 The basis of toxicity in CT and LT

As with the structure of the two toxins, their mode of action is very similar. Initially, one of the five B-subunit monomers binds to a monosialoganglioside molecule (termed G_{M1}) on the target cell membrane (e.g. a mucosal epithelial cell). This primary interaction is sufficient to stabilise the toxin molecule on the cell surface and the other monomers rapidly bind other G_{M1}

molecules with a stoichiometry of 1:1. Once the B-subunit pentamer is fully bound, the toxic A-subunit can enter the cell. The exact mechanism by which this is achieved is unclear. The secondary structure of the A-subunit is far less ordered than the that of the B-subunit (Surewicz *et al.*, 1990) and so might conceivably pass across the cell membrane in a partially unfolded state, with the B-subunit playing no further role. Such a model was proposed by Gill (as described by Stephen & Pietrowski, 1986). However, the results of some *in vitro* experiments on tissue culture cells suggest that both A- and B-subunits enter the cell in endocytic vesicles. Of course, these findings might be irrelevant to the situation *in vivo*. Whatever the debate over the method of entry into the cytoplasm, it is widely agreed that the target for the A-subunit, which causes the toxicity, is a regulatory "G" (guanidine-binding) protein, part of the stimulatory complex associated with membrane-bound adenylate cyclase. This stimulatory complex is part of the cell's physiological apparatus for increasing intracellular cyclic adenosine monophosphate (cAMP) levels in response to various signals (e.g. hormones). The A-subunit is cleaved, yielding A_1 (23kDa) and A_2 (5kDa) fragments. Binding of the A_1 fragment to the activated form of this G protein, (termed $G_s S$), prevents hydrolysis to the inactivated state. As a result, the stimulatory complex cannot be inhibited and adenylate cyclase is irreversibly activated, leading to high levels of cAMP. The next steps are not well-defined but it is clear that these high intracellular cAMP levels have a multitude of effects. One of these is thought to be the phosphorylation by cAMP-dependent kinases of membrane proteins which act as ion channels, resulting in de-regulation of ion-exchange and secretion in enterocytes, the clinical effect of which is severe diarrhoea.

1.6 The Immunogenicity of CT and LT

In many ways CT is an extremely well-characterised molecule. However, there is one area which remains poorly-defined: the molecular basis of the mucosal immunogenicity of CT and LT.

Historically, CT was a good choice of antigen to study, being available in a highly purified form in relatively large amounts. Furthermore, a model already existed for testing the protective effects of anti-toxin antibody (the ligated rabbit ileal loop model). The probable requirement for CT antigens in any effective human oral cholera vaccine underlined the significance of such research.

Early studies demonstrated that cholera toxoid was capable of provoking a response after intraperitoneal priming and oral boosting (Pierce & Gowans, 1975) and that native CT had interesting immunomodulatory properties *in vivo* (Kateley *et al.*, 1975). These latter workers showed that i.v. inoculation of small doses of CT (down to 0.1μg) could, depending on the time of administration relative to immunisation, enhance or suppress the mouse serum antibody response to sheep red blood cells (SRBC). The authors suggested that these effects were due to increased cAMP levels in lymphocytes resulting from adenylate cyclase activation or that the cytotoxic nature of CT released mitogenic macromolecules from dead cells. This work supplemented an earlier brief report (Northrup & Fauci, 1972) that CT could have an adjuvant effect on the mouse response to SRBC and that this adjuvanticity was destroyed by heating CT prior to inoculation. Pierce (1978), in experiments with CT, CT-B and cholera toxoid established that efficient oral priming of rats could only occur if the antigen was in a form which could bind to G_{M1}. Thus either holotoxin or CT-B could stimulate vigorous local responses after feeding but cholera toxoid had no such effect, despite retaining its immunogenicity when given parenterally. Furthermore, this work demonstrated that the holotoxin was about ten times more immunogenic (by molar ratio) than the B-subunit alone. Presumably this was a reflection of the adenylate cyclase activation brought about by the toxic A-subunit. The third important finding of this research was that local immunologic memory could persist for 8 months after oral priming.

This line of research was continued by Elson and Ealding (1984*a*,*b*). They compared the local and systemic responses of mice fed CT or Keyhole Limpet haemocyanin (KLH), a protein which is highly immunogenic when given intravenously. They showed that feeding 5mg of KLH did not induce any antigen-specific intestinal antibody and caused only a small increase in

similar serum antibody titres. In contrast, 10μg of CT was sufficient to induce CT-specific sIgA and high anti-CT serum levels of both IgA and IgG isotypes. This correlated with the detection of helper T cell activity in mucosal (Peyer's patches, mesenteric lymph nodes) and systemic (spleen) lymphoid tissue. More significantly they found that if CT was co-fed with KLH, a KLH-specific sIgA response could be induced, together with greatly enhanced serum anti-KLH titres. That is, co-administration of CT could overcome oral tolerance to an unrelated antigen. This phenomenon has become known as "mucosal adjuvanticity".

Thus the evidence that CT has a strong adjuvant effect on the mucosal reponses to unrelated and physically unconnected antigens is very strong. Equally, there is good evidence that CT and the other antigen must be given by the same route and approximately at the same time (Lycke & Holmgren, 1986). It is less clear if CT-B also has this property. There are some unequivocal reports (Tamura *et al.*, 1989), which established that anti-viral serum titres were higher in mice which received an influenza virus haemagglutinin (HA) vaccine intranasally in conjunction with CT-B than in mice which received vaccine alone. They also demonstrated far higher nasal sIgA concentrations in these mice, but did not prove its specificity. The question of whether CT-B can act as a mucosal adjuvant is of some importance. The answer has obvious implications for the design of subunit vaccines aimed at stimulating mucosal responses and may reveal an insight into the molecular mechanism of the mucosal adjuvanticity of CT. For example, is adenylate cyclase-activating activity essential? This was thought to be the case initially. However, it became obvious that CT-B did behave in a manner qualitatively similar to CT *in vivo*, in that it can stimulate both secretory and serum responses after oral inoculation and does not induce oral tolerance (Pierce, 1978; Elson & Ealding, 1984*a,b*). Further *in vitro* studies showed that CT-B shared other properties with the holotoxin. Woogen and his co-workers (Woogen *et al.*, 1987) were able to prove that CT-B, whilst not as efficient as CT, behaved similarly in that either molecule could inhibit mitogen- or antigen-induced T cell proliferation or anti-IgM-mediated B cell proliferation. This suppressive effect could be abolished if the toxin or B-subunit was pre-incubated with G_{M1}. One distinction which the authors did note was that only CT could inhibit LPS-driven B cell proliferation. They concluded that the binding

of CT or CT-B to the lymphocyte cell surface could inhibit the activation mechanism leading to proliferation and, in addition, a further mechanism involving adenylate cyclase activation could also have a suppressive effect.

The report by De Aizpurua and Russell-Jones (1988), in their review of mucosal immunogens, established that several substances could elicit systemic responses after oral inoculation. These included *E. coli* adhesins (the pili K99 and 987P from animal ETEC strains), influenza vaccine (containing haemagglutinating activity) and numerous lectins, such as Pokeweed mitogen (PWM) and concanavalin A (con A). Furthermore, these authors showed that if the K99 pili or LT-B were fed to mice in conjunction with various sugars, known to be responsible for the receptor-binding activity of the immunogens, then the serum responses to these molecules could be effectively abolished (mirroring the results *in vitro*, using CT-B mixed with G_{M1}-containing liposomes, obtained by Woogen *et al.*, 1987).

When all these findings are viewed in conjunction, it would seem that at least part of the mechanism of mucosal immunogenicity of CT rests on its ability to bind G_{M1}, and so this property is shared by CT-B and LT-B. One explanation put forward which combines the known data into a coherent model, is that binding to receptor molecules on the surface of enterocytes by-passes the normal antigen-sampling procedure occurring in Peyer's patches, which is that process responsible for maintaining oral tolerance. Blocking of the binding sites on the mucosal immunogens (by sugars or whole ganglioside molecules) forces the molecules to be processed via Peyer's patches and so results in unresponsiveness (De Aizpurua & Russell-Jones, 1988).

It is difficult to determine the significance of studies *in vitro* on the effects of CT and CT-B on T and B cells (Woogen *et al.*, 1987, Elson & Solomon, 1990, Anastassiou *et al.*, 1990, Dugas *et al.*, 1991) to the immunogenic mechanism *in vivo*. On the basis of his own published findings Elson appears to suggest that native CT or CT-B can bind to the surface of T cells, which inhibits T cell proliferation and that in some way T suppressor cells (presumably involved in the maintenance of oral tolerance) are inhibited preferentially over T helper cells, the end result being an anti-CT or anti-CT-B response. A number of factors make it difficult to ascertain what might occur *in vivo*; the far greater surface area of ileal enterocytes would suggest that

they could adsorb considerably more antigen (via receptor-mediated endocytosis) than the M cells of the FAE. However, there is a much higher concentration of immunocompetent cells in GALT, in a microenvironment which is probably very different to that experienced by IEL or lymphocytes in the lamina propria. Secondly, as stated earlier, there is evidence for the presentation of antigen by enterocytes *in vitro*, but no evidence if this occurs or not *in vivo*. Finally, Elson found *in vitro* that only native CT or CT-B exerted inhibitory effects on T cells. However, it is conceivable that enterocytes or other cell-types might process such antigens before they are encountered by lymphocytes.

The mechanism of mucosal immunogenicity outlined by De Aizpurua and Russell-Jones (1988) is not necessarily inconsistent with the published research (Guyon-Gruaz *et al.*, 1986) claiming to obtain serum anti-CT antibody after oral immunisation of mice with synthetic peptides corresponding to either amino acids 30-50 or 50-75 of the CT-B sequence. The 30-50 peptide contains the Arg-35 residue reputed to be involved in receptor-binding, thus this peptide may have retained some affinity for G_{M1}. The authors do not present any data on this point. Oral immunisation with the other peptide required four inoculations to elicit a serum response, despite the authors having selected the peptide for its putative immunogenicity.

1.7 Chemical conjugation and genetic fusion of antigens with CT-B and LT-B

1.7.1 CT-B/antigen conjugates and fusion proteins

In the light of the considerable body of evidence suggesting that CT-B (and probably therefore LT-B) possess at least some of the intrinsic mucosal immunogenicity of the holotoxin, it is not surprising that several attempts have been made to utilise this property so as to enhance the mucosal response to unrelated antigens.

The feasibility of this technique was demonstrated by the coupling of horseradish peroxidase (HRP) to CT-B, using glutaraldehyde (McKenzie & Halsey, 1984). The resulting

conjugate was shown to retain some G_{M1}-binding capability. 50µg of CT-B coupled to 30-90µg of HRP was used to inoculate mice intraduodenally. The serum and secretory anti-HRP responses of these mice were compared to those of mice immunised with a non-conjugated mixture of CT-B and HRP, or HRP alone. The authors found that chemical linkage of HRP to CT-B was necessary to obtain optimal sIgA and serum IgG anti-HRP titres.

A similar approach was employed (Bessen & Fischetti, 1988, 1990) in an attempt to protect mice against colonisation by group A streptococci. Synthetic peptides, corresponding to conserved sequences of streptococcal M protein, were covalently linked to CT-B and used to inoculate mice intranasally. It was shown that, despite immunised animals mounting only a poor salivary IgA response, such a procedure could protect mice against pharyngeal colonisation after intranasal challenge by live streptococci.

Again, covalent coupling of CT-B to an antigen, namely *S. mutans* surface protein antigen I/II (Ag I/II), was used to elicit mucosal and systemic responses in orally inoculated mice, as judged by the presence of Ag I/II-specific antibody-secreting cells in salivary glands, mesenteric lymph nodes and spleen and by the presence of specific serum antibody (Czerkinsky *et al.*, 1989).

The findings of McKenzie and Halsey (1984), concerning the need for CT-B to be covalently coupled to the antigen of interest, are in direct contrast with the results obtained by Tamura and his co-workers (Tamura *et al.*, 1988, 1989), who experimented with the enhancement, by CT-B, of the antibody response to an intranasally inoculated influenza virus vaccine. In common with many other reports on the subject, they found that CT-B was less efficient than the holotoxin at enhancing responses. However, they also discovered that, in their model, CT-B could act as a mucosal adjuvant simply by being mixed with the vaccine. The CT-B used in this study was supplied by Sigma Chemical Company and was not contaminated with A-subunit as determined by SDS-PAGE. It may well be that the respiratory and intestinal immune systems react differently to CT-B, otherwise it is difficult to reconcile the data of the two research groups. Alternatively, it may be significant that different antigens were studied.

A more sophisticated technique than chemical conjugation is to create genetic fusions,

consisting of CT-B or LT-B structural genes linked to the sequence encoding the antigen of interest. This has been achieved independently by several researchers (Guzman-Verduzco & Kupersztoch, 1987, Schödel & Will, 1989, Dertzbaugh & Macrina, 1989 and Clements, 1990). Such an approach has several advantages over chemical coupling. Genetic fusions are much more versatile; antigens or short peptide fragments defining epitopes of any desired sequence can, in theory, be fused to the carboxy or amino termini of the carrier molecule. The products of such fusions would also have a precisely defined composition, which would be consistent from batch to batch. In addition, chimeric proteins could, if desired, be delivered by live oral vaccines (Schödel *et al.*, 1990*a,b*).

Dertzbaugh *et al.*, (1990) have reported the construction of a vector which expresses residues 345-359 of the glucosyltransferase B (gtf B) from *S. mutans* as a fusion with the amino terminal of CT-B. Both moieties of this fusion protein retained their antigenicity (as determined by immunoblotting) and, more importantly, their immunogenicity, eliciting gut IgA and serum IgG responses when fed to mice.

1.7.2 LT-B/antigen fusion proteins

One of the earliest fusion plasmids reported was constructed by Guzman-Verduzco & Kupersztoch (1987). They successfully fused a sequence encoding *E. coli* ST to the 5' end of the LT-B structural gene. The LT-B moiety retained antigenicity *in vitro* in ELISA and Western blot analyses. Unfortunately, the ST moiety retained toxicity, causing fluid accumulation in the suckling mouse model (Gianella, 1976). The immunogenicity and possible pentamerisation of the chimaera were not investigated. ST was an attractive choice of antigen to couple to LT-B, being poorly immunogenic by itself. Any molecular combination which could elicit ST- and LT-neutralising antibodies in the gut raised the possibility of producing a single vaccine to protect against enterotoxigenic *E. coli*-mediated diarrhoeal disease.

Another ST/LT-B gene fusion was made by Clements (1990), in which a synthetic

oligonucleotide corresponding to part of the ST gene, was joined to the 3' terminal of the LT-B structural gene. This approach avoided the need to eliminate the leader peptidase recognition site at the amino terminal of LT-B, which is necessary in fusions to the 5' terminal of the LT-B coding sequence, to prevent cleavage of fused epitopes along with the LT-B leader peptide. Clements investigated the hybrid protein and found that the ST toxicity was effectively abolished, whilst mice immunised intraperitoneally with crude or purified peparations of the molecule could generate ST- or LT-B-specific serum antibody. However, whether such a fusion protein could elicit a response when given orally is doubtful; the author presented data implying no multimer-formation or G_{M1}-binding occurred.

Another plasmid vector had been described previously (Schödel & Will, 1989). This contained a polylinker region at the 3' end of the LT-B gene. A number of sequences encoding surface (sAg) or core (cAg) antigens from human (HBV) or Woodchuck (WHV) hepatitis B viruses were inserted into this vector, creating several chimaeric proteins. The authors established that the antigenicity of both moieties in the fusion protein was retained. However, no data were presented on multimer-formation or G_{M1}-binding, so it is unclear if these important attributes were also retained by the hybrid molecules. When mice were fed live attenuated strains of *Salmonella dublin* expressing these fusion proteins, they developed LT-B-specific serum antibody after priming, the titres of which could be boosted by a second oral inoculation. However, no virus-specific serum antibody was detected in these animals.

Subsequently the same group constructed a plasmid (pFS20) in which two over-lapping T cell epitopes of HBVcAg (amino acid residues 120-140) and a B cell epitope from HBVsAg (amino acid residues 133-140) were expressed in a single fusion protein with LT-B (Schödel *et al.*, 1990*a*). When mice were fed live attenuated *S. dublin* producing this polypeptide they developed serum anti-LT-B. In addition, mice of the H-2^b haplotype (C57Bl/10) generated splenic T cells which proliferated *in vitro* in the presence of a synthetic peptide corresponding to residues 121-145 of HBVcAg. However, neither these mice, nor Balb/c mice (H-2^d), developed serum antibody against the B cell epitope. Interestingly, essentially the same peptide (133-143), when fused to the carboxy terminal of HBVcAg, was able to generate serum

antibody when presented to mice by attenuated bacteria, using the same strain, dose and route of inoculation (Schödel *et al.*, 1990*b*).

It is clear from the published data that attempts at immunisation using LT-B fusion proteins are very promising, although initial experiments have had mixed success. However, the ability of these fusion proteins to form pentamers (which appears to be essential for binding to G_{M1}) is, where reported, often shown to be diminished or totally abolished. If current theories on mucosal immunogenicity are correct, this could substantially reduce their efficacy.

1.8 Aims of the project

The aim of this project was to investigate methods of improving the immunogenicity of peptide antigens inoculated via mucosal surfaces. To this end, a plasmid vector was constructed which would allow the in-frame insertion of peptides at the 3' terminal of the LT-B structural gene, in such a way that the resulting fusion proteins retained the ability to pentamerise and bind to G_{M1}.

Additionally, having constructed such a fusion vector, it was intended to insert appropriate peptides and to confirm the identity of the fusion plasmids by DNA sequence determination. The resultant chimaeric polypeptides were to be characterised *in vitro* using SDS-polyacrylamide gel (SDS-PAGE), immunoblotting and G_{M1}-linked ELISA analyses.

Finally, the immunogenicity of these fusion proteins in mice was to be investigated, both as partially-purified protein and by expression in a suitable live attenuated bacterial host.

CHAPTER 2

Materials and Methods

2.1 Bacterial strains

A number of strains were employed in this project. These are detailed in Table 2.1.

Table 2.1 The bacterial strains employed in this study

Organism	Strain	Relevant phenotype	Reference
E. coli	HB101	K12/B hybrid	Bolivar & Backman, 1979
E. coli	TG1	F'	Carter *et al.*, 1985
S. typhimurium	LB5010	r^-m^+	Maskell *et al.*, 1987
S. typhimurium	SL1344	aroA$^-$ aroD$^-$	Dougan *et al.*, 1988
B. pertussis	CN2992	Sm^r	Roberts *et al.*, 1990

2.2 Media for bacterial growth

Luria broth (L-broth), Luria agar (L-agar), top agar and Cohen & Wheeler agar (CW agar) were all prepared by the media production unit at Wellcome Research Laboratories using standard formulae.

L-broth

1.0% w/v tryptone

0.5% w/v NaCl

0.5% w/v yeast extract

L-agar was as above, solidified with 1.5% Bacto agar. Minimal medium was made in the laboratory according to the formula below:

1.5% w/v agar

0.6% w/v Na_2HPO_4

0.3% w/v KH_2PO_4

0.1% w/v NH_4Cl

in distilled water, adjusted to pH7.4 and autoclaved. 1M $MgSO_4$, 20% (w/v) glucose, 0.1% (w/v) thiamine and 1M CaCl, were prepared and sterilised separately. 200µl, 1ml, 1ml and 10µl respectively of these solutions were added to 100ml of the molten agar prior to pouring the plates.

2.2.1 Media supplements

Where appropriate, media were supplemented with antibiotics according to Table 2.2.1. Stock solutions of antibiotics were prepared in distilled water, sterilised by passage through a 0.22µm filter (Millipore, UK) and stored in aliquots at -20°C. CW blood agar plates contained 10% defibrinated horse blood (Tissue Culture Services Limited, UK).

Table 2.2.1 Antibiotics used to supplement culture media

Antibiotic	Stock solution	Working concentration
ampicillin	200mg/ml	100μg/ml
streptomycin	20mg/ml	100μg/ml

2.3 Culture conditions

E. coli and *S. typhimurium* were routinely cultured at 37°C on L-agar plates or in L-broth. *E. coli* TG1 was maintained on minimal medium plus thiamine. *B. pertussis* was grown on CW blood agar plates at 35°C. Stock cultures of all strains were stored in aliquots in liquid nitrogen.

2.4 Plasmids

Plasmid pBRD026, the starting-point for the constructions described in this thesis, was made by Maskell *et al.*, (1987), by sub-cloning a 600 base-pair *Eco*R1 fragment (carrying the porcine LT-B subunit cistron) from pEWD299 (Dallas *et al.*, 1979) into the *Eco*R1 site of pBR322. The resulting plasmid (pBRD026) expresses LT-B constitutively from the P1 ("anti-tet") promoter.

The fusion vector, pFV1, was made by the insertion of specially-designed linker oligonucleotides at the 3' terminus of the LT-B structural gene. LT-B fusion constructs were created by the further addition of oligonucleotides at unique sites within the linker sequence, as described in section 3.4.

2.5 DNA manipulations

2.5.1 Agarose gel electrophoresis of DNA

DNA in gel loading buffer was subjected to preparative and analytical electrophoresis in horizontal gels consisting of 0.8% (w/v) agarose in Tris/borate/EDTA (TBE) buffer containing 0.5μg/ml ethidium bromide.

5x gel loading buffer

30% v/v glycerol

0.25% w/v bromophenol blue

50mM Tris in distilled water

TBE buffer

1.08% w/v Tris

0.55% w/v boric acid

0.1% w/v EDTA in distilled water

2.5.2 Phenol extraction and ethanol precipitation of DNA

DNA in aqueous solution was treated with an equal volume of Tris-buffered phenol/chloroform, to remove proteinaceous impurities, then with an equal volume of chloroform, to remove residual phenol. DNA was precipitated from aqueous solution by the addition of sodium acetate (pH4.5) to a concentration of 0.3M and the addition of 2.5 volumes of pre-cooled absolute ethanol. The mixture was held at -20°C for 10 minutes and the precipitated DNA pelleted by centrifugation in a bench centrifuge for 5-10 minutes. Salt was removed by washing the pellet in cold 70% ethanol. The pellet was spun again for 2-3 minutes and the supernatant poured off. The DNA was dried under vacuum in an Uniscience "univap" concentrator. Once dry, the

pellet was dissolved in an appropriate volume of sterile distilled water and stored at -20°C.

Tris-buffered phenol/chloroform

100ml phenol

96ml chloroform

4ml isoamyl alcohol

(phenol supplied by BRL Gibco, Gaitesburg, Maryland, USA, rest supplied by BDH, UK), equilibrated with 10mM Tris-HCl pH8.0

2.5.3 Gel purification of DNA

DNA was subjected to electrophoresis as described in 2.5.1. Fragments of interest were excised from the gel and purified according to the "freeze-squeeze" technique of Tautz & Renz (1983). The gel slice was immersed in 1ml of buffer (0.3M sodium acetate, 1mM EDTA pH7.0) for 30 minutes in the dark at room temperature. The gel piece was dried on Whatman 3mm paper, transferred to a fresh eppendorf tube and frozen in a dry ice/ethanol bath for 20 minutes. The gel slice was then transferred to a Spin-X column (Costar, Massachusetts, USA) and spun for 10 minutes in a bench centrifuge. The gel matrix was retained by the filter in the column (a low-binding 0.22μm cellulose acetate membrane). The DNA passed through in solution and was purified according to the method detailed above (2.5.2).

2.5.4 Synthesis of oligonucleotides

Generally, synthetic oligonucleotides were prepared by Hugh Spence and Martin Carrier at Wellcome Research Laboratories using a Milligen 7500 DNA synthesizer (Millipore, UK). The

resulting oligonucleotides were purified by high pressure liquid chromatography (HPLC) and lyophilised. About 1 $O.D._{260nm}$ unit of the oligonucleotides was dissolved in 200µl of sterile distilled water. 100µl of this was diluted with a further 400µl of distilled water and the absorbance at 260nm, relative to a blank of water alone, was determined using quartz cuvettes and an LKB Ultrospec II spectrophotometer. (One $O.D._{260nm}$ unit of an oligonucleotide was taken to coontain approximately 40µg). The $O.D._{260nm}$ was also determined to detect contamination, which was found to be negligible.

About 10picomoles of each oligonucleotide were treated with 0.5-1.0 units of T4 polynucleotide kinase (Boehringer Mannheim), used according to the suppliers instructions, in 1x kinase buffer. Complementary oligonucleotides were then mixed in an eppendorf tube, boiled for 5 minutes and the tube left in the water bath which slowly cooled to room temperature, allowing the oligonucleotides to anneal.

10x kinase buffer

0.5M Tris-HCl pH7.6

0.1M $MgCl_2$

50mM dithiothreitol

1mM EDTA

2.5.5 Ligations

2.5.5.1 Preparation of vector DNA

Plasmid DNA for use in ligations with oligonucleotides was incubated with the appropriate restriction endonuclease(s) together with calf intestine alkaline phosphatase (Boehringer) at a concentration of 1 unit/µg DNA/hour. The reaction products were separated by agarose gel electrophoresis as outlined earlier. The desired band was purified as described in 2.5.3. and

resuspended in 20µl of sterile distilled water. A small sample (~2µl) was run on an agarose gel to quantify the DNA concentration in the preparation.

2.5.5.2 Ligation reactions

20-50ng of prepared vector was used per ligation with a series of dilutions of oligonucleotides (ranging from neat to 1/10,000). "Sticky-end" ligations were carried out at 15°C overnight in a total volume of 20µl, including 2µl of T4 DNA ligase (Gibco, BRL) and 4µl of 5x ligase buffer (from the same supplier) together with 1µl of 10mM ATP, (the rest of the reaction volume consisting of vector preparation and sterile distilled water). A control reaction (with prepared vector but no oligonucleotides) was also performed. Ligation reaction products were used to transform bacteria as described below.

2.5.6 Transformation of bacteria

2.5.6.1 Preparation of competent *E.coli*

E. coli was transformed according to the method of Cohen et al., (1972) as described by Sambrook *et al.*, (1989). Competent cells were prepared by diluting an overnight culture 1/100 in fresh L-broth and growing at 37°C in an orbital shaker until an $O.D._{.550}$nm of 0.4 was attained. The cells were harvested by centrifugation (~3,000g for 5 minutes), washed once in ice-cold 50mM $CaCl_2$ and resuspended in half the original culture volume in cold $CaCl_2$ and left on ice for 1 hour. The cells were then harvested as before and resuspended in 1/25 of the original culture volume.

2.5.6.2 Transformation conditions

20-50ng of DNA in a volume of 10µl was added to 200µl aliquots of competent cells in

eppendorf tubes. These were left on ice for 1 hour. Control experiments were performed in which aliquots of competent cells were treated with a known amount (~25ng) of plasmid DNA (e.g. pBR322, pUC18) or received no DNA at all. The suspensions were heat-shocked in a 42°C water-bath for 2 minutes and then put back on ice momentarily. 1ml of L-broth was added to each tube and the cells were allowed to grow for 90 minutes at 37°C. 100µl of each sample was spread over L-agar plates containing appropriate selective antibiotics and incubated at 37°C overnight.

2.5.6.3 Transformation of *S. typhimurium* LB5010

Competent cells of LB5010 were prepared using essentially the same technique as that for HB101. However, after harvesting the culture, the cells were initially resuspended in 1/2 the original culture volume in ice-cold 0.1M $MgCl_2$ and left for 30 minutes on ice. Next, the cells were harvested in 1/2 the original culture volume in cold 0.1M $CaCl_2$ and left for a further 30 minutes in ice. After this step the cells were suspended in 1/25 the original culture volume in cold 0.1M $CaCl_2$ and the procedure was the same as that for the transformation of *E. coli*.

2.5.7 Transduction of Salmonella strains

2.5.7.1 Preparation of high-titre lysates

Smooth vaccine strains of Salmonella are difficult to transform by conventional methods; however they can readily acquire foreign DNA by transduction with the generalised transducing phage P22. LB5010 was transformed with plasmids of interest as detailed in 2.5.6.3. Cultures with the transformed phenotype were grown overnight at 37°C. Duplicate 200µl aliquots of these cultures were infected with different dilutions (10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}) of a stock

suspension of P22. These were incubated statically at 37°C for 1 hour and then mixed with 3ml of molten top agar and spread onto L-amp plates. Once set these plates were incubated at 37°C for approximately 7hrs, by which time confluent plaques were apparent on some plates. 3ml of T2 buffer were then added to those plates exhibiting near confluent lysis. The plates were left to soak overnight at 4°C. The top agar was then scraped off and vortexed vigorously with the T2 buffer. The agar was removed by centrifugation for 15 minutes at 27,000g and a few drops of chloroform added to kill the remaining cells in the lysate. Any surviving cells were removed by passage through a 0.22μm filter.

T2 buffer

70mM NaCl

30mM K_2SO_4

15mM Na_2HPO_4

10mM KH_2PO_4

0.5mM $MgSO_4$

0.05mM $CaCl_2$

0.1% w/v gelatin

2.5.7.2 Transduction of *S. typhimurium* SL1344

200μl aliquots of a stationary phase overnight culture of SL1344 *aroA*$^-$ *aroD*$^-$ were infected with different volumes (0-40μl) of the previously prepared P22 lysate. The aliquots were left at 37°C for 1 hour for the phage to adsorb to the bacteria. After this incubation 100μl of each aliquot was spread onto L-amp plates supplemented with 5mM EGTA (which purges the phage from the culture by preventing viral readsorption) and incubated overnight at 37°C to give rise to colonies with the expected transduced phenotype.

2.5.8 Small scale preparation of plasmid DNA from *E. coli*

Small scale preparations ("minipreps") of plasmid DNA were obtained using a modification of the Birnboim & Doly (1979) alkaline lysis method as described by Sambrook *et al.*, (1989). Colonies with the desired phenotype were picked into 3ml of L-broth containing selective antibiotics and grown overnight at 37°C in an orbital shaker. 1.5ml of this culture was transferred to an eppendorf tube and the cells pelleted by centrifugation for 2 minutes in a bench centrifuge. The supernatant was removed and the remainder of the culture was added to the tube. These cells were pelleted as before and the supernatant removed. The pellet was dried and resuspended, with vigorous vortexing, in 100µl of solution 1 and left at room temperature for 5 minutes. 200µl of solution 2 was added and the mixture incubated on ice for 5 minutes. 150µl of solution 3 was added and, following a further 5 minute incubation on ice, the suspension was spun for 5 minutes in a bench centrifuge. 400µl of the cleared supernatant was transferred to a fresh eppendorf tube containing 1ml of cold absolute ethanol. The mixture was held at -20°C for 10-20 minutes and the precipitated DNA was collected by spinning for 5 minutes in a bench centrifuge. The DNA pellet was dried, resuspended in 400µl of solution 4 and purified as outlined earlier. RNA in the samples was removed by digestion with 20µg/ml RNase A for 30 minutes at 37°C.

Solution 1

0.1M EDTA

30mM Tris-HCl

Solution 2

1% w/v SDS

0.2M NaOH

Solution 3

3m NaOAc pH4.8

Solution 4

0.1M NaOAc

50mM Tris-HCl pH8.0

2.5.9 Large scale preparation of plasmid DNA from *E. coli*

Large scale preparations of plasmid DNA were performed using essentially the same method as above, scaled up 100 fold, except that prior to phenol/chloroform extraction samples were treated with 1mg/ml proteinase K (Boehringer) for 1 hour at 56°C.

2.5.10 Transformation of *E. coli* with M13 DNA

DNA sequences of interest were sub-cloned into the replicative form of M13 vector mp19 (Messing & Vieira, 1982), obtained from Pharmacia (Sweden). Competent TG1 cells were prepared as described previously. These were transformed with 10µl of M13 ligation mixtures and then added to 3ml aliquots of molten top agar containing 200µl of late log phase TG1 seeding culture, 50µl of 20mg/ml X-gal solution and 10µl of 0.1M IPTG. This mixture was poured over an L-agar plate and incubated at 37°C overnight. Recombinant M13 plaques were colourless whilst wild-type plaques were blue.

X-gal solution

20mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactoside
in dimethyl formamide, stored at -20°C

IPTG solution

0.1M isopropyl β-D-thiogalactopyranoside
in distilled water, stored at -20°C

2.5.11 Preparation of single stranded DNA from M13 plaques

Recombinant M13 plaques were picked into 2ml of 1/100 dilutions of *E. coli* TG1 overnight cultures. These were grown for 6 hours at 37°C in an orbital shaker. Bacterial cells were then pelleted in a bench centrifuge. 1ml of the supernatant was transferred to a fresh eppendorf tube and the phage precipitated by the addition of 200µl of 20% polyethylene glycol (PEG 6000) in 2.5M NaCl. Purified single stranded DNA was obtained from the pelleted phage by phenol/chloroform extraction etc. as detailed in 2.5.2.

2.5.12. DNA sequence determination

DNA sequence determination from single stranded DNA templates was achieved using a modification of the dideoxy chain termination method (Sanger *et al.*, 1979); that of Tabor and Richardson (1988) employing altered T7 polymerase. Chain elongation was initiated with custom-made oligonucleotide primers, complementary to a region 50bp upstream (5') of the LT-B stop codon. Sequencing reactions were executed with reagents from the "Sequenase" version 2.0 kit (United States Biochemical Corporation, Cleveland, Ohio, USA) and with α-^{35}S-dATP from Amersham International (Amersham, Bucks, UK).

The reaction products were heated to 95°C for 5 minutes just prior to loading onto pre-heated 6% (w/v) acrylamide/7M urea wedge gels. These were run at 60 watts constant power for ~2 hours, fixed in 10% methanol/10% acetic acid in water for 45-60 minutes, dried under vacuum and put up for autoradiography with Kodak X-Omat S 100 film. Autoradiographs were developed with a Fuji RGII automatic x-ray film processor.

2.6 Analysis of *E. coli* periplasmic fractions

2.6.1 Preparation of periplasmic fractions of *E. coli*

Periplasmic fractions were obtained from *E. coli* cultures as described by Hirst *et al.*, (1984*a*). Briefly, cultures were grown in L-broth to late log phase ($O.D._{.600nm}$ ~1.0). Cells were collected by centrifugation and washed once in ice-cold PBS. The cell pellet was then resuspended in 1/25 of the original culture volume in 0.3M sucrose buffered with 0.1M phosphate to pH7.6. Ethylene diamine tetra-acetic acid (EDTA) and lysozyme were added to a final concentration of 5mM and 20µg/ml respectively. The suspensions were left on ice for 20-25 minutes with occasional agitation and then centrifuged at 20,000g for 15 minutes to pellet the sphaeroplasts. The supernatant containing the periplasmic proteins was stored at -20°C.

2.6.2 β-Galactosidase and β-Lactamase assays

These assays were performed upon the periplasmic and cellular fractions obtained by the above procedure. To assay β-galactosidase, 100µl of a soluble periplasmic fraction or a cell sonicate (obtained by sonicating cell pellets in 1ml of sphaeroplasting buffer on ice for 1 minute, in 10 second pulses, using a standard MSE sonicator and probe) were added to 900µl of reaction buffer and mixed by vigorous vortexing with 20µl of toluene. The samples were incubated at 37°C for 40 minutes with occasional vortexing. The reaction was started by the addition of 200µl of OPNG solution and was allowed to proceed for varying times (30 seconds to 2 minutes) before quenching with 500µl of 1M Na_2CO_3. The absorbance at 420nm of the samples was read using an LKB II ultrospec spectrophotometer. A graph of absorbance against time was drawn and the gradient determined to give the initial reaction rates for the periplasmic and cellular fractions. The results for each fraction were expressed as a percentage of the total enzyme activity.

β-galactosidase reaction buffer

60mM Na_2HPO_4

50mM 2-mercaptoethanol

40mM NaH_2PO_4

10mM KCl

1mM $MgSO_4$

OPNG solution

4mg/ml ortho-Nitrophenyl β-D-galactoside

in distilled water

To assay ß-lactamase activity, 100µl of periplasmic or cellular fractions were mixed with 900µl of ß-lactamase assay buffer in a cuvette. The absorbance at 482nm was monitored, using a spectrophotometer and chart recorder, as the reaction proceeded and the initial reaction rate determined from the gradient of the plot. Again, the results for each fraction were expressed as the percentage of the total enzyme activity.

ß-lactamase assay buffer

600µl distilled water

200µl 0.2M phosphate pH7.0

100µl Nitrocefin (Difco) at 0.1mg/ml in

0.1M phosphate pH7.0

2.7 SDS-PAGE

Periplasmic proteins were separated on SDS polyacrylamide gels under reducing conditions (Laemmli, 1970). Preparations were mixed with an equal volume of 2 x final sample buffer and loaded, with or without prior boiling, onto 12.5% acrylamide gels, with molecular weight

standards run in parallel (Rainbow protein markers from Amersham International). These were run overnight at ~8mA constant current. Gels were stained with 0.1% (w/v) Coomassie Brilliant Blue R250 dissolved in 10% (v/v) acetic acid/40% (v/v) methanol/50% (v/v) distilled water and destained in the same solution in the absence of Coomassie Brilliant Blue.

2 x final sample buffer

10% v/v glycerol

5% v/v 2-mercaptoethanol

3% w/v SDS

0.01% w/v bromophenol blue

1 x SDS-PAGE running buffer

1.4% w/v glycine

0.3% w/v Tris

0.1% w/v SDS

2.8 Immunoblotting

Some resolved periplasmic protein samples were electroblotted onto nitrocellulose. Transfer was accomplished overnight using 1 x Western Blot buffer (see below) in a Bio-Rad Trans-Blot cell at a constant current of 120mA. After blocking sites of non-specific protein binding (with 3% BSA in PBS), the nitrocellulose filters were washed 3 times in PBS and probed with a variety of antisera (e.g. rabbit polyclonal LT-B-specific antiserum, BB05 mouse monoclonal) at appropriate dilutions (see below) in 0.1% BSA in PBS for 1-2 hours at room temperature. After 3 more washes in PBS, anti-rabbit or anti-mouse immunoglobulins conjugated to Horseradish peroxidase (Dakopatts, Glostrup, Denmark) were used as the second antibody. Blots were incubated with these conjugates (diluted 1/1000 in 0.1% BSA in PBS) for 1-2 hours at room

temperature. The blots were further washed (5 times) in PBS and then developed with 30mg 4-chloro-1-naphthol (Sigma, Poole, U.K.) dissolved in 10mls methanol/50mls PBS/30µl H_2O_2 for 10-20 minutes. Similar protocols were followed for colony blots when screening colonies for the expression of desired antigens: colonies were transferred onto circular nitrocellulose filters and lysed by inverting them over chloroform for 30minutes. Filters were moistened with PBS and blotted against Whatman 3mM paper and excess cellular debris removed by washing in PBS. The procedure was then exactly the same as for Western blots.

1 x Western blot buffer

190mM glycine

25mM Tris

20% v/v methanol

Antibodies and antisera

All antisera and antibody preparations were diluted before use, by the stated amount, in PBS containing 0.1% BSA.

anti-LT-B - a polyclonal rabbit anti-LT-B antiserum, raised by Maskell *et al.*, (1987). Used at 1/500.

anti-HRV - a polyclonal mouse anti-peptide antiserum, raised by M. Francis (Wellcome Research Laboratories). Used at 1/150 after absorption with *E. coli* lysate (Biorad, Watford, UK).

anti-114 - a polyclonal rabbit anti-peptide antiserum, raised against amino acid residues 364-383 of influenza virus A/Okuda/57 nucleoprotein coupled to Keyhole limpet haemocyanin (KLH), a gift from X.M. Gao (Institute of Molecular Medicine, John Radcliffe Hospital, Oxford).

BB05 - a mouse monoclonal, raised from mice immunised with *Bordetella bronchiseptica*, reactive with P.69 from *B. pertussis* (Novotny *et al*., 1985). Tissue culture supernatant used at 1/20.

VP2-specific monoclonal antibodies - these were raised by P. Barnett and N. Parry (Wellcome Research Laboratories) against a peptide containing amino acid residues 24-33 (a T-cell epitope) and 156-170 (a B-cell epitope) from VP2 together with an added cysteine residue. Three such monoclonals were employed:- 2.7.4.2, 8.5.5.1 and 9.2.5.3. (Barnett and Parry, unpublished observations). Concentrated tissue culture supernatants were used at 1/100.

2.9 G_{M1}-linked ELISAs

To investigate the receptor-binding activity of LT-B-fusion proteins, periplasmic fractions were assayed using a G_{M1}-linked ELISA, essentially as described by Svennerholm & Holmgren (1978). In these assays, 96 well microtitre plates were coated with 1µg/ml G_{M1} (Sigma) in PBS by incubation overnight at 4°C. Unbound material was removed prior to use by washing 3 times in PBS. Serial dilutions (varying from 2 fold to 5 fold) of the periplasmic fractions were added to the wells and incubated at room temperature for 1 hour. Again, unbound material was removed by 3 washes with PBS. Plates were then probed with anti-LT-B for 2 hours at 37°C, washed 3 times in PBS and further probed with an appropriate second antibody (as described for immunoblotting). Purified LT-B (a gift from T. Hirst) was used as a positive control at 2µg/ml in PBS. The absorbance of the wells at 450 or 492nm was read on a Titertek multiscan reader (Flow laboratories).

2.10 Immunological assays

Mice were inoculated with partially purified LT-B fusion protein or with an attenuated strain of *S. typhimurium* expressing LT-B fusion proteins. The immune responses of mice inoculated in such a manner were evaluated in a number of ways.

2.10.1 Serum ELISAs

96 well flat-bottomed microtitre plates (Costar, Massachusetts) were coated with 50µl/well of the appropriate antigen preparation (generally 1µg antigen/ml PBS) at 4°C overnight. Plates were washed twice in PBS, blocked with 3% BSA (incubating at 37°C for 1 hour, 200µl/well) and washed again in PBS (three washes). Serial dilutions (usually 3 fold or 5 fold) of control and test serum samples were made in 0.1% BSA in PBS. 50µl of these dilutions were added per well. Plates were then incubated at 37°C, typically for 2-3 hours. The plates were washed three times in PBS/0.05% Tween 20 (PBS.T), before the addition of the appropriate horseradish peroxidase-conjugated second antibody (50µl/well). After further washing with PBS.T (five washes), the plates were developed with 0.4mg/ml ortho-phenylenediamine dihydrochloride (OPD) dissolved in ELISA substrate buffer (see below), 50µl being added to each well. Reactions were stopped by the addition of 12% H_2SO_4 (100µl/well). Absorbance at 450nm or 492nm was read using a Titertek Multiscan reader (Flow laboratories) or an Anthos htll programmeable plate reader.

ELISA substrate buffer

50ml contains:-

12.2ml 0.1M citric acid

12.8ml 0.25M Na_2HPO_4

25ml distilled water

20µl 30% H_2O_2

20mg OPD

2.10.2 ELISPOT assay

The enzyme-linked immunosorbent spot (ELISPOT) assay, devised by Czerkinsky *et al.* (1983), was used to study the local immune response in the respiratory tract of animals immunised intranasally. Lungs were removed from mice aseptically and chopped into a fine paste with a scalpel. This was suspended in "digestion buffer", comprising 0.5 units/ml Collagenase (Boehringer) and 0.25mgs/ml DNAse I (Boehringer) in PBS/10mM $MgCl_2$, (1-2ml per pair of lungs). This mixture was incubated for 1 hour at 37°C with gentle agitation (on an orbital shaker set at 80rpm). Following digestion the suspension was passed through a 40 gauge mesh (Sigma) to remove the larger pieces of debris. The cell suspension was washed extensively in PBS and enriched for lymphocytes on a "Lymphoprep" (Nycomed Pharma AS, Oslo, Norway) gradient: 5ml of cell suspension were carefully layered onto 10mls of Lymphoprep and spun at 3,000rpm for 12 minutes at room temperature in a Sorvall RT6000B centrifuge. The cells were recovered from the interface, washed again in PBS and resuspended in Click's medium (supplemented with 10% foetal calf serum, penicillin/streptomycin and L-glutamine at the appropriate concentrations). The number of viable lymphocytes in the preparation was determined with a Neubauer chamber and 0.5mls were added to the wells of a 24 well tissue culture plate (Costar). The plates were incubated at 37°C in an atmosphere of 5% CO_2 for a minimum period of 3hrs. After thorough washing in PBS.T, the plates were treated for 2 hours at 37°C with isotype-specific goat anti-mouse immunoglobulins. This was followed by 3 washes in PBS.T and incubation for 2 hours at 37°C with alkaline phosphatase conjugated to rabbit anti-goat immunoglobulins. The plates were extensively washed with PBS.T and finally developed with 1mg/ml 5-bromo-4-chloro-3-indolyl phosphate dissolved in 2-amino-2-methyl-1-propanol buffer (all purchased from Sigma). Discrete spots could then be counted (using a binocular dissecting microscope), which corresponded to the number of antigen-specific antibody secreting cells in each well.

2.10.3 *B. pertussis* colonisation assay

To determine the protective effect (if any) of inoculation with LT-B69, mice were challenged with an aerosol of *B. pertussis* (as described in Chapter 6). Protection was assayed by comparing the viable count of *B. pertussis* isolated from the lungs of infected mice in the vaccinated and control groups. To perform these counts lungs were removed aseptically from 2-3 mice per group and homogenised in 5ml of PBS. Serial tenfold dilutions of the homogenate were made in PBS. 20ul drops of these were transferred to CW/blood agar plates, allowed to dry and incubated at 35°C for 4-6 days to allow colonies to form. In addition to the above assays, mice inoculated with *S. typhimurium* SL1344 *aroA*⁻ *aroD*⁻, harbouring plasmids directing the expression of various LT-B fusion proteins, were examined for the development of splenic cytotoxic T cells specific for the heterologous epitopes fused to LT-B. The protocol used to investigate cytotoxic T cell responses are described separately in chapter 4.

CHAPTER 3

Construction and characterisation of LT-B fusion proteins

3.1 Introduction

The starting-point for the plasmid constructions described in this thesis was pBRD026 (Maskell *et al.*, 1987), a map of which is shown in Figure 3.1.1. This plasmid expresses porcine LT-B constitutively under the control of the P1 "anti-tet" promoter of pBR322. The *Spe*I site located at the termination codon at the 3' end of the LT-B gene was used to clone in an oligonucleotide encoding a specific sequence of amino acids defining a "hinge" which would permit the in-frame coupling of foreign peptides to the LT-B polypeptide. This hinge sequence oligonucleotide was designed to incorporate restriction sites for *Bgl*I and *Spe*I (Figure 3.1.2). In addition, the oligonucleotides contained a one base pair mismatch which prevented the reformation of one of the *Spe*I sites and, when inserted in the correct orientation, abolished the stop codon at the end of the LT-B gene. Thus translation proceeds through the 3' end of the LT-B gene and the oligonucleotide linker to the stop codon at the reformed *Spe*I site. Oligonucleotides encoding epitopes can be cloned into the unique *Bgl*I and *Spe*I sites, allowing expression of the epitopes as fusions to the carboxy terminus of LT-B.

However, there is considerable evidence to show that fusion of foreign peptides to LT-B can profoundly alter the behaviour of the protein. Sandkvist and her collleagues demonstrated that even slight alterations, such as the addition of 6 or 17 amino acids, to the carboxy terminus of LT-B could radically change the properties of the molecule such that it no longer formed pentamers and could not bind to G_{M1} (Sandkvist *et al.*, 1987). Subsequently, Iida and his co-workers (Iida *et al.*, 1989) presented data to suggest that a single mutation, at position 64 in the LT-B amino acid sequence, could interfere with pentamerisation, subunit A-B interactions and G_{M1}-binding.

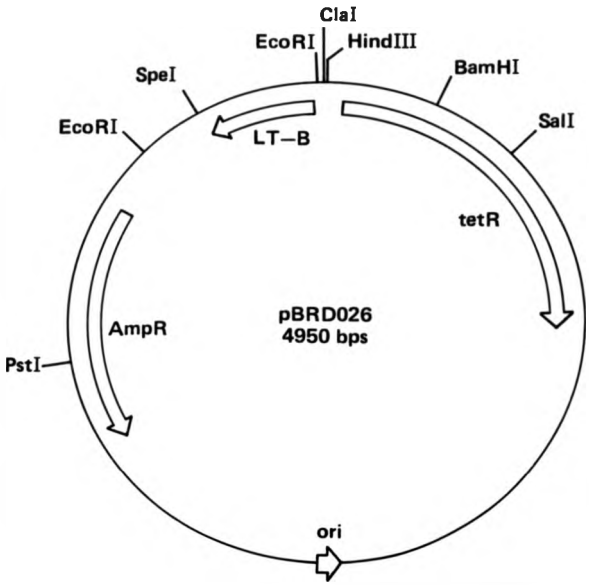


Figure 3.1.1 A map of pBRD026, showing the origin of replication and the LT-B coding region, together with the unique *Spe*I site at the 3' end of the LT-B gene.

Thus it was clear that fusion of peptides to LT-B might alter the characteristics of the carrier molecule. Most importantly, the ability of LT-B to bind G_{M1}, and hence (according to current models) its mucosal immunogenicity, might be diminished or even totally abolished. Careful attention to the design of the hinge region was therefore necessary to reduce these difficulties. Thus a number of important features were incorporated into these sequences.

Firstly, where possible, amino acids with large charged or aromatic side chains were avoided. Secondly, the hinge amino acid sequence was made glycine/proline-rich. Such glycine/proline motifs have been identified in several other proteins (Matsushima *et al.*, 1990) where they are thought to have an important structural role in defining functional domains. Furthermore, the hinge regions of both IgG_1 (Edelman *et al.*, 1969) and IgG_3 (Michaelsen *et al.*, 1977) are known to be rich in proline. It is probable that the small molecular radius of glycine and proline minimizes structural constraints on the polypeptide. More significant perhaps, was the choice of codons specifying these residues, which are only rarely utilised in *E. coli* (Sharp & Li, 1986). Such rare codons are thought to create pauses during translation which allow for the correct folding of the nascent polypeptide into functional domains (Purvis *et al.*, 1987). The *cis-trans* isomerism of proline may also play a role in the creation of these translational pauses. Thus it is hypothesised that there is a brief temporal separation of the translation of the LT-B mRNA from the translation of the fused peptide. This allows the LT-B moiety to assume its native conformation without structural constraints being imposed by the peptide.

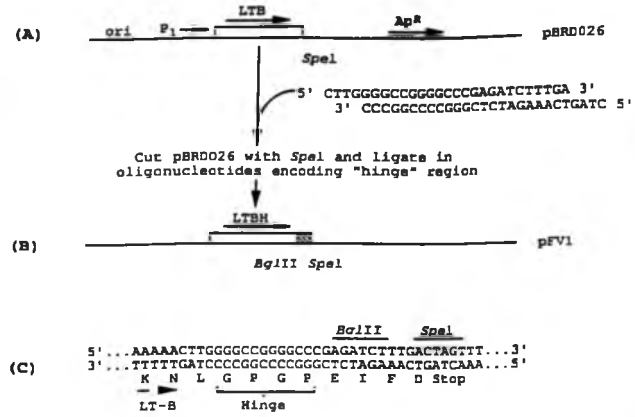


Figure 3.1.2 The strategy used to construct the LT-B fusion vector, pFV1, from pBRD026. (A) Plasmid pBRD026 was cleaved with *SpeI* and synthetic oligonucleotides, with the sequence shown, were inserted to form pFV1 (B). The DNA and corresponding amino acid sequences are shown (C). The inclusion of unique *BglI* and *SpeI* sites allows the insertion of oligonucleotides, (with complementary 5' and 3' ends), encoding heterologous epitopes at the 3' end of the LT-B gene.

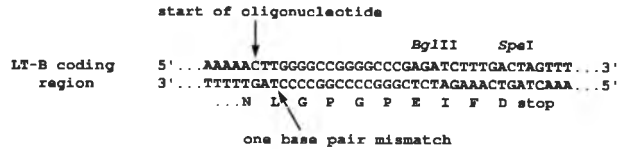
3.2 Construction of the LT-B fusion vector, pFV1

pBRD026 was linearised with *Spe*I, treated with calf intestine alkaline phosphatase and gel-purified. Synthetic oligonucleotides with the desired sequence were prepared by John Keyte (School of Biological Sciences, University of Nottingham). These were incubated with T4 polynucleotide kinase in the presence of ATP, annealed and ligated into *Spe*I-cleaved pBRD026, as described in Chapter 2. The ligation products were used to transform *E. coli* HB101. Miniprep plasmid DNA was obtained from ~36 ampicillin-resistant transformant colonies. This was digested sequentially with *Bgl*I and *Sal* and the products separated on an agarose gel. Recombinant plasmids containing the oligonucleotide insert (with the unique *Bgl*I site) gave rise to 2 bands, ~1.0 and 4.0kb, whereas plasmids without the insert generated a single band only (~5.0kb) corresponding to linearised plasmid. Plasmids which gave the desired restriction pattern with *Bgl*I/*Sal* digests were checked for the retention of an *Spe*I site by digestion with that enzyme.

Plasmids with the expected digestion pattern were investigated further by subcloning a 2.0kb *Pst*I/*Sal* fragment into M13 mp19 RF DNA. Single stranded DNA was prepared and sequenced as detailed in 2.4. In this way the sequence of the synthetic oligonucleotide was confirmed and the sequence across the junctions of the construct could be determined, revealing the orientation of the insert. One such transformant containing correctly inserted oligonucleotides was isolated and termed pFV1, the sequence of which is illustrated in Figure 3.2.

3.3 Selection of foreign epitopes to fuse to LT-B

Having successfully created a vector suitable for the expression of foreign epitopes as carboxy terminus fusions to LT-B the logical next step was deciding which epitopes to insert into the vector. The following criteria were used in the selection:-



The determined DNA sequence across the junctions of the construct.

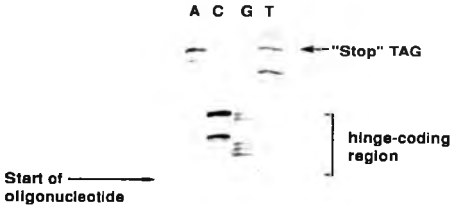


Figure 3.2 The predicted and determined DNA sequence across the junctions of pFV1.

1) the epitopes should be at least moderately well-characterised in other models, preferably by researchers at Wellcome as this would allow access to valuable reagents (such as antisera, monoclonal antibodies, synthetic peptides) and information,

2) the epitopes should be derived from antigens from pathogens of mucosal surfaces, because of the nature of the mucosal immunogenicity of LT-B discussed in the introduction,

3) the antigens from which the epitopes were derived should be the targets for protective immune responses,

4) the epitopes used should encompass a number of different types, ideally both B-cell and T-cell, from bacterial and viral antigens, to investigate as many aspects as possible of the immunogenicity of the resulting fusion proteins,

5) the amino acid and/or nucleotide sequence of the epitope must be available, otherwise the correct oligonucleotides could not be synthesised,

6) the epitope should be mapped to a detailed level (e.g. less than 30 amino acid residues) to allow the entire epitope to be encoded by an easily synthesised oligonucleotide.

Using these criteria, the following epitopes were selected:-

B-cell epitopes

a) The monoclonal antibody BB05 binding-site from P.69, a *B. pertussis* surface-associated protein. Antibodies to this antigen have been shown to protect piglets against *B. bronchiseptica*-mediated disease (Novotny *et al.*, 1985).

b) An epitope present in the major capsid protein (VP2) of human rhinovirus type 2 (HRV 2), (Clarke *et al.*, 1991). A large panel of monoclonal antibodies were raised against a synthetic peptide derived from the VP2 sequence and used to define the epitope (Barnett & Parry, unpublished observations).

T cell epitopes

a) peptide 110 - a peptide from influenza A virus A/Okuda/57 nucleoprotein (NP), which acts as a target for cytotoxic T lymphocytes (CTL) in BALB/c mice with the H-2^d haplotype (Taylor *et al.*, 1987).

b) peptide 167 - a peptide from the same protein which is recognised by T helper (Th) cells in B.10 S mice (H-2^s haplotype), (Gao *et al.*, 1989).

c) peptide 114 - again, a peptide from nucleoprotein, which is a target for CTL in B6 (H-2^b) mice (Gao, unpublished observations).

3.4 Construction of the LT-B/epitope fusion plasmids

The procedure followed was essentially the same for the construction of all the recombinant fusion plasmids. Large scale preparations were made of pFV1. These were digested sequentially with *Bgl*I and *Spe*I (in the appropriate buffers) and gel-purified. Oligonucleotides (synthesised by Hugh Spence and Martin Carrier at Wellcome Research Laboratories) were incubated with T4 polynucleotide kinase in the presence of ATP, annealed and ligated into the vector preparations as described in 2.5.5. The sequences of these oligonuceotides are shown in Figures 3.4.1-3. These oligonucleotide sequences were designed to incorporate restriction sites. Where possible these were unique. This would allow restriction digest patterns to be used to screen for recombinant plasmids with the desired insert. Recombinant plasmids containing oligonucleotide inserts were obtained and were analysed by sub-cloning into M13 mp19 prior to dideoxy sequencing as before. As two restriction sites were used to clone in the oligonucleotides, only one orientation of insertion was possible. DNA sequencing was considered essential to preclude the possibility of an error in the synthesis of the

```
                                    "hinge"
LT-B coding  5'...AAAAACTTGGGGCCGGGGCCCGAGATCGCTCCGCAGCCG
  region     3'...TTTTTGAACCCCGGCCCCGGGCTCTAGCGAGGCGTCGGC
                   N  L  G  P  G  P  E  I  A  P  Q  P

                                                    NarI
GGTCCGCAGCCGCCGCAGCCGCCGCAGCCGCAGCCGGAGGCGCCGGCT
CCAGGCGTCGGCGGGCGTCGGCGGCGTCGGCGTCGGCCTCCGCGGCCGA
 G  P  Q  P  P  Q  P  P  Q  P  Q  P  E  A  P  A


                          CCGCAGCCGGACTAGTTT...3'
                          GGCGTCGGCCTGATCAAA...5'
                           P  Q  P  D  Stop
```

The determined DNA sequence across the junctions of the construct.

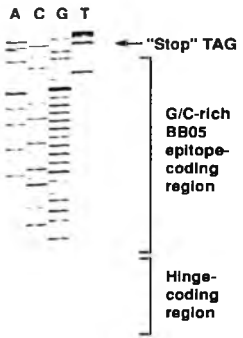


Figure 3.4.1 The predicted amino acid sequence and the predicted and determined DNA sequences across the junctions of the pFV1::69 construct.

```
                                    "hinge"
LT-B coding      5'...AAAAACTTGGGGCCGGGGCCCGAGATCGTTGCT
   region        3'...TTTTTGATCCCCGGCCCCGGGCTCTAGCAACGA
                        N  L  G  P  G  P  E  I  V  A

                          NaeI
      CACAAATCTTGCCTGCCGGCTTGCGTTTACGGTCCGGACTAGTTT...3'
      GTGTTTAGAACGGACGGCCGAACGGCAAATGCCAGGCCTGATCAAA..5'
       H  K  S  C  L  P  A  C  V  Y  G  P  D  Stop
```

The determined DNA sequence across the junctions of the construct

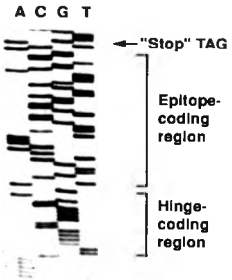


Figure 3.4.2 The predicted amino acid sequence and the predicted and determined DNA sequences across the junctions of the pFV1::167 construct.

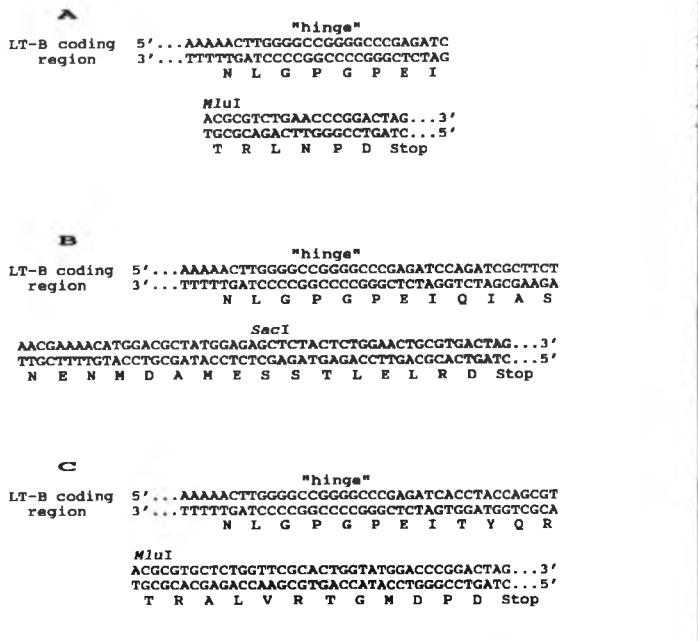


Figure 3.4.3 The DNA and amino acid sequences across the junctions of the pFV1::VP2 (A), pFV1::114 (B) and the pFV1::110 (C) constructs.

oligonucleotides and to confirm the in-frame insertion of the epitope-encoding sequences. The resulting recombinant plasmids are detailed in Table 3.4.

Table 3.4 The chimeric proteins encoded by the various LT-B fusion plasmids

Recombinant plasmid	Recombinant polypeptide	Heterologous Epitope
pFV1	LT-BH	None ("hinge")
pFV1::110	LT-B110	p110, T cell
pFV1::114	LT-B114	p114, T cell
pFV1::167	LT-B167	p167, T cell
pFV1::69	LT-B69	BB05, B cell
pFV1::VP2	LT-BVP2	VP2, B cell

3.5. *In vitro* characterisation of the fusion proteins in periplasmic fractions of *E. coli*

The initial step in the *in vitro* characterisation of the different LT-B chimerae was to ascertain whether these proteins retained the properties of native LT-B: transport to the periplasmic space and assembly into pentamers. To answer this question, periplasmic fractions were prepared from *E. coli* cultures harbouring the various plasmids detailed in Table 3.4, as described in Chapter 2. Periplasmic fractions from HB101 (pFV1) and HB101 (pFV1::69) were obtained. The samples were loaded directly onto an SDS-polyacrylamide gel, or boiled for 10 minutes first, and separated by running the gel overnight. The results are shown in Figure 3.5. It was found that the products of pFV1 (LT-BH) and pFV1::69 (LT-B69), are soluble and transported into the periplasm of *E. coli*, where they are reasonably stable.

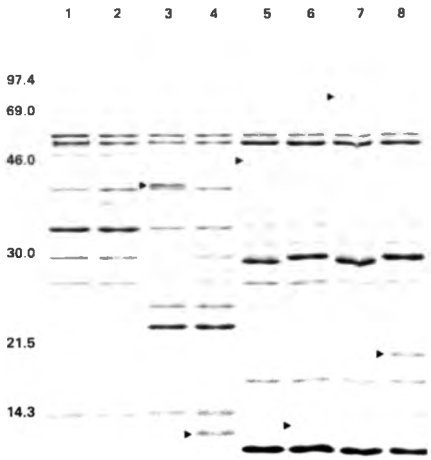


Figure 3.5 SDS-PAGE analysis of periplasmic fractions from *E. coli*. Lane 1, HB101 (unboiled); lane 2, HB101 (boiled); lane 3, HB101 (pMMB68) (unboiled); lane 4, HB101 (pMMB68) (boiled); lane 5, HB101 (pFV1) (unboiled); lane 6, HB101 (pFV1) (boiled); lane 7, HB101 (pFV1::69) (unboiled); lane 8, HB101 (pFV1::69) (boiled). The LT-B- and LT-B69-specific bands are arrowed. Numbers on the left indicate the positions of molecular weight standards with the size (in kiloDaltons) shown.

Furthermore they assemble into pentamers which dissociate into the constituent monomers on boiling. Pentameric LT-BH migrates as a polypeptide of 46 kDa (Hirst *et al.*, 1988) and appears to have a rather higher M, than that of native human LT-B (hLT-B), as expressed by the construct pMMB68 (Förste *et al.*, 1983 and Sandkvist *et al.*, 1987). The monomeric polypeptide migrates as a protein of ~13 kDa and also appears to have a higher M, than that of monomeric hLT-B. The calculated M, of hLT-B is just under 12.0 kDa, corresponding to a 55 kDa pentamer. That of LT-BH is 13 kDa, equivalent to a pentamer of about 60 kDa. These differences are just 1.0 kDa and 5.0 kDa, thus the fact that these can be detected shows the considerable resolution which can be achieved with SDS-PAGE, even on a non-gradient gel.

The fusion protein, LT-B69, runs as a pentamer of ~90 kDa and as a monomer of ~18 kDa. This is a reflection of the coupling of the BB05 epitope, which has a calculated M, of 3 kDa. However the simple addition of 3 kDa to the molecular weight of the protein is insufficient to explain its low mobility. As noted by See & Jackowski (1989), a number of proteins with high proline content have abnormally high molecular weights as determined by SDS-PAGE (Starr & Offer, 1983). The same is true of the P.69 protein from *B. pertussis*, from which the BB05 epitope was taken. Although the protein has a calculated molecular weight of 60 kDa (Makoff *et al.*, 1990), it has an apparent mobility of 69 kDa. This discrepancy is thought to be due to the high proline content, which is particularly notable in the region of the BB05 epitope. Thus it would seem that the high proline content of the BB05 epitope affects the migration of LT-B69 as a whole.

The level of expression of the chimeric proteins was low (just a few % of total protein in periplasmic fractions) but no lower than might be expected in comparison to other proteins expressed from the P1 ("anti-tet") promoter (e.g. Maskell *et al.*, 1987).

One other feature of interest is apparent in Figure 3.5. In tracks 5-8 a prominent low M, (<14.3 kDa) polypeptide is apparent which is not present in tracks 1-4. Whilst all the samples were prepared from *E. coli* HB101 cultures, the samples in tracks 1-4 were prepared from an HB101 strain used at Leicester University, whilst the samples in tracks 5-8 were prepared from the HB101 strain in use at the Wellcome Research Laboratories. Thus there is at least one

distinction between the two strains: the presence or absence of this polypeptide in the periplasmic space. There may well be other differences.

That these proteins were truly of periplasmic origin was confirmed by performing β-galactosidase assays upon the periplasmic and cellular fractions (obtained in the course of the experiments) as described in Chapter 2. β-lactamase assays were also performed on these fractions to estimate the proportion of periplasmic proteins released. Typical results are shown below in Table 3.5.

Table 3.5 Relative enzyme activities in the cellular and periplasmic fractions of *E. coli*

	% β-galactosidase activity	% β-lactamase activity
pFV1 cells	100	52
pFV1 periplasm	0	48
pFV1::69 cells	100	61
pFV1::69 periplasm	0	39

β-galactosidase is an enzyme whose distribution is restricted solely to the cytoplasmic compartment, and therefore it was only detected in the cellular sonicate. Its complete absence from the periplasmic fractions is evidence that the integrity of the inner membrane is not impaired by the lysozyme/EDTA treatment. Therefore any proteins present in these fractions must be periplasmic or membrane-associated in nature. It has been shown previously that hLT-B is truly periplasmic in nature and not membrane-associated (Hirst *et al.*, 1984*a*).

In contrast, β-lactamase is efficiently transported to the periplasmic space. Clearly, this is essential if the enzyme is to be protective against β-lactam antibiotics, which act on penicillin-binding proteins present in the periplasm. The results of the β-lactamase assays demonstrate that, in general, rather less than half of the total cellular pool of β-lactamase is released by the lysozyme/EDTA treatment. Thus, if one assumes all β-lactamase to have a periplasmic location,

the sphaeroplasting protocol employed yields less than fifty percent of total periplasmic protein. However, it does ensure that there is no contamination by cytoplasmic protein in the samples.

3.6 Immunoblotting

Periplasmic fractions were prepared from *E. coli* HB101 cultures harbouring the novel constructs detailed in Table 3.4. These were subjected to SDS-PAGE and transferred to nitrocellulose. The filter was probed with anti-LT-B polyclonal antiserum. The results are shown in Figure 3.6.1. As experienced previously with LT-B69, all the fusion proteins formed pentameric complexes, ranging in apparent molecular weight from about 60 kDa to 97 kDa (i.e. higher than would be expected from LT-B or LT-BH alone). Upon boiling these bands disappear simultaneously with the formation of new bands which migrate with apparent molecular weights ranging from ~13 kDa to 18 kDa, which correspond to the constituent monomers of the fusion proteins (again, higher than would be expected from monomers of LT-B or LT-BH). It would seem that the pentameric form of the fusion proteins is subject to degradation - the bands are diffuse and poorly defined. In contrast the monomeric polypeptides form quite well defined bands. This is slightly surprising because many of the epitopes of CT-B and LT-B are known to be heat-labile and are destroyed by boiling (Kazemi and Finkelstein, 1990). Possibly the boiling results in just a few nearly identical stable forms of the respective polypeptides (i.e greater homogeneity), leading to greater localisation of the material on the gel and corresponding blot. An alternative explanation for the faint staining of the bands (if not their diffuse nature) is that the addition of the heterologous epitopes to the C terminal affects the antigenicity of the LT-B moiety, perhaps masking some of the LT-B epitopes when present in a pentameric complex. Upon boiling of the protein, yielding the constituent monomers, these epitopes are unmasked and made available for binding anti-LT-B antibody.

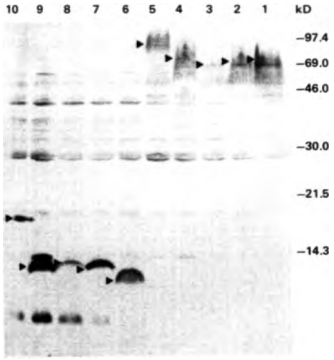


Figure 3.6.1 Immunoblot analysis of periplasmic fractions of *E. coli*. Fractions were prepared as described in 2.7.1. These were subjected to SDS-PAGE and electroblotted onto a nitrocellulose filter. This was probed with a polyclonal LT-B-specific antiserum as detailed in 2.9. Lanes 1 and 6, HB101 (pFV1::VP2); lanes 2 and 7, HB101 (pFV1::110); lanes 3 and 8, HB101 (pFV1::167); lanes 4 and 9, HB101 (pFV1::114); lanes 5 and 10, HB101 (pFV1::89). Lanes 1-5 loaded with unboiled samples, lanes 6-10 loaded with boiled samples. The numbers on the right refer to pre-stained molecular weight standards with the size shown in kiloDaltons.

The identity of the LT-B69 and LT-BVP2 fusion proteins was confirmed in subsequent immunoblotting experiments using sera specific for the respective fused epitopes. Figure 3.6.2 shows periplasmic fractions from HB101 (pFV1) and HB101 (pFV1::69) probed with the BB05 mAb. This monoclonal antibody is seen to react solely with the sample prepared from HB101 (pFV1::69). The high avidity of this reagent enables detection of a wide range of breakdown products in the unboiled sample. The majority of the material however is present as pentameric LT-B69, with an apparent molecular weight of ~90 kDa, as detected by the LT-B-specific antiserum. Upon boiling only a single band is detected. This equates to the 18 kDa band similarly detected with LT-B-specific antiserum and represents monomeric LT-B69. Thus the presence of the LT-B carrier molecule does not appear to inhibit the antigenicity of the BB05 epitope, at least in a denatured form in a Western blot.

Similarly, periplasmic fractions were prepared from HB101 (pFV1) (boiled sample only) and HB101 (pFV1::VP2) (both boiled and unheated samples). These were resolved by SDS-PAGE and transferred to a nitrocellulose filter. This filter was probed with a VP2 peptide-specific polyclonal antiserum (a gift from M. Francis, Wellcome Research Laboratories). The results are shown in Figure 3.6.3. It can be seen from this figure that the serum does not react with the HB101 (pFV1) extract nor, surprisingly, with the unboiled HB101 (pFV::VP2) extract. In contrast, the boiled extract gives a band at the expected position, which is readily bound by the antiserum. Why this should be so is unclear. It may be that, unlike the LT-B69 fusion, there is some sort of steric hindrance in the pentameric complex (even in the denatured form), which prevents the VP2 epitope from being exposed. In this respect it might be significant that the sequence of the added epitope is very short (just 6 extra amino acid residues), whereas the fused epitopes of the other LT-B chimerae are quite long (20-30 extra amino acid residues) in comparison.

Similar experiments, using polyclonal antisera specific for the 110 or 167 epitopes, failed to detect the respective fusions on Western blots, despite the increased molecular weight apparent in blots probed with anti-LT-B antiserum. Possible explanations for this observation are discussed in the following section.

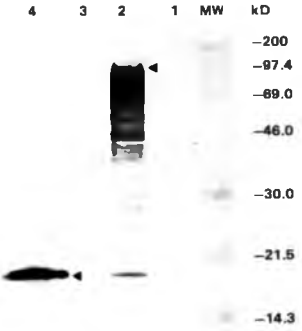


Figure 3.6.2 Immunoblot analysis of periplasmic fractions of *E. coli* HB101 pFV1 and HB101 pFV1::69. Periplasmic fractions were obtained as described in 2.7.1, subjected to SDS-PAGE and electroblotted onto a nitrocellulose filter (as detailed in 2.8 and 2.9). This filter was probed with the mouse monoclonal antibody BB05. Lane 1, HB101 (pFV1) (unboiled); lane 2, HB101 (pFV1::69) (unboiled); lane 3, HB101 (pFV1) (boiled); lane 4, HB101 (pFV1::69) (boiled). The positions of pre-stained molecular weight standards are shown on the right.

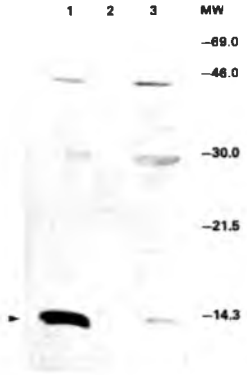


Figure 3.6.3 Immunoblot analysis of periplasmic fractions from *E. coli* HB101 (pFV1) and HB101 (pFV1::VP2). Samples of periplasmic proteins were prepared, separated by SDS-PAGE and electroblotted onto nitrocellulose as described previously. The filter was probed with a polyclonal VP2 peptide-specific antiserum. Lane 1, HB101 (pFV1::VP2) (boiled); lane 2, HB101 (pFV1::VP2) (unboiled); lane 3, HB101 (pFV1) (boiled). Numbers on the right refer to the position of standards with the molecular weight shown in kiloDaltons.

3.7 G_{M1}-linked ELISAs

The observation that the various LT-B chimeric proteins formed pentamers was encouraging because it is believed that only LT-B in the pentameric form has a high affinity for G_{M1} (which in turn is believed to be central to the immunogenicity of LT-B). Svennerholm & Holmgren (1978) devised a G_{M1}-linked ELISA method to investigate G_{M1}-binding by CT and LT. This method was adapted (as described by Maskell *et al.*, 1987) to study the affinity for G_{M1} of the various LT-B fusion proteins. In one such experiment, periplasmic extracts were prepared from *E. coli* HB101, HB101 (pFV1) and HB101 (pFV1::69). These were adjusted to an equal concentration of total protein, and added to G_{M1}-coated or blank, uncoated 96 well flat-bottomed microtitre plates. The samples were serially diluted 1:3 down the plate using PBS as the diluent and then probed with rabbit anti-LT-B antiserum (the assay method is described in detail in Chapter 2). The absorbance was read and plotted against dilution. The results are shown in Figure 3.7.1. This shows that on plates not coated with G_{M1}, very little LT-B could be detected whether the antigen in question was LT-BH or LT-B69. Equally, there was no antibody bound if a periplasmic fraction from HB101 alone was applied to a G_{M1}-coated plate. If, however, fractions from HB101 (pFV1) or HB101 (pFV1::69) were applied to a G_{M1}-coated plate LT-B was readily detected, indicating that LT-BH and LT-B69 both retain the ability to bind G_{M1}. Purified LT-B (a generous gift from T. Hirst, University of Leicester) was included at 1µg/ml as a positive control. Thus, as was experienced with the immunoblotting experiments, the presence of the BB05 epitope at the C-terminal of LT-B does not interfere with the antigenicity of the LT-B moiety nor, more importantly, does it greatly inhibit binding of G_{M1}.

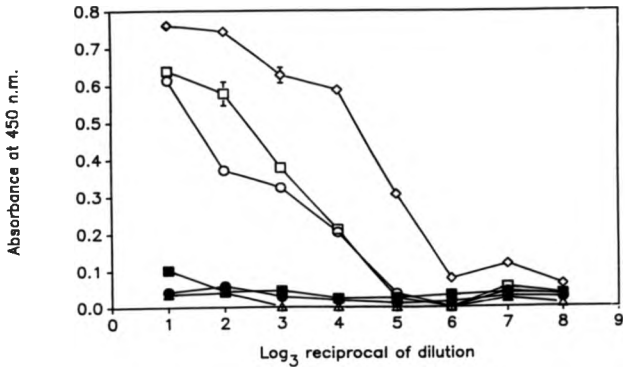


Figure 3.7.1 The ganglioside-binding activities of LT-BH and LT-B69 were investigated by an ELISA technique. Periplasmic fractions from *E. coli* were obtained by the method previously described, adjusted to an equal concentration of total protein and applied to G_{M1}-coated microtitre plates (open symbols) or uncoated blank plates (filled-in symbols). These were then probed with polyclonal anti-LT-B antiserum and processed following standard ELISA protocols. HB101 (△, ▲); HB101 (pFV1) (○,●); HB101 (pFV1::69) (□,■) and purified LT-B alone (◇).

In a similar assay (the results of which are shown in Figure 3.7.2), the antibody probe used was the monoclonal antibody BB05. As expected, no reactivity was observed from HB101 (pFV1) extracts on either blank or G_{M1}-coated plates. In contrast, LT-B69 could be detected easily, but only on a G_{M1}-coated plate, once again demonstrating the specificity of the assay. Furthermore, this proves that the LT-B carrier molecule does not significantly interfere with the antigenicity of the BB05 epitope in a nearly native state (the fusion protein is presumably in a slightly different conformation if bound to G_{M1} than if in free solution) whilst the earlier immunoblotting experiment with the same mAb probe had shown that this was true if the protein was highly denatured.

Similarly, periplasmic fractions from HB101 (pFV1) and HB101 (pFV1::VP2) were compared. These were added to G_{M1}-coated plates and probed with one of three mouse monoclonal antibodies (termed 2.7.4.2, 8.5.5.1 and 9.2.5.3 respectively). These were prepared by P. Barnett (Wellcome Research Laboratories; Barnett & Parry, personal communication), having been raised against a peptide consisting of amino acid residues 24-33 (a T cell epitope) and 156-170 (a B cell epitope) of VP2, together with an added C terminal cysteine residue to facilitate coupling of the peptide to keyhole limpet haemocyanin (KLH). Two of these monoclonals (2.7.4.2 and 8.5.5.1) could detect the LT-BVP2 fusion protein on ganglioside coated plates (Figure 3.7.3, graphs A and B) whilst the other (9.2.5.3) could not (data not shown). This difference may be due to the first two antibodies recognising a particular peptide conformational epitope which is conserved in the fusion protein whilst the latter antibody recognises a conformational epitope, unique to the peptide-KLH conjugate against which it was raised, which is not represented in the LT-BVP2 fusion (when bound to G_{M1}). In a similar experiment, a polyvalent VP2 peptide-specific antiserum (described in 3.6), was used as a probe. The results are shown in Figure 3.7.3, graph C. It is clear from these results (Figure 3.7.3, graphs A, B and C) that the short VP2 peptide sequence is present in the LT-BVP2 fusion and is accessible to antibody when the molecule is in a near native state. In addition, an assay was performed on the same periplasmic fractions using the rabbit polyclonal anti-LT-B antiserum as the probe. The results are shown in Figure 3.7.3, graph D.

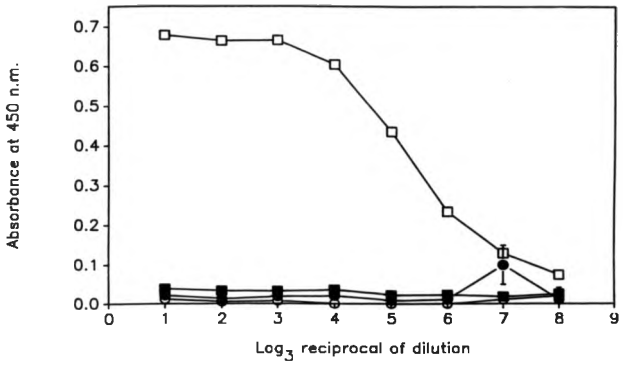


Figure 3.7.2 The identity and ganglioside-binding activity of the LT-B69 fusion protein was confirmed by means of an ELISA (described in 2.10). Periplasmic fractions were prepared from *E. coli*, adjusted to an equal concentration of total protein and allowed to react with microtitre plates coated with G_{M1} (open symbols) or microtitre plates left uncoated as blanks (filled-in symbols). The plates were then probed with the monoclonal antibody, BB05. HB101 (pFV1) (○,●); HB101 (pFV1::69) (□, ■).

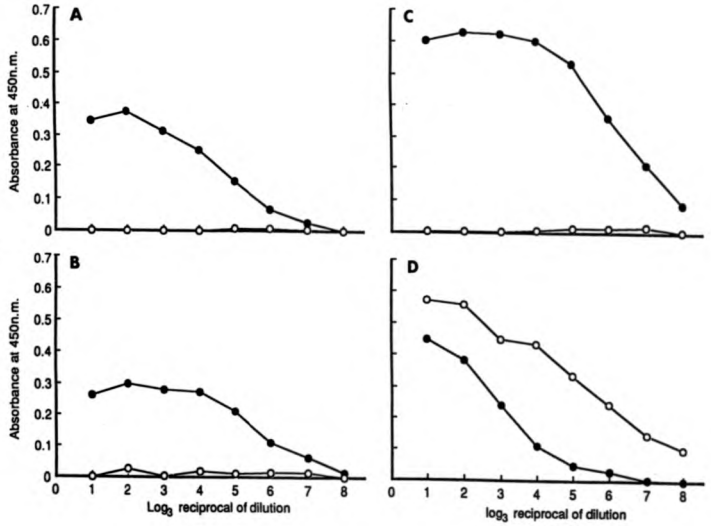
Figure 3.7.3 The identity and G_{M1}-binding activity of LT-BVP2 was confirmed in a number of ELISAs. Periplasmic fractions were prepared from *E. coli* HB101 (pFV1::VP2) and control samples were prepared from HB101 (pFV1). These were adjusted to an equal concentration of total protein, applied to G_{M1}-coated microtitre plates and probed with a variety of antibodies. HB101 (pFV1::VP2) (●) and HB101 (pFV1) (○) were probed with:-

A) VP2 peptide-specific monoclonal antibody 1.2.5.3

B) VP2 peptide-specific monoclonal antibody 8.5.5.1

C) VP2 peptide-specific polyclonal antiserum

D) LT-B-specific polyclonal antiserum


A
B
C
D
Absorbance at 450n.m.
Absorbance at 450n.m.
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0 1 2 3 4 5 6 7 8
Log3 reciprocal of dilution
log3 reciprocal of dilution

There is noticeably less binding of the anti-LT-B antibodies by LT-BVP2 than by LT-BH. This may be due to diminished affinity for G_{M1} as a result of the presence of the heterologous fused epitope. An alternative explanation is that there is an equal amount of bound antigen but less binding of the LT-B-specific antibodies. This seems unlikely because such an effect is not marked with the other LT-B fusion proteins in which the fused epitopes are much larger than that in LT-BVP2. Other G_{M1}-linked ELISAs were performed on periplasmic fractions prepared from *E. coli* HB101 pFV1::114 and control samples from HB101 (pFV1). These samples were probed with a peptide 114-specific polyclonal rabbit antiserum (a kind gift from X.M. Gao, Institute of Molecular Medicine) (Figure 3.7.4, graph A) or polyclonal anti-LT-B antiserum (Figure 3.7.4, graph B). The peptide 114-specific antiserum detected LT-B114 on a G_{M1}-coated plate, but not LT-BH, thus confirming the identity of the fusion protein. Anti-LT-B antiserum reacted equally well with LT-BH and LT-B114, indicating that the LT-B114 fusion protein and LT-BH had comparable G_{M1}-binding activities, assuming they were bound to by anti-LT-B antibody with equal affinity.

As previously experienced in immunoblotting experiments, polyclonal antisera specific for p110 or p167 failed to detect the respective fusion proteins in ELISAs (data not shown). The DNA sequence of pFV1::110 and pFV1::167 was confirmed previously so one would expect correct expression of the fusion proteins. Therefore, it would seem that the likely explanation for this failure to detect expression is that the LT-B110 and LT-B167 fusions are more susceptible to degradation than the other fusion proteins, such that short fragments containing the relevant epitopes run off the bottom of gels during SDS-PAGE (and so are not present on the resulting blots) and are unable to bind to G_{M1} (and so are not detected in G_{M1}-linked ELISAs). Alternatively, the major epitope-specific antibodies in the antisera may not have recognised the heterologous epitopes within the context of the LT-B fusion protein (whilst they did bind to the synthetic peptides, corresponding to the epitopes, when adsorbed onto microtitre plates).

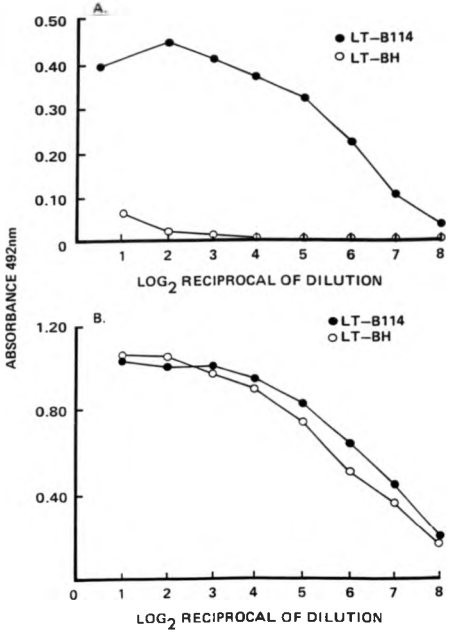


Figure 3.7.4 The identity and G_{M1}-binding activity of LT-B114 was confirmed by means of a G_{M1}-linked ELISA. Periplasmic fractions were prepared (as described previously) from *E. coli* HB101 (pFV1) and HB101 (pFV1::114). These were applied to G_{M1}-coated microtitre plates and probed with heterologous epitope-specific (graph A) or LT-B-specific (graph B) antisera.

3.8 Summary

The results obtained from the various immunoblotting experiments and ELISAs performed in order to determine the behaviour of the LT-B chimeric proteins *in vitro* are summarised in Table 3.8 below.

Table 3.8 Confirmation of the identities and G_{M1}-binding properties of the various LT-B fusion proteins

Recombinant Protein	Detection by:-			
	Immunoblot		G_{M1} ELISA	
	LT-B	Epitope	LT-B	Epitope
LT-BH	+	N.D.	+	N.D.
LT-B110	+	-	+	-
LT-B114	+	N.D.	+	+
LT-B167	+	-	+	-
LT-B69	+	+	+	+
LT-BVP2	+	+	+	+

N.D. = Not done, + = detected, - = not detected

The results obtained from the *in vitro* characterisation of the fusion proteins show that certain additions to the C terminal of LT-B do not necessarily lead to the loss of properties associated with the native molecule, namely accumulation in the periplasmic space of *E. coli*, formation of pentamers and the ability of the pentameric complex to bind to its ganglioside receptor. It is impossible to say to what extent the preservation of these properties is dependent upon the inclusion of the hinge region between the carrier molecule and the fused epitope. One way to determine the significance of the hinge might be to design oligonucleotides corresponding to the sequences of the fused epitopes, such that they could be ligated in directly at the *Spe*I site of pBRD026. One could then compare the resulting fusion proteins with those expressed by pFV1 and its derivatives.

Another observation which can be made is that the chimeric LT-B monomers are capable of pentamerisation in the absence of the A-subunit. This phenomenon has been noted previously for native LT-B (Sandkvist *et al.*, 1987). Indeed, it would be interesting to discover whether the chimeric LT-B molecules were still capable of interacting with the A-subunit. The same authors (Sandkvist *et al.*, 1987) found that the addition of seven amino acids to the C terminal of LT-B prevented the molecule from immunoprecipitating in the presence of A-subunit-specific antiserum (when co-expressed with A-subunit by *E. coli*), whilst the altered molecule was readily precipitated by anti-A/B-subunit antiserum, implying that the A-subunit would no longer associate with the altered B-subunit. It might be argued that, should LT-B fusion proteins ever be used in the production of human or animal vaccines, an inability of the B-subunit to associate with A-subunit is to be preferred so as to prevent the possibility of toxicity (even though it is difficult to envisage conditions where there might be contamination by exogenous toxin A-subunit).

A further point to be considered is the location of the C terminal of LT-B in terms of whether it is surface-exposed or buried within the molecule. Considering the significance of the C terminal in subunit-A/B interactions it would seem probable that the C terminal is surface-exposed. Certainly the G_{M1}-linked ELISA results obtained using heterologous epitope-specific antibody probes would seem to indicate that, in general, fusion to LT-B has little effect on the

accessibility or antigenicity of the epitopes. Indeed, the surface-exposed nature of the C terminal of LT-B has recently been confirmed in the structure of the molecule reported by Sixma *et al.*, (1991).

CHAPTER 4

Inoculation of mice with attenuated *S. typhimurium* sp. harbouring LT-B fusion protein expression plasmids

4.1 Introduction

A number of approaches have been employed in an attempt to stimulate mucosal immune responses against bacteria. The simplest involve the use of the oral route to administer antigens which elicit good systemic responses when given parenterally, such as killed whole cell bacterial vaccines. Numerous studies have been performed using potential vaccines against such bacterial pathogens as *Streptococcus mutans* (Bonta *et al.*, 1979) and *Vibrio cholerae* (whole cell vaccine given in conjunction with purified antigen; Svennerholm & Holmgren, 1986).

Alternatively, mucosal immunity can be generated by the administration of live, spontaneously-arising, attenuated mutant strains, such as the avirulent T_{2a} Istrati strain of *Shigella flexneri* (Istrati *et al.*, 1967) or the attenuated streptomycin-dependent (SmD) strain of the same organism (Mel *et al.*, 1971). Considerable effort has been devoted to the development of an attenuated *Salmonella typhi* strain suitable for use as a live oral vaccine against typhoid fever in humans. Several attenuated strains of other species of *Salmonella* have also been evaluated, with varying degrees of success. For instance, temperature-sensitive strains, unable to grow at the temperature of the host, have been reported for *S. enteritidis* (Fahey & Cooper, 1970*a,b*) and for *S. typhimurium* C5 (Hormaeche *et al.*, 1981). As with *Shigella*, SmD strains have also been described, notably attenuated strains of *S. typhi* (Reitman, 1967; Mel *et al.*, 1974). The Reitman strain was found to be safe and efficacious in humans, but practical problems prevented its adoption as a vaccine (Levine *et al.*, 1976). Germanier and Furer (1975) subjected *S. typhi* strain Ty2 to chemical and ultraviolet mutagenesis and isolated a *galE* mutant. The *galE* gene encodes the enzyme uridine

diphoshate (UDP) galactose-4-epimerase, which catalyses the conversion of UDP-glucose to UDP-galactose. UDP-galactose is an essential component of smooth lipopolysaccharide (LPS), thus *galE* mutants can only make rough LPS, a characteristic associated with attenuation. (If exogenous galactose is present, the *galE* mutants can convert it into UDP-galactose via galactose-1-phosphate, so allowing the synthesis of smooth LPS. However, exogenous galactose is toxic because galactose-1-phosphate accumulates intracellularly, resulting in cell lysis). The *galE* mutant obtained by Germanier, *S. typhi* Ty21a, was found to be safe and protective in human volunteers, affording protection in two large field trials (in Egypt and Chile) ranging from 96-67%, (Wahdan *et al.*, 1982 and Levine *et al.*, 1987 respectively). However, subsequent work (Hone *et al.*, 1988) proved that the *galE* mutation was not the major attenuating lesion in *S. typhi* Ty21a. Partly as a result of the uncertainty surrounding the genetic basis of attenuation in the strain and partly because of a number of practical considerations (vaccine instability, variable efficacy and the requirement for multiple doses), it was not widely adopted for routine vaccination.

This experience highlights the advantage of a rather different approach (which has already been used with considerable success by a number of research groups), that of utilising recombinant DNA technology to introduce defined mutations into the genome of pathogenic organisms, so as to bring about attenuation in a rational manner. This method is more advantageous than evaluating spontaneous or chemically-induced mutants because the introduction of defined deletion mutations at different, widely-separated loci on the bacterial chromosome makes reversion an extremely unlikely possibility. In addition, it allows for a wide spectrum of attenuation, from full virulence to complete avirulence, depending on the deletions made. Thus an optimal level of attenuation can be achieved, at which the organism does not cause any symptoms but persists for long enough in sufficient numbers to elicit strong immune responses. Amongst the genes which have been made the targets for deletion or inactivation in *Salmonella* spp. are *cya* and *crp* (Curtiss & Kelly, 1987), *phoP/phoQ* (Miller *et al.*, 1989), *htrA* (Johnson *et al.*, 1990), *ompR* (Dorman *et al.*, 1989) and several of the *aro* genes (Hoiseth & Stocker, 1981, Dougan *et al.*, 1988). These latter genes encode the essential enzymes

controlling the biosynthesis of aromatic amino acids, and the compounds dihydroxybenzoate (DHB) and p-aminobenzoic acid (PABA) - essential intermediates in the synthesis of nucleotides.

As well as the obvious benefits of an oral typhoid vaccine, such attenuated strains of *Salmonella* have been demonstrated to be ideal carriers of heterologous antigens to both the systemic and the secretory immune systems of experimental animals (reviewed by Dougan & Tite, 1990). This is a reflection of the course of infection which results from oral inoculation of certain strains of mice with *S. typhimurium*. The organism penetrates to deep tissues (such as the spleen) after specific invasion of the GALT (Carter & Collins, 1974). Thus immunocompetent cells from both the systemic and the local immune systems directly encounter antigen. As a result, such bacterial vectors are capable of stimulating serum and secretory antibody responses and cell mediated immunity. There are reports of avirulent *Salmonella* strains having been used to elicit secretory and humoral responses in mice to *E. coli* LT-B and K88 fimbriae (Maskell *et al.*, 1987 and Stevenson & Manning, 1985 respectively), *Shigella sonnei* O-antigen (Black *et al.*, 1987) virulence determinants from *Streptococcus mutans* (Curtiss *et al.*, 1986) and cell mediated immune responses to influenza virus nucleoprotein (Tite *et al.*, 1990). The use of live attenuated *Salmonella* as carriers of foreign antigens has several attractive features. The organism is well-characterised and amenable to genetic manipulation. Potentially protective antigens from many pathogenic viruses and bacteria have been identified. These can be cloned and introduced into *Salmonella*, allowing the organism to express the protective antigens from several pathogens simultaneously. The resulting recombinant multivalent vaccine strain could be given orally, stimulating mucosal, humoral and cell-mediated responses. Thus one oral inoculation could, in theory, provide immunity to several different diseases.

4.2 Materials and Methods

4.2.1 G_{M1}-linked ELISA on *S. typhimurium* lysates

G_{M1}-linked ELISAs were performed on whole cell lysates of *S. typhimurium* SL1344 *aroA*$^-$ *aroD*$^-$ harbouring various constructs essentially as described previously (this thesis and Maskell *et al.*, 1987). Five $O.D._{600nm}$ units of an overnight culture were pelleted by centrifugation in an eppendorf tube. The supernatant was removed and the tube carefully dried with absorbent paper. The tube was then knocked vigorously to loosen the pellet. Bacterial cell envelopes were disrupted by resuspending the pellet in 500µl of 10% (w/v) sarkosyl (sodium salt of N-lauroylsarcosine, obtained from Sigma, U.K.) and left at room temperature for 30-45 minutes with occasional gentle agitation. 1ml of L-broth was added to dilute the viscous solution and replicate 50µl aliquots were applied to a G_{M1}-coated 96 well microtitre plate. The assay procedure was then as that followed for *E. coli* periplasmic fractions, described in Chapter 2.

4.2.2 Inoculation of mice

4.2.2.1 Preparation of bacterial inocula for intravenous infection of mice

Single colonies of SL1344 *aroA*$^-$ *aroD*$^-$ harbouring various constructs were inoculated into L-broth and grown statically overnight at 37°C. 1ml aliquots of these were then stored in liquid nitrogen. The next day, the viable count of this stock culture was determined by thawing an aliquot and diluting it in PBS to 10^{-6} and 10^{-7}. 1ml of the 10^{-6} and 2ml of the 10^{-7} dilutions were incorporated into triplicate samples of molten L-agar and used to pour plates. These were dried and incubated overnight at 37°C and the number of colony-forming units (cfu) determined.

When required to inoculate mice, an aliquot was thawed and diluted in PBS to approximately $1x10^7$ cfu/ml and $1x10^6$ cfu/ml. Mice were inoculated with 0.1-0.2ml of one of

these suspensions into the tail vein. The exact inoculum dose was determined by incorporating an aliquot of surplus inoculum into molten L-agar and counting as before.

4.2.2.2 Oral inoculation of mice with *S. typhimurium*

To prepare inocula for oral infection of mice, frozen aliquots of stock cultures were used to inoculate 2 x 250ml bottles of L-broth which were incubated statically overnight at 37°C. Cells were harvested by centrifugation (~11,000g for 20 minutes) and the pellet resuspended in 4mls PBS. This procedure typically yielded a suspension with a count of ~$5x10^{10}$ cfu/ml.

Oral inoculation was performed with a gavage tube: a blunt 2 inch, 18 gauge needle with a smoothed 2mm wide metal sheath over the end. Mice were lightly ether-anaesthetised and held by the skin between the eyes. They were allowed to swallow the needle which passed into the stomach. 200µl of inoculum was administered from a 1ml syringe attached to the needle. The exact dose delivered was determined as before by incorporating some of the inoculum into molten L-agar and performing a viable count.

4.2.3 Determination of growth curves in infected mice

After inoculation with *S. typhimurium*, mice (generally four per group) were sacrificed at various time-points. Their spleens and livers were removed aseptically into separate sterile stomacher bags (Seward Medical Ltd.). 10mls of sterile distilled water were added to each bag and the organs homogenised for 3 minutes in a Colworth Stomacher. Serial tenfold dilutions of the homogenates were made in PBS. 1ml of these dilutions were incorporated into aliquots of molten L-agar, with and without ampicillin and used to pour plates. The agar was allowed to set and the plates were then incubated overnight at 37°C. Alternatively, serial tenfold dilutions of the homogenates were made directly in 0.9ml aliquots of molten agar. Replicate 100µl drops

of these dilutions were dispensed with a micropipette onto a Petri dish and allowed to set. The dishes were sealed with parafilm to prevent the drops from drying excessively. The next day colonies were counted with the aid of a colony counter. In this way, the total number of live *S. typhimurium* in these organs could be determined together with the proportion of those bacteria which retained the ampicillin resistant phenotype associated with carriage of the LT-B fusion vector plasmids.

4.2.4 T cell assays

The generation of cytotoxic T cells specific for the 110 and 114 epitopes in the corresponding LT-B fusion proteins was investigated in mice which had been inoculated intravenously with SL1344 *aroA*$^-$ *aroD*$^-$ harbouring the appropriate constructs. At various times (generally 3-4 weeks) after priming or boosting, 2 mice per group were sacrificed by cervical dislocation and their spleens removed aseptically into 10mls of sterile PBS. Single cell suspensions were prepared by gentle grinding between frosted glass slides. The cells were washed twice in PBS and cultured ($1-2 \times 10^7$) in 10mls of Click's medium (supplemented with penicillin/streptomycin, L-glutamine and 0.5% normal mouse serum) together with normal spleen cells (5×10^6) or normal spleen cells which had been infected (*in vitro* with allantoic fluid containing infectious influenza A/Puerto Rico/8/34 [PR8] virus) as stimulator cells. After incubation for five days (at 37°C in an atmosphere of 5% CO_2) the cultured cells were washed and serially diluted three fold. These dilutions were added to the wells of a 96 well U-bottomed microtitre plate (Costar). Appropriate target cells (either P815 or EL4 tumour cell lines) were radiolabelled by incubation at 37°C for 1 hour with 300µCi of ^{51}Cr-containing sodium chromate (Amersham, U.K.). The targets were then extensively washed and incubated for a further hour with 200 haemagglutinating units (HAU) of PR8 virus or 100µg of the relevant peptide (110 or 114). Control target cells were not exposed to antigen of any kind. A constant number of target cells were added to the effector cells in the microtitre plate such that the maximum effector:target

ratio was about 20:1. The plate was subjected to centrifugation (1,500 rpm for 5 minutes in a Sorvall RT6000B centrifuge) and incubated for 6 hours, after which time the culture supernatant was harvested. The cytolytic activity of the cultured effector cells was measured by the amount of radiolabel released. Spontaneous release of radiolabel was determined by incubating labelled targets in isolation. Maximum release was achieved by lysing target cells with PBS containing 1% triton.

4.3 Results

4.3.1 Expression of LT-B fusion proteins in *S. typhimurium*

Novel plasmid constructs were introduced into the smooth strain of *S. typhimurium*, SL1344 *aroA* ⁻ *aroD* ⁻, by means of the two-step transformation/transduction method described in sections 2.5.6.-7. Whole cell lysates were prepared (as described in 4.2.1) and analysed by G_{M1}-linked ELISA, using LT-B-specific antiserum. The results are shown in Figure 4.3.1.1. It was found that LT-B and the various LT-B fusion proteins were expressed in *S. typhimurium* SL1344 *aroA* ⁻ *aroD* ⁻ in a form capable of binding to G_{M1} and reacting with anti-LT-B antiserum. Similarly, when the monoclonal antibody BB05 was used as a probe in such assays, LT-B69 expression could be detected in the strain carrying the appropriate plasmid (Figure 4.3.1.2). These findings are consistent with those described previously in Chapter 3. Moreover, an auxotrophic attenuated strain of *S. typhimurium*, SL3261, was shown previously to express LT-B under the influence of the same P1 "anti-tet" promoter (Maskell *et al.*, 1987). Nevertheless, these experiments were necessary to ascertain that expression of these molecules in a different host organism did occur and did not prevent the LT-B fusion proteins from binding to G_{M1}.

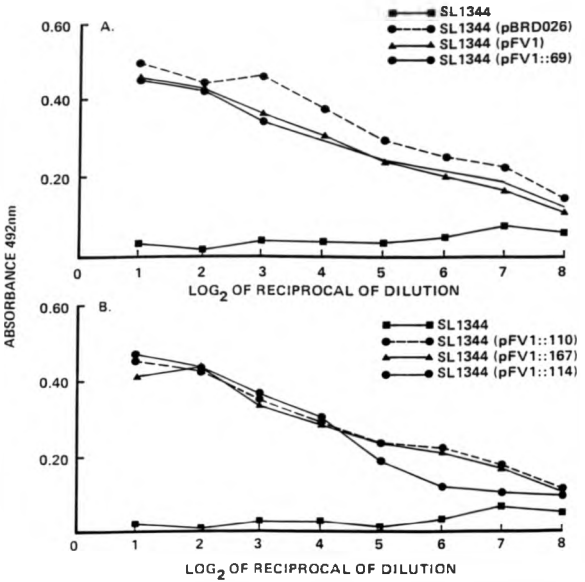


Figure 4.3.1.1 The G_{M1}-binding activity of LT-B and various LT-B fusion proteins in whole cell lysates of *S. typhimurium*. Whole cell lysates were prepared from *S. typhimurium* SL1344 (the parental strain) and from strains harbouring plasmids directing the expression of LT-B or LT-B fusion proteins. These were probed in a G_{M1}-linked ELISA with a polyclonal anti-LT-B antiserum. The results are shown in graphs A and B above.

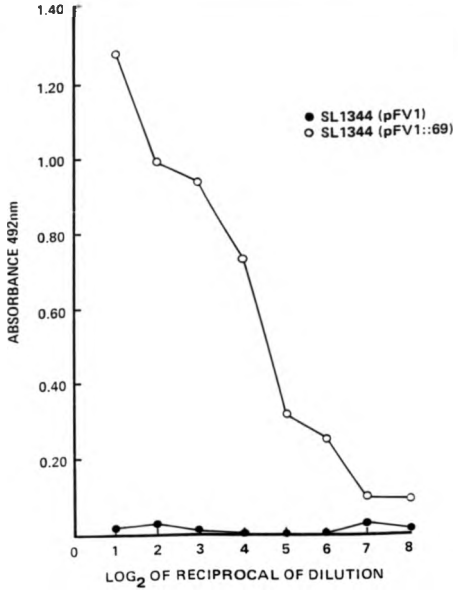


Figure 4.3.1.2 Detection of LT-B69 in a G_{M1}-linked ELISA. Whole cell lysates were prepared from *S. typhimurium* SL1344 pFV1 and SL1344 pFV1::69. These were assayed in an ELISA using the monoclonal antibody BB05 as a probe.

4.3.2 *In vitro* assay of stability of constructs in *S. typhimurium* SL1344 *aroA*⁻ *aroD*⁻

One aim of this project was to express the LT-B fusion proteins in attenuated strains of *Salmonella*, as Maskell *et al.*, (1987) had previously demonstrated that native LT-B expressed from pBRD026 (i.e. expression driven by the same promoter at low-level) in *S. typhimurium* SL3261 could elicit systemic and secretory anti-LT-B antibody responses when given to mice orally.

An important characteristic of *Salmonella spp.* which allows them to act as efficient carriers of heterologous antigens (reviewed by Dougan & Tite, 1990) is the ability of the organism to persist in moderate numbers in the reticulo-endothelial system (RES) after infection of a suitable host (O'Callaghan *et al.*, 1987). Unfortunately, under the strong selective pressures operating *in vivo*, plasmid stability in such strains is often poor (e.g. Tite *et al.*, 1990) once the bacteria are removed from the selective antibiotic present in *in vitro* cultures. Thus, before inoculating mice with *S. typhimurium* SL1344 *aroA*⁻ *aroD*⁻ harbouring the various fusion constructs, the stability of the plasmid in the bacterium *in vitro*, in the absence of ampicillin, was determined.

Single colonies from L-amp plates were inoculated into 3ml of L-broth without ampicillin. These were incubated at 37°C in an orbital shaker overnight. The next day, 30µl of this culture were used to inoculate 3ml of fresh L-broth which was incubated overnight (again, at 37°C in a shaker). The remainder of the culture was diluted to $1/10^7$ and $1/10^8$ in PBS. Duplicate 2ml aliquots of each dilution were incorporated into samples of molten L-agar, with and without ampicillin. The colonies were counted the next day and the percentage of colonies retaining ampicillin-resistance was determined. This process was repeated for the 2nd and 3rd passages. The results are shown below in Table 4.3.2. From these data it is apparent that after 3 passages, (representing a considerable number of bacterial generations, estimated to be about 20-30), all the plasmids are well maintained.

Table 4.3.2 The relative stability of LT-B fusion plasmids *in vitro*

Construct	% Stability after passage number		
	1	2	3
pBRD026	85	92	85
pFV1	54	54	46
pFV1::110	100	87	97
pFV1::167	32	23	20
pFV1::69	100	100	100
pFV1::114	81	73	79

The table shows the percentage of bacterial cells in the culture with an ampicillin-resistant phenotype after serial passage *in vitro*, an index of the percentage of cells retaining the LT-B fusion expression plasmids.

4.3.3 *In vivo* evaluation of plasmid stability

In one such typical experiment, 30 C57Bl/6 mice were inoculated intravenously with about 10^6 cfu of SL1344 (pFV1) or SL1344 (pFV1::114). Four mice per group were sacrificed at regular intervals and their livers and spleens removed aseptically into stomacher bags. The total number of *Salmonellae* in those organs, together with the number which remained ampicillin resistant, was determined (as described in 4.2.3). The results are shown in Figure 4.3.3.1. (graphs A and B). Furthermore, the persistence in spleens and livers of SL1344 *aroA* ⁻ *aroD* ⁻ carrying these constructs appeared essentially unaltered from the behaviour of the parental strain (Dougan *et al.*, 1988), illustrated in Figure 4.3.3.2. Immediately after inoculation, there is a decline in the numbers of recoverable bacteria, presumably due to non-specific host-defence mechanisms. After a lag phase of about 24 hours, the inoculum enters a replicative phase. There is a net increase in bacterial count, peaking around days 10-14. By this point, specific immune mechanisms have become effective and the viable count slowly declines, the bacteria being finally cleared by about 6-7 weeks post-infection. The graphs in Figure 4.3.3.1 show that both pFV1 and pFV1::114 are stable *in vivo* in the absence of antibiotic selective pressure, despite the lower stability of pFV1 in *in vitro* assays.

However, in a similar experiment BALB/c mice were fed *S. typhimurium* SL1344 harbouring the plasmid pFV1::69. In contrast to the results obtained with SL1344 (pFV1::114), no ampicillin-resistant *Salmonellae* could be isolated from the livers or spleens of the mice by 3 weeks post-infection (data not shown), despite pFV1::69 being maintained to a high degree in bacterial cultures *in vitro* (see Table 4.3.2). It would appear therefore that behaviour of the plasmid in bacterial cultures *in vitro* is not necessarily a good indicator of whether the plasmid is likely to segregate in a bacterial population in an animal host, at least in this model.

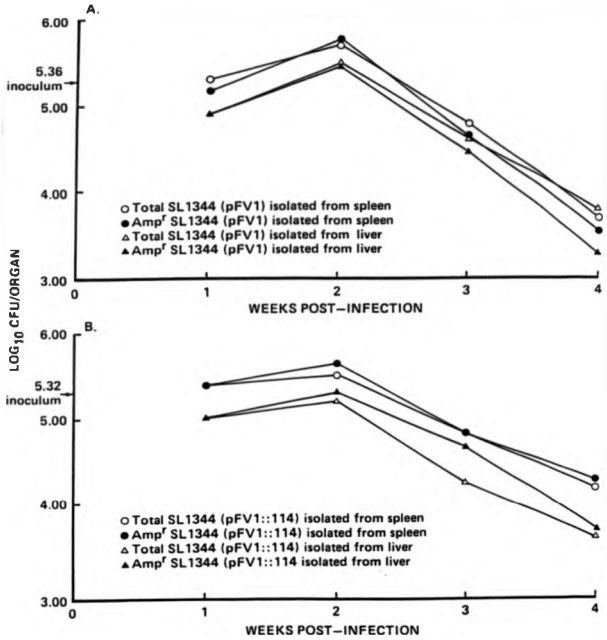


Figure 4.3.3.1 The stability *in vivo* of plasmids pFV1 and pFV1::114. Mice were inoculated intravenously with *S. typhimurium* harbouring the above plasmids. Mice were sacrificed at regular intervals and the number of viable *Salmonellae* in the liver and spleen was determined, together with the number of bacteria which retained ampicillin resistance. Points represent the mean group value (n=4), +/- 1 SD.

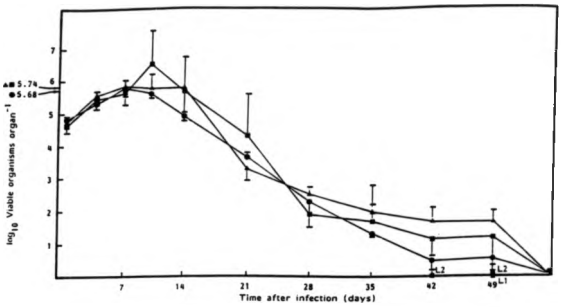


Figure 4.3.3.2 The colonisation of the livers of Balb/c mice following i.v. infection with *S. typhimurium aroA*$^-$ (●), *S. typhimurium aroC*$^-$ (▲) and *S. typhimurium aroA*$^-$ *aroC*$^-$ (■). Each point represents the geometric mean +/- two standard errors (n=4). Reproduced from Dougan *et al.*, 1988.

4.3.4 Immunological evaluation following inoculation with *S. typhimurium* harbouring LT-B fusion protein expression plasmids

4.3.4.1 Serum antibody responses

The immune responses of mice in the above experiment were investigated. Individual serum samples were taken from 4-5 mice per group and tested, by antigen-specific ELISA, for antibody to LT-B and to the 114 epitope, corresponding to amino acid residues 364-382 of influenza A virus nucleoprotein. As expected from the results of previous work (Maskell *et al.*, 1987), mice in both groups mounted a detectable serum antibody response against LT-B after priming. This was observed to increase slightly after the mice were given a second inoculation (see Figure 4.3.4, graph A). Mice in a control group inoculated with SL1344 alone did not develop serum anti-LT-B antibody. In addition, mice which were inoculated with SL1344 (pFV1::114) were found to mount a weak serum antibody response to the 114 peptide epitope (Figure 4.3.4, graph B). This was partially obscured by an increase in the background response. This was presumably due to a serological cross-reaction between the 114 peptide epitope and one or more Salmonella antigens as both the group inoculated with the parental strain and the group inoculated with SL1344 (pFV1) showed a similar increase in response after boosting. Mice inoculated with SL1344 *aroA*$^-$ *aroD*$^-$ (pFV1::110) however, did not develop serum antibodies against peptide 110 (corresponding to amino acid residues 147-161 of influenza A virus nucleoprotein), despite the persistence for several weeks of ampicillin-resistant bacteria in the infected mice (data not shown).

4.3.4.2 Cytotoxic T lymphocyte responses

Unfortunately, a cytotoxic T lymphocyte (CTL) response to the CTL epitope present in the sequence of peptide 114 could not be detected in C57Bl/6 mice infected with *S. typhimurium* SL1344 *aroA*$^-$ *aroD*$^-$ (pFV1::114), using either influenza A virus (PR8) or the corresponding

synthetic peptide as the recall antigen. In contrast, in the same assays, spleen cells from mice which had been infected intravenously with PR8 virus contained a population of cytotoxic T cells which were reactive against either PR8-infected or peptide-pulsed target cells. In a similar experiment, BALB/c mice inoculated with SL1344 *aroA*$^-$*aroD*$^-$ (pFV1::110) failed to mount a detectable CTL response to the peptide 110 epitope. Whilst there is uncertainty over the expression of LT-B110 (which could not be detected using 110-specific antisera in immunoblots or G_{M1}-linked ELISA), no such doubt exists concerning the expression of LT-B114. Some speculations on the possible causes of this lack of T cell reactivity to the 114 peptide epitope are discussed in the following section.

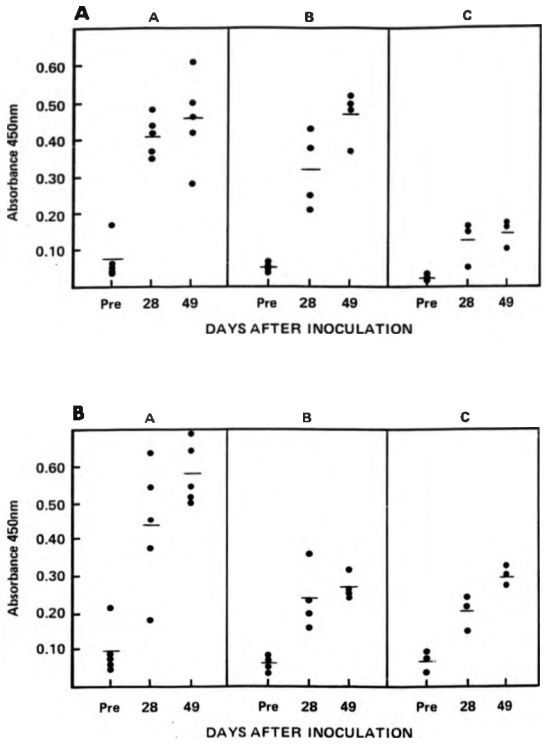


Figure 4.3.4. The primary (day 28) and secondary (day 49) serum antibody responses to LT-B (top graphs) and peptide 114 (lower graphs) of mice immunised intravenously with (A), *S. typhimurium* SL1344 (pFV1::114); (B), SL1344 (pFV1) or (C) SL1344 alone. The mean values are represented by a horizontal bar (n=4 or 5).

4.4 Summary

A number of reasons could be envisaged to explain the failure to detect heterologous epitope-specific T cell responses in mice immunised with attenuated *Salmonella*. Firstly, the peptide sequence might well behave differently when fused to LT-B than when present as an intrinsic part of influenza virus nucleoprotein. Thus nucleoprotein might be degraded by antigen-processing cells so as to yield the antigenic peptide fragment, peptide 114. However, the same amino acid sequence, when present in LT-B, might not generate the epitopic fragment. Similarly, it is possible that expression by *Salmonella typhimurium* may prevent the epitope being processed in the normal way. For instance, despite being essentially an intracellular parasite, (influenza virus similarly being an obligate intracellular parasite), the presence of bacterial antigens might lead to the preferential formation and/or recognition of other peptide fragments. Alternatively, a fragment might be formed which contains the epitope sequence but, because of its composition (i.e. incorporating LT-B sequences), is in a form unable to associate with the MHC molecules of the H-2^b haplotype.

A second explanation could be that there are T cell epitopes in LT-B which are excessively immunodominant. Thus, even if the 114 epitopic fragment is formed and presented, the response of cytotoxic T cells which recognise it might be masked or swamped by the response to other immunodominant epitopes. This could be easily tested by repeating the experiment and performing an assay for CTL directed against LT-B.

This failure to detect CTL responses to T cell epitopes fused to LT-B contrasts with the experience of Schödel *et al.*, (1990*a*,*b*). These workers constructed a plasmid vector directing the expression of one B cell epitope from hepatitis B virus (HBV) pre-S(2) surface antigen (amino acid residues 133-140) and two overlapping T cell epitopes from HBV core antigen (amino acid residues 120-140) fused to the C terminal of LT-B. The resulting fusion protein was expressed in an attenuated *aroA*$^-$ strain of *S. dublin* SL1438, which was used to inoculate mice. After 3 oral doses splenic T cells specific for the fused hepatitis B virus core antigen (HBVcAg) T cell epitopes could be detected. Thus there are a number of differences between this previous work and the current study reported here, including route and number of inoculations, bacterial host strain and

T cell epitopes in the fusion protein. Furthermore, the above authors present no data concerning the G_{M1}-binding activity of the fusion protein. This might be significant if, as outlined above, binding to G_{M1} alters the processing pathway followed by the antigen.

As described previously, the plasmid pFV1::69 was found to segregate in populations of *S. typhimurium* SL1344 *aroA*$^-$ *aroD*$^-$. One possible solution to the problem of instability of plasmids expressing heterologous antigens in bacterial vectors is to integrate the relevant DNA, encoding the heterologous antigen, into the chromosome of the bacterial host. Chromosomal insertion vectors have been described which contain genes encoding heterologous polypeptides flanked by sequences which are homologous to those of the host bacterium's chromosome. A low frequency double crossover event allows homologous recombination to occur such that the heterologous antigen is then expressed from the chromosome. This approach has been used successfully to introduce sequences encoding tetanus toxin C fragment into the chromosome of *S. typhimurium* (Strugnell *et al.*, 1990). The technique has the advantage of eliminating problems of plasmid instability. However, as there is only one chromosome per bacterial cell, levels of expression of the heterologous antigen are generally lower than those which can be achieved with high copy number expression plasmids.

CHAPTER 5

The partial purification of LT-B69

5.1 Introduction

An alternative to the use of attenuated *Salmonella* spp. to achieve immunisation of mucosal surfaces is to directly inoculate mice orally or intranasally with purified proteins. Such an approach is feasible with LT-B fusion proteins because of the properties of LT-B discussed in Chapter 1. This chapter describes the purification of the LT-B69 fusion protein.

A number of methods have been described for purifying LT-B and/or CT-B. One of the most efficient appears to be affinity chromatography, which utilises the G_{M1}-binding activity of the toxin B-subunits to retain them on a G_{M1}-coated inert matrix (Tayot *et al.*, 1981). This technique has also been successfully employed to purify a CT-B fusion protein (Dertzbaugh *et al.*, 1990).

In preliminary experiments, attempts to purify LT-B69 from periplasmic fractions of *E. coli* HB101 (pFV1::69) by this method were unsuccessful. In view of the data showing that LT-B69 retains G_{M1}-binding activity, the most likely explanation for this is that the chemical derivatisation of the G_{M1} (which involves reflux boiling for several hours with 10M Potassium hydroxide) did not occur correctly or that the lysoG_{M1} was not properly coupled to the inert matrix (Spherosil, Pharmacia).

LT accumulates in the periplasm of *E. coli* whereas *V. cholerae* will secrete both CT and LT into the extracellular milieu (Hirst *et al.*, 1988, Witholt *et al.*, 1988). Thus if LT-B fusion proteins can be expressed in an aloxigenic strain of *V. cholerae* (so as to avoid contamination by CT), they might be secreted into the medium, in the same way as the native toxin. This potentially simplifies purification into a process of concentration, as very little of other bacterial products are transported extracellularly from *V. cholerae*. (Indeed, one interesting question is

what selective advantage is provided by cholera toxin secretion, in view of the effort expended by the bacterium to export the toxin to the external medium? It may be that the symptoms of severe diarrhoea, which CT induces, facilitate the spread of the organism). Thus one approach, described below, was the expression of pFV1::69 in an atoxigenic strain of *V. cholerae*, strain TRH7000 (kindly provided by T. Hirst; Hirst *et al.*, 1984*b*), the results of which are detailed in 5.3.

Ultimately, the method of choice used to purify LT-B69 was to separate the proteins from periplasmic extracts of HB101(pFV1::69) by means of ion-exchange chromatography. Whilst this did not achieve the high purity associated with affinity chromatographic techniques, it was simple to perform and did not require the use of low pH conditions (which might denature certain LT-B fusion proteins) to dissociate LT-B69 from the column, which is necessary when using G_{M1}-affinity protocols.

5.2 Materials and Methods

5.2.1 Bacterial strains and plasmids

V. cholerae TRH7000 is an attenuated strain, being atoxigenic and auxotrophic for thymine, but is moderately resistant to polymixin B (PB). Typically, the strain was grown at 30°C on L-agar supplemented with thymine at 100 µg/ml and PB at 100 units/ml.

pFV1 and pFV1::69 have been described previously (this thesis). pRK2013 encodes all the *tra* gene functions from the broad-host-range plasmid RP4 and has been described previously (Figurski and Helinski, 1979). Thus in a tripartite mating involving *E. coli* HB101 (pFV1)/(pFV1::69), HB101 (pRK2013) and *V. cholerae* TRH7000, pRK2013 can supply *tra* gene functions *in trans*, mobilising the pBR322-derived pFV1 plasmids into the *V. cholerae* strain.

5.2.2 Tripartite Bacterial Mating

E. coli cells containing the donor plasmids were scraped from a fresh L-amp plate to form a thick suspension in 0.5mls of L-broth. Similarly, a thick suspension was made of *E. coli* HB101 (pRK2013) cells. A slightly less thick suspension was made of *V. cholerae* TRH7000. Equal volumes (~100µl) of each suspension were mixed together and 50µl drops were put onto L-agar/thymine plates. These were incubated at 30°C for 2-6 hours to allow conjugation to proceed. The cells were then scraped from the plates into 0.5mls of L-broth. Serial tenfold dilutions of this suspension were made in L-broth and streaked out for single colonies on L-agar plates supplemented with thymine, PB and ampicillin (at 100µg/ml), (L-TPA plates) to select for *V. cholerae* cells which had taken up the donor plasmids (encoding ampicillin-resistance). Plates were incubated for 24 hours at 30°C. The resulting colonies were picked onto fresh L-TPA plates and also onto L-TPA plates supplemented with kanamycin at 50µg/ml. *V. cholerae* colonies which acquired the donor plasmids but which had lost the pRK2013 "helper" plasmid (pRK2013 is unstable in *V. cholerae*) had the phenotype PB^R, Amp^R and Km^S. Thus they will grow on L-TPA plates but not on L-TPA plates containing kanamycin. These colonies were screened for the expression of LT-B and/or BB05 by the use of colony immunoblots. Positive-reacting colonies were streaked out on selective media and held at 4°C.

5.3 Results

5.3.1. Secretion of LT-BH and LT-B69 from *V. cholerae* TRH7000

The secretion of LT-BH and LT-B69 from *V. cholerae* TRH7000 was investigated by analysing cellular and extracellular fractions from cultures of *V. cholerae* (pFV1) and *V. cholerae* (pFV1::69) using a G_{M1}-linked ELISA, as described in Chapter 2. The results are represented graphically in Figures 5.3.1.1-3.

Figure 5.3.1.1 shows that small amounts of LT-BH can be detected in the culture

supernatant after just 6 hours of culture. This is despite the fact that levels of expression in *V. cholerae* from the P1 "anti-tet" promoter are presumably no higher than in *E. coli*. This extracellular LT-BH is unlikely to have been released from lysed or damaged cells as the amount of LT-BH remaining cell-associated at this time-point is very small. Thus one can be reasonably confident that the protein reached the medium by active export rather than by a passive "leakage". After 24 hours of culture there is much more cell-associated LT-BH and very much more free LT-BH in the medium: if one considers the relative values for the absorbance at the first dilution, the figure for the medium fraction is about 3 fold higher than that for the cellular fraction. This increase over time could be due to the stability of the protein resulting in a time-dependent accumulation and/or the greater cell density after a more prolonged period of culture.

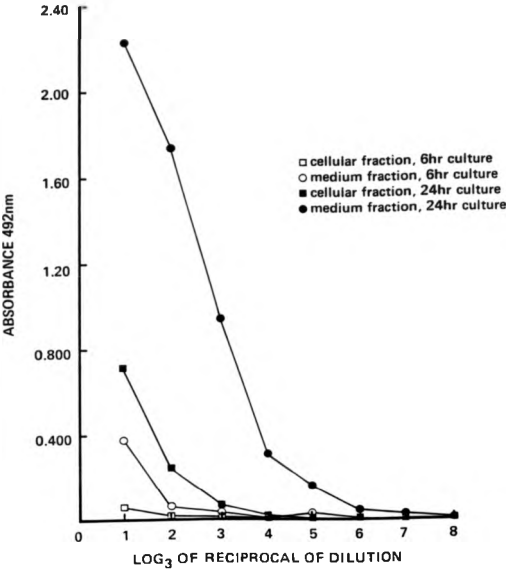


Figure 5.3.1.1 The secretion of LT-BH from *V. cholerae* was investigated by means of G_{M1}-linked ELISA. *V. cholerae* TRH7000 pFV1 was grown at 37°C in L-broth. At two time points (6 hours and 24 hours after initaition of culture), 200µl of culture was sampled. The cells were pelleted by centrifugation and disrupted by sonication in an equal volume of PBS. Aliquots of the cellular fractions prepared in this way, together with samples of the culture supernatant, were applied to a G_{M1}-coated microtitre plate and probed with LT-B-specific antiserum in an ELISA.

The situation appears slightly different with respect to the secretion of LT-B69, as illustrated by Figure 5.3.1.2. After 6 hours of culture, virtually all LT-B69 is still intracellular. After 24 hours however, the data are essentially as those obtained for LT-BH in that there is far more LT-B69 in the medium than remains cell-associated (again, with respect to the absorbance of the first dilution in the ELISA, the figure is about 3 times greater for the extracellular fraction than for the cellular fraction). The total amounts of G_{M1}-bound LT-B epitopes, as judged by the sum of the absorbances for cellular and medium fractions, is essentially the same for LT-BH and LT-B69 at both time-points. Thus it would seem that the presence of the BB05 epitope at the C-terminal of LT-B does have some effect on the secretion of the protein from *V. cholerae* TRH7000 but does not affect the G_{M1}-binding properties of the molecule, relative to LT-BH. When the same assay was performed on cellular and medium fractions from cultures of *V. cholerae* TRH7000 (pFV1::69), using the mAb BB05 as a probe, the results shown in Figure 5.3.1.3 were obtained. Surprisingly, at both 6 and 24 hours there was considerably more G_{M1}-binding BB05-specific epitope within the cellular fraction than in the medium, which appears contradictory to the presence of large amounts of LT-B epitope in the medium after 24 hours, as shown by Figure 5.3.1.2. The probable explanation for this is that the BB05 epitope is being cleaved from the LT-B moiety by a protease activity and so cannot be detected in a G_{M1}-linked ELISA, (it is therefore not surprising that LT-B69 secreted from *V. cholerae* binds to G_{M1} with an affinity comparable to that of LT-BH). The fact that any intact extracellular LT-B69 can be detected implies that this proteolysis occurs very soon after, rather than before or during, export from the bacterial cell. This phenomenon has been experienced with other LT-B fusion proteins secreted from *V. cholerae* (Schödel *et al.*, 1991). In the light of these findings it was considered too difficult to attempt to purify LT-B69 from cultures of *V. cholerae* (pFV1::69).

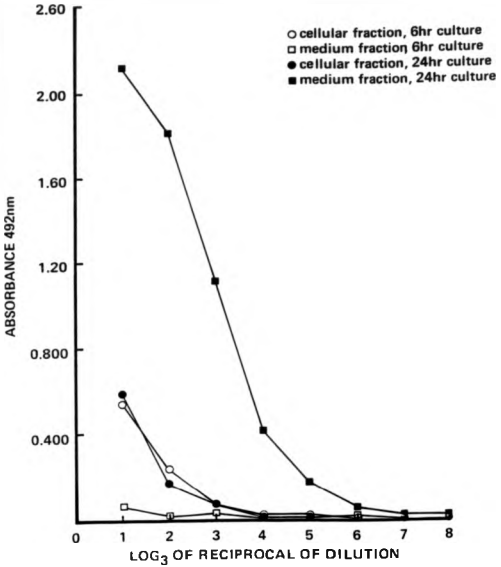


Figure 5.3.1.2 The secretion of LT-B69 from *V. cholerae* was investigated by means of G_{M1}-linked ELISA. Cellular and medium fractions were prepared (as described previously) from cultures of *V. cholerae* TRH7000 pFV1::69 at 6 and 24 hours after the initiation of culture. These samples were analysed in a G_{M1}-linked ELISA, using LT-B-specific antiserum as a probe.

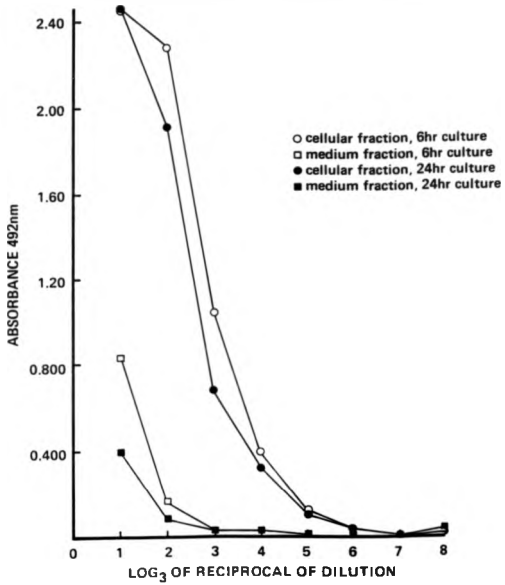


Figure 5.3.1.3 The secretion of LT-B69 from *V. cholerae* was investigated by means of a G_{M1}-linked ELISA. Cellular and medium fractions were prepared (as described previously) from cultures of *V. cholerae* TRH7000 pFV1::69. These samples were analysed in a G_{M1}-linked ELISA, using the monoclonal antibody BB05 as a probe.

5.3.2 Partial purification of LT-B69 from periplasmic fractions of *E. coli*

For the partial purification of LT-B69 fusion protein, large scale periplasmic fractions were made, as detailed in Chapter 2, from 2 litre batches of *E. coli* HB101 (pFV1::69) overnight cultures. These preparations were concentrated 20-25 fold using an Amicon Diaflo equipped with an XM50 membrane (Amicon Corporation, Maryland, USA). The retentate was dialysed overnight against 50mM NaCl buffered to pH8.6 with 25mM Tris-HCl. The dialysate was then loaded onto a DEAE Trisacryl column (Pharmacia, Sweden) equilibrated with the same buffer. After extensive washing (for about 1 hour) the bound protein was eluted with a 50-250mM NaCl gradient, buffered to pH8.6 as before. The A_{280nm} of the eluate was continuously monitored with a UV-1 Single Path Monitor (Pharmacia) and a Servoscribe 210 recorder (Camlab, Cambridge, UK). A typical absorbance profile is shown in Figure 5.3.2. Fractions were collected and analysed by dot-blotting onto nitrocellulose and probing with the BB05 monoclonal antibody, followed by a mouse Ig-specific rabbit antibody conjugated to horseradish peroxidase and developed with a suitable substrate. Positive-staining fractions were further analysed by SDS-PAGE. Those with the highest purity of LT-B69 were pooled. These pooled fractions were further concentrated 10-20 fold using Centricon 30 ultrafiltration tubes (Amicon), by centrifugation at ~6,000g for 30 minutes. The total protein content was determined using the Pierce BCA microtitre assay. The material was then stored in 0.5ml aliquots and maintained at -20°C until required.

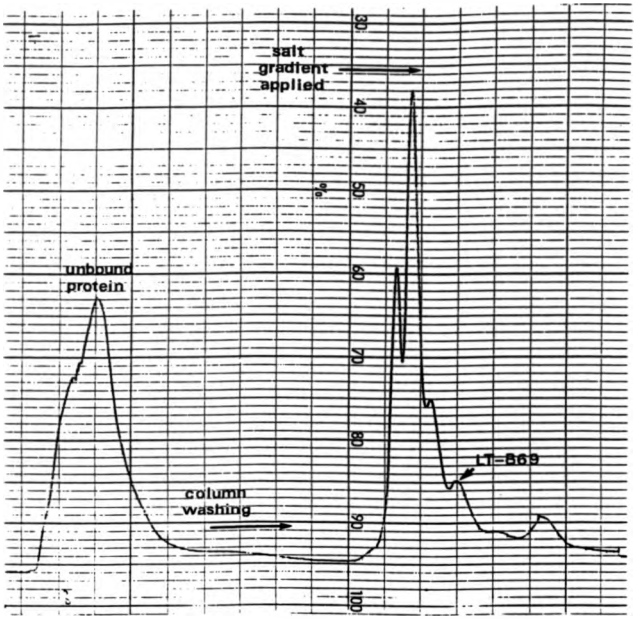


Figure 5.3.2 A typical absorbance profile obtained during the partial purification of LT-B69 from concentrated periplasmic fractions of *E. coli* HB101 pFV1::69. About 50% of the protein in the extract did not bind to the Trisacryl column and passed straight through. After washing for ~1hr in 50mM NaCl/25mM Tris-HCl pH8.6, a 50-250mM NaCl gradient (at the same pH) was applied. The LT-B69-containing fractions (arrowed) were eluted towards the end of the run.

5.3.3 Characterisation of partially purified LT-B69

The purification was monitored by analysing samples before and after purification by means of SDS-PAGE, as shown in Figure 5.3.3.1. Clearly, the pooled fractions are by no means pure. There are several contaminating proteins, the main contaminant running with an apparent M_r of ~29kDa. However, there is considerable enrichment for LT-B69, of which none appears to be lost by passing straight through the ion-exchange column (as judged by SDS-PAGE). It is apparent that some degradation takes place during the purification. In the unboiled sample there are 2 well-represented bands with apparent M_r values of ~68kDa and 90kDa and a fainter band which migrates with an apparent M_r of 97kDa, which presumably represent variously degraded forms of the fusion protein. All three bands have indistinct margins, implying breakdown products with a range of molecular weights rather than a few discrete forms of LT-B69. Upon boiling, all of these multimeric forms of LT-B69 disappear and a single, sharply-defined, new band, corresponding to monomeric LT-B69, is formed at a position equating to an M_r of 18kDa. Thus, with respect to pentamer formation and dissociation into monomers upon boiling, the partially purified fusion protein behaves identically to LT-B69 in crude *E. coli* periplasmic fractions.

It was clearly important to ensure that as well as forming pentameric complexes the partially purified protein could still bind to G_{M1}. Thus the affinity for G_{M1} of the partially purified material was compared with crude *E. coli* periplasmic fractions containing LT-B69 by means of a G_{M1}-linked ELISA.

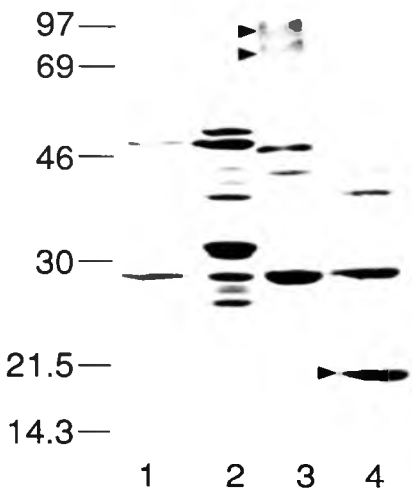


Figure 5.3.3.1 SDS-PAGE analysis of periplasmic fractions from *E. coli* HB101 pFV1::69, before and after purification of LT-B69. Lane 1, total periplasmic protein from HB101 pFV1::69, as loaded onto DEAE Trisacryl column; lane 2, unbound proteins which flowed straight through the column, lane 3, pooled LT-B69-containing fractions eluted from the column (unboiled); lane 4, pooled LT-B69-containing fractions eluted from the column (boiled). Bands containing LT-B69 are arrowed. Relative molecular mass markers (in kiloDaltons) are shown on the left.

Purified preparations of LT-B69 were diluted in PBS so as to give 1/10 and 1/20 of the total protein content of an *E. coli* (pFV1::69) periplasmic fraction, and applied to a G_{M1}-coated microtitre plate. The results are shown in Figure 5.3.3.2. From this one can conclude that the purification process does not abolish the G_{M1}-binding properties of LT-B69. Furthermore, the graph suggests that the purification achieves an enrichment of LT-B69 of between 10 and 20 fold, the actual figure lying towards the upper limit of that margin.

It is generally believed that monomeric LT-B has only a very low affinity for G_{M1} (Iida *et al.*, 1989, Sixma *et al.*, 1991). Whilst this is a reasonable assumption, it is difficult to find much evidence for it in the literature. For this reason, the G_{M1}-binding properties of LT-B69 were further investigated by G_{M1}-linked ELISA, using either unheated partially purified LT-B69 or an equal amount of the same preparation which had been subjected to boiling for 10 minutes. The samples were then probed with the BB05 mAb. This was the probe of choice because some of the epitopes of CT-B are thought to be conformational and heat-labile (David Lewis, personal communication). By contrast, the BB05 mAb recognises a linear sequence and will bind to denatured LT-B69 very efficently in an immunoblot. The results are shown below in Figure 5.3.3.3. It was found that boiling LT-B69 does prevent the fusion protein from binding to G_{M1}. Unfortunately, this does not provide direct evidence that the monomeric nature of boiled LT-B69 is responsible: an equally valid explanation is that boiling causes unfolding of the LT-B polypeptides and that this denaturation destroys the conformation necessary for G_{M1}-binding activity. This problem is a difficult one to overcome because it requires obtaining monomeric LT-B in a native form, yet monomers of LT-B associate very readily into pentamers.

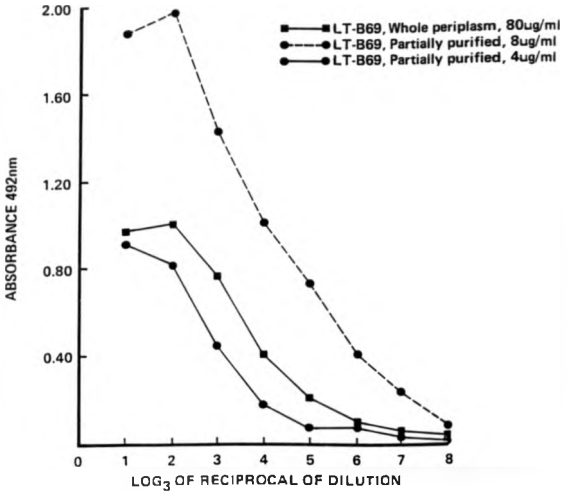


Figure 5.3.3.2 The G_{M1}-binding activity of LT-B69 is preserved during the purification process. Samples of partially purified LT-B69 were adjusted to a concentration of total protein of 4μg/ml or 8μg/ml. A crude periplasmic extract was prepared from *E. coli* HB101 pFV1::69 and adjusted to a concentration of total protein of 80μg/ml. The G_{M1}-binding activity of these samples was then compared in a G_{M1}-linked ELISA, using the monoclonal antibody BB05 as a probe.

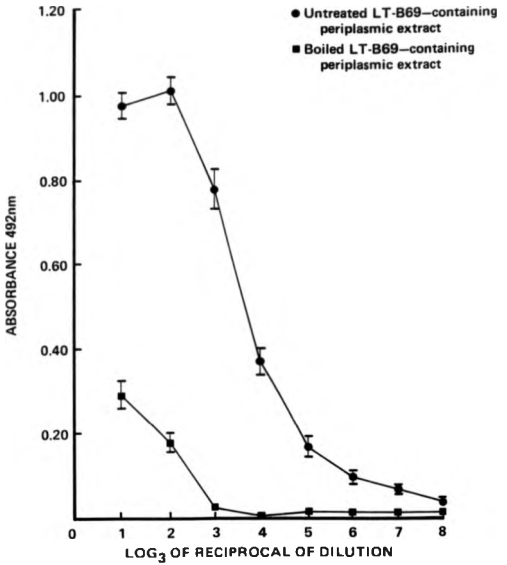


Figure 5.3.3.3 The G_{M1}-binding activity of boiled and unboiled partially purified LT-B69. Equal concentrations of partially purified LT-B69 were applied, either boiled or unheated, to a G_{M1}-coated microtitre plate and probed with the monoclonal antibody BB05.

5.4 Summary

The transport of LT and LT-B in *E. coli* and *V. cholerae* has been studied quite extensively. Witholt *et al.*, (1988) established that when expressed in *E. coli*, LT-B is released into the periplasm as early as 13-14 seconds after the initiation of synthesis. Within that period, the nascent polypeptide is transported to and across the cytoplasmic membrane, during which process the 21 residue N-terminal signal sequence is proteolytically cleaved. Once released into the periplasmic space, the polypeptide is oxidised, allowing a disulphide bridge to form between residues 9 and 86 (Sixma *et al.*, 1991). The final processing step is the formation of B-subunit pentamers and, in the presence of LT-A, holotoxin.

Processing of LT in *V. cholerae* is broadly similar, with cleavage of the signal sequence peptide during transit across the cytoplasmic membrane. The B-subunit monomers then transiently enter the periplasm prior to oligomer and holotoxin formation (Hirst & Holmgren, 1987*a*). The major difference is that once assembled in the periplasm of *V. cholerae*, LT or pentameric LT-B are then further transported across the outer membrane into the extracellular medium. Thus *V. cholerae* must possess at least one extra pathway, which is absent in *E. coli*, which is necessary to facilitate secretion. Secretion of LT-B can occur in the absence of LT-A. The reverse however is not true (Hirst *et al.*, 1984*b*). This suggests there must be information inherent in LT-B (and CT-B) which is preserved in the two recombinant proteins LT-BH and LT-B69. This final step in the pathway is extremely interesting as it appears that toxin secretion involves translocation of the molecule in a folded, stable quaternary structure. Transport of a large protein across the outer membrane would seem to represent a considerable problem to the bacterium. Quite how this is brought about in *V. cholerae* is unclear. Perhaps a B-subunit-specific protein in the outer membrane recognises the protein and facilitates the process.

Experiments were performed which suggested that possible degradation of LT-B69 occurred when the protein was expressed in *V. cholerae*. These findings are in agreement with the experience of Schödel *et al.*, (1991) who found that, whilst one fusion between LT-B and sequences from hepatitis B virus (HBV) middle surface antigen was secreted into the

supernatant of *V. cholerae* cultures, two similar LT-B fusion proteins were retained intracellularly or rapidly degraded once they became extracellular. For this reason other methods of purification were selected. However, in the longer term there are a number of possible ways of overcoming this problem. For instance, it might be possible to construct a strain of *V. cholerae* lacking the protease(s) involved, if the gene(s) responsible were identified. Alternatively, the kinetics of expression of the protease(s) and LT-B69 might be different (only 2 time-points were investigated). Thus at some point during the culture it might be possible to isolate undegraded LT-B69 from the culture supernatant.

With regard to the actual protocol used to purify LT-B69 from *E. coli* periplasmic fractions, the technique was clearly not ideal as LT-B69 represented only about 38% of the total protein in the final sample. However, the concentration of LT-B69 in the starting material was low (~2% of total protein), thus the process achieved approximately a 17 fold enrichment (which agrees with the data obtained from G_{M1}-linked ELISAs performed on crude and partially purified material). If the basic fusion vector (pFV1) was re-designed (e.g. so as to utilise a stronger promoter) such that higher levels of expression resulted, the purification of the associated fusion proteins would be greatly facilitated.

The incorporation of further purification steps could probably increase the purity of LT-B69. However, this might well cause greater degradation than was observed with the relatively simple purification protocol employed. The degree of degradation is of importance because any diminution of G_{M1}-binding by the LT-B moiety, or separation of the fused epitope from LT-B, could reduce the immunogenicity of the heterologous epitope, whereas for the requirements of intranasal immunisation experiments (described in the following chapter), the absolute purity of the preparation was of less significance.

CHAPTER 6

Immunisation with partially purified LT-B69

6.1 Introduction

Having obtained partially purified LT-B69 in a pentameric form able to bind G_{M1}, as described in the preceding chapter, it was obviously desirable to investigate the immunogenicity of the fusion protein. Rather than attempting to inoculate mice by the more conventional routes (i.e. sub-cutaneously or intramuscularly), it was decided to inoculate mice intranasally. The rationale for this approach was as follows; firstly, the rationale of the project was the considerable immunogenicity of LT-B when applied to mucosal surfaces (far greater than that of "conventional" immunogens, such as KLH). As discussed in Chapter 1, the greater T helper cell:T suppressor/cytotoxic cell ratio of bronchus-associated lymphoid tissue (compared to gut associated lymphoid tissue), amongst other reasons, might make the conditions for obtaining an antibody response more favourable in the respiratory tract than in the gut. Secondly, *B. pertussis* is a pathogen of the respiratory tract, thus any local response to the BB05 epitope in the respiratory tract might be of considerable significance: there is some evidence to suggest that the current parenterally inoculated vaccine protects against the disease, that is, the symptoms of pertussis (for example by generating an anti-toxin serum antibody response), but does not protect against infection. Thus the current vaccine does little to prevent transmission of the causative organism. A third consideration is that very little research has been published regarding the immune response to CT-B or LT-B following intranasal inoculation, the work of Bessen & Fischetti (1988, 1990), Tamura and his co-workers (Tamura *et al.*, 1988, 1989) and Takahashi *et al.*, (1991) being the main exceptions.

6.2 Materials and Methods

Aerosol infection of mice with *B. pertussis*

Stock cultures of *B. pertussis*, stored in liquid nitrogen, were thawed and spread onto CW blood agar plates containing streptomycin. These were incubated for 3-4 days. The bacteria were then transferred into PBS using sterile dacron swabs (Baxter Healthcare Corporation, Illinois, USA). The $O.D._{650nm}$ of the resulting suspension was adjusted to ~0.55. This corresponds to a count of ~4 x 10^8 cells/ml. An aerosol was created from this suspension, using a CR60 high flow nebuliser (Medic-Aid, Pagham, Sussex, UK). The nebuliser was equipped with a System 22 "turret turbo" (Medic-Aid) which, according to the manufacturer's specifications, generated an aerosol in which 85% of the particles were less than 5.27μm in diameter (with an absolute minimum size of 2.3μm diameter). Mice which had been previously inoculated with LT-B69 fusion protein or control preparations were exposed to this aerosol for 30 minutes, exactly as described by Roberts *et al.*, 1990. Immediately afterwards some animals were sacrificed and viable counts performed on homogenates of their lungs to determine the size of the inoculum which had seeded in the lower respiratory tract. Aerosol infection of mice using this technique has been shown to produce a fairly uniform degree of initial colonisation (Sato *et al.*, 1980) and has been adopted by a number of researchers (Novotny *et al.*, 1985, Shahin *et al.*, 1990*b*, and Kimura *et al.*, 1990).

6.3 Results

6.3.1 Preliminary experiment involving intranasal immunisation with partially purified LT-B69

Groups of 5 BALB/c mice were lightly ether-anaesthetised and primed intranasally with 20μg of LT-B or LT-B69 in a volume of 40μl of PBS. The inoculum was dispensed in 2-3 drops from

a micropipette into the nostrils. The mice were boosted 4 weeks later with the same dose of antigen and by the same route. Serum samples were taken at various time points and either pooled or analysed individually, the titres of LT-B-specific and P.69-specific antibody being determined by means of an antigen-specific ELISA, as described in 2.10.1. Titres were defined as the reciprocal of the dilution of serum giving half the maximal absorbance at 450nm. The results are shown in Figures 6.3.1.1 and 6.3.1.2.

Figure 6.3.1.1 shows that high titres of anti-LT-B serum antibody developed in mice inoculated with either LT-B or LT-B69. The primary response appeared to be slower in mice which received the LT-B69 fusion protein. However, after boosting the anti-LT-B titres were essentially the same in both groups. From this it is clear that the BB05 epitope had little, if any, effect on the immunogenicity of the LT-B moiety. Figure 6.3.1.2 shows that P.69-specific serum antibody could be detected in mice immunised intranasally with LT-B69 but not in the control group. The kinetics of this anti-P.69 response were those of a typical low level primary response followed after boosting by a more rapid secondary response attaining a higher titre.

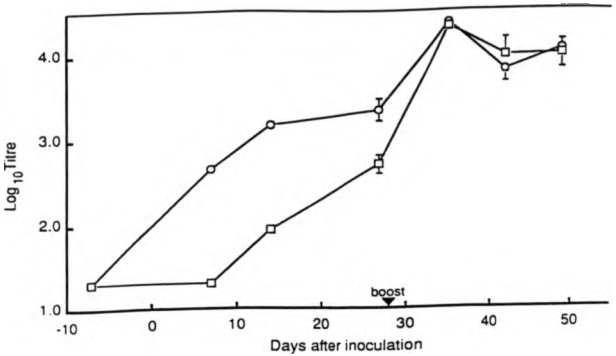


Figure 6.3.1.1 Serum antibody response of mice immunised with LT-B or LT-B69. BALB/c mice were immunised intranasally with 20µg of LT-B (O) or LT-B69 (□) and boosted 4 weeks later by the same route. Pooled or individual serum samples were obtained on the days shown. Anti-LT-B titres were determined by antigen-specific ELISA and represent the reciprocal of the serum dilution giving half maximal absorbance. Where sera were assayed individually, the points represent the mean values (n=5), +/- 1 S.D.

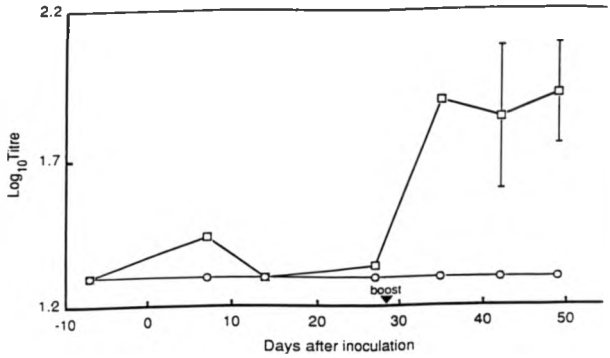


Figure 6.3.1.2 Serum antibody response of mice immunised intranasally with LT-B (○) or LT-B69 (□). Anti-P.69 titres were determined, as described previously, for serum samples obtained on the days shown. Where sera were assayed individually, the points represent the mean values (n=5), +/- 1 S.D.

Three weeks after boosting the mice were sacrificed and ELISPOT assays were performed on homogenates of their lungs as described in 2.10.2. LT-B-specific ASC were detected in both groups of mice, as shown in Figure 6.3.1.3. These were either of the IgG or IgA isotypes. No LT-B-specific IgM-secreting cells were detected. This was not surprising as the assay was measuring a secondary response, thus one would expect a certain degree of maturation of the response and concomitant antibody class-switching. A low number of P.69-specific ASC were detected in mice immunised with LT-B69 (Figure 6.3.1.4), but not in the control group. These P.69-specific ASC were mostly of the IgG isotype but some IgM-secreting cells were also found. This might reflect a true difference in the immunogenicity of the BB05 epitope compared to LT-B but it is more probably a distortion due to the low number of ASC detected. Subsequent experiments showed that the lung immune response to P.69 was essentially of the IgG and IgA isotypes, like the response to LT-B.

These results were extremely encouraging. Although the serum antibody titres specific for P.69 were quite low compared to the anti-LT-B titres, the detection of a serum antibody response to P.69, following intranasal immunisation with LT-B69, was very significant. Mice inoculated intranasally with two doses (each of 10μg) of purified P.69 developed P.69-specific ASC in their lungs but failed to mount an anti-P.69 serum antibody response (M. Roberts, unpublished observation). Thus coupling of the BB05 epitope to LT-B was shown to enhance the immunogenicity of the heterologous epitope, at least with regard to serum antibody responses.

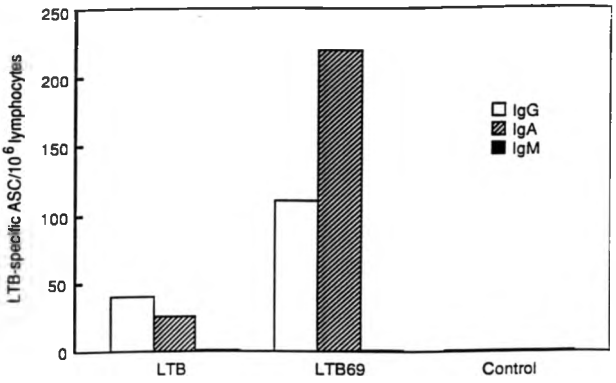


Figure 6.3.1.3 Anti-LT-B antibody-secreting cells (ASC) in the lungs of intranasally immunised mice. BALB/c mice were inoculated with LT-B or LT-B69 as described previously. Three weeks after boosting, lymphocytes were isolated from the lungs and antigen-specific, isotype-specific ELISPOT assays were performed to enumerate the LT-B-specific ASC. The antigen-specificity of the assay was demonstrated by the lack of reactivity in the control wells containing medium without antigen.

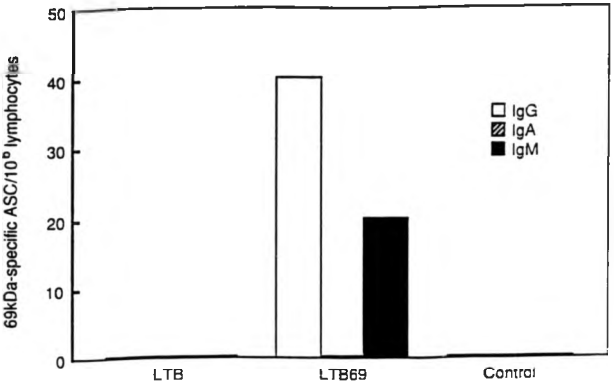


Figure 6.3.1.4 P 69-specific antibody-secreting cells (ASC) in the lungs of mice immunised intranasally with LT-B or LT-B69. ELISPOT assays were performed as described previously.

6.3.2 Immunisation schedules for aerosol challenge experiment

Eight groups of mice (10 BALB/c males per group) were primed on day 0 (and boosted 3 weeks later) with the antigen(s) and by the routes shown in Table 6.3.2.1 below, prior to challenge with an aerosol of *B. pertussis*, as described in 6.2.

Most groups were inoculated intranasally so as to study further the immunogenicity of LT-B69 when applied to mucosal surfaces. As a comparison, two groups were inoculated intramuscularly. The contrasting routes of inoculation might also have yielded information regarding the mechanism of protection (if any) against colonisation by *B. pertussis*, following an aerosol challenge. Another group included for comparison was inoculated with a synthetic peptide (peptide 683, see Figure 6.3.2.1 and Charles *et al.*, 1991, synthesised by D. Cambell, Wellcome Research Laboratories), corresponding to the BB05 epitope sequence, linked covalently to the carrier molecule KLH (which is not a mucosal adjuvant). Two methods of potentially enhancing the immune response to LT-B69 were also investigated. One method involved the incorporation of a small amount of CT (0.1μg) into the inoculum, which has been demonstrated to be an effective mucosal adjuvant when given to mice orally (Elson & Ealding, 1984*a*) or intranasally (Tamura *et al.*, 1989). The other method selected involved treating mice intranasally, 2-3 hours prior to immunisation, with 1mg of G_{M_1} (Sigma) in drops with a total volume of 40μl. Such a protocol has been used successfully to increase the immune response of mice immunised intranasally with particulate antigens containing CT-B (Martin Ford, Wellcome Research Laboratories, personal communication). It is assumed that enhancement of the immune response occurs because exogenous G_{M_1} is taken up by the epithelial cells lining the respiratory tract, thereby increasing the concentration of ganglioside on the epithelial surface. This in turn facilitates the subsequent binding of molecules with an affinity for G_{M_1} (such as LT-B).

Table 6.3.2.1. Immunisation protocols used for mice in a *B. pertussis* aerosol challenge experiment

Group	Immunisation schedule
1	20μg LT-B69[1], intramuscularly (i.m.)
2	20μg LT-B69 plus 0.1μg CT, i.m.
3	20μg LT-B69, intranasally (i.n.)
4	1mg G_{M1} i.n. pre-treatment, then 20μg LT-B69 i.n.
5	20μg LT-B69 plus 0.1μg CT, i.n.
6	1mg G_{M1} i.n., then 20μg LT-B69 plus 0.1μg CT i.n.
7	20μg peptide 683/KLH conjugate[2], i.n.
8	20μg irrelevant protein[3] plus 0.1μg CT, i.n.

[1], this figure represents the amount of LT-B69 in the inoculum; as the fusion protein constituted about 1/3 of the total protein, the amount of total protein in the inoculum was ~60μg.

[2], an amount sufficient to contain 20μg of peptide, (rather than 20μg of conjugate), was used.

[3], this irrelevant protein consisted of *E. coli* periplasmic proteins collected during the partial purification of LT-B69. This material did not bind to the Trisacryl column used in the purification and so passed straight through. The protein content was assayed by the Pierce method, adjusted to 0.5 mg/ml and stored in aliquots at -20°C.

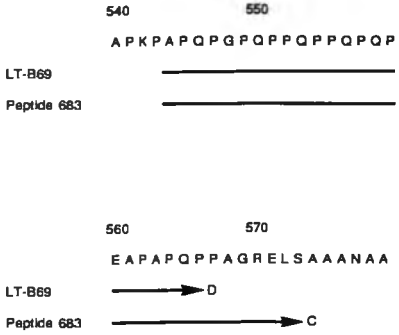


Figure 6.3.2.1 The amino acid sequence of P.69 in the region of the BB05-binding epitope, showing the precise sequence of peptide 683, which was conjugated to KLH and used as a control immunogen in the intranasal immunisation experiments. The numbers refer to the amino acid residues of the mature (i.e. signal peptide-processed) form of P.69. The LT-B fusion differs from the *B. pertussis* sequence at position 567 (pro→asp) and peptide 683 differs at position 574, having a carboxy terminal cysteine to facilitate coupling to carrier molecules.

Figure 6.3.2.2 shows a sample of the material used as the LT-B69 inoculum (compare Figure 5.3.3.1). This gel was subjected to further analysis by means of a densitometer. The plots of the unheated and the boiled samples are shown in Figures 6.3.2.3 and 6.3.2.4. The peaks representing pentameric and monomeric LT-B69 are arrowed. Figure 6.3.2.3 shows three peaks (constituting 34.6% of the total absorbance of the track) in the unheated sample, which are absent from the boiled sample. These represent pentameric LT-B69 and two slightly degraded breakdown products. Figure 6.3.2.4 illustrates the presence of a new peak in the boiled sample, corresponding to monomeric LT-B69, constituting 38.4% of the total absorbance of the track (i.e. about the same as the sum of the three bands in the unheated sample). Using these data, in conjunction with the results of a an assay of the total protein concentration of the sample, it was possible to determine the dose (in µg) given to the mice.

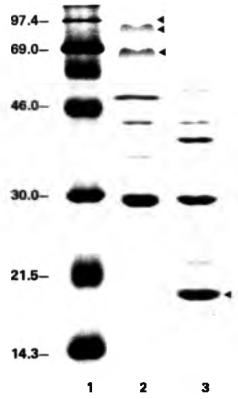


Figure 6.3.2.2 SDS-PAGE analysis of LT-B69 partially purified from periplasmic fractions of *E. coli* HB101 pFV1::69. This material was used to inoculate mice intranasally prior to aerosol challenge with *B. pertussis*. Lane 1, relative molecular mass standards (with mass shown in kiloDaltons on the left); lane 2, partially purified LT-B69 inoculum (unboiled); lane 3, partially purified LT-B69 inoculum (boiled). Bands containing LT-B69 are arrowed.

Figure 6.3.2.3

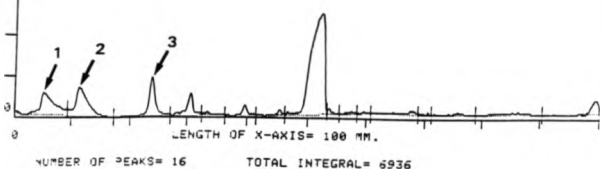
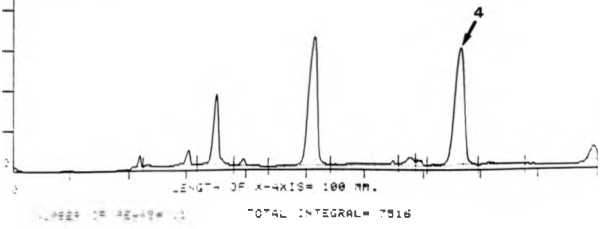


Figure 6.3.2.4



Figures 6.3.2.3-4 Densitometry analysis of lanes 2 (top) and 3 (above) of the gel shown in Figure 6.3.2.2. Peaks 1-3 represent pentameric LT-B69 and its associated breakdown products. Peak 4 represents monomeric LT-B69.

6.3.3 Aerosol challenge ELISPOT data

Table 6.3.3 (below) shows the number of LT-B-specific antibody secreting cells (ASC) in lungs of mice 7 days after boosting intramuscularly or intranasally with LT-B69.

These tables contain a great deal of data which require careful analysis. Firstly, a number of points should be raised concerning their interpretation. The assays were not standardised. The IgG-specific conjugate might have had a rather higher affinity than the IgA-specific conjugate, or vice versa. Thus direct comparisons of cell numbers secreting antibody of different isotype are not strictly valid, so where such differences are small, speculation is dangerous.

Secondly, some of the groups received antigen adjuvanted with cholera holotoxin. Even though the mass of CT given (0.1µg) was very small compared to the antigen dose (20µg), representing less than 1% of the total protein in the inoculum, because of the serological cross-reactivity between CT and LT-B, there is the probability that not all of the LT-B-specific ASC detected in these assays were truly directed against LT-B. It is evident from the results obtained from group 8 (which received irrelevant antigen together with CT) that this effect is not major but does contribute to the total number of ASC detected.

Table 6.3.3 The number of LT-B-specific ASC in the lungs of mice inoculated intranasally or intramuscularly with LT-B69

Group	ASC / 10^6 lymphocytes		
	IgG	IgA	IgM
1	N.D.	N.D.	N.D.
2	N.D.	N.D.	N.D.
3	2.4×10^3	2.1×10^3	N.D.
4	3.8×10^3	7.4×10^3	0.3×10^2
5	9.4×10^3	1.4×10^4	0.3×10^2
6	2.2×10^4	8.6×10^3	0.9×10^2
7	0.9×10^2	N.D.	N.D.
8	0.3×10^3	1.6×10^3	N.D.

(N.D. = not detected)

No P.69-specific ASC were detected except in groups 5 and 6, shown below (number/10^6 lymphocytes):

Group	IgG	IgA
5	2	5
6	1	3

Another observation which can be made concerning the number of LT-B-specific ASC in the lungs of mice in group 8 is that the inclusion of CT in the inoculum appears to disproportionately enhance the IgA response. Thus there are about 8 fold more LT-B-specific IgG-secreting cells in the lungs of mice from group 3 (which received no CT) compared to those of group 8 (which did receive CT), whereas for ASC of the IgA isotype the difference is only 1.3 fold. A similar comparison can be made between groups 3 and 5, where mice in group 5 were immunised by the same route with exactly the same inoculum (save the incorporation of CT) as mice in group 3. The increase in number of IgG-secreting cells was 3.9 fold whilst the magnitude of the IgA response was increased ~6.5 times. This observation is in agreement with the results of Chen & Strober (1990) who found that oral administration of CT could disproportionately augment the IgA response to co-administered influenza virus.

Bearing in mind the previously stated caveat concerning comparisons involving absolute numbers of ASC of different isotypes it is none the less apparent that, in all groups, ASC of the IgM isotype were either not found or were present only in very small numbers. This is not surprising. The assay was investigating a secondary response, thus one would expect a certain degree of maturation of the response, concomitant with class-switching. Equally, the preponderance of IgA- and IgG-producing cells, and their approximate equivalence in number, is in agreement with the findings of Brandtzaeg (1988), concerning the distribution of IgA-, IgG- and IgM-secreting cells in the normal lower respiratory tract of humans.

Another observation which can readily be made is the complete lack of antigen-specific ASC in the lungs of those mice inoculated intramuscularly. These mice did mount a systemic response, as evidenced by their serum antibody titres (see Figures 6.3.4.1. and 6.3.4.2). This is evidence that the lung ELISPOT assay is a true index of a local response and that there was no significant contamination of the samples by, for example, peripheral blood lymphocytes (the mice having been exsanguinated prior to removal of the lungs).

Clearly, the number of LT-B-specific ASC in the lungs of immunised mice is far higher than that of P.69-specific ASC. This is probably due to the greater number of B cell epitopes in LT-B and the cross-reactivity of LT-B with CT. Even so, it is somewhat surprising, considering the

results obtained from the preliminary experiment, in which small numbers of P.69-specific ASC were elicited without the need for CT adjuvant (Figure 6.3.1.4). This is possibly due to a greater degree of degradation in the fusion protein inoculum, observed by SDS-PAGE, leading to a loss of G_{M1}-binding activity by the LT-B pentamer and to cleavage and/or degradation of the BB05 epitope. This low number of anti-P.69 ASC is reflected in the non-appearance of anti-P.69 sIgA in the lung washes of intranasally immunised animals (data not shown). Nevertheless, it is significant that intranasal inoculation of peptide 683, corresponding to amino acid residues 544-574 of the mature P.69 sequence, failed to elicit either P.69-specific ASC or P.69-specific serum antibody (see Figure 6.3.4.2, below).

6.3.4 Antigen-specific serum antibody levels

6.3.4.1 Anti-LT-B serum antibody levels

An increase in the levels of anti-LT-B serum antibody could be detected in all groups which received the LT-B69 fusion protein, whether the route of administration was intramuscular or intranasal (see Figure 6.3.4.1). This increase was only slight after priming but after boosting significant increases were observed with the peak response generally occurring 1-2 weeks after boosting. Neither group 7 (immunised with a peptide from P.69) nor group 8 (immunised with an irrelevant *E. coli* protein preparation) displayed substantially increased LT-B-specific serum antibody levels, although some mice in group 8 showed a small response to the CT adjuvant. The presence of CT in the inoculum caused a marked enhancement of the anti-LT-B antibody response, irrespective of the route of inoculation, bringing about a 2 fold increase in the mean absorbance in ELISAs. By contrast, pre-treatment of mice with intranasally applied G_{M1} had little, if any effect on LT-B specific antibody levels. These results are broadly in line with the lung ELISPOT data in that groups 5 and 6 both had the highest number of LT-B-specific ASC in the lung and both had the greatest amount of circulating antibody.

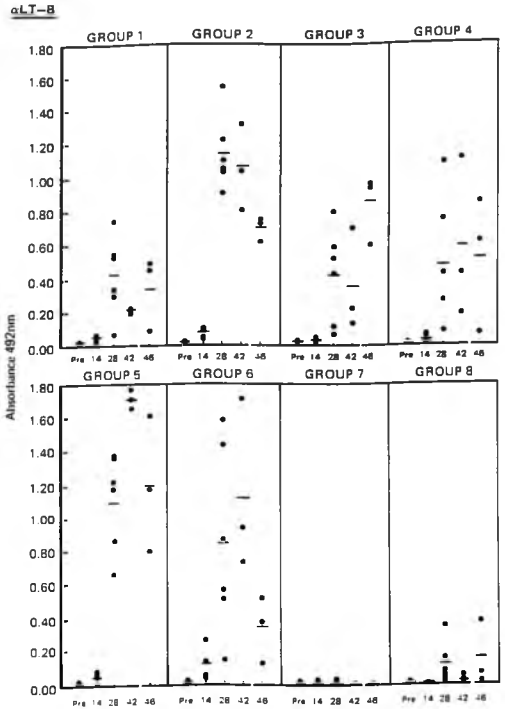


Figure 6.3.4.1 The LT-B-specific serum antibody response following immunisation with LT-B69, with or without various adjuvants (see Table 6.3.2.1). Individual serum samples were obtained on the days shown, diluted 1/1000 and analysed by ELISA. Mean values for each time point are represented by a horizontal bar.

6.3.4.2 Anti-P.69 serum antibody levels

A number of general points can be made concerning the levels of P.69-specific serum antibody in the mice (shown in Figure 6.3.4.2). Firstly, the background levels of absorbance in the ELISA assays was inexplicably high for all groups, despite the use of specific pathogen-free mice (uninfected by *B. bronchiseptica*). Secondly, the overall amount of P.69-specific antibody was considerably lower than that for LT-B. This may be explained by recent epitope-mapping experiments (Charles *et al.*, 1991) with P.69, which suggest that the sequence of P.69 inserted as a fusion with LT-B contains a single epitope - that recognised by the monoclonal antibody BB05. Thirdly, there was no enhancement of anti-P.69 serum antibody levels following challenge with *B. pertussis*, despite the exposure to large amounts of P.69 that such a challenge would involve. This is not surprising. The region of P.69 present in LT-B69 does not contain any T helper cell epitopes (J. Tite and M. Roberts, personal communication). Thus immunisation with LT-B69 would not generate any memory T helper cells specific for P.69. Therefore, even in challenged mice which had been inoculated with LT-B69, P.69-specific ASC received T cell help as a primary response.

Mice in group 8, which received irrelevant protein, failed to mount an anti-P.69 response. Mice in group 7, which received the P.69 peptide intranasally, appeared to mount a very slight response, as did groups 1 and 3, which were immunised with unadjuvanted LT-B69 fusion protein intramuscularly and intranasally respectively.

As with the anti-LT-B response, inclusion of small amounts of CT in the inoculum significantly enhanced the amount of anti-P.69 serum antibody, whether given intramuscularly (group 2) or intranasally (group 5). This is confirmation of the adjuvanticity of CT; interpretation of the results is facilitated by the lack of serological cross-reactivity between CT and P.69, unlike CT and LT-B.

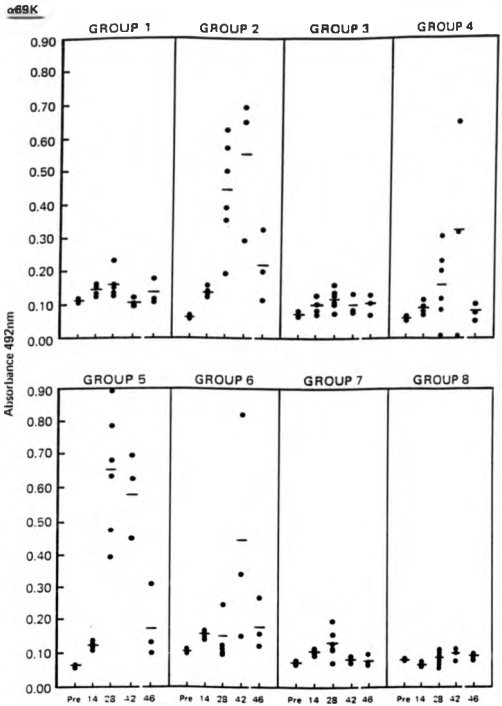


Figure 6.3.4.2 The P.69-specific serum antibody response following immunisation with LT-B69, with or without various adjuvants. Individual serum samples were obtained on the days shown, diluted 1/540 and analysed by means of an ELISA. Mean values for each time point are represented by a horizontal bar.

Pre-treatment of mice with G_{M1} appears to have a more significant effect on anti-P.69 titres than anti-LT-B titres, although the situation where both G_{M1} pre-treatment and CT adjuvant were used (group 6) appears to dampen the response relative to CT adjuvant alone (illustrated to a lesser extent by the anti-LT-B serum antibody levels). It may well be that insufficient time was allowed between the administration of G_{M1} and the inoculum, allowing exogenous unbound ganglioside to adsorb part of the inoculum.

The detection of P.69-specific ASC in the lung and P.69-specific serum antibody illustrates the successful use of an LT-B chimeric protein to stimulate both local and systemic immune responses to a genetically fused epitope delivered intranasally. This finding is significant because intranasal immunisation of mice with recombinant P.69 purified from *E. coli* or native P.69 purified from *B. pertussis* fails to stimulate serum anti-P.69 antibody (M. Roberts, personal communication).

6.3.5 Viable *B. pertussis* counts in lungs of infected mice

Seven days after boosting, all the mice were challenged with an aerosol of *B. pertussis*, as described in Chapter 2. Immediately after exposure to the aerosol, 4 unimmunised indicator mice were sacrificed and viable counts performed on homogenates of their lungs, to determine the size of inoculum which had seeded in the lungs of the exposed mice. The results are shown below in Table 6.3.5.1.

Table 6.3.5.1 Initial colonisation in the lungs of mice inoculated with an aerosol of *B. pertussis*

Mouse	Viable count (cfu/pair of lungs)	Mean +/- 1 S.D.
1	5.5×10^4	$10.5 \pm 4.9 \times 10^4$
2	7.0×10^4	
3	15.0×10^4	
4	14.3×10^4	

On days 7 and 11 after challenge, three mice per group were sacrificed and viable counts of *B. pertussis* in the lungs of the mice were determined, as described in 6.2. The results are shown in Table 6.3.5.2. Previous work (Roberts *et al.*, 1990) had shown that, with the size of inoculum used, assaying at these time points should establish if any protection had occurred, being around the peak of bacterial growth in the lungs of unimmunised mice. Unfortunately, there was considerable intra-group variation in the counts. This, coupled with the small size of the groups, makes it difficult to interpret the data.

It would appear that no protection was afforded by inoculating mice intranasally with peptide 683/KLH conjugate (group 7) relative to those mice inoculated with a mixture of irrelevant proteins (group 8). Similarly, no protective effect was apparent in those mice given partially purified LT-B69 intranasally, with or without G_M, pre-treatment (groups 3 and 4 respectively), although in the latter case 3/6 mice were not detectably colonised at the time-points studied.

Table 6.3.5.2 Colonisation of the lungs of mice 7 and 11 days after challenge with an aerosol of *B. pertussis*

Group	Mean cfu/animal (1 S.D.)	
	Day 7	Day 11
1	$4.6\ (3.7) \times 10^4$	$2.3\ (0.7) \times 10^4$
2	$<1.1^1\ (1.0) \times 10^4$	$2.5\ (2.7) \times 10^4$
3	$4.6\ (3.1) \times 10^4$	$5.4\ (7.0) \times 10^4$
4	$<1.5^2\ (2.5) \times 10^4$	$<5.3^1\ (8.4) \times 10^4$
5	$<5.0^2\ (9.0) \times 10^3$	$<2.0^1\ (2.0) \times 10^3$
6	$<3.0^2\ (4.0) \times 10^3$	$2.0\ (1.8) \times 10^4$
7	$1.9\ (0.6) \times 10^4$	$3.3\ (3.3) \times 10^4$
8	$1.6\ (1.3) \times 10^4$	$3.1\ (1.6) \times 10^4$

[1], 1/3 mice without detectable numbers of bacteria

[2], 2/3 mice without detectable numbers of bacteria

Counts were performed as described in Section 2.10.3. Serial tenfold dilutions in PBS were made of the lung homogenates. Five replicate 50µl drops were added to the surface of a CW blood agar plate (containing streptomycin) and colonies counted after 3-5 days incubation. The mean cfu/animal for each group was then determined. A "less than" symbol is included where no colonies were detected in any of the replicate drops from one or two mice in that group.

It might be argued that protection is apparent in group 6 (G_M, pre-treatment, LT-B69 plus CT I.N.) at the earlier time-point, with only 1 mouse colonised and by comparatively low numbers (~5 fold fewer than the mean value for group 8). However, no significant protection against colonisation is obvious at day 11. It would seem that only group 5 is consistently protected (LT-B69 plus CT I.N., no G_M, pre-treatment), with only a total of 3/6 mice colonised on the two time-points. At day 7, the mean count is more than 3 fold less than the control group and at day 11 there is over 1 $\log_{10}$ difference between the counts. It may be significant that the highest levels of P.69-specific serum antibody were observed in group 5 (Figure 6.3.4.2)

Whilst there is good evidence for protection in only 1 group (i.e. group 5), it seems suggestive, when viewing the results as a whole, that the lowest degree of colonisation occurred among those groups which received the fusion protein I.N. with CT (groups 5 and 6) or intramuscularly with CT (group 2), that is, in those groups with the greatest P.69-specific lung ASC and/or serum antibody responses. This would suggest that the BB05 epitope can stimulate a protective immune response if immunisation involves a suitable adjuvant. Previously it has been shown that immunisation with P.69 can protect mice against intracerebral challenge, and that passive immunity can be mediated by the transfer of BB05 mAb (Novotny *et al.*, 1985). Thus the results presented here would imply that responses to the BB05 epitope can also protect mice against an aerosol challenge. Ideally, to confirm these findings, such an experiment should be repeated with larger groups to reduce the significance of intra-group variation. However, these results are consistent with the recently published work of Charles and his colleagues (Charles *et al.*, 1991). In their study, a fusion was made between the gene specifying the hepatitis B virus core antigen (HBcAg) and synthetic oligonucleotides encoding amino acid residues 537-566 of P.69. The resulting fusion protein was expressed in yeast, purified and used to immunise mice intramuscularly. Mice treated in this way had viable counts of *B. pertussis* 10 fold lower than mice immunised with HBcAg alone. This protective effect was enhanced by the inclusion of incomplete Freund's adjuvant in the inoculum.

6.4 Summary

6.4.1 The mechanism of protection

As the degree of protection achieved was very limited it is difficult to say with confidence what the mechanism of protection might be. However, numerous other reports have shown a strong correlation between immunity to P.69 and protection from disease in mice infected with *B. pertussis* (Novotny *et al.*, 1985, Shahin *et al.*, 1990*b*, Charles *et al.*, 1991 and Fairweather *et al.*, 1990). There are two questions which are of interest; is protection a result of local or systemic immunity (or a combination of the two)? and what is the effector mechanism of the response?

6.4.2 Local or systemic immunity?

As the strongest evidence of protection is seen in group 5 (inoculated with LT-B69 fusion protein together with CT) and to a lesser extent in group 6 (pre-treated with G_{M1} and then inoculated with LT-B69/CT), one might conclude that, when using LT-B69 as an immunogen, intranasal immunisation is necessary to protect mice against aerosol challenge, suggesting that local responses are most pertinent. This would agree with the observation that P.69-specific ASC could only be detected in these groups. However, at day 11, there is little difference between groups 1,2 and 6, with regard to colonisation by *B. pertussis*, the former groups having been inoculated intramuscularly. It should be remembered that the two arms of the immune system do not operate in a mutually exclusive manner. Thus a case could be made for the involvement of systemic responses, a position which is supported by the serum antibody levels of the respective groups. Significantly, it has been found that in mice immunised subcutaneously with recombinant P.69 purified from *E. coli*, circulatory antibody specific for P.69 can protect against lung colonisation following challenge with an aerosol of *B. pertussis* (M.

Roberts, personal communication).

6.4.3 Possible effector mechanisms

As stated above, it has already been demonstrated that antibody directed against the BB05 epitope can be protective against *B. pertussis* aerosol challenge in a mouse model. It is possible therefore that specific antibody, whether sIgA or serum IgG, is the effective mediator of immunity in the experiment reported here.

In the aerosol challenge model, growth of bacteria is restricted to the respiratory tract. Systemic antibody, if it is to exert an effect on the course of the infection, must therefore leave the circulation and transudate into the tissues of the respiratory tract. There is evidence that i.v. administration of anti-P.69 monoclonal antibodies can lead to their detection in the lungs within 1-6 hours (Shahin *et al.*, 1990*b*) and can protect infant mice against aerosol challenge.

There are at least two mechanisms of protection one could envisage. P.69 is known to be surface-exposed (Charles *et al.*, 1989) and has been shown to play a role in the binding of *B. pertussis* to human epithelial cells (HEp2 cells) *in vitro* (Roberts *et al.*, 1991). P.69 contains an arginine-glycine-aspartate (RGD) tripeptide sequence which has been implicated in the binding of mammalian cells to P.69 (Leininger-Zapata *et al.*, 1989). Such RGD motifs have been demonstrated to play a role in the binding of several other proteins to their receptor molecules (e.g. Ruoslahti, 1988; Fox *et al.*, 1989). Thus it is possible that antibody binding to P.69 may cause a structural rearrangement or result in steric hindrance, leading to inhibition of binding to the host epithelium. However, recent evidence suggests that P.69 appears to be irrelevant to infection of the murine respiratory tract by *B. pertussis* (Roberts *et al.*, 1991): insertion mutants of *B. pertussis*, defective for P.69 expression, were able to colonise mice as efficiently as the parental strain following aerosol inoculation. It seems unlikely therefore that P.69-specific antibodies protect by preventing adherence to host respiratory tract epithelium cells, at least in mice. An alternative explanation is that binding of antibody may result in opsonisation, with

improved phagocytosis of the bacteria by those phagocytic cells with F_c receptors (such as alveolar macrophages), or may lead to complement-mediated lysis of *B. pertussis* cells.

6.4.4 Improving the protective efficacy of LT-B69

As mentioned in section 6.3.4.2, LT-B69 contains no T helper cell epitopes from P.69. Clearly, this represents one way in which the protective efficacy of LT-B69 could be enhanced. Such an approach has already been employed successfully to increase the antibody response to a peptide epitope from foot and mouth disease virus (FMDV), (Francis *et al.*, 1987*a,b*). Furthermore, there is no reason why the T helper cell epitope should have to be derived from P.69; any *B. pertussis* T helper cell epitope might suffice as long as the B cell and T cell epitopes are present on the same immunogenic structure (i.e. in this instance, on the same bacterial cell). This phenomenon ("inter-molecular - intra-structural help") has been described previously for influenza virus (Russell and Liew, 1979) and hepatitis B virus (Milich *et al.*, 1987) and such a mechanism might well be operable where the structure is a bacterium rather than a virion. Candidate HLA class II-restricted human T cell epitopes have been described in the S1 subunit of pertussis toxin (De Magistris *et al.*, 1989). The one major constraint on this approach is that the resulting fusion protein must still be capable of binding G_{M1}.

CHAPTER 7

General Discussion

7.1 Summary

It has been established by a number of workers that the immunogenicity of certain peptides, when administered to mucosal surfaces, could be enhanced by the addition or chemical coupling of CT-B or LT-B to the peptide sequence (see 1.7.1). Furthermore, a number of plasmid expression vectors have been constructed which allow genetic fusions to be made to CT-B or LT-B. However, the fusion proteins which are expressed from such plasmids are often sub-optimal because they do not retain the properties of native LT-B, being unable to pentamerise or bind to G_{M1}, the toxin receptor - characteristics which are probably a prerequisite for maximum immunogenicity.

This thesis describes the construction of an LT-B fusion protein expression plasmid, which incorporates several features (e.g. the use of codons comparatively rarely utilised by *E. coli*) designed to allow insertion of oligonucleotides encoding peptide sequences at the 3' end of the LT-B gene such that the resulting fusion protein is more likely to retain the properties of native LT-B. A number of such fusion proteins were produced and all, including the largest fusion protein (comprising a 31 amino acid residue C terminal extension), were found to form pentameric structures, be transported to the periplasmic space of *E. coli* and to retain affinity for G_{M1}.

Additionally, data presented here show that the fusion of LT-B to a peptide sequence can exert a mucosal adjuvant effect after intranasal inoculation of mice, resulting in both increased serum antibody levels and a greater local antibody-secreting cell response compared to immunisation with the peptide conjugated to an inert carrier protein (KLH).

7.2 The structure of LT - a rational basis for empirical observations

Recently, Sixma *et al.*, (1991) published a putative three-dimensional structure for porcine LT, based on X-ray crystallographic studies. This is shown below in Figures 7.2.1-2.

The LT-B monomers were found to consist of one short amino-terminal α helix (residues 5-9), a large central α helix (residues 59-78) and two three-stranded β sheets. These monomers are arranged in a circle around a central pore, much as earlier models predicted (Sigler *et al.*, 1977). They are so positioned that the β sheets of one monomer lie adjacent to the β sheets of its neighbours. The central pore is bounded by many charged amino acid residues (e.g. arginine, lysine) and numerous salt bridges are believed to be formed between these and the charged amino acid residues present in the A2 domain of the A-subunit, which inserts into the pore.

This model is particularly attractive because it explains several experimental observations. For instance, although degraded by boiling and low pH, the pentameric LT-B is reasonably stable in a range of environmental conditions. In the model of Sixma *et al.*, (1991) over 1/3 of the surface area of each monomer is buried by pentamer formation. This figure is higher than most comparable multimeric proteins and probably contributes to the observed stability. Despite this strong association between monomers, only a small number of interactions is believed to occur. According to the current model, one such interaction is between alanine at position 64 in one monomer polypeptide and a methionine residue at position 31 in an adjacent monomer. This would explain the finding of Iida *et al.*, (1989) that a single mutation (ala 64 → val 64) at this position can inhibit pentamerisation.

Furthermore, the G_{M1}-binding site in the present model is thought to reside in a pocket, which is formed by amino acid residues derived from adjacent monomers. This agrees with previous suggestions (e.g., Iida *et al.*, 1989) that LT-B must be in multimeric form to bind to G_{M1}. This is supported by the results obtained in this study concerning the affinity for G_{M1} of boiled and unheated samples of partially purified LT-B69.

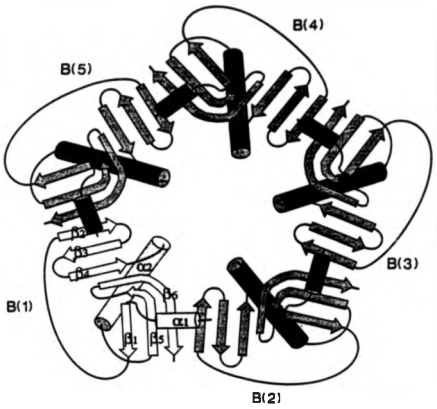


Figure 7.2.1 A schematic diagram of the secondary structure of the LT-B subunit and its pentameric arrangement. α_1:residues 5-9, β_1:15-22, β_2:26-30, β_3:37-41, β_4:47-50, α_2:59-78, β_5:82-88, β_6:94-102. Numbering of the B-subunits is anti-clockwise when viewed towards the A-subunit (reproduced from Sixma *et al.*, 1991).

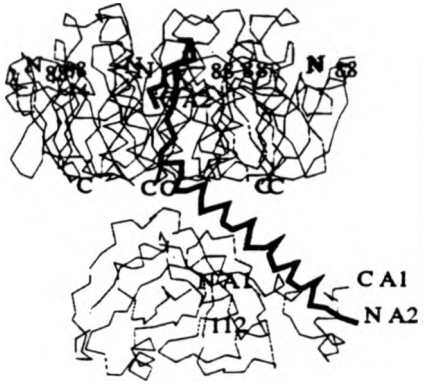


Figure 7.2.2 The three-dimensional structure of LT holotoxin. CA1 represents the last residue of the A1 subunit, NA2 the first residue of the A2 subunit. N and C show the positions of the amino and carboxyl termini respectively of the five B-subunit monomers. 88 represents the location of residue trp88, believed to comprise part of the G_{M1}-binding pocket (reproduced from Sixma *et al.*, 1991).

7.3 The structure of LT - implications for the improved design of LT-B fusion proteins

If the model of the LT-B pentamer proposed by Sixma *et al.*, (1991) is substantially correct, the amino-termini of the LT-B monomers are buried within the pentamer, whilst the carboxy-termini are at the "bottom" of the molecule and point away from the structure. Thus the addition of extra amino acids to the N-terminal might well abolish G_{M1}-binding ability by preventing pentamer formation whereas, because of the location of the C-termini, extensions can be made at these points without interfering with B-subunit - B-subunit interactions. This would support the observations made in this thesis concerning the G_{M1}-binding properties of various LT-B C-terminal fusion proteins. Thus, in the example of LT-B69, up to 31 amino acid residues could be accommodated without a significant deleterious effect on G_{M1}-binding.

Such extensions to the carboxy-termini probably would prevent association with the A-subunit (as experienced by Sandkvist *et al.*, 1987) due to steric hindrance interfering with the normal interactions between the A-subunit and the B-subunit pentamer (see Figure 7.3.2). However, in the context of potential use as mucosal adjuvants free from toxicity, this does not constitute a disadvantage. It is clear that C-terminal additions, within certain limits, should also permit LT-B/ganglioside interactions to take place unimpeded; the proposed G_{M1}-binding pocket is a considerable distance (in molecular terms) from the carboxy-terminal of the monomer.

Thus the C-terminal offers several advantages as a location for the addition of heterologous epitopes, which can be readily effected by the insertion of relevant DNA sequences at the unique *Spe*I site at the 3' end of the LT-B gene. One other favourable location for the accommodation of heterologous epitopes might be the surface-exposed loop, represented by residues 50-64, which has already been found to be immunogenic (Jacob *et al.*, 1985).

7.4 Future developments

One possible development to exploit immunogenic LT-B fusion proteins is to express them in suitable bacterial hosts, such as attenuated strains of *Salmonella* spp., which have proven efficacy as experimental oral vaccines. Such an approach has been described both in this thesis and by other researchers (Schödel *et al.*, 1990a,b) but the results have been rather disappointing thus far. It might be that synthesis within a bacterial vector alters the nature of the initial interactions between the LT-B fusion protein and the immune system. Thus, rather than the LT-B fusion protein being absorbed across a mucosal epithelium into, for example, Peyer's patches or bronchus-associated lymphoid units after oral or intranasal inoculation, the first interactions between the immune system and LT-B present in the periplasmic space of *Salmonellae* might occur with macrophages in the RES which phagocytose the bacteria. This might influence the ensuing immune response. Thus whilst expression in vaccine strains of *Salmonella* spp. may be suitable for stimulating good cell mediated responses to a particular immunogen, it is not necessarily ideal for all LT-B fusion proteins if they possess intrinsic mucosal immunogenicity.

An alternative approach might be to involve the holotoxin in some way; nearly all the comparative studies which have been described show that CT is more immunogenic than CT-B (Pierce, 1978, Lange *et al.*, 1978, Lycke and Holmgren, 1986, Chen and Strober, 1990 and van der Heijden *et al.*, 1991). Presumably the same is true for LT and LT-B. It would seem highly desirable therefore if one could produce an altered form of toxin A-subunit which retained immunological activity without toxicity, comparable to the Cross Reacting Material (CRM) mutant forms of diphtheria toxin (Pappenheimer *et al.*, 1972, Uchida *et al.*, 1973) and pertussis toxin (Pizza *et al.*, 1989). This would have considerable potential as an adjuvant for vaccines delivered orally or intranasally. However, as explained previously, toxin B-subunits extended at the C terminal are unlikely to associate with A-subunit due to steric hindrance. Thus a modified non-toxic A-subunit would have to be added as exogenous holotoxin (with native B-subunits) rather than as an integral part of a holotoxin with chimeric B-subunits. (Alternatively,

it might prove possible to engineer the modified non-toxic A-subunit such that it can associate with chimeric B-subunits).

One of the first examples of such an approach was described by Finkelstein *et al.*, (1971) who produced "procholeragenoid" - a stable, high molecular mass aggregate of A- and B-subunits, formed by heat-treatment of CT. Procholeragenoid had only 5% of the toxicity associated with CT but was still highly immunogenic when given to mice orally (Fujita and Finkelstein, 1972). However, this level of toxicity is unacceptable for use in human vaccines.

Similarly, Liang *et al.*, (1989) demonstrated that glutaraldehyde treatment of CT could diminish toxicity about 1,000 fold whilst the toxoid generated in this way retained some immunogenicity. However, this was still far from ideal because toxicity was not totally abolished and the adjuvant activity was substantially curtailed. Recently, Tsuji *et al.*, (1990) reported their findings concerning a hydroxylamine-induced mutant form of LT. DNA sequence analysis revealed a single base change, resulting in one amino acid substitution in the A-subunit at position 112 (glu112→lys112). This mutant had greatly reduced toxicity but the mucosal immunogenicity or adjuvanticity of the molecule was not described. It may well be that an effective compromise between toxicity and adjuvanticity proves exceptionally difficult to obtain as it seems that adenylate cyclase activation is an intrinsic part of both the toxin and adjuvant mechanisms. However, the recent determination of the structure of LT (Sixma *et al.*, 1991) should allow for a more rational approach (e.g. the logical application of site-directed mutagenesis) which should facilitate the task.

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