CHAPTER 4

DISCUSSION

Life is short, art long, opportunity fleeting, experience treacherous, judgment difficult.

Hippocrates
(460-370 BC)
Greek Physician and
"Father of Medicine."

Discussion

This study identifies three immunodominant epitopes within the GroEL gene i.e. EGQDRGYSY, YSYNKETGE AND GKGTEEKEK. When the peptides synthesized were screened with a monoclonal antibody to GroEL, there was significantly high binding to the peptide KGGKGTEEK, which contains a common overlapping sequence to the peptide GKGTEEKEK, one of the three immunodominant peptides (Panchanathan et al., 1998).

The cross-reactivity of GroEL of *E.coli* and other enteric pathogens, depending on the percentage homology of genomic sequences might cause problems when interpreting results of peptide synthesis and when attempting to develop diagnostic tests based on HSPs (Appassakij et al., 1987). Given GroEL of *S.typhi* has a high percentage of homology with that of *E.coli* (Lindler and Hayes, 1994), there is bound to be a high degree of cross reactivity in the antibody response to either protein. This will show as a high background in the reactivities of the peptides. A very useful way around the problem of high background is to increase the affinity threshold of the test to a point where only reactivities now remaining are those that result from the linear epitopes. This is achieved by performing the antibody-peptide interaction in solution, and, after separating the bound from the unbound peptide, capturing the complex in a suitable manner and now detecting the presence of antibody (Tribbick et al., 1991).

It has been demonstrated that heat shock proteins are induced and expressed in *S. typhi* following thermal stress and these entities are the target of the host immune response as indicated by the presence of antibodies in sera from patients with typhoid

fever (Tang et al., 1997). The work in this study confirms the important role of heat shock proteins in the pathogenesis of typhoid fever and further defines the linear B cell epitopes, showing that they are within the GroEL gene.

The development of the chemistry of grafting, synthesis and cleavage and scaling up of pin size has resulted in the high quality of peptides synthesized, and the feasibility of screening of thousands of peptides in the effort to map both the linear-defined epitopes (B cell epitopes) (Rodda et al., 1986; Getzoff et al., 1987), and of helper- and cytotoxic-T cell epitopes (Mutch et al., 1991; Burrows et al., 1994). In evaluating the long-term stability of the peptides to repeated ELISA testing and repeated cycles of de-protection, Geysen et al. (1987) found the usefulness of sets of peptides to be limited to 30-60 tests. With that in mind, the pins in this study were subjected to no more than 50 reactions before screening was stopped.

Limitations on the systematic use of peptides include the cost and the magnitude of the facilities required to produce a significant number of peptides for testing. Peptides are often coupled to a solid surface or a carrier molecule in order to allow detection of binding interactions between the peptide and a protein. This coupling procedure can significantly alter the presentation of the peptide, even to the extent of masking potentially reactive amino acid side chains.

There are many ways in which the conformational state of an antigen is "seen" by the immune response. The demonstration that a particular peptide reacts with a proportion of the antibodies present in a given serum provides little information as to which amino acids within that peptide are essential for interaction. The role of individual amino acids is readily determined from the reaction of the same serum with the set of

peptides comprising all possible single residue replacements within the original sequence (Geysen et al., 1985). Those residues for which replacement by dissimilar amino acids results in a complete loss of reactivity with the antibody are postulated to be either the contact-residues in intimate contact with the antigen-combining site of the antibody, or amino acids which fulfil an essential structural role in folding of the peptide.

Though of lower resolution, ELISA and Western Blotting with polypeptide fragments of an antigen can recognize discontinuous epitopes which would not be represented in a panel of synthethic peptides (Kelly et al., 1995).

A simple modification to reduce the background of the reactivities of the sera was by using small baths, any container that would fit the pins, in the various reactions, in place of microtiter plates. Ensuring that there was enough solution to cover the gears, and a wave motion is set up (shaker), greater mixing takes place in the bath, so diffusion rate limitations which apply to the antibody in the well are not as pronounced as in the bath. A second and probably more significant effect is that each pin-bound peptide only removes, from the solution, antibody that is specific for the peptide. When the only antibody solution available to the pin is the relatively small volume of the well of a microtiter plate, this places a limit on the total amount available, and the binding of the antibody to the pin significantly lowers the concentration of antibody in the solution, thereby slowing the rate of binding. However, with the large volume of antibody solution available to the pins in the bath technique, the concentration of each of the antibodies will decrease much less, and more total antibody can bind. A greater volume of antibody solution (30-50ml) is used in the bath technique, as compared with 20ml if the solution is dispensed into microtiter plates. However, the sensitivity increase and better signal-to-

background ratio mean that that a greater dilution of the antibody can be used in the test, actually conserving precious antibody. The only time it is not appropriate to use the bath method is when individual pins need to be tested with different antibody preparations in a single assay.

In order to limit the number of peptides to be tested in this study, those present in the hydrophilic regions were selected on the assumption that most of the antigenic epitopes will be found on the surface of the protein and that relatively hydrophilic regions are more likely to be on the surface (Moore, 1992). Immunogenic epitopes , on the other hand, need not be present on the surface , as it was found with the foot-and-mouth disease virus (Meloen and Barteling, 1986).

T cells recognize continuous epitopes: the T cell receptor (TCR) recognizes processed antigen in the form of short peptides produced by proteolysis of the native antigen. These peptides: 7-9 amino acids long in the case of CTL recognition; 12-24 amino acids long for helper T cells - are presented to the T cell by the MHC - encoded molecules, human leukocyte antigens in humans. The epitopes recognized by B cells are sometimes continuous but are more often discontinuous in that they involve side-chain contact residues on adjacent peptide chains because, unlike the T cell, the B cell recognizes native antigen on the surface of the infectious agent. The chances of having more than a very few sequential amino acids exposed on the surface of a protein are very low because hydrophobic amino acids tend to be buried within the core of the protein. For protein antigens, antibodies typically recognize 14-21 amino acids on the surface of the protein. The relevant epitopes, are, therefore, by necessity, frequently discontinuous,

and are made up of amino acids that are juxtaposed when peptide chains fold as the protein assumes its native conformation (Delves et al., 1997).

Synthetic approaches to T-cell epitope determination have recently been developed that complement the search for natural T-cell epitopes and the investigation of the preferences of the different MHC alleles for particular motifs in cognate peptide sequences. Positional scanning approaches with random synthetic peptide libraries allow comprehensive surveys of the sequence requirements for peptide selection by MHC molecules and for induction of T-cell responses. Synthetic T-cell epitopes can be determined independently of the knowledge of the natural T-cell antigen. This opens new perspectives for the development of synthetic vaccines, TCR antagonists and MHC blockers (Walden, 1996).

Identification of T cell epitopes using the Multipin Peptide Technique requires multiple overlapping peptides encompassing a protein to be assayed, which in humans, is limited by volume of donor blood. Honeyman et al. (1998), in an effort to overcome this problem, successfully used an artificial neural network model to predict peptides that bind to a MHC Class II molecule, and found this method to be both sensitive and specific.

The evolution of vaccine strategies has seen a move from the whole organism to recombinant proteins, and further towards the ultimate in minimalist vaccinology, the epitope. The epitope-based approach is compelling as only a relatively tiny, but immunologically relevant sequence is often capable of inducing protective immunity against a large and complex pathogen (Suhrbier, 1997).

Different routes of immunization may induce different patterns of T and B cell epitope recognition, and this may have implications for the design of vaccines (Brookes et al., 1995; Partidos et al., 1995)

Whitton et al. (1993) developed the "string-of -beads" concept, where 2 epitopes, each presented on a different major histocompatibility complex (MHC) class I background, can be linked in tandem to confer protection upon mice of both MHC haplotypes. They later extended this approach by expanding the immunogenic scope in 2 ways: first, by the introduction of T helper (Th) and B cell antibody epitopes alongside CTL epitopes and second, by including immunogenic sequences from a variety of infectious agents, five viruses and one bacterium. (Ling-Ling et al., 1997)

The capacity of linear peptides to neutralize or protect against pathogens has been reported previously (Francis et al., 1987; Boersma et al. , 1993 ; Langeveld et al. , 1994 ; Tabatabai and Pugh , 1994; Gilleland and Gilleland, 1995 ; Hughes and Gilleland, 1995 ; Robinson et al., 1995 ; Taubman et al., 1995). A classic example of linear peptides inducing a protective response against an infectious disease is that of the virus causing foot and mouth disease (Francis et al., 1987; Boersma et al., 1993). Furthermore, Chong et al. (1992) demonstrated that peptides derived from the S1 subunit of the pertussis toxin were capable of inducing antibodies with the ability to neutralize the enzymatically active site of this protein. However, failures to protect with linear peptides have been reported (Chong et al., 1992, 1993, 1995). Linear peptides in either a free, conjugated, or combined form derived from the P1 and P2 proteins of *Haemophious influenzae* were immunogenic in animals but failed to confer protection). The low immunogenicity of a

small peptide can be attributed to the lack of an appropriate T-helper lymphocyte epitope contained within the peptide (Chong et al., 1993; 1995).

An obstacle to epitope vaccine design is the polymorphism of MHC Class I molecules that, together with other proteins in the antigen procesing pathway, determine which peptides are displayed on the cell surface. Consequently, different individuals present different sets of peptides to CTL depending on their genetic make up (Hanke et al., 1998).

The HtrA protein has been identified as a virulence factor in *Salmonella typhimurium*, *Yersinia entreocolitica* and *Brucella abortus* (Johnson et al., 1991; Elzer et al., 1996; Li et al., 1996). The HtrA (or DegP) protein of *Escherichia coli* has been shown to be essential for survival of the organism at all temperatures of $>42^0$C. It is a stress response protein belonging to the E -dependent family of heat shock proteins (HSPs) (Chuang and Blattner, 1993). It is not related to either the 32 -regulated HSPs such as DnaK or DnaJ or the 70-regulated HSPs such as Hsp60, Hsp70, and Hsp90 (Lipinska et al., 1989). The *S. typhimurium* HtrA protein is almost 89% identical to *E.coli* HtrA but is not induced by heat shock, although it is induced by oxidative stress (Johnson et al., 1991). The exact role of HtrA in the bacterial pathogenesis remains to be determined; however if HtrA serves as a target for bactericidal antibodies or opsonic activity, it must be accessible to antibodies. Since HtrA is an apparently periplasmic protein, it is not evident how such antigen-antibody interactions can occur. It is possible that during infection, some surface expression of HtrA is induced as part of a stress response mechanism, e.g. fever. Other intracellular HSPs have been shown to become surface expressed under physiological stress conditions and have been implicated as

adhesion factors (Ensgraber and Loos, 1992; Evans et al., 1992; Hartmann and Lingwood, 1997). H91A, produced by site-directed mutagenesis of the *htrA* gene of *H.influenzae*, is currently used in the genesis of a vaccine for otitis media, now in phase I clinical trials.

HSPs, major targets of both antibody and T cell responses, have been implicated in experimental and human autoimmune arthritis and autoimmune insulin-dependent diabetes (Young and Elliot, 1989; Lydard and van Eden, 1990). The 65-kDa hsp of *S. typhimurium* plays an important role in the pathogenesis of reactive arthritis (Herman et al., 1990). HSPs are the target of the immune response in a broad spectrum of infections because HSPs are abundant under stress conditions e.g. in macrophages (*S .typhi, S. typhimurium*). Even in the absence of heat shock, HSPs are the most abundantly expressed proteins, indicating that uptake of bacteria by macrophages may reflect a stress situation resulting in the expression of bacterial heat shock proteins.

The importance of HSPs in the pathogenesis of disease is complex but its involvement has been established e.g. deletion of the *htrA* gene in *S.typhi* has resulted in attenuation of the organism and its subsequent use in vaccine trials (Tacket et al., 1997). A GroEL homolog of *Helicobacter pylori* has been found to be bound to urease, a recognized virulence factor. GroEL of *S.typhimurium* is responsible for the binding of the bacterium to the intestinal mucus (Ensgraber and Loos, 1992). Hsp60 is also selectively displayed on the surface of virulent *Legionella pneumophila* and plays an important role in invasion of HeLa cells (Garduño et al., 1998).

Altered expression of HSPs has been extensively documented in association with a diverse array of diseases including ischemia and reperfusion damage, cardiac

hypetrophy, fever, inflammation, metabolic diseases, infection, cell and tissue trauma, aging and cancer (Przepiorka and Srivastava, 1998). An important feature of HSPs is their role in the cytoprotection and repair of cells and tissues against the deleterious effects of stress and trauma. Over-expression of one or more hsp genes is sufficient to protect against otherwise lethal exposures to heat, cytotoxic drugs, toxins and tumor necrosis factor- α (TNF-α) (Meldrum, 1998).

Further work to be done along the lines of this study would include fine characterization of the epitopes, screening more typhoid sera, comparing sera from different geographical regions, or sera collected at different phases of the disease. It would also be interesting to see whether the identified epitopes are part of, and/or have common sequence motifs with T cell epitopes of *S.typhi*.