THE GENETICS OF THE IMMUNIZATION RESPONSE
TO TRIPLE ANTIGEN

A thesis submitted for the degree of

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in the

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by

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DECLARATION

This thesis embodies the results of a research project performed in the Department of Human Biology, John Curtin School of Medical Research, Australian National University from March, 1980 to September, 1983 whilst I was in receipt of an Australian National University Postgraduate Scholarship.

The experimental work and analytic data contained in this thesis is my own work. I have acknowledged any assistance I have received from other parties in the Acknowledgements.

DIANE P. RYAN

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ABSTRACT

The aims of this thesis were to examine adverse reactions and normal responses to a suboptimal immunizing dose of Triple Antigen vaccine administered to an unrelated neonatal population. Humoral primary responses to immunization and cellular responses following in vitro sensitization to individual components of the vaccine (B. pertussis, tetanus and diphtheria toxoids) were assessed. These responses were correlated with epidemiological variables, including adverse reaction to the vaccine and with a range of blood genetic markers, including HLA types.

This protocol provides two approaches for the study of immune response genes in man, by examining risk factors in adverse reactions to Triple Antigen and by examining immune response polymorphisms.

Firstly, logistic regression analysis, based on survey information regarding 507 infants, revealed that a parental history of allergy, breastfeeding and administration of the vaccine in partitioned doses increased the risk of an adverse reaction. A maternal history of adverse reaction to tetanus toxoid was also predictive in some infants.

The in vitro and in vivo immune response parameters estimated in this study did not correlate with an adverse reaction nor with its risk variables. Similarly, there was no association with any of the blood genetic markers. Despite these lack of associations, there is considerable potential in an immunization reaction model as a means of studying the role of genetic factors in a disease syndrome.
Secondly, bimodality in immune response parameters was established and examined for association with genetic markers determined in umbilical cord blood. The blood genetic markers used were those known not to be transferred placentally.

Bimodality was observed in the IgM response to *B. pertussis* and tetanus toxoid in 107 infants and in measurable lymphoproliferation following incubation with *B. pertussis*, tetanus and diphtheria toxoids in 53 umbilical cord cell cultures. IgG response to *B. pertussis* was negligible while the tetanus toxoid IgG response was influenced by the method of vaccine administration.

A strong association was found between HLA-DR5 and positive in vitro lymphoproliferation to *B. pertussis*. This association suggested that this HLA-DR antigen was either involved in the initial recognition of *B. pertussis* by unprimed cells or else with enhanced cellular response to a cross-reacting antigen.

The red cell genetic marker, esterase D, and the HLA cross-reacting group, A3-CREG, were associated with non-antigen specific IgM response. No marked association was noted between any genetic marker and an antigen-specific IgM response to either *B. pertussis* or tetanus toxoid probably reflecting the multigenic nature of in vivo antibody control. However, the *B. pertussis* IgM response was associated with antigenic combinations involving HLA-Bw44 inferring that response either required the complementation of MHC antigens or that a heterogeneous form of HLA-Bw44 was involved.
This study has provided strong support for the role of genetic factors in primary humoral responses and in vitro lymphoproliferation to foreign antigens by demonstrating that immune response genes in man are associated with the major histocompatibility complex.
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CHAPTER 1

Section (i) Introduction

This thesis investigates the immunogenetics of primary humoral and cell mediated responses to components of Triple Antigen vaccine, Bordetella pertussis and the toxoids of Corynebacterium diphtheriae and Clostridium tetani, in an Australian neonatal population.

Immunogenetics is the study of inherited differences in immune response, a field of study initiated in humans by Landsteiner, (1928) who described the importance of ABO incompatibility in blood transfusions. The early work in red blood cell genetic systems was extended, initially in experimental animals, into the role of major histocompatibility antigens in tissue graft rejection. Further developments in immunogenetics included study of immune response genes after associations were observed between histocompatibility antigens, susceptibility to particular diseases and inherited ability to respond differentially to foreign antigens. With regard to this latter development, inherited differences were observed in both in vivo and in vitro humoral and cell-mediated responses to specific antigens, including simple polypeptides. Before discussing development of studies which have correlated heterogeneous immune responses with immune response gene polymorphisms, some of the terms used throughout this thesis will be defined.

A gene is defined as a segment of DNA which is able to perform a specific function, in particular to direct the synthesis of a given polypeptide or protein. Genes are linearly arranged on a chromosome and the position a gene occupies is called a locus. Different types of the same
gene arising by mutation are called alleles. Loci which are positioned closely together on a chromosome are termed 'linked' and the alleles of these loci may be inherited from parent to offspring as a unit. A chromosomal segment consisting of closely linked and possibly functionally related genetic loci is called a genetic complex, the most notable being the major histocompatibility complex of man and animals.

The measurable consequence of a humoral immune response, both in vivo and in vitro, is the production of protein molecules or antibodies by immunocompetent cells of B (bursa equivalent) lymphocytic lineage specific for the inducing antigen which may be a microbial product, a chemical substance or cell surface structures from another individual of the same species (alloantigens) or different species (xenoantigens). The antibody produced may be of different immunoglobulin classes such as IgM, IgG, IgE, IgA or IgD. A cell-mediated immune response concerns the cellular recognition and destruction of antigen and involves the proliferation of activated T (thymus processed) lymphocytes which is measured in vivo as delayed-type hypersensitivity and in vitro by morphological change, increased uptake by the dividing cells of radioactive thymidine or increased release of non-cellular factors in the cell culture.

Section (ii) Historical aspects

The antibody response to a variety of antigens in experimental animals was assumed to be under some form of genetic control since a definite correlation existed between the quantitative responses of parents and offspring (Schiebel, 1943, Stern et al. 1956, Sobey and Adams, 1961). The actual mode of inheritance was unknown until Levine and Benacerraf
(1965) in Guinea Pigs and McDevitt and Sela (1965) in mice showed, using differing haptenic determinants on poly-L-lysine conjugates, that the antibody response was transmitted as an unigenic Mendelian dominant trait. Subsequent experiments by Benacerraf, McDevitt and their coworkers (see Benacerraf and McDevitt, 1972) using inbred strains of guinea pigs and mice, linked the control of this specific antibody response to the species-specific major histocompatibility complex (MHC). Up to this time, the MHC was known to exist as a single, large chromosomal region consisting of a number of linked loci which coded for the tissue cell surface glycoproteins present on all nucleated cells and was responsible for strong allograft rejection (see Bodmer et al. 1970). The mapping of the genes regulating antibody response, or the immune response (Ir) genes, to this region extended the biological importance of the MHC beyond the initial concept of histocompatibility. Research into the functional importance of the MHC and its gene products has accelerated over the past twenty years particularly after the observation by Lilly and coworkers (1964) in mice that resistance to Gross Leukaemia virus was influenced by MHC genes (which he later postulated (Lilly, 1971) controlled the immune response to the virus) and the subsequent finding by Amiel (1967) of an association between an MHC antigen (4c) and Hodgkin's disease, a neoplastic disorder, in man. This, however, was not the first reported association between a genetic locus and disease in man. The ABO, Rh, Lewis and other blood groups, red cell enzyme systems and haptoglobin have all been associated with variety of diseases (see Mourant et al. 1977 and Chakravartti, 1967).
The discovery of associations between the MHC region and disease in both man and animals has stimulated research into the possible mechanisms involved in disease processes. A great diversity of diseases has been significantly associated with the MHC. Many are chronic or recurrent in nature and most are assumed due to aberrations in the immune response such as the autoimmune or allergy syndromes (see Dausset and Svejgaard, 1977). In some cases an underlying infectious aetiology is suspected, for example, in ankylosing spondylitis and Klebsiella (Ebringer et al. 1978; 1979) or multiple sclerosis and measles virus (see Jersild et al. 1973, Fog et al. 1977, Walker et al. 1982) and the disease syndrome itself may be triggered by an abnormal immune response to the precipitating agent. Therefore, the assumption arises that immune response genes regulating the response to the infectious agent are probably involved in the pathogenesis of these diseases.

Prior to elaborating on the involvement of Ir gene effects in disease, the structure and function of the MHC will be examined in detail.

Section (iii) Structure and function of the MHC

The H-2 system of the mouse has been the most extensively studied vertebrate MHC. This system will be examined and its homology with the human MHC will be discussed.

H-2 system

The murine MHC is located on chromosome 17 and encodes three different classes of protein molecules which are involved in the immune response (Klein, 1975, Shreffler and David, 1975, Klein et al. 1981). Class I and II molecules are both cell surface glycoproteins which differ, however,
FIGURE 1.1 Genetic map of the major histocompatibility complex of mouse and man.
MOUSE: H-2 REGION

H-2K  H-2I  H-2S  H-2D  Qa2  Qa3  Tl  Qa1

K  Aα Aβ Eβ  Eα  Ss Slp  D  L

chromosome 17

MAN: HLA REGION

GLO  HLA-D  Complement Region  HLA- HLA- HLA- Qa/Tl

SB  DC  DR  C2  BF  C4A:C4B  B  Cw  A  like

centromere

chromosome 6
in intrinsic structure, tissue distribution and functional significance. Class III molecules comprise the serum components of the blood complement system. The localisation of genetic regions in the murine MHC is presented in Fig. 1.1.

Class I molecules, coded by the H-2K and H-2D regions, are the classical transplantation antigens which can be detected by alloantisera or cytotoxic T cells. The molecular structure of this class consists of 45,000 MW glycosylated single polypeptides covalently bound in situ with a \( \beta_2 \) microglobulin encoded by a genetic locus on chromosome 2 (Michaelson, 1981, Ploegh et al. 1981). Class I molecules are found on the surface of all nucleated cells in the body (for review see Klein, 1975).

The function of these ubiquitous molecules, apart from a general role as the antigenic targets in allograft rejection cell-mediated cytotoxicity, is to act as restriction elements in the response of cytotoxic T cells to cells which are virally infected, haptenated or carry different minor histocompatibility antigens (See Doherty and Zinkernagel, 1975, Paul and Benacerraf, 1977). This function demonstrates the important concept of MHC restriction. For example, as presented by Zinkernagel and Doherty (1974) T cells will only recognize and kill a viral-infected cell if the viral product to which they have been sensitized is associated with the same Class I antigens (H-2K or H-2D gene products) present on the original immunizing cell. It is still debatable whether the cytotoxic T cell recognises an antigen-induced modification of the Class I molecule (altered self, single receptor) or else shows dual specific-
ity where both the MHC antigen and the antigen are recognised individually although in combination for activation of the T cell (see Doherty et al. 1976, Thomas et al. 1977, Zinkernagel and Doherty, 1979).

Class II molecules are encoded by the H-2I region. This region originally was divided by the analysis of different aspects of the immune response in intra H-2 recombinant inbred mouse strains into five subregions I-A, I-B, I-J, I-E and I-C (Murphy, 1980). Klein et al. (1981) disputes the existence of I-J, I-B and I-C and recent experiments, using cDNA clones and 'chromosomal walking' techniques, have shown that I-J and I-B subregions are not located in the H-2I region (Steinmetz et al. 1982a). The I-A and I-E subregions encode for the immune region associated (Ia) antigens or Class II molecules (Klein et al. 1981). Structurally, these molecules are cell membrane associated sialoglycoprotein heterodimers composed of a 33,000-35,000MW chain and a 28,000-31,000MW chain which are non-covalently linked (Uhr et al. 1979). The structural peptides of the dimers \( A_\alpha', A_\beta \) and \( E_\gamma \) chains are encoded by the I-A subregion and \( E_\alpha \) by I-E (Steinmetz et al. 1982a). A further non-MHC coded invariant chain is also associated with the structure (Jones et al. 1979). Class II molecules have a very limited tissue distribution being found on B lymphocytes, subpopulations of T cells, macrophages, blood monocytes, reticuloendothelial cells and spermatozoa (Ploegh et al. 1981). I-J region gene products are believed to code for soluble T cell factors and cell surface antigens on T cells and macrophages which suppress immune function (suppressor factors and cells) (Murphy et al. 1976, Niederhuber et al. 1979, Ochi et al. 1982). The I-B and
I-C subregions are involved in the control of the immune response towards certain antigens (Lieberman and Humphrey, 1972, Benacerraf and Germain, 1978).

The function of the I region is to control and regulate the immunological response of an individual (see Munro and Waldmann, 1978). Class II molecules act as MHC restriction molecules on macrophages and B cells and regulate the recognition of foreign antigen by helper, delayed type hypersensitivity (DTH) and suppressor T cells (Nagy et al. 1981). The quantitative difference in the humoral or cellular immune response to certain antigens which results from the interaction between immunocompetent cells is controlled by immune response genes whose gene products are the Ia antigens (Lerner et al. 1980, Klein et al. 1981). Dominantly inherited responsiveness or non-responsiveness to an antigen is determined by the effectiveness of a given combination of antigen and Class II molecule in stimulating responder or suppressor T cells. For example the mechanisms suggested for non-responsiveness include (i) the inability of antigen presenting cells (APCs) of the non-responder haplotype to present foreign antigens to T cells in immunogenic form (Rosenthal, 1978, Benacerraf, 1978) and (ii) the absence from the T cell repertoire of specific clones able to recognise the antigen in the context of certain MHC antigen (Von Boehmer et al. 1978, Ishii et al. 1981) (iii) active stimulation of antigen specific suppressor cells (Blanden, 1980). Similar mechanisms have been proposed for responsiveness (Blanden, 1980, Benacerraf, 1981) and are probably controlled by Ir genes. Ir genes, therefore, may have a specific and non-specific effect on immune response as
suggested in a workshop meeting by van Rood (see Immune response genes, 1980). It is still debated if some genes may encode for part of the T cell receptor (acceptor hypothesis) (Taussig et al. 1975, Krammer and Eichman, 1977) although Steinmetz et al. (1982a) believe that only a restricted number of Ir or Ia genes exist and not a vast array which would be needed for all possible T cell receptors.

Class III molecules are components of the complement cascade. The H-2S region, situated between H-2I and H-2D, encodes for the murine equivalent of the complement factor C4 (also called the Ss-Slp system) (Shreffler, 1976). Klein et al. (1981) believes that H-2S should not be considered part of H-2 complex because it neither resembles structurally nor does it control similar traits associated with the rest of the complex. However, its functional role in the immune response or its importance in the mapping of immune response or disease susceptibility genes in the MHC region cannot be disputed.

Two regions telomeric to H-2D, Qa and Tla encode Class I related antigens which are expressed on certain classes of lymphoid cells (Weiss et al. 1983). There is a high degree of homology between these antigens and the classical Class I antigens encoded by H-2K and H-2D (Margulies et al. 1982, Steinmetz et al. 1982b). The function of these molecules is not yet understood.

The functional significance of the H-2 region is presented in Table 1.1.

**HLA System**

The MHC region in man is situated on the short arm of
TABLE 1.1

FUNCTIONS OF THE H-2 COMPLEX

<table>
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<th>1-J</th>
<th>1-E</th>
<th>1-C</th>
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<tr>
<td>REJECTION OF ALLOGRAPHS</td>
<td>+++</td>
<td>++</td>
<td>-</td>
<td>-</td>
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<td>CELL-MEDIATED LYMPHOLYSIS</td>
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<td>MIXED LYMPHOCYTE REACTION</td>
<td>+</td>
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Adapted from Klein et al. (1981).
chromosome 6 (Breuning et al. 1977) and is called the HLA region (see Fig. 1.1). The region's name derives from the classical transplantation antigens coded by this chromosomal segment which were detected serologically on white blood cells (Human leucocyte associated or HLA antigens). This group of antigens, designated HLA-A, HLA-B and HLA-Cw structurally resemble the Class I antigens of H-2 being single polypeptides covalently bound to $\beta_2$ microglobulin which, in man, is encoded by chromosome 15 (Goodfellow et al. 1975, Barnstable et al. 1978). HLA-A, -B and -Cw antigens are distributed on all tissue cells (see Amos and Kostyu 1980) and are similar to the murine molecules in that they are the antigenic targets for allograft rejection (Morris et al. 1978) and can restrict the cytotoxicity of killer cells against a variety of antigens (McMichael et al. 1977, Dickmeiss et al. 1977, Goulmy et al. 1977; 1979).

The human equivalent of the murine I region, despite limited evidence as to the location of Ir genes in man, is the HLA-D region which lies centromeric to the HLA-B region and is bounded by a red cell enzyme locus, glyoxylase (GLO) (Olaisen et al. 1976). The gene products of these human and murine regions are similar both structurally and functionally as will be discussed.

The genetics and postulated structure of the HLA-D region has recently undergone reassessment. Originally antigen definition of the region's gene products was based on cellular reactivity in a mixed lymphocyte reaction (MLR). This defined the HLA-Dw antigens believed coded by the HLA-Dw locus which controlled the primary stimulatory determinants (reviewed by Termijtelen et al. 1982). Later
serological equivalents of HLA-Dw were discovered which were termed HLA-D related or -DR antigens (Bodmer et al. 1977, Nousiainen et al. 1980, De Marchi et al. 1980) although some authors, in particular Suciu-Foca and coworkers (1979, 1980) and Sachs and associates (1981), dispute this relationship and believe the cellular and serological antigens derive from separate loci. Further serologically defined HLA-D region loci were postulated including MB, MT, BR, DC, DS, LB and Te (Duquesnoy et al. 1979, Park et al. 1978, 1980, Tanigaki and Tosi, 1982). The association between established HLA-DR specificities and these new loci suggested strong interrelationships between these non HLA-DR loci and that they may be representative of alleles at a single locus (Shackelford et al. 1981, 1982, Strominger, 1983). This locus is now generally referred to as HLA-DC. An additional locus HLA-SB has been mapped to the HLA-D region by primed lymphocyte typing and is now believed to code for minor stimulatory determinants (Shaw et al. 1981, 1982). It is highly likely from preliminary work in molecular genetics with human HLA-DC and HLA-SB cDNA clones that more loci await definition (Hurley et al. 1983).

The HLA-DR, HLA-DC and HLA-SB coded molecules resemble in both tissue distribution and structure the class II molecules of the mouse. Both man and murine Ia antigens are dimeric sialoglycoprotein molecules with a heavy or \( \alpha \) chain and a highly polymorphic light or \( \beta \) chain (Silver and Ferrone, 1979, Shackelford and Strominger, 1980). HLA-DR has been shown to be homologous to murine I-E antigens (Springer et al. 1977, Allison et al. 1978) and HLA-DC (Bona and Strominger, 1982, Goyert et al. 1982) and
possibly HLA-SB (Hurley et al. 1982, Strominger, 1983) are homologous to I-A antigens. The antigens are primarily found on B lymphocytes although HLA-DR have a similar distribution to that of mouse Ia (Hart et al. 1981). This homology between murine and human Ia antigenic structure, which is further strengthened by sequence homology (Allison et al. 1978) adds to the existing evidence that Ir genes may be found in the HLA-D region.

In similarity to the murine system, HLA-D region antigens have demonstrated MHC restriction in cell-cell collaboration in the generation of an immune response in vitro. The presentation of PPD (Bergholtz and Thorsby, 1977; 1978; 1979, Haar and Heron, 1982), tetanus toxoid (Kurnick et al. 1980) influenza (Lamb et al. 1982a; 1982b, Eckels et al. 1982) or herpes simplex virus (Berle and Thorsby, 1980, Stewart et al. 1981) by macrophages to responder T cells in vitro required HLA-DR identity between the APC and T cell before lymphocytic proliferation occurred. The in vitro proliferation could also be blocked by specific HLA-DR antisera which further emphasised the functional aspect of these molecules (see Thorsby et al. 1982). These experiments provide direct proof for the involvement of HLA antigens in the immune response which is further implied by the association between HLA-gene products with a variety of diseases (Dausset, 1981).

The closely-linked structural genetic loci for the serum complement components properdin Factor B (Bf), C2, C4A (Rodgers) and C4B (Chido) are assumed to be situated between HLA-B and HLA-D region (see Arnason et al. 1977, Meo et al. 1977, O'Neill et al. 1978a; 1978b). Unlike the confirmed by Carroll et al. 1983, Nature 307: 237.
rest of the known MHC region, this chromosomal segment is highly conserved with negligible crossing over and in general its inheritance is equivalent to that of a single genetic unit (Awdeh et al. 1983).

Section (iv) Polymorphism and linkage disequilibrium

An important characteristic of the MHC Class I and Class II molecules is their extreme polymorphism. Up to 1982, in man, there were approximately 20 defined specificities of HLA-A antigens and 32 HLA-B antigens reported in Caucasoid populations although there are numerous reports on heterogeneity being found in established specificities (see Terasaki, 1980). The polymorphic component of Class I molecules are contained in differing polypeptide sequences in the primary structure of the molecule (Lopex de Castro et al. 1982). The $\beta_2$ microglobulin is invariant (Ploegh et al. 1981).

The HLA-D region, with its multiple loci also has a great capacity for the generation of polymorphic molecules. In the HLA-DR antigens and presumably also in the HLA-DC and HLA-SB antigens, the main polymorphic determinants are contained in the $\beta$ chain of the dimer (Silver and Ferrone, 1979, Shackelford and Strominger, 1980) although considerable variability can be contributed by the $\alpha$ chain which is a similar situation to that found in the mouse (Uhr et al. 1979, Steinmetz et al. 1982a, Strominger, 1983). Further polymorphism may be derived from the combination of different $\alpha$ and $\beta$ chains especially in the heterozygote although association between the polypeptide chains of different loci has not been detected (Jones et al. 1981, Huber et al. 1981, Ozato and Sachs, 1982).
Point mutation, gene duplication (see Ceppellini et al. 1967) and gene conversion (Lopez de Castro et al. 1982, Weiss et al. 1983) may be the physical mechanisms responsible for the generation of this polymorphism. However, these events do not explain why the number of different alleles is so high in the MHC compared to other genetic systems or why most individuals (90%) are heterozygous for HLA-A, -B, -C and -D region loci.

Selective pressures favouring heterozygosity in the MHC may account for the maintenance of this polymorphism (see Bodmer et al. 1972). Selection may be for a complex individuality marker system to prevent mutual fusion or parasitism between members of the same species (Burnet, 1973) or extrapolating from the concept of MHC restriction and the involvement of MHC molecules in antigen recognition, it would be of an evolutionary advantage to a species if the size of the repertoire of recognition molecules for environmental antigens, in particular infectious agents, was extremely large (Jerne, 1971, Doherty and Zinkernagel, 1975, Zinkernagel, 1978). Alternatively, the MHC polymorphism could be secondary because of its close linkage with another polymorphic locus such as T/t in the wild type mouse which controls surface structures important in development (Snell, 1968, Bodmer, 1972a, Bennet, 1975). Recent papers have suggested a similar locus may exist in man (Amos et al. 1975, Fellous et al. 1982, Awdeh et al. 1983).

MHC genes are inherited from parent to offspring as a haplotype which represents the combination of alleles from various loci present on a chromosomal segment.
Despite the presence of recombination during genetic meiosis which may rearrange the parental allelic combination inherited, certain combinations of antigens occur more often together than expected by random association in a population. This 'linkage disequilibrium' is characteristic of particular HLA alleles such as HLA-Al and HLA-B8 in caucasoids. Different racial populations exhibit distinctive antigenic combinations (see Bodmer and Bodmer, 1978).

Several hypothesis have been advanced for this phenomenon of linkage disequilibrium. Genetic drift, recent mutation, non-random mating and migration with population stratification and associated 'founder effect' could explain such disequilibrium in small racial groups. (see Cavalii-Sforza and Bodmer, 1971, Bodmer and Thomson, 1977). However, the strength of certain antigen combinations in a population suggests a selective advantage for these gene linkages as has been argued by Bodmer (1972b). Selective pressures exerted by infectious diseases such as the epidemics of smallpox, bubonic plague and influenza serotypes may be responsible for the existence of specific genes which confer the ability to respond effectively to these infective agents (Motulsky, 1960, Marsh et al. 1980). Complementation between two or more genes may be required for an adequate immune response to the pathogen, especially if individual gene expression is inadequate, as has been found in mice (Dorf and Benacerraf, 1975, Melchers and Rajewsky, 1975) and suggested by Greenberg et al. (1981) to exist for the response to streptococcal antigen, so the presence of these genes linked on a haplotype would have a survival advantage and therefore be preserved.
Alternatively, another gene regulating the immune response (immune response or disease resistance gene) in linkage to these antigenic combinations, may be the component selected for resulting in non-specific co-selection of the common chromosomal segment (known as the 'hitch-hiker effect'; see Kan and Dozy, 1978) and the apparent disequilibrium. Recently the inheritance of a non-random association between MHC alleles, especially HLA-B and DR and the serum complement loci forming an extended MHC haplotype in Caucasoid families has been hypothesized as indicating the presence of a genetic locus equivalent to that of T/t in the wild mouse or Segregation Distorter (S.D.) in Drosophila melanogaster wild populations (Awdeh et al. 1983). It was implied in this report that the linkage of this extended haplotype (from HLA-A to the GLO locus) was due to either suppression of recombination or else due to the haplotype 'hitch-hiking' on the same chromosomal segment as a segregation distortion gene.

Section (v) The MHC and Disease

Major evidence for the existence of human Ir genes in the MHC has so far been indirect with most proof arising from the observed association between certain diseases and the HLA region (see Dausset, 1981). The strongest associations are now being found with the gene products of the HLA-D region according to Svejgaard et al. (1980; 1983) and Stastny et al. (1983) in the most up-to-date reviews of this area of research. These associations have stimulated considerable interest into the possible mechanisms involved in disease pathogenesis with wide ranging implications in the diagnosis, prognosis and genetic counsel-
ling for certain diseases (for example from Brewerton and Albert (1977) the diagnostic value of HLA-B27 in ankylosing spondylitis and other arthropathies) and has also highlighted the genetics of these diseases with particular emphasis on the postulated involvement of immune response genes (Dausset, 1977).

However, finding a significant MHC association with a disease syndrome does not automatically lead to increased knowledge of the pathogenic processes involved. Few diseases such as the enzyme, complement component or blood coagulation factor deficiencies have a measurable, clear-cut pathogenic mechanism with a definable genetic aetiologic factor (see Jersild et al. 1976, Vogel and Motulsky, 1979). Instead the majority of diseases studied in association studies are complex immunopathologic syndromes of unknown aetiology such as, for example, diabetes mellitus and systemic lupus erythematosus (reviewed extensively by Dausset and Svejgaard, 1977). In these diseases there are wide variations in clinical signs such as organ involvement, severity of symptoms and age of onset although the basic diagnostic criteria of the disease, for instance, persistent hyperglycaemia in diabetes mellitus (Creutzfeldt et al. 1976) is the same. In such studies MHC associations have helped define the heterogeneity present in these disease syndromes (Stastny et al. 1983). For example juvenile or insulin dependent and maturity onset or insulin independent diabetes mellitus, previously differentiated by age of onset, are now established by the strong HLA-DR associations in IDDM (Wolf et al. 1983) and the lack of association in NIDDM (in Caucasoids) (Nerup et al. 1974) as different
disease entities with the same diagnostic sign (Cudworth and Festenstein, 1978). Similarly, "classical" and drug induced SLE syndromes have been divided by their different HLA-DR associations (Svejgaard et al. 1980). However, the significance of these associations is still clouded. Many hypothetical mechanisms have been proposed to explain these interesting findings (see Svejgaard et al. 1975, Stastny et al. 1983) but no established linkage between the MHC antigen and a major pathologic defect responsible for the disease has been clearly demonstrated.

Environmental factors play an important part in the genetic study of disease syndromes (Buckley, 1979). Practically, the presence of environmental phenocopies (see Votel and Motulsky, 1979) can diminish the strength of association between the MHC antigen and the genetically determined syndrome. This is a particular problem when diagnostic criteria are not comprehensive. However, many diseases require an environmental 'trigger' before a pathologic syndrome develops. The most obvious example of this interaction is the initiation of an atopic syndrome by an allergen (King, 1979). In other diseases, especially those with a suspected immune defect, the interaction is more subtle and may be purely hypothetical, for example, the involvement of measles virus in the pathogenesis of multiple sclerosis (Fog et al. 1977) or coxsackie B virus and IDDM (Yoon et al. 1979). The non-occurrence of disease in an individual known by family study to be susceptible probably results from non-exposure to this triggering agent. This situation also decreases any true association which may exist between MHC antigens and the disease.

Therefore, the probability of finding a high concor-
dance between a disease and a single genetic factor is remote when the presence of disease heterogeneity and variable environmental components are taken into consideration. A notable exception is the HLA-B27 associated seronegative arthropathies, in particular, ankylosing spondylitis (AS) (Brewerton et al. 1973, Dick et al. 1974, Aho et al. 1973, Friis and Svejgaard, 1974). Although positive evidence is lacking in most instances, it has been hypothesised that preceding infection with entero bacteria such as Yersinia, Salmonella or Klebsiella may trigger AS (see Brewerton and Albert, 1977) and that the disease may result from a reduced immune response to these infectious agents which could arise from crossreactivity between HLA-B27 and the infective bacterial strains (Seager et al. 1970, Geczy et al. 1980) or else from the presence of a defective immune response gene associated with HLA-B27 (Nikbin et al. 1976). Therefore, the initiation of the pathogenic processes of AS, although not all the observed pathology, is associated with a single MHC gene.

Another similar example is gluten-sensitive enteropathy (GSE) a small intestinal disorder involving massive epithelial cell destruction due to wheat gluten toxicity (Strober, 1976) which was initially associated with HLA-B8 (Falchuk et al. 1972, Stokes et al. 1973) and later with the combination of HLA-DR3, the HLA-D region antigen in known linkage disequilibrium with HLA-B8, and HLA-DR7 (De Marchi et al. 1979, Betuel et al. 1980). HLA-B8, however, has also been associated with elevated gluten specific antibody titres (Scott et al. 1974) and positive in vitro lymphocyte transformation to wheat gluten (Cunningham-Rundles et al. 1979) in both GSE patients and normal controls. Combined with
the observation that GSE patients have a greater frequency
and titre of gluten antibody than normal controls (Alarcon-
Segovia et al. 1964, Taylor et al. 1964), the association
of HLA-B8 with both GSE and the immune response to gluten,
the aetiopathological agent, strongly suggests that the HLA-B8
associated gluten-specific immune response plays an import-
ant role in the initiation of the disease process. However,
not all GSE patients have HLA-B8 nor all HLA-B8 positive
have the disease which necessitates the presence of other
disease-susceptibility factors, possibly as represented
by the HLA-DR associations, for GSE to occur (Strober, 1977,

These two examples indicate that, despite obvious
heterogeneity in the genetic predisposition to a disease,
certain determinable facets, in particular the immune
response to the pathogenic agent, can be related to the
observed MHC association. Thus a better understanding of
at least part of the pathogenesis of the disease and the
involvement of MHC genes can be achieved. Extrapolating
from this observation, the study of MHC associations with
specific immune responses to suspected aetiological agents
may be predictive of those diseases which have a demonstra-
ted immunological basis and postulated infectious or envi-
ronmental cause. This supposition relies upon the exist-
ence of immune response genes, similar to that of the mouse,
controlling immune responses to environmental antigens in
man. The current state of this research pertinent to this
study will now be examined.
Section (vi) MHC and immune responses to environmental antigens

In contrast to the experimental protocol of Ir gene investigations in the mouse and guinea pig (see Benacerraf and Germain, 1978) the search for Ir genes to particular antigens in man has been hampered by both the ethical restrictions placed upon the immunization of non-biologic antigens in human investigations and the genetic heterogeneity present in the outbred human population. However, one approach in the study of human Ir genes is to examine associations between MHC antigens and the immune response to naturally occurring antigens. The reason for this approach is that an immune response gene to an environmental antigen, in particular an infectious agent, is more likely to be found in an outbred population since this antigen may have placed great selection pressure on this population in the past resulting in the reasonably high population frequency of the specific response gene (Boettcher, 1975, Marsh et al. 1980). Associations have been reported between the HLA region antigens or haplotypes and specific humoral responses to a variety of environmental antigens including natural and attenuated viruses, bacteria or their byproducts (see Kaslow and Shaw, 1981) and allergens such as rye grass (Marsh et al. 1980) and cedar pollen (Sasazuki et al. 1983) in both population and family studies. HLA-associated genetic control of the magnitude of skin lesions due to delayed-type hypersensitivity reaction to intradermal injections of microbial antigens has also been reported (Buckley et al. 1973, Morris et al. 1977). However, the study of in vivo humoral responses in many may have the same difficulties as studying MHC associations with disease syndromes. First, there is the possibility of polygenic control obscuring a specific Ir gene effect. The antibody
response to a complex antigen, that is, an antigen with many determinants, is controlled by multiple genes including MHC mediated effects (Feingold et al. 1978, Mouton et al. 1979). The use of a limiting dose of such an antigen or else immunization with simple unideterminant molecules are needed to clarify a MHC genetic effect (Munro and Waldman, 1978). However, in human investigations, where exposure to the study antigen is by natural or ethical immunization, neither of the necessary antigen conditions are achieved and the situation is similar to that of a complex antigen. Ir gene effects may still be observed in this case in inbred congenic strains of mice (Benacerraf and Germain, 1978) although not necessarily in human volunteers with non-equivalence of their non-MHC loci. This is exemplified by the study of Haverkorn et al. (1975) who could not show, in monozygotic and dizygotic twins, apart from perhaps measles virus, that a clear-cut HLA linked control existed for the antibody response to routine ethical vaccines. This result may be interpreted as either no specific response gene existing for the tested antigens or a specific genetic control existed but was obscured by the effect of other genetic loci.

A second disadvantage of this type of in vivo study is the vagaries of environmental exposure to the test antigen. When the response following natural infection is measured, the variability in response may reflect differences in exposure rather than a genetically controlled response. Similarly, immunization with a 'natural' antigen might result in a variety of primary and booster responses depending upon previous natural exposure to the antigen with corresponding differences in the measured level of response. This situation highlights the advantages in the
animal model where the controlled administration of a synthetic antigen is used.

Another approach in Ir gene investigations is to study certain facets of an in vitro analogue of an in vivo immune response to a specific environmental antigen. Pioneered in mouse studies (Shevach and Rosenthal, 1973, Schwartz et al. 1976), these experiments examine the action of Ir genes at the cellular level. In human studies, they have been used to test for the presence of postulated Ir genes and the functional significance of cell surface Ia antigen. The most commonly used approach is the T cell proliferation assay, reportedly the analogue of delayed type skin hypersensitivity (Oppenheim and Rosenstreicher, 1976) which measures the antigen recognition ability of T cells. Several studies, using this assay, have found HLA associations with bimodality in response to specific antigens such as Streptokinase-streptodornase (SK-SD) (Greenberg et al. 1975), tetanus toxoid (Sasazuki et al. 1978), Candida antigen (Nose et al. 1980), vaccinia virus (de Vries et al. 1977), streptococcal cell wall antigen (Sasazuki et al. 1982, Nishimura and Sasazuki, 1983), schistosomal cell wall antigen (Sasazuki et al. 1980b) and synthetic polypeptides (Hsu et al. 1981). In this latter study Hsu et al. (1981) used T-GAL and H-GAL to both sensitize and challenge human lymphocytes in vitro. Therefore, this in vitro study not only circumvented the ethical restrictions on in vivo immunizations in human studies and expanded the potential repertoire of antigens which could be tested under controlled conditions for possible Ir gene involvement, but also guaranteed uniformity of antigen exposure generally not
found in in vivo studies. Reliance upon environmental exposure or artificial immunization to the study antigen still formed the basis of the other studies mentioned.

Family studies used by Greenberg et al. (1980), Sasazuki et al. (1982) and Hsu et al. (1981) established that responsiveness to the tested antigen was linked to the HLA region and these three studies subsequently hypothesized the existence of antigen-specific Ir genes. Isolation of the particular chromosomal segment coding for this responsiveness or, in the case of Sasazuki and coworkers, non-responsiveness, was hampered by the handful of families showing recombination between the hypothesized immune response gene and various HLA loci. On the basis of one intra-HLA recombinant family, Hsu et al. (1981) mapped the response genes to both T-GAL and H-GAL in the HLA-A and -B chromosomal segment which concurred with the work of Taussig (1978) who mapped T-GAL 'acceptor gene' close to the HLA-A locus. The equivalent locus in the mouse, Ir-1, has been mapped to the H-2I region. The remaining two studies dealing with the immune response to bacterial antigens did not have recombinant family data although Sasazuki et al. (1983), in the absence of confirmatory data, has claimed that the gene controlling low responsiveness to streptococcal wall antigen was in linkage disequilibrium with a HLA-DC allele.

Section (vii) Relevant background to the aims of this thesis

Section (vii.1)

This thesis investigates the possible presence of immune response genes controlling the primary humoral and cell mediated immune response to Bordetella pertussis
(B. pertussis) and the formalin inactivated toxins (toxoids) of Corynebacterium diphtheriae (C. diphtheriae) and Clostridium tetani (Cl. tetani) in an unrelated Australian Caucasoid population. These immune response genes could be hypothesized to exist in this population because before the introduction of immunization programs, both B. pertussis (the aetiological agent of whooping cough) and C. diphtheriae (disease agent of diphtheria) produced major epidemics in Australia (Feery, 1981). Variable individual susceptibility to infection by these bacteria is well known ranging from inapparent infections to fulminating disease syndromes, possibly culminating in death, which suggests the possible involvement of differential genetic factors in the population (see Davis et al. 1976a; 1976b). The case of immune response genes specific to Cl. tetani or its toxin is less clear. This ubiquitous organism does not have the communicability of the other two bacteria and, apart from the highly fatal neonatorum tetanus syndrome, which results from umbilical cord contamination at parturition, no recorded natural epidemic has ever occurred. In an unimmunized, untreated individual with tetanus, an effective toxin neutralizing response sufficient for survival is not generated and, if treated, no residual protective response is observed (see Davis et al. 1976b). Therefore, only artificial immunization affords protection against this disease and any specific Ir gene effect, if one exists, would probably control response to the toxoid rather than the toxin.

The population selected for this study were infants who were two to five months of age at the time of antigen administration. The main advantageous characteristic of
this population compared to the older children and adults used in other similar studies is its immunological naivety which is essential in a study on the primary immune response to infectious agents. The possibility of transplacental sensitization to the study antigens, in utero, cannot be discounted and therefore a preimmunization sample, in this study umbilical cord blood, is necessary to either confirm or deny that previous sensitization has occurred. Environmental exposure to the toxoid antigens or original bacterial toxins is highly unlikely though there is a slight probability that contact with *B. pertussis* might occur. However, infections by this organism at such an early age are extremely serious and would not go unnoticed. By contrast, inapparent infections in older children are not unusual not only to *B. pertussis* but to a variety of other infectious organisms. The measurement of preimmunization antibody titres, in this latter case, may not indicate that the individuals have been sensitized to the infectious agent, especially if the antibody response is not persistent, and reintroduction of the agent would lead to a strong recall response.

The major disadvantage of investigations using neonatal population(s) is the lack of availability of biological samples for testing. The method utilized in this study to circumvent this problem is presented in the Research Design (Chapter 2, Section ii).

The study antigens were administered under controlled conditions to all members of this neonatal population by means of the ethical vaccine known as Triple Antigen in Australia and as DPT (Diphtheria-Pertussis-Tetanus) in the
United Kingdom, United States of America and Europe. A major drawback in previous studies investigating the immune response to ethical vaccines was that the vaccine dose administered was given to achieve optimum protective immune response and not maximal response to a specific antigen determinant which was the role of the limiting dose conditions of animal Ir gene investigations (Benacerraf and Germain, 1978). In Australia, adequate protective response to the components of Triple Antigen is achieved after the administration of the vaccine as three individual doses in a primary course. Each injected dose given after the initial dose has an enhancing effect on the immune response produced by the preceding dose (see Davis et al. 1976d). Therefore, the immune response to the initial immunizing dose is not maximal and may correspond to a limiting dose. The humoral response to *B. pertussis* and tetanus toxoid following initial Triple Antigen dose was studied in this thesis.

The cellular response to *B. pertussis*, tetanus and diphtheria toxoid was measured following incubation of these antigens in relevant umbilical cord mononuclear cultures. Under the assumption that neonatal cells would be naive to the test antigens, the presence of an antigen-specific cellular response occurring in these cultures would represent primary in vitro sensitization of these cells and indicate the probable existence of specific antigen recognition molecules coded by immune response genes.

Deviances in the frequency of particular MHC and red cell enzyme genetic markers were examined in the differential humoral and cellular immune responses to the bacterial antigens in this population. Population studies of this
type in general are biased towards the association of a measured trait with an increased frequency of a certain genetic marker, particularly of the MHC antigens where a decreased frequency is less likely to reach significance since the average population frequency of the individual MHC antigens is very low (Svejgaard et al. 1974). Observed response-genetic marker associations in population studies may either detect heterogeneity in the population sample due to racial stratification, with the measured character being more prevalent in a certain racial group, or indicate the direct causation of the response by the allele showing the frequency deviation or else suggest linkage equilibrium between the marker allele and the gene(s) coding from the response (Svejgaard et al. 1975). Careful selection of the experimental population precludes the deviations being due to racial heterogeneity. The latter two explanations cannot be separated in a population study as this separation requires family data involving recombinations in the genetic region under study (Day and Simons, 1976). However, a population study cannot establish genetic linkage between a marker gene and the response gene. Neither can it detect loose linkage between these two genes nor polygenic control of the measured response especially if only one gene is linked to the genetic marker locus (Svejgaard et al. 1975). Again, in this situation, family data may be more informative. A population-based study has been chosen for this thesis because of the impossibility in trying to measure a primary response to the studied bacterial antigens, equivalent to that sought in naive neonates, in all family members.
Therefore, using the previously mentioned techniques, this section of the thesis intends to examine the existence of human immune response genes and, if present, localise these hypothesized genes by association study to specific chromosomal regions; in particular, to certain MHC regions.

Section (vii.2)

This thesis also examines the predictive value of the measured immune responses to the individual bacterial antigens and their corresponding genetic marker associations in determining the occurrence of an adverse reaction to Triple Antigen vaccine. Of the children immunized with Triple Antigen, a certain proportion will experience an adverse reaction following immunization. This reaction may range from mild behavioural irritability and local reaction to serious neurological disturbances (see Cody et al. 1981, Feery, 1982). These reactions are believed to have an immunological basis and most have been ascribed to the B. pertussis component of the vaccine (Cody et al. 1981, Miller et al. 1982). In the mouse and the rat, two 'disease syndromes', histamine sensitization (Munoz and Bergman, 1968) and experimental allergic encephalomyelitis (EAE) (Levine and Sowinski, 1973), have been associated with the injection of B. pertussis organisms. In the first syndrome, histamine sensitization, variability in the effect of Histamine Sensitizing Factor (HSF), a cell surface antigen of B. pertussis, in sensitizing inbred strains of mice to the lethal effects of histamine, has been observed. In EAE the variability is contained in the efficacy of B. pertussis as an adjuvant in the host immune response to central nervous system myelin protein, the
severity of the neurological damage produced depending upon
the magnitude of the myelin-specific response (Lennon and
Byrd, 1973, Bernard et al. 1976). Susceptibility to both of
these syndromes is genetically coded with possible involve­
ment of MHC linked genes (Wardlaw, 1970, Levine and Sowwin­
ski, 1974, Bernard, 1976) although debate on the extent of
this involvement remains unresolved (Ovary et al. 1973,
will be hypothesized in this thesis that the adverse
reaction to Triple Antigen may also be a 'disease' syndrome
due to B. pertussis and that susceptibility to this syn­
drome is genetically linked. The heterogeneity of this
'disease' model will be examined and association between
specific genetic markers, the immune response to the various
components of Triple Antigen Vaccine and their corresponding
genetic marker associations, if any, will be sought.
CHAPTER 2

Research design, materials and methodology.
Section (i) Research design

Over a four month period, from June to early October, 1980, a 10 ml sample of umbilical cord blood was collected from the majority of infants born in two of Canberra's major maternity hospitals, Royal Canberra and Woden Valley. Collection was performed by the attending obstetrician and the cord blood transferred to the John Curtin School of Medical Research for separation and storage. In this way, a large initial subject base with sufficient sample per subject was created.

In the Australian Capital Territory (A.C.T.) children are immunized according to National Health and Medical Research Council (NH&MRC) recommendations. At the time the study was proposed these recommendations consisted of a primary course with Triple Antigen being given at two, four and six months of age and Diphtheria-Tetanus 12 months later. 85 per cent of all immunizations performed in the A.C.T. are given by health workers in Capital Territory Health Commission Immunization Clinics. Modifications to the recommended NH&MRC regime instituted in these clinics will be discussed in Chapter 3.

Most children born in Canberra are immunized by these clinics and, under normal circumstances, return to the same clinic for each injection. Therefore, children whose cord blood was collected at the beginning of the study period would be returning to the health clinics for the four month injection of the primary Triple Antigen course at the end of the cord collection period.

To retrieve as many of the original cohort as possible, each major immunization clinic was visited over the next
four months from mid-October, 1980, to early February, 1981. At the clinic, each mother returning with a child for the second Triple Antigen injection was invited to participate in the survey, interviewed and, with parental informed and written consent, a blood sample via a heel prick was taken from the child for use in antibody determination. Sufficient information was collected at birth and at the interview to accurately match the cord and heel blood samples.

The preceding protocol has the major advantage that the collection of all data (with the obvious exception of the cord blood) could be performed by one person whilst still gaining maximum benefit. However, at the interviewing stage, on two days a week, two major immunizing clinics were operating simultaneously necessitating a second person to conduct the interviews and heel sample collection on these "double" days. Clinic protocol and the implementation of the survey questionnaire will be discussed in Chapter 3.

Section (ii) Media and reagents

All chemical reagents used were of analytical standard and purchased from Ajax Chemicals, Sydney, Australia unless otherwise indicated.

Diphtheria toxoid (purified)

100 Limes flocculation units (Lf) per ml.
Contains 0.01% thiomersal. (Commonwealth Serum Laboratories - CSL - Parkville, Melbourne, Australia).

Tetanus toxoid (partially purified)

100 Lf per ml. Contains 0.01% thiomersal (CSL).
Bordetella pertussis (bacterial cell suspension)

40 International Opacity Units (I.O.U.) per ml.
Contains 0.01% thiomersal (CSL).

Foetal Calf Serum (FCS)
Sterile solution, mycoplasma free (Flow Laboratories).
Complement content heat inactivated by immersion of stock bottle in 56°C water bath for 30 mins.

Normal Saline
9g sodium chloride (NaCl) dissolved in 1 litre of distilled water (0.9% solution).

Phosphate Buffered Saline (PBS)
8.5g sodium chloride (NaCl).
1.07g disodium hydrogen phosphate (Na$_2$HPO$_4$. anhyd).
0.39g sodium dihydrogen phosphate (NaH$_2$PO$_4$. 2H$_2$O)
dissolved in 1 litre of distilled water.

Hanks Buffered Salt Solution (Hank's BSS)
Solution 1:
100g sodium chloride (NaCl).
5g potassium chloride (KCl).
1.25g magnesium sulphate (MgSO$_4$.7H$_2$O).
1.25g magnesium chloride (MgCl$_2$. 6H$_2$O).
1.75g calcium chloride (CaCl$_2$)
12.5g glucose (C$_6$H$_{12}$O$_6$).
dissolved in 1 litre of distilled water.

Solution 2:
3.76g disodium hydrogen phosphate (Na$_2$HPO$_4$. 2H$_2$O).
3g potassium dihydrogen phosphate (KH$_2$PO$_4$)
dissolved in 1 litre of distilled water.

Final mixture:
4 parts solution 1 to 1 part solution 2.
**RPMI medium**

10.4g RPMI 1640 powder containing 1-glutamine (GIBCO, Biocult, Glasgow, Scotland).
dissolve in 1 litre of distilled water.
sterilize by millipore filtration.
store at 4°C.

**HEPES buffer**

238.3g N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid (HEPES, Sigma Chemical Co., St. Louis, USA) dissolved in 700 mls double distilled water (NaOH)
titrater pH of solution to 8.1 with 5N sodium hydroxide
make up to 1 litre with double distilled water
sterilize by millipore filtration
store at 4°C.

**RPMI - antibiotic culture medium**

RPMI medium

2g sodium bicarbonate (NaH$_2$CO$_3$).
100,000 units penicillin (Benzylpenicillin sodium, CSL).
100,000µg streptomycin (Streptomycin sulphate, GLAXO)
25ml HEPES buffer
sterilize by millipore filtration
store at 4°C.

**AET solution (2%)**

2g 2-aminoethylisothylisothiouronium bromide hydrobromide (AET, Sigma Chemical Co., St. Louis, USA) dissolve in 100mls distilled water
titrater to pH 8.0 with sodium hydroxide (NaOH).

**Eosin yellow stain**

Solution 1:

25g eosin-yellow powder (Hopkins and Williams, England).
dissolve in 500mls distilled water (5% solution)

Solution 2:
2.55g sodium chloride (NaCl)
dissolve in 100mls Hank's BSS (2.55% solution)

Final mixture
3 parts solution 1 to 1 part solution 2

Formalin
Formaldehyde Stock 40% solution (Labser Pty. Ltd.,
Peakhurst, N.S.W., Australia).
Titrate to pH 7.0 with 5N sodium hydroxide (NaOH)
Buffer with excess calcium carbonate (CaCO₃)

ELISA Coating Buffer
1.50g sodium carbonate (Na₂CO₃)
2.93g sodium bicarbonate (NaHCO₃)
0.20g sodium azide (NaN₃, Natriumazid, Fluka, AG Buchs
SG, Switzerland)
dissolved in 1 litre distilled water
pH adjusted to 9.6
Store at 4°C, replace every 2 weeks.

ELISA Wash Buffer
Phosphate buffered saline (PBS) containing
0.05% v/v Tween 20 detergent

ELISA Diluting Buffer
Phosphate buffered saline (PBS) containing:
0.05% v/v Tween 20 detergent
0.25% w/v Bovine serum albumin
0.01% w/v Sodium azide (NaN₃)

Urease-conjugated sheep antihuman IgG antibody
Diluted for use in assay with ELISA Diluting Buffer.
Detects all classes of human IgG (CSL).
Urease-conjugated sheep antihuman IgM antibody

Urea with bromocresol purple, pH of 4.8 in sterile solution

Stored at 2°-8°C (CSL).

Phytohaemagglutinin

Lectin (Sigma chemical Co., St. Louis, USA)
diluted to test dilutions with normal saline and stored in the short term at -20°C.

McCoy's Transport Medium

6.5g McCoy 5A medium, dried (GIBCO)
100,000 units penicillin (Benzylpenicillin, CSL)
0.1g Streptomycin (Streptomycin sulphate, Glaxo)
8mg Gentamycin (Sigma Chemical Co.)
2.5ml foetal calf serum (Flow Laboratories)
3.0ml pH indicator Phenol Red (0.5%)
500ml Double distilled water
6ml HEPES buffer

Adjusted to pH 7.0 with 5N NaOH.

Filtered with 0.2-0.45µm filter.

Stored at 4°C.

Section (iii) Antigen preparation

The individual antigenic components of Triple Antigen vaccine were purchased in special product form from CSL with each preparation containing 0.01% thiomersal as a preservative. Thiomersal (ethylmercurithiosalicylate) is a mercurousulphur compound with extreme cytotoxicity and the capacity to neutralize the enzyme urease. Since these antigen suspensions were to be used as additions to cell culture and, also as the adsorbed antigen in an enzyme linked immunosorbent assay (ELISA) utilizing an urease
conjugate antibody, the preservative, as a precaution, was removed from the preparations by dialysis.

Both tetanus and diphtheria toxoids were dialysed against normal saline for a period of 36-48 hours at 4°C and in the dark to minimize deterioration. The protein content of both toxoids was determined by the Lowry protein estimation technique (Lowry et al. 1951) before and after dialysis to ensure that no substantial loss or dilution of the toxoids occurred during the process. Bovine serum albumin (1mg per ml; Armour Pharmaceutical, Eastbourne, England) was used for the construction of the protein concentration standard curve. A decrease of 20-30% occurred in both toxoids' estimated protein content during dialysis. The final protein concentrations were tetanus toxoid 0.2mg protein per ml and diphtheria toxoid 0.6mg protein per ml. The dialysed preparations then underwent millipore filtration using a 0.4µm filter (Sartorius Membranfilter, Göttingen) before being aliquoted as 1 ml amounts into sterile screw-top glass bottles (FBG-Trident Ltd., Temple Cloud, Avon) and stored at 4°C away from light.

B. pertussis cell suspension was dialysed against phosphate buffered saline (PBS) under aseptic conditions to prevent any microbial contamination. B. pertussis concentration was estimated after dialysis by visual counting of the organisms under a microscope before being aliquoted and stored as described for the toxoids.

New batches of toxoid and cell suspension were dialysed every three weeks, when in use, to minimize possible contamination and breakdown which might be accelerated with handling.
Section (iv) Cord blood collection, separation, storage and retrieval

(a) Collection

Cord blood from the umbilical artery after the delivery of the placenta, was collected into 10 ml heparinized plastic screwtop tubes (John's Products) by the attending obstetrician and kept at room temperature until transported to John Curtin School. Upon arrival, each sample was identified by the child's date of birth, mother's name and date of birth with home address and obstetrician's name, if available. A code number which was cross-referenced to the available data, was given to each sample. Information concerning the condition of each sample on arrival (for example, if it had been mistakenly refrigerated during its progress from labour ward to delivery point or, if insufficient mixing or delayed transfer to the heparinized tube had resulted in clotting), the date of collection and the date of actual delivery to JCSMR was noted.

(b) Separation

After centrifugation at 2000 rpm for 10 mins (all centrifugation were performed in a Clements GS200 bucket centrifuge), the plasma, buffy coat and red cell layers, were removed from each collection tube.

Plasma was aliquoted as 1ml quantities into sterile glass vials (FBC-Trident Ltd.) and stored at -20°C. Erythrocytes, after two washes in 0.9% saline at 200 rpm for 10 mins, were similarly aliquoted and stored.

White cell separation was performed following the technique of Boyum (1968) with appropriate modifications. The buffy coat, transferred to a 15 ml glass test-tube
(Assistent, West Germany) was diluted 1:6 with warm normal saline (cold saline causes excessive contamination of the white cell layer with autologous rosetting red cells). Six mls of the blood-saline suspension was carefully layered onto 4 mls of Ficoll-Paque gradient (Pharmacia Fine Chemicals, Upsala, Sweden) containing 5% normal saline in glass test tubes. This gradient combination proved most effective in removing residual RBC, mostly immature reticulocytes, without significant loss of the lymphocyte-monocyte population.

The mononuclear layer was harvested from the gradient interface, washed once in 10% foetal calf serum (FCS) in PBS (all washing steps performed at 2000 rpm for 4 minutes) and then resuspended in 8 mls of 10% FCS-PBS. Platelets were removed from the preparation by underlaying the suspension with 3 ml of a 20% sucrose in distilled water solution and centrifuged at 1000 rpm for 15 mins, gradually increasing the initial speed so as not to disturb the sugar gradient. Contaminating platelets remained at the interface and were discarded with the supernatant. The cell pellet was washed once in 10% FCS-PBS and resuspended in appropriate volume of the same medium for counting in a haematocytometer (Bright Line, Spencer, USA)

(c) Freezing

Cryopreservation was performed as a modification of the technique suggested by Amos (1977). Following counting, the cell pellet was washed once and resuspended, on ice, in cold 30% FCS in Hanks Balanced Salt Solution (Hanks BSS) to a concentration of 7-10 x 10⁶ cells per ml. Chilled 20% Dimethylsulphoxide (DMSO, Mallinckrod, Kentucky, USA) in
Hanks BSS was added slowly by dropwise addition to the cell suspension with constant agitation until the cell and DMSO mixture were of equal volume (final DMSO concentration of 10%). This mixture was aliquoted as 2 ml amounts into chilled polypropylene screwtop ampoules (NUNC, Denmark) and placed into partially perforated invaginations of a polystyrene tray in an -80°C Revco freezer. Over a 24-36 hour period the ampoules in the polystyrene tray undergo freezing at a rate of approximately 1°C per minute until -70°C is reached. The ampoules were then transferred to storage boxes in a liquid nitrogen freezer (-196°C) for long-term storage.

(d) Thawing of mononuclear cells

Thawing procedure used was a modification of that suggested by Amos (1977). The NUNC ampoules were retrieved from nitrogen storage and placed in a 37°C water bath until partially thawed. The contents were transferred to a 10 ml plastic capped test tube (Disposable Products) and diluted with 10 ml cold 30% FCS in either Hanks BSS or RPMI (depending on the ultimate use of the cells) which was added in a slow dropwise method (similar to that used when freezing) so that the DMSO concentration was gradually decreased from 10% to less than 1%. DMSO at room temperature is toxic to cells. Cell suspension was immediately washed and the supernatant discarded. After one further wash in the appropriate media the cell pellet was ready either for preparation in cell culture or for HLA typing.

Section (v) Cell culture

The suggested SEOPF method of cell culture for cell mediated tests (Tissue Typing Handbook, 1976) was modified
for use in the cord cell stimulation studies. To minimize possible bacterial and fungal contamination, the thawing and initial preparation of the mononuclear cells was performed in a laminar flow unit except when the cells were transferred in sterile closed plastic tubes for passage to and from the centrifuge for the washing steps.

(a) Mononuclear cell preparation

After thawing, the cell pellet was resuspended in 30% FCS in RPMI medium and a 50µl aliquot taken for counting. The aliquot was mixed 1:1 with 0.1% aniline blue for determination of viability whilst the cells were being counted. Trypan blue was originally used for viability testing but proved toxic to the cord cells. The cell suspension was washed once and resuspended in 10% FCS in RPMI-antibiotic medium to a live cell concentration of $1 \times 10^6$ cells per ml.

(b) Cell culture technique

The cultures were performed in lidded 96 well round bottomed tissue culture plates (Limbro). 150µl of adjusted cell suspension was aliquoted into each well using an Oxford pipetter (Oxford Sampler System, USA) ($1.5 \times 10^5$ cells per well). 50µl of test antigen suspension (diluted in RPMI media) was added to the appropriate well and adequately mixed using the pipetter. "Unstimulated" controls received 50µl of RPMI media per well. Triplicate cultures were set up for each combination of antigen dilution (or media if unstimulated) and individual cell sample. Each cell sample was tested against various dilutions of each antigen: Bordetella pertussis $4 \times 10^8$, $2 \times 10^8$ and $1 \times 10^8$ organisms per culture; Tetanus toxoid 4µg, 2µg, 1µg protein per culture; Diphtheria toxoid
14µg, 7µg, 3.5µg and 1.75 g per culture. The determination and rationale behind using these specific doses is given in Chapter 5. The cultures were incubated at 37°C in an atmosphere of 5% CO₂ in air with 99% humidity for 7 days. During the last 16 hours of the incubation the cultures were pulsed with 1.0µCi titrated thymidine (New England Nuclear) delivered in 50µl of RPMl media.

On the 7th day, the cultures were harvested by means of an automated cell harvester (Skatron, Flow Laboratories, Scotland) with the cells being collected onto perforated glass fibre disc sheets (Titertek, Flow Laboratories, Scotland). The disc sheets were dried for 30 mins in a 60°C incubator and the individual discs were then removed with blunt-nosed tweezers and placed in plastic scintillation vials (Zinnser, West Germany). Five to seven mls of a scintillation "cocktail" containing 0.5% 2,5-diphenyloxazole (PPO) in xylene was added to each vial and the disintegrations read in a liquid scintillation gamma counter (Packard, USA). All results were corrected for the efficacy of the machine used (approximately 64%).

Section (vi) HLA typing
(a) Preparation of AET-treated sheep red blood cells.

The technique used was a modification of the original method by Pellegrino et al. (1975). Fresh sheep venous blood was collected 1:1 in Alsever's solution. Erythrocytes were washed five times in normal saline at 2000 rpm for 10 mins. 10 mls of a 2% solution of 2-Aminoethylisothiouronium Bromide hydrobromide (AET, Sigma Chemical Co., St. Louis, USA) in distilled water (pH 8.0) was added to 2 ml of packed sheep red blood cells. The suspension was mixed and incubated in a 37°C water bath for 15 mins.
The sensitized red cell pellet (AET-SRBC) was then washed five times in normal saline. For use in T and B lymphocyte separation a 1% solution of sensitized sheep RBC in foetal calf serum was used.

(b) T and B lymphocyte separation

Cord mononuclear cells were thawed as previously described in 10 ml plastic pop-top test tubes. After the initial wash in cold 30% FCS in Hanks BSS, the cells were resuspended in 3 ml of the same medium and mixed with an equivalent volume of 1% AET-SRBC solution. This mixture, after incubation in a 37°C waterbath for 15 mins, was centrifuged at 2000 rpm for 1 min before being incubated at 4°C for 1½ to 2 hours. Following this latter incubation, the cell pellet was carefully resuspended so as not to disturb any formed T lymphocyte-SRBC rosettes and layered using a wide bore pipette (a cut down Pasteur pipette) onto 4 ml of Ficoll-Paque gradient. The gradient was centrifuged at 2000 rpm for 20 mins and the B lymphocyte-monocyte layer harvested from the gradient interface and washed once in 10% FCS in Hanks BSS in small plastic tubes (Disposable Products). The B lymphocyte cell pellet after one wash in 10% FCS in Hanks BSS was resuspended in sufficient medium for counting.

The supernatant in the gradient tube was discarded and 10 ml of 0.83% Ammonium Chloride (NH₄Cl) in distilled water, an osmotic RBC-lysing agent, was added to the remaining T lymphocyte-SRBC pellet. This mixture was incubated at 37°C for 10 mins before centrifugation at 200 rpm for 4 mins. The T cell pellet was resuspended in one ml of 10% FCS in Hanks BSS and layered onto one ml
of Ficoll-Paque in a small plastic tube (Disposable Products). The gradient tube was spun at 2000 rpm for 20 mins. This second gradient step was necessary despite the potential loss of a reasonable number of T lymphocytes which might occur. Its dual function was to remove the SRBC debris after the lysis treatment and also to reduce the number of dead cells originally in the thawed preparation which would have passed through the first gradient to settle with the rosetting T cells. Without this step, the debris and dead cells would hinder the legibility of the final HLA typing tray.

The T cell layer was harvested from the gradient interface, washed once in 10% FCS in Hanks BSS and resuspended in sufficient media for counting. After counting, 10% FCS in Hanks BSS was added to both B and T lymphocyte enriched preparations to give a final cell concentration of 2.0 to 2.5 x 10^6 cells per ml.

(c) Microlymphocytotoxicity test

Typing for HLA-A, -B, -Cw and -DR antigens was performed using the standard lymphocytotoxicity test (Naiad Manual, 1977) which will be briefly described. Previously prepared 60 well microtest trays (Microlitre Nunclon Delta, Nunc, Denmark) containing 1µl HLA antisera overlayed by 5µl paraffin oil (Tromoxal Medicinal Liquid Paraffin B.P., Sigma Co. Ltd., Clayton, Victoria, Australia) per well and stored at -80°C, were thawed at 4°C before use. Using single barrel (Hamilton Repeating Dispensers, Hamilton Reno, Nevada, USA) or multiple six barrel dispensers (Terasaki Multiple Repeating Dispenser, Hamilton Reno, Nevada, USA) 1µl of test cell suspension at a concentration of 2-2.5 x 10^6 cells per ml was added to each well. After
appropriate incubation time, 5µl of rabbit complement (Pel Freez Biologicals, Rogers, Arkansas) was added and the tray further incubated. Ten minutes before the end of this second incubation, 1µl of eosin-yellow stain per well was added. The assay was stopped and the results preserved by the addition of 2µl formalin per well. Incubation times for the test cell-antisera interaction and rabbit complement incubations were half hour and one hour for HLA-A, -B and -Cw determinations and one hour and two hours for HLA-DR. All incubations were performed at 26°C.

Following the application of a glass coverslip (Lomb Cover Glass No. 1 thickness, Lomb Scientific Co., Sydney, Australia) each well was read by means of an inverted phase contrast microscope (Olympus Model CK, Olympus Optical Co. Ltd., Tokyo, Japan) and scored according to the criteria presented in Table 2.1. A positive result in a well indicated that the test cells carried the HLA antigen against which the well's antisera was directed.

The following antigens were typed for:

**HLA-A locus:** 1, 2, 3, 9 (w23,w24), 10 (25,26), 11, w19, 28
29, w30/31, 32, w33.

**HLA-B locus:** 5, (w51,w52,w53), 7, 8, 12 (w44,w45), 13, 14,
15, 16, 17, 18, 21, (w49,w50), 22 (w54,w55,w56),
27, 35, 37, 40, (w60,w61), 41.

**HLA-Cw locus:** 1, 2, 3, 4, 5, 6.

**HLA-DR locus:** 1, 2, 3, 4, 5, w6, 7, w8.

**HLA-B public antigens:** w4, w6.

The antisera used to define these antigens were mainly from local sera (Canberra), arising from pregnancy iso-immunization or from blood donors of unknown immunizing history, supplemented by Australian regional and imported
### TABLE 2.1

**SCORING SYSTEM USED TO EVALUATE THE ANTIGEN STATUS IN INDIVIDUAL WELLS OF HLA TYPING TRAY**

<table>
<thead>
<tr>
<th>SCORE</th>
<th>PERCENTAGE OF STAINED (DEAD) CELL PER WELL</th>
<th>+ANTIGEN STATUS OF WELL</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>NOT TESTED</td>
</tr>
<tr>
<td>1</td>
<td>0-10%</td>
<td>NEGATIVE</td>
</tr>
<tr>
<td>2</td>
<td>11-20%</td>
<td>DOUBTFUL NEGATIVE</td>
</tr>
<tr>
<td>4</td>
<td>21-40%</td>
<td>DOUBTFUL POSITIVE</td>
</tr>
<tr>
<td>6</td>
<td>41-80%</td>
<td>POSITIVE</td>
</tr>
<tr>
<td>8</td>
<td>81-100%</td>
<td>STRONG POSITIVE</td>
</tr>
</tbody>
</table>

+ indicates whether the particular antigen, against which the well antiserum is directed, is present on the test cell.
HLA antisera. The local antisera used correlated well, in prior testing, with the regional and overseas sera of established typing.

Section (vii) Plasma and red cell genetic markers

(a) Properdin factor B typing

The differentiation of the allelic variants of the properdin factor B system was performed using the immunofixation electrophoretic method on acrylamide gel originally described by Alper et al. (1972). Cord blood plasma was used to test for the alleles.

(b) RBC enzyme genetic markers

The estimation of red cell enzyme genetic markers, using starch gel electrophoresis according to the methods described by Harris and Hopkinson (1976), was performed on the cord blood haemolysates in the laboratory of Dr N.M. Blake, Department of Human Biology, J.C.S.M.R. The enzyme systems tested were malate dehydrogenase (MDH), phosphoglucomutase-1 and -2 (PGM1, PGM2), superoxide dismutase (SOD1), acid phosphatase-1 (ACP1). 6-phosphogluconate dehydrogenase (6PGD), lactate dehydrogenase (LDH), adenylate kinase (AK1), adenosine deaminase (ADA), glutamate pyruvate transaminase (GPT), phosphoglycolate phosphatase (PGP), esterase D (ESD) and glyoxalase (GLO).

Section (viii) Heel prick collection

Heel pricks were collected from four to seven month old infants. This was the recommended method of blood collection for this age group. A small incision was made in the outer heel edge by means of an automated lancet device (Autolet, Owen, Mumford Ltd., Oxford, England). The blood was collected into 110µl heparinised capillary
tubes and mixed by agitation with a magnetic flea. The tubes were sealed at both ends with plasticine prior to even division so that they could fit into an haematocrit centrifuge (Readacrit). After centrifugation, the packed red cell portion of the tube was severed by means of a small file and discarded. The plasma was then aspirated into a 0.4ml Beckman plastic stoppered tube (Beckman, USA) and stored at -196°C.

Section (ix) Enzyme linked immunosorbent assay (ELISA)

Antibody estimation in the heel prick and cord blood samples was performed using an indirect microplate ELISA method (Voller and Bidwell, 1975).

(a) IgG estimations

The microELISA method used was a modification of Voller et al. (1978) which will be described briefly.

Test antigen diluted in Coating Buffer (B. pertussis 2 x 10⁸ organisms per ml or tetanus toxoid 16µg protein per ml) was incubated in 200µl volumes in V bottom 96 well microtitre plates (V plate disposable, Cooke Engineering Co., Alexandria, Vancouver, Canada) which were sealed by Parafilm (American Can Co., Greenwich, Connecticut, USA) for two to three hours at 37°C. Following incubation, the non-attached antigen was gently aspirated by a vacuum pump and the trays were thrice washed by immersion for three minute periods in Wash Buffer before being air dried. Heel prick or cord blood plasma were diluted in Diluting Buffer (see Chapter 4 for dilutions used) and 100µl of individual dilutions were added to the microtitre plate wells. The trays were resealed and incubated for ½ hour at 37°C. The excess plasma was removed from the tray wells by a quick
flick of the tray before the tray was washed as previously described. 100µl of urease conjugated anti human IgG antibody diluted 1:150 in Diluting Buffer was added to each well, the trays resealed and incubated for a further ½ hour at 37°C. The conjugated antibody used detected all classes of human IgG. The final dilution of the conjugate used in the assay had been previously determined by chequerboard titration using antigen-specific positive and negative plasma as specified by the manufacturer (CSL).

After removal of the conjugate by flicking, the trays were washed three times in Wash Buffer followed by three further washes in distilled water. This final water wash removed residual Wash Buffer which would have adversely affected the pH sensitive enzyme substrate. Urease substrate was added in 100µl amounts to each well after which the sealed tray was incubated for another ½ hour at 37°C. The colour change in the trays which was readily detected visibly (Fig. 2.1.) was read immediately following incubation by an automated microELISA spectrophotometer. Dual wavelengths 570Å and 630Å were used to minimize colour density fluctuations due to the use of the V-shaped microtitre trays. The 'blank' control well for the spectrophotometer contained PBS.

(b) IgM estimations

The microELISA method used was similar in experimental protocol to that used for the IgG estimations except for variations in antigen dilutions (B. pertussis 1 x 10⁶ organisms per ml and tetanus toxoid 8µg protein per ml) and optimal incubation times and conditions for the plasma, conjugate and substrate addition steps. Plasma was incu-
FIGURE 2.1 IgG antibody measurement using urease-conjugated antibody in an ELISA method. The microtitre tray has been separated into sections, each block of six wells corresponding to one child and the respective cord sample. Antibody levels to *B. pertussis* and tetanus toxoid in each sample were tested on the same tray. Antibody negative wells are coloured yellow to yellow-grey. Antibody positive wells range from light to dark purple in colour.
bated in the trays for one hour at 37°C. Following the washing steps, urease conjugated sheep antihuman IgM antibody (CSL) diluted 1:30 with diluting buffer was incubated in sealed trays for 2 hours at room temperature (18-22°C). After the final water wash, urease substrate ((CSL) was incubated initially for 1/2 hour at 37°C and then the trays were refrigerated overnight (16 hours) before being read in the spectrophotometer the next morning. This delay in reading did not increase background colour excessively but proved essential for the separation of positive and negative results since the actual levels of IgM from the heel prick samples compared to adult positives were very small.

Section (x) Statistical Methods
(a) Tests of significance
(i) Data presented as individual counts in a 2 x 2 contingency table.

Contingency tables (see Armitage, 1971) have been used in this thesis to tabulate the occurrence of a particular characteristic such as an epidemiologic variable or HLA antigen in the various bimodal immune response groups delineated in this study.

The tests of significance used to compare differences in the distribution of a characteristic between the response groups are:

Chisquare test \( \chi^2 \) (Armitage, 1971),

utilizing Yates' correction for discontinuity when the tested sample size is small (see Yates, 1934).

Fisher's exact probability (Armitage, 1971)

This is the only reliable test of significance when one or more of the expected values of a cell in a 2x2 contingency table is less than 5.
Probability correction for HLA association data

When HLA frequencies between two subpopulations are compared and there is no prior hypothesis relating to a previously observed difference in HLA antigen frequency, then it is usual to regard each HLA antigen comparison as independent observations in a multiple Chisquare test and multiply the probability due to each antigen comparison by the number of HLA antigens tested (Svejgaard et al. 1974). This method was instituted to prevent the incorrect rejection of a true null hypothesis that the antigen frequencies between the two subpopulations are equal because it is known (see Morrison, 1976) that in any group of 20 comparisons, one comparison would be expected to show a significant difference at the 5% level by chance alone.

(ii) Data presented as combined groups

For detailed explanation on the statistical meaning of population and sample means (μ, \( \bar{x} \)), variances (\( \sigma^2, S^2 \)), standard deviations (σ, S) and standard error of the mean (\( \frac{\sigma}{\sqrt{N}}, \frac{S}{\sqrt{n-1}} \)) see Armitage (1971).

In this thesis, the sample population means, for example the mean age of a positive IgM responder group, are treated as independent observations of the mean of the original population from which the sample originated. In the comparison of two group means (\( \bar{x}_1, \bar{x}_2 \)) for example the mean age of the positive and negative IgM responder groups to B. pertussis, the null hypothesis tested is that the samples are independent observations of the same original population. The test of significance used, Student's t test, for the
comparison of two independent means, describes the chance of a discrepancy in the two sample means \((\bar{x}_1, \bar{x}_2)\) as large or larger than that observed if the observed difference is due to sampling variation. The procedure for this test has been adequately described by Colton (1974).

In Chapter 5, to eliminate individual cell samples which had undergone excessive transformation in the unstimulated 'control' cultures, a test for outliers, utilizing a kurtosis statistic was implemented. To conduct the test, the mean counts per minute (cpm) of the unstimulated cultures of each cell tested were organised in ascending order of magnitude. Beginning with the two lowest cpm values, the kurtosis statistic \(b_2\) was calculated. The formula for this statistic is

\[
b_2 = n \frac{\left[ \sum (X_i - \bar{x}) \right]^4}{\left[ \sum (X_i - \bar{x})^2 \right]^2}
\]

(Mendell et al. 1977)

where \(n = \) size of sample tested

\(X_i = \) individual unstimulated cpm value of the \(i\)th sample

\(\bar{x} = \) mean cpm value of all \(n\) samples

\[
\bar{x} = \frac{\sum_{i=1}^{n} X_i}{n}
\]

\(b_2\) was recalculated as each sample's mean cpm was added in ascending order to the population of sample mean cpm already in the analysis until the addition of an extra mean cpm increased the value of \(b_2\) above the critical value for a probability less than 0.05. Significant values for \(b_2\) \((p<0.05)\) for each sample size are documented (D'Agostino and Tjeitgen, 1971). The cell sample causing this signifi-
cant trend was rejected from the analysis since its unstimulated culture value was far removed from the mean values of the majority of cell samples tested.

(b) Logistic regression

In Chapter 3, logistic regression models generated by the computer package GLIM (see Breslow and Day, 1980, Baker and Nelder, 1978) were used to determine the value of various components of the child's personal and family history in predicting an adverse reaction to Triple Antigen.

The logistic regression model is written as

\[
\log \frac{P(\text{adverse reaction}/x)}{1 - P(\text{adverse reaction}/x)} = b_0 - b_1 x
\]

where adverse reaction is the dependent variable

\(x\) is the independent explanatory variable which, in this study, may represent breastfeeding at the time of Triple Antigen immunization, administration of the vaccine as partitioned doses or parental history of allergy.

\(b_0\) represents the intercept and \(b_1\) the slope of the regression line describing the relationship between \(\log P/(1-P)\) and \(x\).

The predicted probability of reacting adversely to Triple Antigen is

\[
P(\text{adverse reaction}/x) = \frac{\exp(b_0 - b_1 x)}{1 - \exp(b_0 - b_1 x)}
\]

for a value of the independent variable \(x\).

Test statistics which in GLIM terminology are called 'scaled deviances' provide assessments of which independent variable or interaction between these variables contribute
significantly to the probability of having an adverse reaction. These statistics are estimated using a maximum likelihood method (Baker and Nelder, 1978).
CHAPTER 3

Immunization clinic survey: components of an infant's personal and family history as risk variables determining an adverse immunization reaction to Triple Antigen.
INTRODUCTION

Bordetella pertussis is the etiological agent of whooping cough, a serious respiratory disease of infants and young children. In the absence of specific immunization, pertussis epidemics can occur with mortality confined mainly to infants less than six months of age (see Miller et al. 1982). The introduction of pertussis vaccine into Australia in 1920 and the subsequent incorporation of the combined vaccine Triple Antigen into infant immunization programmes since 1953, has markedly reduced the incidence and, in particular, the mortality attributed to the disease in this country (Feery, 1981).

Despite the observed efficacy of Triple Antigen in providing satisfactory protection against serious B. pertussis infection (Miller and Fletcher, 1976, Baraff et al. 1978, Church, 1979), there is considerable controversy, especially in the United Kingdom (Miller et al. 1982) and Sweden (Strom, 1960, 1967, Taranger, 1987) concerning its possible toxicity. Reports of serious neurological sequelae such as convulsions, collapse, residual mental deficit and, in rare cases, death occurring after Triple Antigen immunization (see Stewart, 1979) were instrumental in the declining vaccine acceptance rate in England culminating, in 1977-79, in the worst whooping cough epidemic that country had experienced in 25 years (see Miller et al. 1982). No specific pertussis-related neurologic syndrome has been adequately defined (Miller et al. 1981) although one of the major opponents to the use of the pertussis immunization, Stewart (1977), has suggested the presence of a "pertussis-reaction syndrome". There has been much
conjecture, extrapolated from animal models, on the possible mechanism of *B. pertussis* vaccine-induced neurologic damage. Hypotheses advanced have ranged from *B. pertussis* induced hypersensitivity reaction in brain tissue in children with existing neurologic disease similar to the syndrome of experimental allergic encephalitis in rats and mice (Wardlaw, 1970, Miller *et al.* 1982), with encephalopathy arising from the combination of *B. pertussis* administration and sensitization to bovine serum albumin (Steinman *et al.* 1982) or to a direct, though undefined, neurotoxic effect of the vaccine (Ross and Bellman, 1979). Proof is lacking as to the exact mechanism. However, in the animal models on which the former two hypotheses were based, an association was observed between the specific genotype of the animal and susceptibility to the particular syndrome, with one report (Steinman *et al.* 1982) suggesting that susceptibility to the adverse reaction to *B. pertussis* in humans may be under genetic control.

In contrast to the debate concerning the neurologic sequelae, there is consensus in the literature that less severe reactions can follow the use of Triple Antigen and that, by comparative studies between the use of DPT (Diphtheria-Pertussis-Tetanus) and DT (Diphtheria-Tetanus), the majority of these reactions are due to the pertussis component of the vaccine (Stewart, 1977, Griffith, 1978. Barkin and Pichichero, 1979, Katz and Wilfert, 1979, Cody *et al.* 1981, Feery, 1982). These reactions include local manifestations such as pain, swelling and redness at the injection site and systemic reactions including fever, malaise, fretfulness, drowsiness and persistent
or high pitched unusual crying (Cody et al. 1981). Depending on the report and the dose of the vaccine received, the incidence of these reactions varies markedly from 31.8% of children receiving a fourth dose of DPT (Feery, 1982) to 93% of total children receiving DPT (Barkin and Pichichero, 1979). Variation in reactogenicity has also been noted between DPT vaccines from different manufacturers (Griffith, 1978) as well as between DPT used in different countries (Miller et al. 1982) which may partly explain the difference in reaction rates between reports.

The occurrence of reactions in children receiving the toxoid vaccine (DT) suggests that an unknown percentage of children with reactions after receipt of Triple Antigen, may be reacting in response to components of the vaccine other than B. pertussis. Reactions following vaccination with tetanus toxoid are well documented (Cooke et al. 1940, Brindle and Twyman, 1962, White, 1973, Facktor et al. 1973, Jacobs et al. 1982) with the reaction syndrome as described by Levine and Edsall (1981) and Jacobs et al. (1982) in adults being very similar to the less severe DPT and DT reactions reported by Cody et al. (1981), Barkin and Pichichero (1979) and Feery (1982). High tetanus antitoxin titres present at the time of vaccination was implicated as increasing the risk of a reaction occurring in members of a "reaction prone group" (Levine and Edsall, 1981) even though this group was only a small minority of total persons with high antitoxin-titres. Reactions to diphtheria toxoid are rare under the age of five years (Hofman and Lansberg, 1972) and no immunization reaction syndrome specific for diphtheria, apart from a short
febrile episode in older children and adults (McComb and Trafton, 1950, Smith and Wolnisty, 1962) has been reported. Local irritant reactions resulting from the alum adjuvant of toxoid vaccines including DT or faulty injection technique are not uncommon (Hofman and Lansberg, 1972). Cellular hypersensitivity reactions to the mercury containing preservative thiomersal used in DT and DPT vaccines have also been reported (Jacobs et al. 1982) resulting in local manifestations consisting of oedema, tenderness, redness and skin vesiculation at the injection site. Therefore, reactions to Triple Antigen may not be simply due to susceptibility to one component (e.g. B. pertussis) but may reflect reaction to a composite of components.

Host factors, as well as vaccine factors, play an important part in determining an individual's response to immunization. Apart from possible genetic differences in immune response to immunization (discussed in Chapters 4 and 6), the risk of an immunization reaction may also be influenced by an individual's health status. The United Kingdom Joint Committee on Vaccination and Immunization in 1977 (Editorial, British Medical Journal, 1977) recognised that certain aspects of a child's personal and medical history may increase the possibility of a serious reaction occurring after Triple Antigen administration. Therefore, the following contraindications to pertussis immunization were recommended to minimize the risk of a serious reaction (i) any child with a history of neonatal seizures, convulsions or cerebral irritation or has a history or family history of epilepsy or other diseases of the nervous system; (ii) the occurrence of developmental
or other defects in child; (iii) any local or general reaction to a preceding dose of Triple Antigen vaccine; (iv) any febrile illness, particularly respiratory until the child had fully recovered. The presence of personal or family history of allergy, although previously suspected as a contraindication (Sen et al. 1972) is no longer considered sufficient cause for exclusion from vaccination with its importance as a risk factor being left to the discretion of the administering doctor. Recent recommendations on childhood immunization by the Australian National Health and Medical Research Council (Childhood Immunization Schedule, 1981) closely follow the guidelines set by the 1977 Joint Committee, although their application may vary depending on the policies of individual Australian State or Territory Health Commissions. In the Australian Capital Territory, at the time this study was undertaken, children presenting to the Health Commission for Triple Antigen immunization with allergy or a close family history of allergy (in parents, siblings, grandparents, aunts and uncles) were receiving partitioned doses of Triple Antigen with the obvious implication that, in the A.C.T., allergic history was considered a possible risk factor in the occurrence of an immunization reaction.

In Chapter 2, the approach taken to the research project was delineated in the Research Design. The aim of this present chapter is to (i) describe the purpose of the survey undertaken and its relevant background; (ii) define the main epidemiologic variables which will be referenced in the rest of the project; (iii) examine the
results of the survey in relation to the effect of the defined variables in determining the risk of an immunization reaction occurring.
METHODS

Section (i) Aim of the survey

The main purpose of the survey questionnaire was to collect pertinent data on the immunization, personal and family history of the children who had cord samples collected in the hospital cohort study. The survey period extended from mid-October, 1980 to the end of February, 1981, in an effort to contact as many of the original cohort who had received their first Triple Antigen immunization and were returning to health clinics for the second injection. It was not considered practicable to extend the survey beyond the four month period since the effort and time expended for the additional number of children traced was not justified.

Section (ii) Background to Triple Antigen immunization in the Australian Capital Territory late 1980 to early 1981

According to Capital Territory Health Commission (CTHC) records, 85% of all immunizations in the A.C.T. were performed at the Health Commission child health clinics. The immunization regime followed in these clinics is based upon the recommendations of the NH&MRC childhood immunization schedule (Childhood Immunization Schedule, 1981). At the time of this present study, the schedule was slightly modified by the policies formulated by the CTHC. In accordance with the NH&MRC schedule, the primary course of Triple Antigen immunization was given at two, four and six months of age with a Diphtheria-Tetanus booster (DT) administered at a 12 month interval following the primary course. Each Triple Antigen dose consisted of a 0.5ml combination of 25 Limes flocculating (Lf) units of puri-
fied diphtheria toxoid, 5Lf units of purified tetanus toxoid, 20,000 million heat killed \textit{B. pertussis} organisms and 0.1% w/v thiomersal preservative administered deep subcutaneously in the deltoid muscle. Both Triple Antigen (DPT) and DT vaccines were manufactured by Commonwealth Serum Laboratories (CSL), Parkville, Australia.

At the first visit to the immunization clinic the attending parent, usually the mother, completed an information card which contained, apart from the baby's name, birth date and home address, a series of yes/no questions concerning the presence of particular allergies or neurological disorders in either the child or its close relatives.

Severity of an allergic condition in the parent or a relative was classified by the attending health care sister as 'severe' or 'mild' on the basis of whether treatment was required or not. The suitability of the child for Triple Antigen immunization was judged by the following criteria. If an existing allergic condition or a contraindicated neurologic syndrome was present in the child or else the neurologic condition occurred in the child's parents, sibs or parental first degree relatives, the choice of attending a general practitioner for the entire primary immunization course or the administration of DT instead of DPT at the health clinic was offered. If the child had an allergic condition which was not presently active or else asthma, hayfever, eczema or other non-atopic allergies occurred in the child's parents or sibs or a 'severe' allergic syndrome occurred in the parent's first degree relatives, a partitioned dose of Triple Antigen was offered with one fifth of the first immunizing dose given
initially and if no 'adverse reaction' was noted by the parent, the remainder of the first dose was given one week later. If an 'adverse reaction' such as a painful swollen injection site, fretfulness with a high fever or the occurrence of a high pitched scream or a combination of these manifestations occurred, the choice of finishing the primary course with DT or attending a general practitioner for the remainder of the primary course was given. It must be stressed that the reporting of familial allergy and its possible diagnosis by the parent at the initial clinic interview was on hearsay grounds only. Also, owing to confusion by some parents about the meaning of the term "neurological", misleading information was sometimes given to the health care sister.

Section (iii) Survey Protocol

The selection procedure of parents to participate in the survey was determined by the work protocol of the individual immunization clinics. Ideally, the health care sisters asked all parents returning their children for the four month immunization if they would like to participate in a "survey on immunization". If the parent consented then the survey interview was conducted by myself or Dr S. Serjeantson depending on the clinic in a private room or the waiting room of the clinic. A heel prick sample was taken from the child at the same time as the interview, after obtaining written informed consent from the parent.† An initial trial questionnaire was tested with a few parents (five) to evaluate the suitability of the questions and the interview technique. The original questionnaire was subsequently modified slightly and trial

†Approved by JCSMR Clinical Research Committee (Appendix I)
information was not used in the survey.

Section (iv) Interview procedure

The survey was in the form of an interview with the interviewer marking the appropriate box or making specific notes on a questionnaire form as the questions were presented to the parent. An example form is presented in the Appendix II. Personal particulars on the child and exact immunization information were extracted from the immunization record card of each child. Information on the mother's name, date of birth and the hospital in which she was confined, was recorded which, together with the residential suburb, facilitated matching the survey history with the cord blood previously collected.

To establish if a reaction had occurred after the first immunization, the question, 'Did you notice anything unusual about your child after the first injection', was posed with particular care not to prompt for the report of a reaction. If the answer was positive, specific questions on the initial time the reaction was noticed, duration and need for medication were asked. There was no probing as to the possible physical manifestations which might have occurred in addition to those that the parent had already mentioned as this line of questioning may have prompted the reporting of a suggested reaction type as fact.

Regardless of whether a reaction occurred or not, specific questions concerning the presence and subjective severity of asthma, hayfever, eczema, food, drug and a range of other allergic syndromes in 'close relatives of
the child', (parents, sibs, grandparents, aunts, uncles and cousins) were asked. The presence of treatment or specific diagnostic tests, for each condition, especially sensitization tests, was discussed with the interviewee. Information was also sought on any history of illness or disease in the child, the approximate period of time the child was breastfed, maternal vaccination history including the vaccine used and the approximate year received, history of herpetic viral infections in the mother and the presence of any neurological history in any member of the child's family including second degree relatives. This last question was presented as specific neurological entities such as fits, epilepsy or developmental defects so as to maximize understanding of the term. The occurrence of a reaction following Triple Antigen in sibs or other family members was also investigated with an attempt made to classify the type of reaction as local, systemic or neurological such as convulsions or collapse.

The immunization clinic survey located 29% of the 908 neonates with collected cord blood samples and included an additional 242 infants not present in the original cohort because they were either born at a Canberra hospital not covered during the initial study or were born outside the Australian Capital Territory. The omission of 71% of the cord blood cohort, although higher than expected, was not considered serious since the aim of the survey was not to describe the epidemiology of immunization reactions in the Australian Capital Territory. Subsequent examination of attendance records at the individual health clinics covered revealed that
many children had not yet returned for their second immuniza-
tion, a few had finished their primary immunization
course before the survey had begun and others had been
referred to private practitioners. The exact numbers for
each group could not be determined as not all attendance
records were available. Other children were undoubtedly
lost to follow-up due to parents moving interstate since
Canberra's population is considerably mobile.

In general, parental cooperation in the survey was
good although a few were excluded due to language diffi-
culties or inability to participate owing to other commit-
ments. The final survey sample of 507 respondents was
considered adequate for the correlation of interview in-
formation with the occurrence of an immunization reaction.

Section (v) Definition of Terms

Immunization reactor status

The definition of an immunization reactor was based
on similar definitions occurring in the CSL handbook
(1979) and the reports by Cody et al. (1981) and Barkin
and Pichichero (1979).

A child was classified as an immunization reactor if
either a local manifestation with or without a systemic
reaction, a behavioural change or a combination of both
occurred after the first immunization or, in a few cases,
after the initial partitioned dose.

(a) A local reaction was defined as redness, swelling
(approximately the size of a 50c piece), pain with or
without bruising at the injection site with nodule form-
ation possibly occurring in a delayed fashion. This local
reaction occurred within 8 hours after injection and lasted
overnight or longer (approximately 12 hours). The systemic reaction, if it accompanied the local reaction, consisted of fretfulness, fever (reported by the mother as the baby was 'hot to touch') vomiting, although this may have been regurgitation, and sleeplessness. A systemic reaction alone did not constitute an immunization reaction unless medical advice was sought and received or else it was considered important enough at the health clinic after a test dose of DPT for DT to be recommended for the remainder of the primary immunization course.

(b) Behavioural changes occurring after immunization were classified as persistent screaming, prolonged drowsiness or collapse. A persistent screaming episode consisted generally of a high pitched cry, which started two to six hours after injection and continued for a period of two hours or longer. During this time the baby was inconsolable and needed paediatric sedatives. A child could also suffer a period of unusual drowsiness beginning within two to six hours after immunization and lasting overnight or longer, in one case up to two days. The child is reported to be listless, unresponsive to surroundings and has to be repeatedly woken to be fed. One child suffered collapse immediately after injection wherein he became cold and clammy to touch and lost consciousness for a few minutes. He recovered fully a few hours later.

(c) Exacerbation of eczema or the appearance of a body rash occurring within a few days of the immunization was considered in this study as a reaction.
Parental Allergy

The allergic syndromes interpreted as components of parental allergy were the atopic diseases including allergic rhinitis, asthma and atopic eczema and food allergies known to be IgE mediated (for discussion on food allergies see Nagy and Klass, 1979). Only the presence of these particular allergies in the child's parents were considered significant since the information sought was first-hand and would tend to be more reliable than the hearsay reporting of similar syndromes in distant family members. However, the establishment of specific allergic syndromes via an interview and with no recourse to actual diagnostic testing has limitations (de Weck, 1977). Misreporting may occur even if the scope of allergies is limited to reagin-mediated conditions. For example, atopic rhinitis (hay-fever) may be confused with nonatopic (infectious) or vaso-motor rhinitis (Connell, 1979), contact eczema may be reported as atopic eczema and it has been reported that only 40% to 50% of asthmatics suffer from IgE mediated attacks (Keslin, 1979). Unfortunately such phenotypically similar syndromes cannot be completely differentiated on the basis of an interview although the degree of misclassification can be partly offset by careful selection of survey histories. The positive parental allergy group included the children whose parent (or parents) - (a) had an atopic syndrome for which a professional opinion was sought, for example, the use of sensitization tests to establish the cause; (b) had more than one allergic syndrome with similar syndromes being reported in other family members; (c) had diagnosed food allergies of the type known to be IgE mediated and (d) were without
precise diagnosis or family history but had hayfever which
was seasonal in nature for which treatment was required. The
"no parental allergy" group included children whose parents,
to the best of their knowledge, had never suffered from an
reagin-mediated syndrome.

Section (vi) Statistical analysis

The responses to the questionnaire were numerically
coded onto a computer data file. Each child, its immuniza-
tion history and the corresponding family data were identi-
fied by an individual number prefix. Initial analysis
involving enumeration of reported variables and associa-
tions between factors in the family history and immuniza-
tion reactor status was performed using the FREQUENCY and
CROSSTABS subprogrammes of the computer package SPSS
(Statistical Package for the Social Sciences 19). Most
associations were confirmed by chi-square analysis utilizing
Yates correction for discontinuity. The programme package
GLIM was used to generate logistic regression models to
analyse the importance of relevant predictive variables in
determining immunization reactor status. The relevant
theory of logistic regression has been given in Chapter 2.
RESULTS

Section (i) Response to Triple Antigen Immunization

Of the 507 respondents to the survey, 483 children had received Triple Antigen for all or part of their first immunization, 20 children had received CDT and four children had not received any immunization. Four children who, after receiving Triple Antigen, had contracted whooping cough within a fortnight of immunization, were removed from the following analysis concerning reactions to Triple Antigen. Of the remaining 479 Triple Antigen recipients, 279 (58.2%) had been immunized with partitioned doses.

Overall, in the Triple Antigen group, 46.3% of respondents claimed that their child had clearly reacted to the vaccine or else had behaved abnormally in the period immediately following immunization. Table 3.1 shows the profile of responses amongst these reported reactions. The data in this table is arranged so that each response in the Reaction class subgroup corresponds to one child. However, a child could show more than one response.

Minor behavioural and systemic changes, such as fretfulness and mild fever, were commonly reported (26.5% and 14.2% respectively in all Triple Antigen recipients). Individually these symptoms cannot be satisfactorily regarded as a reaction since a multitude of causes not associated with the actual immunization, for example the journey to and from the immunization clinic or high ambient temperatures (immunizations were taking place during early and mid summer) could be responsible. By contrast the appearance of a local reaction (17.3%) or the occurrence of a high pitched scream (7.3%), drowsiness (1.7%) or collapse
TABLE 3.1

REPORTED REACTION SYNDROMES IN CHILDREN RECEIVING TRIPLE ANTIGEN. RAW DATA FROM SURVEY

<table>
<thead>
<tr>
<th>NATURE OF SURVEY REPORTED REACTION</th>
<th>NUMBER OF CHILDREN WITH REPORTED REACTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>REACTION SYNDROME</td>
<td></td>
</tr>
<tr>
<td>1. LOCAL (ALL TYPES)</td>
<td>83</td>
</tr>
<tr>
<td>2. BEHAVIOURAL CHANGE</td>
<td></td>
</tr>
<tr>
<td>(i) PRETFULNESS</td>
<td>127</td>
</tr>
<tr>
<td>(ii) HIGH PITCHED SCREAM</td>
<td>35</td>
</tr>
<tr>
<td>(iii) DROWSINESS</td>
<td>7</td>
</tr>
<tr>
<td>(iv) COLLAPSE</td>
<td>1</td>
</tr>
<tr>
<td>3. SYSTEMIC REACTION</td>
<td></td>
</tr>
<tr>
<td>(i) FEVER</td>
<td>65</td>
</tr>
<tr>
<td>(ii) VOMITING (REGURGITATION)</td>
<td>3</td>
</tr>
<tr>
<td>(iii) ECZEMA</td>
<td>6</td>
</tr>
<tr>
<td>(iv) BODY RASH</td>
<td>3</td>
</tr>
<tr>
<td>4. REACTION OCCURRED ON FIRST PART OF PARTITIONED DOSE</td>
<td>10</td>
</tr>
</tbody>
</table>

+ TOTAL NUMBER OF CHILDREN WITH REACTION REPORTED IN SURVEY 222

* Corresponds to the number of children reported to have experienced one or more of the listed reaction syndromes.
(0.2%) in Triple Antigen recipients can be reliably attributed to immunization with ten (2.1%) reacting to the initial partitioned dose. The occurrence of eczema or an extensive body rash following immunization may indicate the exacerbation of a previously existent condition or else be an individual response to one of the components of the vaccine.

According to the criteria for the selection of immunization reactors presented in Methods, 99 (20.7%) of the 479 Triple Antigen recipients were classified as experiencing a significant reaction following immunization, the remainder of recipients being defined as non reactors.

The possibility that the incidence of reported immunization reaction could be affected by biases in interviewing technique, differences between health clinics and maternal parity was examined. There was no difference between the interviewers in the incidence of reported immunization reactions (20.5% compared to 20.7%; P>0.1) nor in the rate of positive parental allergy (66.5% compared to 64.8%; P>0.1) (Table 3.2). Similarly, the number of immunization reactors interviewed in each clinic were similar indicating that there was negligible bias in the selection of reactors by individual health clinic sisters for the interview (Table 3.2). The parity of the mother was considered as a possible bias variable because it is likely that primipara mothers may report an event as unusual because they have, in the main, had no previous experience of how a child could react to immunization. This did not prove to be correct, however, as 16.7% of 203 primipara mothers reported a reaction in their child which is not significantly different from the 23.2% reaction rate reported
TABLE 3.2

COMPARISON OF PERCENTAGE OF TOTAL IMMUNIZATION REACTORS, LOCAL REACTORS AND CHILDREN WITH POSITIVE PARENTAL ALLERGY BETWEEN INTERVIEWERS AND INDIVIDUAL HEALTH CLINICS. DATA SHOWN FOR TRIPLE ANTIGEN RECIPIENTS ONLY

<table>
<thead>
<tr>
<th>SURVEYED CLINICS BY INTERVIEWER</th>
<th>SURVEY PARTICIPANTS (NO. PER CLINIC)</th>
<th>TOTAL REACTORS (% PER CLINIC)</th>
<th>LOCAL REACTORS (% PER CLINIC)</th>
<th>POSITIVE PARENTAL ALLERGY (% PER CLINIC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTERVIEWER 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KALEEN</td>
<td>60</td>
<td>20.0</td>
<td>16.7</td>
<td>71.6</td>
</tr>
<tr>
<td>PHILLIP</td>
<td>66</td>
<td>16.7</td>
<td>15.2</td>
<td>69.7</td>
</tr>
<tr>
<td>WESTON</td>
<td>34</td>
<td>17.6</td>
<td>17.6</td>
<td>67.7</td>
</tr>
<tr>
<td>KAMBHAH</td>
<td>105</td>
<td>24.8</td>
<td>19.0</td>
<td>63.8</td>
</tr>
<tr>
<td>KIPPAX 1</td>
<td>52</td>
<td>19.2</td>
<td>17.3</td>
<td>61.5</td>
</tr>
<tr>
<td>INTERVIEWER TOTAL</td>
<td>317</td>
<td>20.5</td>
<td>17.3</td>
<td>66.6</td>
</tr>
<tr>
<td>INTERVIEWER 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KIPPAX 2</td>
<td>79</td>
<td>22.7</td>
<td>17.7</td>
<td>69.6</td>
</tr>
<tr>
<td>CIVIC</td>
<td>83</td>
<td>19.3</td>
<td>16.9</td>
<td>60.2</td>
</tr>
<tr>
<td>INTERVIEWER TOTAL</td>
<td>162</td>
<td>20.9</td>
<td>17.2</td>
<td>64.8</td>
</tr>
<tr>
<td>TOTAL CLINICS</td>
<td>479</td>
<td>20.7</td>
<td>17.3</td>
<td>66.0</td>
</tr>
</tbody>
</table>
by 272 multipara mothers. Therefore, the immunization reaction rates observed were due to variables not associated with the manner of reporting.

Section (ii) Technique of vaccine administration

The incidence of local reaction to a vaccine can be influenced by the site of administration and the individual technique of the operator. In all clinics, the vaccine was given deep subcutaneously. However, since individual clinics had their own operators who were in attendance each week, if the incidence of local reactions was influenced by administration technique, then the reaction rates would be expected to be different between the individual immunization clinics. Table 3.2 shows that the proportion of local reactors in each clinic covered by the survey was equivalent.

Section (iii) Administration of a partitioned dose

Of the immunization reactors, 76.8% had received a partitioned dose of Triple Antigen for their first immunization compared to only 51.6% of non-reactors (Table 3.3). This difference in the proportion of children receiving partitioned doses between the reactor and non-reactor groups was highly significant (P<0.001).

Section (iv) Parental allergy

Each child in the survey was assigned to either the positive or no parental allergy groups as defined by the criteria presented in the methods section. The distribution of parental allergy in the immunization reactor and non-reactor groups was then examined and, as shown in Table 3.3, 81.8% of the reactor group had allergic parents compared with 62.8% in the non-reactor group (P<0.001).
<table>
<thead>
<tr>
<th>COMPONENT</th>
<th>TOTAL INDIVIDUALS IN EACH COMPONENT SUBGROUP</th>
<th>IMMUNIZATION REACTORS</th>
<th>( p ) VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>PARTITIONED DOSE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GIVEN</td>
<td>272</td>
<td>76</td>
<td>27.9</td>
</tr>
<tr>
<td>SINGLE DOSE ONLY</td>
<td>207</td>
<td>23</td>
<td>11.1</td>
</tr>
<tr>
<td>PARENTAL ALLERGY</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>POSITIVE</td>
<td>316</td>
<td>81</td>
<td>25.6</td>
</tr>
<tr>
<td>NEGATIVE</td>
<td>161</td>
<td>18</td>
<td>11.2</td>
</tr>
<tr>
<td>BREASTFEEDING AT TIME OF IMMUNIZATION</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BREASTFED</td>
<td>280</td>
<td>73</td>
<td>26.1</td>
</tr>
<tr>
<td>NOT BREASTFED</td>
<td>185</td>
<td>23</td>
<td>12.4</td>
</tr>
</tbody>
</table>

\( p \) value of corrected \( \chi^2 \) value of the comparison between the distribution of the positive component in reactors compared to the similar distribution in non-reactors.
Section (v) Breastfeeding status

465 mothers (97.1%) gave sufficient data on the duration of breastfeeding with 60.2% of the children being breastfed at the time of immunization. In the immunization reactor group 76.0% (73 of 96) were being breastfed when the first immunization was given compared to 56.1% (207 of 369) of non-reactors. This association between breastfeeding and immunization reaction is highly significant (P<0.001) (Table 3.3)

Section (vi) Sex differences

Table 3.4 specifies the number of boys and girls in the total sample and in both reactor status groups. Overall 245 boys and 234 girls were given Triple Antigen. This corresponds to a sex ratio in the total sample of 1.05 (boys to girls). However, the sex ratio in the two reaction groups differs considerably with an increased number of male children in the reactor group (ratio 1.48). This increase is not significantly different from the sex ratio of 0.96 in the non-reactor group (χ² (corrected) = 3.15; P>0.05).

Among the 99 immunization reactors, 32 of the 59 boys (54.2%) exhibited a persistent screaming or drowsiness episode compared to only 8 of the 40 girls (20.0%) in the same group. This increase in male persistent screamers is significant (χ² = 10.23; P<0.01), implying that, in this study, boys were more likely to experience this particular behavioural disturbance.
TABLE 3.4

DISTRIBUTION OF IMMUNIZATION REACTORS AND NON-REACTORS ACCORDING TO SEX

<table>
<thead>
<tr>
<th>IMMUNIZATION REACTOR STATUS</th>
<th>SEX</th>
<th>SEX RATIO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MALE</td>
<td>FEMALE</td>
</tr>
<tr>
<td>REACTOR</td>
<td>59</td>
<td>40</td>
</tr>
<tr>
<td>NON-REACTOR</td>
<td>186</td>
<td>194</td>
</tr>
<tr>
<td>TOTAL IMMUNIZED CHILDREN</td>
<td>245</td>
<td>234</td>
</tr>
</tbody>
</table>

\* Comparison of sex distribution in immunization reactors and non-reactors:

\[ \chi^2 \text{(corrected)} = 3.15, p > 0.05 \]
Section (vii) Neurologic history

Despite initial questioning by health clinic workers on the presence of familial neurologic history, 35 children with a history of convulsion or epilepsy in either a parent, sib or parental sib or grandparent were given Triple Antigen. The main reasons why these respondents were not detected before immunization were because they either did not understand the meaning of the term 'neurological disease' or else did not consider that 'family history' encompassed more than the immediate nuclear family of the child. Overall 22.9% of these 35 children were immunization reactors with six of the eight children experiencing either a high pitched scream or drowsiness episode. This incidence of scream or drowsiness episodes was not significantly different from that of children with similar behavioural disturbances without a positive neurologic history (positive history, 17.1%; negative history, 7.7%. \( \chi^2 \text{(corrected)} = 2.68, P > 0.5 \)). There was no sex difference between these reactors, the sex ratio being approximately 1:1.

Section (viii) Maternal and sib vaccination history

The response to the question on previous maternal vaccination was poor with only 67.1% recalling the vaccine used and the year administered. Only ten mothers of these respondents reported having an adverse reaction to a previous vaccination. Tetanus toxoid, in particular, accounted for seven of these maternal reactions. Two mothers experienced anaphylactoid episodes whilst the remaining five reported local discomfort and limb immobility around the injection site. The children of six tetanus reactors were reported as having a reaction after the first
immunization. Four experienced severe local and febrile episodes accompanied by a high pitched scream and two had a red swelling at the injection site resulting in hard nodule formation in one. A severe local reaction also occurred in two sibs of one of these children after they had received their respective Triple Antigen vaccinations. Although not included in the main analysis, one child, whose mother had experienced anaphylaxis to tetanus, showed major behavioural disturbances including screaming followed by severe local and systemic signs after an injection of DT, not Triple Antigen. Two sibs responded to Triple Antigen and DT in a similar manner. It is apparent from these last two cases that an immunization reaction in a sib may be a predictor for the occurrence of a similar reaction in a subsequent child. Of the children receiving Triple Antigen, 272 had one or more older sibs for which immunization history was available. A reaction recalled as either a severe local reaction, screaming or drowsiness episode or convulsion was reported for at least one older sib in 12.9% of these 272 families. Three children each had two sibs who reacted to previous Triple Antigen immunization whilst one child had three reacting sibs. In the 272 infants with older immunized sibs 13 (20.9%) of 63 immunization reactors had at least one sib with a reaction to Triple Antigen whilst 22 (10.5%) of 209 non-responders had a similar history. This difference approaches but does not reach significance \( \chi^2 \) (corrected) = 3.56 1 d.f.; P>0.05. However, the four children who had two or three sibs with an immunization reaction were also immunization reactors.
Section (ix) Risk factors determining an immunization reaction following Triple Antigen

Immunization reactor status in this study has been shown to be significantly associated with the administration of partitioned doses of Triple Antigen vaccine, a positive parental history of allergy and the presence of breast-feeding at the time of immunization and influenced by maternal reaction to tetanus toxoid immunization. A series of logistic regression models, generated by the computer program GLIM, were used to determine the goodness of fit of various combinations of these variables to the data. Only those variables for which information was present for the majority of individuals were included. This excluded maternal vaccination history from the analysis. The GLIM program generates a scaled deviance value which is the assessment of the goodness of fit of the current model under study relative to the complete model which includes all possible combinations of entered variables and fully explains the data. The scaled deviance of the current model, therefore, gives a measure of acceptability of the model under study. Since the distribution of the data entered is binomial (the independent variables are discrete positive or negative variables) the scaled deviance is distributed as an approximate chi-squared distribution with degrees of freedom equivalent to the number of explanatory variables in the complete model minus the number of linearly independent parameters in the current model.

Each variable such as parental allergy (ALL), partitioned dose (PD) or breast-feeding (BF), was entered into the model in a stepwise additive fashion as shown in Table 3.5. This table shows the scaled deviance generated by
each variable addition, the likelihood of the model given
the original data ($p_1$ value), the net deviance due to each
variable (corresponding to a $\chi^2$ value) and its relative
significance as a predictor ($p_2$ value). The null model
defines a group effect due to the data without any expla­
atory variables.

With immunization reactor status as the dependent
variable ($y$ variate), the variables of parental allergy,
partitioned doses and breast-feeding were found to have
individual significant effects as they were entered into
the model. However, the significance of parental allergy
or partitioned doses differed depending upon
which predictor was entered into the model first. This
indicates that these two terms are non-orthogonal and that
they are strongly correlated in the original data. There­
fore, it is difficult to separate their individual effects
as direct or indirect resulting from this correlation with
possibly one term being the strong risk variable.

No significant two or three-way interaction was found
between ALL, BF or PD. Since after the additive model of
ALL+BF+PD was entered the corresponding scaled deviance was
not significantly different from the complete model,
($\chi^2 = 4.399$, $P > 0.3$) the three variables of breast-feeding,
parental allergy and partitioned dose combined in an indivi­
dual have the greatest predictive effect, in this study, on
determining immunization reactor status.
TABLE 3.5

RESULTS OF LOGISTIC REGRESSION ANALYSIS DETERMINING THE SURVEY VARIABLES WHICH ARE RISK FACTORS FOR THE OCCURRENCE OF AN ADVERSE REACTION TO TRIPLE ANTIGEN. ALL DATA GENERATED BY THE COMPUTER PROGRAM GLIM

<table>
<thead>
<tr>
<th>SURVEY VARIABLE</th>
<th>SCALED DEVIANCE OF TEST MODEL</th>
<th>d.f</th>
<th>P1</th>
<th>NET DEVIANCE DUE TO VARIABLE</th>
<th>P2</th>
</tr>
</thead>
<tbody>
<tr>
<td>NONE</td>
<td>42.25</td>
<td>7</td>
<td>&lt; 0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BF</td>
<td>28.84</td>
<td>6</td>
<td>&lt; 0.001</td>
<td>13.41</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>(1) + ALL</td>
<td>16.18</td>
<td>5</td>
<td>&lt; 0.01</td>
<td>12.66</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>+ PD</td>
<td>4.39</td>
<td>4</td>
<td>&gt; 0.3</td>
<td>11.78</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>(2) + PD</td>
<td>10.01</td>
<td>5</td>
<td>&lt; 0.1</td>
<td>18.83</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>+ ALL</td>
<td>4.39</td>
<td>4</td>
<td>&gt; 0.3</td>
<td>5.61</td>
<td>&lt; 0.05</td>
</tr>
</tbody>
</table>

+ degrees of freedom
++ the survey variables tested in the logistic regression model
BF breastfeeding status at time of immunization
ALL parental history of allergy
PD partitioned dose of Triple Antigen administered

Each variable was added in a stepwise manner into the model. See text for explanation of scaled deviance, net deviance and probability (p1, p2) values.

Model (1) in which ALL was added before PD and Model (2) in which PD was added before ALL after initial entry of BF. Note the differences in net deviances for ALL and PD in each model.
DISCUSSION

The survey presented in this chapter has examined causative factors in the occurrence of adverse reactions following the first immunizing dose of Triple Antigen. The majority of the untoward reactions reported were of the "less serious" type (Cody et al. 1981) consisting of a range of local reactions, with or without systemic effects, and behavioural disturbances such as persistent screaming and drowsiness. Apart from the occurrence of collapse and prostration in one child, no serious neurological sequelae, such as convulsions, were recorded.

It has been confidently shown in the results that the rate of reported reactions was not biased by the selection of the participating interviewees, the investigative approach of the interviewer, differing maternal experience with childhood immunization or the technique of vaccine administration at the individual health clinic (see Table 3.2). However, the overall rate of immunization reactors in the Canberra study (21%) was lower than that of 32% reported by Feery (1982) for children receiving a fourth dose of DPT and considerably less than the rate of 93% given by Barkin and Pichichero (1979). The discrepancy in reaction rate between the present and reported studies is primarily due to the conservative assessment used for the determination of immunization reactor status in the Canberra series although differences in reactogenicity of the vaccine used, especially in comparison with overseas studies, cannot be disregarded. The reported occurrence of minor behavioural or systemic changes in the absence of other reaction syndromes were not classified as
'immunization reactors' in the present study because it was shown by Barkin and Pichichero (1979) that similar symptoms could occur in the unvaccinated child. These minor reactions, which included fretfulness and mild fever, were the most commonly reported symptoms in studies which claimed immunization reaction rates in excess of 90%.

The possible role of familial allergy as a risk factor in serious immunization reaction, such as the neurological sequelae, has been disputed in the recent medical press (Davies, 1981, Hull, 1981, Preston, 1982), although the guidelines of the United Kingdom's 1977 Joint Committee on Vaccination and Immunization (Editorial, British Medical Journal, 1977) specified that, whilst an allergic history was not a contraindication, it was left to the discretion of the physician administering the vaccine if a child with a positive history was given Triple Antigen. The caution suggested by the Joint Committee has been translated to practical significance in the A.C.T. where it is the policy of the Capital Territory Health Commission to offer, at considerable expense, a partitioned dose of Triple Antigen to children with a family history of allergy. In view of the lack of scientific evidence to confirm or deny its importance, the Canberra study has provided the first formal investigation into the significance of allergic family history as a predictor of adverse reactions to Triple Antigen.

Ideally, allergy present in the child or first degree relatives should define "familial" allergy since the probability of a child with allergic parents inheriting and expressing an allergic predisposition has been estimated
as being between 10% to 40% (Lubs, 1971). Since most of the children included in the study population were aged 4 to 6 months, the likelihood of detecting, by questionnaire or in vitro testing, all potentially allergic children at this early age was small and, therefore, the presence of parental atopic or otherwise IgE-mediated allergies (as defined in Methods) was used in the analysis to indicate a possible allergic predisposition in the child.

Atopic allergy and other IgE mediated allergies have long been known to have a familial tendency (Cooke and Vander Veer, 1916, Black and Marsh, 1976) which is now believed to be partly mediated by IgE regulator genes (Gerrard et al. 1978, Rao et al. 1980) and allergen-specific MHC-linked immune response or suppression genes (reviewed by Marsh et al., 1981). Other immunological mechanisms giving rise to an allergic syndrome such as intrinsic basophil defects (Radermecker et al. 1982) or variations in suppressor T cell function (Canonica et al. 1979, Martinez et al. 1979, Schuster et al. 1979, Lydyard et al. 1981) may also be inherited.

The results showed a strong positive association between an adverse reaction to Triple Antigen and parental allergy (Table 3.3) which was further emphasized by the logistic regression analysis (Table 3.5). This observed association invites interesting speculation as to the mechanism involved in the pathogenesis of these local, systemic and behavioural adverse reactions.

There has been considerable controversy in the past as to whether a history of atopy predisposed an individual to an adverse reaction to drugs such as penicillin or to
vaccination (see Ettlin et al. 1981). Atopic individuals are assumed to possess either a state of generalised immune hyper-responsiveness, especially with respect to IgE production, to any foreign antigen (Marsh et al. 1980), or some intrinsic immunological defect not found in non-atopics such as faulty basophil regulation which predisposes the atopic individual to release more readily the pharmacological agents involved in the pathogenesis of both an allergic and inflammatory reaction (Radermecker et al. 1982). Therefore the child's allergic background, as indicated by parental history, may predispose the child to be more susceptible to "allergens" in the vaccine and thus be directly responsible for the observed adverse reaction.

Alternatively the immunization reaction may result from specific pharmacological effects of B. pertussis, which simulate several pathognomic facets of an allergic reaction, acting upon the child's allergic predisposition. In mice and rats, the excellent adjuvanticity of B. pertussis vaccine for IgE production is well known (Pauvels et al. 1979, Jarrett et al. 1980) and the importance of IgE in the initiation of an allergic reaction cannot be overstated (review by Ishizaka and Ishizaka, 1978). Also, a B. pertussis cell surface component, Histamine Sensitizing Factor (HSF), can induce susceptibility to the pharmacological action of histamine, in particular to the lethal effects in normally histamine-resistant strains of mice (Munoz and Bergman, 1968). Although the efficacy of HSF in its histamine-sensitizing capacity is unknown in man, increased skin sensitivity to histamine has been
reported in children following both natural and vaccinal exposure to *B. pertussis* (Sanyal, 1960, Mathov, 1962). These skin effects may be HSF mediated. It could, therefore, be speculated that an adverse reaction to Triple Antigen may be influenced by *B. pertussis* induced IgE production and sensitization to histamine especially in a potentially allergic individual who would be genetically susceptible to these effects.

However, Table 3.3 also showed that children receiving a partitioned dose of Triple Antigen were also more likely to have an adverse reaction to the vaccine. According to CTHC policy most children with a family history of allergy, in particular hayfever, asthma or eczema, receive a partitioned dose for the first immunization. Therefore, there is an obvious correlation between a family history of allergy and the receipt of a partitioned dose in the survey data and this correlation is further emphasised by these two variables' non-orthogonality in the logistic regression analysis. Since, in this study, there were very few occurrences (17 children) where a partitioned dose was administered to a child with a negative family history of allergy, it is difficult to elucidate if the risk for an adverse reaction is due to the individual effect of both variables, an interaction between them or simply the confounding effect of both variables.

If the administration of a partitioned dose does influence the occurrence of an adverse reaction, then it could be speculated that the initial injection may sensitize the individual to a component in the vaccine to which he or she may react upon completion of the dose. This
'sensitization' would be more effective if the individual also had an allergic predisposition thus constituting a relationship between allergic history and partitioned doses in the pathogenesis of an adverse reaction. Therefore it would appear that a less serious reaction to Triple Antigen in allergic children may be aggravated by the administration of the vaccine as a partitioned dose. However, since the primary purpose of this partitioned dose is to minimize the probability of serious neurological sequelae occurring in children believed to be at risk, there is a need for a carefully designed study in which non-allergic children are given partitioned doses to determine any possible deleterious effects due to this putative sensitization to the vaccine.

In the Canberra survey, maternal adverse reactions to tetanus toxoid were highly predictive of an adverse reaction to Triple Antigen although this group comprised only a small subset of all reactions. This association with maternal vaccination history indicates a possible susceptibility to the toxoid or a common component present in both the single toxoid or Triple Antigen vaccines being inherited by the child. In two reported cases with a positive maternal reaction history, from the survey, the survey child and its sibs reacted to either Triple Antigen or DT. However, reactions to tetanus toxoid can also have an immunological basis which is not necessarily genetically determined, for example, accidental hyper-immunization with the toxoid (Trinca, 1976, Levine and Edsall, 1981, Jacobs et al. 1982), and thus not all positive maternal or familial reaction histories will be
predictive.

In general, the predictive value of sib immunization reaction history, proved to be very poor for determining reaction rate on the first immunization. Inadequate recall of the older sib's immunization history by the mother may have diminished the value of this variable.

Although the parental allergy and maternal reaction data implicates genetic factors in immunization reactions, the possible influence of environmental factors was reflected in the relationship between breastfeeding at the time of immunization and reaction. Whilst the advantage of breastfeeding for potentially allergic children has been stressed by various medical publications and health advisory centres (Chandra, 1979, Harland, et al. 1979, Saarinen, 1979, Watkins et al. 1979), the significant association between immunization reaction and breastfeeding was not due to a spurious relationship between breastfeeding and family history of allergy in this data. These two variables were clearly independent in the logistic regression analysis. Therefore, breastfeeding appears to increase the risk of an adverse reaction occurring, although it cannot be disregarded that breastfeeding mothers may be more likely to recognise and record a reaction than those that bottle feed. Otherwise, the passage of factors in the breastmilk to the child at the time of immunization may explain this association. The nature of these transmitted factors is open to conjecture. It is highly unlikely that the factors are reaginic antibodies since it has been shown that IgE is only present in colostrum in very small quantities and in negligible amounts in later breastmilk (Underwood...
et al. 1976, Turner et al. 1977). However, the passage of antigen-sensitized maternal lymphocytes or helper factors in the breastmilk has been documented (Mohr, 1973, Schlesinger and Covelli, 1977, John et al. 1976) and these components are believed to be responsible for sensitization of neonates to particular antigens even though no previous contact with the antigens by the infant was possible (Schlesinger and Covelli, 1977). A similar mechanism of sensitization to components of Triple Antigen may be operating in this present study especially if the breastfeeding mothers have been previously exposed, naturally or artificially, to the vaccine antigens. This form of sensitization may also affect the immunological response of the child to the same antigens following immunization. This aspect is further examined in Chapter 4.

A history of neurological disease in the immediate or close family of the immunized child, reported for 35 individuals in this study, did not appear to predispose the child to a serious adverse reaction such as the neurological sequelae (Stewart, 1979, Miller et al. 1981). However, six of the eight immunization reactors in this group experienced an episode of either high pitched screaming or drowsiness. Dick (1974) believes that these syndromes, in particular the "scream", are a direct effect of the vaccine on the central nervous system and are thus less severe, short-term neurological effects. However, the overall incidence of this form of immunization reaction between children with or without a positive family history of neurological disease was not significantly different and, therefore, from these data it cannot be stated that neurologic family history is pre-
dictive of susceptibility to these behavioural disturbances. Of the total number of immunization reactors, high pitched screaming was reported more frequently in boys reactors than girls. If this form of reaction does originate from the central nervous system, then the elevated frequency of boys could indicate that this sex may have an increased susceptibility to cerebral irritation as has been previously observed by Wallace (1974).

It was not the intention of this survey in examining the rate of adverse reactions after Triple Antigen immunization to make any value judgements on either the benefits or safety of pertussis vaccines. This topic is still an area of considerable debate with much conflicting evidence (Stewart, 1979; 1981, Miller et al. 1981, Cody et al. 1981, Feery, 1982). Instead, this survey has studied the predictive value of components in the immunized child's family history in determining the occurrence of an adverse reaction. It must be stressed, at this stage, that "immunization reaction" in this study includes the physical manifestations defined in Methods and the subsequent findings cannot be extrapolated to the serious neurological sequelae assumed to be caused by pertussis vaccination. By the use of a logistic regression model the Canberra study has shown that the risk of an adverse reaction occurring after the first Triple Antigen immunization is increased if the child has a parental history of atopic allergy, has received the first immunizing injections as a partitioned dose and is being breastfed at the time of immunization. Furthermore, though not included in the final model since there were comparatively few cases, maternal adverse reaction to
tetanus toxoid has a predictive value.

In summary, an adverse reaction to Triple Antigen appears to be due to a combination of factors some of which are under genetic control whilst others are clearly environmental, for example, breastfeeding. It is likely that the immunological responses to Triple Antigen, both humoral and cell mediated, are correlated with the same genetic and environmental factors which result in an adverse reaction. This question will be further examined in Chapters 4 and 5.
CHAPTER 4

Primary humoral response of infants to components of Triple Antigen.
INTRODUCTION

The purpose of this present chapter is to assign the study population to responder groups on the basis of a primary humoral response to two antigenic components of Triple Antigen, *B. pertussis* and tetanus toxoid.

Previous reports have demonstrated that, in man, individual differences in antibody response can occur after exposure to a range of microbial antigens including *Salmonella adelaide* flagellin (Wells et al. 1971, Whittingham et al. 1980), tetanus toxoid (MacLennan et al. 1973, Edsall et al. 1975, Rivat et al. 1978), rubella vaccine (Spencer et al. 1977, Kato et al. 1978, Harcourt et al. 1979), influenza A vaccination (Spencer et al. 1976), measles and diphtheria toxoid (Haverkorn et al. 1975), *Haemophilus influenzae* (Pandey et al. 1979) and meningococcal polysaccharide vaccination (Pandey et al. 1982). To allow clear grouping of responders and non-responders in this study, a low dose of antigen was used, the rationale being that only the most immunogenic component of the antigen would be presented. This step is essential if the antibody response to a complex antigen is studied (Benacerraf and McDevitt, 1972, Gasser and Silvers, 1974). The first immunizing dose of Triple Antigen was chosen for this present study as it approximated this low antigen dose condition.

The response of an individual to any form of immunization is affected by both technical and host factors (Edsall, 1966, MacLennan et al. 1973). The technical aspects were standardised in this study to deep subcutaneous administration with a single or partitioned dose of Triple Antigen as
directed by the Capital Territory Health Commission's Infant Immunization Guidelines of 1980 and late 1981. Host factors (reviewed by Edsall et al. 1975) are important in determining the character and magnitude of the antibody response and include genetic constitution, age, sex and, to a lesser extent, hormones and nutrition. Since genetic constitution is the factor under study, both age and sex would be important confounding effects on the expression of genetically-determined differential antibody response. The relevant background of these two factors will be examined as they effect the methodology employed in this section.

(i) Age

The age range of children in the survey receiving the first immunizing dose of Triple Antigen was two to seven months. Two significant factors influence the immune capability of children at this age. First is the stage of maturity of the child's immune response with respect to its ability to recognise and respond to simple and complex antigens (Osborn et al. 1952, Smith and Eitzman, 1964) and second is the influence of maternal factors transferred either in the cord blood (de Sant'Agnese, 1949, Peterson and Christie, 1951, Talmage, 1957, Albrecht et al. 1977) or in the breastmilk (Mohr, 1973, John et al. 1976, Schlesinger and Covelli, 1977).

In the absence of maternal antibody, the response to immunization, either naturally acquired or artificially induced, in the neonate is characterised by the production of immunoglobulin M (IgM) (Stiehm and Fudenberg, 1966, Gathings et al. 1981). This is evolutionarily the most primitive of the antibody classes (Marchalonis and
Edelman, 1965) and is the principal immunoglobulin produced in the classical primary humoral response (Rowley and MacKay, 1969). The level of IgM produced in infants, even though it is the major immunoglobulin synthesized, is only a fraction of normal adult levels. In the two to six month age range the IgM levels in these infants have reportedly ranged from 20-30% adult level (Stiehm and Fudenberg, 1966) to 35-50% adult level (Buckley, et al. 1968).

Physiological maturity, as indicated in infants by chronological age, appears to have little effect on the anti-toxoid response to diphtheria and tetanus (Osborn et al. 1952) although it is reportedly crucial for response to B. pertussis (Miller et al. 1949, Barrett et al. 1962, Provenzano et al. 1965). By measuring pertussis agglutinins, Barrett et al. (1962) found that infants less than three months of age were inferior in response compared to older infants (three to six months) after the first immunization to Quadruple vaccine (Diphtheria-Tetanus-Pertussis-Poliomyelitis) and concluded that these younger infants were less able to process the highly complex B. pertussis antigen repertoire.

The presence of maternal antibody in the neonate specific for the immunizing antigen poses a multifold problem when measuring an infant's antibody response thus necessitating the investigation of only paired cord and post-vaccinal plasma samples in this study. Most of the IgG present in the newborn is maternal in origin (Martensson and Fudenberg, 1965). This has been verified by determination of the immunoglobulin heavy chain allotypes (Gm) in both cord and neonatal blood which showed
that the developing infant's Gm phenotype can be masked by this maternal presence for many months (Morell et al. 1971, Williams, 1979).

The rate of disappearance of maternal IgG from the infant's circulation depends upon the particular type of IgG transferred across the placenta and the initial level of this immunoglobulin idiotype at birth (Barrett et al. 1962). Maternal antimeasles IgG can be present in infants older than twelve months (Balfour and Amren, 1978, Albrecht et al. 1977) whilst the persistence of B. pertussis maternal antibody is very short lived (Brown and Kendrick, 1960, Provenzano et al. 1965). Therefore, the amount of maternal antibody to a particular disease at any point in time can be highly unpredictable.

Circulating maternal antibody can depress or even inhibit an infant's IgG response to a specific antigen and this effect is well documented for measles (Albrecht et al. 1977, Balfour and Amren, 1978), poliomyelitis (Perkins et al. 1961, John et al. 1976) and B. pertussis (Barrett et al. 1962, Abbott et al. 1971, Preston, 1977). This depressive action of maternal antibody is believed due to either the development of a "tolerance" state in the infant which is highly selective for the specific antigen (Provenzano et al. 1965) or the result of neutralization of the active immunizing principle by the maternal antibody in vivo (Brown and Kendrick, 1960).

Passive immunity to B. pertussis, as already mentioned, is rapidly eliminated in most cases before the infant is immunized (Barrett et al. 1962). However, inhibition of specific IgG response still occurs in these infants with

The effect of circulating maternal IgG on initial IgM response of the infant has not been documented.

(ii) Sex

Circulating levels of IgM and IgG are reportedly higher in adult females (Allansmith et al. 1969, Stoop et al. 1969, Michaels and Rogers, 1971). The higher antigen-specific antibody response in females has been linked to the X chromosome (Rhodes et al. 1969, Grundbacher, 1972, Purtito and Sullivan, 1979) with proportionately higher antibody responses occurring in conditions where there is excessive duplication of the X chromosome (Nurmi, 1982). Hormonal influences are also believed to be involved although there is considerable conjecture concerning the effect of oestrogens on serum immunoglobulin levels (Eidinger and Garrett, 1972, Sljivic and Warr, 1973, Kenny et al. 1976). This sex effect may be less pronounced in infants since Michaels and Rogers (1971) could not find a significant difference in the geometric mean antibody response between the sexes in infants although it was quite evident in later childhood.
Section (i) Epidemiological Data

All information concerning immunization protocol, parental history of allergy, maternal vaccination status and the child's age, sex, immunization and breast-feeding history was obtained from the survey presented in Chapter 3.

Section (ii) Study population

Heel prick and cord plasma samples, retrieved from liquid nitrogen storage, were used in the microELISA technique previously described in Chapter 2, Section (IX).

The heel prick samples were selected by the following protocol:
(a) Only heel prick samples with corresponding cord samples were tested;
(b) an equivalent number of each sex was represented in each assay;
(c) each heel prick sample had been collected six to eleven weeks after the first immunization. According to Rowley and MacKay (1969) IgM levels of a primary response reach a plateau over this period;
(d) all samples to be tested against B. pertussis were from children immunized with Triple Antigen at 9 weeks of age or older to minimize the possibility of immunological immaturity affecting response to this antigen.

Section (iii) Determination of optimum plasma dilutions
(a) Optimum plasma dilutions for IgM estimation.

The B. pertussis and tetanus toxoid determinations were performed on the same tray. Plasma dilutions tested were 1:25, 1:50, 1:100. The two B. pertussis positive convalescent plasmas were from children who had confirmed
whooping cough and the three negative plasmas were from children who had received DT only. The tetanus toxoid positive plasma was from a child who had received Triple Antigen less than six weeks before blood collection and the negative plasma from a child who was unvaccinated.

(b) Optimum plasma dilutions for IgG heel prick and cord plasma estimations.

The heel prick and cord plasma samples were tested against B. pertussis and tetanus toxoid on the same tray. The following plasma dilutions were tested: B. pertussis heel prick 1:80; 1:160; 1:400; tetanus toxoid heel prick 1:200; 1:400; 1:800; 1:1600; B. pertussis and tetanus toxoid cord 1:100; 1:400. The positive and negative plasmas used for the heel prick IgM determinations were used also in this assay. Fifteen cord sera were used for both B. pertussis and tetanus toxoid estimations of optimum dilutions. Eight had positive tetanus vaccination histories. Pertussis contact history was unknown for the 15 cord samples tested.

Section (iv) Determination of antibody response in survey heel prick samples

The volume of the heel prick plasma collected during the survey ranged from 5-30µl. These limited volumes confined testing to only two of the three Triple Antigen components, B. pertussis and tetanus toxoid.

The plasma dilutions used in these assays were the dilutions which gave greatest discrimination between positive and negative control samples in the previous section.
(a) IgG estimation

105 heel prick samples were diluted 1:80, 1:160 for estimation of response to B. pertussis and 1:200, 1:400 for the tetanus toxoid assay. The matched cord plasma was tested at 1:400 for both antigens.

(b) IgM estimation

72 heel pricks were tested against B. pertussis and 83 against tetanus toxoid. All heel pricks and the corresponding cord samples were diluted 1:40.

(c) Assay and plasma controls

Each microELISA tray, whether for IgG or IgM estimation, contained the following technical and plasma controls: antigen-substrate; antigen-conjugate-substrate; antigen-Diluting buffer-substrate, PBS blank for the microELISA spectrophotometer, negative plasma control for B. pertussis and tetanus toxoid and, for adult tetanus, IgG and IgM estimations only, positive control.

The negative control for heel prick antibody estimation for both B. pertussis and tetanus toxoid was a plasma sample from an unvaccinated 4 month old child with no maternal history of tetanus vaccination or B. pertussis contact. This negative maternal history was verified by negative cord response to both antigens. There was no suitable positive age control for the heel prick samples for both antigens.

The negative control for cord tetanus and B. pertussis antibody level was cord plasma from a mother with no vaccination history or contact with B. pertussis. The positive control for IgG tetanus was adult plasma with a positive vaccination history for tetanus and an estimated
titre of 1:3200 using the microELISA method.

Section (v) Definition of positive antibody response

For the calculation of an antibody response at a selected plasma titre, the absorbance value of each test sample, measured at 630Å-570Å dual wavelength, was compared to the absorbance value of the negative control over the same wavelength settings. In IgM estimations, if the ratio of the absorbances of test sample to negative control was 2 or greater then the test sample was considered positive at the 1:40 dilution. This level corresponded to an obvious colour change in the test well after overnight refrigerated incubation with an absorbance value of at least 0.4. For IgG estimations, a positive response occurred if the ratio was greater or equal to 3. This corresponded to an absorbance value of 0.9 or higher.

Section (vi) Statistical methods

The strength of associations between antibody response and epidemiological variables were computed using either Chi-square test utilizing Yates' correction or Fisher's exact test where applicable. Correlation values \( r \) were computed from the relevant Chi-square by the following equation: \( r = \frac{X^2}{N} \) where \( N \) is the size of the sample tested. Group means were compared using Students' \( t \) test.
RESULTS

Section (i) Determination of plasma dilutions

The criterion for selection of specific plasma dilutions was to allow maximal use from the limited volume of heel prick material available. No attempt was made to estimate the actual titres of the plasma samples.

From evidence presented in the introduction, it can be predicted that both the IgM and IgG primary response to \textit{B. pertussis} would be small in magnitude despite the high sensitivity of the microELISA technique. Fig 4.1 compares the IgM and IgG responses to \textit{B. pertussis} in children convalescing after whooping cough infection with the same responses in children who had received DT vaccination only. The absorbance values of the two IgM positive samples (Fig 4.1a), despite decreasing rapidly between the 1:25 and 1:50 plasma dilutions, are distinctly elevated above the absorbance values of the negative samples over the same dilution range. No absorbance activity was detected in either the positive or negative samples at the 1:100 dilution. There was also no non-specific prozone effect at the lesser dilution in the negative plasma samples. For the subsequent assays, the plasma dilution for detecting IgM response to \textit{B. pertussis} was set at 1:40.

Separation of the absorbance values of the IgG response to \textit{B. pertussis} between the two extreme whooping cough exposure histories (Fig. 4.1b) was maximal at 1:80 and still evident at 1:160 except that the absorbance of one positive sample was not obviously different from that of the negative samples.
FIGURE 4.1  Humoral response to B. pertussis in heel prick plasma. Diagram (A) shows the IgM response of children with positive (◇) and negative (◆) B. pertussis contact histories. Heel prick plasma was diluted 1:25 to 1:100 with Diluting Buffer. Diagram (B) depicts the IgG response of the same samples. In this case heel prick plasma was diluted 1:80 to 1:400. The ELISA trays were read at dual absorbance values (Abs) of 570Å and 630Å.
Graphs A and B show the absorbance (Abs.) levels at different plasma dilutions. Graph A includes dilutions of 1:25, 1:50, and 1:100, while Graph B includes dilutions of 1:80, 1:160, and 1:400.
The antibody response to tetanus toxoid was predicted to be greater than that to *B. pertussis* owing to the comparatively less complex structure of the toxoid. The IgM response to tetanus toxoid, shown in Fig. 4.2a is very similar in kinetics to the IgM response to *B. pertussis*. The absorbance value of the positive samples, however, does not decrease as rapidly over the 1:25 to 1:50 dilution range. The separation of positive and negative response is adequately defined over this dilution range. For convenience, the same plasma dilution as that chosen for *B. pertussis* (1:40) was subsequently used for the testing of an IgM response to tetanus toxoid.

Fig 4.2b shows that sufficient definition of a differential IgG response to tetanus toxoid between the toxoid vaccination positive sample and the unvaccinated negative sample occurred at the 1:200 and 1:400 dilutions. Both dilutions were used in the subsequent assay for detecting IgG response to tetanus toxoid. A minimum of 10µl heel prick plasma volume was sufficient for all of the above IgG and IgM estimations.

Fig 4.3 shows the response of cord plasma to tetanus and *B. pertussis*. At the low dilution (1:100) all cord samples had high absorbance values for both antigens. This implied the presence of a cord factor which probably was nonspecifically binding to both the microtitre tray and the conjugate leading to this false positive response. A higher dilution was therefore essential to lessen this nonspecific prozone effect. At 1:400 a differential cord response resulted for both antigens and was used in the subsequent assay.
FIGURE 4.2 Humoral response to tetanus toxoid in heel prick plasma. Diagram (A) shows the IgM response of children with positive () and negative (♦) vaccination history for tetanus toxoid. Heel prick plasma was diluted 1:25 to 1:100 with Diluting Buffer. Diagram (B) shows the IgG response of the same two samples. In this case, heel prick plasma was diluted 1:200 to 1:1600. The ELISA trays were read at dual absorbance values (Abs) of 570Å and 630Å.
FIGURE 4.3  Humoral response in umbilical cord plasma to
(A) B. pertussis and (B) tetanus toxoid. The
cord samples were tested at two dilutions
1:100 and 1:400 for both antigens. Note the
clustering of the majority of the samples
tested at high absorbance values (1.3 to 1.5)
at dilution 1:100. The ELISA trays were read
at dual absorbance values (Abs) of 570Å and
630Å.
Section (ii) Cord antibody response

Of 105 cord plasma samples tested, 59 (52.21%) were positive at 1:400 for the IgG response to tetanus toxoid and 24 (21.24%) were IgG positive for *B. pertussis*. No cord sample was positive for IgM at 1:40.

The cord tetanus toxoid IgG positive response indicated a specific antibody response since a positive tetanus vaccination history recorded at interview was significantly associated with the positive tetanus cord IgG response ($\chi^2_1$ (corrected) = 8.00; $p<0.01$). However, there was insufficient history on maternal *B. pertussis* exposure to make the same comparison.

Section (iii) Infant's IgM response to *B. pertussis*

Fourteen of the 72 heel prick samples tested showed a positive IgM response to *B. pertussis* at 1:40 dilution. The test samples were then separated into positive and negative response groups and compared for the following variables: age at first immunization, sex, method of vaccine administration, cord IgG response to *B. pertussis*, breastfeeding history at time of immunization alone and in combination with cord IgG response, immunization reactor status and parental history of allergy. The results of these comparisons are presented in Table 4.1.

The age and sex distribution between the two groups were equivalent. The method of vaccine administration variable compared the administration of a partitioned dose one week before the remainder dose to that of a partitioned dose given one month or longer before. These two regimes proved not significantly different in their effect on the IgM response and were subsequently combined for comparison.
TABLE 4.1

DISTRIBUTION OF POSSIBLE CONFOUNDING VARIABLES IN THE TWO IgM RESPONSE GROUPS TO B. PERTUSSIS

ALL COMPARISONS ARE NON SIGNIFICANT (P>0.05) *

<table>
<thead>
<tr>
<th>CONFOUNDING VARIABLE</th>
<th>IgM RESPONSE TO B. PERTUSSIS AT 1:40 TITRE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>POSITIVE</td>
</tr>
<tr>
<td></td>
<td>N*</td>
</tr>
<tr>
<td>SEX OF CHILD</td>
<td></td>
</tr>
<tr>
<td>Male children</td>
<td>14</td>
</tr>
<tr>
<td>VACCINE ADMINISTRATION</td>
<td></td>
</tr>
<tr>
<td>Total receiving</td>
<td>14</td>
</tr>
<tr>
<td>Partitioned dose</td>
<td>14</td>
</tr>
<tr>
<td>Partitioned dose given one week before</td>
<td>10</td>
</tr>
<tr>
<td>CORD B. PERTUSSIS TITRE</td>
<td></td>
</tr>
<tr>
<td>Positive titre at 1:400</td>
<td>14</td>
</tr>
<tr>
<td>BREASTFEEDING</td>
<td></td>
</tr>
<tr>
<td>Total breastfed</td>
<td>14</td>
</tr>
<tr>
<td>Breastfed, positive cord titre</td>
<td>8</td>
</tr>
<tr>
<td>PARENTAL ALLERGY</td>
<td></td>
</tr>
<tr>
<td>Positive history</td>
<td>14</td>
</tr>
<tr>
<td>IMMUNIZATION REACTOR STATUS</td>
<td></td>
</tr>
<tr>
<td>Reactor</td>
<td>14</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>N</th>
<th>WEEKS (+1 S.D++)</th>
<th>N</th>
<th>WEEKS (+1 S.D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>12.27+2.52</td>
<td>58</td>
<td>12.18+2.94</td>
</tr>
</tbody>
</table>

* number tested
++ standard deviation

* distribution of specified confounding variable in positive IgM group compared to the distribution of same variable in negative IgM group.
with the children receiving a single immunizing injection. Again there was no difference in effect of the method of vaccine administration.

The possibility of sensitization or suppression due to the presence of maternal factors transferred by cord or breastmilk was examined. Seven heel pricks had cord samples IgG positive for B. pertussis but there was no abnormal distribution of these samples in either the positive or negative group. Breastfeeding with or without a positive IgG cord response was also equivalent between the two groups.

There was no association between immunization status and IgM response group. Similarly, the proportion of positive parental allergic histories was approximately the same in both groups.

Section (iv) Infant's IgM response to tetanus toxoid

Nineteen heel prick samples showed a positive IgM response to tetanus toxoid at 1:40 dilution whilst 64 samples were negative. Similar to the B. pertussis IgM results, the heel prick samples were separated into two IgM response groups and compared with the same epidemiologic variables. The results in Table 4.2 were similar in trend to the B. pertussis results (Table 4.1) with no epidemiologic variable showing a significantly different distribution between the positive and negative IgM response groups.

Only three B. pertussis IgM positive responders showed a positive IgM response to tetanus toxoid. Therefore, there was no significant association between the B. pertussis and tetanus toxoid IgM positive reresponse groups
TABLE 4.2

DISTRIBUTION OF POSSIBLE CONFOUNDING VARIABLES IN THE TWO IgM RESPONSE GROUPS TO TETANUS TOXOID

ALL COMPARISONS ARE NONSIGNIFICANT (P>0.05) *

<table>
<thead>
<tr>
<th>CONFOUNDING VARIABLE</th>
<th>IgM RESPONSE TO TETANUS TOXOID AT 1:40 TITRE</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>POSITIVE</td>
<td></td>
<td>NEGATIVE</td>
</tr>
<tr>
<td></td>
<td>% POSITIVE</td>
<td>WITH VARIABLE</td>
<td>% POSITIVE</td>
</tr>
<tr>
<td></td>
<td>WITH VARIABLE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SEX OF CHILD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male children</td>
<td>19</td>
<td>63.2</td>
<td>64</td>
</tr>
<tr>
<td>VACCINE ADMINISTRATION</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total receiving</td>
<td>19</td>
<td>57.9</td>
<td>64</td>
</tr>
<tr>
<td>partitioned dose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Partitioned dose given one week before</td>
<td>11</td>
<td>72.7</td>
<td>41</td>
</tr>
<tr>
<td>CORD TETANUS TOXOID TITRE</td>
<td>17</td>
<td>52.9</td>
<td>58</td>
</tr>
<tr>
<td>Positive titre at 1:400</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BREASTFEEDING</td>
<td>18</td>
<td>61.1</td>
<td>63</td>
</tr>
<tr>
<td>Total breastfed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breastfed, positive cord titre</td>
<td>11</td>
<td>54.5</td>
<td>36</td>
</tr>
<tr>
<td>PARENTAL ALLERGY</td>
<td>19</td>
<td>68.4</td>
<td>64</td>
</tr>
<tr>
<td>Positive history</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IMMUNIZATION REACTOR STATUS</td>
<td>19</td>
<td>31.6</td>
<td>64</td>
</tr>
<tr>
<td>Reactor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AGE AT FIRST IMMUNIZATION</td>
<td>19</td>
<td>12.54+4.17</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>11.35+2.41</td>
</tr>
</tbody>
</table>

+ number tested
++ standard deviation
*

distribution of specified confounding variable in positive IgM group compared to the distribution of same variable in negative IgM group.
Section (v) IgG response to tetanus toxoid

Owing to the difficulty in defining an infant's IgG response in the presence of maternal IgG, all children with positive cord responses to tetanus toxoid were removed from the analysis. Only 46 children had a negative cord tetanus toxoid IgG response. Nineteen children had a detectable IgG response at 1:200 dilution whilst 26 were negative (one sample was not measured due to a technical error). The two response groups were then compared for age at first immunization, sex, method of vaccine administration, breastfeeding, immunization reactor status and parental history of allergy (Table 4.3).

The mean age of the positive response group was higher than that of the negative response group ($t = 2.58$, $p<0.05$). This increase was due to the inclusion of four children in this group who were over four months of age at the first immunization. There was also a significant increase in the number of children receiving a partitioned dose of Triple Antigen in the positive response group ($p = 0.016$). No other variable reached significance.

Six children who were IgM positive for tetanus toxoid were also IgG positive for the same antigen. There was, however, no significant correlation between the two tetanus antibody positive groups ($r = 0.068$).

Section (vi) IgG response to B. pertussis

Excluding the three children who had confirmed or suspected whooping cough and those children who had a positive cord titre to B. pertussis to eliminate the possibility of non-response being due to suppression,
### TABLE 4.3

**DISTRIBUTION OF POSSIBLE CONFOUNDING VARIABLES IN THE TWO IgG RESPONSE GROUPS TO TETANUS TOXOID**

**SIGNIFICANT COMPARISONS ARE INDICATED***

<table>
<thead>
<tr>
<th>CONFOUNDING VARIABLE</th>
<th>IgG RESPONSE TO TETANUS TOXOID AT 1:200 TITRE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>POSITIVE</td>
</tr>
<tr>
<td></td>
<td>N⁺</td>
</tr>
<tr>
<td>SEX OF CHILD</td>
<td></td>
</tr>
<tr>
<td>Male children</td>
<td>19</td>
</tr>
<tr>
<td>VACCINE ADMINISTRATION</td>
<td></td>
</tr>
<tr>
<td>Total receiving</td>
<td>19</td>
</tr>
<tr>
<td>Partitioned dose</td>
<td></td>
</tr>
<tr>
<td>Given one week before</td>
<td>16</td>
</tr>
<tr>
<td>BREASTFEEDING</td>
<td></td>
</tr>
<tr>
<td>Total breastfed</td>
<td>19</td>
</tr>
<tr>
<td>PARENTAL ALLERGY</td>
<td></td>
</tr>
<tr>
<td>Positive history</td>
<td>19</td>
</tr>
<tr>
<td>IMMUNIZATION REACTOR</td>
<td></td>
</tr>
<tr>
<td>STATUS</td>
<td></td>
</tr>
<tr>
<td>Reactor</td>
<td>19</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>WEEKS (±1 S.D)⁺⁺</th>
<th>N</th>
<th>WEEKS (±1 S.D)⁺⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGE AT FIRST IMMUNIZATION</td>
<td>19</td>
<td>13.35±4.93</td>
<td>26</td>
<td>10.57±2.09²</td>
</tr>
</tbody>
</table>

* number tested

⁺⁺ standard deviation

* distribution of specified confounding variable in positive IgG groups compared to the distribution of same variable in negative IgM group.

1 Fisher's exact test: \( p = 0.016 \)

2 Student's t test: \( p < 0.01 \)
only one sample of the 81 samples tested had a positive titre at 1:80.
DISCUSSION

Previous studies on the humoral response to B. pertussis containing vaccines in young children (Macaulay, 1981, Barrett et al. 1958; 1959, Provenzano et al. 1965, Brown and Kendrick, 1960) have concentrated on age-related efficacy of the vaccine rather than on individual differences between the vaccine recipients. These reports, though different in intent, have been useful in emphasizing the variables such as age at immunization and the presence of maternal antibody which may have an effect on the antibody response of two to seven month old children.

Before the advent of radioimmunoassay and ELISA, experimental techniques, such as indirect haemagglutination (Provenzano et al. 1965, van der Does-van den Berg et al. 1981), complement fixation (Bradstreet et al. 1972, Macaulay, 1979) or toxin neutralisation tests (Bainton et al. 1979) were used to estimate antibody titres to components of combined pertussis vaccines. The definition of a specific IgG and IgM response in a single test sample was determined by the use of specific antisera and radial diffusion in agar gels (Buckley et al. 1968) or else the sample was treated with mercaptoethanol (Rowley and MacKay, 1969, Whittingham et al. 1980) or ultracentrifuged (Macaulay, 1981) to separate the individual immunoglobulins. These techniques for total and specific idiotypic antibody estimation were clearly unsuitable for this present study since these methods required a plasma sample of considerably larger volume than that available from the heel prick samples. Instead a microELISA technique utilising urease-conjugated sheep antihuman immunoglobulin in an indirect
sandwich method was established and implemented. This technique was an adaptation of the original macroELISA and required only small test volumes (100µl per test). The minimum volume of the original undiluted heel prick sample necessary for the completion of all the immunoglobulin estimations, that is the IgG and IgM response to both *B. pertussis* and tetanus toxoid, was 10µl. The assay was simple and rapid to perform with the complete assay for IgG from the time of antigen-coating to spectrophotometer reading being finished within five and one half hours. It also had the capability of processing many samples at the same time. In comparison with radioimmunoassay, the enzyme conjugate did not pose a health hazard for the assay operator nor did it require stringent storage requirements as would be necessary for a radioactive probe.

The experimental protocol developed for this study efficiently measured post-vaccinal IgM response even though Ruuskanen *et al.* (1980) using a similar micro-ELISA system could find no detectable levels. The sheep antihuman IgM conjugate proved specific for IgM and did not show false positives in the presence of suspected high IgG titres such as those present in cord plasma and the adult tetanus toxoid positive control. There was no indication that the presence of IgG hindered IgM estimation since both immunoglobulins could be measured in the same heel prick sample.

This study has examined the presence of a differential antibody response at a specific IgM or IgG titre following initial exposure to *B. pertussis* and tetanus. No child in the final analysis had a reported history of
whooping cough or previous contact with tetanus toxoid vaccination. Division of the children into positive and negative responder groups was possible for IgM response to B. pertussis and tetanus and for IgG response to the toxoid. Only one individual had a positive IgG response to B. pertussis.

The association of the confounding variables of age and sex with the IgM response to both antigens was unremarkable. With the removal of children less than 9 weeks of age from the B. pertussis study group to eliminate the possibility of reported immunological immaturity affecting the processing of this antigen (Barrett et al. 1962), there was no significant correlation between the age at the time of first immunization and IgM antibody response to both B. pertussis and tetanus toxoid. There was no sex difference between the two IgM response groups for both antigens. However, since actual titres to each antigen were not measured, the results of Michaels and Rogers (1971) indicating that there was no difference in the geometric mean antibody response between the sexes in infancy could not be confirmed.

The effect of maternal antitetanus IgG on the child's IgG response to tetanus toxoid, was not examined because without serial studies of antibody titres it is not possible to differentiate maternal and infant IgG response. There was no evidence for the presence of non-specific factors in the breastmilk affecting IgG antibody response to tetanus vaccination (Table 4.3) as has been reported following poliomyelitis vaccination (John et al. 1976). Table 4.3 did show, however, a marked relationship between
the administration of a partitioned dose and a positive IgG response to tetanus toxoid (p=0.016). This association may reflect a causal relationship between these two factors with the positive IgG response indicating a booster response arising from the priming effect of the initial partitioned dose. Interestingly, there was no significant difference in the positive IgG response group between the children who received the partitioned dose one week apart and those in which the time delay was one month or greater.

Alternatively, the higher rate of IgG responses to tetanus toxoid children in receipt of partitioned vaccination, may be a spurious association due to a sampling artefact which resulted from the inclusion of older infants in the positive IgG response group. There is a significant age difference between the positive and negative IgG response groups (p<0.01) and this difference is attributable to four infants in the positive IgG response group being older than four months of age at the time of first immunization. These four children had expressed minor "allergic" conditions such as a temporary skin rash at initial presentation for immunization and were therefore offered a partitioned dose by the health clinic which was administered after the allergic manifestation had passed. This delay in offering immunization increased the age at which these children had received their full first immunization. Upon removal of these four children from the analysis, the association between the effect of partitioned dose and age on positive IgG response, became nonsignificant (partitioned dose p>0.05; age : p>0.05). The other variables tested
remained nonsignificant.

In contrast to the IgG response to tetanus toxoid, there was no correlation between the presence of a partitioned dose and a positive or negative IgM response in either the B. pertussis or tetanus toxoid results (Table 4.1, 4.2). The possibility of previous sensitization or suppression due to the transfer of maternal antibody or factors in the cord blood or breastmilk affecting the infant's IgM response to both antigens was also not supported by the results (Tables 4.1, 4.2).

By elimination of the confounding variables known to affect infant antibody response there is a strong indication that the observed differential primary IgM response to a B. pertussis and tetanus toxoid may be due to genetically determined differences between the children rather than the result of environmental effects (Allansmith et al. 1969).

Only three children gave a positive IgM response to both antigens. This implies that the response of the majority of positive IgM responders to B. pertussis and tetanus toxoid was antigen-specific rather than reflecting an increased propensity to produce IgM to any antigen. Ruuskanen et al. (1980), although measuring secondary IgG responses to DPT, reported a similar phenomenon with no significant correlation between the antibody responses against the individual vaccine antigens.

The grouping of differential IgM responders in this study therefore appears to approximate that of murine studies where differences in responder status are due to the presence of specific immune response genes.
(Benacerraf and McDevitt, 1972, Payne, 1977) even though it has been otherwise suggested by Kaslow and Shaw (1981) that the multigenic mouse model of Biozzi (Biozzi et al. 1971) may be more appropriate for human antibody response to infectious agents.

Neither immunization reactor status nor the variables which were highly significant for the determination of a reaction correlated with responder status in any antigen or antibody group. Therefore, the mechanism which recognises B. pertussis or tetanus toxoid for an IgM or IgG response is not responsible for the generation of an immunization reaction. It is possible that IgE reagin response may reflect immunization reactor status more accurately (Nagel et al. 1979, Matuhasi and Ikegami, 1982). Antigen-specific IgE, under suitable experimental conditions, can be increased after injection of the suitable allergen (Levine and Vaz, 1970, Marsh et al., 1980; 1981). IgE serum levels must be determined before and after immunization in order to determine the presence of a response since it is known that allergic persons, including young children, have basal IgE titres higher than the normal non-allergic population (Hoffman et al. 1975, Wilcox and Marsh, 1978). However, the experimental protocol used in this study was not amenable to the measurement of a specific IgE response. The level of IgE in cord sera, used as the preimmunization samples, does not always reflect the possible allergic tendency of the infant (Bazaral et al. 1971, Michel et al. 1980, Croner et al. 1982) probably due to the difficulty in determining the quantities present (Johannson, 1968). Differential basal
IgE levels are reportedly more predictive in children older than six weeks (Bazaral et al. 1971). Other components of the protocol, such as the method of antigen administration (deep subcutaneous) and the length of time before the test blood sample was collected (Levine and Vaz, 1970), would hinder optimal measurement of specific IgE even if a suitable preimmunization sample was available.

This study has successfully defined bimodality in the humoral response of infants receiving primary Triple Anti-gen immunization. Although non-genetic effects may explain the difference between the tetanus IgG response groups, it has been demonstrated that the two response groups for both B. pertussis and tetanus toxoid IgM determination are homogeneous with respect to age, sex, method of antigen administration, history of parental allergy, breastfeeding and immunization reactor status. Therefore, further investigation of any genetic difference between the IgM responder groups for both antigens can be confidently undertaken. These results will be presented in Chapter 6.
CHAPTER 5

Lymphoproliferative response of umbilical cord lymphocytes following in vitro exposure to components of Triple Antigen.
INTRODUCTION

Antigen-induced lymphoproliferation is primarily regarded in man as an in vitro cellular secondary response arising from previous antigen exposure (Mills et al. 1966; Oppenheim et al. 1968; Alford et al. 1976; Oppenheim and Rosenstreich, 1976; Fleer et al. 1976; Rasanen, 1980). A few reports (Seldin and Rich, 1978; Ellner et al. 1979; Young and Engleman, 1980; Treves et al. 1981) state that it can also represent an in vitro primary response. The criterion used in these latter reports for the differentiation of in vitro secondary and primary responses was based on the time of peak proliferation after the initial addition of antigen to culture, with secondary or recall response peaking at four to six days of culture (Oppenheim and Rosenstreich, 1976; Ellner et al. 1979) and the response due to primary in vitro sensitization occurring from seven days onwards (Seldin and Rich, 1978). Ellner et al. (1979) also noted a decreasing trend in the magnitude of the peak lymphoproliferative response from secondary to primary response and concluded that this difference in kinetics and magnitude of response might be attributed to a difference in the number of antigen-sensitive lymphocytic subsets in the primary and secondary culture. A further distinction has been reported between primary and secondary responses in their relative requirements for antigen presenting cells. Treves et al. (1981) found that the primary cellular response to protein purified derivative of tuberculin (PPD) was dependent upon the presence of macrophages in culture whilst this dependency was not absolute in cultures of PPD.
primed cells. This differential dependency requirement for macrophages has similarly been found for primary and secondary mixed lymphocyte cultures (Eckels et al. 1981).

Umbilical cord lymphocytes are considered an immunologically protected cell population with negligible previous antigenic contact (Leiken and Oppenheim, 1971; Hsu et al. 1981; Handzel et al. 1980; Rasanen et al. 1980; Campbell et al. 1974; Hayward, 1981) and are, therefore, used as non-responder controls in cellular studies on in vitro secondary responses in adults (Hsu et al. 1981; Rasanen et al. 1980; Mogensen and Andersen, 1981). However, cellular stimulation in cord lymphocyte cultures, after the addition of whole or soluble microbial antigens, has been repeatedly reported and has been ascribed to the inherent mitogenicity of the microbial preparation used (Andersen et al. 1977; Morse et al. 1977; Ringden et al. 1977; Sakane and Green, 1978; Kasahara et al. 1980; Räsänen et al. 1980), the mitogenic potential of a media inclusion, in particular foetal calf serum (Johnson and Russell, 1976; Alford et al. 1976; Rosenthal and Möller, 1979) or to previous, though highly speculative, sensitization resulting in a cellular secondary response. This sensitization reportedly may arise from maternal contamination, in the form of cellular components or soluble mediators, occurring transplacentally (Russell, 1975; Barnewton et al. 1976; Gallagher et al. 1981) or the cord lymphocytes may become sensitized following prenatal infection or exposure to the microbial agent (Brody et al. 1968; Aase et al. 1972; Horten et al. 1976; Alford et al. 1976; Ruben et al. 1975). There have been a few reports where antigen-induced lympho-
proliferation has occurred in cord lymphocyte cultures though not in the matched maternal lymphocyte culture. This phenomenon has either remained unexplained (Russell, 1975), ascribed to maternal unresponsiveness as a consequence of pregnancy (Rubin et al. 1981), the result of a genetic difference in the neonate's immune capability due to its paternal genetic contribution (Gill et al. 1979), a non-specific mitogenic effect (Gallagher et al. 1981), technical errors leading to false-positives in the cord culture (Gallagher et al. 1981) or due to the response of the cord lymphocyte cultures to primary in vitro sensitization (Ellner et al. 1979).

The emphasis of this portion of the study is on the occurrence of a lymphoproliferative response possibly arising from primary in vitro sensitization of cord mononuclear cells to the antigenic components of Triple Antigen: B. pertussis, tetanus toxoid and diphtheria toxoid. By the elimination of known confounding environmental factors, this study intends to show that the cord cell population can be partitioned into positive and negative responder groups on the basis of antigen-induced lymphoproliferative response.

(i) Technical aspects

According to the Research Design protocol outlined in Chapter 2, the responding cell population in the lymphoproliferative assay consists of cryopreserved umbilical cord cells. This section will briefly examine the nature of umbilical cord cells and the effect that cryopreservation may have upon mononuclear cells.
(a) Umbilical cord cells

Cord mononuclears have been reported to show increased
(Carr et al. 1972; Campbell et al. 1974; Eife et al. 1974;
Yu et al. 1975; Alford et al. 1976) similar (Leiken et al.
1968; Schecter et al. 1977) or decreased (Jones, 1969;
Ayoub and Kasakura, 1971) lymphoproliferative responses to
mitogenic stimulation by phytohaemagglutinin (PHA), compared
to normal adult controls, resulting in conflicting reports on
the immune competence of these cells based upon this immune
function test. In contrast, after antigenic stimulation,
whether considered as a primary or secondary response, the
general consensus is that cord mononuclears have a low
lymphoproliferative potential (Ellner et al. 1979; Gill
et al. 1979; Rasanen, 1980). Recent papers have affirmed,
by the use of tests of cellular immune function (reviewed
by Andersson et al. 1981) that neonatal cells are function­
ally immune competent (Pirofsky et al. 1973; Granberg et al.
1976; Stiehm et al. 1979; Handzel et al. 1980) although
in vitro antibody synthesis by cord cells may be impaired
(Miyagawa et al. 1980; Andersson et al. 1981; Gathings

Most cord blood cell samples possess the ability to
transform spontaneously in culture which has been confirmed
both morphologically (Pulvertaft and Pulvertaft, 1966;
Jones, 1969) and by the uptake of radioactive thymidine
(Carr et al. 1972; Prindull et al. 1975; Alford et al.
1976; Gill et al. 1979). This characteristic occurs in
only 1% of healthy Caucasoid adults (Bord et al. 1959;
Pulvertaft and Pulvertaft, 1966; Prindull et al. 1975)
although it can be a feature of some pathologic and physio-
logic states (Prindull et al. 1975). Spontaneous transformation in cord cells has been postulated as the result of foetal responses to maternal lymphocytes in the circulation (Carr et al. 1972), congenital rubella cellular infection (Marshall et al. 1970), differences in the maturity of functional mononuclear sub-populations in cord blood (Alford et al. 1976; Gill et al. 1979), persistent low level stimulation in prenatal life (Carr et al. 1972) or the presence of mitotically active stem cells, of either the myeloid or erythroid germ line, in cord blood (Prindull et al. 1975).

The presence of suppressor mechanisms in cord blood is well documented (Wolf et al. 1977; Hayward and Lydyard, 1978; Durandy et al. 1979; Wilson and Remington, 1979; Andersson et al. 1981; Fischer et al. 1982). Foetal suppressor cells or the liberation of suppressor factors are reported to regulate cord blood suppression (Oldstone et al. 1977; Abedin and Kirkpatrick, 1980; Tosato et al. 1980) but these suppressive mechanisms are more effective in suppressing the immune function in adult cell cultures rather than neonatal cell cultures (Andersson et al. 1981).

(b) Cryopreservation of cells

The use of frozen mononuclears as responder cells in culture is not a widely used practice. Barclay and White (1976) studied the use of frozen responder cells and concluded that the blastogenic response of the frozen lymphocyte closely parallels its fresh counterpart. Following mitogenic stimulation the lymphoproliferative response of the frozen cell may be greater than fresh cells although antigenic stimulation may result in a lower response by
the frozen cell culture compared to a fresh cell culture (Barclay and White, 1976). Callery et al. (1980) showed that most cellular functions were not affected by cryopreservation providing optimum freezing and thawing techniques were employed.

(ii) Nature of the antigen

Although there have been no substantiated reports of mitogenicity occurring after the addition of tetanus or diphtheria toxoid to culture, the B. pertussis organisms contain Lymphocytosis Promoting Factor (LPF) which is mitogenic to both murine (Kong and Morse, 1977a, 1977b) and human (Morse et al. 1977, Andersen et al. 1977) mononuclears including cord blood cells. LPF forms part of the surface antigenic complex, now termed pertussigen (Arai and Munoz, 1981). Andersen et al. (1977) found that organisms from four Danish B. pertussis vaccine strains non-specifically stimulated the seven cord mononuclear preparations they were tested against. A significant correlation was found in Andersen's report between the response to B. pertussis and Escherichia coli in both adult and cord samples and it was hypothesised that the non-specific response may reflect crossreactivity between components of B. pertussis and the antigens of other micro-organisms similar to that found with Pseudomonas aeruginosa (Høiby, 1975) rather than due purely to a mitogenic effect. Mogensen and Andersen (1981) reported that the lymphocyte response to the seven microbial preparations they tested (BCG, Mycoplasma pneumoniae, Herpes simplex, E. coli, Haemophilus influenzae, Salmonella typhi and tetanus toxoid) was a conglomerate response to both antigen-specific components and cross-reacting or polyclonally-activating moieties. Lower
antigenic concentrations appeared to eliminate the non-specific component of the microbial preparations, resulting in an antigen-specific response (Mogensen and Andersen, 1981). The cord lymphocyte response to low doses of *B. pertussis* has not been clarified.
METHODS

Section (i) Epidemiological and antibody data

This data has been previously presented in Chapter 3 and Chapter 4 respectively.

Section (ii) Selection of umbilical cord cell samples for culture

Cell samples from an equivalent number of immunization reactor and non-reactor children were selected. After this crude selection, the following criteria were applied in a stepwise fashion for choosing the final experimental sample.

(a) Material available

Three or more storage vials per sample was the minimum criterion so that sufficient mononuclear material remained for HLA typing although, in some cases, not sufficient for testing against all antigen doses used and, in most cases, for retesting.

(b) Foetal haemoglobin estimation

Samples from cord blood containing less than an estimated 10% adult haemoglobin level were selected to minimize, if not eliminate, the potential of maternal admixture at collection.

(c) Viability of cells after freezing

The recovered cell sample had to exhibit greater than 70% viability as assessed by 0.1% aniline blue exclusion test before it was used for culture. Methods employed to reduce the percentage of dead mononuclears, in general, randomly affected the condition and cellular composition of the sample.
Section (iii) Evaluation of freezing and thawing technique

Fresh venous blood was collected in heparinised vacuum tubes from two adult volunteers of known health and immunization history. Mononuclear cells were obtained using the buoyant density gradient Ficoll-Hypaque method (Boyum, 1968). Each cell suspension was equally divided with one half transferred to McCoy's transport media and left overnight at room temperature whilst the other half was adjusted to a cell concentration of $1 \times 10^7$ cells per ml and cryogenically stored as described in Chapter 2. After 24 hours, the frozen cell preparations were thawed aseptically and adjusted to a cell concentration of $10^6$ live cells per ml in RPMI tissue media. The cells left at room temperature were examined for viability before being similarly prepared for culture. Each half sample was aliquoted in 150µl volumes with its pair into the same culture tray and tested, in duplicate cultures, against PHA : $0.8\mu l$, $0.4\mu l$, $0.1\mu g/ml$ and *B. pertussis* : $1.6 \times 10^9$, $8 \times 10^8$, $4 \times 10^8$ organisms per ml. The cultures were incubated for 72 hours then pulsed and harvested as previously described (Chapter 2).

Section (iv) Comparison of response of adult and cord cryopreserved mononuclear cells to PHA

Cryopreserved cell samples of two adults and 21 cord bloods were thawed and prepared for culture (as previously described in Chapter 2) and, depending upon the concentration of cells retrieved, tested against a range of PHA doses : $0.01\mu g$, $0.1\mu g$, $0.2\mu g$, $0.3\mu g$, $0.4\mu g$, $0.6\mu g$, $0.8\mu g$ and $1.6\mu g/ml$ in duplicate cultures. The adult samples and 15 cord samples were incubated for 72 hours and six cord samples were incubated for 120 and 169 hours with a thy-
midine pulse during the last 16 hours of culture before harvesting.

Section (v) Determination of optimal culture conditions for differential response to B. pertussis, tetanus and diphtheria toxoid.

Seven cord cell samples, after thawing, were halved and each half added to dual sets of culture trays which received identical culture media and antigen dilutions. The antigen dilutions used were: B. pertussis $20 \times 10^8$, $4 \times 10^8$, $1 \times 10^8$ organisms per culture; tetanus toxoid: $10\mu$g, $5\mu$g, $2\mu$g, $1\mu$g, $0.5\mu$g protein per culture; diphtheria toxoid: $35\mu$g, $17.5\mu$g, $7\mu$g, $3.5\mu$g, $1.75\mu$g protein per culture. Each dilution was administered in 50µl of tissue culture medium.

Cell concentration used was $10^6$ live mononuclear cells in 150µl tissue culture media aliquots. One set of the matched trays were incubated for 120 hours, the other set for 168 hours.

Section (vi) Culture conditions for separation of responders and non-responders to B. pertussis, tetanus and diphtheria toxoids

The culture conditions have been previously described in Chapter 2. Sixty cord cell samples were selected for culture by the criteria stated in section (ii). Tested antigen dilutions were: B. pertussis $4 \times 10^8$, $2 \times 10^8$, $1 \times 10^8$ organisms per culture; tetanus toxoid: $4\mu$g, $2\mu$g, $1\mu$g protein per culture; diphtheria toxoid $14\mu$g, $7\mu$g, $3.5\mu$g and $1.75\mu$g protein per culture and were added to triplicate cultures.
Section (vii) Statistical methods

Results were expressed as either mean corrected counts per minute (cpm) or the log 10 equivalent of this value. The raw cpm for each culture was corrected for the efficacy of the liquid scintillation counter used, the background culture cpm was subtracted from this corrected value and the mean and standard deviation of the respective triplicate antigen or unstimulated cultures computed. Stimulation indices (S.I.) were calculated as follows:

\[
\text{mean corrected cpm for test antigen culture} - \frac{\text{mean corrected cpm for unstimulated cultures}}{} \]

Since low lymphoproliferative responses and, therefore, low mean corrected cpm of test antigen cultures was anticipated from the use combination of umbilical cord cell, frozen mononuclear cells and primary in vitro sensitization, a cellular response would be masked by a high unstimulated culture value. Therefore, a test for outliers involving the kurtosis statistic (Mendell et al. 1977) was applied to the ranked order of the mean corrected cpm for the unstimulated cultures to remove from the analysis those cord cell samples which had undergone unnecessarily high spontaneous transformation whilst in culture. A S.I. value greater than 2.5 was considered a positive response to the particular test antigen providing the antigen culture mean value was also greater than three standard deviations from the unstimulated culture mean.

Comparison of group means was computed using Student's t test. All associations between lymphoproliferation responder status and epidemiological or antibody data was performed using Chi-square test utilizing Yates correction for discontinuity or Fisher's exact test where applicable.
Section (i) Evaluation of freezing and thawing technique

Fig. 5.1 presents the \( \log_{10} \) mean cpm of fresh and cryopreserved adult mononuclear cell cultures after incubation with PHA or *B. pertussis* for 72 hours. The range of PHA concentrations chosen were purposely suboptimal so that any immune disfunction occurring after cryopreservation could be detected. Fig. 5.1(a) shows that there is no significant difference between the \( \log_{10} \) mean cpm at each PHA dose for the fresh and frozen cell cultures from both the adults tested.

No lymphoproliferation occurred in the *B. pertussis* cultures for both adults at any of the concentrations tested (Fig. 5.1(b)). This implied that the bacterial preparation used, the vaccine strain of *B. pertussis*, in this assay does not possess any potent mitogenic or polyclonal effect which might be detectable in a 72 hour culture.

The similarity between the log mean cpm of the unstimulated cultures and also the lymphoproliferative responses to the suboptimal PHA doses in the fresh and frozen cell cultures of both adults infers that the cryopreservation and thawing technique employed has no significant effect on the immune capability of the cell samples to survive and respond in culture.

Section (ii) Comparison of response of cryopreserved adult and cord cell samples to PHA

The results of the lymphoproliferative response expressed as stimulation indices of the two adult and 21 cord cell samples to suboptimal doses of PHA are shown in Fig. 5.2. Limitations on the size of the cell sample
available restricted the testing of the 19 cord cell samples to only two doses of PHA. Fig.5.2(a) shows the response of the two adult samples and 15 cord samples after incubation for 72 hours whilst the response of six cord samples following incubation with PHA for 120 and 168 hours is shown in Fig.5.2(b).

In comparison to the two adult samples (Fig.5.2(a)) which were progressively stimulated by the increasing doses of PHA, the cord cell samples showed a wide variation in their ability to respond to PHA after 72 hours incubation. At the PHA dose level of 0.8µg/ml against which all the cell samples were tested and showed the greatest variation in response, eight of the cord blood samples had a stimulation index greater than 2.5 (range 2.81-98.2) whilst the remaining seven showed no response (S.I. range 0.97-1.91). At the lower concentration of 0.2µg/ml, four of the eight PHA responders still had a significant response (S.I. range 3.38-94.3).

The six cord cell samples incubated for 120 and 168 hours (Fig.5.2(b)) with PHA had a similar range in lymphoproliferative response at the 0.2µg/ml and 0.8µg/ml dose levels. The widest variation again occurred at the 0.8µg/ml concentration. Comparing the stimulation indices of the cord cells in the 120 hours culture to their counterparts in the 168 hour culture, two samples had a significantly increased response with a three-fold and ten-fold rise in S.I. respectively and four samples were unchanged in response to three remaining responders, although the S.I. was reduced in two and the third unresponsive.
FIGURE 5.1 Lymphoproliferative response of adult fresh and cryopreserved mononuclear cells to (A) suboptimal doses of PHA and (B) varying doses of *B. pertussis* after 72 hour incubation. Results expressed as the logarithm (base 10) of the mean counts per minute of triplicate unstimulated (unstim) or antigen added cultures.
A

LOG CPM

10

● FRESH

--- sample 1

◆ FROZEN

--- sample 2

unstim 0.08 0.2 0.4 µg/ml PHA

B

LOG CPM

10

● FRESH

--- sample 1

◆ FROZEN

--- sample 2

unstim 4 8 16 x10^8 B.PERTUSSIS
Response of adult and umbilical cord cryopreserved mononuclear cells to PHA. Diagram (A) depicts the lymphoproliferative response in two adult and fifteen umbilical cord cell culture after 72 hour incubation with PHA. (i) Results expressed as the logarithm (base 10) of the mean counts per minute of triplicate unstimulated (unstim) or mitogen added cultures. The two adult samples and two of the umbilical cord samples were exposed to five dose levels of PHA whilst the remaining thirteen cord samples were exposes to 0.2µg and 0.8µg doses only (ii) Results expressed as stimulation indices (S.I.) for all samples tested for dose levels 0.2µg and 0.8µg only.

Diagram (B) shows the lymphoproliferative response of six umbilical cord samples after 120 hour and 168 hour incubation with 0.2µg and 0.8µg doses of PHA. (i) Results expressed as the logarithm (base 10) of the mean counts per minute at each dose. (ii) Results expressed as stimulation indices (S.I.).
Section (iii) Determination of optimal culture conditions

The criterion for the selection of the culture variables such as cell concentration per well, antigen dilution and length of incubation was the combination of variables which gave maximal separation of the cord cell samples into differential lymphoproliferative response groups to B. pertussis, tetanus and diphtheria toxoids. Cell concentration, however, was not flexible because of the limitations set by sample availability. The concentration used for culture was set at $10^6$ live cells per ml which, not only allowed a sufficient number of antigen cultures to be performed per cord blood sample, but was also highly comparable to established antigen culture protocols in other studies.

Dose concentrations for B. pertussis, tetanus and diphtheria toxoids were selected to be suboptimal in effect, the rationale being to lessen the possibility of a heterogeneous response to the antigen preparation masking any differential antigen-specific response which might occur. Since 'suboptimal dose' is an arbitrary classification depending upon the specific assay, the initial dose range used in these test cultures were determined by comparison with previous studies, in particular Andersen et al. (1977), which had used the same antigens at optimal dilutions in cord cell cultures.

Matched cord blood cultures, identical for culture media, cell concentration, antigen dose range and tested cord cells, were incubated simultaneously for 120 and 168 hours to establish approximately the period of peak proliferation in these cultures under these limiting conditions. The
results expressed as mean corrected cpm per antigen dose for the six cord cell cultures are represented diagramatically in Fig.5.3 for the three antigen preparations tested and as stimulation indices in Fig.5.4 for *B. pertussis* and tetanus toxoid.

In Fig.5.3, the unstimulated culture mean cpm responses for the cord cells, after 168 hours incubation, are higher than in the 120 hour culture with five of the six samples having a mean unstimulated value greater than 450 cpm compared to only two samples in the 120 hour culture. The overall effect of these higher unstimulated culture cpm would be to decrease the corresponding stimulation index values of the antigen cultures of the 168 hour cultures compared to the five day cultures. However, in the 120 hour *B. pertussis* and tetanus toxoid culture results (Fig. 5.4) none of the six tested cord samples reached a S.I. greater than 2. In the corresponding 168 hour cultures, despite the higher unstimulated culture values, S.I.S. of 2 and greater were achieved. The obvious conclusion from this result is that the lymphoproliferation in the antigen addition culture, as measured by thymidine uptake, is considerably higher in the 168 hour than 120 hour culture. This is confirmed in Fig.5.3 where the maximal cpm in the 168 hour *B. pertussis* and tetanus toxoid are 1900.5 and 1300 cpm respectively compared to 980 and 630 cpm respectively in the five day cultures. Another observation from Fig.5.3 is that the overall cpm values in the antigen cultures are not very large which may be a characteristic of the responder cell type, i.e. cryopreserved umbilical cord mononuclear cells.
FIGURE 5.3 Lymphoproliferative response of cryopreserved umbilical cord cells to (A) B. pertussis, (B) tetanus toxoid and (C) diphtheria toxoid. Matched antigen cultures of varying doses of the incubated individual antigens for 120 hour and 168 hour. Results expressed as mean counts per minute of triplicate unstimulated (unstim) and antigen added cell cultures.
A

![Graph A](image)

**MEAN CPM**

$\times 10^3$

- **120 hours**
- **168 hours**

B

![Graph B](image)

**MEAN CPM**

$\times 10^3$

- **120 hours**
- **168 hours**

$\times 10^8$ B. PERTUSSIS

$\mu g/ml$ TETANUS TOXOID
Lympoproliferative response of cryopreserved umbilical cord cells to (A) *B. pertussis* and (B) tetanus toxoid. Same antigen cultures presented in Fig. 5.3 except that the results are expressed as stimulation indices (S.I.) rather than mean cpm. S.I. greater than 2.5 considered as indicating significant cellular stimulation. Diphtheria toxoid results not presented as no significant results.
Fig. 5.3 also shows that, after 168 hours incubation, there is a bimodal distribution in cpm response in the *B. pertussis* culture, especially in those cultures which received $4 \times 10^8$ organisms per culture. Three cord samples had a mean cpm value greater than 1100 cpm whilst the remaining three samples had mean cpm values less than 400 cpm. No such division occurred in the 120 hour culture. There is a suggestion from the *B. pertussis* results that there may be a range of antigen concentration to which the individual cord cell will respond. One cell which responded, though not significantly, to the $20 \times 10^8$ *B. pertussis* dose in 168 hour culture (mean cpm 1900.5, S.I. 1.9) did not respond with a similar magnitude to any other dose. In contrast, another sample showed negligible response to the $20 \times 10^8$ dose (mean cpm 1510, S.I. 4.0) in the 168 hour culture.

Four cord cell samples were tested against the tetanus toxoid dilutions and three against diphtheria toxoid. In Fig. 5.4 it can be seen that one sample responded to three of the five tetanus dilutions, whilst the other three samples tested showed no response. There was no response to diphtheria toxoid in the small sample tested. Since the 168 hour incubation period offered the best discrimination of differential response to two of the three antigenic preparations tested, it was chosen for the rest of the study.

Section (iv) Differential lymphoproliferative response to *B. pertussis*, tetanus and diphtheria toxoid

The mean corrected cpm of each triplicate unstimulated culture for the 60 cord cell samples tested, was subjected to the test for outliers to remove those cells from the analysis.
which were excessively transformed. Six samples had unstimulated culture means greater than 2000 cpm. On their step-wise removal from the outlier test, the kurtosis statistic was insignificant at the 5% level of significance. Despite the high unstimulated cpm values, the mean cpm of the corresponding antigen cultures were very small for these six cord cell samples.

Of the 54 cord cells remaining in the analysis, 12 samples had stimulation indices greater than 3 for the *B. pertussis* cultures whilst one and two cord samples showed significant lymphoproliferation in the tetanus and diphtheria toxoid cultures respectively. Only one cord sample was stimulated by more than one antigen, i.e. *B. pertussis* and tetanus toxoid.

Since the number of cord samples with positive response (S.I.>2.5) to tetanus and diphtheria toxoid concentrations is very small, the rest of the analysis will concentrate on the *B. pertussis* results. The cord cells with S.I. >2.5 to one or more tested doses were termed positive responders to *B. pertussis* whilst the samples with S.I.<2.0 to all doses of *B. pertussis* were negative responders.

Lymphoproliferative response to *B. pertussis* was not affected by the sex of the child, a history of positive parental allergy nor immunization reactor status (Table 5.1) Response could not be attributed to a positive cord titre to *B. pertussis* as no child in the positive response group was positive for *B. pertussis* maternal antibody. However, the distribution of positive and negative cord titres between the two lymphoproliferative response groups
was equivalent (Table 5.1) so a positive cord titre was also not correlated with negative response to *B. pertussis*. Positive IgM antibody response to *B. pertussis* in those cord samples with matched heel pricks was not associated with either positive or negative lymphoproliferative response (Table 5.1). Neither was a lymphoproliferative response to PHA after 72, 120 or 168 hours incubation correlated with either of the *B. pertussis* response groups (Table 5.1).
TABLE 5.1
DISTRIBUTION OF POSSIBLE CONFOUNDING VARIABLES IN THE TWO
LYMPHOPROLIFERATIVE RESPONSE GROUPS TO B. PERTUSSIS

ALL COMPARISONS ARE NONSIGNIFICANT (P>0.05) *

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^+ number tested

* distribution of confounding variable in positive lymphoproliferative response group compared to the distribution of same variable in negative IgM group.

1 correlation between positive IgM response and positive lymphoproliferative response: \( r = -0.11 \)

2 correlation between response to PHA at any incubation and positive lymphoproliferative response: \( r = -0.19 \)
DISCUSSION

This study has used a novel experimental technique, utilizing cryopreserved umbilical cord mononuclear cells as responder cells in a lymphoproliferative assay, to investigate the response of non-primed cells to antigenic exposure. The cryopreservation and thawing methods employed, as evaluated in a test assay using paired fresh and frozen adult mononuclear samples, did not measurably affect the viability or functional responsiveness to suboptimal doses of PHA during a 72 hour culture period. Recovered umbilical cord samples, exposed to a similar suboptimal PHA dose range, exhibited a great variability in proliferative response, as measured by stimulation indices, which was not observed in the recovered adult cultures. The cord samples responses ranged from levels of high proliferation at specific doses to non-response over the entire dose range, independent of the time of incubation (Fig. 5.2). Overall approximately 47% of cells tested showed no response (SI<2) to any of the PHA doses in the assay. In studies utilizing adult volunteers impairment or non-response to PHA at suboptimal doses is reportedly indicative of immunodeficiency (Fitzgerald, 1971). Therefore, it could be inferred that a proportion of the cord samples are immune defective possibly due to cellular immaturity as has been previously proposed for reported low PHA responsiveness in cord cells (Jones, 1969; Ayoub and Kasakura, 1971). However, there is sufficient evidence to show that PHA responsiveness can be demonstrable in 10-12 week old foetuses (Stites et al. 1975) and that cord cells are immunologically mature in most cellular functions (Handzel
et al. 1980). Failure of young infants to respond to PHA has also been attributed to neonatal cellular function being affected by previous viral infection, in particular congenital rubella (Montgomery et al. 1967), prematurity or low birth weight (Leino et al. 1981), malnutrition (Whittle et al. 1980) and prenatal exposure to anaesthesia, antibiotics or other drugs (Hauser and Remington, 1982). However, Simons et al. (1968) and Fitzgerald (1971) have reported individual variation in response to PHA in healthy adults which was highly dose dependent and fluctuated according to various environmental factors such as time of blood collection and hormonal cycle. Division of PHA responsiveness on the basis of testing with a single or narrow range of PHA doses was condemned in these two reports as being highly inaccurate for the determination of immunodeficient individuals. In this present study, it is highly likely that the individual differences in PHA kinetics observed in this population of cord cells was artificially produced by the low doses of PHA employed. Although impairment of cellular function, both intrinsic and environmentally induced, cannot be totally disregarded, Table 5.1 shows that cell samples with apparent depressed PHA responses proliferated in the presence of B. pertussis implying that the non-response to PHA in these cases is unlikely to be due to immune incompetence of the cord cells.

In the preliminary test antigen cultures (Fig.5.4) a differential cellular response to B. pertussis and tetanus toxoid was demonstrable in a proportion of cord cell samples in the seven day cultures compared to a lack of significant proliferation (SI<2.0) by identical samples
when incubated with the same antigens for five days. Therefore, all subsequent antigen assays were incubated for seven days to test for the presence of antigenic stimulation.

The results revealed two lymphoproliferative response levels to *B. pertussis* and tetanus and diphtheria toxoids, the responder group containing cord samples with stimulation indices greater than 2.5 to one or more concentrations of the tested antigen and a non responder group with stimulation indices less than 2.0 to all antigenic doses. Only the *B. pertussis* group, by virtue of the number of responding samples, was used for subsequent investigation on associations between lymphoproliferative response and epidemiological variables since the tetanus and diphtheria toxoid response groups, with one and two individuals respectively, were of insufficient size to have statistical significance.

Apart from the differences in response to the antigenic stimulus, both *B. pertussis* response groups were similar with respect to the proportion of males and females and parental history of allergy in each group. Cellular stimulation was not associated with maternal *B. pertussis* titre as no responder had a positive cord titre. However, in the 38 non responder samples tested only four had a positive cord titre. This difference in the number of cord positive samples between the two response groups was not significant (Table 5.1). The presence of a positive cord titre possibly would not have affected lymphoproliferative response as it has been shown in the report of Gill et al. (1979) who, using tetanus
toxoid, Candida, Varicella, Mumps and streptodornase-streptokinase antigens demonstrated that maternal exposure to an environmental antigen did not affect the offspring's immune capability to the same antigen.

The measured lymphoproliferative response in *B. pertussis* responders was specific for the antigenic preparation and not attributable to stimulatory substances in the culture media, in all cases except one the corresponding diphtheria and tetanus toxoid cultures showing no similar proliferative response. Evidence for the lack of previous sensitization of the cord cells (Table 5.1) combined with the observation that the peak proliferative response occurred in cultures of greater than five day duration, indicates strongly that the cellular response of the responder cells could be the result of primary in vitro sensitization. However, other interpretations of these data, especially as to the nature of the proliferative response, may also exist. Bacterial cell surface antigens mitogenic to human adult and neonatal lymphocytes, in addition to the LPS moiety of *B. pertussis*, have been reported for many bacterial species by Banck and Forsgren (1978). This report, however, made a distinction between an 'early' non-specific mitogenic response and a 'late' occurring specific T cell response to bacteria such as *Mycobacterium tuberculosis* and *B. pertussis* in adult cells. In this present study, the data showing bimodality of cord cell responsiveness to *B. pertussis* in the seven day culture (Fig. 5.3) in combination with the non-responsiveness to the *B. pertussis* antigenic preparation in the initial adult 72 hour cultures (Fig. 5.1) suggest that the response is more likely
antigen-specific rather than a purely mitogenic phenomenon. It cannot be concluded that this response is 'primary' since peak proliferation occurring in 7-9 day cultures has also been interpreted as a late recall response to a complex antigen, resulting from the low dose of antigen delaying the time at which proliferation of the sensitized cells in the culture population can be detected (Young and Engleman, 1980). In the cord cell cultures of the present study there is no evidence for previous sensitization to \textit{B. pertussis} in the responder cells to justify the presence of a secondary response to this antigen. However, the priming of the cells by a more ubiquitous bacterium whose surface antigens cross react with that of \textit{B. pertussis}, such as \textit{E. coli} as suggested by Andersen et al. (1977), is theoretically possible. In a similar vein, Rubin et al. (1981) found that the proliferative response to two gram-negative bacteria (\textit{Klebsiella} and \textit{Pseudomonas}) were closely correlated in cord lymphocyte cultures suggesting that the cell mediated response could be directed against similar antigenic determinant or activating substances on gram-negative bacteria. Therefore, the possibility that the cellular response observed in the \textit{B. pertussis} cord cell cultures are a result of some antigenic crossreaction and thus secondary in nature, cannot be disregarded. However, with the restricted cellular material preventing definitive establishment that true primary \textit{in vitro} stimulation has occurred, by the demonstration of accelerated culture kinetics upon restimulation as described by Hensen and Elferink (1979), the uncertainty as to the nature of response does not detract from the findings that a differential response can be found in \textit{B. pertussis} stimulated
cord cell cultures.

Lymphoproliferative responses to tetanus toxoid and diphtheria toxoid by cord lymphocytes have been previously recorded (Ellner et al. 1979, Gill et al. 1981). The responses to these less complex antigens in this study probably are the result of primary in vitro stimulation since cross reactivity between these toxoids and other naturally occurring antigens in Western developed countries is unknown and the previously mentioned reports have found no evidence to suggest that sensitization to the toxoids occurred in utero. Interestingly, in the data of Leiken and Oppenheim (1971), despite stating that diphtheria and tetanus toxoids "usually have no effect on unimmunized new­borns" and that there was no overall antigenic response (mean cpm value) in the combined cord cell cultures, showed that three of 12 cord samples tested responded (according to their criteria of a SI greater than 3 being statistically significant) to diphtheria toxoid whilst two of 9 cord samples responded to tetanus toxoid after five day culture. The difference in kinetics and the number of toxoid stimulated samples in this latter report, compared to the present study, may be a function of a difference in responding cell type use (fresh cord mononuclears were used by Leiken and Oppenheim (1971)) and the antigen dose employed in culture. It was unfortunate that the sample sizes of the two toxoid positive response groups were insufficient in this present study for further detailed study.

The lack of correlation between the lymphoproliferative response and IgM antibody response to B. pertussis in this study (Table 5.1) was not an unexpected finding since dis-
parity between antibody production and cellular proliferation has been previously documented as occurring in both man (Greenberg et al. 1975, De Vries et al. 1977, Broff et al. 1981) and animals (Ben Sasson et al. 1974, Ellner and Rosenthal, 1975). This phenomenon has been attributed to either dissimilarities in the antigenic moieties recognised by the B cells in vivo and the proliferative T cells in vitro (Benacerraf, 1978, Thomas and Shevach, 1978) or to qualitative differences in antigen presentation by macrophages with the culture T cells recognizing and responding to antigen-induced alterations in self major histocompatibility antigens on the antigen presenting cell instead of to the actual antigen (Rosenthal, 1978, Broff et al. 1981).

In the main analysis, immunization reactor status did not correlate with the lymphoproliferative response. This infers that the antigenic determinants recognized by proliferating cells in vitro are not predictive of an in vivo immunization reaction. Measurement of an in vitro IgE response to B. pertussis would have provided an interesting variable for correlation with reactor status. An assay for determining antigen-specific IgE levels in cell culture using solid phase radioimmunoassay has been described by Nonaka et al. (1981) and successfully applied by Sasazuki et al. (1983) in a study of in vitro IgE response to Japanese cedar pollen in adult Japanese. However, this assay system is still undergoing further development (Kimata et al. 1983) since there is considerable evidence in the literature that the significance of the measured IgE response is greatly influenced by the experimental conditions of the assay (reviewed by Sampson and
Buckley, 1981). The extensive experimental refinements required to adapt this assay system to measure a primary \textit{in vitro} response in cord cells was considered beyond the scope of this thesis. Cord IgE levels, which are believed to be neonatally derived (Miller \textit{et al.} 1973), ranges from 0.1 to 5.5 international units (i.u.) per ml with a mean level of 0.2-0.3 i.u. per ml (Michel \textit{et al.} 1980, Mendoza \textit{et al.} 1982). This mean value is one three hundredth the IgE level of a normal non-atopic adult. Since it has been observed that \textit{in vitro} IgE production can closely parallel \textit{in vivo} IgE level (Turner \textit{et al.} 1981) if the \textit{in vivo} level of a normal adult is 600 picograms (see Turner \textit{et al.} 1981), then theoretically the average neonatal level would be approximately 2 picograms which is far below the resolution of all the assays so far developed (see Kimata \textit{et al.} 1983). Therefore, an assay of extreme sensitivity would be required to detect any variation in \textit{B. pertussis}, tetanus or diphtheria toxoid induced IgE response in cord cell cultures.

In summary, this portion of the study has defined a differential cord cell lymphoproliferative response to \textit{B. pertussis} organisms \textit{in vitro}, which is not associated with a child's subsequent response to immunization with the same organism, the presence of parental allergy or the child's sex. The observed cellular response is more likely an antigen specific phenomenon, although the exact nature of the response is in doubt, rather than a non-specific mitogenic effect. Therefore, this study has recognised variation in an \textit{in vitro} antigen specific lymphocytic response in an unrelated population of similar environmental experience which strongly suggests that this
difference is genetically determined. This hypothesis is further considered in Chapter 6.
CHAPTER 6

Genetic markers measurable in umbilical cord blood: association with parameters of the immunization response to Triple Antigen.
Genetic control of antibody response has been demonstrated in mice (McDevitt and Tyan, 1968, Benacerraf and McDevitt, 1972), rats (Gunter et al. 1972, Amerding et al. 1972), guinea pigs (Ellman et al. 1970) and rhesus monkeys (Dorf et al. 1975) as the consequence of immune response (Ir) or immune suppression (Is) gene action. By the use of highly inbred animal strains (Klein, 1975) and the planned immunization of precisely defined synthetic polypeptides or low doses of naturally occurring antigens of restricted heterogeneity, these original studies mapped Ir and Is genes to the genetic region coding for the species-specific major histocompatibility complex (MHC). Further studies, particularly on the mouse MHC (H-2) utilizing intra H-2 recombinant strains, located Ir genes in the appropriately named immune (I) region (reviewed by Benacerraf and Germain, 1978). The I region also codes for murine immune associated (Ia) antigens which are cell surface allo-antigens known to stimulate in vitro mixed lymphocyte reaction (MLR) and are involved in communication between immunocompetent cells including antigen recognition by T lymphocytes (reviewed by Niederhuber, 1978). Recent reports have shown that in the mouse Ia antigens are equivalent to the Ir or Is gene products (Lerner et al. 1980, Longo and Schwartz, 1981, Steinmetz et al. 1982a).

Postulation that Ir genes may exist in man arose following the discovery of significant associations between certain diseases and the serologically defined class I antigens (HLA-A, B and C) of the human MHC on chromosome 6 (Svejgaard et al, 1975). It was subsequently predicted
and proved, with notable exceptions such as of HLA-B27 with ankylosing spondylitis and HLA-A3 and idiopathic haemochromatosis, that stronger disease associations generally occurred with HLA-D region antigens, the human homologue of the murine I region (reviewed by Svejgaard et al. 1983). The human class II antigens coded by this region (HLA-DR), are assumed equivalent to the murine Ia antigens having a similar tissue distribution, being found on B lymphocytes, sub-populations of T cells, macrophages, sperm and endothelial cells, and also show considerable structural amino acid homology with the murine molecules (Thorsby et al. 1977, Allison et al. 1978, Fu et al. 1978, Moen et al. 1980, Goyert et al. 1982). Similar also to Ia antigens, HLA-DR identity between immuno-competent cells is necessary for effective antigenic presentation and in vitro lymphocytic response (Hirschberg et al. 1980).

However, despite the similarity between the murine and human MHC region and the circumstantial evidence for their existence, it is very difficult to demonstrate Ir genes in man. Unlike murine studies, human investigation deals with an outbred, genetically heterogeneous population of varying environmental experience. Although this heterogeneity may be minimized by investigation of large family pedigrees, or isolated consanguineous populations, there are exacting ethical restraints on human immunization with non-physiological antigens, such as those used for the original definition of animal Ir genes. There is also no recourse for prospectively testing hypotheses in informative progeny as can be achieved in animal investigations. Consequently, studies of human
Ir gene action initially relied on examining antibody response polymorphisms following natural or artificially-induced sensitization to environmental and microbial antigens. Statistical associations have been reported between the HLA region and specific antibody responses to a variety of antigens including influenza A (Spencer et al. 1976), measles virus (Jersild et al. 1973, Haverkorn et al. 1975, Ilonen et al. 1977), rubella vaccination (Kato et al. 1978; 1983), Salmonella adelaide flagellin immunization (Whittingham et al. 1980), wheat gluten (Scott et al. 1974) and various allergens (reviewed by Marsh et al. 1981) although after correction for the number of HLA antigens tested the majority of these reported associations, with the exception of the allergen studies, are no longer significant.

In contrast to the controlled immunization protocol of animal Ir gene investigations on antibody response, the actual number of sensitizing exposures to the immunogen under study in the previously cited human reports is unknown especially when an environmental antigen is involved. In studies on the primary antibody response to a natural antigen, absolute naivety to the immunizing antigen in adult volunteers cannot be guaranteed even in the absence of a detectable preimmunization titre. With adult subjects there is always the possibility that previous contact with the antigen may have occurred, although the resultant antibody response was of short duration and therefore not measurable at the time of the study or else there may have been previous sensitization to epitopes of the immunogen under study due to exposure to another environmental antigen containing similar
moieties. Thus the measured humoral response may reflect differing environmental exposure rather than a genetic difference. In comparison, it is highly unlikely with the limited environmental experience of neonates that previous sensitization to most ethical vaccine antigens occurs. Neonates are therefore ideal subjects for investigations of primary humoral responses to a controlled immunization regime such as that achieved in the present study.

The ethical limitations on in vivo immunization in human investigations have been instrumental in the establishment of alternative in vitro assays for the study of immune response polymorphisms. Antigen-specific T cell proliferation in vitro has been shown to be under MHC linked Ir gene control in experimental animals (Shevach et al. 1972, Lonai and McDevitt, 1974, Schwartz and Paul, 1976). Differential cellular proliferative responses after incubation with test antigen have been significantly associated with the HLA region including recall cellular responses to streptococcal antigens (Greenberg et al. 1975, Sasazuki et al. 1980a), schistosomal antigens (Sasazuki et al. 1980b), synthetic polypeptides (Hsu et al. 1981), vaccina virus (De Vries et al. 1977) and tetanus toxoid (Sasazuki et al. 1978). Using family pedigree analysis two studies (Greenberg et al. 1980, Hsu et al. 1981) have claimed the existence of Ir genes controlling cellular responses linked to familial HLA haplotypes with Hsu et al. (1981) on the basis of one intra-HLA recombinant family, suggesting that in vitro responsiveness to the synthetic polypeptides (H,G)-A-L and (T,G)-A-L maps closer
to HLA-B than the HLA-D region. Subsequently, Sasazuki et al. (1983) has demonstrated the linkage of a possible immune suppression (Is) gene for lymphoproliferative response to streptococcal cell wall antigen to the HLA-D region. However, despite these studies indicating linkage of polymorphic responses to the human MHC, insufficient recombination events between the HLA complex and differences in cellular immune function have been observed in man to positively map these immune responses to a specific HLA region.

In murine studies, non MHC Ir genes affecting immune responsiveness to specific antigens are known to exist although, apart from those in association with immunoglobulin allotypes, the majority are not well characterised (Klein, 1975). In human investigations, significant associations have been found between polymorphic antibody responses to Salmonella adelaide flagellin (Whittingham et al. 1980), meningococcal polysaccharide (Pandey et al. 1982) and Haemophilus influenzae (Pandey et al. 1979) with immunoglobulin heavy and light chain allotypes. However, in both human and animal studies, there is a strong suggestion that the observed immune response to a particular antigen is the additive result of either more than one MHC complementing Ir gene (Jones et al. 1978, Lafuse et al. 1980) or to a combination of MHC and non MHC Ir gene influences (Marusic et al. 1982).

In this study the immunization response to limiting doses of three complex antigens in an unrelated neonatal population has been examined. As outlined in the previous three chapters, individual differences in susceptibility
to an adverse immunization reaction, the ability to produce a specific IgM titre to *B. pertussis* and tetanus toxoid and a lymphoproliferative response to *B. pertussis* have been observed. These differential responses reflect a range of different immune functions which theoretically could be controlled by an equally variable range of MHC and non-MHC mediated mechanisms. It is, therefore, the purpose of this chapter to investigate the association of specific genetic marker systems with each differential response.

**Technical aspects**

Two criteria determine the usefulness of a genetic marker locus in the investigation of its association with an immune function. First, the marker locus must be polymorphic in the population under study and, second, the individual alleles must be easily distinguishable under the experimental method used to elucidate them. The genetic markers satisfying these criteria and, subsequently, used in this present study, were the HLA A, B, Cw and DR loci antigens, the Properdin Factor B complement system (BF) and the electrophoretically defined red cell enzyme systems, all of which were determined using umbilical cord blood.

There are several limitations arising from the use of cord blood. Maternal contamination via the transplacental passage of genetic factors or during the collection of the sample at birth can lead to the masking of a foetal phenotype, in particular the immunoglobulin heavy chain allotype (Gm) alleles (Morell et al. 1971), or the occurrence of unusual phenotypes such as leucocyte chimerism (O'Reilly et al. 1973) or an over-
abundance of Gm phenotypes (Williams, 1979, Hayward, 1981) arising from the combination of maternal and foetal phenotypes in the same sample. Although maternal admixture at the time of cord blood collection can be detected by the estimation of the adult:foetal haemoglobin proportion in the cord sample, paired maternal cord samples are required to evaluate a foetal phenotype when the factors are passed transplacentally (Hayward, 1981). Therefore, owing to the absence of an appropriate maternal sample in this study, the polymorphic systems known to be transferred across the placenta, primarily the immunoglobulin allotypes, were not examined. Quantitative differences between adult and cord red cell enzyme and complement genetic markers reportedly due to decreased production of these factors by the foetus have been noted (Alper et al. 1972, Roach et al. 1981) although, with the well known exception of adult and foetal haemoglobin (Wood et al. 1977), there have been few reported qualitative differences.
METHODS

Section (i) Sample selection and measurement of genetic markers

Collected umbilical cord samples from 238 survey selected children were assayed for the red blood cell genetic marker systems, properdin Factor B and, in samples with sufficient stored cellular samples, HLA antigens. The methods for the measurement of the above genetic systems have been presented in Chapter 2, Sections (vi) and (vii).

The same genetic marker systems were measured in an adult population which consisted of 382 randomly selected Red Cross blood bank donors from the Canberra region. Both the red blood cell and Factor B determinations were performed by the laboratory of Dr N.M. Blake. The HLA antigen data for 95 individuals were provided by the laboratory of Dr S. Serjeantson.

Section (II) Statistical Methods

Gene frequencies for the red blood cell enzyme and Factor B systems were determined by gene counting (Ceppellini et al. 1955) whilst the HLA results were presented as antigen frequencies. Significant gene or allelic deviations between the various bimodal response groups were determined by Chi-square analysis. The HLA results were corrected for the number of antigenic comparisons (See Chapter 2, Section x). Net counts per minute (Net cpm) was calculated as follows: mean cpm in triplicate antigen stimulated culture minus mean cpm in triplicated unstimulated culture.
RESULTS

Section (i) Determination of HLA region, BF and red blood cell genetic markers in cord blood and adult donors

A comparison was made between the distribution of genetic markers assayed in cord blood and that of the same genetic factors in an adult population to test for possible qualitative differences in the cord blood markers.

The adult donors were from the same geographical area (Canberra City and environs) and similar Caucasoid background as the parents of the survey children. Identical techniques were used for the measurement of the polymorphic red blood cell enzyme and BF complement systems in both adult and cord samples and most of the same antisera were employed in the microlymphocytotoxicity test for the determination of HLA loci antigens.

(a) Non-chromosome 6 genetic markers

Apart from the inconvenience in handling caused by the presence of Wharton's Jelly in the red cell haemolysates, there was no unusual difference in the electrophoretic allelic systems between the adult and cord samples. The gene frequencies of the cord blood enzyme systems approximated closely that of the adult population (Table 6.1).

(b) Non-HLA chromosome 6 markers

There was no significant difference between the adult and cord glyoxylase and Factor B allelic frequencies (Table 6.2). Technically the electrophoretic mobility of the Factor B alleles differed slightly in cord blood with the BF*F to BF*S ratio (the distance from the origin of
TABLE 6.1

COMPARISON OF GENE FREQUENCIES OF THE POLYMORPHIC NON-CHROMOSOME 6 GENETIC MARKERS BETWEEN CANBERRA CORD BLOOD AND CANBERRA BLOOD BANK DONORS

<table>
<thead>
<tr>
<th>GENETIC MARKER</th>
<th>CHR. ASSIGNED</th>
<th>ALLELE</th>
<th>CORD BLOOD</th>
<th>ADULT DONORS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NUMBER TESTED</td>
<td>GENE FREQ.</td>
<td>NUMBER TESTED</td>
</tr>
<tr>
<td>PGM1</td>
<td>1</td>
<td>PGM1*1</td>
<td>235</td>
<td>0.755</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PGM1*2</td>
<td></td>
<td>0.245</td>
</tr>
<tr>
<td>6PGD</td>
<td>1</td>
<td>6PGD*A</td>
<td>238</td>
<td>0.968</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6PGD*C</td>
<td></td>
<td>0.032</td>
</tr>
<tr>
<td>AKI</td>
<td>9</td>
<td>AK1</td>
<td>238</td>
<td>0.979</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AK2</td>
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</tr>
<tr>
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<td></td>
<td></td>
<td>ACP1*B</td>
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<td></td>
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<td></td>
<td></td>
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<td>PGP*3</td>
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TABLE 6.2

COMPARISON OF GENE FREQUENCIES OF NON-HLA CHROMOSOME 6
GENETIC MARKERS IN CANBERRA CORD BLOOD AND
CANBERRA BLOOD BANK DONORS

<table>
<thead>
<tr>
<th>GENETIC MARKER</th>
<th>ALLELE</th>
<th>CORD BLOOD</th>
<th>ADULT DONORS</th>
</tr>
</thead>
<tbody>
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<td></td>
<td>NUMBER TESTED</td>
<td>GENE FREQ.</td>
<td>NUMBER TESTED</td>
</tr>
<tr>
<td>GLO</td>
<td>GLO*1</td>
<td>227</td>
<td>.438</td>
</tr>
<tr>
<td></td>
<td>GLO*2</td>
<td>190</td>
<td>.562</td>
</tr>
<tr>
<td>FACTOR B</td>
<td>BF*F</td>
<td>190</td>
<td>.184</td>
</tr>
<tr>
<td></td>
<td>BF*S</td>
<td>190</td>
<td>.797</td>
</tr>
<tr>
<td></td>
<td>BF*F0.7</td>
<td>190</td>
<td>.005</td>
</tr>
<tr>
<td></td>
<td>BF*SO.7</td>
<td>190</td>
<td>.013</td>
</tr>
</tbody>
</table>
the gel to the F electrophoretic band divided by the origin
to the S electrophoretic band distance) being marginally
decreased (average cord ratio 1.18) compared to the adult
ratio (average adult ratio 1.29). One cord sample (desig­
nated 849 in Fig. 6.1) did not resemble any of the commonly
known BF*S variants by having a S:S-variant ratio smaller
than other cord S:S1 ratios. Neither did it approximate
the adult S:S1 ratio. It is possible that this is a new
Factor B rare variant or else it may represent an immature
allelic form. Further investigation of these anomalies
was beyond the scope of the study.

(c) HLA loci genetic markers

The comparison of HLA-A, -B and -Cw antigens between
adult and cord samples is presented in Table 6.3 and the
HLA-DR results in Table 6.4.

The following antigenic splits were identified in both
cord and adult samples using the available antisera: Aw23,
Aw24, (A9); A25, A26 (A10); Bw44, Bw45 (B12); Bw49, Bw50
(B21); Bw55, Bw56 (B22); Bw60, Bw61 (B40). Initially
slight difficulty was encountered in interpreting the HLA­
A and -B antigen typing of some cord cells because of
extra reactivity on the individual typing trays. Following
analysis of known antisera inclusions and subsequent HLA-DR
typing of these cells, this reactivity was found to be the
result of HLA-DR specificities in the HLA-A and -B antisera
being detected by the higher proportion of B cells present
in cord cell preparations rather than due to any possible
maternal contamination.

The HLA-A and -B antigen frequencies of the cord
samples were in good agreement with the Canberra blood bank
Factor B in umbilical cord plasma. Photograph showing the common BF alleles, BF*F and BF*S, and the rare variants, BF*F_{0.7} and BF*S_{0.7}, detectable in umbilical cord plasma. Note the sample designated 849 which has a normal 'S' band and an unusual 'S' variant which is electrophoretically faster than the 'S_{0.7}' variant.
Bf cord blood
14.5.82
adults (Table 6.3). There was a depression in the frequency of All (p (uncorrected) = 0.05) and B22 (Bw55+Bw56) (p (uncorrected) = 0.04) in the cord blood samples, both of which lose significance after correction for the large number of antigens tested.

The HLA-Cw antigens were very poorly defined in both the cord and adult samples which reflects the paucity of good antisera for definition of this region. HLA-Cw3 and -Cw4 were easily detected in both adult and cord samples in comparable frequencies (Table 6.3).

Comparison of the HLA-DR antigen frequencies (Table 6.4) shows that, whilst there is no difference between the distribution of individual HLA-DR antigens in the cord and adult samples, there is a large increase in the frequency of HLA-DR blank in the cord samples (p (corrected) <0.05). The calculated HLA-DR blank in Table 6.4 equates to the presence of one definable DR antigen in an individual. Of the cord bloods 48.2% of samples had a single DR antigen compared to 27.3% of adult donors. Since there is a close correspondence between the frequency of the other measured genetic markers in both Canberra adults and neonates, it is unlikely that the increased DR-blank represents either increased homozygosity at the HLA-DR loci in cord specimens or an increased frequency of undefined HLA-DR antigens. However, it may be due to the weak antigenic expression of certain DR antigens, a possibility which will be considered further in the Discussion.
<table>
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<tr>
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<th>HLA ANTIGEN</th>
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<th>ADULT DONOR (N=95)</th>
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<td>a.f(%)</td>
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+ antigen frequency ++ results not available * p(uncorrected) = 0.05
** B22+Bw55+Bw56 : p(uncorrected) = 0.04
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<tr>
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<td>23</td>
<td>20.5</td>
</tr>
<tr>
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Section (ii) Comparison of genetic marker frequencies in the differential response groups

(a) Immunization reactor status

No gene frequency deviations in the non-HLA genetic markers between the reactor and non-reactor groups were observed (Table 6.5 and Table 6.6). However, in the HLA results (Table 6.7) the immunization reactor group had an increased antigenic frequency of HLA-A3 (reactors 29%, non-reactors 12.3%) and HLA-B15 (reactors 11.4%, non-reactors 1.5%) with a decreased frequency of HLA-Bw51 (reactors 6.8%, non-reactors 20.0%) and HLA-B blank (reactors 13.6%, non-reactors 33.8%). No deviation retained significance after correction for the number of comparisons in the HLA-A and -B loci. HLA-DR antigen frequencies were equivalent.

(b) IgM response to B. pertussis

Significant allelic deviations between the positive and negative IgM response groups occurred in the red cell enzyme system, esterase D and the HLA-B data. The allele, ESD*2 was not present in the thirteen IgM positive individuals tested although 17 of the 65 IgM negative children assayed for this enzyme system were positive for this allele (gene frequency deviation: p<0.001; phenotype frequency difference: p<0.03) (Table 6.5).

Eight of the twelve (66.7%) HLA typed children positive for B. pertussis IgM (at 1:40) had the HLA-B allele Bw44 compared to only twelve of the 45 HLA typed negative IgM group (26.7%) (Table 6.8). The significance of this observation was lost after correction. There was, however, a significant association between IgM positivity and HLA-Bw44 in male children. All eight of the HLA-Bw44 children
in the IgM positive group were males whilst, of the thirteen IgM negative HLA-Bw44 positive children, only five were boys (p<0.004). In the IgM positive group, five of the eight HLA-Bw44 positive boys were immunization reactors compared to none of the five HLA-Bw44 positive males who were IgM negative.

On examination of glyoxylase (GLO) phenotype distributions in the two IgM response groups, the percentage of GLO heterozygotes in the IgM positive group (16.7%) was significantly less than the corresponding percentage in the IgM negative groups (66.7%). This imbalance in heterozygote number was not reflected in the gene frequencies for the two GLO alleles (Table 6.6) as there was a corresponding rise in both GLO*1 (41.7%) and GLO*2 (41.7%) homozygote groups.

(c) IgM response to tetanus toxoid

Similar to the IgM B. pertussis results, there was a significant decrease in the ESD*2 allele in the IgM positive group (Table 6.5) being present in only one of the seventeen IgM positive individuals tested (gene frequency deviation: p<0.01; phenotype frequency difference: p<0.05).

In the tetanus toxoid IgM positive group, HLA-A3 (positive 46.2%, negative 11.6%) and HLA-B27 (positive 23.1%, negative 2.3%) were increased and HLA-DR blank (positive 14.3%, negative 44.4%) was decreased compared to the IgM negative group (Table 6.9). The corrected deviations were not significant.
TABLE 6.5
GENE FREQUENCIES FOR THE POLYMORPHIC NON-CHROMOSOME 6 GENETIC MARKERS IN THE DIFFERENTIAL RESPONSE GROUPS TO IMMUNIZATION REACTION, IgM RESPONSE TO B. PERTUSSIS AND TETANUS TOXOID AND CMI RESPONSE TO B. PERTUSSIS
(Significant results are underlined)

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<thead>
<tr>
<th>GENETIC MARKER</th>
<th>IMMUNIZATION</th>
<th>IgM RESPONSE</th>
<th>CMI RESPONSE</th>
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<td>REACTION</td>
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<td>TETANUS TOXOID</td>
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<td>POS NEG</td>
<td>POS NEG</td>
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<td>.824 .781</td>
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<td>.000 .138</td>
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*p < 0.001  **p < 0.01
TABLE 6.6

DISTRIBUTION OF NON-HLA CHROMOSOME 6 GENETIC MARKERS IN DIFFERENTIAL RESPONSE GROUPS TO IMMUNIZATION REACTION, IgM RESPONSE TO B PERTUSSIS AND TETANUS TOXOID AND CMI RESPONSE TO B PERTUSSIS

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<th>CMI RESPONSE</th>
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<td>.539</td>
<td>.500</td>
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<td>BF*S</td>
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<td>BF*SO.7</td>
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<td>.013</td>
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+ reactor
++ non-reactor
### TABLE 6.7

**HLA A, B AND DR ANTIGEN FREQUENCIES IN TRIPLE ANTIGEN IMMUNIZATION REACTORS AND NON-REACTORS**

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<th>IMMUNIZATION REACTOR STATUS</th>
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<td>REACTOR a.f. (%)</td>
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<td>NON REACTOR a.f. (%)</td>
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* p (uncorrected) = 0.05
** p (uncorrected) = 0.036
*** p (uncorrected) = 0.035
### TABLE 6.8

HLA A, B AND DR ANTIGEN FREQUENCIES FOR IgM ANTIBODY RESPONSE GROUPS TO B. PERTUSSIS

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<th>HLA ANTIGEN</th>
<th>IgM RESPONSE GROUP</th>
<th>HLA ANTIGEN</th>
<th>IgM RESPONSE GROUP</th>
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</thead>
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<td>NEGATIVE a.f.(%)</td>
<td>POSITIVE a.f.(%)</td>
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<tr>
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<td>50.0</td>
<td>40.0</td>
<td>B7</td>
</tr>
<tr>
<td>A3</td>
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<td>B8</td>
</tr>
<tr>
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<td>B37</td>
</tr>
<tr>
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<td>Bw60</td>
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<tr>
<td>DR-blank</td>
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*p (uncorrected) = 0.012*
## Table 6.9

### HLA A, B AND DR ANTIGEN FREQUENCIES FOR IgM ANTIBODY RESPONSE GROUPS TO TETANUS TOXOID

<table>
<thead>
<tr>
<th>HLA POSITIVE ANTIGEN</th>
<th>IgM RESPONSE GROUP</th>
<th>HLA NEGATIVE ANTIGEN</th>
<th>IgM RESPONSE GROUP</th>
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<tbody>
<tr>
<td>N=13</td>
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<td>N=43</td>
<td>30.2</td>
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</tr>
<tr>
<td>46.2</td>
<td>11.6*</td>
<td>30.8</td>
<td>16.3</td>
</tr>
<tr>
<td>0.0</td>
<td>2.3</td>
<td>38.5</td>
<td>34.8</td>
</tr>
<tr>
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<td>0.0</td>
<td>4.7</td>
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<td>9.3</td>
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<tr>
<td>15.4</td>
<td>7.0</td>
<td>0.0</td>
<td>11.6</td>
</tr>
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<td>7.0</td>
<td>7.7</td>
<td>7.0</td>
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<td>7.7</td>
<td>16.3</td>
</tr>
<tr>
<td>21.4</td>
<td>24.4</td>
<td>B-blank</td>
<td>15.4</td>
</tr>
<tr>
<td>28.6</td>
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<td>23.1</td>
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<tr>
<td>0.0</td>
<td>11.1</td>
<td>7.7</td>
<td>16.3</td>
</tr>
<tr>
<td>35.7</td>
<td>35.6</td>
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<tr>
<td>7.1</td>
<td>2.2</td>
<td>44.4**</td>
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</table>

* $p$ (uncorrected) = 0.011
* * $p$ (uncorrected) = 0.033
(d) Lymphoproliferative response to B. pertussis

Cord cells showing a positive B. pertussis lymphoproliferative response had elevations in the frequencies of HLA-Aw32 (positive 37.5%, negative 2.8%) and HLA-Bw60 (positive 37.5%, negative 8.3%) and no measurable HLA-DR4 antigen (positive 0.0%, negative 46.9%). The most striking result was the presence of HLA-DR5 in six of the eight HLA-DR typed positive responders (75.0%) whilst it occurred in only two of the 36 HLA-DR typed negative responders (6.3%). After correction for the number of comparisons, this latter result remained highly significant (p<0.001) Table 6.10. In Fig. 6.2, the net cpm to B. pertussis in cultures of HLA-DR5 positive cells is compared to HLA-DR5 negative samples. Only one HLA-DR5 positive cell showed a negative net cpm response to B. pertussis. Seven of the non-HLA-DR5 cells, one of which was HLA-DR4 had a positive response, although small in magnitude, whilst 27 had negative net cpm responses. Fourteen of these 27 were HLA-DR4 positive. Therefore, it can be statistically shown that HLA-DR5 is correlated with a positive blastogenic response to low doses of B. pertussis (r = 0.61). The data also suggest that, although it cannot be proven by this experimental sample considering the proportion of HLA-DR blank, HLA-DR4 may be associated with non response to the same antigen dosage.

Section (iii) Joint allelic combinations of chromosome 6 markers and differential response

After the number of comparisons were corrected for, the HLA allelic deviations found by initial 2 x 2 contingency analysis between responders and non-responders in the immunization reaction group, IgM response to B. pertussis and tetanus toxoid and the B. pertussis
### Table 6.10

**HLA A, B and DR Antigen Frequencies for the Lymphoproliferative Response Groups to B. Pertussis**

<table>
<thead>
<tr>
<th>HLA Antigen</th>
<th>CMI Response</th>
<th>CMI Response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>a.f. (%)</td>
<td>a.f. (%)</td>
</tr>
<tr>
<td>HLA-A</td>
<td>N=8</td>
<td>N=36</td>
</tr>
<tr>
<td>A1</td>
<td>37.5</td>
<td>41.7</td>
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<td>47.2</td>
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<tr>
<td>A3</td>
<td>12.5</td>
<td>19.4</td>
</tr>
<tr>
<td>Aw24</td>
<td>25.0</td>
<td>8.3</td>
</tr>
<tr>
<td>A25</td>
<td>0.0</td>
<td>5.6</td>
</tr>
<tr>
<td>A26</td>
<td>12.5</td>
<td>8.3</td>
</tr>
<tr>
<td>A11</td>
<td>0.0</td>
<td>13.9</td>
</tr>
<tr>
<td>A28</td>
<td>12.5</td>
<td>8.3</td>
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<tr>
<td>A29</td>
<td>12.5</td>
<td>11.1</td>
</tr>
<tr>
<td>Aw30/31</td>
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<td>5.6</td>
</tr>
<tr>
<td>Aw32</td>
<td>37.5</td>
<td>2.8*</td>
</tr>
<tr>
<td>A-blank</td>
<td>37.5</td>
<td>22.2</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>15.6</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>N=8</td>
<td>N=32</td>
</tr>
<tr>
<td>DR2</td>
<td>37.5</td>
<td>25.0</td>
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<tr>
<td>DR3</td>
<td>12.5</td>
<td>37.5</td>
</tr>
<tr>
<td>DR4</td>
<td>0.0</td>
<td>46.9**</td>
</tr>
<tr>
<td>DR5</td>
<td>75.0</td>
<td>6.3***</td>
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<tr>
<td>DR6</td>
<td>12.5</td>
<td>9.4</td>
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<tr>
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<td>40.6</td>
</tr>
<tr>
<td>DR-blank</td>
<td>25.0</td>
<td>18.8</td>
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</table>

* p(uncorrected) = 0.015
** p(uncorrected) = 0.014
*** p(uncorrected) = 1.81 x 10^{-4}

p(corrected) = 0.0063
Comparison of the lymphoproliferative response to \textit{B. pertussis} in HLA-DR5 positive and HLA-DR5 negative individuals. Seven of the eight HLA-DR5 positive cells show a positive net response to \textit{B. pertussis} compared to only seven of the 33 HLA-DR5 negative cells. Note the more vigorous response of the six HLA-DR5 positive cells which are also classified as 'responders'. 
NET CPM \times 10^3

\begin{itemize}
  \item responders
  \item non-responders
\end{itemize}

\begin{align*}
\text{negative} & \quad \text{DR5 positive} \\
\text{negative} & \quad \text{DR5 negative}
\end{align*}
lymphoproliferative groups (with the exception of HLA-DR5 and blastogenic response to *B. pertussis*) proved non-significant. This implies that either the observed deviations occurred by chance owing to the large number of independent observations undertaken (the number of HLA antigens per loci) or the antigen deviations may be secondary to a true association between the measured parameters of immune response and a particular HLA haplotype or a gene in linkage with the HLA antigen or haplotype. Lack of family data precludes the investigation of possible haplotype associations with immune response. Therefore, the occurrence of allelic combinations between the individual HLA antigens, Factor B and glyoxylase loci in the four response groups were investigated to test for probable supertypic (HLA cross reacting groups) or haplotypic (different loci combination) associations.

Most significant associations were found between the allelic combinations and the *B. pertussis* IgM response groups (Table 6.11). The combinations of A2 + Bw44, Aw24 + Bw44 and Bw44 + BF*S were significantly increased in the positive IgM responders. Although not reaching significance, DR4 + BF*S and Bw44 + DR4 occurred more often in the *B. pertussis* IgM positive group (38.5% and 36.4% respectively) compared to 18% and 11.1% respectively in the negative group. In addition HLA-DR4 was more likely to be found in combination with GLO*2 (27.3%) than GLO*1 (9.1%) in the positive IgM responders whereas HLA-DR4 was equally divided between GLO*1 (16.3%) and GLO*2 (18.4%) in the negative response group.
On analysis of the known HLA antigen cross reacting groups (CREG) only one CREG proved to be associated with immune response. A3 CREG (HLA-A3, HLA-All) was significantly associated with a positive IgM response occurring in 50% of B. pertussis IgM positive individuals and in 53% of the tetanus toxoid IgM positive group. Three individuals with this CREG’s antigens overlapped between the B. pertussis and tetanus toxoid IgM positive group. By comparison, approximately 18% of non-responders of both B. pertussis and tetanus toxoid response groups had either HLA-A3 or HLA-All.

Interestingly, though non-significant, trends were also noted in Table 6.11. Neither the immunization reactor group nor the B. pertussis IgM positive group had the Aw24+Bw51 combination although it was present in 9.2% of immunization non-reactors and 8.9% of B. pertussis IgM negative responders. A3 + B7 was increased in immunization reactors (reactor 9.1%, non-reactors 4.6%) and, in the positive IgM response groups of B. pertussis (positive 8.3%, negative 2.2%) and tetanus toxoid (positive 15.4%, negative 0.0%).
<table>
<thead>
<tr>
<th>ANTIgenic COMBINATION</th>
<th>IMMUNIZATION REACTOR STATUS</th>
<th>IgM RESPONSE B. PERTUSSIS</th>
<th>TETANUS TOXOID</th>
<th>CMI RESPONSE B. PERTUSSIS</th>
</tr>
</thead>
<tbody>
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<td></td>
<td>R</td>
<td>NR</td>
<td>POS</td>
<td>NEG</td>
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<td>36.4</td>
<td>14</td>
<td>21.5</td>
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<tr>
<td>A2+Bw44</td>
<td>8</td>
<td>18.2</td>
<td>7</td>
<td>10.8</td>
</tr>
<tr>
<td>Aw24+Bw44</td>
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<td>9.1</td>
<td>4</td>
<td>6.2</td>
</tr>
<tr>
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<td>10</td>
<td>22.7</td>
<td>9</td>
<td>13.8</td>
</tr>
<tr>
<td>A3+B7</td>
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<td>9.1</td>
<td>3</td>
<td>4.6</td>
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<td>6</td>
<td>9.2</td>
</tr>
<tr>
<td>Bw44+BF*S</td>
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<td>42.1</td>
<td>17</td>
<td>27.4</td>
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<tr>
<td>Bw44+DR4</td>
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<td>B7+DR2</td>
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<td>5</td>
<td>10.0</td>
</tr>
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<td>7</td>
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<td>17</td>
<td>26.4</td>
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<td>22.2</td>
<td>15</td>
<td>34.1</td>
</tr>
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</table>

1 $p = 0.026$
2 $p = 0.015$
3 $p = 0.005$

+ Number of individuals with specified antigenic combination
++ Percentage occurrence of the specified antigenic combination in each response group
* Probability calculated from 2 x 2 contingency table for each antigenic combination comparing the number of individuals with the combination to those without in the positive and negative responder groups.
DISCUSSION

The close agreement between the cord and adult allelic frequencies for the polymorphic genetic marker systems (Tables 6.1, 6.2, 6.3, 6.4) showed that not only could these non-placentally transferred systems be adequately measured in cord blood but also that the survey selected neonatal population was genetically representative of the normal Canberra adult population. However, slight differences were noted in the Factor B and HLA-DR systems compared to the adult controls. Whilst the minor change in the electrophoretic relationship of the cord Factor B alleles compared to adult ratios did not affect assignment of Factor B phenotypes, the high HLA-DR blank frequency despite similarity in the distribution of specific HLA-DR alleles between adult and neonate requires further examination.

This increase in HLA-DR blank in the cord samples cannot be attributed to putative weak expression of particular HLA-DR antigens at birth, implying a variance in maturation between specific antigens, as there was no observed significant depression in the frequency of any individual HLA-DR antigen compared to the adult population. However, it is possible that the expression of certain HLA-DR antigens is suppressed in the antenatal period and this suppression is still evident at birth resulting in the high blank frequency. Random suppression of HLA antigens neonatally has been previously described by Tiilikainen et al. (1974) and Dumble et al. (1977) although both of these papers described suppression of HLA-A and HLA-B antigens in cord lymphocytes. (The HLA-DR locus was not examined as this system was an ill-defined concept when these reports were written).
By examination of parental HLA typings, incompatible paternal HLA antigens were found to be either lacking (Tiilikainen et al. 1974) or weakly expressed (Dumble et al. 1977) on a proportion of cord lymphocytes. Upon subsequent cord cell culture, Tiilikainen et al. (1974) found that the previously hidden paternal antigens were re-expressed. From this result, this latter report concluded that either the foetopaternal antigens were masked by a maternally-derived non-cytolytic blocking factor, a concept which Dumble et al. (1977) also suggested as explanation for their findings, else that the paternal antigen complexed to maternal antibody was continually shed or pinocytosed from the foetal cell surface resulting in a temporary shortage of the paternal antigenic determinant. Since the importance of HLA antigen incompatibility in allograft rejection is well known (Morris and Ting, 1982), this mechanism of paternal antigen masking probably reflects a survival mechanism whereby the foetus may be protected from maternal immunological defences in utero. Unfortunately, in the Canberra study, without information on parental HLA phenotypes, the possible identity of the suppressed HLA-DR antigen, if present, nor the true frequency of HLA-DR blank allele can be deduced. The overall effect of these non-detected alleles is that only positive associations between individual HLA-DR alleles and an immune response can be determined since a negative association such as the presence of a particular allele and a low response may be due to non-detection of that allele in high responders rather than association with an actual immunological mechanism.
Only the lymphoproliferative response to *B. pertussis* was found to be closely associated with an individual HLA antigen, inferring the regulatory effect of MHC antigens on this assay system. Seventyfive per cent of neonatal cells with a positive blastogenic response to *B. pertussis* had HLA-DR5 whilst this antigen was present in only 6% of non-responders. This marked association between a single HLA antigen and positive response rejects the notion that the observed lymphoproliferation resulted from mitogenic stimulation and strongly suggests, from the lack of heterogeneity in the association, that only one or a few antigenic determinants were involved. However, it is still unclear whether the immunodeterminant(s) involved are unique to *B. pertussis*, implying a primary response arising from *in vitro* sensitzation or due to a cross-reacting moiety present on both *B. pertussis* and a putative bacterium to which the neonatal cells were previously sensitized. If the measured response was secondary, it is unlikely that non-response in the assay primarily resulted from non-exposure to the original microbial agent since a random association between HLA-DR antigens and response would be expected and not a strong correlation with an individual antigen as has been observed in this assay.

Several theories can be advanced to account for the observed association between the *B. pertussis in vitro* lymphoproliferative response and HLA-DR5. The HLA-D region is known to consist of a population of tightly linked loci, including loci coding for DR, SB, DC, other yet to be identified class II antigens and possibly the
human equivalent of Ir genes. In addition to their primary stimulatory function in mixed lymphocyte reaction, several HLA-D region gene products (DR and MT or DC) (Berle and Thorsby, 1982) have demonstrated the ability to act as important MHC restriction elements in cell-cell interactions in in vitro antigen specific proliferation assays and also are believed to be involved in antigen recognition by helper and delayed type hypersensitivity (DTH) T cells in culture. In relation to this latter function, antigen, either by close physical association with the HLA-D gene product or else by modifying the self D region coded molecule, is expressed in an immunogenic form on the surface of an antigen presenting cell (APC) such as a macrophage or monocyte and is able to be recognised by the functional helper or DTH cell. In guinea pigs and mice, certain combinations of I region gene products and antigen on the APC have been shown to be superior in the immunogenic presentation of antigen resulting in a stronger antigen specific blastogenic response (Shevach, 1976; Yano et al. 1978). Recently, Thorsby et al. (1982) reported a similar phenomenon in man whereby T lymphocytes from HLA-D/DR1 heterozygotes sensitized in vitro to PPD showed stronger lymphoproliferative responses in secondary cultures when PPD was presented by allogenic HLA-D/DR1 positive APCs rather than by APCs bearing the second HLA-D/DR antigen present during initial priming. Therefore, a qualitative difference in the ability to present antigen was noted between HLA-D/DR molecules for PPD. In this present study, the combination of the B. pertussis expressed antigen was noted between HLA-D/DR molecules for PPD.
expressed antigen and HLA-DR5 may be more effectively presented as an antigenic unit to the responding culture cells than combinations of this immunogen with other HLA-DR antigens, therefore resulting in the observed difference in proliferative response.

However, there was not a 100% concordance between HLA-DR5 and a positive lymphoproliferative response. This could be due to several possibilities apart from the technical variability that inevitably occurs in in vitro biological systems. Either it could imply structural heterogeneity in the cell surface molecule recognised by the HLA-DR5 antisera as has been previously described for HLA-DR4 (Reinsmoen et al. 1982) or else that HLA-DR5 is in linkage disequilibrium with another HLA-D region locus which has a more direct role in the primary response. The possible interaction between HLA-DR5 and the product of another gene locus for cellular response must also be considered.

Alternatively, the HLA-DR5 gene or a closely linked allele may be involved in another aspect of the lymphoproliferative response either remote from or in addition to a role in antigen presentation. Such aspects may include the production in cell culture of antigen specific helper factors or else influence the T cell repertoire responsible for recognition of the particular B. pertussis antigen.

HLA-DR4 is notably absent from the B. pertussis positive responder group. However, it is not possible to determine, as previously discussed, whether the absence is of functional significance, such as being associated with ineffective antigen presentation or it is the result of random HLA-DR allelic suppression disguising the presence
of HLA-DR4 in the positive responder group.

In contrast to the lymphoproliferative results, there was no statistically significant association between any individual MHC antigen and IgM antibody response to both *B. pertussis* and tetanus toxoid. This result confirms the similar lack of MHC antigen association found in previous reports on antibody response to infective agents. In an outbred population such as man it is unlikely that an in vivo antibody response which is mediated by the complex interaction of various genetic loci could be ascribed to a single genetic factor. Even in inbred animal species, immunized by antigens of restricted heterogeneity, control over the resultant antibody response can be due to a composite of MHC linked Ir genes controlling antigen presentation at the site of injection and the cellular cooperation of B and T cells, and genes coding for immunoglobulin allotypes determining the affinity and specificity of the antibody idiotype produced by the responding plasma cells. During the antibody response to more complex immunogens, as has been described for the Biozzi mouse model (Biozzi *et al.* 1971, Feingold *et al.* 1976, Mouton *et al.* 1979), in addition to the above genetic controls, other MHC or non-MHC loci mediate the proliferation and differentiation of antibody-forming cells or can have a non-specific stimulatory or suppressive effect on the determination of an animal's responder status. This latter situation may more closely approximate the antibody response measured in the Canberra study since the primary injection of Triple Antigen may not be sufficiently limiting to allow a homogeneous antibody response to occur.
Several other factors may also be operating in this present study which could further conceal or negate any genetic marker association being observed. First, the possible effect of the adjuvanicity due to the combination of antigenic components in Triple Antigen on the genetically determined aspects of the antibody response in a certain individual cannot be easily elucidated. This may present a major pitfall in the use of this particular combined vaccine, if not others, in a similar genetic study. Second, it is uncertain if an antigen specific IgM antibody response even to a "T dependent" antigen is controlled by MHC Ir genes since this response may occur without T cell help (Benacerraf and Germain, 1978). There have been no substantiated reports of MHC Ir gene control for any T independent antigen.

However, significant associations were found between combinations of MHC antigens and IgM response especially to B. pertussis. In the B. pertussis group, HLA-Bw44 in combination with both BF*S and HLA-Aw24 were correlated strongly with an IgM response to the B. pertussis antigen with the phenotype involving all three antigens only occurring in the IgM positive responders. The combination of HLA-Bw44 and HLA-A2 was also significant in this group. In addition, although not significant when corrected for the number of chisquare comparisons, there was an overall increase in the frequency of HLA-Bw44 in this group which attained statistical significance in males. There was also an unusually high number of GLO homozygotes in the IgM positive group.

Since it cannot be shown without family data whether
the observed combinations of antigens are in cis or trans-relationship to each other, the function of these combinations in the determination of antibody response can only be speculated. The occurrence of the HLA antigen combinations emphasizes the complexity of the genetic control of the positive IgM response to *B. pertussis*. It could be hypothesized that the MHC antigens involved in the combinations are also involved in the actual antibody response whether as marker antigens for a high responder phenotype to *B. pertussis* or the antigenic combinations may be indicative of complementary MHC linked genes interacting in the control of various aspects of the antibody response. Alternatively, since HLA-A2 and HLA-Bw44 are in known linkage disequilibrium in Australian Caucasoids (Terasaki, 1980), the MHC antigens may indicate the presence of an immune response gene found more commonly on the A2 + Bw44 haplotype. The other antigen combinations of significance in the IgM response, Aw24 + Bw44 and Bw44 + BF*S are not in positive linkage disequilibrium in the general population. This suggests a primary role for HLA-Bw44 in the IgM response to *B. pertussis* with the different combinatorial patterns between the high and low responder groups inferring possible heterogeneity in HLA-Bw44 as has been previously suggested by Tekolf et al. (1982). Interestingly, HLA-B12, the public specificity of HLA-Bw44, was more frequently found in Australian Caucasoids with an increased IgM response to the bacterial surface antigen, *Salmonella adelaide* flagellin (Whittingham et al. 1980). No similar association was found in the IgG antibody response to the same antigen. This latter report conclu-
Although only HLA-B locus antigens were studied, that HLA-B12 may be associated with the mechanisms responsible for the generation of an IgM response. However, in this Canberra study, the increased frequency of HLA-Bw44 was only found in the B. pertussis group and not in the tetanus toxoid IgM positive response group. This finding suggests that HLA-Bw44 may be involved in primary recognition and response, by the production of IgM antibody, to B. pertussis although it is speculative to suggest that this MHC antigen may be also involved in the primary response to other bacterial species though not necessarily to their synthesized products such as exotoxins.

There is evidence in the present report that the serological crossreacting group, A3-CREG may be involved in the generation of an IgM response with HLA-A3 or HLA-All being found in approximately 50% of IgM positive responders to both B. pertussis and tetanus toxoid groups. The crossreacting moiety recognised by various antisera in a CREG is proposed to be a common antigenic structure (the public determinant) which may represent the ancestral structure from which the CREG antigens have arisen by point mutation. This public determinant, therefore, may be the antigen primarily involved in the observed association and could be associated with phenotypes showing lack of T cell help or excessive T cell suppression which are important mechanisms in the generation of an IgM response (Katz and Armerding, 1976).

With the exception of the red cell enzyme esterase D, the non MHC genetic markers were not associated with any immune parameters of response. The similarities in the gene frequencies of these markers between the bimodal
response groups not only indicated the background homogeneity of the response groups but also emphasised that the major contribution to immune response was from the MHC region. However, the esterase D allele ESD*2 was found to be negatively associated with IgM response to both *B. pertussis* and tetanus toxoid. ESD*2 was either absent or at a very low frequency in the IgM positive groups although it had obtained a frequency of approximately 15% in both IgM negative groups. This enzyme has been formally mapped to chromosome 13, an acrocentric chromosome with very few genetic loci assignments. In contrast to the MHC genetic markers with their extensive polymorphisms, it is difficult to confidently define a negative association between an immune response parameter and an allele from a diallelic system in a small population size such as that of the Canberra study. The unusual alignment of alleles could be a consequence of sampling or else may demonstrate a true association with response indicating that certain aspects of the immune response in man, similar to that in animal studies, may be controlled by genetic loci outside those regions coding for the MHC and the immunoglobulin structural genes. Considering the relative scarcity of known genetic loci on chromosome 13, it is reasonable to suggest that an as yet unspecified locus situated close to the esterase D locus and determining some aspect of an IgM response may exist. The results from this study cannot confirm if this putative gene locus may be specific for various microbial agents. A naive population of a much greater size artificially exposed to similar antigens would be required to satisfactorily examine the existence of this
Recent studies investigating immune response in man by Sasazuki and co-workers have reported HLA-linked cell mediated low or non responsiveness to a range of antigens including tetanus toxoid (Sasazuki et al. 1978), schistosomal cell wall (Sasazuki et al. 1980a), streptococcal cell wall (Sasazuki et al. 1980b) and Japanese cedar pollen (Sasazuki et al. 1983). Non responsiveness to these antigens was attributed to dominant genes which segregated with HLA haplotypes in family studies and were believed analogous to the murine immune suppression genes which controlled the induction of suppressor T cells (Debre et al. 1975, Nishimura and Sasazuki, 1983).

In contrast, Greenberg et al. (1980) and Hsu et al. (1981) using in vitro lymphoproliferative response to streptococcal and synthetic polypeptide antigens respectively and Haverkorn et al. (1975) investigating in vivo antibody response to measles antigen have proposed that HLA-linked dominant genes exist which control high responsiveness to these antigens. Hsu et al. (1981) also presented evidence that complementation between two HLA-linked genes may be required for response.

The Canberra series, in agreement with these latter three studies, has found that a positive immune response to B. pertussis was clearly HLA associated with a definite HLA-DR antigen association with in vitro lymphoproliferation and a possible HLA-B antigen association with in vivo IgM antibody response to B. pertussis.
CHAPTER 7

Final Discussion.
The genetic control of immune responsiveness has been well established in man. Pedigree and twin analysis have confirmed familial segregation or sib concordance for basal IgE levels (Gerrard et al. 1978, Rao et al. 1980), IgG antibody response to infectious agents (Haverkorn et al. 1975) and cell mediated responses to synthetic antigens (Hsu et al. 1975) and bacterial cellular components such as streptococcal cell wall antigen (Sasazuki et al. 1980a). However, despite numerous studies which have examined the association between particular HLA region antigens and the humoral or cellular immune response to a variety of infectious (see Kaslow and Shaw 1981), allergic (see Marsh et al. 1981, Sasazuki et al. 1983) and synthetic antigens (Taussig et al. 1978, Hsu et al. 1981) few investigations have presented evidence consistent for the presence of specific human immune response genes (Sasazuki et al. 1983, Hsu et al. 1981, Greenberg et al. 1980; 1981, Haverkorn et al. 1975) paralleling those found in the mouse, rat and guinea pig animal models (Benacerraf and Germain, 1978). The major barrier to this area of research in man has been the complexity of the human experimental model compared with that of the animal systems. This complexity is two-fold and consists of the heterogeneity present in both the genetic background and environmental experience of the study population as well as the technical barriers imposed by the legal and moral ethical restraints placed upon human investigations. Consequently, there has been much criticism, especially concerning in vivo and, to a lesser extent, in vitro studies as to whether the measured polymorphism in immune response parameters reflects
differences in antigenic exposure rather than genetic response.

With the intention to minimize most of the technical difficulties apparent in other population studies, this thesis has presented an experimental model for the detection of immune response polymorphisms in a human population. The model, consisting of an ethnically homogeneous neonatal population, measured both the primary humoral response to a single low dose of an ethical vaccine, Triple Antigen, administered under a controlled immunization regime and the cellular response following in vitro sensitization to the individual components of the vaccine. Previous exposure to the vaccine antigens either did not exist, as indicated by the questionnaire responses or in vitro cord antibody estimations or else, in cases where a positive cord titre to the test antigen indicating possible sensitization occurred, there was no observable effect on either measurable IgM antibody (see Tables 4.1; 4.2) or cellular response (Table 5.1) to the specified antigen.

The entire Canberra study population was exposed to the same antigen dosage, by uniform subcutaneous injection in the proximal arm. Unlike other studies on humoral response using ethical vaccines, this dose was suboptimal in immunizing effect, thus approximating the administration of complex natural antigens in animal studies (Green et al. 1970, Benacerraf and Germain, 1978, Kohno et al. 1982). Administration of the vaccine as partitioned doses did not influence the measurable IgM humoral response to either *B. pertussis* (Table 4.1) or tetanus toxoid (Table 4.2). However, although initial data suggested that
partitioned vaccination may have an effect on the IgG response to tetanus toxoid, the significance of this observation remains unresolved since there was a strong suggestion that it may have been secondary to a sampling effect (Chapter 4).

Measurement of a neonatal-specific IgG response was not satisfactorily achieved in this study when antigen-specific maternal antibody was present. This was a particular problem with tetanus toxoid where over 60% of the test data were discarded due to this uncertainty as to the origin of the IgG response. Lower prevalence of maternal antibody titres were observed with _B. pertussis_, a finding which is consistent with documented evidence of the poor persistence of protective antibody after both natural and induced sensitization by this bacterium (Barrett et al. 1962, Provenzano et al. 1965, Preston, 1977). However, only one neonate in the present study had a measurable IgG response to _B. pertussis_ immunization. The overall poor IgG response to _B. pertussis_ may be due to a combination of factors including the natural propensity of the neonate to produce IgM rather than IgG, the possible weak immunogenicity of the administered _B. pertussis_ dose for an IgG response or the collection time of the heel prick sample being suboptimal for the determination of an IgG response. The latter supposition is the easiest to test and would require several collections after immunization, which was not feasible in this study, to determine when, if ever, the optimal IgG titre occurs.

_In vitro_ techniques in human investigations circumvent the ethical restraints of _in vivo_ testing and, by choosing
a cellular test population with minimal environmental heterogeneity, can investigate more readily the genetic mechanisms involved in immune response. With this aim, this study has used cryopreserved umbilical cord cells as responders in a lymphoproliferative assay. Although cryopreserved responders have been used previously in similar assays (Biddison et al. 1980, Rich et al. 1980) the use of umbilical cord cells to investigate the genetic control of antigen-specific lymphoproliferation is a novel concept. In these cells, the possibility of intrauterine infection or sensitization to the test antigen can be detected from maternal exposure history, measurement of in vitro cord blood humoral response and, ideally, maternal cellular response. The neonatal system is therefore ideal for the measurement of a cellular primary immune response.

In this study bimodality in measurable lymphoproliferation was observed in cell cultures following incubation with B. pertussis, tetanus and diphtheria toxoids (Chapter 5). Although this study did not find evidence that previous sensitization to these three antigens may be responsible for the cellular response, the work of Andersen et al. (1977) suggests that the B. pertussis cellular response may be due to uterine exposure to a cross-reaction antigen. However, the kinetics of the lymphoproliferative response to B. pertussis, with measurable bimodality in response occurring after seven days culture, strongly indicate a primary antigen specific response even though the possibility of a late recall response, postulated by Young and Engleman (1980) to be due to the small number of antigen-specific sensitized cells in the cell culture sample, cannot be disregarded. This question of potential cross-
reactivity and thus the possibility of previous sensitization will always be a problem when a multi-antigenic entity such as a whole bacterium is used as the immunogen for in vitro sensitization. The use of purified cell surface antigens from *B. pertussis* for inclusion in culture may help resolve this dilemma.

The cellular responses to tetanus and diphtheria toxoid were unfortunately not definitive owing to the small number that responded. A larger sample may reveal more informative results for these two antigens.

The selected genetic marker systems to be used for the association study were reliably measured in the neonatal system. There was an indication that the expression of certain HLA-DR antigens on the surface of cord cells may be randomly suppressed which meant partial loss of experimental data with only positive associations between HLA-DR and an immune parameter being informative. Resolution of the foetal phenotype in selected cases would require parental HLA-typing, adding an organisational complication to a prospective population study. Alternatively, future studies using these cells may co-culture suspect cells with tissue culture medium, as performed by Tiilikainen *et al.* (1974) in their study on HLA-A and -B antigens, which might allow re-expression of the suppressed HLA-DR antigen.

The strong association between HLA-DR5 with positive in vitro proliferation to *B. pertussis* in the cord cell cultures concurs with the limited existing experimental evidence in man that in vitro cellular response to bacterial antigens may be under HLA-linked regulatory control. It is impressive that the gene or genes involved in this response are in the HLA-D region and therefore may be
analogous to the murine immune response genes.

Few previous studies have found an association between a specific immune response to a bacterial antigen and a single MHC antigen, in particular an HLA-D region antigen. In the original report on HLA associations with in vitro responses to bacteria, Greenberg et al. (1975) discovered an association between HLA-B5 and high responder status to the streptococcal antigens, streptodornase and streptokinase. HLA-D region antigens were not determined. Sasazuki et al. (1978, 1980b) found, in Japanese, that low in vitro responsiveness to both tetanus toxoid and schistosomal worm antigen were associated with Dw12, the cellular equivalent of a Japanese antigenic subtype of HLA-DR2 (Ohta et al. 1983). The HLA-B antigens in linkage with this HLA-D specificity were also associated with low responsiveness. In Caucasoids, Lehner et al. (1981) described an association between low antigen dose induced helper T cell factor activity to *Streptococcus mutans* and the combined HLA-DR specificities of HLA-DRw6, -DR1, -DR2 and -DR3 in dental caries resistant individuals. This study also found that HLA-DR4 positive cells produced streptococcal specific helper T cell activity upon exposure to extremely high non-physiological doses of antigen and that HLA-DR4 individuals, despite the presence of the 'resistant' DR specificities, were susceptible to dental caries.

The main disadvantage with these and most similar studies is their reliance upon natural infection by the bacterium or else previous immunization by a vaccine strain for sensitization. The work by Sasazuki and co-workers
relies upon hypothetical previous sensitization as an explanation for the generation of the specific suppressor cell which, in later studies by Sasazuki et al. (1983), have been postulated to be the mechanism controlled by the HLA-linked immune suppression gene in his experimental system. Despite assumed ubiquity of the test bacterium in some studies, variation in exposure to the organism must occur which jeopardises the validity of the association data.

The current study removes this uncertainty by investigating primary in vitro sensitization to the test antigen. The observed HLA association, in this primary response, may be instrumental in the initial recognition of the bacterium by the unprimed responding cells. It must be stressed that the nature of the responding cell was not determined in this study so the type of immunocompetent cells involved in response can only be speculated. The possible mechanisms that the HLA-DR5 associated genes might control have been previously discussed in Chapter 6. A further mechanism established in murine studies from the recent literature suggests that Ir gene products may differ both qualitatively and quantitatively in their ability to present antigen on the surface of an antigen presenting cell (Mates et al. 1982). The variability of this difference may be contained in the actual α or β chain of the Ia molecule (McNicholas et al. 1982), Mathis et al. 1983). Another tenable hypothesis for this observed association between HLA-DR5 and lymphoproliferation to B. pertussis is that HLA-DR5 is not associated with immune responsiveness per se but with lack of immune suppression. As will be discussed later, the
cellular proliferation observed in culture may indicate susceptibility to a pathogenic effect of the whooping cough disease. This proliferative ability may be controlled by suppressor mechanisms in a normal individual resistant to the particular disease effect. However, an HLA-DR5 positive individual may not have this protective mechanism and thus be susceptible to either the disease component or else to host mediated damage similar to that observed in the murine lymphocytic choriomeningitis model (Doherty and Zinkernagel, 1974).

The polymorphism observed in the IgM antibody response to both B. pertussis and tetanus toxoid did not have any overall significant MHC association in the neonatal population (Chapter 6). A similar lack of observable MHC association has also been reported by the extremely restricted number of studies dealing with the antibody response to bacterial products (see Kaslow and Shaw, 1981). This result implies that MHC genes may not have any marked control over the in vivo immune response to bacterial products. In this present study this was not an unexpected result for the following reasons (i) the nature of the antibody response. It has been documented in murine studies that, even with the use of T-dependent antigen, an antigen specific IgM response is not under strict Ir gene control as found with the IgG response to the same antigen (Benacerraf and Germain, 1978). (ii) the complexity of the genetic control over an in vivo antibody response. Complementation between several MHC loci have been reported for an antibody response to a synthetic antigen in mice (Dorf and Benacerraf, 1975, Melchers and Rajewsky, 1975) and
possibly man (Hsu et al. 1981) whilst immune response to a complex natural antigen may involve multigenic control which includes both antigen specific and non-specific effects (Feingold et al. 1976, Mouton et al. 1979). (iii) some 'non responders' in this present study may, in fact, be genetically capable of response but possess an immature immune system which is unable to process the complex B. pertussis antigen. An effort was made to limit this effect by elimination of children from the analysis who received their immunization whilst under 9 weeks of age.

All of these above factors would result in the lack of significant MHC associations. However, associations were observed between high IgM response to B. pertussis and antigen combinations involving the HLA-Bw44 antigen. It was implied that this HLA-B antigen was involved in the immune response to B. pertussis and either that complementation with other MHC genes may be required for the positive response, as described by Greenberg et al. (1981) for streptococcal antigen, or else heterogeneity may exist in the HLA-Bw44 antigen (Tekolf et al. 1982) and a subspecificity may be more strongly correlated with response.

Tait et al. (1982) described a similar situation in insulin-dependent diabetes mellitus (IDDM) where a subtype of HLA-DR4 showed a higher relative risk for the disease than HLA-DR4. Alternatively HLA-Bw44 may be in strong linkage with a subtype of another antigen, in particular an HLA-DR specificity, which is primarily involved in response. This was proposed by Tait and his coworkers (1982) why diseases such as IDDM and rheumatoid arthritis, which are associated with the same HLA-DR antigen, have different HLA-B associations.
The public specificity of HLA-Bw44, HLA-B12, was associated in a previous study with an IgM response to the flagellin antigen of Salmonella adelaide (Whittingham et al. 1980). Extrapolating from this observation, this thesis has hypothesized that HLA-B12 or its subtypes may be involved in non-specific immune response to whole bacteria. This view is further supported by reported trends of HLA-B12 associations with post-streptococcal sequelae including rheumatic fever in Caucasoids (Ward et al. 1976) and acute glomerulonephritis in Japanese (Sasazuki et al. 1979, with Haemophilus influenzae infection in children (Tejani et al. 1977) and also with staphylococcosis in family studies (Jacobs and Norman, 1977). Low antibody response to Haemophilus influenzae has also been associated with the immunoglobulin light chain allotype Kml (Pandey et al. 1979) which, as shown by the interesting study of Whittingham et al. (1980), may indicate that recognition and response to the bacterial antigen involves the interaction between MHC and non-MHC genes, in particular those coding for immunoglobulin allotypes.

Non antigen-specific control over the generation of an IgM response was suggested in this study by the significant association of possibly the public antigen of a cross-reacting antigenic group and also a non-MHC locus, esterase D with the IgM antibody response to both B. pertussis and tetanus toxoid. The switch from IgM to IgG synthesis is believed to require specific T cell help (Benacerraf and Germain, 1978) and therefore the two reported associations may be indicative of a deficient helper T cell network (Katz and Armerding, 1976). Since this study involves a neonatal system it might be hypothesized that
these two genetic marker systems may also indicate delayed maturity in the development of an efficient T cell help required for an IgG response rather than a specific defect. This appears a more feasible explanation since IgG non-responder status has not been reported with either A3-CREG or esterase D in adult systems.

It is interesting that a non-MHC locus esterase D, is associated with an antibody response. This may be the first description of a non-MHC immune response determining locus, not in linkage with a known structural immunoglobulin locus, in man. Hypothetically this locus may have a non-specific effect on an individual's immune response and thus be analogous to similar non H-2 associated loci in the mouse (Klein, 1975).

An interesting hypothesis investigated by this study was whether the immune response to the components of Triple Antigen vaccine and any corresponding postulated Ir genes, were predictive of an adverse reaction occurring to the vaccine. The assumptions underlying this hypothesis were that (i) the observed adverse reaction had an immunological basis similar to that described by Vessal and Kravis (1979) for other immunization reactions and was not simply a non-specific 'toxic' effect of the vaccine; (ii) the adverse reaction would be treated as a 'disease' model with all members of the study population being exposed to the "disease-causing" agent, in this case, Triple Antigen vaccine; (iii) the induction of the adverse reactions may involve genetic susceptibility to B. pertussis components as observed in experimental allergic encephalomyelitis (EAE, Levine and Sowinski, 1973) and HSF sensitiza-
(Munoz and Bergman, 1968) in rodents. To briefly recapitulate, susceptibility to both EAE and HSF sensitization is believed to be under genetic control with Linthicum and Frelinger (1981) proposing that complementation between an EAE 'responder' H-2 determined haplotype and non H-2 linked HSF susceptibility gene was necessary for EAE to occur. Previously Wardlaw (1970) had suggested that HSF susceptibility in mice was H-2 linked although this was subsequently disputed by Ovary and his co-workers (Ovary et al. 1973, Ovary and Caiazza, 1975). Background (non-MHC) genes (Gunther et al. 1978, Lindh and Kallen, 1978) and sex-linked genes (Montgomery and Rauch, 1981) have also been implicated in the EAE animal model; (iv) if genetic components were involved in the adverse reaction, their effects would be more likely to be observed following the first immunizing dose. Later immunizing doses may be complicated by the presence of primed immune responses masking specific genetic effects.

A randomly selected neonatal population was surveyed to investigate the presence of adverse reactions following the first immunizing dose of Triple Antigen. Classification of a reaction syndrome was based upon a number of differing physical sequelae to the vaccination including injection site manifestation and behavioural disturbances such as persistent screaming or drowsiness. These reported reactions were neither long lasting nor detrimental to the child involving a limited degree of discomfort for one or two days following immunization. No serious neurological sequelae were reported.

Using epidemiologic variables generated by the survey,
classical contingency analyses, later confirmed by logistic regression modelling, indicated that two 'environmental' or external factors and a possible genetic component were predictive of an adverse reaction to Triple Antigen.

The presence of breastfeeding, more specifically up to and including the time of immunization, was significantly associated with adverse reaction. This variable indicated a strong external effect on reaction and it could only be postulated, in the absence of confirmatory evidence, that specific reactogenic factors may be passed in the breast-milk to the child, the exact nature of these factors being purely speculative. Alternatively, these breast factors may either sensitize the child to the vaccine antigens, as has been suggested by Schlesinger and Covelli (1977) for observed infant cellular response to PPD, or else non-specifically enhance the child's immune response to these antigens (Butler et al. 1979). This hypothetical sensitization to the vaccine antigen or else an enhanced immune response may therefore contribute to the underlying mechanism leading to an immunization reaction.

Children, whose parents have reported suffering from a suspected IgE mediated allergy such as allergic rhinitis or eczema were over-represented in the adverse reaction group. This result inferred that an inherited allergic predisposition may be partly responsible for the reaction. It has been speculated in Chapter 3 that the observed reaction may arise from the allergenic effects of B. pertussis on this assumed allergic predisposition or alternatively, hyper-reactivity to various components in the vaccine as a result of the "generalised immune hyper-
responsiveness" postulated by Marsh et al. (1980) to be associated with the allergic phenotype.

However, there was some doubt whether the positive parental allergy-adverse reaction association was a primary effect or else this finding was due to a close association with the administration of partitioned doses of the vaccine, which comprised the second significant environmental or external predictor in the adverse reaction model. Resolution of this interaction could not be achieved in this study since the majority of children with positive parental allergy histories had received the first immunization as a partitioned dose. To prove that the observed parental allergy association with neonatal adverse reaction is not a localised phenomenon of the Australian Capital Territory due to its policy of Triple Antigen immunization, it would be profitable to study a similar survey population where either all children, regardless of allergic history, received a single, immunizing dose or, to investigate directly whether partitioned doses did have a major influence, randomly administer partitioned and non-partitioned doses to a suitable sample of 'non allergic' children. Although variation in vaccine administration did complicate interpretation of the adverse reaction model, the institution of partitioned immunization into ACT policy was primarily to minimize the chance of serious neurological sequelae occurring. If it could be proven that partitioned doses were responsible, in part, for the adverse reactions reported in this study, the benefit of this vaccine administration scheme in reducing the incidence of potential life-threatening vaccine side effects would more than compensate for the minor discomfort
involved. However, this may not prevent such a reaction after the second immunizing dose. Therefore the most effective solution would be the identification and removal from the vaccine those components that induce severe adverse reactions providing these components do not also induce protective immunity. The development of subcellular or inactivated 'active principle' vaccines, similar to the formalinized toxoids, may help reduce the reaction problems of the present whooping cough vaccine.

Although not included in the final logistic regression model because of the few cases observed, a history of an adverse reaction to tetanus toxoid in a mother was strongly predictive, in this study, of an adverse reaction to Triple Antigen in the child. This association implies not only another instance of possible heritable factors being involved in the reaction syndrome but also that the observed adverse reaction may be genetically heterogeneous with individual differences in susceptibility to the different antigenic component of the vaccine. Sib data, however, proved inconclusive in determining whether the reaction syndrome itself was inheritable.

Neither the immune response parameters to components of Triple Antigen estimated in this study nor deviations in any genetic marker system were associated with an adverse immunization reaction. This result invites much interesting speculation. First, it may indicate that the adverse reaction may not have an immunological basis or, alternatively, that the immune parameters measured in these studies were not indicative of the immune mechanisms which result in an adverse reaction. Other
humoral or cellular assays such as antigen specific IgE response to the vaccine components, in vitro responsiveness to specific B. pertussis components such as HSF or in vitro total or allergen-specific leucocytic histamine release (Radermecker et al. 1982) may be more informative especially if it is proven that an allergic predisposition is a cofactor for the occurrence of an immunization reaction.

The measured polymorphisms in the immune response to the vaccine components observed in this study may not be associated with adverse reaction because they may instead indicate innate responsiveness to the wild type of the vaccinal strains and corresponding susceptibility or resistance to the natural disease. A positive cellular response to B. pertussis in vitro may reflect the cell mediated response to this bacterium or crossreacting counterpart in vivo and thus a superior immune response to the bacterium and possible protection against the disease or else reduced severity of symptoms. Alternatively, if the response was B. pertussis specific and since lymphocytosis is a pathogenic sequelae of whooping cough infection and, to a lesser extent, B. pertussis vaccination (see Davis et al. 1974a) the in vitro responsiveness following incubation with the restricted B. pertussis concentration may be indicative of increased susceptibility or else enhanced recognition of the lymphocytosis promoting Factor (LPF) component of the bacterium (Morse and Kong, 1977). Similarly the IgM response to B. pertussis may be one of the initial defence mechanisms against whooping cough infection and be directed against
the bacterial antigens involved in attachment and invasion of the host rather than those antigens responsible for the adverse reaction. Since infection is by the respiratory route, secretory IgA production may be more informative for determining specific host response although again not immunization reaction.

The lack of overall significant genetic marker associations with adverse reaction may, in the extreme case, be explained by the lack of genetic control for the reaction. However, as demonstrated by the logistic regression model and the data indicating the predictive value of a maternal adverse reaction to tetanus toxoid vaccination, there is a great degree of heterogeneity in the adverse reaction model, in part due to the criteria used for the classification of a reaction which has led, as already has been noted (see Chapter 3), to an inevitable proportion of reactors being misclassified as non-reactors. The strong environmental component of the adverse reaction model (breastfeeding and partitioned immunization) would further disguise or dilute any measurable genetic involvement.

Although not reaching significance, certain trends occurred in the HLA data for differential immunization reactions which paralleled similar trends found in the HLA comparisons between IgM response groups to both *B. pertussis* and tetanus toxoid (Table 6.11). The most interesting result was the observation that the majority of boys positive for both HLA-Bw44 and a high IgM response to *B. pertussis* had adverse reactions. No girl had a similar combination of HLA antigen, immune response and immunization reaction although this absence may be due to
the very small sample size of girls with positive IgM response rather than infer a sex difference. This result, plus the similar trends in the antigenic combinations of Aw24-Bw51 and A2-B7 in the adverse reactions and one or both positive IgM responders, implies that the humoral response to the vaccine components might play a part in the generation of an adverse reaction.

The complexity of the Triple Antigen vaccination model chosen masked any direct association between the particular immune response parameters or genetic marker systems and adverse reaction to the vaccine. However, the use of a simpler model involving an adverse or unusual response following the use of a microbial vaccine, as the means to study immune response gene action in a disease situation, has considerable potential. In a very preliminary study de Vries (1979) observed that low in vitro cellular responsiveness following vaccination with vaccinia virus and the MHC antigen associated with this response, HLA-Cw3 (see de Vries et al. 1977) might be correlated with febris post-vaccinalis, a sequel to smallpox vaccination due to in vivo multiplication of the vaccine virus (Davis et al. 1974c). This post-vaccinal syndrome may be due to poor containment of vaccinia virus in individuals with HLA-Cw3. Unfortunately, further data on this initial association are not available. In a more complete study on congenital rubella syndrome (CRS) in Japanese Kato et al. (1980) found that the mothers of children with CRS were more likely to have deviations in HLA specificities (increased HLA-B15, decreased HLA-Bw22) which he previously found associated with high antibody responses to rubella vaccination in Japanese school-
girls (Kato et al. 1978). Therefore, he postulated that susceptibility to CRS was related to a high responder HLA antigen. In two recent studies, although antigen sensitization occurred via pregnancy isoimmunization and not artificial immunization, Reznikopp-Etievant et al. (1981) and Taaning et al. (1983) found that immunization with the platelet-specific antigen Zwα was strongly associated with HLA-B8 in Zwα negative mothers giving birth to children with isoimmune neonatal thrombocytopenia. Therefore HLA-B8 (and possibly HLA-DR3; see Svejgaard et al. 1983) may be associated with an Ir gene directing a strong immune response against alloantigens and be, therefore, indirectly responsible for the corresponding disease in the child. A similar example is the study of Lehner et al. (1981) on the HLA-DR associations in dental caries resistant and susceptible individuals which has already been discussed.

The most interesting aspect of the preceding four examples is that the occurrence of the disease or adverse reaction under study, be it post vaccinal fever, CRS, isoimmune thrombocytopenia or dental caries, can be predicted by either the immune response to the study antigen or the HLA marker associated with the response. Therefore, prophylactic measures may be undertaken after prospective testing to minimize the occurrence of the disease syndrome in high risk individuals whose risk status has been determined by the presence of these marker systems. However, a complexity arises when more than one disease syndrome is associated with the same genetic marker. Further refinement in the identification of specific MHC products whether by serological methods,
cellular typing methods or by the use of cloned DNA probes and ultimately restriction enzymes to detect the actual gene sequence responsible for the specific polymorphism will be necessary to attempt identification of unique gene products associated with disease states. Alternatively, if many diseases are associated with one specific MHC marker or MHC chromosomal segment, for example, HLA-B8-DR3 haplotype, then a common pathogenic effect may exist in all the associated diseases which is primarily connected with the particular MHC region. For example, HLA-B8-DR3 haplotype has been associated with impaired cellular immunity to both mitogens and specific antigens (McComb and Michalski, 1982. Kallerberg et al. 1981, Greenberg and Yunis, 1978, Ambinder et al. 1981), although this association has not been universally supported (see Cunningham-Rundles et al. 1978), and may be the mechanism relating to this haplotype with a host of autoimmune diseases (see Svejgaard et al. 1983).

It is debatable, in view of the heterogeneity of the experimental model, if more definitive typing of the MHC region antigens may have revealed associations with adverse reactions to Triple Antigen or else delineated significant trends common to both immune parameters and reaction. This study has relied upon the typing of classical T and B cell serological antigens. Although a great many HLA-A and HLA-B antigens were able to be split into their subtypic specificities, this did not occur for HLA-DR. Typing reagents for the complex of HLA-D region antigens, now termed HLA-DC, and those for HLA-SB were either not readily available or lacked definitive classification when the samples in this study were HLA typed. Subse-
quent pooling in statistical analysis of the HLA-DR antigens in known disequilibrium with the HLA-DC specificities did not reveal any increase in the strength of the HLA-D related associations.

However, even if an unique genetic marker is associated with disease this does not automatically lead to elucidation of the actual disease mechanism especially in a genetically heterogeneous disease although genetically homogeneous subsets may be identified which may aid the investigation of these mechanisms. The study of an immunization reaction model may be more beneficial for the elucidation of possible mechanisms. In this case, the immune response associated with the reaction syndrome is known and translation of possible \textit{in vivo} mechanisms to \textit{in vitro} experimental systems may be achieved with the \textit{in vitro} model having the advantage of separating specific control mechanisms from the variety of non-specific effects evident in an \textit{in vivo} system. This model, therefore, has considerable potential in not only studying the existence of immune response genes in man but also could help unravel the \textit{in vivo} function of such genes and their possible involvement in disease syndromes.
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APPENDIX I

Letter of approval from JCSMR Clinical Research Committee for research protocol involving human subjects.
26 August 1980

Dr Susan Serjeantson  
Department of Human Biology  
JCSMR

Dear Dr Serjeantson,

Thank you for your letter dated 11 August 1980. I am glad to tell you that the Clinical Research Committee has now approved the protocol of your research proposal "Genetics of Immune Responses to Pertussis Vaccines".

Yours sincerely,

Carola Parke  
School Secretary
APPENDIX II

Immunization Reaction survey questionnaire performed in Capital Territory Health Commission Immunization Clinics. Questions asked by interviewer, answers interpreted and written on form. Responses later coded and transferred to computer data file.
GENETICS OF IMMUNIZATION REACTION SURVEY

GENERAL INFORMATION
Date of Survey
Health Centre
IMM I.D. number (Laboratory code)
Child's name
Child's sex
Child's ethnicity
Mother's name
Mother's date of birth
Suburb (residential address)

FIRST IMMUNIZATION HISTORY (from clinic record card)
Partitioned dose given: yes/no
- one week before
- other combinations
Triple Antigen or DT for immunization course
Date immunization given
Sabin vaccine given
Date administered
Was first sabin dose retained

IMMUNIZATION REACTION TO TRIPLE ANTIGEN/DT
Partitioned dose
Vaccine given: Triple Antigen/DT
Any reaction: yes/no
When did adverse reaction start: specify
How long did it last: specify
Injection site reaction: yes/no
Reddened and/or itchy: yes/no
Swelling: yes/no
Hard lump formation at site: yes/no
Other, e.g. bruising: specify
Fever, e.g. red cheeks, hot to the touch: yes/no
Malaise: not wanting to eat: yes/no
irritable, fretful: yes/no
vomiting: yes/no
diarrhoea: yes/no
Generalized rash: yes/no
Inconsolable crying or screaming for long period: specify
Swelling, soreness around joints: yes/no
Unusual pattern of sleep: specify
Convulsion: yes/no
If yes, with fever: yes/no
Other symptoms: specify

Single dose or remainder dose
Vaccine given: Triple Antigen/DT
Any reaction: yes/no
When did adverse reaction start: specify
How long did it last: specify
Injection site reaction: yes/no
Reddened and/or itchy: yes/no
Swelling: yes/no
Hard lump formation: yes/no
Other, e.g. bruising: specify
Fever, e.g. red cheeks, hot to touch: yes/no
(iii)

Malaise: not wanting to eat: yes/no
irritable, fretful: yes/no
vomiting: yes/no
diarrhoea: yes/no

Generalised rash: yes/no

Inconsolable crying or screaming for long period: specify

Swelling, soreness around joints: yes/no

Unusual pattern of sleep: specify

Convulsion: yes/no

If yes, with fever: yes/no

Other symptoms: specify

CONCOMITANT ILLNESSES IN THE CHILD: yes/no

Skin: yes/no

Specify if yes:

Gastrointestinal: yes/no

Specify if yes:

Other: yes/no

Specify if yes:

FAMILY HISTORY OF ALLERGY

Mother

Asthma: yes/no

diagnosis: specify

History of asthma in other family members: yes/no

Hayfever, yes/no

diagnosis: specify

History of hayfever in other family members: yes/no

Members affected: specify

Hayfever during pregnancy: yes/no

Skin rash/hives: specify
Other skin conditions: specify
Allergies: yes/no
Food: specify
Drugs: specify
Dander/dust mites: specify
Pollen: specify
Other: specify
History of allergies in other family members: yes/no
Members affected: specify
Year of last vaccination (if known)
Vaccine used
Any reactions: specify
Any history of: cold sores - yes/no
if 'yes', in last 12 months - yes/no
chickenpox yes/no
shingles yes/no
Breastfeeding: yes/no
Period involved: specify

Father
Asthma: yes/no
diagnosis: specify
History of asthma in other family members: yes/no
Member affected: specify
Hayfever: yes/no
diagnosis: specify
History of hayfever in other family members: yes/no
Members affected: specify
Skin rash/hives: specify
Other skin disorders: specify
Allergies: yes/no
    Food: specify
    Drugs: specify
    Dust/dander: specify
    Pollen: specify
    Other: specify

History of allergy in other family members: yes/no
    Member affected: specify

Other children: number
    Sib (different sheet for each sib)

Date of birth
    Sex

Asthma: yes/no
    diagnosis: specify

Hayfever: yes/no
    diagnosis: specify

Allergies: yes/no
    Food: specify
    Drugs: specify
    Dust/dander: specify
    Pollen: specify

Immunization history:
    Vaccine used
    Any reaction

HISTORY OF NEUROLOGICAL DISORDER
Any known disorders in the family: yes/no
    Member affected: specify
    Type of disorder: specify

HEEL PRICK SAMPLE yes/no
    Amount: