MULTIPLE FACTORS SHAPE THE TCR Vβ REPERTOIRE IN HUMANS

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Multiple Factors shape the TCR Vβ Repertoire in Humans

by

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ABSTRACT

Contributions to the human peripheral TCR Vβ repertoire were assessed by a semi-quantitative PCR method. Lymphocytes were expanded by PHA using a stimulation protocol that did not alter the Vβ repertoire. Marked differences in PCR-priming efficiencies of 23 Vβ segments were identified. Significant correlations between priming efficiencies and the observed Vβ repertoire were found and therefore the data were corrected for differences in priming efficiencies. Vigorous statistical analysis showed that non-genetic factors were responsible for significant differences between total T cell Vβ repertoires of monozygotic twin pairs and unrelated pairs. In preliminary analysis, however these influences were misinterpreted as having a genetic basis. No evidence was obtained that genetics (including HLA) influenced the total T cell Vβ repertoires. However, the role of genetics in influencing the Vβ repertoire of CD4+ or CD8+ T cell subsets could be detected at the level of a few Vβs.

Large families were studied to delineate the effects of HLA on the TCR Vβ repertoire. The results of these studies indicated that HLA influenced the Vβ repertoire but that non-HLA factors also have a significant influence.

Further, it was found that particular Vβs (15,16,18) were expressed at a constant level and several Vβs (particularly

10 and 12) were typically highly variable in expression levels. This was true for all individuals irrespective of the genetic background (including HLA) and irrespective of the T cell type. Still other Vβs tended to be highly variable in expression in CD4+ or CD8+ subsets but not in both.

These studies unequivocally document that influences on the Vβ repertoires are complex. Genetics including HLA seem to play a role, however non-genetic influences can also have a major role in determining the TCR Vβ repertoire.

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BL's tests	Bartlett's and Levene's tests
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
CO_2	Carbon dioxide
CPM	Counts per minute
dNTPs	Deoxynucleotide triphosphates
DN	Double positive
DP	Double negative
DTT	Dithiothrietol
EDTA	Ethyldiamine tetraacetic acid
FCS	Fetal calf serum
FTOC	Fetal thymic organ culture
HBSS	Hank's balanced salt solution
HLA	Human leukocyte antigen
HOV	Homogeneity of variance
HPLC	High performance liquid chromotography
IL-2	Interleukin-2
IL-4	Interleukin-4
KDa	Kilodaltons
Mabs	Monoclonal antibodies
$MgCl_2$	Magnesium chloride
MHC	Major histocompatibility complex

mRNA	Messenger ribonucleic acid
MUN	Memorial University of Newfoundland
ng	Nanograms
PBL	Peripheral blood lymphocytes
PCR	Polymerase chain reaction
PHA	Phytohemagglutinnin
qPCR	Quantitative PCR
RPMI 1640	Roswell Park Memorial Institute 1640
RPM	Revolutions per minute
RT-PCR	Reverse transcribed PCR
SCID mice	Severe combined immunodeficiency mice
SP	Single positive
TCR	T cell receptor
µCi	Microcurie
µg	Microgram
µl	Microlitre
VPPMs	Vβ product position markers
WHO	World Health Organization

CHAPTER 1

GENETICS, HLA AND THE T CELL RECEPTOR

The immune system maintains the integrity of an organism despite constant antigenic attack from the environment. The main features of specific immunity are the following; it is specific for antigen, it can discriminate between self and non-self, and can recall previous contact with antigen. Re-exposure to antigen can produce a large and rapid specific immune response. Another important feature of the immune system is its ability to recognize an extremely large repertoire of antigens.

The first definitive delineation of the two main types of immunity, humoral and cell-mediated immunity, came in the early 1900s. Scientists such as Emil von Behring, Shibasabura Kitasato, Karl Landsteiner and others showed that resistance to antigens could be conferred by active components found in serum. These components were termed humors (thus, humoral immunity) and later termed more generally as antibodies (Abbas *et al*, 1991). The cell-mediated specific immune response was firmly established in the 1950s when George Mackaness showed that resistance to *Listeria monocytogenes* could be transferred to other mice with cells but not serum (Abbas *et al*, 1991). With the verification of the uniqueness of the two types of specific response it was important to investigate the origins of the immune response.

In the 1960s, Good *et al* (1962) suggested that the thymus was involved in the development of the immune response. This speculation arose from the observation that thymic abnormalities in children were frequently associated with immunological deficiency disorders. As well, it was shown that removal of the thymus from mice at birth caused a decrease in circulating lymphocytes, impaired graft rejection and reduced antibody production (Roitt, 1977). Experiments in chickens by Cooper *et al* (1965) have been crucial in determining the origin of the antibody-mediated and the cell- mediated responses. Two immunologic sites, the thymus and the bursa of Fabricius, have been recognized in chickens. The thymus or the bursa in newborn chicks was removed and the chicks irradiated to inactivate the peripheral lymphocytes. Testing of the chicks after several weeks showed that bursectomy affected humoral antibody synthesis but did not affect cell-mediated immunity. In contrast, thymectomy affected cell-mediated immunity and had some effect on antibody production. Thus, primitive lymphoid cells were characterized based on the two major sub-populations. Cells dependent on the thymus and responsible for cell-mediated immunity were termed T cells. Bursa dependent, antibody producing cell were termed B cells.

In this report, the cell of interest was the T cell therefore, the subsequent portion of the thesis will deal with T cells and related topics.

T cells can be divided into two subsets, T helper and T cytotoxic cells. T helper cells are needed to help B cells in antibody production. Cytotoxic T cells are involved in cell-mediated immunity but again show the requirement of T helper cells to differentiate into cytotoxic T cells. T cells can be isolated into subsets based on two surface molecules, CD4 and CD8. Expression of CD4 and CD8 tends to correspond with functions of T helper and T cytotoxic cells respectively. In the 1980s (Mosmann *et al*, 1986) it was demonstrated that mouse CD4' T helper (Th) cell clones could be further sub-divided based on distinct patterns of cytokine secretion profiles into Th1 and Th2 cells. Effector functions can be regulated through cytokines that stimulate responses from other cells involved either directly (B cells, macrophages) or indirectly (endothelial cells) in the immune response (IR). Th1 cells produce cytokines such as interleukin (IL)-2, interferon (IFN)-γ and lymphotoxins. These factors are important in cell-mediated immunity. Th2 cells produce cytokines such as IL-4, IL-5 and IL-10, factors needed for the humoral immune responses. The existence of human equivalents was subject to much controversy because isolated T cells could produce both profiles of cytokines (de Vries *et al*, 1991; Anderson *et al*, 1990; Romagnani *et al*, 1991). However, recent studies have successfully isolated the Th1 and Th2 subsets from patients suffering from chronic diseases (Parronchiet *al*, 1991; Hannen

et al, 1991; Yssel *et al*, 1992).

The T cell recognizes antigen through a special membrane bound receptor termed the T cell receptor or TCR. Since the isolation of the cDNA for this receptor in 1984 (Yanagi *et al*, 1984; Hedrick *et al*, 1984), new perspectives have emerged in the subjects of immunology, gene regulation and protein complex assembly. Particular aspects of the T cell antigen receptor were the focus of the thesis. However, before delving into the technicalities of the thesis topic it is essential to consider, in general, the processes involved in deriving T cells and TCRs. In addition, it is important to investigate the molecules involved in helping the T cell recognize antigen.

1.0 Importance of the major histocompatibility complex molecules for T cells: An overview

The developmental history of T cells begins with pluripotent cells from the bone marrow entering the thymus. T cells with a variety of T cell receptors (TCRs) are generated but few mature and enter the peripheral blood. The majority die by apoptosis in the thymus. The death of these cells has been related to specific 'selection' processes mediated by a unique group of molecules encoded by the major histocompatibility complex (MHC). These MHC molecules are also

needed by the mature T cells to carry out their effector functions, particularly antigen recognition.

The T cell response is initiated only when the TCR recognizes antigen presented by the MHC molecules. Moreover, in most cases antigen can only be recognized by the TCR if it is presented by a self-encoded MHC molecule, *i.e.* a MHC molecule particular to that individual. Several studies have shown that a T cell, unlike a B cell, does not recognize unprocessed macromolecules; instead the TCR on a T cell recognizes processed antigenic peptides bound to the MHC molecule (Babbit *et al*, 1985; Buss *et al*, 1987).

It is therefore, important to know the genetics, structure and the functional roles of the MHC molecules to understand their involvement in the T cell selection processes.

1.1 The major histocompatibility complex molecules: The antigen presenters

The major genetic loci whose products are involved in tissue rejection after transplantation are known collectively as the major histocompatibility complex or the MHC. The MHC is divided into three regions termed class I, II, and III. The products of class I and class II genes have a similar overall structure and these molecules are crucial in triggering T

lymphocytes. Class III molecules have differing structures with activities that will not be examined here.

1.1.1 Genomic organization of class I and II loci

The human class I gene region contains three highly polymorphic loci, HLA-A, HLA-B and HLA-C and these encode the major transplantation antigens. These genes extend over 1.5 million bases of DNA on chromosome six. Recent examination of the class I region has shown the existence of additional class I genes whose products also have the potential to present processed peptides. These genes are termed HLA-E, HLA-F and HLA-G and are known as class Ib genes. They exhibit limited polymorphism and can produce what has been termed an unrestricted T cell response (reviewed in Geraghety, 1993). Out of 15 class Ib genes found in humans only HLA-E, F and G are functional.

The human class II genes are located in the HLA-D region that encodes at least six α and ten β chains. Three major encoded loci are DR, DQ and DP. The DR region expresses one α chain gene and up to four β chain genes. The α chain product associates with any of the four β chains. The DP and DQ regions each contain one coding α and one coding β chain gene. Functional heterodimers are produced when the α chain of one locus is associated with a β chain from a second locus, for example, DQα with DQβ produces a functional DQ molecule. The class II region spans about 1 million bases of DNA.

1.1.2 Structure of the class I molecule

MHC molecules are able to bind and present peptides and this function is intimately linked with the structure of these molecules. The MHC class I molecule comprises a heavy chain (45 KDa) non-covalently associated with the 12 KDa β_2-microglobulin. The heavy chain consists of three extracellular domains designated as α_1 (which is located at the N terminal), α_2 and α_3, and a transmembrane domain and a cytoplasmic tail. The extracellular portions $\alpha_{(1-3)}$ of the heavy chain are glycosylated and the degree of glycosylation depends upon the species and the MHC haplotype. The human β_2-microglobulin gene is on chromosome 15 and its product is similar to the constant region domain of immunoglobulin. The β_2-microglobulin polypeptide is non-polymorphic in humans but dimorphic in mice. It is stabilized by an intrachain disulphide.

The three dimensional structure of the class I molecule has been elucidated by X-ray diffraction. The crystal structure of HLA-A2 indicates that the class I molecule folds to form a groove. The α1 and α2 domains form a broad platform of 8 antiparallel β strands with two antiparallel α helices as the sides of the groove. The groove interacts with processed peptides and consists of ridges and pockets that allow for interactions of the class I molecules with peptide side chains. Most variations in the residues of different HLA class I molecules occur within the peptide binding groove or

in areas immediately around the groove. This variation permits the class I molecule to bind different peptides.

1.1.3 Structure of class II molecule

The product of class II genes is a heterodimer consisting of an α and a β chain. The α chain varies from 30-34 KDa, while the β chain varies from 26-29 KDa. These differences in molecular weight are due to increased glycosylation of the α chain. The extracellular regions of each chain can be divided into two domains; α_1 and α_2, and β_1 and β_2. The extracellular domains of each chain are anchored to the cell by a 30 residue transmembrane region while 10-15 residues constitute the cytoplasmic tail. With the exception of the β_2 domain, all domains are *N*-glycosylated. The α_2 and β_2 domains are similar to the class I α_3 domain and have characteristics similar to the immunoglobulin constant domains.

The three dimensional structure of the class II molecule has recently been elucidated from the crystallization of the HLA-DR1 molecule (Brown *et al*, 1993). The structure of the peptide-binding groove of the class II molecules was predicted from the class I structure. The crystallized protein bears out the predicted features; the structure is composed of anti-parallel β-strands with two anti-parallel α-helical regions on either side of the β strands. Between the two α helices there is a deep cleft. The α chain of the class II molecule contributes four anti-parallel β sheets and one α helix and

the β chain contributes the other requirements.

1.1.4 Tissue distribution, assembly and intracellular transport of class I and II molecules

Class I molecules are found on the surfaces of all nucleated cells. Heavy and light chain synthesis occurs in the endoplasmic reticulum (ER). In the ER these chains are assembled into a heterodimer which is unstable at room temperature (Townsned *et al*, 1990; Schumacher *et al*, 1990). The assembly of the heterodimer is essential to the release of the molecule from the ER (Ploegh *et al*, 1981). From the ER, the MHC class I molecules are transported to the Golgi apparatus, then to the trans-Golgi reticulum and eventually to the cell surface (Neefjes *et al*, 1988). The key to the T cell response is the recognition of the peptides presented by the MHC molecules. However, the T cell effector response, CD4' or CD8' T cell mediated effects, depends on the source of the peptides that have been generated and their presentation by the class I or class II molecules. Peptides generated from the degradation of proteins from intracellular parasites or from cells that have undergone transformation are presented to the CD8' cytotoxic T cells which then destroy the infected or transformed cell. The vast majority of peptides generated and presented by class I molecules originate from proteins located

in the nucleus or the cytoplasm and, in exceptional cases proteins from the mitochondria or ER (Henderson *et al*, 1992).

Class II molecules are expressed on a subset of cells in the immune system. This subset includes B cells, macrophages, dendritic cells, thymic epithelium and activated T cells of most species except mice. The levels of class II expressed on these cells are not static but can be upregulated or downregulated (Maffei *et al*, 1987).

For class II MHC molecules it is the α and β chains that make up the peptide-presenting complex on the cell surface. In the cytoplasm however, the class II molecule is assembled in the ER from an α and β chain and a third molecule termed the invariant chain (Lamb *et al*, 1992). *In vitro* studies using this trimer have shown that removal of the invariant chain facilitates peptide binding to class II molecules (Roche *et al*, 1990; Teyton *et al*, 1990). Therefore, one of the functions of the invariant chain may be to prevent premature peptide loading of the class II molecule. Class II molecules are transported from the ER to the Golgi complex (GC) and then to the trans Golgi reticulum (TGR). The invariant chain induces efficient transport of the class II molecules from the ER (Anderson *et al*, 1992). It has also been shown that the class II molecules are released from the ER as a complex of three α, three β and three invariant chains *i.e.*, a 9 subunit complex

(Roche *et al*, 1991). The sorting signal present on the invariant chain causes the class II molecules to be sorted through endocytic routes where they come into contact with peptides (Baake *et al*, 1990; Lamb *et al*, 1991). Class II molecules are kept in the endocytic pathways for 1-3 hours before they appear on the cell surface (Neefjes *et al*, 1990). During this time the invariant chain is degraded by proteases and the class II molecule is free to bind peptides (Neefjes *et al*, 1992). From here the molecule is transported to the surface.

Soluble antigens can be internalized through several pathways: 1) antigen binding to the surface Ig of a B cell, 2) phagocytosis by macrophages of antigen-antibody complexes and 3) fluid phase pinocytosis. The first two processes can concentrate the specific antigen for presentation to $CD4^+$ T cells (Lanzavecchia *et al*, 1988).

Therefore, the functional distinction between MHC class I and class II molecules is at the level of protein assembly and intracellular transport. These differences therefore determine the kind of antigens presented by each class. Because class I MHC molecules come into contact with nuclear and cytoplasmic peptides they are able to bind and present these antigens on their surface. Class I molecules predominantly bind peptides derived from self-proteins, viral proteins, bacterial or protozoan proteins that have penetrated

the cytosol. In contrast, the invariant chain present during assembly of the class II molecule prevents it from binding the cytoplasmic peptides. Therefore, peptide loading for class II molecules occurs in the area where the invariant chain is removed, the endocytic pathway. Class II molecules bind peptides derived from internalized proteins such as surface proteins, soluble proteins, viral, bacterial or protozoal proteins that have entered the cell because of phagocytosis. Thus the peptides presented and the pathways of the molecules to the cell surface are different for class I and II molecules.

The involvement of MHC molecules in peptide binding and surface presentation of these peptides establishes the primary step on the road to immune response by mature T cells. The specificity of the immunological response and immune elimination now depends on the T cells' ability to recognize the antigen and mobilize the correct response. The mature T cells' ability to respond is intimately related to 1) genetics that determine the amino acid sequence of the T cell receptor that is expressed and 2) the cellular selection processes that determine the fate of an immature T cell prior to becoming a mature T cell. Therefore sections 1.2 and 1.3 below will examine the genetic and cellular events responsible for the generation of T cell receptors.

1.2 T cell receptor and repertoire generation: Molecular

perspectives

1.2.1 The receptor

T cells have specific antigen receptors to carry out their functions. The T cell receptor comprises two highly variable glycoproteins, either an α and a β polypeptide chain, or a γ and a δ polypeptide chain. The TCR is noncovalently associated with five other invariant molecules γδεξη(or ζ). These five constitute the CD3 complex (Meuer *et al*, 1983; Brenner *et al*, 1985). More than 95 % of the T cells recognize antigens through the αβ heterodimer found on their surface (Groh *et al*, 1989; Inghirami *et al*, 1990; Brenner *et al*, 1986). The molecular weight (MW) of the α chain ranges from 40-50 KDa and the MW of the β chain ranges from 35-47 KDa. The difference in the MW of the two chains can be attributed to an extra N-linked carbohydrate moiety present on the alpha chain.

The general structure of the TCR is similar to the structure of immunoglobulins and TCRs are members of the immunoglobulin supergene family (Davis *et al*, 88, 90; Wilson *et al*, 1988; Raulet *et al*, 1989; Toyonaga *et al*, 1987; Siu *et al*, 1984). TCR α and β chains are each composed of immunoglobulin-like external domains that are known as the variable and constant domains. The receptor is anchored into the plasma membrane by a transmembrane peptide that contains

one (β chain) and two (α chain) positively charged residues, and a cytoplasmic tail for both the α and β chains. The transmembrane positively charged residues have been implicated in the assembly and intracellular transport of the TCR complex (Abbas *et al*, 1991).

1.2.2 Repertoire generation at the molecular level

The α chain gene is located on chromosome 14 at 14q11 and contains the δ TCR gene complex within the Vα and the Jα regions (Caccia *et al*, 1985). The TCR β gene complex is located on chromosome 7 and includes 70 Vβ and two Cβ segments. Each Cβ segment has a set of Jβ and one Dβ segments (Caccia *et al* 84, 85; Collins *et al*, 1985, Lai *et al*, 1988; Wilson *et al*, 1990; Kimura *et al*, 1986; Concannon *et al*, 1986; Robinson *et al*, 1991; Ferradini *et al*, 1991; Toyonaga *et al*, 1985; Clark *et al*, 1984; Diu *et al*, 1993). The Cβ segments are highly homologous differing only in four amino acids. Vβ and Vα are arranged into multiple families with some families having only single members and others having multiple members. Membership in a family is assigned when a sequence (either cDNA or genomic DNA) exhibits greater than 75% nucleotide homology, in the coding regions, with the members of a particular family. The V(D)J gene segments are rearranged stochastically during T cell ontogeny to form a functional V region gene, this is then transcribed to form pre-mRNA. Subsequently, the V region gene product is joined to a C

region sequence by RNA splicing to form the complete mRNA product. Additional diversities can be generated through somatic insertions of nucleotides at the junctions of germline encoded gene segments during the rearrangement process in the nucleus. The mechanisms including random αβ joining, imprecise joining, and N region additions allow the potential generation of an extremely large repertoire of approximately 10^{15} TCRs that are capable of identifying and binding a limitless universe of antigens.

The β chain locus rearranges prior to the α chain locus. The functionally productive rearranged β product also inhibits the rearrangement of the corresponding allelic locus on the other chromosome. This process is known as allelic exclusion. It was thought that α gene rearrangement was also subject to allelic exclusion, however a recent study has dramatically illustrated that this is not the case and that over 33 % of mature T cells express two TCR α-chains (Padovan *et al*, 1993).

1.3 T cell development: Selection of receptor at the cellular level

Most of the information regarding the development of mature T cells has been derived from studies in mice, particularly from mice that have undergone homologous recombination, which is the addition or deletion of important genes in the genome. It is well known that all hematopoietic cells are derived from one type of pluripotent stem cell which has the ability for self-renewal. A search has been undertaken to find the precursors for pre-T cells, that is cells that have become irreversibly committed to becoming T cells.

1.3.1 Early T-cell progenitors in human bone marrow and fetal liver

The early progenitors migrate from the bone marrow to the thymus. The migration into the thymus is not random but is the result of chemotactic signals emitted from the thymic rudiment. Once in the thymus the stem cells (now called thymocytes) begin to differentiate under the influence of the thymic microenvironment. Although the stem cells present in the thymus develop as T cells, it is evident that these stem cells are still multipotent as they have the potential to develop into other hematopoietic cells *in vitro*. Researchers have attempted to identify cell surface molecules that correlate with commitment of the pluripotent cell to become

an immature T cell. Such surface molecules would then act as markers which allow further characterization of the immature T cell.

CD34, a heavily glycosylated, 120 KDa molecule has proven to be an extremely useful marker for studying human hematopoiesis because it is expressed on pluripotent stem cells (Molgaard *et al*, 1989). Reports using the SCID mouse and *in vitro* fetal thymic organ culture (FTOC) have shown that human fetal CD34$^+$ liver and bone marrow cells can develop into T cells (Peault *et al*, 1991; Baum *et al*, 1991; Barcena *et al*, 1993; Galy *et al*, 1993). Markers such as CD2, CD5, and CD7 are expressed on immature CD34$^+$ thymocytes and on most mature T cells. These markers are often assumed to define T cell commitment, but these markers are also found on other cells (Lanier *et al*, 1992). Some reproducible evidence suggests that CD2$^+$CD34$^+$ and CD7$^+$CD34$^+$ bone marrow cells can develop into CD4$^+$CD3$^+$ and CD8$^+$CD3$^+$ T cells after *in vitro* culture with thymic stromal cells (Tjonnford *et al*, 1993). However, these studies do not provide conclusive evidence that the CD34$^+$ precursor cells are irreversibly committed to becoming T cells as no tests have been conducted to investigate whether these precursors can differentiate into other lineages.

1.3.2 Early immature T cells in the thymus

The seeded thymocytes are shown to be CD3$^-$CD4$^-$CD8$^-$ (triple negatives; TN) expressing the CD34 molecule (Terstappen *et al*,

1992). The known step irreversibly associated with commitment to the T cell lineage is the surface expression of a functionally rearranged β chain. The expression of this product is associated with progression of the cell from the $CD4^-CD8^-$ (double negative DN) to the $CD4^+CD8^+$ (double positive DP) stage and prevents further rearrangement of the other TCRβ locus. In mice it was also discovered that the β chain is linked via a disulfide bond to a 33 KDa molecule termed as 'X' and this complex is associated loosely with the CD3 γ-chain only. Evidence that the appearance of CD4 and CD8 expression comes after CD3 complex expression came from a study in which the addition of anti-CD3 monoclonal antibodies to FTOC resulted in the acceleration of the appearance of DP cells (Levelt *et al*, 1993). It was hypothesized that signals from the CD3 complex may be required for further development to the DP stage. However the appearance of CD4 and CD8 molecules on the surface of the T cell may not necessarily be subsequent to the appearance of the CD3 complex. It was shown that $CD34^+CD4^-CD8^-$ cultured with IL-7 are capable of producing the $CD4^+CD8\alpha^+$ molecules on the cell surface (Hori *et al*, 1991). Thus there appears at least two ways of inducing the change from DN to DP. In humans $TCR\beta^+X^+$ has not been demonstrated. Overall, the most recent data have generated snapshots of information for varied times but the complete picture is not available. The only definite scenario that forecasts the appearance of the DP

cells is the appearance of the TCR β chain on the cell surface.

1.3.3 Production of mature T cells: Role of positive and negative selection

Rearranged β gene expression provides the only marker for the multipotent cells' commitment to the progression toward mature T cells. The appearance of CD4 and CD8 (DP) molecules on cell surfaces occurs after the expression of the β chain. However, very little is known about the mechanisms involved in the progression from DN to the DP stage. The penultimate step to the production of single positive (SP) mature functional T cells is the production of the double positive T cells bearing the functional αβ TCR. From this population of committed cells only those cells with the correct properties go on to develop into SP mature T cells. These properties are defined by binary selection processes that occur in the thymus.

1.3.3A Positive and negative selection

The ability to recognize the universe of antigens comes from the ability of the immune system to produce T cell receptors with diverse specificities. The TCR diversity is first produced without any considerations for self or non-self antigen. However, in the generation of the wide and varied TCR repertoire by combinatorial and diversifying mechanisms, there

has to exist a mechanism whereby the T cells discriminate between self and non-self antigens.

The initial process begins with the migration of the progenitor cells into the thymus. It was noted that less than 5% of all T cells that enter the thymus later enter the ancillary lymphoid tissue (Scollay *et al*, 1980). To avoid potential threats to the self, discriminatory processes eliminate or silence self-reactive T cells and retain T cells specific for non-self. The mechanisms involved in this selection of T cells have been primarily elucidated in transgenic mice (see below), in which binary selection processes have been demonstrated (von Boehmer *et al*, 1990; Blackman *et al*, 1990). These binary processes have been termed positive and negative selection.

Positive selection is the term used when an immature double positive T cell ($CD4^+8^+$) progresses to the mature single positive stage ($CD4^+8^-$ or $CD4^-8^+$) based on the T cell's ability to recognize self-MHC molecules. Class I molecules select for $CD8^+$ subsets and Class II molecules select for $CD4^+$ subsets. T cells show dual recognition of both the antigenic peptide and the polymorphic portions of the MHC molecule. Negative selection is the process by which T cells recognizing self-peptides are deleted by apoptosis thereby yielding tolerance to self. Recent investigations have revealed that self-tolerance in the thymus is also effected by means of clonal

inactivation of self-reactive T cells (Ramsdell *et al*, 1990). Recent evidences have demonstrated that MHC, CD4 and CD8 molecules are involved in the positive and negative selection processes. Therefore, the roles of these molecules are considered in depth in the following sections.

1.4 Role of MHC in positive and negative selection: General review of the evidence from mice

Much of the information comes from the following studies: Kappler *et al*, 1987; Fowlkes *et al*, 1988; Sha *et al*, 1988; Kisielow *et al*, 1988; MacDonald *et al*, 1988; Bill *et al*, 1989; Bill *et al*, 1989; Blackman *et al*, 1989; Liao *et al*, 1989; Kaye *et al*, 1989; Blackman *et al*, 1990; Liao *et al*, 1990; Berg *et al*, 1990.

1.4.1 Initial evidence for the role of self-MHC in T cell receptor selection

The following is a generalized description of some methods used to determine the influence of the MHC. Support for the crucial role of the MHC comes mainly from two lines of evidence in mice. Initial evidence for self-MHC restriction came from studies that produced chimeric mice by lethally irradiating the mice and transferring bone marrow derived hematopoietic stem cells from mice with a partially different MHC haplotype. Mice with the haplotype A and AxB(F1) were lethally irradiated and reconstituted with bone marrow from

AxB(F1) mice. The chimeric mice were immunized and the MHC restriction of the responding T cells was determined. In a cytotoxic T cell assay it was shown that the T cells derived from the AxB mice killed virally infected target cells when the peptides were presented in the context of MHC class I of either haplotype A or B. However, chimeric mice of haplotype A reconstituted with bone marrow from AxB mice only recognized infected cells that had MHC haplotype A. This presented strong initial evidence supporting the concept of self-MHC recognition.

The second line of evidence demonstrated the importance of the thymus in self-MHC restriction. This was accomplished in mice that were chimeras for bone marrow and thymus. In one experiment, mice with haplotype A (host animals) were lethally irradiated and reconstituted with bone marrow from mice with haplotype AxB. The thymuses from the host animals were removed and different combinations of thymuses from donors of either A or B haplotype were transplanted. Before the transplantation the thymuses were lethally irradiated; this killed all the resident macrophages, dendritic and lymphoid cells with the exception of the epithelial cells, which are radioresistant. Mature T cells from host animals transplanted with thymuses of haplotype A were able to proliferate when stimulated with antigen presented by APCs with the same A haplotype. The antigen was unrecognized when presented by APCs with the B

haplotype. The converse occurred in host animals transplanted with thymuses from donors with the B haplotype where T cells recognized antigens only when presented in the context of the B MHC haplotype. These experiments reveal that T cell education to recognize self-MHC originates in the thymus.

1.4.2 Transgenic mice, self-MHC and positive and negative selection

Possibly the best demonstration of important principles involved in the production of mature peripheral T cells comes from studies of transgenic mice. The rearranged genes for the TCR αβ receptor were introduced into the pronuclei of fertilized mouse eggs and the eggs implanted into pseudopregnant females. The TCRs used in the transfection had known peptide and MHC specificities. By using tissue specific promoters, the transfected gene was constitutively expressed only in developing T cells.

The need for self-MHC and the actions of positive and negative selection were shown in a transgenic model where the blastocytes were transfected with a TCR specific for the H-Y antigen. H-Y is a self antigen abundantly expressed in many cell types in male mice but not in female mice. The TCR transgene recognized this particular antigen in the context of the H-2D^b class I MHC. Comparison of thymuses from H-2D^b male and female transgenic mice revealed that there were normal

numbers of immature double positive T cells present in both the sexes. The TCR gene was also transfected into the eggs of mice that had the H-2D^k haplotype. In the thymuses of these H-2D^k transgenic mice, no single positive mature T cells were found. Analysis of the thymic medulla and the peripheral blood in H-2D^b male and female transgenic mice demonstrated that very few mature single positive T cells were present in transgenic males whereas females had normal numbers of single positive T cells expressing this transgene. This experiment conclusively proved the existence of a double selection process and the role of the MHC molecule. The process of positive selection was seen by the absence of immature DP T cells in the H-2D^k mice *i.e.*, since the TCR was unable to recognize self-MHC it could not progress to the DP stage. Without recognition of the H-2D^k MHC these cells were eliminated. The process of negative selection, the elimination of self-reactive T cells, was observed in the male H-2D^b mice as it was found that there were very few mature T cells found in the peripheral blood but normal numbers existed in the female mice of the same haplotype. The T cells recognizing the H-Y antigen in the male mice were eliminated.

Additional evidence for the MHC requirement by T cells was obtained in experiments where either class I or class II expression was eliminated by knockout technology. Here, absence of class I retarded development of CD8$^+$ T cells and a

deficiency of class II did not allow for the development of mature CD4$^+$ T cells. Therefore, it is evident that CD8$^+$ cells need class I expression to mature and CD4$^+$ cells need class II.

From the evidence in mouse models, it has been hypothesized that at the cellular level, positive selection requires a specific interaction between a TCR and self-MHC. This interaction saves immature T cells from certain death. Since T cells selected by positive selection have affinity for self-MHC and affinity for those self-MHC molecules presenting self-peptides, the TCRs with high affinity for self-peptides must be eliminated to render the host self-tolerant (negative selection).

1.4.3 Role of the CD4 and CD8 molecules

As mentioned earlier, mature peripheral T cells can be divided into two major categories expressing CD4 or CD8 molecules. CD4 and CD8 are molecules of the immunoglobulin superfamily and function as coreceptors. CD4$^+$ T cells recognize peptides bound to MHC class II molecules while CD8$^+$ T cells recognize peptides in the context of MHC class I molecules (Swain *et al*, 1983). It has been experimentally demonstrated that these coreceptors need to interact with the same MHC molecule as the TCR for optimal signal transduction (Janeway *et al* 1988, 1992).

It was proposed that T cell surface CD4 and CD8 coreceptor molecules may be directly involved in the selection process (Swain *et al*, 1981). It was shown that the CD4 and CD8 molecules bind to the membrane-proximal domains of the class I and II molecules.

The CD4 and CD8 coreceptors provide the additional avidity necessary for the positive selection of DP T cells. T cells that show zero avidity or high avidity are deleted. Studies in transgenic mice expressing mutant MHC class I molecules that do not bind CD8 show that the MHC binding of the CD8 coreceptor is essential for positive and negative selection (Ingold *et al*, 1991; Killen *et al*, 1992). This means that CD4 and CD8 coreceptors play an important role in the association of the trimolecular complex namely TCR, peptide and the MHC.

Two models have been proposed for the role of CD4 and CD8 molecules in the selection of T cells and in the progression of the T cell from a DP to SP stage. The first model termed the instructional model postulates the following; the selection of a T cell receptor for a particular subset depends on the binding of the CD4 or CD8 co-receptors to the MHC class I or class II molecules respectively (Robey *et al*, 1991; Seong *et al*, 1992). For example, if the TCR present on the immature DP T cell recognizes class I MHC, then the CD8 coreceptor is able to bind to specific domains on the class I MHC. This then

signals the cell to downregulate the production of CD4 molecule and maintain the production of the CD8 molecule. The additional avidity given by the interaction may cause the T cell to be positively selected.

The stochastic model postulates that the CD4 or CD8 coreceptor is shut off randomly without any engagement to MHC molecules. Therefore, positive selection occurs when the correct match between TCR, MHC and the co-receptor exists (Itano *et al*, 1994; Davis *et al*, 1993). For example, if a particular TCR has a specificity for class I self-MHC but the CD8 coreceptor production is shut off randomly, then the inability of the expressed CD4 molecule to interact with the MHC class I molecule would prevent the T cell from maturing and would lead to the deletion of that particular T cell. Thus, interactions between the co-receptor and the MHC molecule can allow for the determination of some of the mature TCR repertoire.

1.5 Implications for HLA influence on TCR in humans

The observation that the murine peripheral TCR V gene repertoire can be governed by such influences has inspired researchers to investigate genetic influences, particularly HLA, on the human TCR repertoire. The human equivalent of mouse H-2, *i.e.* HLA gene regions, may play a role in positive and negative selection and therefore in determining the

peripheral blood TCR repertoire.

1.5.1 Problems associated with studying the human TCR repertoire

1.5.1A Proper representation

Unlike the mouse system, the human population cannot be genetically manipulated in the laboratory. In mouse models researchers were able to develop bone marrow and thymus chimeras. The results of these experiments were quite sound since the genetic variables and environmental conditions were properly controlled. Thus, one problem in the study of human T cell development and the TCR repertoire is the difficulty in controlling for the genetic and environmental variations between individuals.

1.5.1B Methodologies used to study TCR repertoires

There are two general methods used for investigating the mouse and human TCR repertoire. One good method for analyzing Vβ or Vα expression is the use of monoclonal antibodies specific for Vα or Vβ protein sequences on the TCR molecule. In mice the use of monoclonal antibodies (Mabs) has allowed the elucidation of basic immunological principles such as thymic selection (Kappler *et al*, 1987). Antibodies have been used to monitor T cell responses to specific antigens and

superantigens (Herman *et al*, 1990). These also have been used to prevent and treat diseases (Acha-Orbea *et al*, 1988; Chiocchia *et al*,1991; De Alboran *et al*, 1992; Kappler *et al*, 1989).

In contrast, very few Mabs are available for the human TCR V regions, despite the availability of T cell clones for more than ten years. V region specific Mabs have been difficult to generate and the existing panel identifies only 20 - 25 % of the Vβ gene segments and three of the Vα products (Callan *et al*, 1993).

Thus, the alternate method of choice has been PCR amplification of cDNA derived from peripheral blood T cells to determine the V gene repertoire. The mature T cells in the peripheral blood represent cells that have undergone the positive and negative selection processes. The following discussions will briefly consider the kinds of PCR techniques used in TCR repertoire identification and examine the theoretical procedures needed to accomplish quantitative PCR.

1.5.2 Summary

The main points of the above introduction can be summarized;

☞ T cells recognize antigens by a membrane receptor known as the T cell receptor or TCR.

☞ Molecular diversities allow a large and diverse TCR repertoire to be generated in the thymus.

☞ From these large number of T cells only those recognizing self-MHC are selected for by a process known as positive selection.

☞ T cells that recognize self antigens are negatively selected and are eliminated.

☞ Both positive and negative selections are mediated by class I and class II MHC molecules within the thymus.

☞ Evidence for the role of the MHC molecules comes from controlled experiments done in mice.

☞ Researchers have attempted to identify the effects of HLA on the human TCR repertoire.

☞ A common protocol to identify the expressed TCR in peripheral blood is to analyze the cDNA from T cells using PCR techniques.

1.6. Polymerase chain reaction for detection of TCR expression

The polymerase chain reaction (PCR) has been a powerful tool for the study of TCR usage. However, studying the TCR repertoire by PCR has proven to be tremendously challenging. The method is limited by the tremendous diversity of the V region repertoire. More than 70 Vα and 70 Vβ gene segments have been characterized (Wilson *et al*, 1988; Kimura *et al*, 1987; Ferradini *et al*, 1991) and have been classified into at least 29 Vα and 24 Vβ families (Diu *et al*, 1993). This makes it difficult to choose a set of primers that will allow the amplification of all possible TCR V region sequences. Nevertheless, several methods have been established to study the repertoire. Most of the methods along with their disadvantages will be discussed briefly. The method used in this study will be dealt with briefly here and in more detail in the methods section.

1.6.0 Four PCR methods

The four most frequently used methods are anchor PCR, PCR using consensus primers, inverse PCR, and sequence-specific (quantitative) PCR.

1.6.1 Anchor PCR

Anchor PCR involves amplification of cDNA from TCR transcripts using only a single 3' specific primer (Loh *et al*,

1989). The method has been employed to study V region TCR transcripts. A 5' poly-G (guanosine) tail was added to peripheral blood T cell cDNA which was then amplified using a 5' poly C (cytosine) and a 3' primer. Because of the modification of the 5' end, the variability at the 5' end was not an obstacle to PCR amplifications. V gene usage was then studied by cloning and sequencing of amplified products. Anchor PCR was successfully used in elucidating the structure of the rat Vβ genes (Smith *et al*, 1991). The obvious advantage of this method is that with a single PCR reaction all of the TCR cDNA transcripts are identified. The disadvantages are: 1) complication of the procedure due to enzymatic modifications of the cDNA, 2) increased risks of contaminating the PCR reactions by the additional technical modifications, 3) possible interference of residual poly-G primers with the amplification of target cDNA, 4) the requirement for identification of the V region repertoire that all clones obtained be sequenced, and 5) the possibility that rare mRNA may be missed (Uematsu *et al*, 1991).

1.6.2 Consensus primers based PCR

Use of consensus primers to determine the V region repertoire has been less extensive than use of anchor PCR or quantitative PCR. This method uses a degenerate primer that can amplify all of the TCR transcripts present in a sample

(Broeren *et al*, 1991 Howell *et al*, 1991). The degenerate primer is designed to recognize points of homology in all TCR transcripts as substitutions are made in the primer to account for interfamilial diversities. The disadvantages of this method are 1) it is not known if the degenerate primer amplifies all TCR transcripts, 2) since the primer is degenerate, greater homologies with some TCR cDNAs will bias the amplification towards the sequences with increased homologies to the primer, 3) the primer may cross-react with non-specific transcripts, thus when investigating important disease tissues (brain plaque, synovial fluid) false results could be produced and 4) to identify V regions, sequencing has to be done. This method does not permit rapid analysis.

1.6.3 Inverse PCR

Inverse PCR is used after synthesized cDNA has been circularized by enzyme modification. The cDNA is then amplified in opposite directions rather than amplifying the region between the two primers which is usually done for conventional PCR (Uematsu *et al*, 1991). This system has many of the same disadvantages as anchor PCR and efficiency depends on the circularization of the cDNA. Again, since cloning and sequencing are required, rapid analysis is difficult.

1.6.4 Quantitative PCR

Sequence-specific primer PCR involves the amplification of a TCR cDNA sequence by primers flanking the 5' and 3' ends.

The 5' sense primer is specific for the variable region of a TCR β chain while the 3' anti-sense primer is specific for the constant region of the β chain. This method was first described by Choi *et al* (1989) for the study of peripheral blood T cells stimulated by *Staphylococcus aureus* enterotoxins. That study employed 5' sense primers specific for 20 known Vβ families. Since then, other studies have used primers specific for the Vα region to determine the Vα repertoire (see, for example, Oksenberg *et al* 1990). Vβ primers have been used, for example, to study T cell clones reacting with myelin basic protein (Wucherpfennig *et al*, 1990), and have been used to study TCR usage in rheumatoid arthritis, e.g. Davey *et al*, 1993. Choi *et al* (1989) used radiolabelled primers to quantitate the expression of each Vβ family and therefore this PCR method was termed quantitative PCR. With the quantitative PCR technique researchers mentioned above and many others have been able to quantitate the TCR Vβ or Vα region transcripts from many sources.

The advantages of this system are 1) the relative ease of use, 2) rapid analysis and 3) transcripts of interest can be sequenced later. The disadvantages are 1) the use of many primer sets to amplify the families, 2) the inability to detect previously unidentified Vβ families and 3) substantial financial investment.

1.7 Essentials of quantitative PCR

It is pertinent to illustrate the theoretical steps involved in a quantitative PCR (qPCR) reaction as this is the principle technique employed in this study and in many other studies designed to explore the TCR repertoire with known Vβ primers. Figure 1.7a shows all the steps essential in determining the TCR repertoire from a peripheral blood lymphocyte (PBL) sample by this method. The details of the techniques including limitations are further discussed in the methods section.

1.7.1 Use of reference templates

It is difficult to quantitate the expression levels of Vβ or Vα transcripts due to the nature of the PCR reaction itself. Many parameters affect not only the amplification reaction but also the validity of results obtained. Slight shifts in temperature or concentrations of nucleotides, primers or buffers can significantly bias the results. Despite these considerations, qPCR is used by many researchers to measure TCR levels in samples. In order to control for these variables, use of a reference template was introduced by Choi *et al* (1989). This approach involves the use of two additional primers, 5' sense and 3' antisense primers specific for the TCR constant α region and termed Cα primers. Cα primers are added to each reaction tube along with

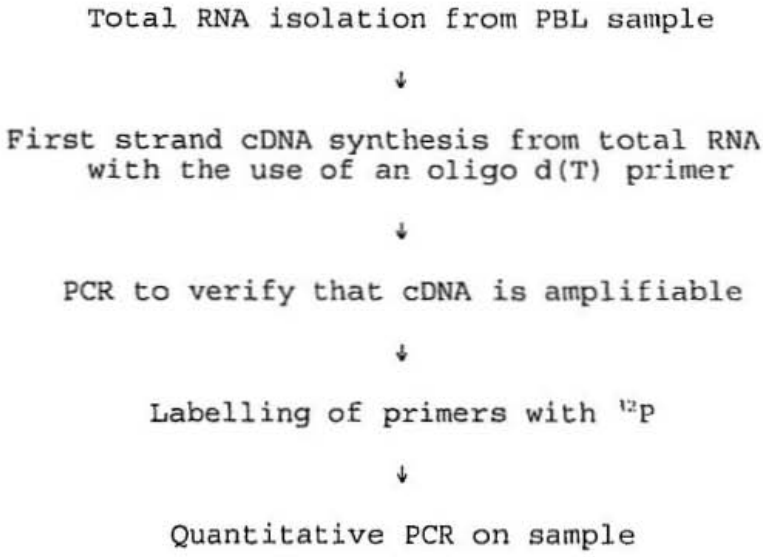


FIGURE 1.7a

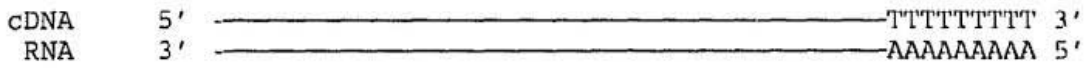
This figure shows the overall steps required for a qPCR

the Cβ and Vβ specific primers. The Cα primer pair is the same for all reaction tubes and it is assumed that the quantity of the α chain transcripts is constant for all PCR reactions involving a particular sample. The rationale for the use of Cα primers is the following: since the TCR is expressed as a heterodimer made of the αβ glycoproteins, the cytoplasm of a mature T cell must have mRNA transcripts for both the α and β chains. As there is only one Cα constant region gene segment available in the germline then, regardless of the Vβ segment associated with the α chain, the Cα gene segment will always be the same. Thus the Cα gene segment functions as a reference that allows for the quantitation of the variable β gene segments present in the peripheral blood. This reference template also functions as an indicator of the reaction conditions in each tube. The theoretical steps for this approach are shown in figures 1.7 b and c.

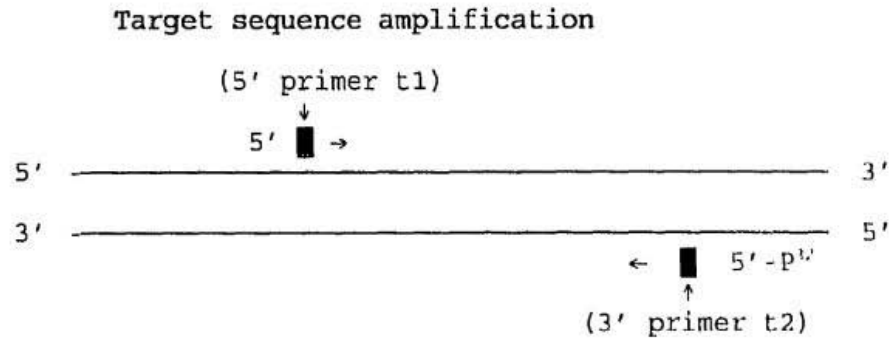
1.7.2 Monoclonal Antibodies versus qPCR

Monoclonal antibodies can be used to quantitate the level of expression of TCR protein on the cell surface. One criticism of mRNA based TCR repertoire analysis is that the level of RNA may not correlate with cell surface expression of the TCR. A second criticism is that small populations of activated cells could contribute large amounts of RNA to the total RNA levels thus biasing the repertoire analysis.

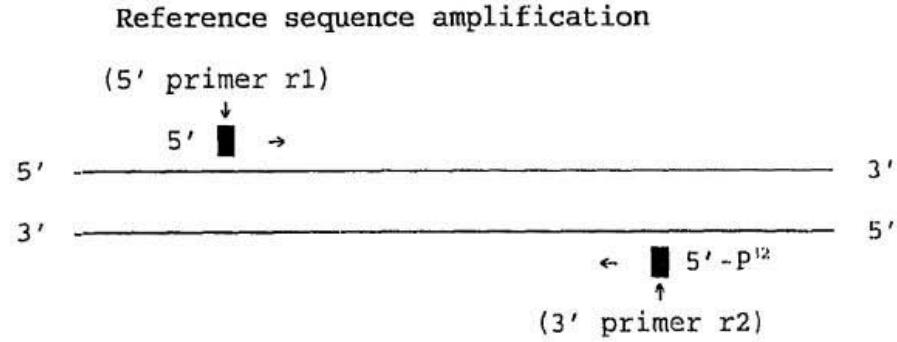
Step 1. After the completion of the first strand synthesis there exists an heteroduplex consisting of cDNA:RNA.



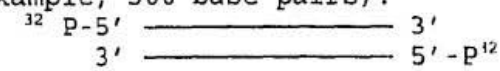
Step 2a. Primer pair (5′ and 3′ specific) specific for this sequence are added into the reaction vessel.



Step 2b. Primer pair is also added for a reference sequence cDNA also present in the same sample. The quantity of this reference sample is always constant. Both the reference and target are co-amplified in the same tube.



Step 3. Target primers t1 and t2 produce an amplificant of certain size (for example, 300 base pairs).



Reference primers r1 and r2 produce a certain size fragment (~600 bps).

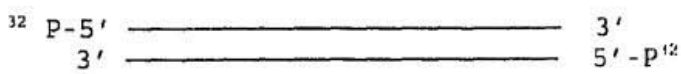
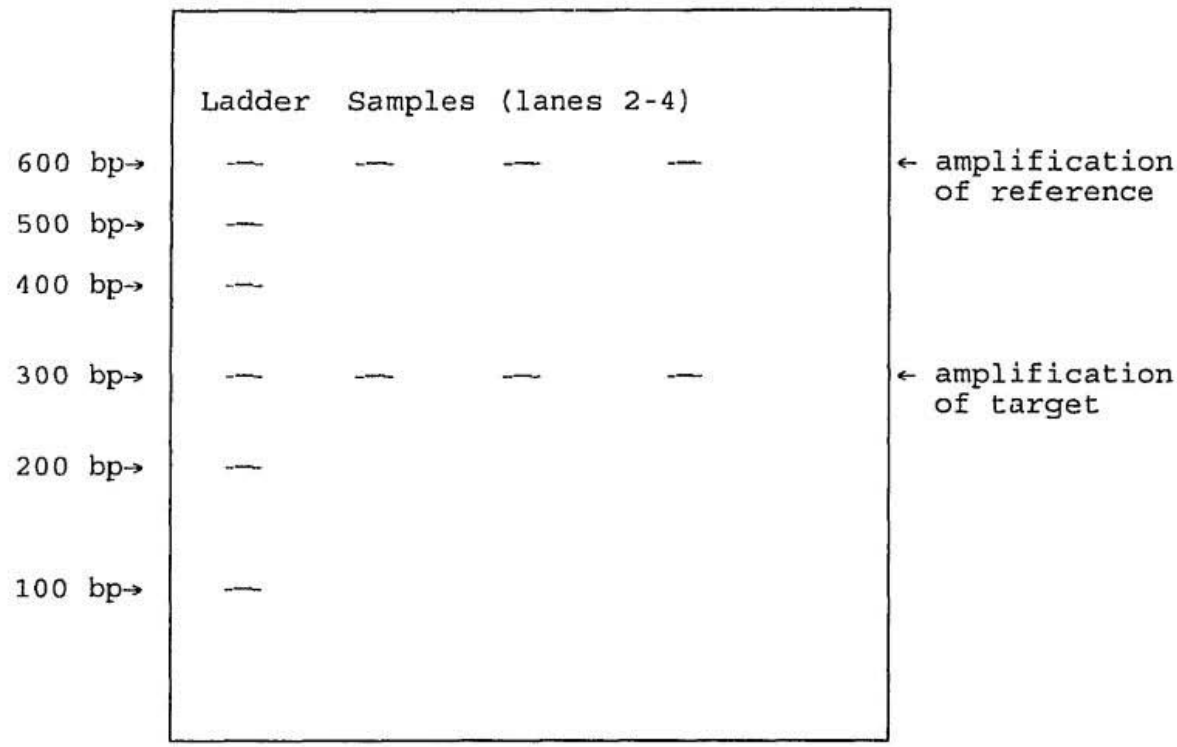


Figure 1.7b
Diagramatic representation of how qPCR is accomplished.

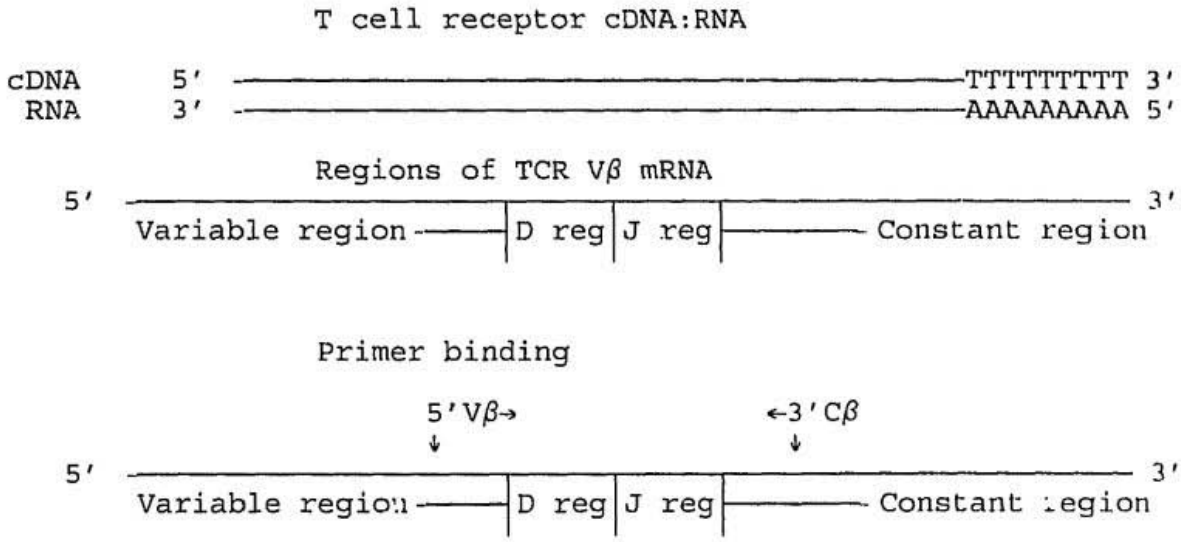
Step 4. Products are electrophoresed on 2% agarose gel and visualized by ethidium bromide staining.



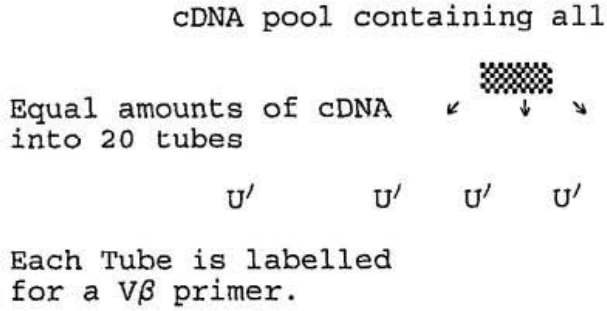
Step 5. The products are excised and counted in scintillation fluid. The counts for the target are normalized to those of the reference.

Normalized Amount present = Target counts / reference counts

Figure 1.7b cont.



Step 1. Peripheral blood TCR cDNA contains many different transcripts representing various Vβ families and their members. Therefore, a) primers specific for 20 families of TCR Vβ are aliquoted into 20 different tubes, and b) equal amounts of cDNA are delivered into each tube.

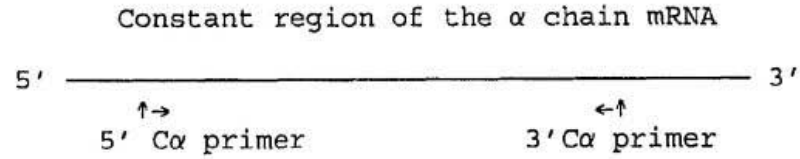


Equimolar amounts of Vβ primers are added to each tube. The 5′ primer represents t1 and 3′ primer, t2. Vβ primers are t1 and Cβ primer represents t2 in figure 1.7b.

Figure 1.7c

Diagramatic representation of qPCR analysis of T cell receptors as described by Choi *et al* (1989).

Step 2. Reference primers are added to each tube. The reference primer for TCR analysis co-amplifies constant region of the alpha chain in the same tube.



Step 3. To quantitate the 3' Cα and 3' Cβ primers are radiolabelled (fig 1.7b).

Step 4. Same as figure 1.7b step 4.

Step 5b. Gels are dried, exposed to x-ray film and excised for counting. Vβ counts are normalized with counts for Cα.

$$\text{Ratio present} = \frac{\text{Vβ counts for a specific Vβ family}}{\text{Cα counts in that same tube}}$$

The entire primer set used represents the ratios of most of the Vβ families found in the sample. Therefore the ratios are added and a proportional profile is calculated;

$$\text{Percent Vβ} = \frac{\text{ratio present of a particular Vβ}}{\sum \text{ of ratio counts from Vβ1-20}} * 100$$

Figure 1.7c cont.

TCR mRNA transcript expression in the cytoplasm has been compared with the cell surface expression of TCRs using available monoclonal antibodies. The results indicate that the relative proportions calculated from amplified products reflect the quantity expressed on the cell surface (Choi *et al*, 1989; Rosenberg *et al*, 1992; Wong *et al* 1993). RNAse protection experiments involving the mouse Vβ repertoire showed no discrepancy between RNA and protein levels (Singer *et al*, 1991; Okada *et al*, 1989). Studies have shown that in normal individuals the level of activated cells is less than 1 % and that activation of T cells does not always increase the level of TCR β mRNA production (Paillard *et al*, 1988 and 1990). These results suggest that significant skewing of the repertoire due to increased levels of mRNA production would be highly unlikely.

1.8. Genetics, HLA and TCR expression in humans

In humans, many qPCR studies have implicated particular TCR V gene families in the pathogenesis of various diseases. The role of HLA molecules in determining the TCR repertoire has also been investigated. As observed in mice it is seen that MHC molecules can play a role in determining the peripheral repertoire but the details of this role have been more difficult to demonstrate because humans are outbred and HLA genes are highly polymorphic.

To control for genetic differences between individuals, various studies have analyzed the TCR repertoire of identical twins and large sibling families (Hawes *et al*, 1993; Akolkar *et al*, 1993; Lovebridge *et al*, 1990; Davey *et al*, 1994, Akolkar-Gulwani *et al*, 1995, Hawes *et al*, 1995). Only a small number have used quantitative PCR (Hawes *et al*, 1993; Alkokar *et al*, 1993; Lovebridge *et al*, 1990). Other studies have used the available monoclonal antibodies (min of 4 and max of 7) to study the more than 24 Vβ families and their associated members (Davey *et al*, 1994; Akolkar *et al*, 1991). Some studies have used anchor PCR to investigate the TCR repertoire in families and identical twins (Loh *et al*, 1989; Rosenberg *et al*, 1992; Vissinga *et al*, 1994)

1.8.0 Quantitative PCR studies investigating the effects of genetics and HLA influences on the human TCR repertoire

The results and the conclusions of three important studies which employed qPCR and were published before or at the time the present study began are presented below.

1.8.1 Lovebridge *et al*, 1991

One of the first studies addressing a genetic contribution was by Lovebridge *et al* (1991). Five monozygotic twin pairs and two pairs of unrelated individuals were investigated. PCR amplifications of cDNA from peripheral blood lymphocytes were performed. The products were run on 2 % agarose gel, blotted to nitrocellulose and probed with a radioactively labelled TCR Cβ-specific oligonucleotide. The Vβ amplification was measured by densitometry and the results were subjected to two-way analysis of variance. The rationale behind the study was that if genetics has played a role in determining the repertoire, then the scores obtained for twin pair comparisons should be more similar than for those obtained by comparing unrelated individuals. The data showed that the level of discordance for two pairs of unrelated individuals was significantly greater than for the five pairs of twins. The authors concluded that the TCR repertoire was primarily generated by genetically determined selection processes. In this study the contribution of HLA was not investigated.

1.8.2 Hawes *et al*, 1993

Four pairs of randomly chosen monozygotic twins were investigated for a genetic contribution to the TCR repertoire. The peripheral blood lymphocytes were sorted into subsets based on the expression of the CD4 or CD8 coreceptors on T cell surfaces. The cells were stimulated with mitogen (PHA) and analyzed for Vβ and Vα expression. The relative expression of each Vβ was calculated and the values for each twin pair were compared. Similarities in the overall patterns between individuals were determined by the correlation value R^2. To facilitate comparisons between unrelated individuals, twin pairs were cross-compared. For example, if twin pair one consisted of members A1 and A2, and twin pair 2 consisted of members B1 and B2 then R^2 comparison of A1 with A2 (A1/A2) and B1/B2 provided scores for 'related individuals' comparisons. Similarly, R^2 comparisons of A1/B1, A1/B2, A2/B1 and A2/B2 provided the scores for 'unrelated individuals' comparison. Statistical comparisons revealed that the R^2 values for the 'related individuals' comparisons were closely correlated while 'unrelated individuals' comparisons had R^2 values that had a greater range. These data allowed the authors to conclude that genetic factors play a role in determining the TCR repertoire. This study also investigated specifically the effect of HLA. It was found that the TCR repertoire correlations for HLA identical but unrelated individuals were

more concordant than for HLA nonidentical individuals. This allowed further the conclusion that the genetic contribution to the repertoire is shaped by HLA dependent mechanisms.

1.8.3 Akolkar *et al*, 1993

Akolkar *et al* (1993) studied large multisibling families. From pairwise comparisons it was found that HLA identical siblings had more similar patterns of V segment frequencies than either totally mismatched or haplo-identical siblings. However, studies of randomly selected, HLA-matched but unrelated individuals did not show any evidence for an HLA based effect on the generation of the TCR repertoire. The authors also claimed that certain Vβs were skewed towards either CD4' or CD8' T cell subsets indicating some sort of preferential recognition involving class I and class II HLA molecules. Akolkar *et al* (1993) used a calculation termed 'delta' scores to calculate the differences in the relative usage of Vβ gene segments. Two individuals were analyzed by pairwise comparison for all parameters studied and the absolute sum of the differences or delta score indicated the level of similarity or difference. Low 'delta' scores indicated individuals with a high degree of similarity whilst a high 'delta' score indicated large differences between the individuals. From their data, the authors concluded that genetics, and especially HLA, play a significant role in

shaping the TCR repertoire in humans.

1.9 Summary

The following is the summary of the major points:

☞ To investigate the TCR repertoire from the peripheral blood in humans, researchers frequently use PCR methods as very few antibodies are available.

☞ The quantitative PCR method is frequently used to assess the TCR repertoire.

☞ Three of a number of studies using the quantitative PCR method specifically deal with the issue of genetics (particularly HLA) and the TCR repertoire.

Chapter 2

RESEARCH HYPOTHESIS, RATIONALE AND OBJECTIVES

The initial intent of this study was to investigate the T cell receptor repertoire from the peripheral blood of patients suffering from multiple sclerosis. The preliminary work required that the contribution of genetics and HLA to the T cell receptor repertoire in healthy individuals be estimated to facilitate the selection of a representative control group. A literature search revealed that very few studies had addressed the issue of genetics and the TCR repertoire. Therefore, it was decided that this study would address this issue in depth in healthy individuals.

2.1 Research hypothesis

Based on published literature it was hypothesized that genetics play the main role in shaping the human peripheral TCR Vβ repertoire and that HLA molecules are the major genetic factors involved.

2.2 Research objectives

This study was designed to investigate the effects of genetics and specifically HLA on the TCR Vβ repertoire in the peripheral blood of identical twins and individuals from large families. The focus of the research was:

- Investigation of the TCR Vβ repertoire in $CD4^+$ and $CD8^+$ T subsets in identical twins by the qPCR method. This approach was used to determine the role of genetics in shaping the TCR repertoire.

- As most of the published HLA-TCR studies deal with T cell subsets, it was important to know if a genetic effect also could be detected in the total TCR repertoire from identical twins.

- Analysis of the total T cell repertoire from large multisibling families to determine the contribution of HLA to the TCR repertoire.

2.3 Rationale

It was decided that the thesis would focus on the TCR Vβ repertoire for the following reasons;

- The generous gift of TCR Vβ primers from Dr. Elizabeth Hodges (Tenovus Laboratory, Southampton General Hospital, Southampton UK).

- The lack of detailed reports on the role of genetics, including HLA, on the TCR Vβ repertoire.

- The focus in the literature on the Vβ repertoire rather than the Vα repertoire.
- The opportunity to standardize a powerful technique which had future potential in the investigations of diseases involving T cell responses.

Chapter 3
Materials and Methods

Seven healthy pairs of monozygotic twins of varying ages from the same geographical location (St. John's, Newfoundland) were studied. Three large families consisting of six, seven, and ten members were also analysed. All subjects were HLA class I and class II typed by serology and the HLA results are shown in table 3.1.

3.1 Preparation of lymphocytes and T cell subsets, and mitogen stimulation

3.1.1 Ficoll-Isopaque isolation of lymphocytes

Between 10 and 30 ml of blood were collected from volunteers in EDTA vacutainer tubes. The blood was diluted 1:1 with PBS at room temperature and layered over Ficoll-Isopaque. The tubes were centrifuged at 2000 RPMs for 25 minutes at room temperature. The lymphocyte layer was removed with a plugged, autoclaved long stemmed glass pipet and the cells washed twice with HBSS. Each wash was followed by a 10 minute spin at 2000 RPMs. After the final wash, the pelleted cells were resuspended in RPMI 1640 with 10% FCS. The cells were aliquoted and stimulated with PHA for 3 days at 37°C in 5% CO_2.

Table 3.1

Class I and II HLA haplotypes of all individuals used in this study

Individuals
Family FH — Haplotypes

	A	B	Bw	C	DR	DRw	DQ
FHLHM	3, 3	7, 35	6, 6	4, 5	1, 11	52,53	1, 3
FHDH	2, 2	44,62	4, 6	3, 5	4, 4	53,53	3, 3
FHLH	2, 3	7, 44	4, 6	5, 5	1, 4	53,53	1, 3
FHPH	2, 3	7, 44	4, 6	5, 5	1, 4	53,53	1, 3
FHKH	2, 3	62,35	6, 6	3, 4	4, 11	52,53	3, 3
FHCH	2, 3	44,35	4, 6	4, 5	4, 11	52,53	3, 3

Individuals
Family FG — Haplotypes

	A	B	Bw	C	DR	DRw	DQ
FGAGM	25,31	35,60	4, 6	3, 4	4, 4	53,53	1, 3
FGGG	1, 3	8, 44	4, 6	3, 4	3, 4	52,53	2, 3
FGAG	3, 25	44,35	4, 4	3, 4	4, 4	53,53	1, 3
FGJG	1, 25	8, 35	4, 6	4, 4	3, 4	52,53	1, 2
FGSF	1, 31	8, 60	6, 6	3, 3	3, 4	52,53	2, 3
FGNaG	1, 31	8, 60	6, 6	3, 3	3, 4	52,53	2, 3
FGCM	1, 31	8, 60	6, 6	3, 3	3, 4	52,53	2, 3
FGDB	3, 25	44,35	4, 4	3, 4	4, 4	53,53	1, 3
FGNG	1, 25	8, 35	4, [illegible]	4, 4	3, 4	52,53	1, 2
FGWG	1, 31	8, 60	6, [illegible]	3, 3	3, 4	52,53	2, 3

Table 3.1 cont.

Individuals
Family FK — Haplotypes

	A	B	Bw	C	DR	DRw	DQ
FKAKM	2, 3	14,18	6, 6	3, 8	2, 3	52,53	1, 2
FKHHK	1, 1	8, 57	4, 6	7, 8	6, 7	52,53	1, 3
FKMAK	1, 3	14,57	4, 6	8, 8	2, 7	53,53	1, 3
FKJPK	1, 3	14,57	4, 6	8, 8	2, 7	53,53	1, 3
FKUK	1, 3	14,57	4, 6	8, 8	2, 7	53,53	1, 3
FKRK	1, 3	14,57	4, 6	8, 8	2, 7	53,53	1, 3
FKHMK	1, 2	57,18	4, 6	3, 8	3, 7	52,53	2, 3

Twin Pairs — Haplotypes

	A	B	Bw	C	DR	DRw	DQ
MB1/MB2	3, 30	44,18	4, 6	4, 5	3, 4	52,53	2, 3
SR1/SR2	2, 3	7, 60	6, 6	3, 7	6, 6	52,52	1, 1
LR1/LR2	2, 24	7, 17	4, 6	7, 7	7, 12	52,53	3, 3
SN/SG	2, 2	51,14	4, 6	8, 8	11,7	52,53	2, 3
SC1/SC2	1, 24	7, 8	6, 6	7, 7	15, 3	52,52	1, 2

Note: Twin pair BB/TB were not available for HLA analysis.

3.1.2 Isolation of $CD4^+$ and $CD8^+$ subsets.

$CD4^+$ and $CD8^+$ T cell subsets were separated from freshly isolated blood lymphocyte preparations (section 3.1.1) from three pairs of identical twins by positive selection using magnetic beads coated with anti-CD4 and anti-CD8 monoclonal antibodies (DYNAL Inc). Magnetic beads are provided at 1.4 X 10^{11} beads/ml. The recommended ratio of bead:cell for mononuclear cell suspensions is 3:1 (DYNAL technical data sheet). For this experiment the ratio used for isolation was 4:1 for both anti-CD4 and anti-CD8 magnetic beads. The steps taken to isolate $CD4^+$ and $CD8^+$ T cells are described in detail by DYNAL. Following the isolation of these T cell subsets by positive selection, the magnetic beads were removed from the T cell surface using DETACHaBEAD (DYNAL). The washed cells were resuspended in RPMI 1640 with 10% FCS and stimulated with PHA for three days at 37°C in 5% CO_2.

3.1.3 Methodological concerns

3.1.3A Crossover contamination

Cross-contamination of blood samples and of lymphocyte populations was excluded. Solutions were pre-aliquoted into centrifuge tubes prior to handling the blood and gloves were washed with 70% ethanol after each sample. Each sample was

capped except when actually being experimentally manipulated. PHA was added with aerosol resistant filtered tips. A new tip was used for each sample being processed to prevent crossover and PHA contamination.

3.1.3B Positive selection

The recommended protocol (DYNAL) for the isolation of CD4' and CD8' T cells suggested the positive selection of one subset be followed by the selection of the other. However, this could lead to a contamination of CD4' T cells by CD8' T cells or contamination of CD8' T cells by CD4' T cells since some residual antibody-coated magnetic beads may remain trapped in the T cell pellet. To exclude this possibility, CD4' and CD8' T cell subsets were isolated from two separate aliquots of the same cell suspensions.

3.1.3C Mitogen stimulation

One potential area of concern in quantitative PCR analysis is the use of mitogens and their effect on the lymphocyte population. A study published by Wong *et al* (1989) indicated that PHA tends to skew the repertoire of the stimulated lymphocyte population. The primary concern with PHA stimulation is that it may skew the TCR repertoire

preferentially or randomly.

3.1.3D Method to investigate Vβ skewing due to mitogen

To address this issue the following study was undertaken in which quantitative PCR was performed on peripheral blood T lymphocytes from three individuals, FS, FHDH and FHLH. For the individual FS, 40 ml of blood were divided into four 10 ml fractions. Total RNA was extracted from lymphocytes before and after PHA stimulation. The lymphocytes were stimulated for three, five and seven days. For individual FHDH, RNA was extracted from unstimulated lymphocytes and from a three day PHA stimulation experiment. An earlier three day stimulation had been done four months before. Lymphocytes isolated from individual FHLH were fractioned into two separate tubes and the T cell receptor profile investigated after three days of stimulation for both fractions.

3.2 RNA preparation and cDNA synthesis

Total RNA was extracted from stimulated cells by the RNAzol B method (Cinna/Biotex Laboratories, Houston, TX). One to two micrograms of total RNA were used for oligo d(T) primed synthesis of first strand cDNA (First Strand cDNA Synthesis Kit, Pharmacia) to yield a total reaction volume of 33 µl.

3.2.1 RNA preparation

Guanidinium Thiocyanate is one of the most effective protein denaturants and is also a strong ribonuclease inhibitor. The RNAzol B method is a recently described method that employs a single step total RNA extraction method replacing the lengthy procedures used previously (Cox *et al*, 1968). RNAzol promotes the formation of complexes of RNA with guanidinium and water molecules. It annuls hydrophilic interactions of DNA and proteins thereby rendering these molecules insoluble in the aqueous phase. The RNA however remains in the aqueous phase and can be easily extracted. Total RNA can be removed and precipitated with high purity and the procedure is faster than earlier described methods (Chomczynski *et al*, 1987).

Cells obtained after PHA stimulation were centrifuged in the same tubes at 2000 RPM for 10 minutes and the supernatant discarded. The use of the incubation tubes for RNA extraction decreased the chance of contamination and loss of cells. Two 500 µl aliquots of RNAzol B were added to each tube. The addition of the first 500 µl of RNAzol served to lyse the cells. Solubilization of the RNA was effected by passing the lysate a few times through the pipet tip. The second 500 µl

aliquot was used to wash the walls of the tube. To the 1.0 ml of RNAzol lysate was added 100 µl of chloroform ($CHCl_3$). The sample was vigorously shaken for 15 seconds then left on ice for 5 minutes. After the addition of chloroform the sample formed two phases, a lower blue chloroform phase and a colourless upper phase. DNA and proteins are found in the interphase and in the organic (chloroform) phase. To achieve distinct separation of the phases, the sample was spun at 12,000 x g at 4°C for 15 minutes. Only 80-85% of the aqueous phase was removed to prevent contamination by the interphase. The aqueous phase was placed into a 1.5 ml sterile Eppendorf tube and cold isopropanol added at a 1:1 ratio. The sample was then left at -20°C overnight. The sample was vortexed at 12,000 x g at 4°C for 15 minutes and a whitish RNA precipitate could be seen at the bottom of the tube.

After the supernatant was discarded, the pellet was washed twice with 500 µl of 75% ethanol. The pellet was dried by the removal of excess alcohol by repeated pipetting and centrifugation. This ensured a rapid drying time (less than one minute), low risk of contamination and prevented excess drying of the pellet. The recommended procedure by the manufacturer was to vacuum dry for 15 minutes but this increased the drying time, increased the risk of contamination

since the tubes were open in the vacuum dryer and, as there was no control over the drying process, the RNA product could be damaged. The pellet was redissolved in 8.0 µl of DEPC treated deionized water. It was kept on ice for 30 minutes to facilitate the solubilization process and then placed at -70°C until further use.

3.2.2 First strand cDNA synthesis

First strand cDNA was synthesized from total RNA by the Gubler-Hoffman method (Gubler *et al*, 1983). First strand cDNA was synthesized with an oligo-dT primer that binds to the poly-A tail of mRNA. This produces a heteroduplex of cDNA:mRNA which was used for PCR reactions.

The isolated total RNA was quantitated and 1-2 µg of total RNA was aliquoted into 500 µl microcentrifuge tubes. The volume of total RNA aliquoted was brought up to 20 µl volume with DEPC treated deionized water. The sample was then heated to 65°C for 10 minutes and cooled on ice for 5 minutes. From the first strand cDNA kit (Pharmacia) a cocktail was made per sample containing 11 µl of Bulk Strand mix (which contained cloned FPLC *pure* Murine Reverse Transcriptase, RNAguard, RNAase/DNAase-Free BSA, dATP, dCTP, dGTP, dTTP), one µl of oligo dT primer (0.20 µg/µl) and one µl of DTT. The cocktail was added to the cooled RNA sample anc incubated at 37°C for 60 minutes. The tube was then placed at -70°C until further use.

3.2.3 Methodological concerns

The main concern was to maintain a practical protocol that ensured no crossover contamination could occur during RNA

extraction and purification, and during cDNA synthesis. To prevent contamination, solvents were pre-aliquoted and different sterile aerosol resistant filtered tips were used for each tube. In addition all microcentrifuge racks were washed with detergent and ethanol after each use to once again prevent cross-over contamination.

3.3 PCR amplifications and primer labelling

3.3.1 PCR amplifications

Amplifications were performed as described previously by Choi *et al* (1989) with modifications. The total volume of cDNA needed for an experiment was added to a master mix to ensure that each reaction tube contained equal amounts of cDNA, *i.e.* one µl of cDNA obtained from the first strand reaction (section 3.2.2) was aliquoted into each reaction tube containing one of the twenty-three Vβ primers. The twenty-three Vβ family specific oligonucleotide primers used for PCR amplifications were a gift from Dr. Elizabeth Hodges (Tenovus Laboratory, Southampton General Hospital, UK) and are derived from the following sources; Vβ primers 2, 3, 4, 5, 7, 9, 10, 11, 12, 14, 16, 18, 19 from Wucherpfennig *et al* (1990) and Vβ primers 1, 5.1, 5.2, 6, 8,13.1, 13.2, 15, 17, 20, 3'Cα antisense, 5'Cα sense and 3'Cβ antisense primers from Choi *et al* (1989). Nineteen of the 23 primers are family specific, that is they recognize and amplify all members of a Vβ family. Four of the Vβ primers (5.1, 5.2, 13.1 and 13.2) are specific for particular Vβ family members. Twenty-three tubes were separately aliquoted with 1 µL (0.165 µM) of each of the 23 Vβ primers. All other reagents were added to the master mix

to maintain the concentrations at a constant level. Expression of each Vβ family was quantitated by co-amplification of 3'Cα antisense and 5'Cα sense primers present in each reaction tube. The final concentrations of the primers in the total reaction volume were 0.165 µM for 5'Cα sense primer, 0.15µM 3'Cα antisense primer, 0.015µM (10^5 CPM) ^{32}P labelled 3'Cα antisense primer, 0.15µM 3'Cβ antisense and 0.015µM ^{32}P 3'Cβ primers. The reaction volume for each amplification was 50µl and contained 2.5 units of Taq DNA polymerase (Promega Corp., Madison, WI), 1 x Promega buffer (supplied), 240µM dNTPs and 1.5mM MgCl . A twenty-fourth tube represented the negative control and to this tube no cDNA was added. The samples were amplified on a Cetus/Perkin-Elmer 480 thermocycler under the following conditions: 95°C melting, 55°C annealing and 72°C extension for 1 min each for 30 cycles.

3.3.1A Vβ Nomenclature

Recently the World Health Organization (WHO) issued a new nomenclature to represent the various Vβ families (WHO-IUIS, 1993). In this study, the WHO nomenclature was not adopted in order to facilitate easier comparisons with most of the relevant published literature in which the new nomenclature

had not been adopted. However, to facilitate drawing of the figures with Jandel's Sigma Plot software , it was necessary to use the terms 5S1, 5S2-3, 13S1 and 13S2 (WHO-IUIS, 1993) for Vβs 5.1, 5.2, 13.1 and 13.2.

3.3.1B Primer Labelling

The initial concentrations of the primers used for the PCR reactions were 8.25 µM for Cα forward primer and Vβ primers, 7.5 µM for Cβ and Cα reverse primers and 0.75 µM for ^{32}P labelled Cβ and Cα reverse primers. The radioactively labelled reverse primers were added at one-tenth the concentration of the unlabelled reverse primers (recommended by personal communication Dr Brian Kotzin, Department of Pediatrics and Medicine, National Jewish Center, Denver, CO 80206). The final concentrations therefore were 0.165 µM for Cα forward primer and Vβ primers, 0.150 µM for Cβ and Cα reverse primers and 0.015 µM for ^{32}P labelled Cβ and Cα reverse primers. The protocol used for labelling was obtained from Maniatis *et al* (1989) and was modified for increased efficiency and yield for the enzyme used. The total volume used for the labelling reaction was 25 µl. The final concentration of the labelled Cα and Cβ primers after labelling and purification was 0.75µM.

Before presenting the labelling and purification protocol, the process used to determine the exact amount of primer to be labelled needs to be discussed. In order to obtain 0.75 µM of labelled primers, 275 ng of Cα and 225 ng of Cβ are required to be labelled in 25 µl of total volume. However, the labelled primers were column purified and there was a loss of primers on the columns. The percent recovery on the columns was 80%. The amounts of primers needed were therefore increased to 343.75 ng for the Cα primer and 281.25 ng for the Cβ primer to compensate for loss as the counts from 1 µl of sample were between 1-3 x 10^4 counts.

Appropriate amounts of Cα and Cβ primers were aliquoted into two separate 500 µl Eppendorf tubes. To each tube was added 1.6 units of T4 polynucleotide Kinase (Pharmacia), 5 µl of 10 x PNK buffer, 7 µl of ^{32}P labelled gamma ATP (3000µCi/ml) and made up to 25 µl with deionized water. The reaction was incubated for 60 minutes at 37°C in a thermocycler. Experimental results showed that counts obtained from reaction mixes incubated in a water bath were consistently lower than those from the thermocycler. The reactions were stopped by increasing the thermocycler temperature to 65°C for 10 minutes. The completed reactions were then purified by Bio-Spin 6 chromatography columns (Bio-

RAD Laboratories, Hercules, CA) used for rapid removal of unincorporated nucleotides from labelling reactions. The columns were first washed thrice with 1 x SSC and then a sample was placed on top of the gel matrix and spun at 2400 RPMs for 6 minutes. The void volume containing the primer was collected and diluted to the required concentration.

3.3.2 Methodological Concerns

3.3.2A Detection of failed Reverse Transcriptase (RT) PCR

The success of the RT-PCR (cDNA synthesis) step is never assured. Before proceeding to the time consuming and expensive qPCR step, it was important to know whether the cDNA being used could be amplified. To determine the usefulness of the cDNA a non-radioactive PCR reaction was always performed with only the 5'Cα forward primer and the 3'Cα reverse primer or with the 5'Cβ forward primer and the 3'Cβ5 reverse primer. If the cDNA is amplifiable with the Cα primers, a 600 base pair band is clearly visible on ethidium bromide stained gels.

3.3.2B Varying primer concentrations during Vβ aliquoting.

One of the crucial components in a PCR reaction is the primer concentration. From the initial manufacturer's concentrations, Vβ primers were diluted to yield a concentration of 8.25 μM in total volumes ranging from 200-400

µl. The Vβ primers were added at one µl per reaction tube. Therefore, it was very important to obtain a homogeneous source of each Vβ primer. In order to accomplish this 10 µl homogeneous aliquots were taken from the 8.25 µM Vβ stock tubes. Subsequent addition of one µl from the 10 µl aliquots for each Vβ primer ensured adequate homogeneity for every qPCR trial. The Cα and Cβ forward and reverse primers were added to the master mix to maintain equal primer concentrations.

3.3.2C Cross-over contaminations

To reduce crossover contamination, all PCR work was done with aerosol-resistant filtered tips. Since the cDNA was present in the cocktail or master mix, aliquoting 23 times can cause the barrel of the pipettor to become contaminated with nucleotides. To avoid this fresh hydrophobic filtered tips were used for each delivery.

3.3.2D Number of PCR cycles

One factor that plays an important role in qPCR reactions is the number of cycles used to amplify target cDNA. The PCR amplification is not 100% efficient. After a number of cycles the products stop accumulating exponentially and PCR amplification enters into a quasi-linear or stationary phase.

This stage of the reaction is known as the "plateau". The causes for the plateau could be any or all of the following; 1. thermal inactivation of Taq DNA polymerase, 2. limiting concentrations of Taq DNA polymerase, 3. reduction in the denaturation efficiency as cycle numbers increase, 4. increased inefficiency in primer annealing as cycles increase, 5. excess substrate accumulation, 6. competition by non-specific products, and 7. product reassociation (Sardelli, 1993). The key point is that if the PCR reaction enters a plateau phase of amplification then the PCR products no longer reflect the initial quantites of cDNA. Thus it becomes necessary to stop the PCR reaction when the product accumulation is still in the exponential phase. To determine the ideal number of cycles the following protocol was followed (as described by Choi *et al*, 1989).

3.3.2D i. Method used to detect plateau phase

The Jurkat cell line which expresses Vβ8 was RNA extracted and analysed for a plateau effect by the standard protocol of qPCR analysis (see above). The PCR reactions were done for 15, 20, 25, 30, 35, 40, 45 cycles.

3.3.2E Primer efficiency

Generally, primer efficiency is determined as the slope of an amplification curve. Reaction conditions and target amounts may cause variability in amplification efficiencies of each primer. The ideal situation would be to determine the efficiency of each primer under reaction conditions normally used to amplify cDNA. The principal limitation in most cases for this type of investigation is the unavailability of homogenous sources of mRNA that are specific for a particular TCR Vβ. For example, quantitation of the priming efficiencies of Vβ8 and Vβ12 are possible because Jurkat (a constitutive Vβ8 producer) and Molt (a constitutive Vβ12 producer) can be used. Cell lines producing various other Vβs are not readily available. However, despite this limitation, a novel technique was introduced here that provided tangible results about primer efficiencies under reaction conditions normally used to amplify cDNA.

3.3.2E i. Method to Detect Primer Efficiencies

A cDNA sample from one donor was non-radioactively amplified with the 23 Vβ and 3'Cβ reverse primers in each of 23 separate reaction tubes. The product for the constant region of the alpha chain was obtained in a separate tube using unlabelled 5'Cα and 3'Cα primers. Primer concentrations

used were exactly the same as described above. The products were chloroform extracted and quantitated on a spectrophotometer. To quantitatively determine primer efficiency, 0.07 μM of each Vβ product was aliquoted into separate tubes and equimolar amounts (0.165 μM) of the corresponding Vβ primers were added. To ensure quantitation and proper representation of individual tube conditions, 0.07 μM of the Cα product (which was amplified separately) was added as an internal control for each tube. To ensure equal aliquoting, the Cα product was added to a master mix along with all other reagents except the individual Vβ products and primers. The amplification done the second time was semi-quantitative. The amplification conditions and concentrations (chemicals, radiolabelled and unlabelled primers) were exactly the same as that of a semi-quantitative reaction employed to investigate a sample. The relative counts for each amplified Vβ product were obtained by the formula:

$$\text{Relative counts (RC)} = \frac{\text{Vβ counts (for a product)}}{\text{Cα counts (in that tube)}}$$

3.4 Semi-quantitative data analysis

3.4.1 Generation of Vβ product position markers (VPPMs)

The method of Vβ analysis used in this study was different from the standard method described by Choi *et al* (1989). When the PCR-amplified Vβ products are loaded onto agarose gels, it is often difficult to visualize the Vβ bands with ethidium bromide staining and therefore the gel has to be exposed to x-ray film to facilitate excision of the Vβs. To help visualize these products, the following procedure was developed: on a cDNA preparation from stimulated PBLs two rounds of non-radioactive amplifications were done using only the Vβ primers and the 3'Cβ reverse primer. After the first non-radioactive amplification, 2µl aliquots were re-amplified with each of the corresponding Vβ primers. The reactions were chloroform extracted and loading dye (50:50 of 10 x TBE:glycerol with 10 mg of bromophenol blue) was added to each of the 23 tubes at a ratio of 2 µl of products to 1 µl of dye. These were then designated as Vβ product position marker or VPPMs. Before agarose gel electrophoresis, 3µl of VPPM corresponding to each of the Vβ products was aliquoted into 23 wells of NUNC plates. Thus, following electrophoresis, the bands are easily visualized thereby making it quicker and easier to exise the radioactive Vβ product bands.

3.4.2 Electrophoresis

10 µl of PCR-amplified Vβ products were mixed with the corresponding VPPMs and loaded onto 2% agarose gel (high melting, Sigma Inc.) which was run at 100 volts for 30 minutes in a Bio-Rad submarine gel apparatus. The bands were visualized with ethidium bromide and excised under UV. The gel slices were allowed to dissolve in scintillation cocktail (Beckman Ready Value) and then counted. Relative amounts were calculated according to the following formula:

$$V\beta\ \% = \frac{V\beta/C\alpha}{\sum V\beta/C\alpha} \times 100$$

Data obtained were normalized based on the Cα present in that tube and from that sample only.

3.4.3 Methodological concerns

3.4.3A Crossover contamination

In excision of Vβ products from agarose gels the most critical step involves removal of the gel slices containing the various products. The slices must be carefully taken out without removing any of the other products adjacent to the one being excised. As well, since the same scalpel is used there

should be no carry over of radioactive counts.

The use of the VPPMs facilitates efficient removal of wet gel slices containing amplified Vβ products; in our experience it is much more difficult to excise dry gels accurately than wet gels. The scalpel was washed thoroughly with strong detergent and rinsed with 75% ethanol after the excision of every product band. New scalpel blades were used for different samples.

3.4.3B Excision of α and β products

Initial excision of Cα and then the excision of Vβ product bands could possibly have increased the number of counts found for the Vβ product. This is because the counts of the Cα product were typically much higher than the Vβ product counts and addition of counts from the Cα product to the Vβ product were possible. To avoid this potential error all of the Vβ product bands were always excised first with washing of the scalpel after every excision (as described in section 3.4.3A). This ensured that the Vβ product counts were not contaminated by the Cα product counts.

3.5 Statistical approaches

All statistical analyses were done using Minitab 10 for

Windows (Minitab Inc., State College, PA, USA).

3.5.1 Removing Artificial Dependency

The Vβ repertoires for total T cells and subsets were compared between individuals by linear regression and the correlation between Vβ repertoires determined by using the Pearson correlation coefficient, r. In calculating the percent contribution of each Vβ to the total Vβ repertoire the value of the last Vβ (in this case Vβ 20) becomes fixed. That is, as the sum of all Vβ percentages must equal 100%, the percentage of the last Vβ becomes dependent on the sum of the other Vβs, *i.e.* artificial dependency is introduced into the data set. Therefore, to obtain an independent sample and an accurate reflection of the linear similarities of Vβ profiles, one Vβ had to be removed from all statistical calculations (personal communication, Dr V. Gadag, Associate Professor of Biostatistics, Division of Community Medicine, Faculty of Medicine, MUN). We chose to remove Vβ20 from all statistical calculations.

3.5.2 Data Analysis of Identical Twins

In this study, CD4' and CD8' subsets were isolated from peripheral blood T cells from three pairs of identical twins.

Six pairs of identical twins were also analysed for the total T cell Vβ repertoire without subset isolation. For data analysis of the CD4+ and CD8+ T cell subsets, one of the twin pairs was assigned to represent an identical twin pair and the other twin pairs were mixed and matched to form unrelated pairs of individuals. For the six twin pairs, four were randomly chosen to represent identical twin pairs and the remaining two pairs were mixed to form two pairs of unrelated individuals. The resulting Pearson r values were converted to Z values using the calculations presented in this section. The Z values were then compared by chi-square analysis using the following formula derived by Dr. V. Gadag (Associate Professor of Biostatistics, Division of Community Medicine, Faculty of Medicine, MUN);

Conversion of Pearson r to Z scores

$$Z = 1.1513 \log ((1+r)/(1-r))$$

($r = (R^2)^{1/2}$, coeffecient of correlation (COC), reflects the nature and strength of the linear relationship between two variables.)

Formula to Compare the different Z scores by Chi-square

Z_{mean} = Sum of all Z_is / number of observations (k)

Z_i = represents individual Z scores for r values being compared

$$X^2 = \Sigma\ (n_i - 3)(Z_{i\ 1}^{K} - Z_{mean})^2$$

n = number of parameters for each individual. For this study the number of parameters are 22 Vβs per person. Therefore for this study the

$$X^2 = 19 \sum_{i=1}^{K} (Z_i - Z_{mean})^2$$

Null Hypothesis (H_0) = all r values obtained are equal

Alternative Hypothesis (H_1) = Not all r values are equal

This formula compares r values from identical twins and unrelated pairs at the same time and the statistical significance of the results was determined using a χ^2 table. Acceptance of the null hypothesis would indicate there is no difference in the r scores obtained from the twin pair and unrelated comparisons. Rejection of the null hypothesis would indicate that there is a difference in the r values. When the

null hypothesis was rejected, the raw data were closely examined to determine if the data pointed to anomalies due to genetics or HLA. The χ^2 statistic was also used to test the equality of r values obtained from all twin pair comparisons.

3.5.3 Statistical Analysis of TCR Profiles from Large Families

For this study three large families were analysed. Only members within a family were compared. The r scores from the comparison of members from each family were grouped into three categories based on the extent of sharing of HLA haplotypes. The three categories for assignment of the family members were the following: HLA-identical, haplo-identical and non-identical groups. Family members could be assigned to more than one HLA category. The father and the mother were assigned to the non-identical groups but each had a partial identity with the children. Some siblings could be assigned to all three categories; for example, siblings had complete, partial and no HLA identity with other siblings and thus contributed to r scores for all three categories.

In the analyses of data from each family, six separate χ^2 were done. The first three χ^2 tests compared the r values obtained within each of the three HLA categories. The next three χ^2 tests compared one group with another; for example,

the r scores from the HLA-identical groups were compared with the scores from the non-identical or the haplo-identical groups.

3.5.4 Analysis of homogeneity of variance tests using Bartlett's and Levene's Tests.

The Bartlett's and Levene's tests for homogeneity of variance investigate whether all the variances for differing parameters, in this case for Vβs, are equal. Acceptance of the null hypothesis means that all variances are equal but acceptance of the alternate hypothesis suggests variances are not equal.

3.6 Derivation of the Test Statistic Used in this Thesis

The statistic used to test the hypothesis that all the non-zero correlation coefficients do not differ significantly is derived as follows. Let the number of pairs of individuals compared be K. For the i-th pair of individuals n_i denotes the number of pairs of % contributions of different Vβs to the total repertoire and r_i denotes the Karl Pearson's correlation coefficient between them. Fisher has shown that for moderate values of the n_i, the statistic $Z_i=(½)\ln[(1+r_i)/(1-r_i)]$ is approximately normally distributed with the mean given by $\zeta=(½)\ln[(1+\rho_i)/(1-\rho_i)]$ and S.E. given

by $1/(n_i-3)$, where ρ_i is the population correlation coefficient corresponding to the i-th pair. Since $n_i=22$ for all the pairs, under the hypothesis that all the ρ_i's are equal, Z_1, Z_2, ..., Z_K can be viewed as a random sample from a single normal population with common mean ζ and variance 1/19. Then, it is well known that the statistics

$$\bar{Z}=\left(\sum_{i=1}^{K} Z_i/K\right) \text{ and } \chi'^2=19\sum_{i=1}^{K}(Z_i-\bar{Z})^2$$

are independent and that χ'^2 is distributed as χ^2 with (K-1) degrees of freedom (Anderson, 1984).

Chapter 4
Results I

Testing of methodological concerns

4.1 The influence of phytohemagglutinin (PHA) stimulation on the Vβ repertoire

An earlier report by Wong *et al* (1992) suggested that PHA preferentially skews the TCR repertoire toward particular Vβs. In section 3.1.3 concerns as to whether PHA skews the TCR Vβ repertoire were addressed. The profiles of TCR repertoire obtained were compared by the Pearson correlation, r. The r value defines the extent of similarity between variables X and Y. A high r value indicates good similarity while a low r value indicates that the data are less similar. The r value for the correlation analysis of unstimulated repertoires of the two individuals FHDH and FS was 0.913. The r value for the comparison of the repertoires of stimulated lymphocytes from individuals FHDH and FS was 0.626, for the comparisons of FS and FHLH the r value was 0.648 and for the comparison of FHDH with FHLH the r value was 0.911. As well, comparison of the unstimulated and stimulated repertoires yielded r values of 0.907 and 0.776 for individuals FHDH and FS respectively. Correlational comparison of two PHA stimulated aliquots of PBL from one individual (FHLH) gave an r value of 0.953. A

comparison of repertoires determined before and after a four month period for individual FHDH gave an r value of 0.911.

4.2 Determination of the exponential and plateau phases of PCR amplification.

It was important to establish the exponential and plateau phases of the PCR reaction so that all PCR amplifications used for semi-quantitative PCR would be within the exponential phase. The results of this experiment are shown in figure 4.2. The curve obtained was quite typical of profiles presented in other studies (Choi *et al*, 1989; Wong *et al*, 1993; Erlich, 1989). It is evident that at 30 cycles the PCR reaction is well within the exponential phase and the plateau phase is not reached until after 35 cycles. Therefore we proceeded to use 30 cycles for all qPCR reactions.

4.3 Results From Testing for Primer Efficiency

A protocol was designed that involved direct quantitation of the efficiency of each primer in order to test the assumption that all Vβ primers amplify cDNA with equal efficiencies. As mentioned in the methods, to accomplish this task, equimolar Vβ products from a first-round PCR

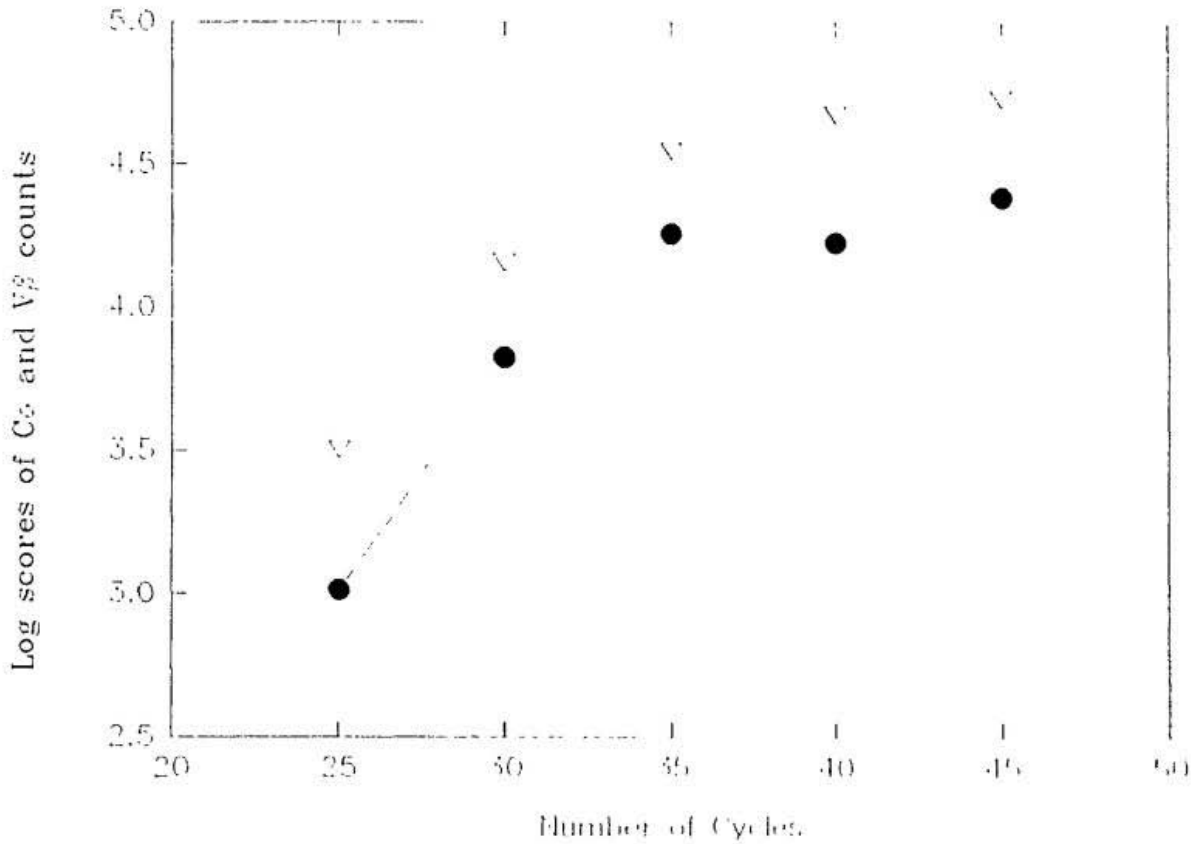


Figure 4.2

Log cpm plot shows the different phases in the experiment to investigate for plateau effect. The ▲ represents log scores for Vβ8 counts and ● represents log scores of Cα counts.

amplification were aliquoted and then re-amplified semi-quantitatively under conditions similar to that used for amplification of cDNA sample prepared from a PBL sample. The experimental rationale was simple. It was reasoned that by providing equimolar aliquots of each Vβ obtained from a first round cDNA amplification, and keeping all primers and reagents at constant concentrations, the only independent variable would be the capacity of the primers to recognize and prime the corresponding segment of cDNA under the reaction conditions. This reaction was done in triplicate. The results are shown in figure 4.3. Ethidium bromide staining and 2 day exposure on x-ray film showed no non-specific or intermediate mRNA product that would affect the results. Comparison of each trial with the mean of all three trials yielded a mean r value of 0.949.

Figure 4.3 shows that there is a hierarchy of priming efficiencies. For the primers derived from Choi *et al* (1989) Vβ 5.1, 8,13.1, 13.2, 20 (figure 4.3) had lower efficiencies than 1, 5.2, 6, and 15. Vβ 17 seemed to be the least efficient primer. For primers derived from Wucherpfennig *et al* (1990) Vβ 3, 12, 15, 19 had low efficiencies with the Vβ 10 primer being the least efficient. In general, primers from Choi *et al* (1989) were much more efficient than those derived

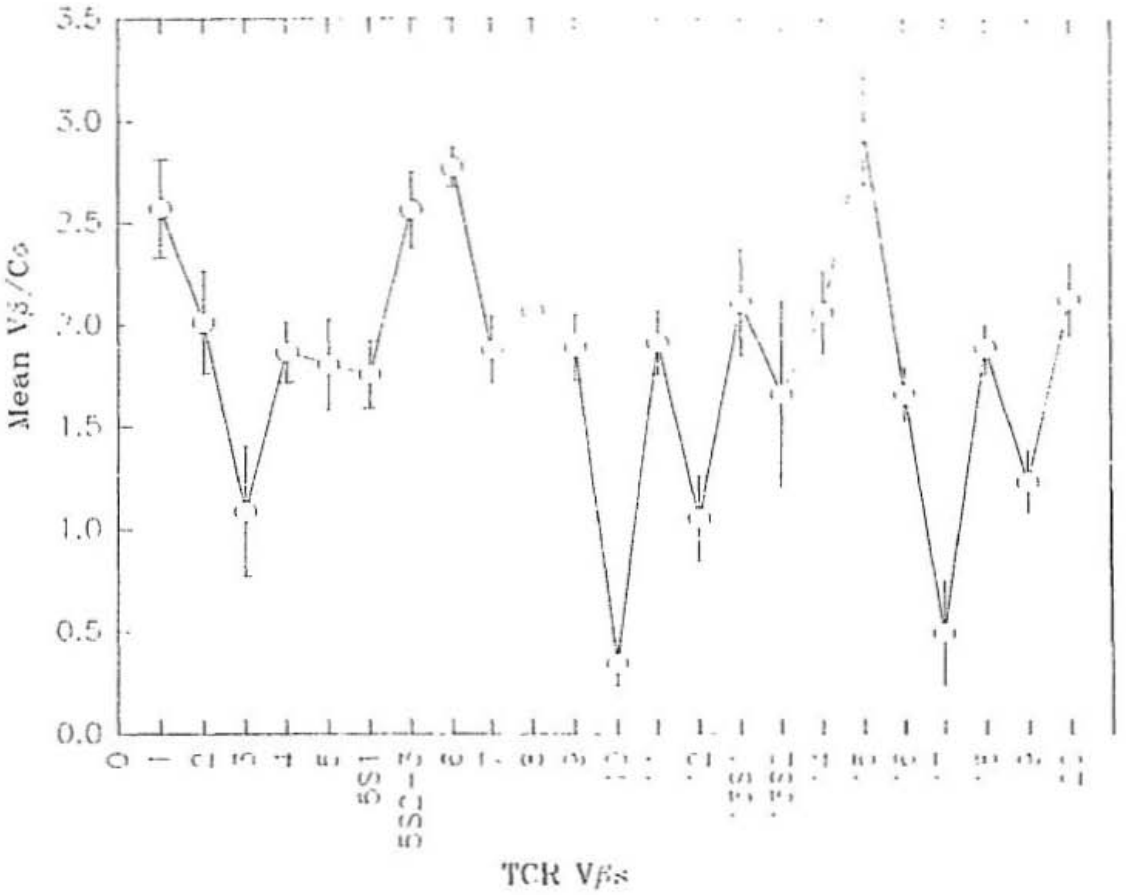


Figure 4.3

Efficiencies of Vβ primers; the mean Vβ/Cα ratios of three experiments for each Vβ is shown ± SD.

from Wucherpfennig *et al* (1990).

4.4 Validation of the use of Vβ Product Position Markers (VPPMs)

Various methods have been employed to quantitate the amplified Vβ products. Some investigators have used autoradiography in conjunction with densitometry (Birnbaum *et al*, 1991) or have excised amplified products from dried gels using x-ray films as templates to excise the Vβ bands (Choi *et al*, 1989; Hall *et al*, 1993). Since the signals for Vβ amplificants are quite weak in comparison to the constant region amplificants, it is frequently necessary to expose the film for 3-7 days to completely identify the Vβ products. The approach described in this investigation circumvents the need for drying, autoradiography and densitometry. This process is simple and much faster and possibly more accurate. This procedure uses V beta product position markers or VPPMs.

The use of the position markers for TCR analysis is novel and increases the speed and accuracy with which a sample can be processed. As with any new method, certain validations are necessary.

To determine whether the positions marked by the VPPMs truly identified the amplified Vβ product position, a sample from one individual (FHLHM) was semi-quantitatively amplified

and processed as described in the methods then electrophoresed in a 2 % agarose gel. After the completion of the electrophoresis a photograph of the wet gel was taken and the gel was dried for 1hr. The VPPMs added were still visible after the gel was dried, therefore a photograph was taken again. The dried gel was exposed to x-ray film (Dupont) for 24 hrs and the film was compared with the pictures of the wet and dried gel (figures 4.4 a and b). In figure 4.4a the top DNA band represents the Cα product and the lower DNA bands represent the likely position of the radioactive Vβ products. Figure 4.4b shows the position of the actual radioactive Vβ product bands. It is clear from the figure that the VPPMs exactly mark the position of the Vβ products.

The time required from the completion of the electrophoresis to excision of the gel was about 30 minutes. An additional 8 hours were required for the gel slices to dissolve in the scintillation fluid. In comparison, to obtain a complete autoradiograph takes at least 3 days.

Preliminary examination using 5 Vβ primers and Cα primers to amplify a sample (FHLHM) showed that the counts from the dried gel were apparently more variable for the 5 Cα product counts than the counts obtained from the wet gel (table 4.4). Therefore, a more extensive test was performed. A cDNA sample

Figure 4.4 a

Ethidium Bromide staining of Vβ product position markers on a dried gel. Lanes 1 and 13 were loaded with DNA ladder. The brighter bands at the top, lanes 2-12 and 14-24, represent Cα products of 600 bps. The bands below the Cα products represent the Vβ product bands.

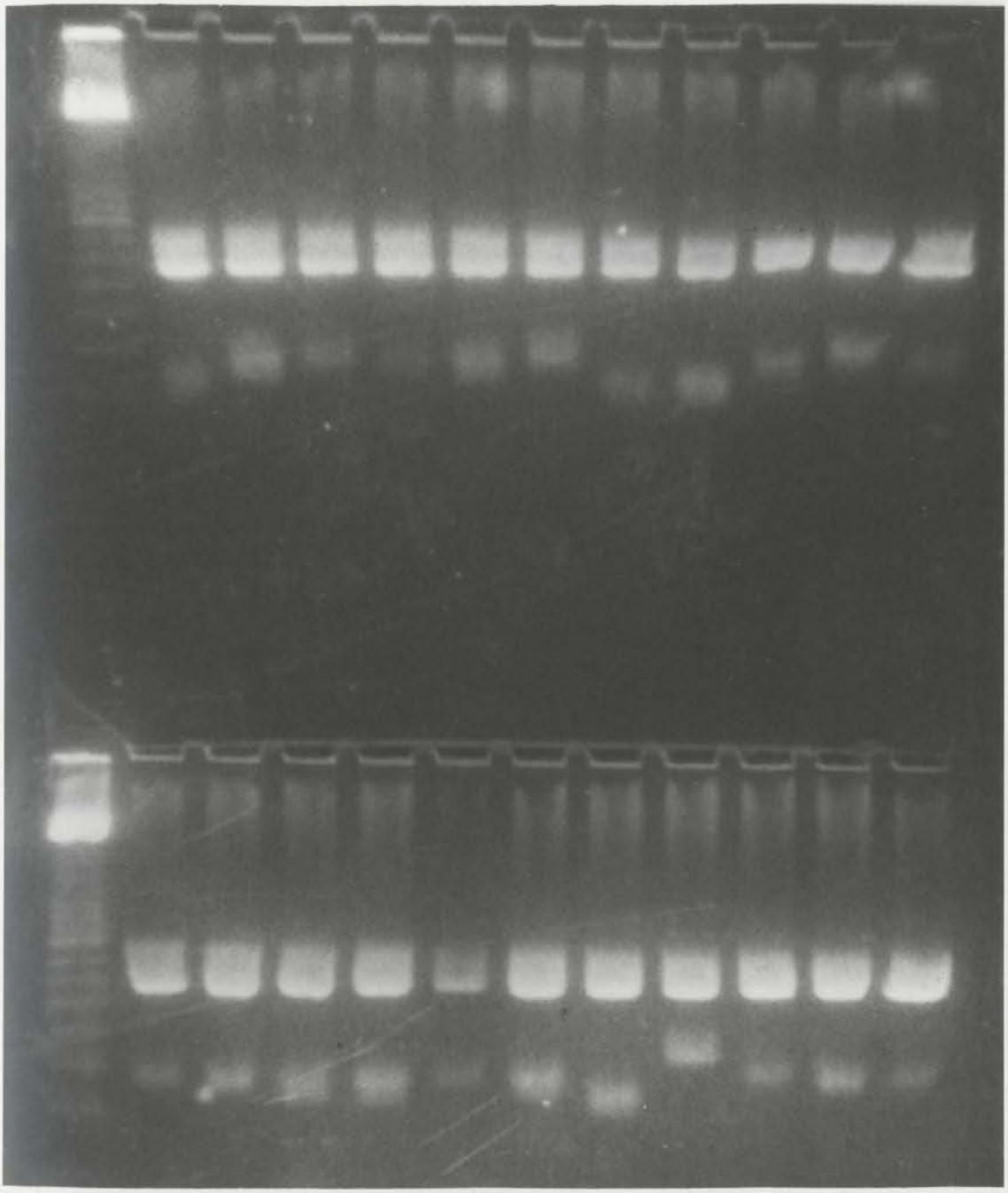


Figure 4.4 b

An x-ray film of the gel from figure 4.4a shows that the locations indicated by the Vβ product position markers corresponds with the locations of the radioactive Vβ products. Again, the darker bands indicate Cα products and the bands below these are the Vβ product bands.

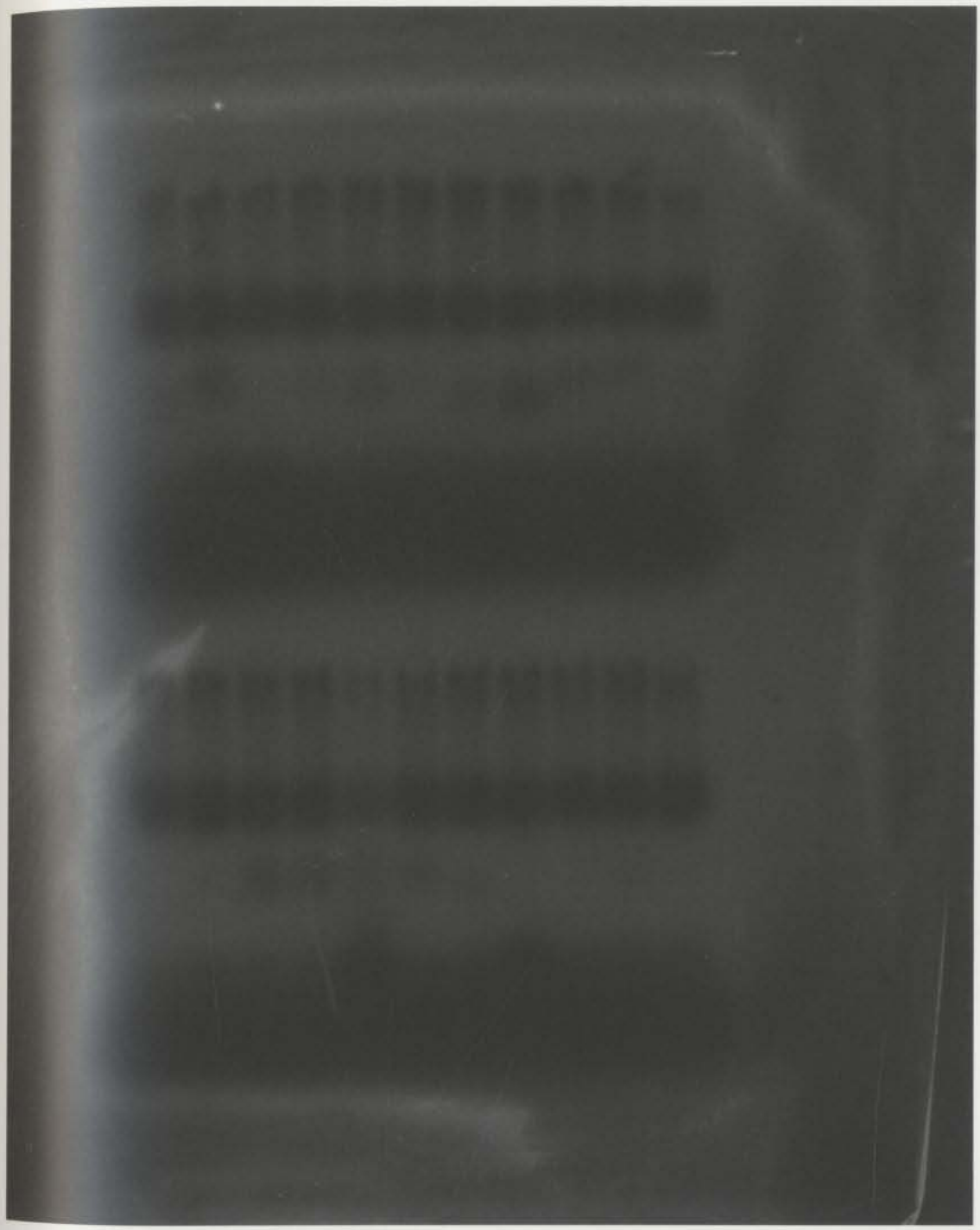


Table 4.4

Preliminary assesment for the consistency of the wet gel excision method versus the dry gel excision method was done on five random Vβs from a random sample (FHLHM). Section A shows the counts from the dry gel excision method and section B shows the counts from the wet gel excision method.

Section A

RAW Cα Counts	Corrected	RAW Vβ Counts	Corrected	Vβ/Cα
28303	28049	7168	6995	0.249385
24254	24000	4475	4302	0.17925
13663	13409	706	533	0.039749
18580	18326	2697	2524	0.137728
17862	17608	2908	2735	0.155327

Section B

RAW Cα Counts	Corrected	RAW Vβ Counts	Corrected	Vβ/Cα
29978	29544	9326	8988	0.304224
28275	27841	10658	10320	0.370676
29758	29324	2182	1844	0.062884
33721	33287	4588	4250	0.127677
34358	33924	7772	7434	0.219137

(SR2) was amplified by qPCR for all 23 Vβs and the products electrophoresed four times. Three of the runs were analyzed as wet gels while the fourth gel was dried and then analyzed. The % Vβ for all four runs is shown in figure 4.4c. The correlation between the three wet gels was >0.95. The correlation between the average wet gel counts and the dried gel was 0.900. This showed that the wet gel method employed here was at least as accurate and reliable as the dry gel method.

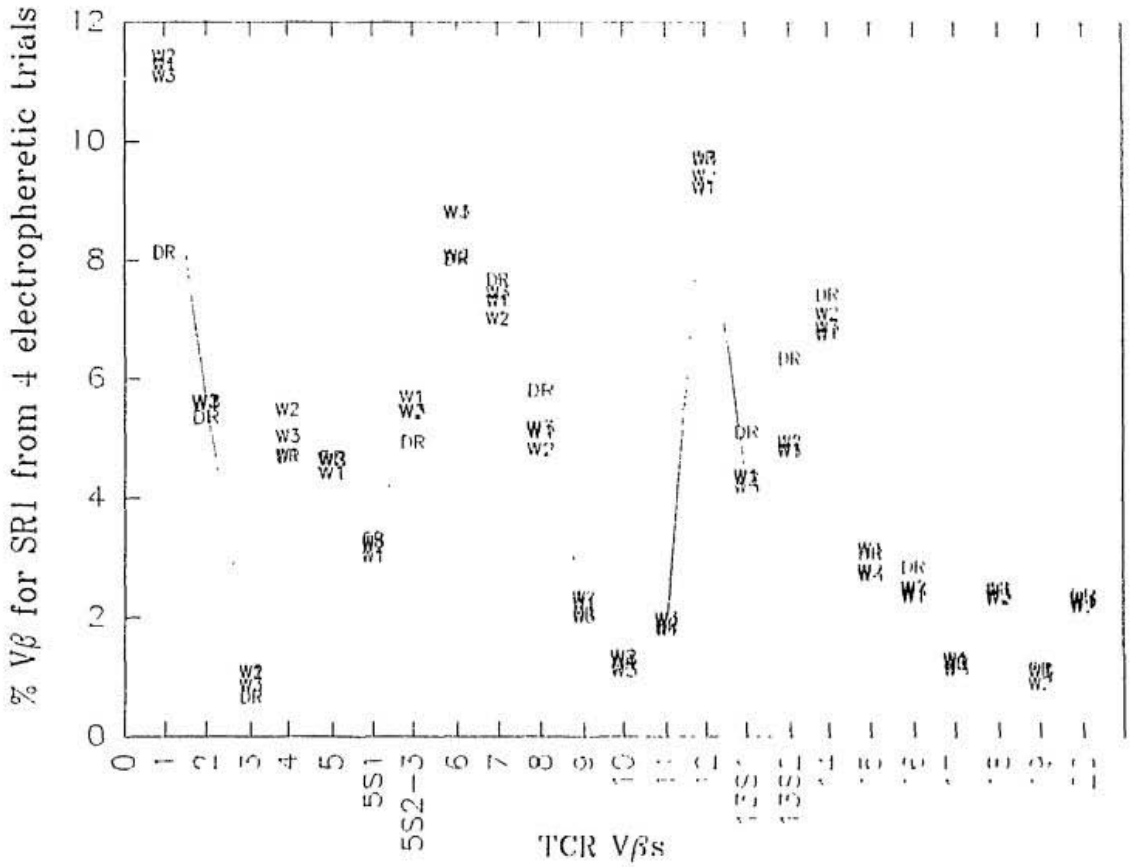


Figure 4.4c

The repeatability of wet gel excision was compared with dry gel excision for individual SR1. Three trials representing percent Vβ by the wet gel method are represented by W1, W2, and W3. The counts from the dry gel method are represented by DR.

Chapter 5

Discussion I

Discussions on the issues pertaining to methods

5.1 PHA use and repertoire skewing

5.1.1 Effect of PHA in this study

The concerns as to the use of PHA to stimulate T cells is whether or not PHA skews the TCR repertoire randomly, or preferentially expands the expression of particular Vβs. The unstimulated repertoires of two individuals (FS and FHDH) were quite similar but PHA stimulation of T cells derived from those individuals caused the TCR repertoires of the two individuals to be quite different. The changes in the TCR Vβ profile cannot be due to preferred stimulation of T cells bearing particular Vβs in both samples, since one would have expected the profiles of the two individuals to be more similar after than before PHA stimulation. Yet, the possibility remains that the changes could have been due to random stimulation. Comparison of individual LH's TCR repertoire in two separate tubes stimulated with PHA indicates that the Vβ profiles are highly similar. This indicates that PHA stimulation does not randomly stimulate TCR repertoire as random stimulation would have led to differences in TCR profiles between tubes for the same individual. These results lead to the conclusion that the repertoire is not randomly or preferentially skewed by PHA. This confirms several earlier

reports (Malhotra *et al*, 1992; Hawes *et al*, 1993, Davey *et al*, 1993) but contrasts with the findings of Wong et al (1993) who report that PHA stimulation preferentially skews the TCR Vβ repertoire. We question this latter conclusion for two reasons. First, the 5-7 day stimulation period used by Wong et al (1993) could have favoured preferential growth/survival of T cells with particular Vβs. We have used a standard 3 day PHA stimulation protocol (Smith *et al*, 1974) Second, and more important, Wong *et al* used a TCR Cβ primer pair to amplify cDNA in reaction tubes separate from the Vβ amplifications and then used the TCR Cβ value obtained in that separate tube to normalize Vβ values. However, when a TCR Cα segment is co-amplified with each of 23 Vβs in separate tubes, the amount of TCR Cα amplified typically varies from 2% to 10% but can vary by 45% between tubes even when all reagents except the Vβ primers are initially prepared in a single tube (master mix) to keep all reagent concentrations constant. Thus, we believe it is essential to co-amplify a reference standard in the same tube as the Vβ segment to control for between-tube amplification differences (see section 5.1.2 below). Any conclusions drawn on the effects of PHA on the Vβ repertoire must, therefore, remain open to question when Vβ values are normalized against Cβ segments amplified in separate tubes. It may be noted in contrast that anti-CD3, when used as a mitogen, does skew the Vβ repertoire (Davey *et al*, 1993).

Evidence for this is convincing as the HPLC method used to measure Vβ products shows only 4% intra- and inter-assay variation (Davey *et al*, 1993).

An important question is why the repertoires for the two individuals, FS and FHDH, were less similar after than before PHA stimulation. Likely the Vβ repertoires prior to PHA stimulation reflected chance usage of a few but similar Vβ families by both individuals in the peripheral blood. Whereas after PHA stimulation of all T cells, the Vβs detected represented a measure of the individual's potential Vβ repertoire (Malhotra *et al*, 1991).

5.2 Validation of primer efficiency data

To validate these data, a number of issues had to be scrutinised. The first issue was the contribution of carryover primers, nucleotides and $MgCl_2$ from the first round amplification. The second issue was the use of 30 cycles to determine primer efficiency as the Vβ targets used for this phase of testing were actually first round amplified products. The issue here was whether the second round PCR amplifications reached the plateau phase prior to 30 cycles. The final issue was the influence of reference primers and PCR product on the primer efficiency reaction.

If the first round PCR had failed completely, then the amount of primer, nucleotides and $MgCl_2$ added to the second

round amplification can be calculated. The concentration of target used in the second round PCR was 0.07 μM. To obtain this concentration the first round products were diluted one in 100. From this one μl was used for the second round amplification. The total PCR volume per Eppendorf tube was 50 μl, therefore the total dilution was one in 5000. Assuming that the first round reaction had failed, all of the unused primers would be transferred to the second round PCR amplification. The initial primer concentration was 0.166 for each Vβ. The contribution of this primer to the second round amplification can be calculated as follows:

$$100 - ((0.166\ \mu M - 0.166/5000) / 0.166\ \mu M) \times 100$$

$$= 0.02\ \% .$$

The same can be calculated for $MgCl_2$ and nucleotides. If the first round reaction was 30 % efficient then the contribution would be 0.014 %. The contribution of the primers, $MgCl_2$ and nucleotides was assessed by spectophotometer as well. A one in ten dilution of the original 50 μl reaction mix yielded an OD value of 0.035 while a hundred fold dilution yielded 0.003 OD reading. Since the first round PCR products were diluted more than 1000 times for the second round PCR, the contribution of reagents from the first round PCR were assumed to be negligible. Note, in this assay cDNA and Taq polymerase were not included.

The concern over the plateau phase was addressed by

testing first round amplified products in a second round PCR reaction. Specifically, Vβ 12 was amplified for 20, 25, 30, 35 and 40 cycles. The results shown in figure 5.2a indicate that amplifications were in the linear phase until 30 cycles.

The validity of the primer efficiency data and the concern as to whether Cα reference product amplification in the same tubes affects the Vβ priming efficiency were addressed in the following manner. An individual's (SR2) cDNA was amplified with Vβ primers only. The Vβ products were purified on a resin that preferentially binds double stranded DNA. This resin was part of the Wizard Prep Kit (Promega) and is used to purify PCR products for sequencing. The end result of this method was purified double stranded PCR product. The purified products were quantitated on a spectophotometer. The reactions for the second round PCR amplifications were done in a similar manner to the second round amplifications in the primer efficiency experiment but with two modifications. The number of cycles was reduced to 28 and no Cα product was added. The cycles were reduced to determine if there would be a difference in the Vβ efficiency profiles. Results are shown in figure 5.2b. The correlation of Vβ priming efficiency profile from this result and the initial experiment (involving 30 cycles) yielded a r value of 0.882.

The high correlation between the two different methods used to determine primer efficiency conclusively proves that

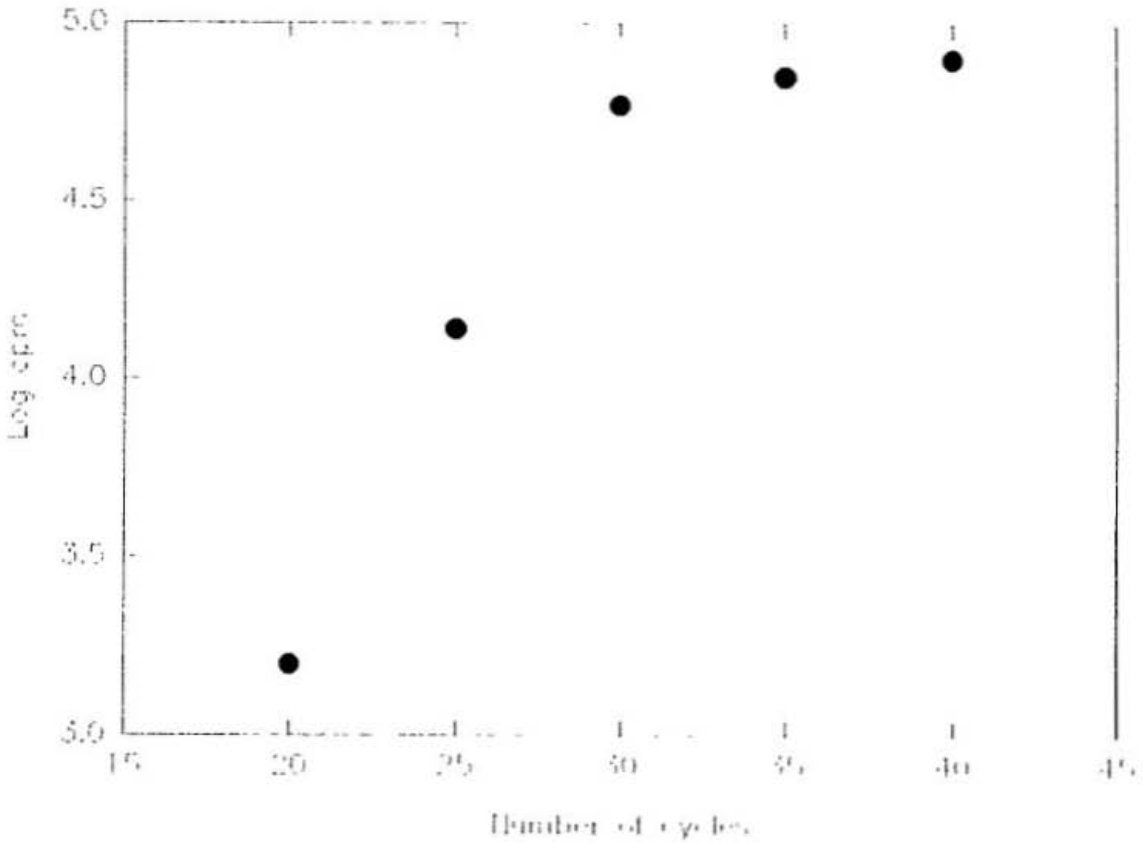


Figure 5.2a

Log cpm plot of Vβ12 at different cycles.

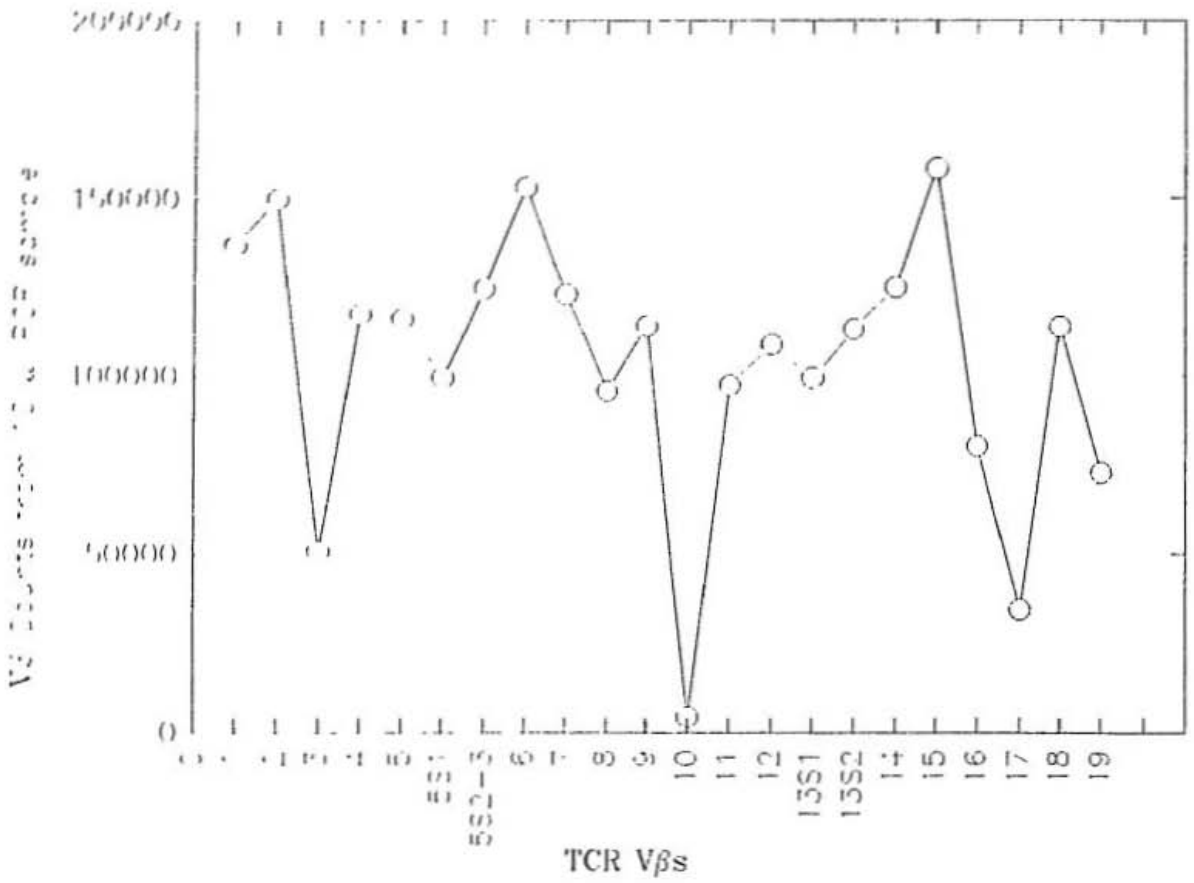


Figure 5.2b

Purified Vβ product segments were amplified to verify the primer efficiency data obtained previously. The amplifications were done at 28 cycles on resin purified Vβ product segments.

there are differences in the abilities of various primers to prime. The correlation between results obtained after 28 cycles and 30 cycles shows that the initial experiments were not influenced by a plateau effect, carry over of reagents or Cα product amplifications.

5.2.1 Importance of primer efficiency data

Again, the general assumption associated with the use of any published set of Vβ primers is one of equal efficiency for all Vβ primers used in an experiment. Based on this assumption some investigators have drawn conclusions as to the relative Vβ expression levels in a tissue from one or more individuals, for example, Akolkar *et al* (1993) and Wucherpfennig *et al*, (1992). The primer efficiency experiments point out that high Vβ values may simply be the result of efficient priming. The previous inability to test the issue of primer efficiency has again been in part due to the lack of homogeneous mRNA sources. However, this is no longer an issue since the question of primer efficiency can be proficiently addressed by the methods used in this study.

5.3 Importance of the use of VPPMs

The importance of the Vβ product position markers is indicated by three points: the ease of use, simplicity of use and cost efficiency. Since the VPPMs are loaded in agarose gels together with the Vβ samples, there are no additional

steps with their use. The VPPMs aid in visualizing the bands immediately and therefore facilitate product excision. Further, since the VPPMs are easily visible, there is no need for autoradiography or densitometry which requires expensive equipment. Also, it is more difficult to excise a dry than a wet agarose gel. As the number of steps associated with use of VPPMs are few, possible loss of Vβ products and the increased probabilities for radioactive exposure and contamination are decreased. The qPCR analysis can be accomplished in a smaller physical space in the laboratory and in a shorter length of time than when following already published methods.

Chapter 6
Results II

Determining the effect of primer efficiency

6.1 Vβ Primer efficiency

In the analysis of the TCR Vβ repertoire it has been assumed that all TCR Vβ primers have equal priming efficiency in PCR reactions (Akolkar *et al* 1993). This assumption is unproved and it was felt important to design a protocol to quantitate Vβ priming efficiencies under the precise reaction conditions normally used to analyze cDNA from a peripheral blood lymphocyte (PBL) sample. Application of this protocol established the priming efficiencies of each Vβ primer. The mean Vβ primer efficiencies from three trials are shown in figure 4.3.

6.1.1 ANOVA and homogeneity of variance tests

The data in figure 4.3 clearly show a marked heterogeneity in priming efficiencies with Vβs 3, 10, 12 and 17 being the least efficiently primed and Vβs 6 and 15 being the most efficiently primed. The latter were primed 5-6 fold more efficiently than the former Vβs. To test if the means for all primers were statistically different a one-way analysis of variance (ANOVA) was performed for the data from the three trials. Results are shown in table 6.1.1. The ANOVA calculations show that the means obtained are significantly different ($p < 0.001$).

Table 6.1.1

The one-way analysis of variance on the data from primer efficiency experiments. The $p < 0.001$ shows that the mean for each of the primers is different.

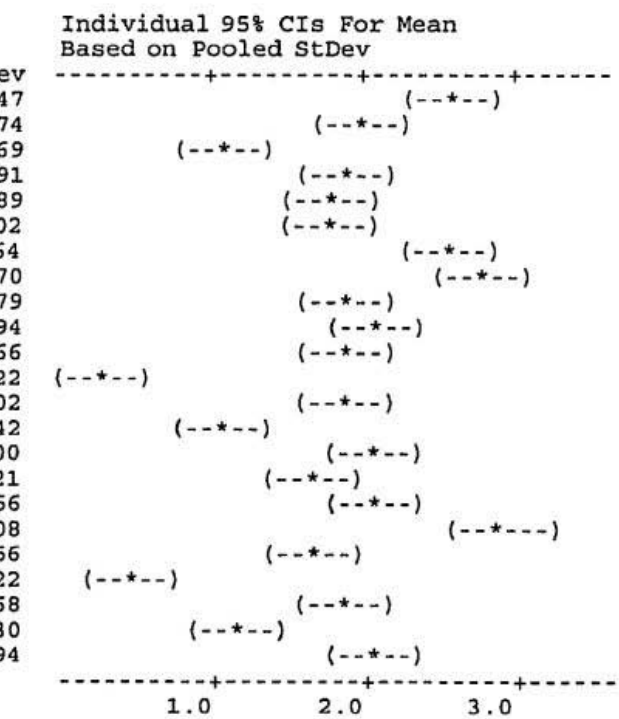
One-Way Analysis of Variance

Analysis of Variance on Primer Efficiency

Source	DF	SS	MS	F	p
Factors	22	27.3561	1.2435	18.62	0.000
Error	46	3.0714	0.0668		
Total	68	30.4275			

Vβs	N	Mean	StDev
1	3	2.5726	0.2947
2	3	2.0133	0.3074
3	3	1.0871	0.3869
4	3	1.8647	0.1791
5	3	1.8033	0.2689
5S1	3	1.7551	0.2002
5S2-3	3	2.5616	0.2264
6	3	2.7720	0.1170
7	3	1.8794	0.1979
8	3	2.0680	0.0294
9	3	1.8896	0.1966
10	3	0.3436	0.1322
11	3	1.9136	0.1902
12	3	1.0530	0.2542
13S1	3	2.1093	0.3200
13S2	3	1.6613	0.5521
14	3	2.0642	0.2456
15	3	2.9499	0.3208
16	3	1.6623	0.1556
17	3	0.4938	0.3122
18	3	1.8911	0.1568
19	3	1.2369	0.1830
20	3	2.1278	0.2194

Pooled StDev = 0.2584



A concern was that in the TCR Vβ analysis, amplification of particular Vβ segments might be more highly variable from experiment to experiment than amplification of other Vβ segments. If so, then the differences in the percent profiles for a particular Vβ from different individuals would not necessarily be due to differences in Vβ target amounts between individuals but due to differences in priming or amplification at different times. To test this possibility, the equality of variance was analyzed by the Bartlett's and Levene's (BL's) tests for homogeneity of variance (HOV). These tests were done to determine whether the qPCR results obtained could be influenced by the variable efficiencies of the Vβ primers.

The BL's tests for HOV showed that the Vβs from the priming efficiency experiment have equal variances (p value =0.997). The worst primer was just as variable in its priming efficiency as the best primer. The standard deviations in figure 4.3 for priming efficiency for all three trials were quite small. Small standard deviations and equal variances for all Vβs suggest that any differences in results obtained for particular Vβs from various individuals would be due to differences in Vβ amounts between individuals.

6.2 Priming efficiency and the TCR Vβ profile

In view of the heterogeneity in Vβ priming efficiencies it was pertinent to know if there was a correlation between

priming efficiencies and the observed profile of the TCR Vβs. In other words, did high priming efficiencies correlate with high percent values for corresponding Vβs and low priming efficiencies correlate with low values for corresponding Vβs.

The % Vβs from all of the individuals were correlated with mean primer efficiency. Thirty out of forty eight comparisons showed a significant correlation. Examples of r values obtained from these comparisons are shown in table 6.2, section A. The critical value for the Pearson correlation r (df=20, α=0.05) is 0.444. Thus, the observed Vβ repertoires are partly dependent on priming efficiencies.

6.2.1 Corrections for dependency on primer efficiency

These findings indicate that in order to interpret quantitative data from an individual's Vβ repertoire the values obtained initially have to be corrected to adjust for differences in the priming efficiency of different Vβs. The mean Vβ/Cα derived for each Vβ primer after three trials (figure 4.3) was used to calculate the correction factors necessary for each Vβ. The correction factors were calculated by dividing an arbitrary integer (five) by the mean Vβ/Cα derived for each primer (table 6.2.1). The uncorrected % Vβ scores obtained were multiplied by the correction factors and new relative scores were calculated by the formula:

Table 6.2

Correlations (r values) between Vβ priming efficiencies and Vβ repertoires using initial (unmodified, Section A) and modified % values (Section B) for all Vβs.

	Pearson r values	
	Section A	Section B
	Initial Vβs	Modified Vβs
Total T cells		
DB2	0.474	0.024
SC1	0.553	0.253
SC2	0.534	0.130
SN	0.419	-0.128
WG	0.106	-0.209
LH	0.417	0.154
$CD4^+$ T cells		
MB1	0.494	0.203
MB2	0.410	0.003
SR1	0.553	0.253
SR2	0.554	0.044
LR1	0.642	0.471
LR2	0.634	0.303
$CD8^+$ T cells		
MB1	0.568	0.247
MB2	0.525	0.124
SR1	0.494	0.020
SR2	0.490	-0.185
LR1	0.659	0.388
LR2	0.631	0.250

Table 6.2.1
The mean Vβ/Cα ratio for each Vβ gene product is shown with the derived correction factors used to correct for differences in Vβ priming efficiencies.

Vβ genes	Mean Vβ/Cα	Factor (5/mean)
1	2.572596	1.943562
2	2.013349	2.483425
3	1.087087	4.599447
4	1.864703	2.681393
5	1.803271	2.772739
5.1	1.75507	2.84889
5.2	2.561621	1.951889
6	2.771964	1.803776
7	1.879368	2.660468
8	2.068019	2.417773
9	1.889556	2.646125
10	0.343586	14.55241
11	1.913559	2.612932
12	1.053029	4.748206
13.1	2.109255	2.370505
13.2	1.661332	3.009634
14	2.064225	2.422217
15	2.94991	1.694967
16	1.662344	3.007801
17	0.493777	10.12603
18	1.891113	2.643945
19	1.236922	4.042291
20	2.127766	2.349882

$$\text{Corrected \% V}\beta = \frac{\text{uncorrected \% V}\beta \text{ x factor}}{\sum \text{uncorrected \% V}\beta \text{ x factor}} \text{ X } 100$$

Note, a calculation made by applying the correction factors to the Vβ/Cα values and then determining the % Vβ contribution yielded the same corrected % Vβ scores as calculations done using the above formula (data not shown).

6.2.2 Effects of corrections

6.2.2A Effect of corrections on correlation with primer efficiency

After all of the TCR Vβ data from all 48 individuals were corrected, the corrected Vβ data were compared with the mean priming efficiency data by correlation analysis. Out of the 48 samples only one now showed significant correlation between priming efficiency and the Vβ repertoire (table 6.2, section B, sample LR1, $CD4^+$ T cells).

6.2.2B Effect of correction on the TCR Vβ profile

Figure 6.2.2 (a and b) shows typical TCR Vβ repertoire profiles obtained from qPCR analysis for two individuals. Correlation analysis of the Vβ repertoires of all individuals before and after the application of correction factors yielded r values ranging from 0.713 to 0.927. This indicates changes in the TCR Vβ repertoire had occurred for each sample after

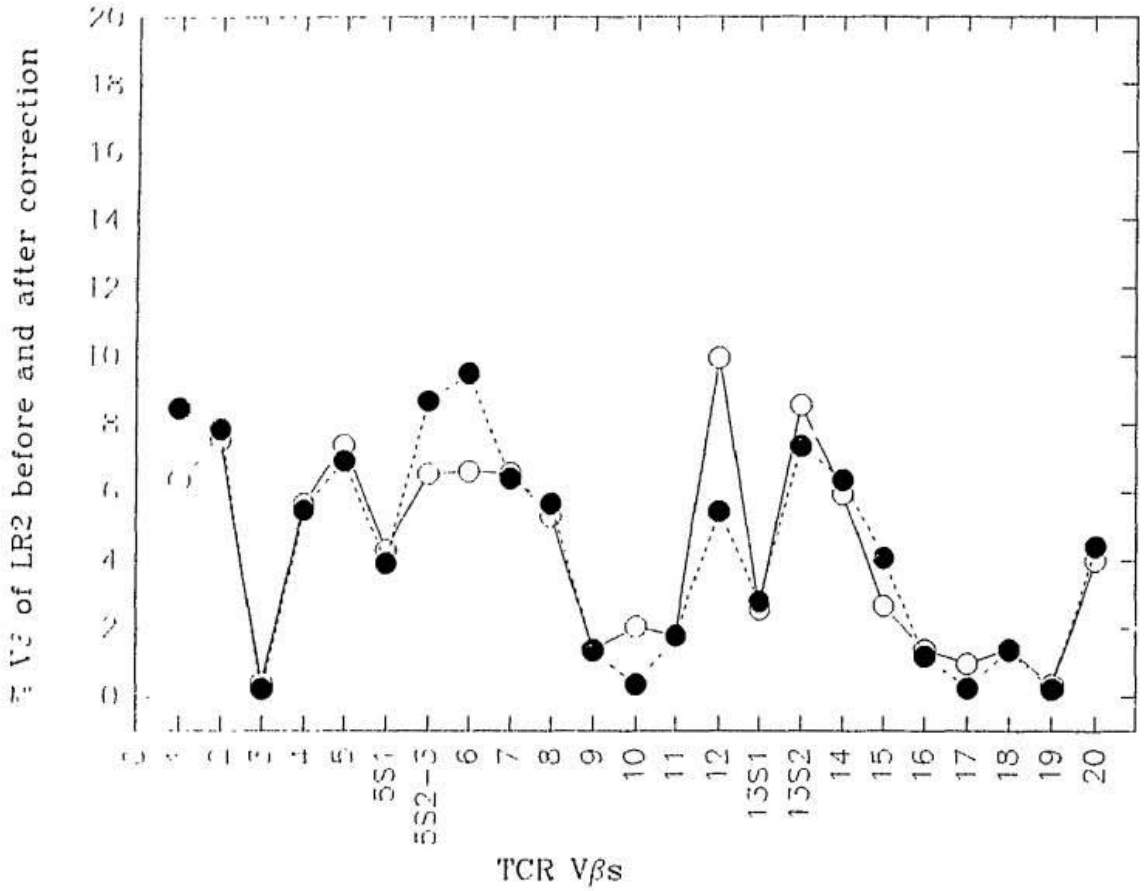


Figure 6.2.2a

The Vβ repertoire of the CD4 subset of an individual (LR2) using data uncorrected (●) and corrected (0) for differences in Vβ priming efficiences.

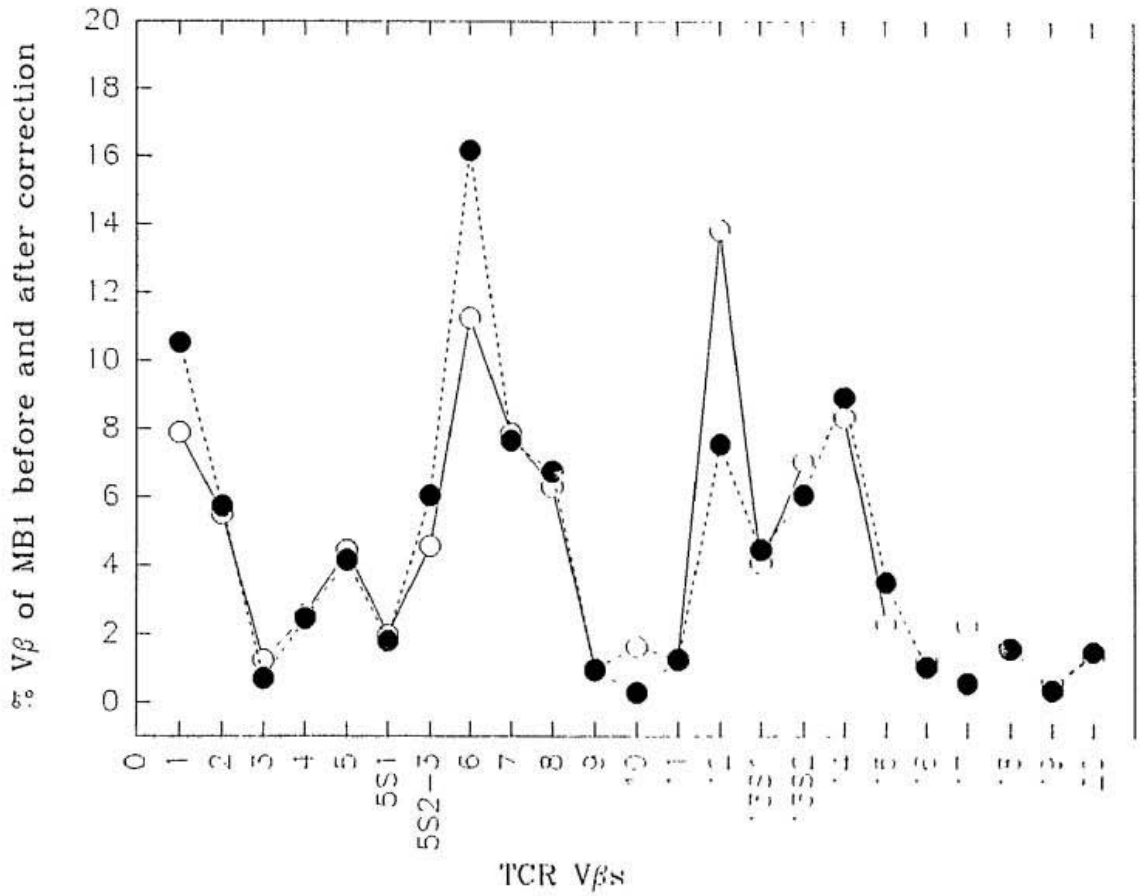


Figure 6.2.2b

The Vβ repertoire of the CD4 subset of an individual (MB1) using data uncorrected (●) and corrected (0) for differences in Vβ priming efficiences.

the data were corrected (figures 6.2.2 a and b).

It is evident from figure 6.2.2, for the particular individuals studied, that correcting for differences in Vβ priming efficiencies caused changes in Vβs 1,6,12 with Vβs 6 and 12 being affected the most. Following correction of the data, the percent values of Vβs 10, 12 and 17 were increased whereas Vβs 1, 5.2, 6 and 15 were decreased. The other Vβs are essentially the same or showed only slight modifications *i.e.*, a change of less than 1%.

6.3 Vβ Percentages: Are they dependent on primer efficiency or initial target amounts?

From the Vβ priming efficiency experiment, Vβ 15 was identified as one of the most efficient primers. Despite the high priming efficiency the levels of Vβ 15 found in cDNAs from all 48 samples in this study were relatively low compared to many other Vβs (figure 6.2.2). In contrast, the priming efficiency of the Vβ 12 segment was low with only Vβs 3, 10 and 17 being less efficiently primed (figure 4.3). However, using the uncorrected data (figure 6.2.2) the amount of Vβ 12 detected is greater than most other Vβs including Vβs 4, 5.1, 5.2, 8, 9, 11, 13.1, 13.2, 15, 16, 18, 19 and 20. All of the latter Vβs are primed more efficiently than Vβ 12, yet the levels of Vβ 12, in the two representative experiments (figure 6.2.2) were higher than the levels of these various Vβs. This

observation coupled with the knowledge of the priming efficiencies of Vβs 12 and 15 argues that for each TCR Vβ, the levels quantitated by qPCR (*i.e.*, uncorrected data) provide an accurate reflection of the amounts of the initial Vβ targets present in a given sample.

CHAPTER 7
Vβ analysis

7.1 General observations of the TCR Vβ profile

The TCR Vβ data from the twin and family studies indicated that few Vβs are expressed at a high level. In fact, calculation of the means for each Vβ in the different groups shows that six Vβs make up between 46%-53% of TCR Vβ repertoire. For the $CD4^+$ T cell subset Vβs 5, 2, 7, 13.2, 6 and 12 made up over 50% of the expressed levels. For the $CD8^+$ T cell subset Vβs 13.2, 14, 1, 6, 7 and 12 contributed 50% of the Vβ repertoire. In total T cells from the twin pairs Vβs 7, 5, 13.2, 2, 6, and 12 made up a mean of 49% of the expressed levels. For the FK family Vβs 5, 7, 2, 6, 13.2 and 12 made up a mean of 53% of the expressed repertoire, for the FH family Vβs 10, 2, 6, 13.2, and 12 accounted for a mean 50% of the Vβ repertoire, and for the FG family Vβs 5.2, 8, 6, 2, 10 and 12 contributed about 46% of the repertoire. The above Vβs were listed in ascending order of contribution, the last Vβ listed contributing most to the repertoire. It is therefore evident that Vβ 12 made the largest contribution to the Vβ repertoire in all the groups and Vβ 6 made consistently high contributions for all of the groups.

7.2 Analysis of the Vβ repertoire of $CD4^+$ and $CD8^+$ T cell subsets using Vβ percentages corrected for differences in Vβ priming efficiencies

7.2.1 Analysis of the data

The Vβ repertoires were compared between individuals by correlation analysis. After excluding Vβ 20 from the correlation analysis, r values were determined for the $CD4^+$ and the $CD8^+$ T subsets from each twin pair and then tested for equivalence (table 7.2.1a). The null hypothesis that all of the r values are equal was not rejected.

The next step was to assess the effect of unrelatedness on the Vβ repertoire. This was done by comparing twin pairs to unrelated pairs. The latter were formed from members of different twin pairs. For example, for the first round of calculation twin pairs MB1/MB2 were studied as the identical twin pair while, SR1/LR1 and SR2/LR2 were assigned as unrelated pairs. This process was repeated for all twin pairs for both $CD4^+$ and $CD8^+$ T cells in all possible combinations. The results are shown in tables 7.2.1b and 7.2.1c. Again, the null hypothesis was that all r values are equal. The critical value from the chi-square table (df=2, p=0.050) is 5.99. The chi-square values obtained from comparisons of the identical twin pairs and unrelated pairs for the $CD4^+$ T subset did not show any evidence for differing r values *i.e.*, none of the comparisons led to rejection of the null hypothesis (table 7.2.1b). The same analysis for the $CD8^+$ T cell subset showed that two of the comparisons of twin pair-unrelated combinations had significant chi-square values (table 7.2.1c).

Table 7.2.1a

Pearson r values and χ^2 comparisons for T cells from twin pairs isolated for CD4' and CD8' T cell subsets.

CD4' T Cells

Twin pairs	r value
MB1/MB2	0.884
SR1/SR2	0.917
LR1/LR2	0.806

CHI-SQUARE COMPARISONS
All twin pairs 1.99

CD8' T cells

Twin pairs	r value
MB1/MB2	0.946
SR1/SR2	0.896
LR1/LR2	0.958

CHI-SQUARE COMPARISONS
All twin pairs 2.23

Critical chi-square value at DF 2, 0.05 = 5.99

Table 7.2.1b

Chi-square analysis of r values for CD4' T cell Vβ repertoire obtained from regression analysis between identical twin pairs and between pairs of unrelated individuals

			Pearson 'r'	Z values
A. Twin pair	LR1/LR2		0.806	1.12
Unrelated Pairs				
	MB1/SR1		0.952	1.85
	MB2/SR2		0.870	1.33
CHI-SQUARE VALUE		5.45		
B. Twin pair	LR1/LR2		0.806	1.12
Unrelated Pairs				
	MB1/SR2		0.930	1.66
	MB2/SR1		0.831	1.19
CHI-SQUARE VALUE		3.28		
C. Twin Pair	MB1/MB2		0.883	1.39
Unrelated Pairs				
	SR1/LR1		0.794	1.08
	SR2/LR2		0.835	1.20
CHI-SQUARE VALUE		0.92		
D. Twin Pair	MB1/MB2		0.883	1.39
Unrelated Pairs				
	SR1/LR2		0.851	1.26
	SR2/LR1		0.747	0.97
CHI-SQUARE VALUE		1.78		
E. Twin Pair	SR1/SR2		0.917	1.57
Unrelated Pairs				
	MB1/LR1		0.779	1.04
	MB2/LR2		0.717	0.90
CHI-SQUARE VALUE		4.72		
F. Twin Pair	SR1/SR2		0.917	1.57
Unrelated Pairs				
	MB1/LR2		0.843	1.23
	MB2/LR1		0.667	0.80
CHI-SQUARE VALUE		5.59		

CRITICAL CHI-SQUARE VALUE (AT $\alpha = 0.05$, DF = 2) = 5.99

Table 7.2.1c

Chi-square analysis of r values for $CD8^+$ T cell Vβ repertoire obtained from regression analysis between identical twin pairs and between pairs of unrelated individuals.

		Pearson 'r'	Z values
A. Twin Pair	LR1/LR2	0.961	1.96
Unrelated Pairs			
	MB1/SR1	0.858	1.29
	MB2/SR2	0.864	1.31
CHI-SQUARE VALUE	5.55		
B. Twin Pair	LR1/LR2	0.961	1.96
Unrelated Pairs			
	MB1/SR2	0.823	1.17
	MB2/SR1	0.888	1.41
CHI-SQUARE VALUE	6.26		
C. Twin Pair	MB1/MB2	0.945	1.78
Unrelated Pair			
	SR1/LR1	0.826	1.18
	SR2/LR2	0.832	1.19
CHI-SQUARE VALUE	4.54		
D. Twin Pair	MB1/MB2	0.945	1.78
Unrelated Pairs			
	SR1/LR2	0.898	1.46
	SR2/LR1	0.724	0.92
CHI-SQUARE VALUE	7.30		
E. Twin Pair	SR1/SR2	0.892	1.43
Unrelated Pairs			
	MB1/LR1	0.942	1.76
	MB2/LR2	0.942	1.76
CHI-SQUARE VALUE	1.32		
F. Twin Pair	SR1/SR2	0.892	1.43
Unrelated Pairs			
	MB1/LR2	0.948	1.79
	MB2/LR1	0.897	1.46
CHI-SQUARE VALUE	1.47		

CRITICAL CHI-SQUARE VALUE (AT $\alpha = 0.05$, DF = 2) = 5.99

The comparisons involving the twin pair LR1/LR2 with unrelated pairs MB1/SR2 and MB2/SR1 produced a chi-square value of 6.26 (table 7.2.1c). Similarly, the twin pair comparisons of MB1/MB2 with unrelated pairs SR1/LR2 and SR2/LR1) produced a chi-square value of 7.30. Preliminary statistical evidence seems to point toward a possible genetic contribution to the $CD8^+$ T cell subset repertoire but no evidence was obtained for a genetic influence on the $CD4^+$ T cell subset Vβ repertoire.

7.2.2 Analysis of the CD4 and CD8 data in a manner similar to Hawes *et al* (1993)

The statistical calculations provided by both Akolkar *et al* (1993) and Hawes *et al* (1993) are fundamental in establishing a role for genetics and HLA in shaping of the T cell Vβ receptor repertoire. Both papers show that comparisons of unrelated individuals show a wider range of values than comparisons involving related individuals. Here, the data obtained in this study are presented in a manner similar to Hawes *et al* (1993).

The data were compared by the R^2 statistic to assess similarity. Each individual in a twin pair was compared with other unrelated pairs to yield R^2 scores to show the effect of unrelated comparisons. The R^2 data from twin pair and unrelated comparisons from this study are shown in figure 7.2.2a and the results of Hawes *et al* (1993) are shown in

figure 7.2.2b.

7.2.2A Intuitive concerns associated with the data

The values obtained from the comparisons of unrelated individuals showed a wide range while values from the comparison of twins showed a narrower range for both studies. However, the comparisons of unrelated pairs also yielded some R^2 values that are higher than those values obtained from the comparisons of the related twins. If the greater identity of the values obtained from twins is due to genetics, what would explain the higher R^2 values obtained from comparisons of unrelated individuals? From the data of Hawes *et al* (1993) the R^2 value for the $CD4^+$ subset of Twin pair 3 = 0.914 but an unrelated comparison yielded a R^2 of 0.937. Can the larger variations in the values for R^2 in the unrelated comparisons actually validate the claim of strong genetic influence in the twin pair comparisons?

7.2.2B Statistical concerns associated with the data

A wider spread of data is not necessarily proof of a genetic influence since the data have not been tested statistically. In this study, the method of testing used by Akolkar *et al* (1993) was not employed as their test score calculated was not statistically derived and therefore cannot be tested by rigorous mathematical analysis. This allows the data to be interpreted subjectively only (as done by Akolkar

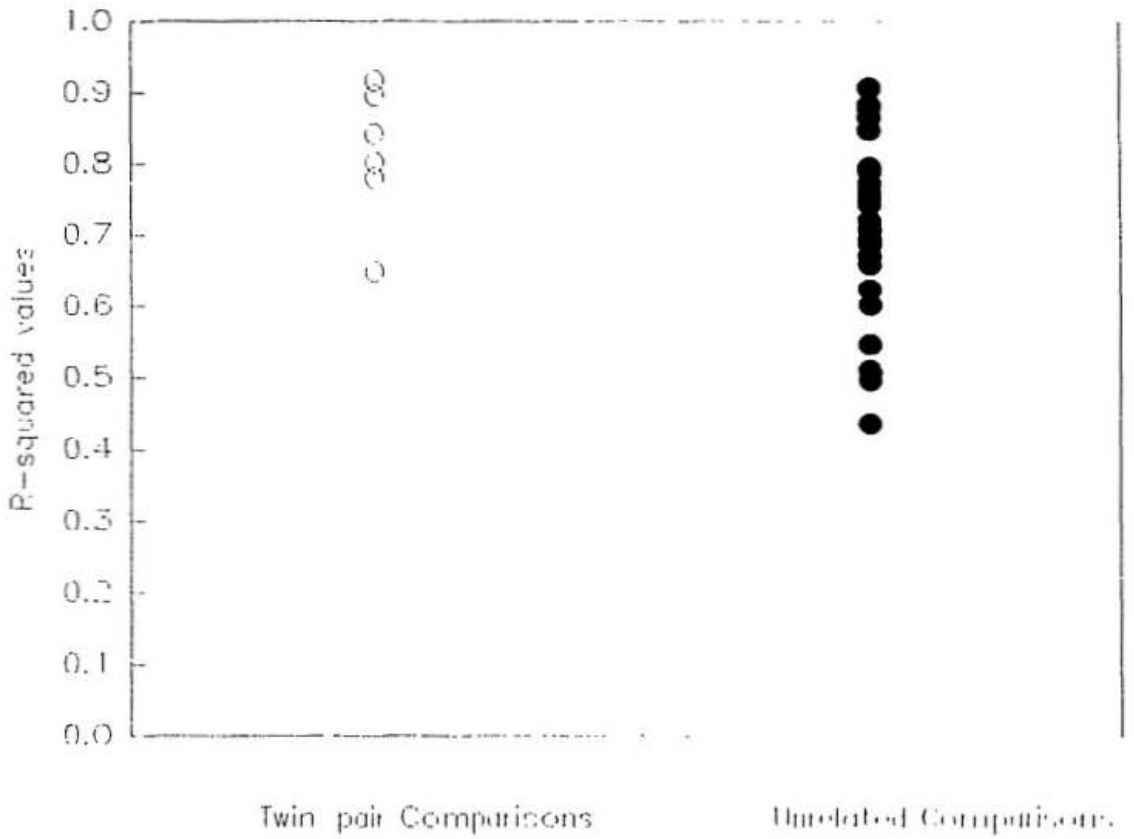


Figure 7.2.2a

R^2 values from the comparisons of twins and unrelated individuals from this study.

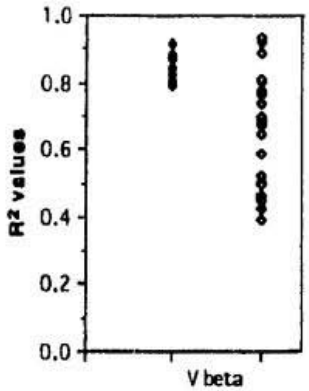


FIGURE 2. The distribution of the correlation values (R^2) of the $\alpha\beta$ TCR V gene usage patterns are represented for the comparisons between monozygotic twins (*solid symbols*) and unrelated combinations (*open symbols*). As seen by the distribution of the values for the twins compared with that of the unrelated combinations, the twins show a higher level of concordance, particularly for the Vβ where all the values are > 0.800 ($R^2 = 1.000$, being a perfect correlation and $R^2 = 0$, being no correlation). The Vα correlation is also higher between twins than unrelated individuals, albeit to a lesser extent than that of the Vβ.

Figure 7.2.2b

R^2 values from the comparisons of related twins and unrelated individuals from the study by Hawes *et al* (1993).

et al, 1993). For the study conducted by Hawes *et al* (1993), the significance of the R^2 test statistic was not tested to determine if the wider range of values observed from the comparison of the unrelated individuals versus the twins was statistically significant. A common statistical misconception is to suggest that an increase in correlation from 0.10 to 0.20 is equivalent to an increase in correlation from 0.60 to 0.70 or that a value of 0.80 represents twice the relationship indicated by a value of 0.40 (Kirk, 1978). Thus, would it be valid to assume that the mean correlation of 0.833 is greater than 0.638 (Hawes *et al*, 1993; twin pair versus unrelated pair comparisons for $CD8^+$ T cells)?

7.2.2C Statistical comparisons of the R^2 values

There are no tests available to statistically calculate whether or not there are significant differences between R^2 values. For example, how different are R^2 values 0.430 and 0.640 or 0.780 and 0.920? The R^2 value is a derivative of the Pearson correlation value r. Even though R^2 values can not be compared, there is a test available to investigate statistically significant differences in the r value (which can directly be obtained from the R^2 value). Correlation values were compared using a form of Z test. The formula is the following:

$$Z = \frac{Z_1 - Z_2}{\quad}$$

σ

$$Z_1 = 1.1513 \log ((1+r_1)/(1-r_1))$$

$$Z_2 = 1.1513 \log ((1+r_2)/(1-r_2))$$

$$\sigma = (1/(n_1 - 3) + 1/(n_2-3))^{1/2}$$

$r = (R^2)^{1/2}$, coefficient of correlation (COC), reflects the nature and strength of the linear relationship between two variables.

n = number of data points

Sample Calculation comparing two R^2 values

$R_1^2 = 0.771$, $R_2^2 = 0.409$, $n_1 = 23$, $n_2 = 23$

$r_1 = 0.878$, $r_2 = 0.640$

$Z_1 = 1.1513 \times \log (1.878/0.122)$

$Z_1 = 1.1513 \times 1.1873$

$Z_1 = 1.3667$

$Z_2 = 1.1513 \times \log (1.640/0.360)$

$Z_2 = 0.7582$

$Z = (1.3667 - 0.7582)/0.316$

$Z = 1.93$

The null hypothesis was; H_0: $\mu_1 = \mu_2$ (no difference for r_1 and r_2), $\alpha = 0.025$ (two tailed), $Z = 1.96$ and H_1: μ_1N.E μ_2. Therefore, for the above example, the null hypothesis should be accepted as there is no statistically significant difference between 0.409 and 0.771. Application of this formula to the data obtained from Hawes *et al* (1993) shows the

following; comparison of the mean R^2 value of 0.869 for the $CD4^+$ T subset from the comparisons of twins with the mean R' of 0.661 for unrelateds yields a Z value of 1.52. Comparison of the mean R^2 value of 0.833, for the $CD8^+$ T subset from twins with the mean R^2 of 0.638, derived from the comparisons of unrelated individuals, yields a Z value of 1.27. Both Z values were below the critical value of 1.96. Using the Z test formula given above, the minimum R^2 value required to show a difference between comparisons of related and unrelated individuals can be calculated. For the $CD4^+$ T subset the minimum R^2 value for unrelated comparisons would have to be below 0.569 and for the $CD8^+$ T subset the R^2 value has to be below 0.471.

The Z test results from the comparison of the R^2 data obtained in this study are shown in appendix A. The data point out that analysis done in a manner similar to Hawes *et al* (1993), does not reveal any significant evidence for a genetic contribution in $CD4^+$ and $CD8^+$ T cells from monozygotic twins.

7.3 Analysis of the total T cell Vβ repertoire in identical twins and unrelated pairs of individuals

The next part of the study investigated whether the total T cell Vβ repertoires of identical twins were significantly different from the corresponding repertoires of unrelated pairs. Six pairs of identical twins were first analyzed for

their TCR Vβ repertoires without T cell subset isolation. The correlation coefficients (r values) from the comparisons of identical twin pairs are shown in table 7.3a. Z values were calculated from these r values and the null hypothesis of equal r was tested to demonstrate identity of scores from identical twin pair comparisons. Four twin pairs-LR1/LR2, BB/TB, SG/SN and SC1/SC2 were randomly assigned as the identical twin pairs. Twin pairs DB1/DB2 and SR1/SR2 were mixed to yield a comparison between DB1/SR1 and DB2/SR2 or DB1/SR2 and DB2/SR1. Results of this analysis are shown in table 7.3b.

The initial correlation analysis to test the null hypothesis of equal r values of identical twins was rejected (table 7.3a). Twin pair BB/TB had the lowest r value (table 7.3a) among the twin pairs. Subsequent removal of the twin pair BB/TB from the chi-square analysis of the identical twin pairs led to acceptance of the null hypothesis (table 7.3a, section B). The reason for the re-analysis without including the scores of twin pair BB/TB was to identify a possible genetic contribution in the other twin pairs which could have been masked by the lower r values of this twin pair. As well, analysis without twin pair BB/TB determined if the initial results were an exception or the rule in terms of the lack of a detectable genetic influence on the TCR repertoire. In this analysis it was determined that twin pair BB/TB was an

Table 7.3a

Chi-square analysis of r values for total T cell Vβ repertoires obtained from regression analysis between identical twin pairs before and after removal of twin pair BB/TB.

Section A

Twin Pairs	Pearson 'r'	Z scores
LR1/LR2	0.943	1.76
BB/TB	0.729	0.93
DB1/DB2	0.771	1.02
SR1/SR2	0.850	1.26
SG/SN	0.782	1.05
SC1/SC2	0.935	1.70

CHI-SQUARE VALUE 11.97
CRITICAL CHI-SQUARE VALUE AT $\alpha = 0.05$ 11.07

Section B

Comparison of 'r' values without the twin pair BB/TB

CHI-SQUARE VALUE 8.84
CRITICAL CHI-SQUARE VALUE AT $\alpha = 0.05$ 9.49

Table 7.3b

Chi-square analysis of r values obtained from comparisons of total T cell Vβ repertoires of monzygotic twin pairs and unrelated pairs. Section A represents comparisons of unrelated pairs and twin pairs without the removal of twin pair BB/TB. Section B shows chi-square comparisons after the removal of twin pair BB/TB.

Section A

		Pearson 'r'	Z scores
Twin Pairs	LR1/LR2	0.943	1.76
	BB/TB	0.729	0.93
	SG/SN	0.782	1.05
	SC1/SC2	0.935	1.70
(A) Unrelated Pairs			
	DB1/SR2	0.810	1.13
	DB2/SR1	0.654	0.78

OR

(B) Unrelated Pairs			
	DB1/SR1	0.760	1.00
	DB2/SR2	0.690	0.85

CHI-SQUARE COMPARISONS		CRITICAL CHI-SQUARE VALUE
Twin Pairs	10.65	7.81 at DF = 3, α = 0.05
(A) unrelated Pr	1.13	3.84 at DF = 1, α = 0.05
(B) Unrelated Pr	0.21	3.84 at DF = 1, α = 0.05
Twin Pr VS (A)	15.94	11.07 at DF = 5, α = 0.05
Twin Pr VS (B)	15.71	11.07 at DF = 5, α = 0.05

Section B

CHI-SQUARE COMPARISONS Without Twin Pair BB/TB		
Twin Pairs	5.89	5.99 at DF = 2, α = 0.05
(A) unrelated Pr	1.13	3.84 at DF = 1, α = 0.05
(B) Unrelated Pr	0.21	3.84 at DF = 1, α = 0.05
Twin Pr VS (A)	13.91	9.49 at DF = 4, α = 0.05
Twin Pr VS (B)	13.73	9.49 at DF = 4, α = 0.05

exception.

Correlation analysis of the randomly chosen identical twin pairs and the unrelated pairs (created by mixing members of different twin pairs) led to rejection of the null hypothesis of equality of r values (table 7.3b, section A). Comparison of the r values for the four identical twin pairs led to the rejection of the null hypothesis. Again, removal of the BB/TB twin pair caused acceptance of the null hypothesis (table 7.3b, section B). Correlation analysis of the r values for both the unrelated pairs of individuals did not reject the null hypothesis. Subsequent comparison of the three identical twin pairs, without BB/TB, with the two unrelated pairs caused the null hypothesis to be rejected (table 7.3b, section B). The results of the comparisons between unrelated and identical twin pairs after the removal of twin pair BB/TB, provide preliminary evidence for genetic influence on the Vβ repertire, *i.e.* higher r values for the identical twins than the unrelated pairs in the total T cell Vβ repertoire.

7.4 Family studies to determine role of HLA

The third segment of my study was to investigate the effects of HLA on the Vβ repertoire of total T-cells. More particularly I investigated the effect of complete, partial and no HLA identity on the TCR Vβ repertoire. Three multi-sibling families consisting of 6, 7 and 10 members were investigated including both parents in each family. The largest family had the largest age range (from 19-65 years).

7.4.1 Role of HLA on TCR Vβ profiles from family FK

The first family (FK) had seven members, the parental haplotypes were designated AB and CD, four siblings shared the BD haplotypes, and the fifth sibling shared one haplotype with the other children (table 7.4.1, section A). The haplotypes of family FK are shown in table 3.1 and from these the haplotypes A, B, C and D were assigned in table 7.4.1 (section A). All comparisons of the Vβ repertoires between family members gave highly significant r values. Comparison of the Vβ profiles of the parents yielded an r value of 0.907 (table 7.4.1, section B).

Individuals were grouped into three categories in terms of HLA as shown in table 7.4.1, section B. Correlations (r values) in Vβ repertoires were derived for all individuals. For example, in the FK family, the parents FKAK and FKHHK were in the HLA non-identical category. In addition, the parents

Table 7.4.1

Comparisons of HLA identical, haploidentical and HLA non-identical individuals from the family FK: A) Family members and the extent of haplotype sharing is indicated; B) Pearson r values and derived Z scores are indicated for comparisons between HLA identical, haploidentical and HLA non-identical individuals; C) Chi-square statistical comparisons are shown.

Section A.

Individuals	HLA Haplotypes
FKAK (mother)	AB
FKHHK (father)	CD
FKJPK	BD
FKRK	BD
FKUK	BD
FKMK	BD
FKHMK	AD

Section B.

HLA-identical (I)		Haploidentical (P)		Non-identical (N1)	
'r'	Z	'r'	Z	'r'	Z
0.969	2.08	0.854	1.27	0.907	1.51
0.970	2.09	0.820	1.16		
0.944	1.77	0.909	1.52		
0.977	2.23	0.918	1.58		
0.927	1.64	0.927	1.64		
0.956	1.90	0.881	1.41		
		0.901	1.48		
		0.943	1.76		
		0.933	1.68		
		0.861	1.30		
		0.854	1.27		
		0.810	1.13		
		0.884	1.39		
		0.865	1.31		
		0.805	1.11		

Section C.

COMPARISONS (α =0.05)	CHI-SQUARE VALUES	CRITICAL CHI-SQUARE VALUES	
HLA-Identicals	4.64	11.07	(DF = 5)
Haploidenticals	11.12	23.68	(DF = 14)
Non-identical	NA	NA	(DF = 0)
I VERSUS P	40.52	31.41	(DF = 20)
P VERSUS NI	11.34	24.99	(DF = 15)
I VERSUS NI	7.79	12.59	(DF = 6)

were also placed in the HLA haploidentical category for comparisons with the children. Each of the children could be assigned to more than one HLA category as well. The four HLA identical siblings, FKRK, FKJPK, FKUK and FKMAK, not only provided comparisons for the HLA identical category but also for the HLA haploidentical category, since they could be compared with sibling FKHMK who had the AD haplotypes and compared with both parents.

Comparison of the r values (table 7.4.1, section C) derived for the HLA identical and HLA non-identical groups led to acceptance of the null hypothesis at $p = 0.05$, *i.e.* there was no significant difference between the Vβ repertoire profiles of the two groups. Similarly, comparison between the HLA non-identical and the haploidentical groups led to the acceptance of the null hypothesis (table 7.4.1, section C). However, comparison of the r values for the HLA haploidentical and HLA identical groups resulted in rejection of the null hypothesis. Comparison of r values within each group shows that there were no significant differences between the r values within each group. Overall, the finding of a statistically significant difference between the r values for HLA identical and HLA haploidentical groups leads to the preliminary interpretation that HLA plays some role in determining the Vβ repertoires in the members of this family.

7.4.2 Role of HLA on TCR Vβ profiles from family FH

The second family included six members, FHDH, FHLHM, FHLH, LHKH, FHCH and FHPH. Again the parental haplotypes were designated as AB and CD. There were two HLA identical siblings (FHLH and FHPH) and two haploidentical siblings (FHKH and FHCH). Table 3.1 shows the HLA haplotypes of all members of this family. Almost all of the regression values (table 7.4.2, section B) were much higher than the critical value of 0.444 for Pearson r at df of 20.

All correlations derived from members of this family were placed in HLA-identical, haploidentical or non identical categories (table 7.4.2, section B). Chi-square analysis of the r values from comparisons among HLA-identical and HLA non-identical groups led to rejection of the null hypothesis of equality of r values (table 7.4.2, section C). The same result was obtained for chi-square analysis of r values in the haploidentical versus the HLA-identical groups or the haploidentical versus the HLA non-identical groups (table 7.4.2, section C). Chi-square analysis showed that the r values obtained for the haploidentical and the HLA non-identicals were different within each group. Since, there was only one r value for the HLA-identical group, this value was not tested further. These preliminary results indicate that HLA shapes the Vβ repertoire in this family.

Table 7.4.2

Comparisons of HLA identical, haploidentical and HLA non-identical individuals from the family FH: A) Family members and the extent of haplotype sharing is indicated; B) Pearson r values and derived Z scores are indicated for comparisons between HLA identical, haploidentical and HLA non-identical individuals; C) Chi-square statistical comparisons are shown.

Section A.

Individuals	HLA Haplotypes
FHLHM (mother)	AB
FHDGH (father)	CD
FHLH	AC
FHKH	BD
FHPH	AC
FHCH	AD

Section B.

HLA-identical(I)		Haploidentical(P)		Non-identical (NI)	
'r'	Z	'r'	Z	'r'	Z
0.756	0.987	0.332	0.35	0.365	0.38
		0.685	0.84	0.718	0.90
		0.738	0.95	0.857	1.28
		0.484	0.53		
		0.933	1.68		
		0.564	0.64		
		0.517	0.57		
		0.795	1.08		
		0.447	0.48		
		0.230	0.23		
		0.539	0.63		

Section C.

COMPARISONS (α =0.05)	CHI-SQUARE VALUES	CRITICAL CHI-SQUARE VALUES	
HLA-Identical	NA	NA	(DF = 0)
Haploidentical	53.72	18.31	(DF = 10)
Non-identical	7.70	5.99	(DF = 2)
I VERSUS P	32.14	19.68	(DF = 11)
P VERSUS NI	39.34	22.36	(DF = 13)
I VERSUS NI	7.95	7.81	(DF = 3)

7.4.3 Role of HLA on TCR Vβ profiles from family FG

The third family consisted of 12 members, of whom ten were available for testing. The parental haplotypes were designated as AB and CD for the mother and father respectively. The haplotypes of the individuals are given in table 3.1. The correlations for all members in this family are shown in table 7.4.3 (section B). Out of 44 regression comparisons 17 were above the critical r value of 0.444. Four of the eight siblings shared the BD haplotypes, two shared the AC haplotypes and two shared the AD haplotypes. Only siblings sharing the AC haplotypes showed a good correlation at 0.791 (table 7.4.3, section B, HLA-identical). In contrast, siblings sharing BD or AD haplotypes had very poor correlations, *i.e.* low r values (table 7.4.3, section B, HLA-identical).

Comparison of the correlation values obtained within each group led to rejection of the null hypothesis for the haploidentical and HLA non-identical groups. This indicated that the r values obtained for these groups are variable. The chi-square test to compare the r scores of HLA-identical individuals with haploidenticals did not lead to the rejection of the null hypothesis. Comparison of the r scores from both the HLA haploidentical and HLA-identical groups with the non-identical group led to rejection of the null hypothesis.

Table 7.4.3

Comparisons of HLA identical, haploidentical and HLA non-identical individuals from the family FG: A) Family members and the extent of haplotype sharing are indicated; B) Pearson r values and derived Z scores are indicated for comparisons between HLA identical, haploidentical and HLA non-identical individuals; C) Chi-square statistical comparisons are shown.

Section A.

Individuals		HLA Haplotypes	Individuals	HLA Haplotypes
FGAG	(mother)	AB	FGJG	AD
FGGG	(father)	CD	FGAG	AC
FGWG		BD	FGNAG	BD
FGNG		AD	FGSF	AC
FGCM		BD	FGDB	BD

Section B.

HLA-identical(I)		Haploidentical(P)		Non-identical (NI)	
'r'	Z	'r'	Z	'r'	Z
0.322	0.33	0.059	0.06	0.150	0.15
0.094	0.09	0.554	0.62	0.399	0.42
0.260	0.27	0.179	0.18	0.486	0.53
0.500	0.55	0.601	0.70	0.883	1.39
0.403	0.43	0.095	0.10	0.162	0.16
0.350	0.37	0.056	0.06	0.371	0.39
0.441	0.47	0.162	0.16	0.302	0.31
0.791	1.07	0.696	0.86	0.816	1.15
		0.591	0.68	0.145	0.15
		0.815	1.14		
		0.524	0.58		
		0.746	0.96		
		0.218	0.22		
		0.370	0.39		
		0.657	0.79		
		0.014	0.01		
		0.323	0.34		
		0.214	0.22		
		0.305	0.32		
		0.388	0.41		
		0.104	0.10		
		0.408	0.43		
		0.391	0.41		
		0.617	0.72		
		0.689	0.85		
		0.426	0.46		
		0.444	0.48		
		0.540	0.60		

Table 7.4.3 cont.

Section C.

COMPARISONS (α =0.05)	CHI-SQUARE VALUES	CRITICAL CHI-SQUARE VALUES	
HLA-Identicals	10.95	14.07	(DF = 7)
Haploidentical	47.12	40.11	(DF = 27)
Non-identical	30.76	15.51	(DF = 8)
I VERSUS P	47.13	55.76	(DF = 35)
P VERSUS NI	78.33	55.76	(DF = 36)
I VERSUS NI	42.00	26.29	(DF = 16)

The rejection of the null hypothesis within the haploidentical and HLA non-identical groups could be traced to two r values for each group. Within the HLA haploidentical group correlations between FGAG and FGJG, and FGSF and FGJG yielded r values of 0.815 and 0.746 respectively. Removal of these high r values produced a chi-square value of 32.46 (critical 37.65, df 25, α = 0.05) and therefore led to acceptance of the null hypothesis for the haploidentical group. Within the HLA non-identical group, correlation comparisons between individuals FGAG and FGSF, and FGDB and FGSF produced r values of 0.883 and 0.816 respectively. Removal of these values from the chi-square test yielded a value of 2.60 (critical 11.07, df 5, α = 0.05) and led to acceptance of the null hypothesis.

The results of these comparisons accentuate the importance of examining the data closely after statistical comparison. The spread of r values for all three HLA based groups was not very different. Most of the r values were quite low. However, from the initial chi-square analysis within each group, the acceptance of the null hypothesis within the HLA-identical group and the rejection of the null hypothesis within each of the two remaining HLA based groups would have argued for an HLA effect. However, closer examination of the data revealed that the initial rejection of the null hypothesis for haploidentical and non-identicals was due to

the few high r values, r values that indicated greater similarities in profile between individuals. Following the removal of these high r values, chi-square analysis of all other r values from all three groups combined led to the acceptance of the null hypothesis (χ^2 = 30.44. At df = 40, α = 0.05, critical value = 55.76). This means that overall in the three groups there were very similar r values. However, since the r values are low the results indicate that the correlations between individuals in all three groups are poor, *i.e.* within each group the individuals have markedly different Vβ repertoires.

The r values obtained from the comparison of the HLA-identical group with the other two groups indicated that there is some evidence for an HLA effect for individuals with the AC haplotypes but not for individuals with the BD or the AD haplotypes. Here, the parsimonious interpretation is that HLA influences the Vβ repertoire in the FG family but this is evident only for comparisons with individuals having the AC haplotypes.

Comparison of the TCR Vβ percent values within the FG family are interesting. Among the siblings, FGWG who bears the BD haplotypes, had Vβ 8 levels of 27% while other siblings including those bearing the BD haplotypes, had Vβ 8 levels at 3-6%. In sibling FGCM (who also bears the BD haplotypes) and in the father there was an over-representation of Vβ 10 at 22%

and 17% respectively. The levels of Vβ 10 in other family members were between 0.3% and 9.4%. In another BD identical sibling (FGNG) Vβ 13.1 was over-represented at 15% while all others had Vβ 13.1 levels between 0 and 4%. Sibling FGJG had no detectable levels of Vβ 17 while other family members had levels between 3-8%.

7.5 The Bartlett's and Levene's tests (BL's) for homogeneity of variance (HOV)

7.5.1 The procedure for BL's tests for HOV

Figures 7.5 a-c show the mean ± SD of the TCR Vβ profile for the $CD4^+$ and $CD8^+$ T subsets and total T cells for identical twins studied. It is obvious from these graphs that the percent levels of expression of certain Vβs are relatively constant. Other Vβs seem to show wide fluctuations and others appear to have variances in between the two extremes. For example, in the $CD4^+$ and the $CD8^+$ T subsets, despite varying HLA backgrounds and cell types, the levels of Vβ 11 seem to be relatively constant unlike levels of Vβ 10. The Bartlett's and Levene's (BL) tests were used to determine statistically whether there indeed were differences in the variances of the different Vβs. The MINITAB statistical package actually uses both the Bartlett's and Levene's tests to analyze homogeneity of variance but only the Levene's test is relevant as it tests any data that are continuous. In the Levene's test, Bonferroni

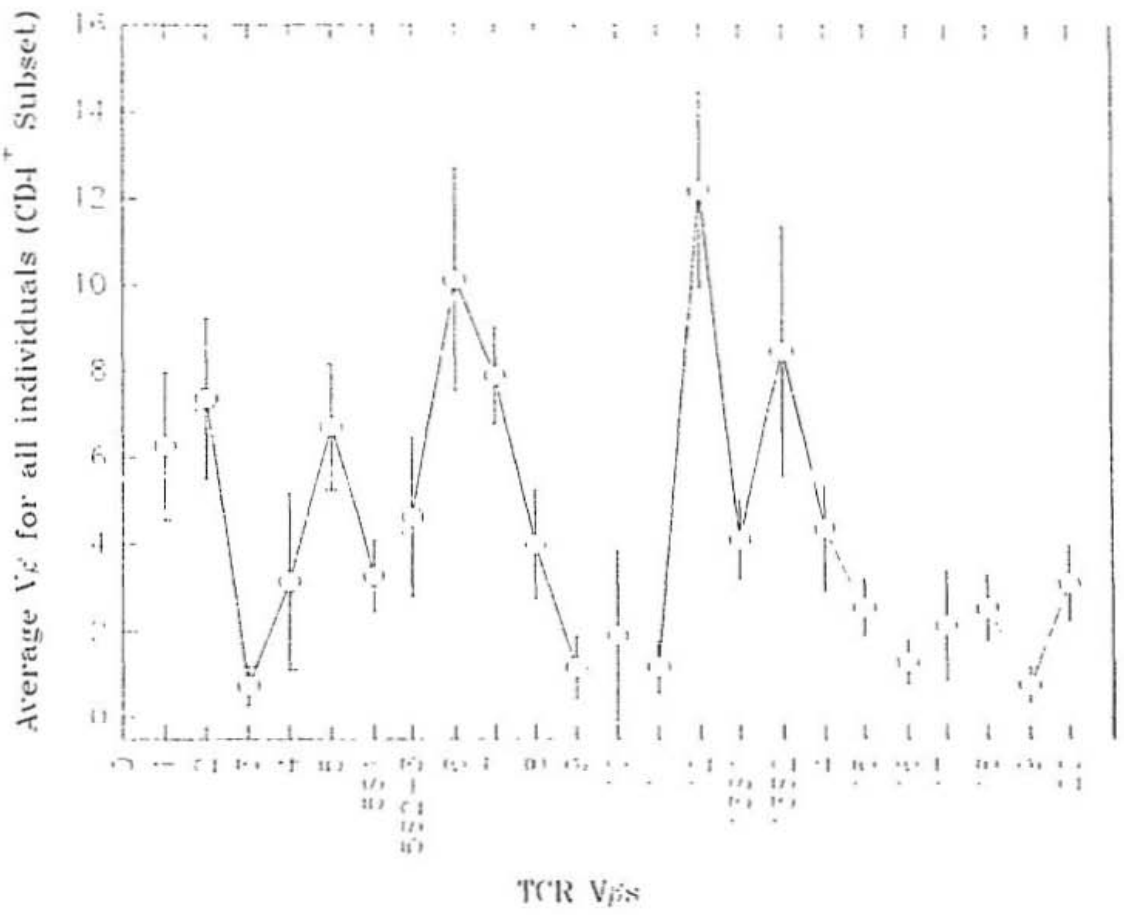


Figure 7.5a

The mean Vβ repertoires of CD4+ T cells from the three identical twin pairs studied (± SD).

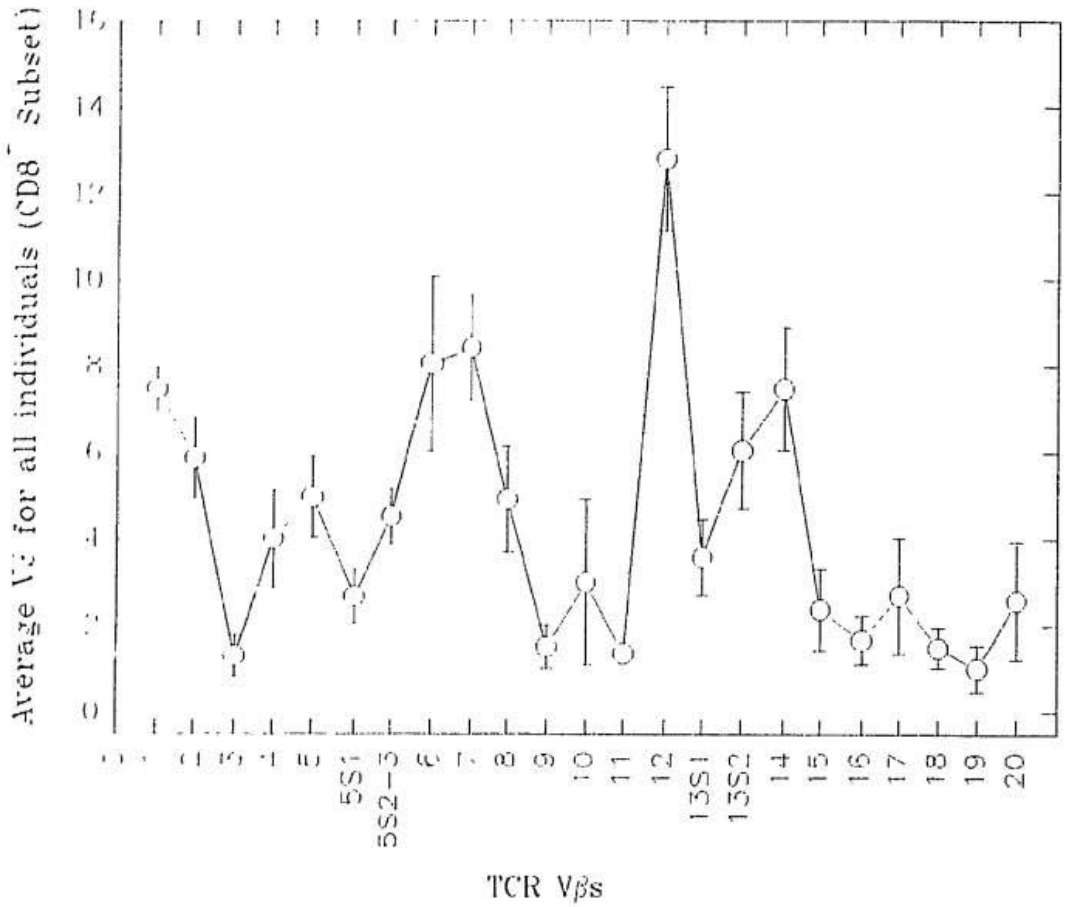


Figure 7.5b
The mean Vβ repertoires of CD8⁺ T cells from the three identical twin pairs studied (± SD).

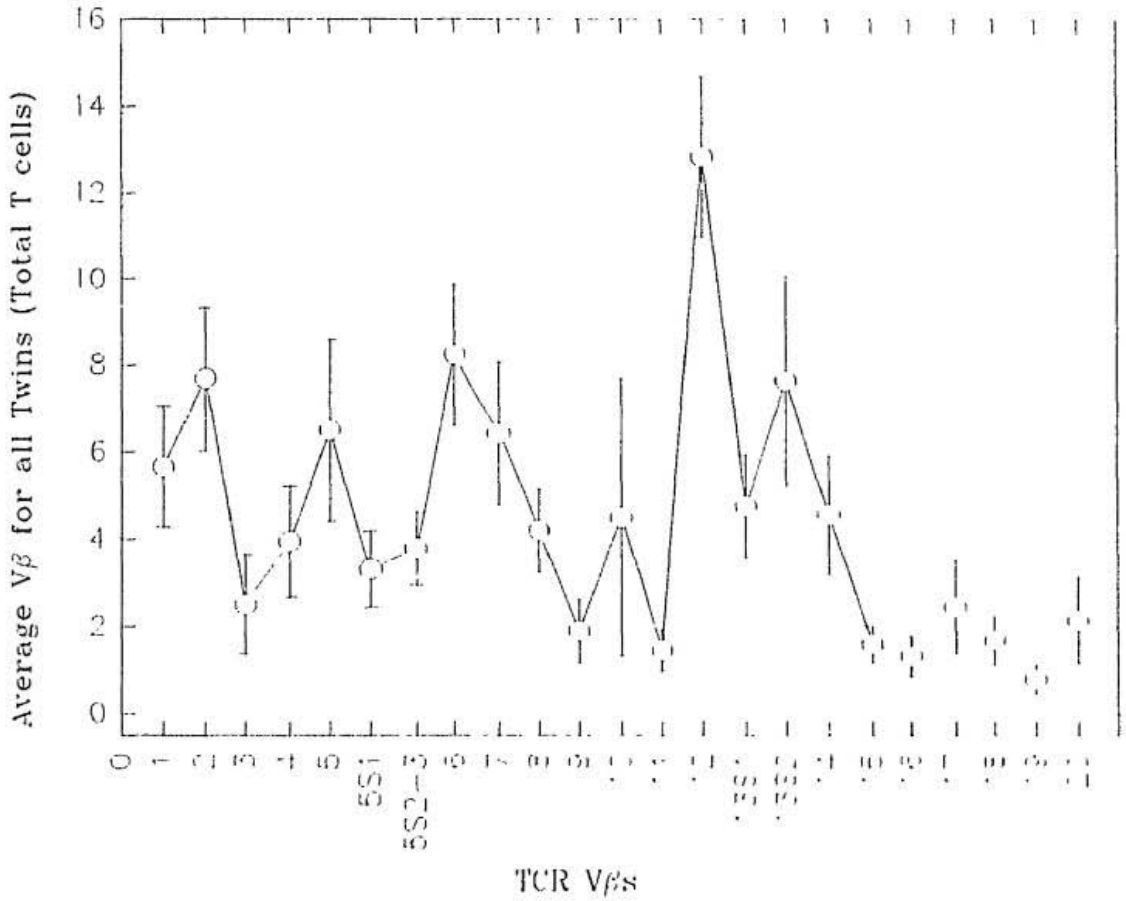


Figure 7.5c
Mean Vβ values from the total T cell Vβ repertoires of six identical twin pairs studied (± SD).

95% confidence intervals were calculated for the standard deviations of each Vβ. Please note that a sample MINITAB calculation is shown in appendix B.

7.5.2 Analysis of the CD4+ T cell subset

7.5.2A Grouping of Vβs with variable variances

The analysis of the TCR Vβ data for the CD4+ subset produced a p value of 0.014 (figure 7.5.2). This means that certain Vβs have variances which are significantly different from those of other Vβs. From figure 7.5.2 the Vβs could be divided into three broad groups based on the magnitude of the Bonferroni confidence intervals (CI). Group one consisted of the Vβs with the lowest CIs and these included Vβs 3, 9, 11, 15, 16, 18 and 19. Group three included Vβs 1, 2, 4, 5.2, 6, 10, 12, and 13.2 and these Vβs had the highest CIs. Group two included Vβs 5, 5.1, 7, 8, 13.1, 14 and 17 and these Vβs had CIs that were between those of groups one and three (table 7.5.2). Each of the three groups was then tested for internal homogeneity of variance (HOV). The p values for groups 1-3 were 0.712, 0.870 and 0.899 respectively (appendix C, figure C1-C3 and table 7.5.2). The high p values indicate that there are no significant variations in the level of Vβs within each of these groups. Group 1 had a variation in CIs from 0.7%-1.2%, group 2 had variation in CIs from 1.7%-2.9%, and group 3 had CIs ranging from 3.1-5.0%.

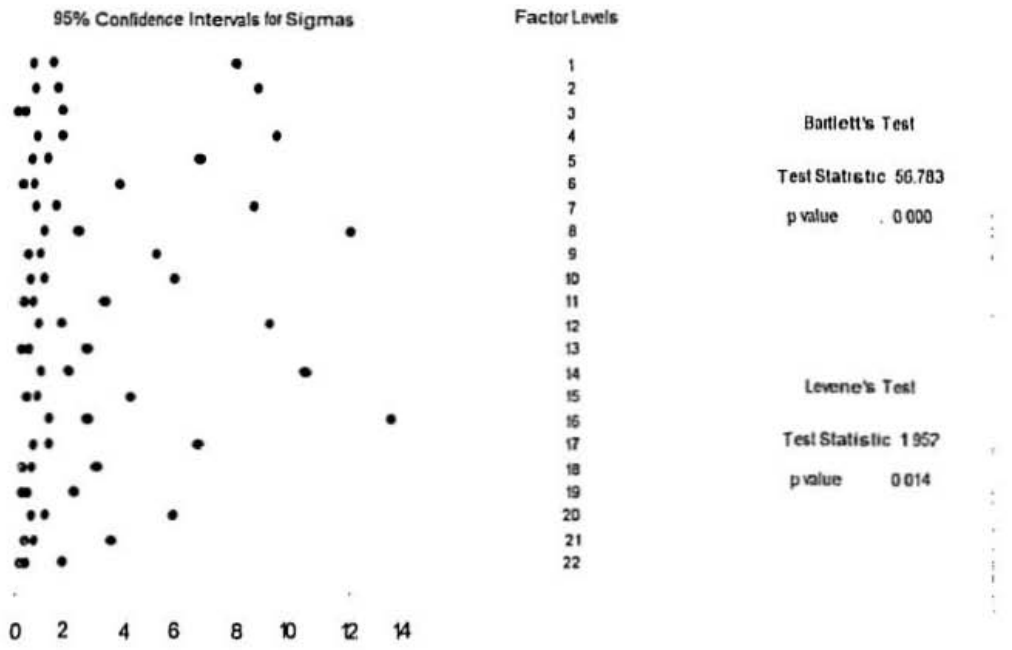


Figure 7.5.2

Bartlett's and Levene's (BL's) tests of homogeneity of variance (HOV) as determined for all Vβs from the CD4' T' cell subset. Factor levels 1 - 22 correspond to Vβs 1 - 19 respectively; Vβ20 is excluded from the analysis (see Results).

Table 7.5.2

Bartlett's and Levene's tests of homogeneity of variance for the CD4' T cell subset.

Groups	95% CI's for SD'	Vβs represented
1	0.7 - 1.2	3, 9, 11, 15, 16, 18, 19
2	1.7 - 2.9	5, 5.1, 7, 8, 13.1, 14, 17
3	3.1 - 5.0	1, 2, 4, 5.2, 6, 10, 12, 13.2

Group comparisons	P value
1	0.712
2	0.870
3	0.899
1 versus 2	0.000
1 versus 3	0.000
2 versus 3	0.001

Conclusion:

Variance of groups: 1 < 2 < 3

Next, the groups were cross-analyzed *i.e.*, the standard deviation of each group was compared with the standard deviation of the other groups (appendix C, figures C4-C6 and table 7.5.2). This comparison of all three groups showed significant differences in variance between each group ($p < 0.001$, for each comparison). These results provide statistical evidence that in the $CD4^+$ T cell subset Vβs can be categorized into Vβs of low variance, moderate variance and high variance.

7.5.2B Implications of the homogeneity of variance test for the $CD4^+$ T cell subset

The results of the Levene's test suggest that Vβs found in group three are more variable among the members of the three twin pairs than other Vβs. This variation could be due to either genetic or non-genetic differences among the three identical twin pairs. Therefore, each Vβ value in group three was compared among the identical twins to identify the causes of the high variance of expression.

The levels of Vβs 1 and 2 were relatively constant for all twin pairs with the exception of MB1/MB2. The levels of Vβs 1 and 2 for twin MB1 was 7.1% and 8.7% while the levels for MB2 were 3.3% and 4.0%. The other twins had levels between 5.7% and 8.5%. This particular distribution is well within the CIs for Vβs in group two. Since the differences in the expression of Vβs 1 and 2 were only found within one identical

twin pair (MB1/MB2), the different levels for these Vβs within this pair must be due to non-genetic causes. The Vβ 8 levels for twin pair LR1/LR2 were 5.8% and 5.7%. The levels of Vβ 8 for the other twin pairs were between 1.4% and 2.3%, a range of only 0.9%. The differences again were found in only one twin pair (LR1/LR2) and the levels of Vβ 8 were the same for both individuals of this twin pair. Since the Vβ 8 levels are different for other twin pairs it can be assumed that genetic factor(s) played a role in determining the Vβ 8 levels for this twin pair. In the same twin pair LR1/LR2, the levels of Vβ 5.2 were 6.5% and 7%. The levels for the other twins ranged from 2.2% to 4.5%. A range of 2.3% would have categorized Vβ 5.2 in group two. As the levels are constant in the LR1/LR2 twin pair it could be hypothesised again that genetic factors contributed to the higher levels of Vβ 5.2 in both members of this pair.

The levels of Vβ 6 and 10 were at different levels in identical twin pairs. For example, Vβ 6 levels for LR1 was 12% while the identical twin LR2 had a level of 6.6%, and the Vβ 10 level for individual MB1 was 0.9% while for the identical twin member MB2 Vβ 10 was 4.7%. Similar differences for Vβs 6 and 10 were found for the other twin pairs as well. Thus, this leads to the conclusion that differences in the levels of Vβ 6 and 10 among identical twin pairs may be due to non-genetic causes.

The levels of Vβ 12 seemed constant within twin pairs but different between the twin pairs; for MB1/MB2 14.3% and 14.6%, for SR1/SR2 12.0% and 13.1% and for LR1/LR2 9.2 and 9.9%. Since, the levels seem relatively constant within each twin pair but different between twin pairs, it is likely that levels of Vβ 12 are influenced by genetic factors in each twin pair.

The levels of Vβ 13.2 for twin pairs SR1/SR2 and LR1/LR2 ranged from 4.0% to 8.6%, however, the levels for twin pair MB1/MB2 were 11.3% and 11.8% respectively. Again, since the levels of Vβ 13.2 within the twin pair MB1/MB2 were relatively constant but different within the other twin pairs it can be concluded that these differences in Vβ 13.2 expression are due to genetic effects.

7.5.3 Analysis of the CD8⁺ T cell subset

7.5.3A Grouping of Vβs with variable variances

Initial analysis of Vβ data from the CD8' subset for all individuals allowed broad identification of three groups of Vβs with apparently different variances (table 7.5.3). The groups were separated as described for CD4' T cells (figure 7.5.3). Group 1 represented those Vβs with the lowest variance and group 3, those with the highest variance. Vβs represented in each group were; group 1: 1, 3, 9, 11, 16, 18, and 19, group 2: 2, 4, 5, 5.1, 5.2, 7, 8, 13.1, and 15 and group 3: 6,

Table 7.5.3

Bartlett's and Levene's tests of homogeneity of variance for the CD8' T cell subset.

Groups	95% CI's for SD	Vβs represented
1	1.9 - 3.2	1, 3, 9, 11, 16, 18, 19
2	1.6 - 2.5	2, 4, 5, 5.1, 5.2, 7, 8, 13.1, 15
3	2.9 - 5.1	6, 10, 12, 13.2, 14, 17

Group comparisons	P value
1	0.692
2	0.768
3	0.949
1 versus 2	0.823
1 versus 3	0.002
2 versus 3	0.000

Conclusion

Variance of groups: 1 = 2 < 3

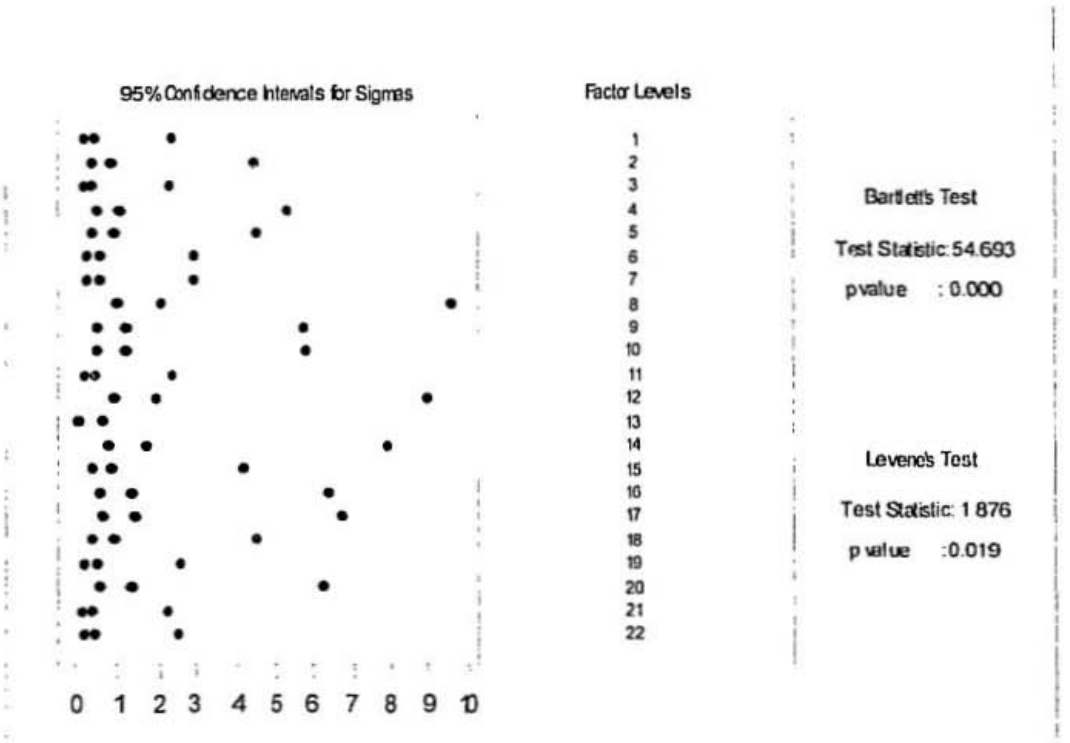


Figure 7.5.3

Bartlett's and Levene's (BL's) tests of homogeneity of variance (HOV) as determined for all Vβs from the CD8' T cell subset. Factor levels 1 - 22 correspond to Vβs 1 - 19 respectively; Vβ20 is excluded from the analysis (see Results).

10, 12, 13.2, 14, and 17. Groups 1-3 were then tested for internal homogeneity of variance (appendix D, figures D1-D3). The p values were 0.692, 0.768, 0.949 for groups 1-3 respectively. Variations for Vβs in group 1 ranged from 1.9% to 3.2%, group 2 from 1.6% to 2.5% and group 3 fluctuated from a low of 2.9% to a high of 5.1% (table 7.5.3). Next the three groups were cross compared; group 1 was compared with group 2 and 3, and group 2 was compared with group 3 (appendix D, figures D4-D6). The results indicated that there was no difference between the variances of group 1 and 2, but comparison of groups 1 and 2 with 3 showed significant differences. The p values for the comparison of 1 with 3 and 2 with 3 were 0.002 and 0.000 respectively. Thus for the $CD8^+$ subset there were two distinct groups of Vβs, groups 1 and 2 with low variance and group 3 with high variance.

7.5.3B Implications of the homogeneity of variance test for the $CD8^+$ T cell subset

The percent values of each Vβ for group three (table 7.5.3) were compared among the twin pairs to determine the causes of the greater variances in that group. The levels of Vβ 6 in twin pair LR1/LR2 was 9.3% and 8.3%. In twin pair SR1/SR2 Vβ 6 levels were 7.1% and 5.5%. The levels of Vβ 6 in twin pair MB1 and MB2 differed more; MB1 had 11.3% and MB2 had 7.0%. These differences in twin pair MB1/MB2 must be of non-

genetic origin. The levels of Vβ 10 within identical twin pairs ranged from 0.9% to 6.4%, thereby suggesting that the Vβ 10 levels observed may have been influenced by non-genetic causes.

The levels of Vβ 12 are variant mainly because of the twin pair SR1/SR2 who had levels of 12.6% and 15.2%. Twin pairs MB1/MB2 had Vβ 12 levels of 13.8% and 13.2% and LR1/LR2 had levels of 10.4% and 11.7%. The differences within the twin pairs MB1/MB2 and LR1/LR2 were small and the levels within each pair seem to have a genetic basis.

Levels of Vβ 13.2 within twin pairs MB1/MB2, SR1/SR2 and LR1/LR2 were relatively constant at 7.0% and 7.3%, 4.2% and 5.0%, and 7.4% and 5.4%. The variation within each twin pair would have placed Vβ 13.2 in group one for the HOV test. However, the differences in Vβ 13.2 between MB2 and SR1 caused this Vβ to be listed in group three. The levels within each twin pair were relatively constant but the levels were different between the twin pairs. Thus, the differences between the twin pairs are likely due to genetic factors. The same analysis applies to Vβs 14 and 17. For Vβ 14 the levels in twin pairs MB1/MB2, SR1/SR2 and LR1/LR2 were at 8.3% and 7.9%, 5.7% and 5.7% and 8.9% and 8.4%. The levels in the individual pairs are relatively similar but, between the twin pairs the levels are different especially between SR1/SR2 and LR1/LR2 thus, suggesting that genetics influenced the percent

levels of Vβ 14. For Vβ 17 the levels for twin pairs MB1/MB2 and LR1/LR2 ranged between 1.6% and 2.3%. However, the levels for SR1 and SR2 were at 4.2% and 4.6% again, arguing for a genetic explanation for the variation between SR1/SR2 and the other twin pairs.

7.5.3C Implications on Pearson r analysis of identical twin pairs and unrelated pairs for the CD8⁺ T cell subset.

There were two comparisons for the CD8⁺ subset that caused the null hypothesis to be rejected in the chi-square test (table 7.2.1 c). This means that the r values for the unrelated pairs and twin pairs were different. Re-analysis established that rejection of the null hypothesis was due to the low r values found in the unrelated pairs. For the two situations in which the null hypothesis was rejected, the unrelated pairs were MB1/SR2 and SR2/LR1. SR2 was common to both unrelated pairs and it was considered possible that the differences in these comparisons were the result of highly variant Vβs from SR2 as determined by BL's tests for HOV. Comparison of the data for the three individuals, MB1, LR1 and SR2, implicated two potential Vβs, 6 and 10. The Vβ 6 levels in MB1, LR1 and SR2 were 11.3%, 9.3% and 5.5% and the levels of Vβ 10 were 1.6%, 0.9% and 6.4% respectively. Exclusion of Vβ 6 from the correlation analysis for these particular individuals did not produce any significant difference in the

r values. However, removal of Vβ 10 from the correlation analysis led to acceptance of the null hypothesis as there was an increase in the value of r between unrelated pairs. The level of Vβ 10 found in the other twin (SR1) was 3.5% and this level was comparable to the levels found in the siblings MB2 and LR2. Since there were no differences in correlation analyses when using SR1 in unrelated pairs, and the levels of Vβ 10 were different in the members of the twin pair SR1/SR2, it can only be concluded that the null hypothesis was rejected due to non-genetic causes affecting the levels of Vβ 10 in twin pair SR1/SR2.

7.5.4 BL's tests of HOV for the total T cell repertoire from identical twin pairs

7.5.4A Grouping Vβs by variances

The BL's tests for HOV was performed on the total T cell repertoire of identical twins (figure 7.5.4). The initial $p < 0.001$ indicated a high degree of heterogeneity of variance for the Vβ levels. The Vβs could be split broadly into 4 different groups (table 7.5.4a). The p values for HOV within these groups were 0.350, 0.937, 0.897 and 0.492 for groups 1-4 (appendix E, figures E1-E4). From the Bonferroni CIs, the values for group 1 ranged from 0.4%-0.7%, for group 2 from 1.2% to 1.6%, for group 3 from 1.8%-2.6% and for group 4 from 3.1% to 5.1%. Cross-comparison of all groups yielded $p < 0.001$ *i.e.*, there were four different levels of variance found for

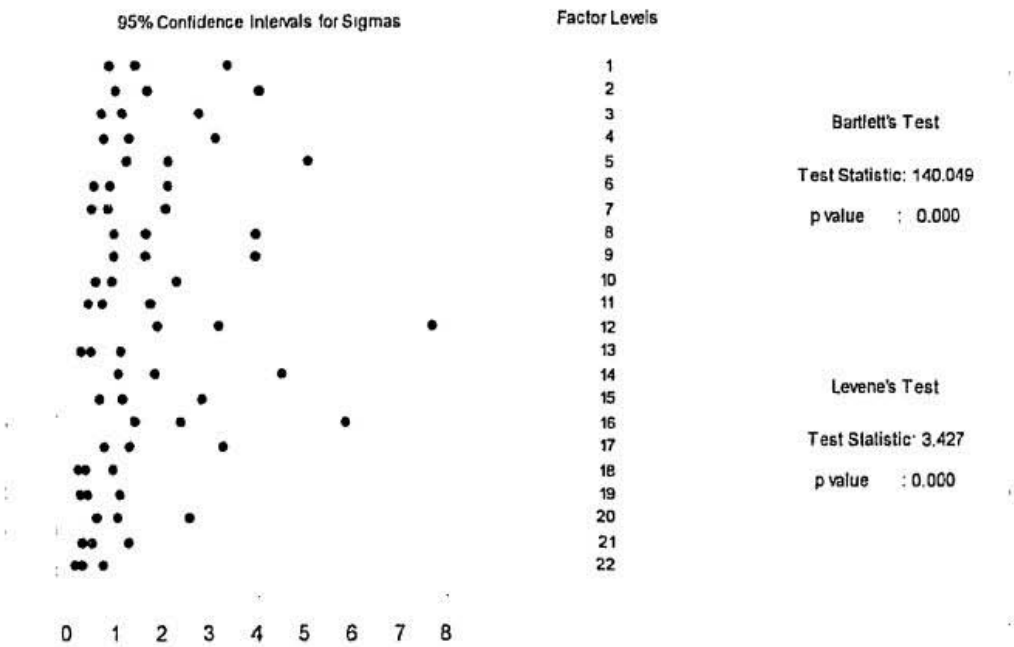


Figure 7.5.4

Bartlett's and Levene's (BL's) tests of homogeneity of variance (HOV) as determined for all Vβs from total T cells of identical twins. Factor levels 1 - 22 correspond to Vβs 1 - 19 respectively; Vβ20 is excluded from the analysis (see Results).

Table 7.5.4a

Bartlett's and Levene's tests of homogeneity of variance for the total T cell Vβ repertoire of monozygotic twin pairs.

Groups	95% CI's for SD'	Vβs represented
1	0.4 - 0.7	11, 15, 16, 18, 19
2	1.2 - 1.6	3, 5.1, 5.2, 8, 9, 13.1, 17
3	1.8 - 2.6	1, 2, 4, 6, 7, 14
4	3.1 - 5.1	5, 10, 12, 13.2

Group comparisons	P value
1	0.350
2	0.937
3	0.879
4	0.492
1 versus 2	0.000
1 versus 3	0.000
1 versus 4	0.000
2 versus 3	0.000
2 versus 4	0.000
3 versus 4	0.000

Conclusion

Variance of groups: 1 < 2 < 3 < 4

the Vβs; group 1 < group 2 < group 3 < group 4 (appendix E, figures E5-E10).

7.5.4B Implications of HOV on the Vβ repertoire for total T cells in identical twins

The reasons for the variability of group four Vβs were investigated by studying the percent of each Vβ for the identical twin pairs. The levels of Vβ 5 were relatively constant for all individuals (4.4% - 7.0%) with the exception of DB2 (12.6%). DB1, the identical twin of DB2 had a level of 7.0% *i.e.*, the variations in Vβ 5 levels in this pair must have been caused by non-genetic factors. The levels of Vβ 10 were widely different for all individuals and ranged from 0.0% to 11.8%. The differences within twin pairs suggest the changes were due to non-genetic causes.

The levels of Vβ 12 seemed to fluctuate widely and ranged from 10.2% to 15.9%. Despite the range, it is not clear if the differences were due to genetic and/or non-genetic causes. The reasons for the high variance of Vβ 13.2 could be traced to two twin pairs - DB1/DB2 and SG/SN. For twin pair DB1 and DB2 the Vβ 13.2 levels were 9.0% and 5.2% respectively. For twin pairs SG and SN, Vβ 13.2 levels were 13.6% and 6.9%. As the variance levels were not consistent within both individuals of a twin pair, the changes within twin pairs are likely to have been due to non-genetic causes.

7.5.4C Implications on correlation analysis of twin pairs and unrelated pairs for the total T cell analysis.

Randomly chosen twin pairs assigned as unrelated pairs and twin pairs produced r values that also led to rejection of the null hypothesis (table 7.3b, section A). Testing the r values from all the identical twin pairs also led to rejection of the null hypothesis (table 7.3a, section A). This rejection was subsequently shown to be due to the low r value from the BB/TB twin pair comparison (table 7.3b, section B).

Using the HOV test to identify the highly variant Vβs it was found that most Vβs differed by less than 2.6% between individuals with the exception of Vβ 10 which, differed by 8.8% for BB and TB. The removal of Vβ 10 from the comparison of BB/TB increased the correlation coefficient from 0.729 to 0.873. Re-analysis by the chi-square test led to acceptance of the null hypothesis (7.5.4b). It was found that correlations involving the individual BB with other unrelated individuals caused the r value to be quite low, but this was not the case for correlations involving the twin TB with other unrelated individuals.

The results of the chi-square test of the significance of the comparison between unrelated pairs, DB1/SR2 and DB2/SR1 or DB1/SR1 and DB2/SR2 (table 7.3b, section B), with identical twin pairs led to rejection of the null hypothesis. If a non-genetic cause could affect the correlation between identical

Table 7.5.4b

Chi-square analysis of r values obtained from comparisons of total T cell Vβ repertoires of monzygotic twin pairs and unrelated pairs after the removal of Vβ10 for twin pair BB/TB and Vβ5 for all unrelated comparisons.

	Pearson 'r'	Z scores	
Twin Pairs LR1/LR2	0.943	1.76	
BB/TB	0.873	1.35	(without only Vβ 10)
SG/SN	0.782	1.05	
SC1/SC2	0.935	1.70	
(A) Unrelated Pairs			
DB1/SR2	0.821	1.16	
DB2/SR1	0.776	1.04	
OR			
(B) Unrelated Pairs			
DB1/SR1	0.764	1.00	
DB2/SR2	0.799	1.10	

CHI-SQUARE COMPARISONS		CRITICAL CHI-SQUARE VALUE
Twin Pairs	6.22	7.81 at DF = 3, α = 0.05
(A) Unrelated Prs	0.14	3.84 at DF = 1, α = 0.05
(B) Unrelated Prs	0.01	3.84 at DF = 1, α = 0.05
Twin Prs VS (A)	9.81	11.07 at DF = 5, α = 0.05
Twin Prs VS (B)	10.71	11.07 at DF = 5, α = 0.05

pairs BB and TB, then the differences between unrelated pairs and identical twins could also be due to non-genetic causes. The r value for the comparison of DB1 and DB2 was 0.771 while the comparison for SR1 and SR2 gave an r value of 0.850. The lower r value for DB1 and DB2 suggested there were more differences in Vβ expression between these individuals than between SR1 and SR2. All Vβs from group 3 were compared for the DB1/DB2 twin pair and it was found that the % value for Vβ 5 differed for these two individuals by 5.6%. Removal of this Vβ from the correlation analysis caused an increase in the r value of 0.771 to 0.849. Subsequent comparison with SR1 and SR2 caused the r values to increase. The chi-square test between related twin pairs and unrelated pairs led to acceptance of the null hypothesis, *i.e.* there was equality of r values for both groups (table 7.5.4b), when Vβ 5 was removed from the correlation analysis.

7.6 Bartlett's and Levene's tests for HOV on large families

7.6.1A BL's HOV on family FK

The Bartlett's and Levene's tests were performed on the three families. The HOV analysis on Vβs for family FK yielded a p value of 0.155. The Vβs for this family could be divided broadly into four groups based on Bonferroni CIs. Tests of HOV within each group produced p values of 0.891, 0.705, 0.935 and 0.989 for groups 1-4 suggesting homogeneity of variance within each group (table 7.6.1a). The results from the cross comparison of the groups can be summarized by the following mathematical expression; variance of group 1 = variance of group 2 < variance of group 3 < variance of group 4.

7.6.1B Implications of variant Vβs on data from family FK

The percent levels of the three most variant Vβs from this family were compared among all members. It was found that the levels of Vβ 1 for the father (FKHHK) was 0.3% while in the rest of family this Vβ ranged from 5.7-7.0%. There were no detectable levels of Vβ 10 in the mother (FKAK) but in the other members of the family Vβ 10 ranged from 0.79 to 4.7%. Vβ 12 for FKHMK was at 18% while in the rest of the family this Vβ ranged from 13-14%. Unfortunately, it cannot be determined whether the levels of Vβ 1 in FKHHK, Vβ 10 in FKAK or Vβ 12 in FKHMK were influenced mainly by HLA or non-HLA factors as there were no other individuals with identical

Table 7.6.1a

Bartlett's and Levene's tests of homogeneity of variance for the total T cell Vβ repertoire from the members of the FK family.

Groups	95% CI's for SD*	Vβs represented
1	2.0 - 3.0	3, 6, 9, 11, 15, 16, 17, 18, 19
2	1.6 - 2.6	2, 4, 5.2, 7, 13.1, 14
3	2.0 - 3.7	5, 5.1, 8, 13.2
4	4.0 - 8.5	1, 10, 12

Group comparisons	P value
1	0.891
2	0.705
3	0.935
4	0.989
1 versus 2	0.401
1 versus 3	0.045
1 versus 4	0.000
2 versus 3	0.062
2 versus 4	0.000
3 versus 4	0.004

Conclusion

Variance of groups: 1 < 2 = 3 < 4

haplotypes to permit comparisons. As well, since the C haplotype was not inherited by any of the children, it is possible that the very low level of Vβ 1 in the father was influenced by the C haplotype.

7.6.1C Implications of the HOV tests on HLA effects for family FK

From the initial chi-square analysis some evidence for an effect of HLA was found. Comparison of the r values from the HLA-identical group with those from the haploidentical group led to rejection of the null hypothesis (table 7.4.1a) thus, suggesting a possible HLA effect. Re-analysis of the data for all members without the most variant Vβs is shown in table 7.6.1b. The r values, in comparison to table 7.4.1, are much higher for all groups in table 7.6.1b (section B). Cross comparison of the three HLA categories of individuals now led to acceptance of the null hypothesis for all comparisons. Earlier comparison of the HLA-identical group with the haploidentical group led to the acceptance of the null hypothesis. Without the three influential Vβs (1, 10 and 12) the data do not seem to show any evidence for an HLA effect on the remaining Vβs.

7.6.2A Bartlett's and Levene's tests for HOV on family FH

The Levene's test on the Vβs from the second family

Table 7.6.1b

Comparisons of HLA identical, haploidentical and HLA non-identical individuals from the family FK without the group 3 Vβs (1, 10 and 12) that were the most variant in this family: A) Family members and the extent of haplotype sharing is indicated; B) Pearson r values and derived Z scores are indicated for comparisons between HLA identical, haploidentical and HLA non-identical individuals; C) chi-square statistical comparisons are shown.

Section A.

Individuals	HLA Haplotypes		
FKAK (mother)	AB	FKRK	BD
FKHHK (father)	CD	FKUK	BD
FKJPK	BD	FKMK	BD
FKHMK	AD		

Section B.

HLA-identical (I)		Haploidentical (P)		Non-identical (NI)	
'r'	Z'	'r'	Z'	'r'	Z'
0.951	1.77	0.848	1.20	0.947	1.73
0.955	1.81	0.861	1.25		
0.958	1.85	0.925	1.56		
0.965	1.94	0.946	1.72		
0.940	1.67	0.948	1.74		
0.967	1.97	0.901	1.42		
		0.848	1.20		
		0.910	1.47		
		0.899	1.41		
		0.857	1.23		
		0.848	1.20		
		0.839	1.17		
		0.929	1.59		
		0.889	1.36		
		0.851	1.21		

Section C.

COMPARISONS (α =0.05)	CHI-SQUARE VALUES	CRITICAL CHI-SQUARE VALUES	
HLA-identicals	1.07	11.07	(DF = 5)
Haploidentical	8.68	23.68	(DF = 14)
NON-IDENTICAL	NA	NA	(DF = 0)
I VERSUS P	23.73	31.41	(DF = 20)
P VERSUS NI	10.49	24.99	(DF = 15)
I VERSUS NI	1.23	12.59	(DF = 6)

investigated produced a p value of 0.055. The Vβs could be divided into four groups but group 4 consisted of only one Vβ, 13.1. Cross-comparisons (table 7.6.2a) of group 1 with group 2 and with group 3 showed significance ($p < 0.000$) for both comparisons. Comparison of group 2 with group 3 showed significance ($p < 0.001$). The variances in group 1 ranged from 1.2 to 1.8%, in group 2 from 1.84% to 3.2%, in group 3 from 3.9 to 7.7% and in group 4 from 4.9% to 23.9%.

7.6.2B Implications of HOV tests on the family FH data

The percent level of each Vβ in groups 3 and 4 were compared for all family members. The levels of Vβ 13.1 in two siblings were much higher than in the rest of the family. Siblings FHLH and FHCH had 15 and 24% Vβ 13.1 respectively, while the rest of the family had levels between 3.6-5.1%. The % variations found for group 3 Vβs (4, 10, 12 and 17) were comparable to those of the most variant Vβs of group 4 (1, 10, 12) from the first family, FK. The level of Vβ 4 from sibling FHPH was 8% whereas the other family members had levels between 2 and 4%. The levels of Vβ 10 ranged from 0.00 to 11.90% and Vβ 12 levels ranged from 10 to 18%. Vβ 17 levels in FHDH were at 17% while the other members were between 2.1 to 4.4%.

Siblings FHLH and FHPH are HLA-identical. The levels of Vβ 13.1 in FHLH were not reflected in the HLA-identical

Table 7.6.2a

Bartlett's and Levene's tests of homogeneity of variance for the total T cell Vβ repertoire from the members of FH family.

Groups	95% CI's for SD'	Vβs represented
1	1.2 - 1.80	1, 5, 5.1, 5.2, 8, 9, 11, 15, 16, 18, 19
2	1.84 - 3.2	2, 3, 6, 7, 13.2, 14
3	3.9 - 7.7	4, 10, 12, 17
4	4.9 - 23.9	13.1

Group comparisons	P value
1	0.847
2	0.970
3	0.531
4	NA
1 versus 2	0.000
1 versus 3	0.000
1 versus 4	0.000
2 versus 3	0.000
2 versus 4	0.000
3 versus 4	0.699

Conclusion

Variance of groups: 1 < 2 < 3 = 4

sibling FHPH and similarly, the higher levels of Vβ 4 in FHPH were not reflected in FHLH. Therefore, the differences in Vβs 4 and 13.1 expression are likely due to non-HLA influences on the Vβ repertoire.

The levels of Vβ 10 were only similar for FHLHM and FHKH at 7.9% and 7.8%. The other individuals, including the HLA-identical siblings, showed fluctuating levels of Vβ 10 (0%-11%). Therefore, the best explanation is that non-HLA factors are the cause of these differences between Vβ expression, and in particular for the differences in HLA-identical siblings. The levels of Vβ 12 ranged from 13.4% to 16.2% in the family members with the exception of FHDH (9.8%). The different level in FHDH could be due to an HLA effect but this cannot be determined as there was no other family member with HLA identity to FHDH. The level of Vβ 17 in FHDH was 13.1% while in the rest of the family the levels ranged between 2%-3.3%. Again, it is difficult to determine if the differences in Vβ 17 expression were due to HLA or to non-HLA factors in the absence of an individual having HLA identity with FHDH.

7.6.2C Implications of HOV tests on HLA effects for family FH

It is possible that the initial chi-square calculations that demonstrated an effect of HLA on the Vβ repertoire were affected by the highly variant Vβs, *i.e.* Vβs 4, 10, 12, 13.1 and 17. To determine the effect of these Vβs on the

correlation analyses, these variant Vβs were removed from the analysis and the data were re-analyzed by the chi-square statistic (table 7.6.2b). The results were similar to the initial chi-square analysis; comparison of HLA-identical and HLA non-identical groups with the HLA haploidentical group led to rejection of the null hypothesis (table 7.6.2b, section C). However, comparison of HLA-identical with HLA non-identical groups led to acceptance of the null hypothesis. Further analysis of the data showed that the null hypothesis was rejected for the comparison between the HLA haploidentical and HLA-identical groups due to poor correlations between the father and the children. The correlations of the father and the mother with the children were tested (table 7.6.2c) and were found to be statistically different by chi-square analysis.

7.6.3 HOV Analysis of Family FG

Similar tests were performed on the third and largest family (table 7.6.3). The Vβs were divided broadly into four groups - the first group consisting of the least variant Vβs had variations of 0.8 to 1.7%, group 2 Vβs had variations of 2.6 to 3.7% and group 3 Vβs of 3.6 to 7.7%. The range of variation for the fourth and most variant group, consisting of Vβs 2, 8, 13.1, and 10, was 4%-14%. The percent variation for group 2 Vβs in this family (FG) was comparable to the %

Table 7.6.2b

Comparisons of HLA-identical, haploidentical and HLA non-identical individuals from the family FH without the group 3 Vβs that were the most variant in this family: A) Family members and the extent of haplotype sharing is indicated; B) Pearson r values and derived Z scores are indicated for comparisons between HLA identical, haploidentical and HLA non-identical individuals; C) chi-square statistical comparisons are shown.

Section A.

Individuals	HLA Haplotype
FHLHM (mother)	AB
FHDGH (father)	CD
FHLH	AC
FHKH	BD
FHPH	AC
FHCH	AD

Section B.

HLA-identical(I)		Haploidentical(P)		Non-identical (NI)	
'r'	Z'	'r'	Z'	'r'	Z'
0.929	1.56	0.636	0.71	0.406	0.41
		0.856	1.21	0.930	1.57
		0.907	1.43	0.910	1.45
		0.602	0.66		
		0.885	1.32		
		0.898	1.38		
		0.575	0.62		
		0.850	1.19		
		0.903	1.41		
		0.526	0.55		
		0.936	1.62		

Section C.

COMPARISONS (α =0.05)	CHI-SQUARE VALUES	CRITICAL CHI-SQUARE VALUES	
HLA-Identicals	NA	NA	(DF = 0)
Haploidenticals	24.06	18.31	(DF = 10)
Non-identical	13.14	5.99	(DF = 2)
I VERSUS P	27.12	19.68	(DF = 11)
P VERSUS NI	37.30	22.36	(DF = 13)
I VERSUS NI	2.24	7.81	(DF = 3)

Table 7.6.2c

Chi-square analysis of r values from comparisons of mother and father with the respective children for the FH family. These correlations were obtained after the removal of the most variable Vβs.

Pearson r	
Mother	Father
0.856	0.636
0.885	0.602
0.850	0.575
0.936	0.536

	CHI-SQUARE VALUES	CRITICAL CHI-SQUARE	
Father	1.67	7.82	(DF = 3)
Mother	0.15	7.82	(DF = 3)
Mother versus Father	17.99	14.07	(DF = 7)

Table 7.6.3

Bartlett's and Levene's tests of homogeneity of variance for the total T cell Vβ repertoire from members of the FG family.

Groups	95% CI's for SD*	Vβs represented
1	0.8 - 1.7	13.2, 15, 16, 18
2	2.6 - 3.7	1, 3, 4, 5, 5.1, 5.2, 6, 7 9, 11, 12, 14, 17, 19
3	3.6 - 7.7	2, 13.1
4	4.9 - 10.3	8, 10

Group comparisons	P value
1	0.333
2	0.941
3	0.524
4	0.674
1 versus 2	0.000
1 versus 3	0.000
1 versus 4	0.003
2 versus 3	0.010
2 versus 4	0.034
3 versus 4	0.974

Conclusion

Variance of groups: 1 < 2 < 3 = 4

variation of Vβs from group 3 or 4 from other families. High variations were found for Vβs 9, 11 and 19. These Vβs, in samples from the other two families were relatively invariant. Cross comparisons of groups 1-4 indicated that the variance of group 1 < variance of group 2 < variance of group 3 = variance of group 4.

With this family, because of the number of Vβs that were highly variable in expression, it is not possible to delineate HLA effects at the level of individual family members. However, it is apparent that there are HLA effects found in the comparisons of the profiles of individuals with the AC haplotypes.

CHAPTER 8

DISCUSSION II

8.1 Primer efficiency and the TCR literature

Few data are available on the efficiency of Vβ primers yet Vβ primer sets have been used to assess the relative tissue expression of various Vβs (Paliard *et al*, 1991; Howell *et al*, 1991; Scottini *et al*, 1991; Ben-Nun *et al*, 1991; Davies *et al*, 1991; Geursen *et al*, 1993; Oksenberg *et al*, 1990; Davey *et al*, 1993; Birnbaum *et al*, 1992; Hodges *et al*, 1994; Wucherpfennig *et al*, 1990). One study was done by Choi *et al* (1989) to determine the efficiency of a few primers. Hall *et al* (1992) attempted to maximize primer efficiency by maintaining narrow ranges of hybridization conditions for Vβ primers but it was not possible to achieve perfect conditions for all primers. In addition, the latter study did not provide any tangible analysis of primer efficiency ie, a pictorial representation of how each primer behaved under the usual PCR amplification conditions. Moreover, as we and others already have an existing panel of primers, it would be expensive to replace these by a primer set with narrow hybridization temperature ranges. Therefore, it was felt to be more practical to discern the limitations of our already existing panel of TCR Vβ primers.

The results provide a very definite picture of how primers behave under the usual reaction conditions. When an equal number of target molecules was provided for all Vβ

primers, there definitely was a hierarchy in priming efficiencies. In all three trials the Vβs 6 and 15 primers had the highest efficiencies. In addition the uncorrected data on Vβ repertoires from various individuals showed significant correlations with the primer efficiency ratios. This result suggests that values obtained from quantitative PCR analysis partly reflect the efficiencies of the primers used. The immediate conclusion that can be drawn is that comparisons between Vβs within an individual tissue sample are invalid. For example, if Vβs 1 and 2 are detected at higher levels than Vβ 3, using uncorrected data, one cannot conclude that the former Vβs are expressed at higher levels than the latter Vβ. Comparisons made between samples from different individuals would be valid using uncorrected data. For example, if Vβ 6 is detected at higher levels in individual A than in individual B, one can conclude that Vβ 6 is expressed at higher levels in A. Therefore, in tissue lesions, or in fluids obtained from any disease state, the apparently dominant TCR Vβ(s) need not be the disease inducing Vβ(s) but may just be the better primed Vβ(s). An important exception consists of studies in which Vβ expression in diseased tissues is compared with Vβ expression in blood, as the latter provides an appropriate control for Vβ expression in the affected tissues.

Various researchers have used different Vβ primer sets (Oksenberg *et al*, 1990, 1992; Wucherpfennig *et al*, 1990; Choi

et al, 1989; Hall *et al* 1993). The efficiencies of the primers making up each of the primer sets are unknown and often assumed to be equivalent. As mentioned in the methods, the Vβ primers obtained for this study came from two different sources. Comparison of the various primer efficiencies (figure 4.1) suggests that highly inefficient primers come from both sources. It is likely that other primer sets will also show marked variation in priming efficiencies. Thus, the different conclusions reached as to the normal levels of expression of particular Vβs are likely related at least in part to variation in Vβ primer efficiencies in different studies (Akolkar *et al*, 1993; Hawes *et al*, 1993).

It could be argued that the observed differences in primer efficiencies were due to different optimal annealing temperatures for the different Vβ primers. For the primers used in this experiment, we used the same annealing temperatures (55 °C) as described by Choi *et al* (1989) and Wucherpfennig *et al* (1990) (the source of our primers).

The study conducted by Lovebridge *et al* (1991) considered the possibility of varying primer efficiencies. The protocol developed in this study allows for correction of the differences in priming efficiencies and facilitates comparisons within an individual without results being influenced by primer efficiencies. Experiments therefore need not be confined to comparisons of values obtained from the

same blots as described by Lovebridge *et al* (1991) and different samples can be compared to facilitate more extensive data analysis.

8.2 Shaping of the TCR Vβ repertoire

The mechanisms responsible for shaping the human peripheral blood T cell Vβ repertoire are not completely defined but both genetic and non-genetic factors have been implicated from the mouse models. These include genetically determined polymorphisms, positive and negative selection events in the thymus, and post-thymic shaping of the repertoire by antigens and superantigens.

In humans the relative contributions of genetic and non-genetic factors have been difficult to define and several studies have used twins and large multi-sibling families to clarify the factors influencing the human TCR Vβ repertoire (Akolkar *et al*, 1991; Hawes *et al*, 1993; Akolkar *et al*, 1993). From the reports that used qPCR, a strong role for genetics including HLA has been claimed. These claims are based, for the most part, on data that have not been subjected to rigorous statistical analysis. In particular Hawes *et al* (1993) argue that genetics including HLA are the predominant influence on the Vβ repertoire. These conclusions are based on differences in the ranges of R^2 correlations obtained in twins versus unrelated individuals. Data obtained in the present

study were analyzed using the same statistics, i.e. R^2 correlations. The results of these calculations was remarkably similar to those obtained by Hawes *et al* (1993). However, rigorous statistical testing, using a Z test for comparing correlational values, showed that the R^2 differences found between comparisons of twins and unrelated pairs were statistically insignificant not only for the data in the present study but also for the available data from Hawes *et al* (1993).

8.2.1 Statistical approaches in this study

In this report the data were statistically assessed by regression and the Pearson coefficient of correlation, r. The Pearson correlation was used as the initial statistical process of comparison for the following reasons; 1) normalization of the Vβ data to find percent expression causes other statistical comparisons to be inapplicable, 2) linear association of two separate data sets was assessed and 3) use of correlation analysis allowed the implementation of the chi-square test to measure the significance of r values obtained for unrelated comparisons and to draw some mathematically definite conclusions.

In contrast to other studies mentioned, this study undertook to examine in detail the reasons why differences occur in Vβ repertoires between unrelated and related individuals. An r value is calculated by a formula that uses

the sum of squares between variables X and Y. Since the strength of the relationship between X and Y is determined by the overall similarities, differences in the levels of individual Vβs within identical twin pairs and unrelated pairs cannot be assessed by this method. Thus, in this report the Bartlett's and Levene's tests for homogeneity of variance were used to identify particular Vβs that are highly variable in their expression between individuals. Once the variant Vβs were identified in total T cells and T cell subsets from twins or in each family, the results were used to assess whether differences in expression of each of these Vβs were due to genetic or non-genetic factors. By directly comparing individuals, the Vβ findings could be interpreted as being influenced by genetic or non-genetic factors. This is in contrast to the mentioned literature where first level statistical differences were assumed to have a genetic origin. In addition, I was also able to determine the contribution of these Vβs to the Pearson r value when comparing the TCR Vβ profile from different individuals.

As mentioned previously, the statistics used in the literature to analyze the Vβ repertoire are varied. In this report, the X^2 (chi-square) statistic to test whether the Vβ repertoires of different individuals are different, was derived by Dr V. Gadag (Division of Community Medicine, MUN). Correlation analysis and the Bartlett's and Levene's tests

were used in combination to provide a systematic analysis of data obtained from the qPCR analysis.

8.2.2 General observations of the TCR Vβ profile from all samples

In this study it was found that six Vβs typically contribute approximately 50 % of the Vβ repertoires of total T cells and both T subsets. Vβs 6 and 12 were among the highest contributors present in all three categories (CD4$^+$ and CD8$^+$ T subsets and total T cells). Vβs 3, 9, 10, 11, 16 and 19 were found at very low levels for most individuals. Using the method of anchor PCR, Rosenberg *et al* (1992) found that Vβ 6, 4 and 12 were the most commonly used Vβs, while Vβs 3, 10, 11, 15, 18, 19 and 20 were consistently found at low levels. These results, with the exception of the levels of Vβ 4, are similar to those in the present study. The levels of Vβs 15, 18 and 20 were found to be less than 5 % in this study as well as in the study by Rosenberg *et al* (1992).

8.2.3 Factors shaping the repertoire in the CD4$^+$ and CD8$^+$ T subsets

The chi-square analysis of the CD4$^+$ T subset data shows that no differences exist between the r values obtained from the comparisons of unrelated pairs and identical twins. For the CD8$^+$ T subset, some of the initial chi-square tests showed statistically significant differences between unrelated and twin pairs suggesting the possibility of genetic contributions

to the CD8$^+$ T cell Vβ repertoire. However, further analysis (using the data from BL's HOV analysis) showed that one Vβ level in a single individual, SR2, had influenced the data and led to the preliminary conclusion of a genetic contribution to the CD8$^+$ T cell Vβ repertoire. It was shown that the level of Vβ 10 in SR2 was non-genetically modulated and that subsequent removal of this Vβ rendered the differences between unrelated pairs and identical twins insignificant. Therefore, for both CD4$^+$ and CD8$^+$ T subsets there was no evidence for a significant effect of genetics on the overall TCR Vβ repertoire in contrast to what has been reported by others, eg Hawes *et al*, (1993) and Akolkar *et al*, (1993). A more complete statistical analysis by Hawes *et al* (1993) or Akolkar *et al* (1993) would also have allowed them to conclude that genetics does not make a major contribution to the Vβ repertoire, or probably more likely that a genetic contribution at the Vβ family level is difficult to detect with the qPCR method.

Closer analysis of the present data revealed some evidence for a genetic effect on particular Vβs in both CD4$^+$ and CD8$^+$ T subsets. From the Bartlett and Levene's (BL's) tests of homogeneity of variance (HOV), Vβs 6, 10, 12 and 13.2 were the most variant Vβs for both CD4$^+$ and CD8$^+$ T cells. For the CD4$^+$ T cell subset it was also shown that Vβs 1, 2, 4, and 5.2 were among the most variant Vβs while Vβs 14 and 17 were among the most variant Vβs for the CD8$^+$ T cell subset.

Qualitative analysis of these Vβ data reveals some interesting observations. In section 7.6.2B it was shown that for the CD4' T subset, the levels of Vβs 4, 5.2, and 13.2 were influenced by genetics in particular individuals and Vβ 12 levels were genetically influenced in all three twin pairs. For the CD8' subset, the levels of Vβ 13.2, 14, 17 and 20 were shown to be influenced by genetics. Evidence for genetic effects on the Vβ repertoire were therefore found in particular T subsets, in specific Vβs and in distinct twin pairs. Nevertheless, it is difficult to attribute genetic contributions to the Vβs with complete certainty as it is possible that the Vβ repertoires of members of a twin pair could have been equally affected by exposure to the same antigen.

8.2.4 Effects shaping the total T cell repertoire

Initial analysis of all of the r values obtained from the comparison of the total T cell Vβ repertoires from six identical twin pairs led to rejection of the null hypothesis that all r values are equal. This indicates that there are differences in r values obtained from Vβ repertoire analysis among identical twin pairs. Removal of one twin pair, BB/TB, from the chi-square analysis led to acceptance of the null hypothesis, i.e. the total T cell Vβ repertoires of twin pairs were then equal. It was found that the low r value in the BB/TB twins was due to different Vβ 10 levels between the

members of this twin pair. Comparison of r values from identical twin pairs with r values from unrelated pairs led to rejection of the null hypothesis that twins and unrelated pairs have similar total T cell Vβ repertoires. It was discovered that the differences were due to varying levels of Vβ 5 in members of the DB1/DB2 twin pair . Non-genetic factors were attributed as causing the differences since the levels of Vβ 5 and 10 were different in genetically identical twin pairs. It should be noted that only detailed analysis of the data uncovered the influence of non-genetic events on the TCR Vβ profile. In the studies by Hawes *et al* (1993) or Akolkar *et al* (1993), the contributions of non-genetic events were not thoroughly investigated and Vβ differences were entirely attributed to genetic and HLA effects.

8.2.5 Identifying skewing patterns toward either the $CD4^+$ or $CD8^+$ T cell subset.

Antibody studies have been crucial in identifying Vβs that show skewed usage in particular T cell subsets. Hawes *et al* (1993) have used *t* tests to identify skewing while Akolkar *et al* (1993) have used $CD4^+/CD8^+$ T cell ratios to determine skewing from qPCR analysis of Vβ. Statistical comparison of one twin member with the other, for example MB1 with MB2, is not possible with the *t* test. In identifying possible Vβs that are skewed toward the MB1/MB2 twin set, the *t* test compares

the mean value for a particular Vβ from this twin pair with the mean values of the same Vβ from the two other twin pairs LR1/LR2 and SR1/SR2. The use of *t* tests to identify such skewness may fail for the following reason. The homogeneity of variance analysis for the $CD4^+$ T subset showed that in the twin pair MB1/MB2, Vβs 1, 2, 8 and 10 were present at different levels. For MB1 the Vβ levels were the following; for Vβ 1 - 7.1 %, Vβ 2 - 8.7 %, Vβ 8 - 5.0 %, and Vβ 10 - 0.9 % for MB2, Vβ 1 - 3.34 %, Vβ 2 - 4.0 %, Vβ 8 - 2.13 % and Vβ 10 - 4.7 %. The mean values for MB1 and MB2 were for Vβ 1 - 5.2 %, Vβ 2 - 6.35 %, Vβ 8 - 3.6 % and for Vβ 10 - 2.8 %. In order for *t* tests to show skewing the mean values for one twin pair must be different from the other twin pair for the same Vβs. For twin pair SR1 and SR2 the mean values for the Vβ are the following Vβ 1- 6.2 %, Vβ 2 - 8.4 %, Vβ 8 - 4.4 % and Vβ 10 - 1.8 %. The means for the same Vβs in twin pair LR1/LR2 are Vβ 1 - 7.4 %, Vβ 2 - 7.4 %, Vβ 8 - 4.0 % and Vβ 10 - 1.0 %. The use of *t* tests to identify these Vβs did not find these values to be significantly different at $\alpha = 0.05$ (two tailed). It is evident from these results that there are differences at the levels of individual Vβs within identical twins but these differences will not be detected with the *t* test.

8.3 Effects shaping the TCR Vβ repertoire in large families

The purpose of analyzing large families was to determine

the effect of HLA on the Vβ repertoire. Theoretically the families offered an ideal situation to analyze the different effects of sharing two, one or no HLA haplotypes on the Vβ repertoire.

The family FH shows good evidence of an HLA contribution to the TCR Vβ repertoire. Family FG shows evidence for an HLA effect for the individuals with the AC haplotypes. However, family FK seemed to show some initial evidence for HLA effects but the latter effects were subsequently shown to be mostly due to non-HLA influences. For all families, the influence of non-HLA factors on particular Vβs were also shown.

In this study non-HLA influences on the Vβ repertoire were identified but whether these non-HLA influences are genetic and/or non-genetic cannot be determined. Further, there are additional complexities that cannot be completely addressed. The lack of clearly identifiable HLA effects may stem from the fact that individuals with identical HLA haplotypes need not be genetically identical as are monozygotic twins. Conversely, high correlations in Vβ repertoires between individuals who are HLA non-identical could have been due to shared genes outside the HLA region. There were clear instances of individuals with disparate HLA haplotypes with similar levels of particular Vβs and individuals with shared HLA haplotypes having different levels of particular Vβs.

Added to this complexity is the influence of non-genetic events such as those uncovered in the analysis of twins. The level of contribution of these events cannot be determined for the family analysis since these would be included in the broad category of non-HLA events.

From the homogeneity of variance analysis of the FH and FK families and the twins it was found that very few Vβs were highly variable in different individuals. Most Vβs such as Vβs 7, 9, 11, 14 and 19 had low variability in levels of expression. However, these particular Vβs showed high variability in expression in the FG family. The most variant Vβs of groups 3 and 4 were excluded from the correlation analysis in the FG family but their removal did not result in any significant differences in the chi-square analysis (data not shown). It is also interesting to note that the variability of group 2 Vβs in the FG family was comparable to the variability of group 3 and/or 4 Vβs from the other families and twins studied. Thus, it would require the removal of almost all of the Vβs to assess for an HLA effect on the repertoire for this family.

Compared to the data from all other individuals analyzed in this study, some unusual levels of particular Vβs were detected in the FG family: Vβ 2 in individuals FGJG and FGAG were at 15%; Vβ 8 in individual FGWG was 27%, Vβ 10 in FGCM was at 22%; FGJG had no detectable levels of Vβs 17 or 19, and

individual FGGG had Vβ 10 levels at 17 %. Superantigens can cause preferential stimulation and subsequent loss of expression of specific Vβs (Choi *et al*, 1989, 1991). It remains unknown as to whether the unusual levels of these Vβs are related to superantigens.

The levels of Vβs 15, 16 and 18 were relatively constant for all individuals including those in the FG family. Whether these particular Vβs are constitutively expressed at constant levels remains to be determined.

8.4 Concerns over repeatability and technical variations

It is difficult to determine in these experiments the extent to which technical variations affected the results. However, there is good evidence that the results are highly reproducible. It was shown that analysis of the Vβ repertoire from one individual in two different culture tubes stimulated with PHA on the same day gave a r value of 0.950. As well, the wet to dry gel comparisons showed that three wet gels could be analyzed with results having r values greater than 0.970. Other studies have used the same cDNA stock to show repeatability (Hawes *et al*, 1993). Further, there is indirect but convincing evidence to suggest there was a minimal contribution of technical variations to the results. First, from all of the analyses involving $CD4^+$, $CD8^+$ and total T cells in twins and families it was found that the number of highly variant Vβs were between 3 and 8 of the 23 tested Vβs. Of

these, Vβs 10 and 12 appeared consistently among the most variant Vβs. Furthermore, with the exclusion of the FG family, the expression levels of Vβs 3, 5.1, 5.2, 7, 8, 9, 11, 14, 15, 16, 18 and 19 (12 of 23 Vβs) were within 0.48 - 2.5 % CIs from the homogeneity of variance analysis. This then suggests either that errors consistently occur for the same Vβs for all individuals or that errors are quite small. Secondly, if technical error was common the results from the analysis of all groups should have shown similar findings to those in the FG family in which many Vβs showed marked variation between individuals. All of the Vβs found in group 2 of the FG family homogeneity of variance analysis would be grouped into the most variant groups for the other families or for the twin analysis. Thus, if technical errors had influenced the results in a major way one would have expected wide variations between all individuals and not just between the members of the FG family.

8.5 Similarity of TCR Vβ repertoires

The most striking observation from the study of the $CD4^+$ and $CD8^+$ T cell subsets was that very few Vβ were expressed at high levels and most individuals had comparable TCR Vβ repertoires. Comparing the total T cell repertoires from family FK and the $CD4^+$ and the $CD8^+$ T cell repertoires of the twins yielded r values greater than 0.890, for most comparisons. Comparisons of the total T cell repertoire with

both the CD4+ and CD8+ T cell repertoires also showed similar profiles with high correlations. Similar observations were made by Akolkar *et al* (1993) and Moss *et al* (1992).

A possible explanation for the similarities in Vβ repertoires between individuals is that the influence of HLA/genetics on the repertoires is not readily detected because the Vβ primers used were inappropriate. The primers used in this study and by others are for the most part Vβ family primers designed to recognize and amplify as many family members as possible within a single Vβ family. The Vβ counts detected for a particular family are directly proportional to the initial target amounts of constituent family members and not proportional to the target amounts of the individual Vβ member of that family. It is therefore possible that the sum of counts for a Vβ family from different individuals may be quite similar. For example, if for the Vβ 6 family, individual A expresses Vβ 6.1, 6.4 and 6.5 (but not 6.3 or 6.7) at levels of 30 %, 50 % and 20 % respectively and individual B expresses Vβ 6.1, 6.3 and 6.7 (but not 6.4 or 6.5) at levels of 60 %, 20 % and 20 %, the counts will not reflect HLA/genetic selection events but will show that the relative amounts of Vβ 6 are equal in the two individuals. Evidences of HLA/genetic influences on the Vβ repertoire would become apparent only when all individual Vβ family members are analysed individually

Genetic causes other than HLA may have roles in shaping the Vβ repertoire. A recent report by Donahue et al (1994) on Vβ 3 expression levels in peripheral blood has pointed to the inheritance of a defective Vβ 3 allele that results in low Vβ 3 expression. Posnett *et al* (1994) have shown that the there is a correlation between the expression levels of Vβ 3 and the inheritance of a defective recombination signal. A study by Jores *et al* (1994) have shown that the human fetal thymus contains large numbers of specific types of Vβ segments. These large pools do not correlate directly with the number of Vβ members in a particular Vβ family nor with the proximity of Vβ gene segments to the Dβ regions. All of these observations point to the existence of novel mechanisms that determine the levels of TCR Vβ expression in peripheral blood.

Chapter 9

Conclusions

This study has uncovered some fundamental principles relevant to analysis of the Vβ repertoire that have not been adequately discussed or researched before. It has been assumed by some investigators that the TCR Vβ primers have equal amplification efficiencies in PCR reactions but this has been shown conclusively not to be the case. Some investigators have used different primer sets without conclusive evidence that these are equal in efficiency to other published primer sets and have then compared the data obtained with the different primer sets. It is now apparent that it is important to carry out preliminary studies on any set of TCR primers (Vβ or Vα), to determine their relative priming efficiencies. The advantage of using this approach is twofold. First, after determining the efficiency of a set of primers, differences in primer efficiencies can be corrected for, thus providing a more accurate measure of an individual's Vβ repertoire. Second, if all the published primer sets are investigated and their relative efficiencies determined, then a primer set with equal efficiencies of all primers can be chosen to investigate the TCR Vβ or Vα repertoire.

The efficiency of Vβ primers was shown experimentally to correlate with Vβ target amounts. After correcting for differences in Vβ priming efficiencies the data no longer

showed dependence on primer efficiency.

Analysis of identical twins showed that non-genetic causes can play a significant role in determining the Vβ repertoire in individuals. It was also shown that comparing profiles without considering non-genetic contributions can lead the researcher to the erroneous conclusion that genetics explain differences in the Vβ repertoire between individuals. The influence of genetics on particular Vβs was identified by rigorous statistical analysis.

The family studies showed evidence for an HLA effect on the Vβ repertoire but also revealed that this effect can be completely masked by non-HLA factors. Previous studies have indicated a dominant role for HLA in shaping the peripheral Vβ repertoire and the role of non-genetic events was interpreted as being minor. However, this study has shown that the effects on the TCR Vβ repertoire are multiple and include not only genetic but non-genetic factors and also HLA and non-HLA factors. The term non-HLA is used rather loosely and could reflect genetic or non-genetic contributions.

Future studies to investigate the role of genetics including HLA in shaping the TCR repertoire must consider several factors as yet only partially dealt with here and in the literature. Future studies must consider such parameters as age, sex, ethnicity, and inheritance patterns and would provide valuable information on how the peripheral human TCR

repertoire is generated and maintained.

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Appendix A

Comparison of R^2 values, obtained from the twin pair and unrelated individuals comparison, by the Z test.

The Pearson r values derived from the comparisons of related and unrelated individuals were tested using a form of Z test described in the results (section 7.2.2B). First, all of the r values are converted to Z scores (also described in section 7.2.2B). The Z scores are not compared randomly but done in the following manner (Dr. V. Gadag, Dept. of Medical Biostatistics, MUN). Let twin pair A1/A2 be the related pair and twin pairs B1/B2 and C1/C2 are identical twin pairs but unrelated to twin pair A1/A2. Pearson r score from the comparison of profiles from A1 and A2 represents a score due to "relatedness", while comparison of individuals A1 or A2 with B1, B2, C1 and C2 represents scores due to "unrelatedness". All r scores are converted to Z scores. The Z score obtained from the comparison of A1/A2 is compared with the Z scores resulting from the comparison of A1 or A2 with the unrelated individuals B1, B2, C1 and C2. Pearson r scores derived from the comparisons of unrelated individuals, for example B1 and C1, are not compared with the r score from A1 and A2. This method of analysis would be done for all three twin pairs. The results from this study are given below in table A.

Table A

The Z scores from the comparison of related twins with unrelated individuals are shown below. The twin pair being compared is shown in bold face. From the table below, for the CD8 subset, the Z test comparison of the r value from MB1/MB2 and the r value from MB1/SR1 yield a Z score of 1.58. This value is less than the critical value of 1.96 (df = 22, α = 0.05). For the CD4 subset, the same comparison yields a score of 1.47.

	CD8		CD4	
	Comparisons		Comparisons	
	MB1/MB2		**MB1/MB2**	
	MB1/	MB2/	MB1/	MB2/
SR1	1.58	1.17	1.47	0.63
SR2	1.95	1.49	0.85	0.17
LR1	0.64	1.35	1.14	1.93
LR2	0.20	0.30	0.53	1.59
	SR1/SR2		**SR1/SR2**	
	SR1/	SR2/	SR1/	SR2/
LR1	1.01	1.84	1.60	1.97
LR2	0.08	0.94	1.02	1.21
MB1	0.47	0.85	0.91	0.28
MB2	0.07	0.40	1.19	0.73
	LR1/LR2		**LR1/LR2**	
	LR1/	LR2/	LR1/	LR2/
MB1	1.01	0.58	0.26	0.36
MB2	1.73	0.67	1.04	0.71
SR1	2.49	1.56	0.15	0.44
SR2	3.13	2.41	0.52	0.24

Appendix B

Sample calculations using the Bartlett's and Levene's tests for homogeneity of variance.

Sample analysis of data by Bartlett's and Levene's test

Data: TCR Vβ data for 5 Vβs from six individuals

vβS	1	2	3	4	5
SAMPLE					
A	4	5	10	20	5
B	5	5	2	1	6
C	4	5	15	10	5.5
D	4.5	6	2	0	7
E	4	5.5	15	2	6
F	5.5	5.5	0	3	6.5

Each column containing the values for particular Vβ is stacked. In this example, columns containing Vβs 1-5 are stacked on top of each other with values for Vβ1 on the top. All of the values for Vβ1 are represented as 1 (or level 1), Vβ2 as 2 (or level 2) and so on. The variances are calculated for each level and compared to all other levels. For example level 1 is compared with 2 - 5. Equalities of all variances can be determined from the test statistic calculated (Figure B1).

If the variances are statistically different, those levels with comparable (done subjectively) variances are grouped together. Based on figure B1, Vβ 1, 2 and 5 with smaller variances are grouped into the first category. Vβs 3 and 4 with seemingly large variances are grouped into the second category. These groups are then tested for internal homogeneity (figure B2, B3). Next, the two different groups are tested to ensure that the selection has indeed

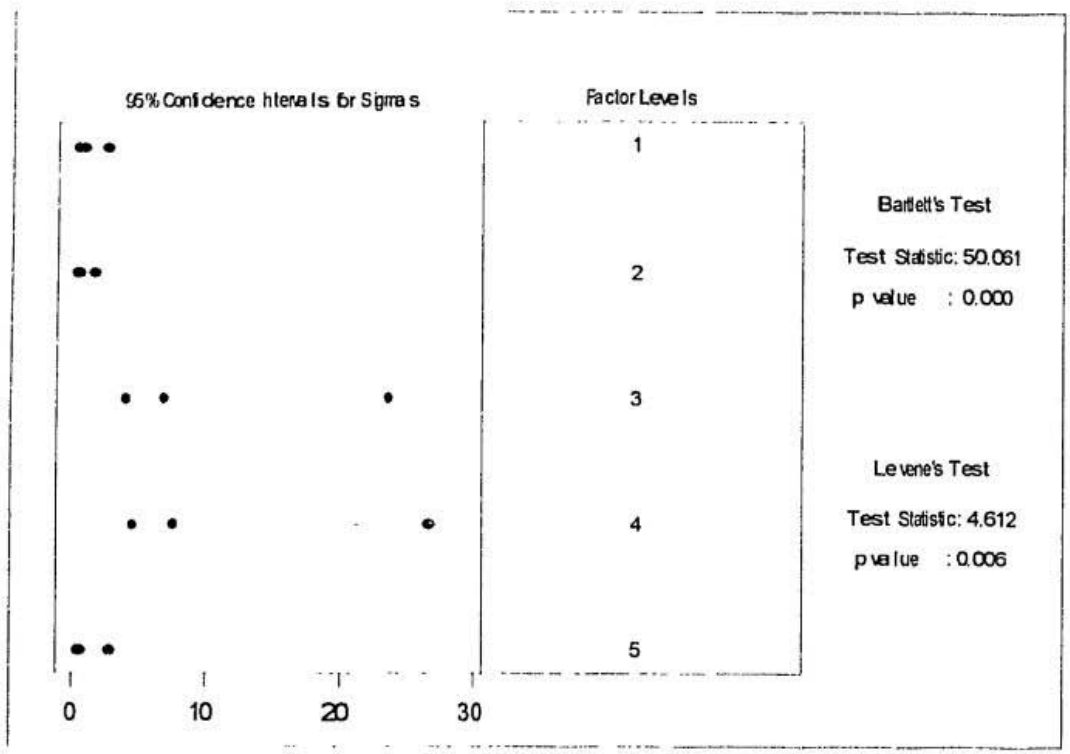

Figure B1

Calculation of homogeneity of variance for all values for the five Vβs. The p value of 0.006 indicates variable variances.

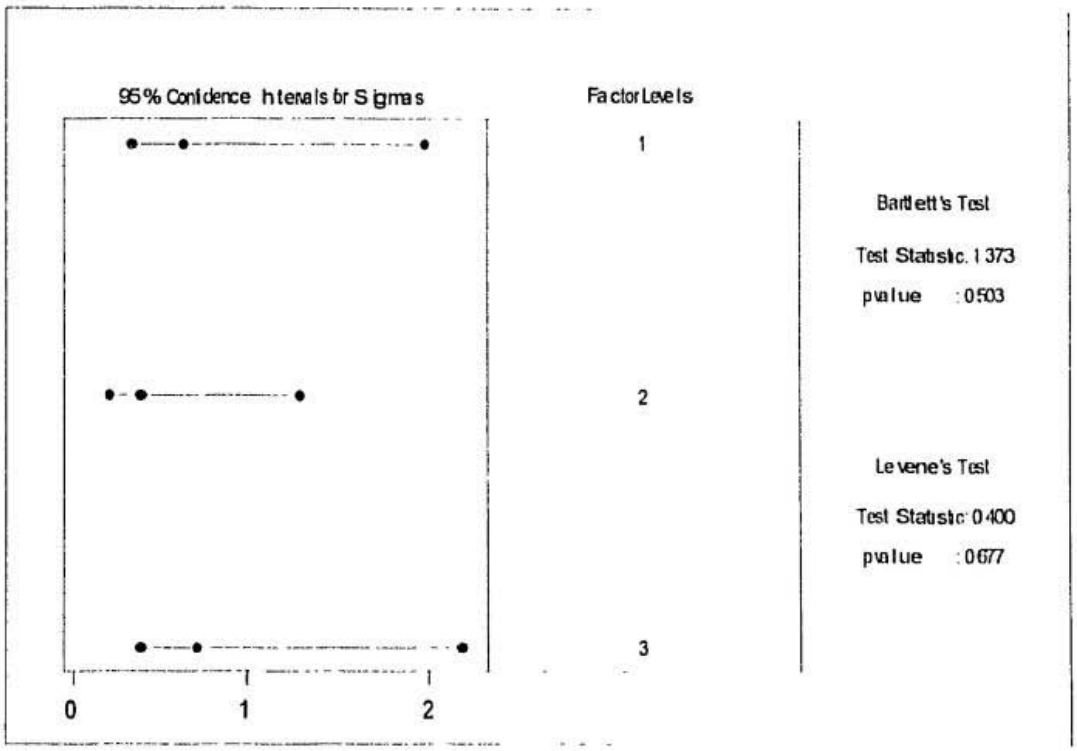

Figure B2

From figure B1, Vβs 1, 2 and 5 seemed to have the lower variances and were grouped together and tested for homogeneity of variance.

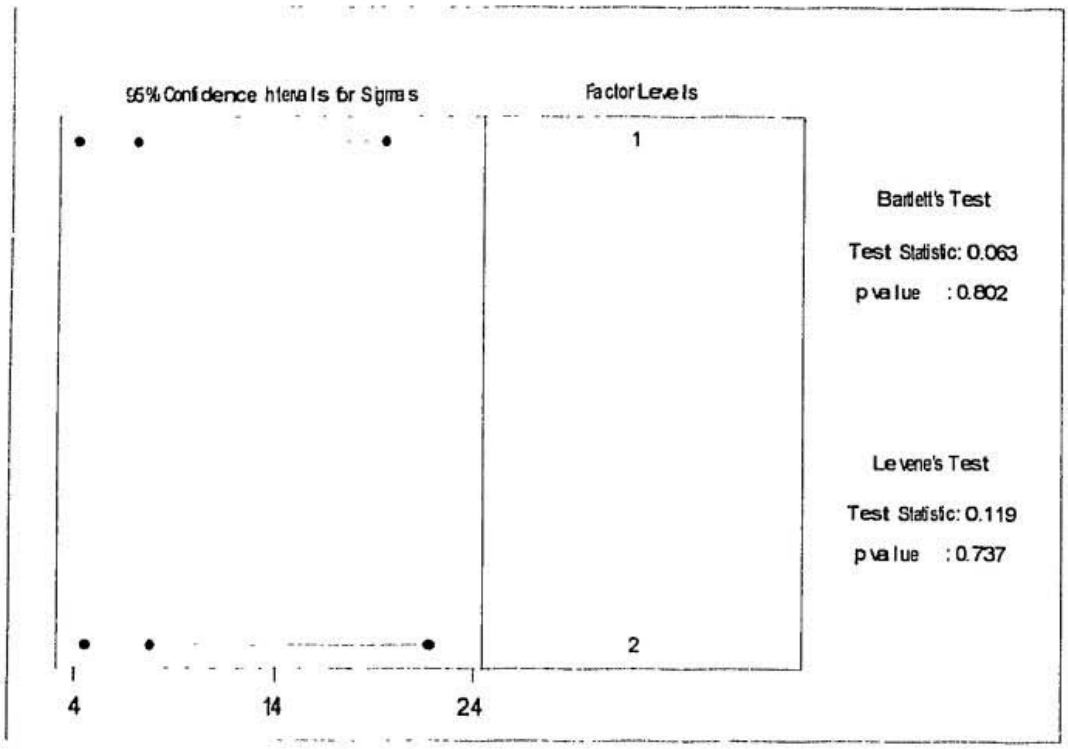

Figure B3

From figure B1, the variances of Vβs 3 and 4 seemed similar therefore were grouped and tested for homogeneity of variance.

isolated groups with variable variances (figure B4). The p value of 0.002 indicates that the groups have variable variances.

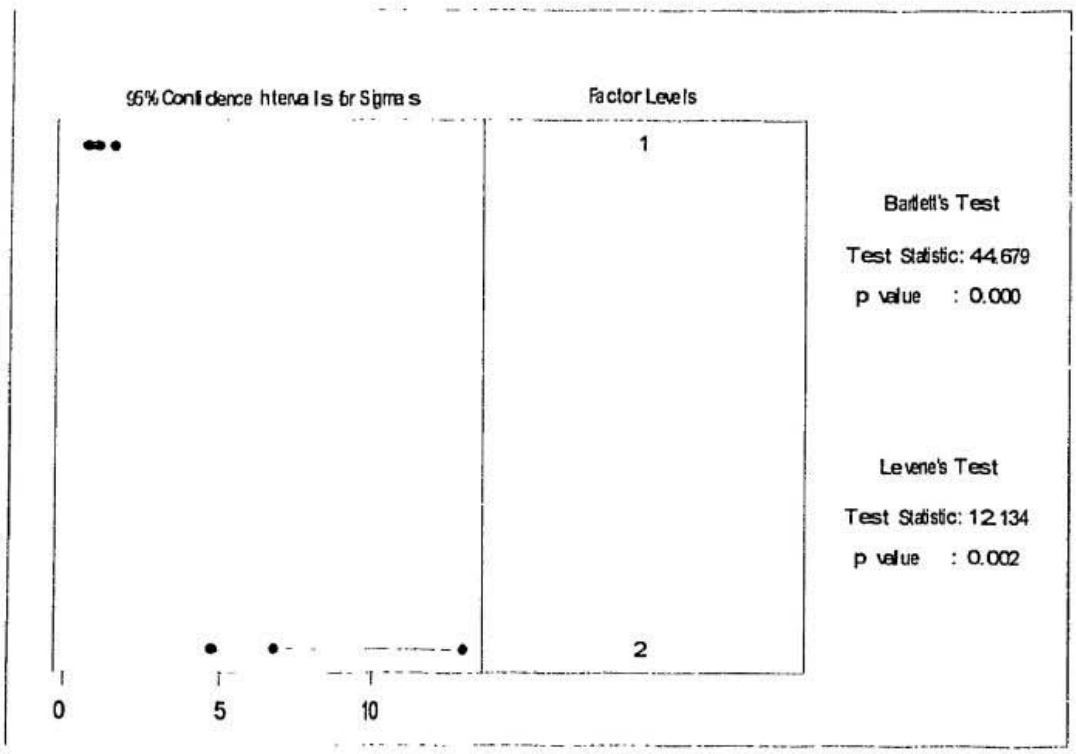

Figure B4

The two groups of Vβs from figures B2 and B3 were tested to ensure that the variances were different.

Appendix C

Figures from Bartlett's and Levene's for CD4' T subset

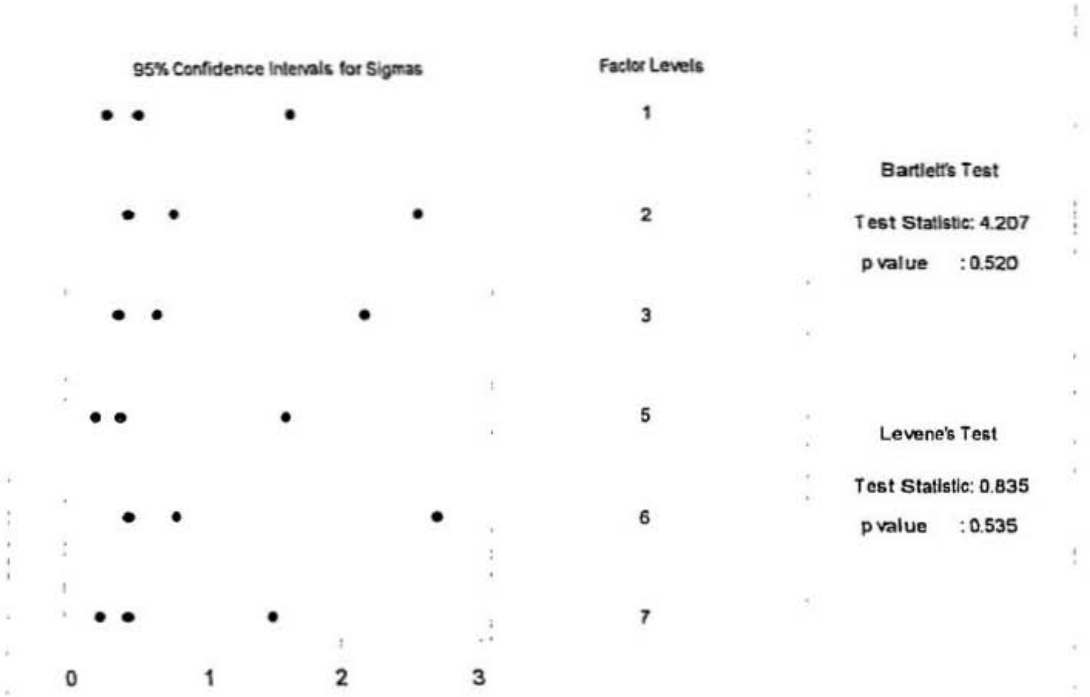

Figure C1

Bartlett's and Levene's (BL's) test of homogeneity of variance (HOV) as determined on group 1 Vβs from the CD4' T cell subset. Factor levels 1 - 7 correspond to Vβs 3, 9, 11, 15, 16, 18, and 19.

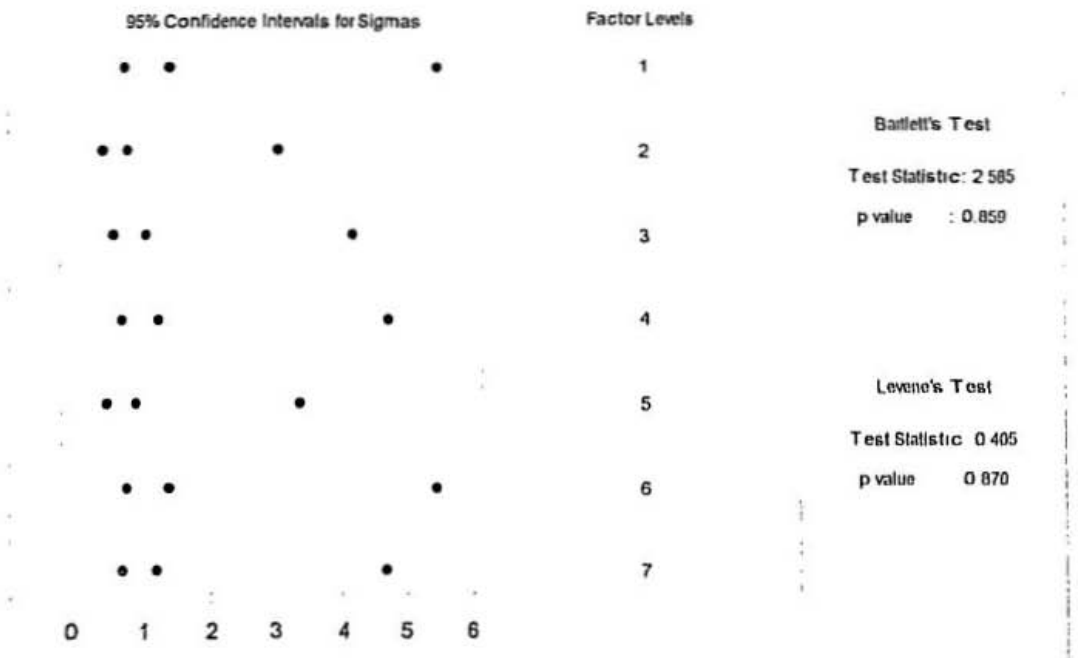

Figure C2

Bartlett's and Levene's (BL's) test of homogeneity of variance (HOV) as determined on group 2 Vβs from the CD4+ T cell subset. Factor levels 1 - 7 correspond to Vβs 5, 5.1, 7, 8, 13.1, 14, and 17.

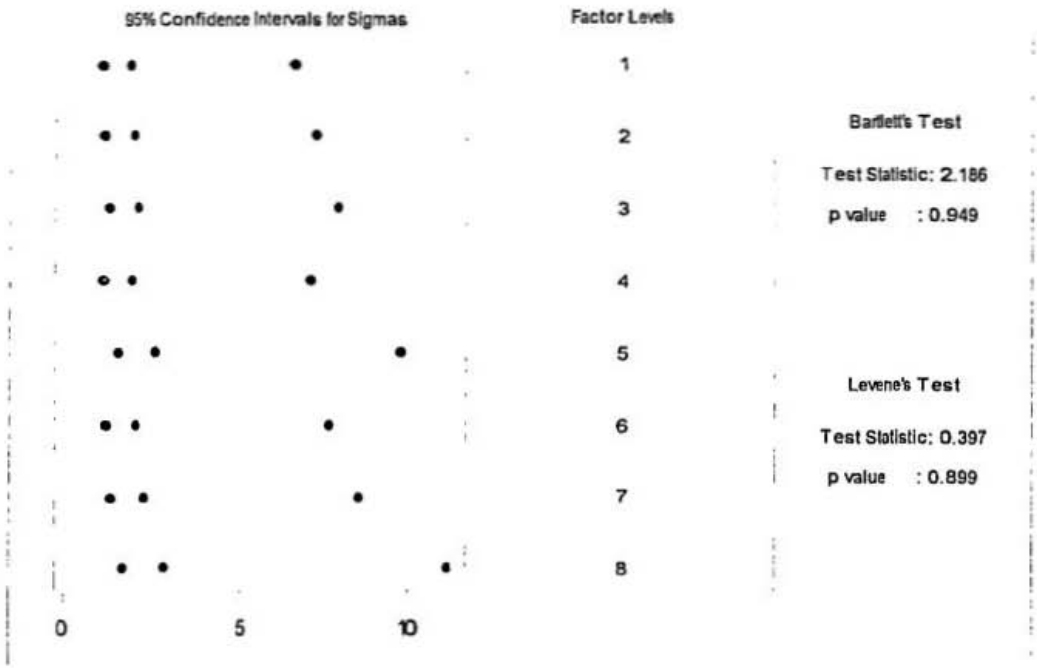

Figure C3

Bartlett's and Levene's (BL's) test of homogeneity of variance (HOV) as determined on group 3 Vβs from the CD4+ T cell subset. Factor levels 1 - 8 correspond to Vβs 1, 2, 4, 5.2, 6, 10, 12 and 13.2.

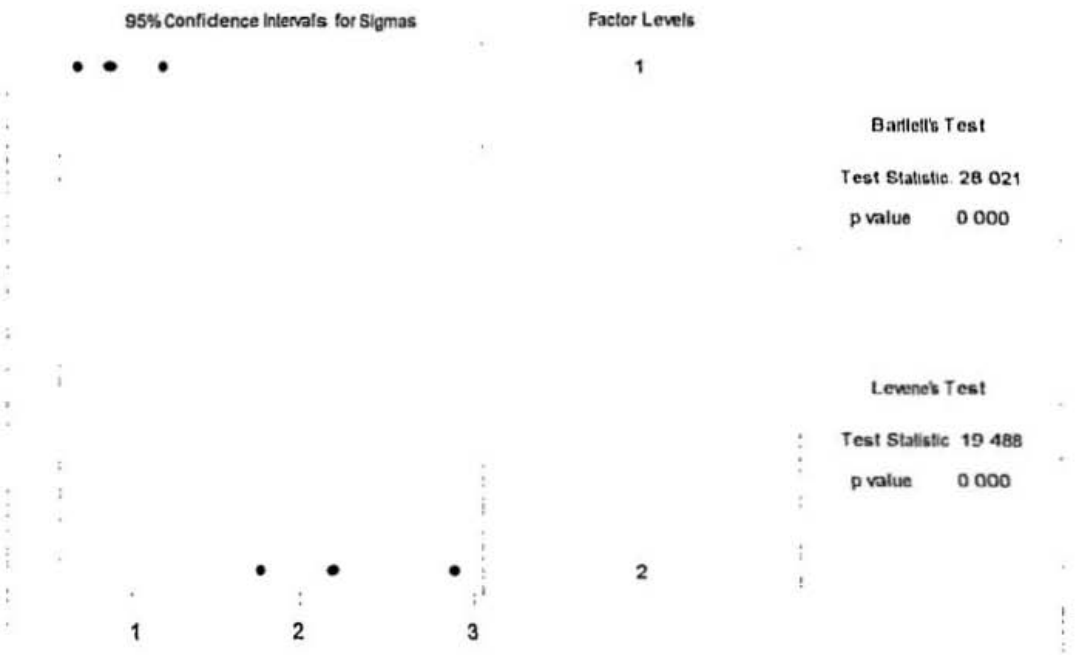

Figure C4

Bartlett's and Levene's test of homogeneity of variance to compare the variances of Vβs in group 1 with those Vβs in group 2 from the CD4+ T cell subset. Factor level 1 corresponds to the combined variances of all Vβs in group 1 and factor level 2 corresponds to the combined variances of all group 2 Vβs.

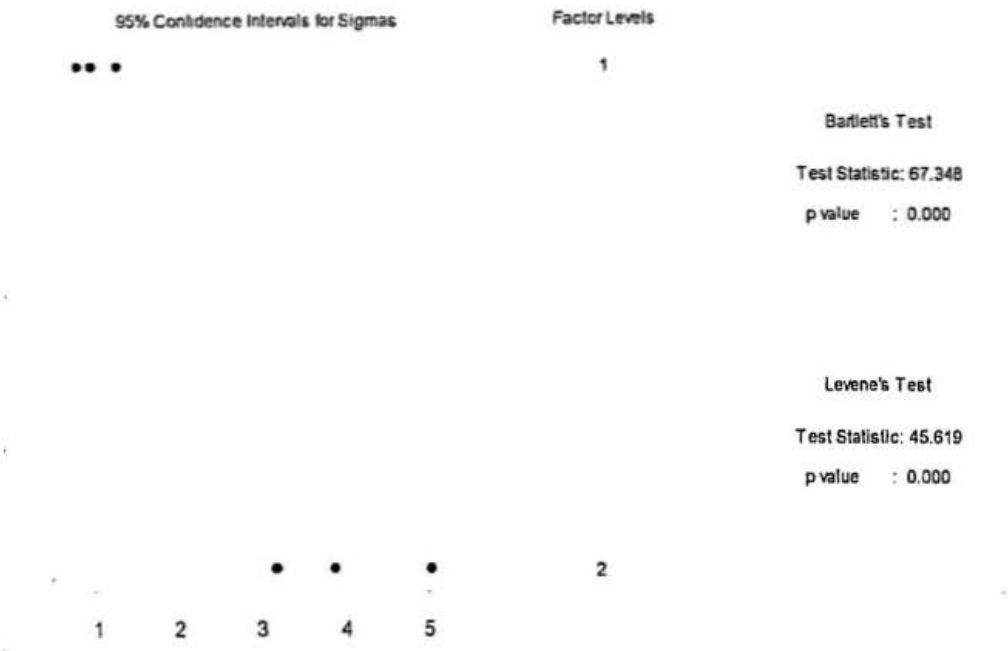

Figure C5

Bartlett's and Levene's test of homogeneity of variance to compare the variances of Vβs in group 1 with those Vβs in group 3 from the CD4⁺ T cell subset. Factor level 1 corresponds to the combined variances of all Vβs in group 1 and factor level 2 corresponds to the combined variances of all group 3 Vβs.

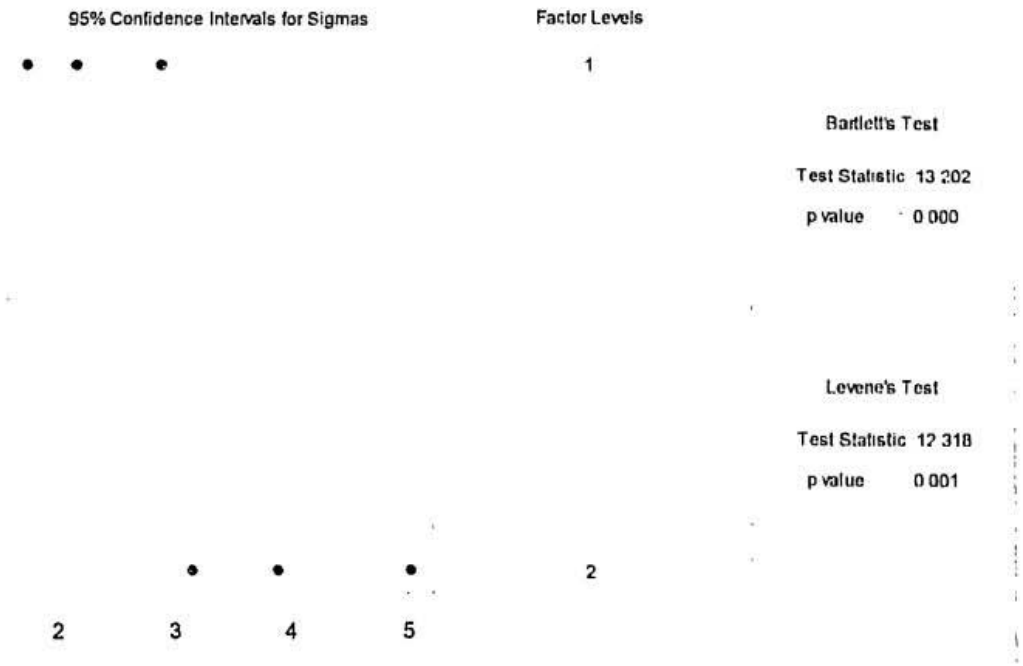

Figure C6

Bartlett's and Levene's test of homogeneity of variance to compare the variances of Vβs in group 2 with those Vβs in group 3 from the CD4+ T cell subset. Factor level 1 corresponds to the combined variances of all Vβs in group 2 and factor level 2 corresponds to the combined variances of all group 3 Vβs.

Appendix D

Figures from Bartlett's and Levene's for $CD8^+$ T subset

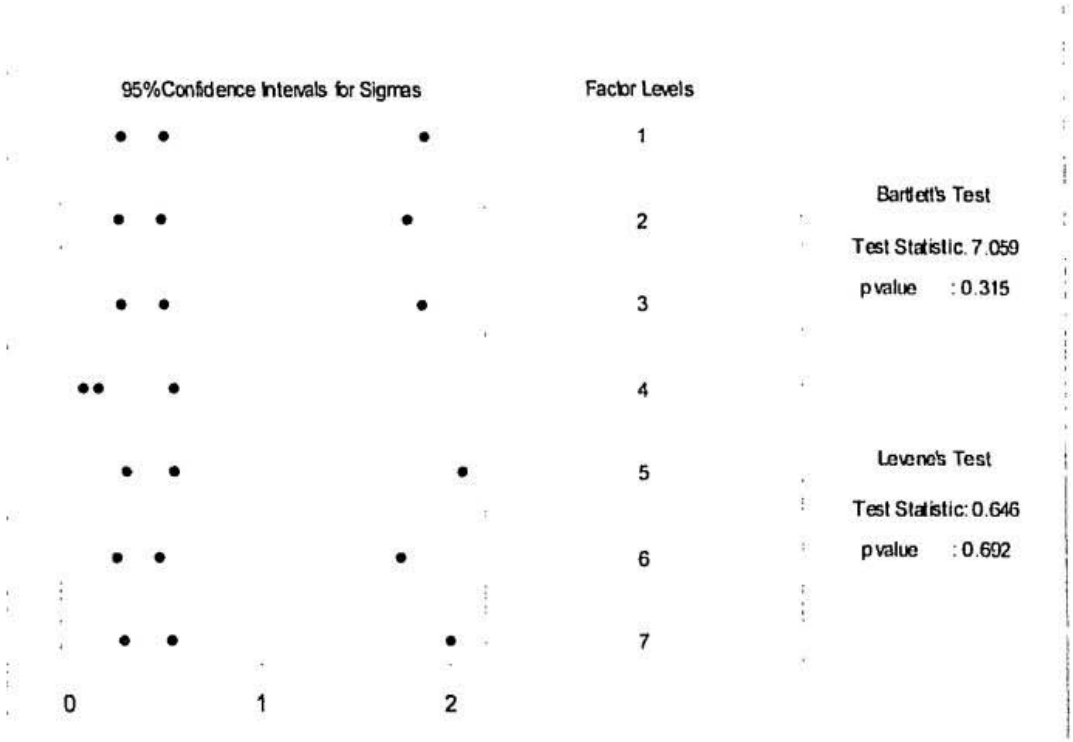

Figure D1

Bartlett's and Levene's (BL's) test of homogeneity of variance (HOV) as determined on group 1 Vβs from the CD8' T cell subset. Factor levels 1 - 7 correspond to Vβs 1, 3, 9, 11, 16, 18, and 19.

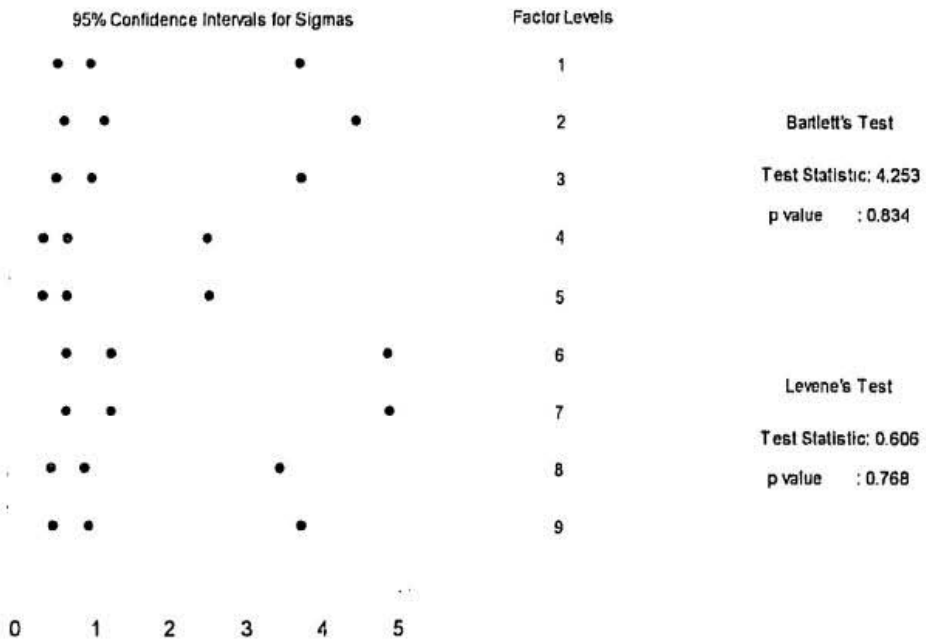

Figure D2

Bartlett's and Levene's (BL's) test of homogeneity of variance (HOV) as determined on group 2 Vβs from the $CD8^+$ T cell subset. Factor levels 1 - 7 correspond to Vβs 2, 4, 5, 5.1, 5.2, 7, 8, 13.1, and 15.

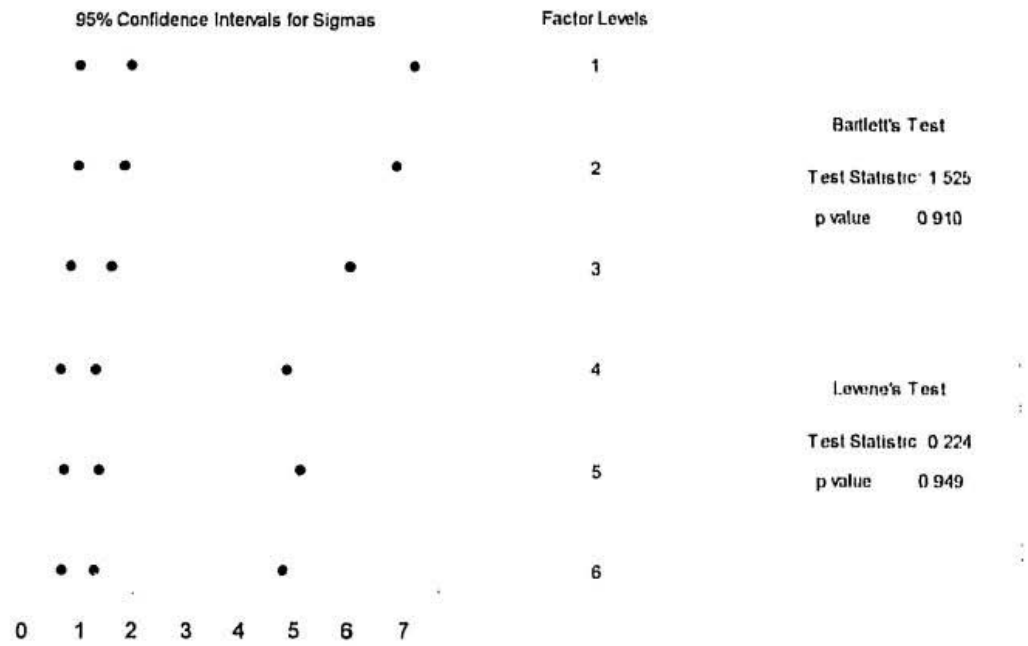

Figure D3

Bartlett's and Levene's (BL's) test of homogeneity of variance (HOV) as determined on group 3 Vβs from the CD8' T cell subset. Factor levels 1 - 8 correspond to Vβs 6, 10, 12, 13.2, 14 and 17.

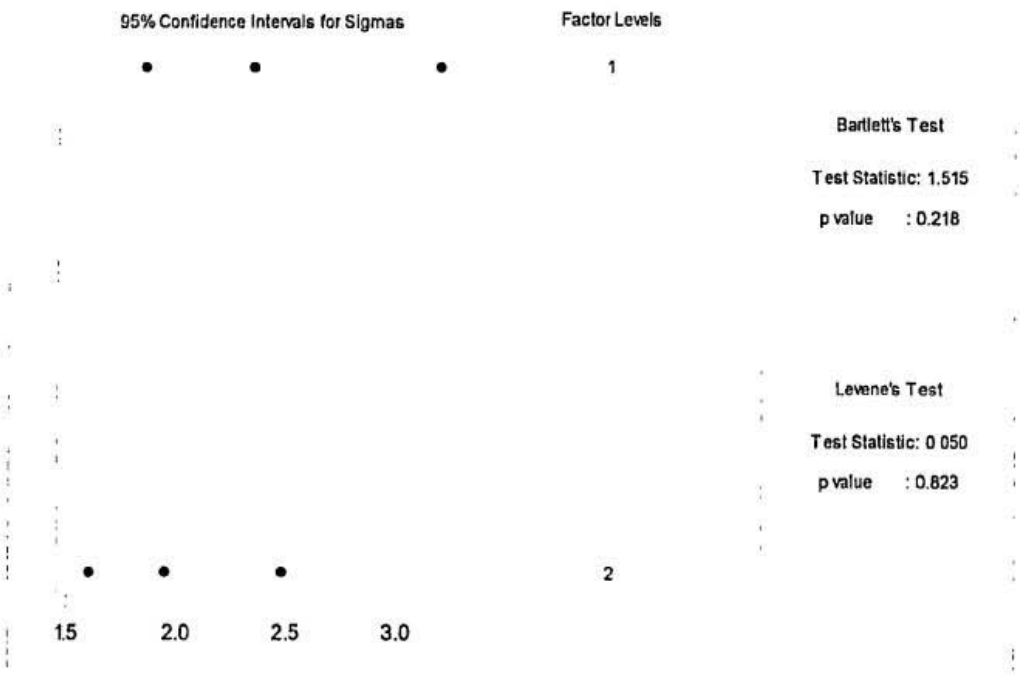

Figure D4

Bartlett's and Levene's test of homogeneity of variance to compare the variances of Vβs in group 1 with those Vβs in group 2 from the CD8⁺ T cell subset. Factor level 1 corresponds to the combined variances of all Vβs in group 1 and factor level 2 corresponds to the combined variances of all group 2 Vβs.

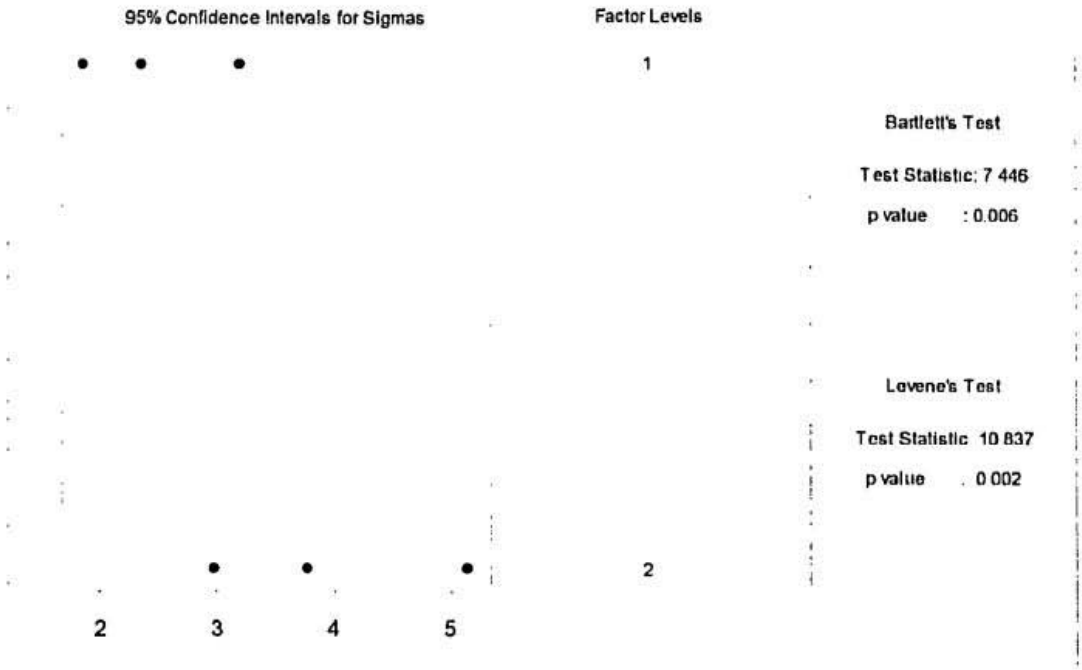

Figure D5

Bartlett's and Levene's test of homogeneity of variance to compare the variances of Vβs in group 1 with those Vβs in group 3 from the $CD8^+$ T cell subset. Factor level 1 corresponds to the combined variances of all Vβs in group 1 and factor level 2 corresponds to the combined variances of all group 3 Vβs.

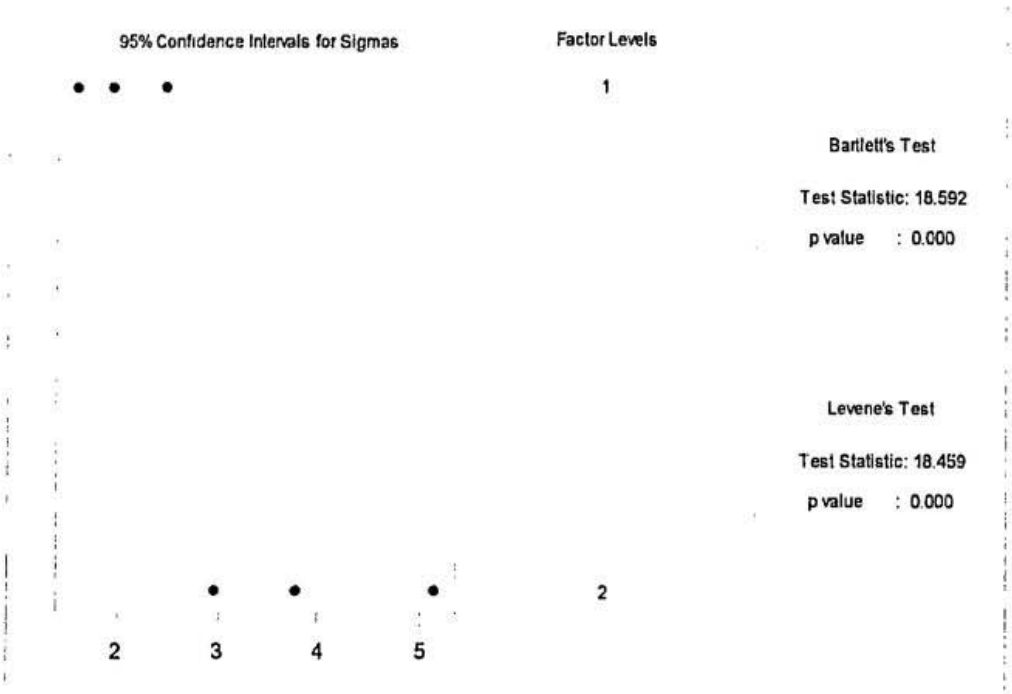

Figure D6

Bartlett's and Levene's test of homogeneity of variance to compare the variances of Vβs in group 2 with those Vβs in group 3 from the CD8' T cell subset. Factor level 1 corresponds to the combined variances of all Vβs in group 2 and factor level 2 corresponds to the combined variances of all group 3 Vβs.

Appendix E

Figures from Bartlett's and Levene's for unisolated T cells from monozygotic twins

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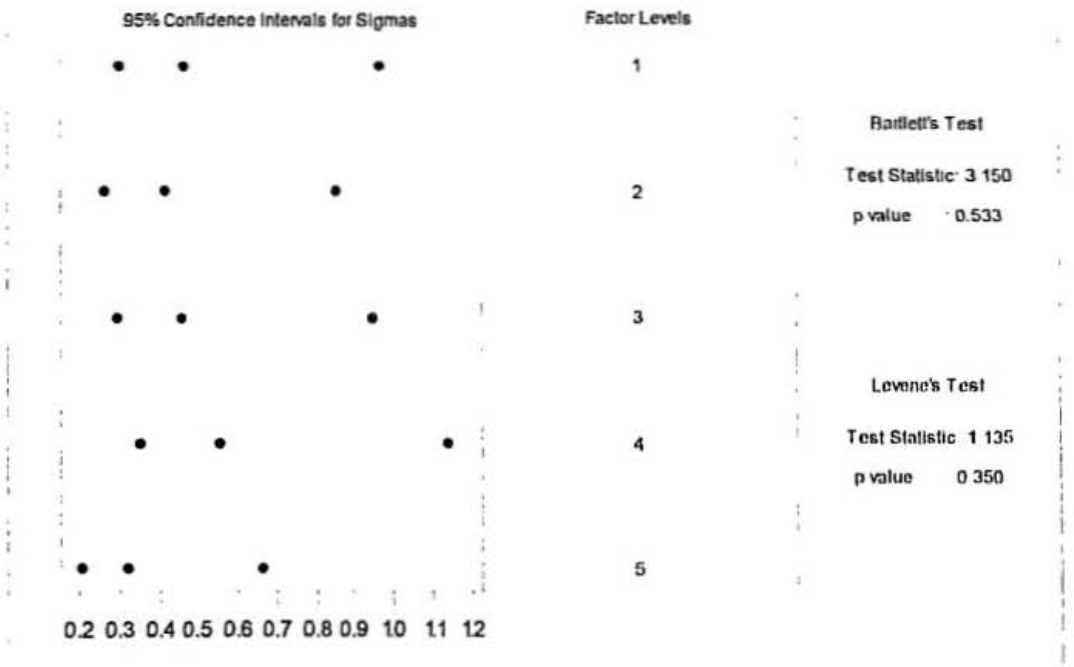

Figure E1

Bartlett's and Levene's (BL's) test of homogeneity of variance (HOV) as determined on group 1 Vβs from total T cells of identical twins. Factor levels 1 - 5 correspond to Vβs 11, 15, 16, 18, and 19.

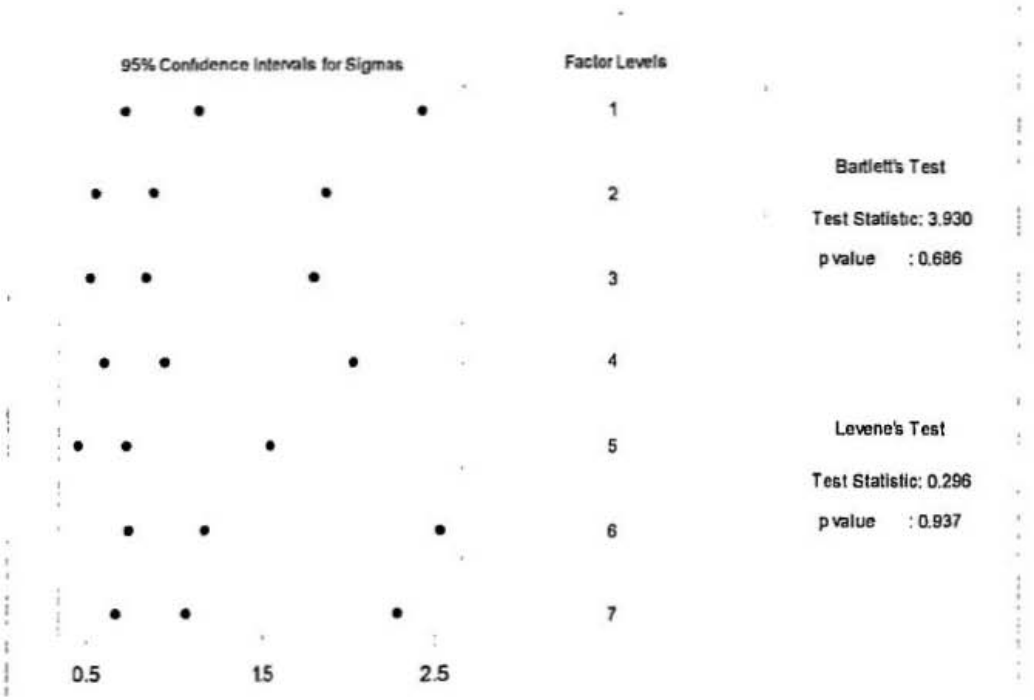

Figure E2

Bartlett's and Levene's (BL's) test of homogeneity of variance (HOV) as determined on group 2 Vβs from total T cells of identical twins. Factor levels 1 - 7 correspond to Vβs 3, 5.1, 5.2, 8, 9, 13.1, and 17.

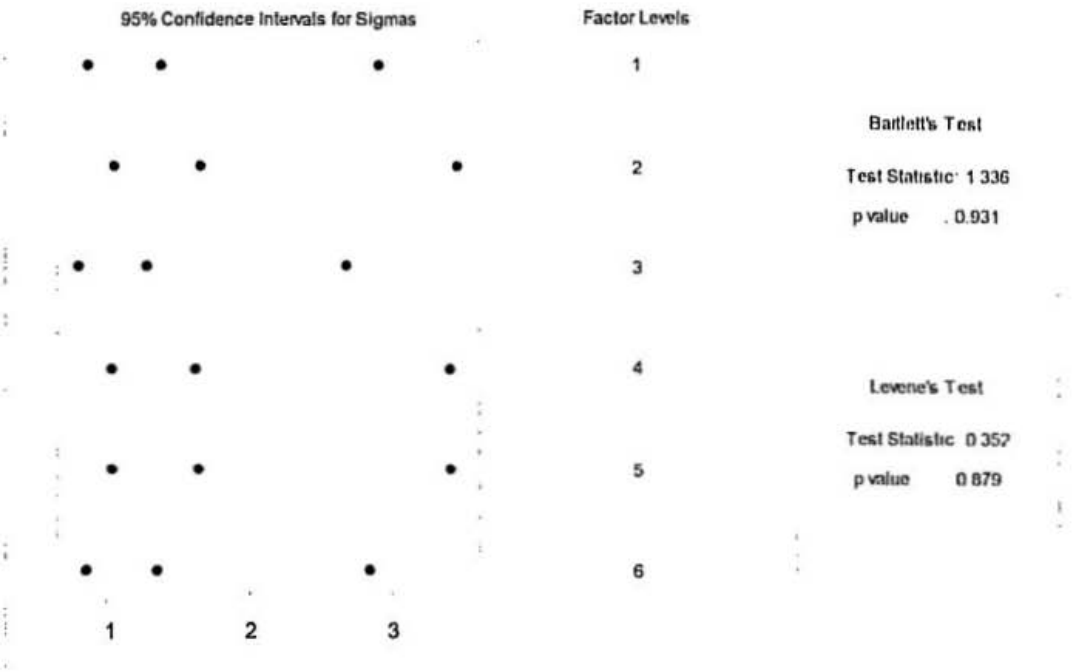

Figure E3

Bartlett's and Levene's (BL's) test of homogeneity of variance (HOV) as determined on group 3 Vβs from total T cells of identical twins. Factor levels 1 - 6 correspond to Vβs 1, 2, 4, 6, 7, and 14.

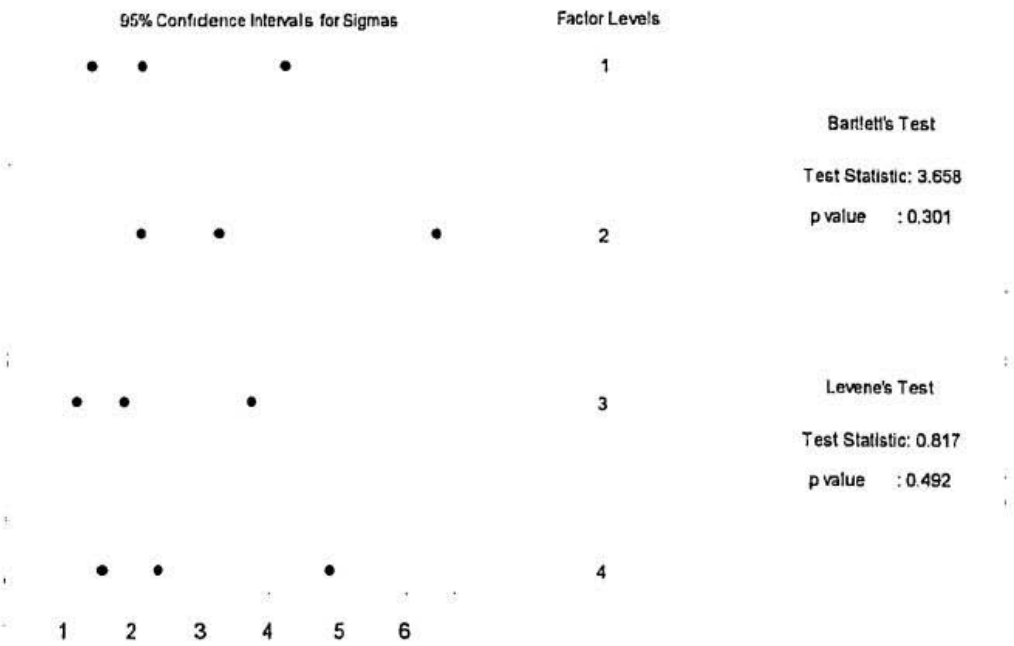

Figure E4

Bartlett's and Levene's (BL's) test of homogeneity of variance (HOV) as determined on group 4 Vβs from total T cells of identical twins. Factor levels 1 - 4 correspond to Vβs 5, 10, 12 and 13.2.

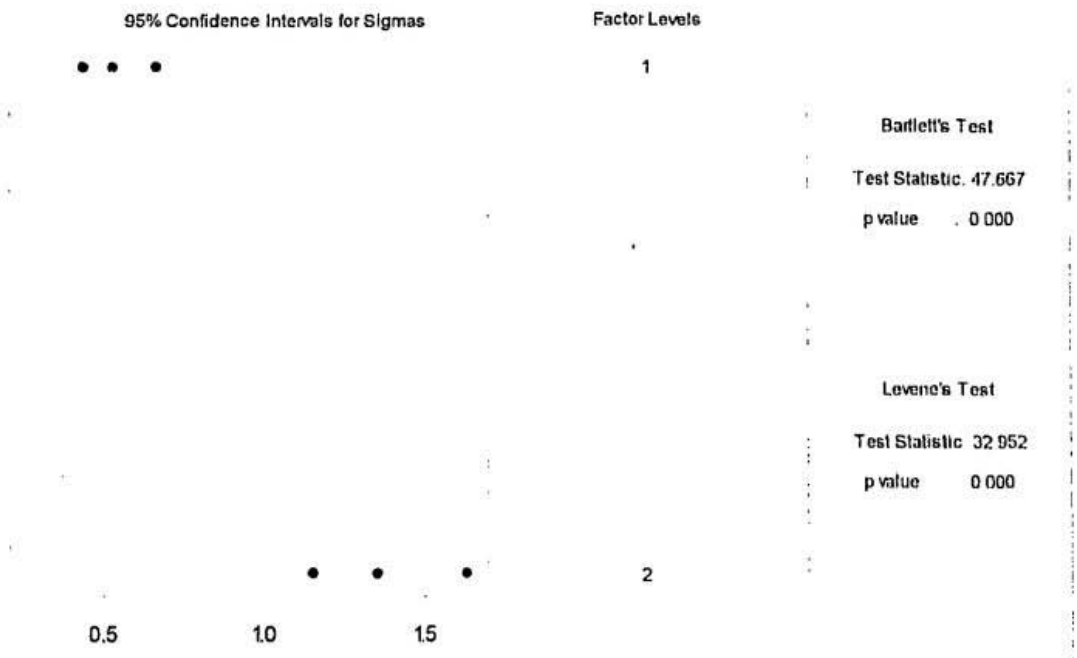

Figure E5

Bartlett's and Levene's test of homogeneity of variance to compare the variances of Vβs in group 1 with those Vβs in group 2 from total T cells of identical twins. Factor level 1 corresponds to the combined variances of all Vβs in group 1 and factor level 2 corresponds to the combined variances of all group 2 Vβs.

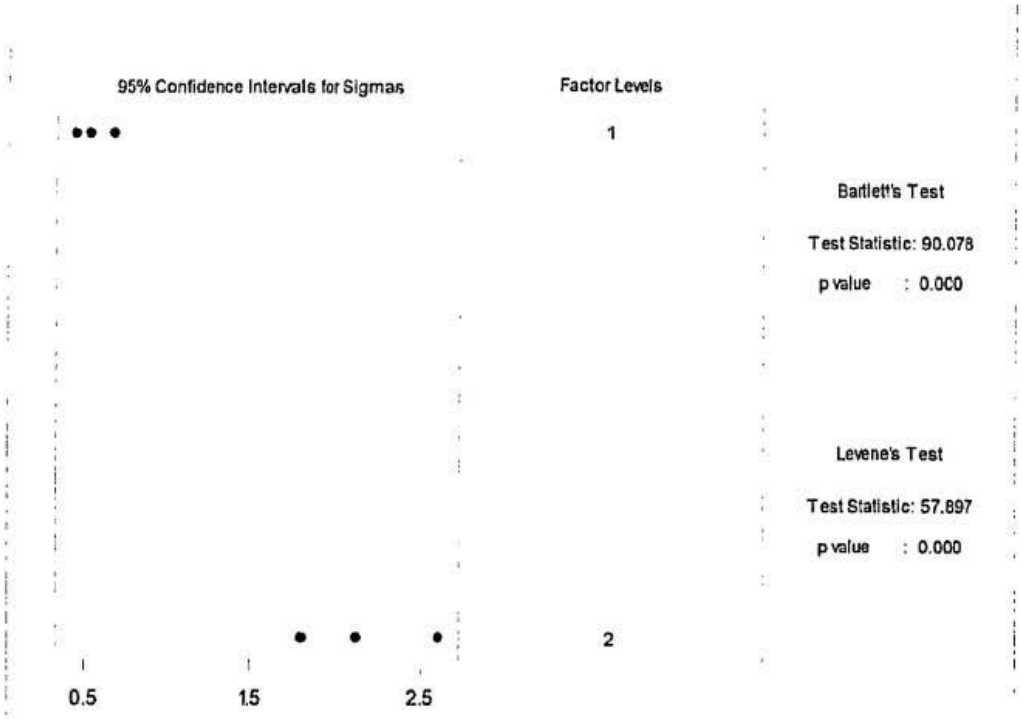

Figure E6

Bartlett's and Levene's test of homogeneity of variance to compare the variances of Vβs in group 1 with those Vβs in group 3 from total T cells of identical twins. Factor level 1 corresponds to the combined variances of all Vβs in group 1 and factor level 2 corresponds to the combined variances of all group 3 Vβs.

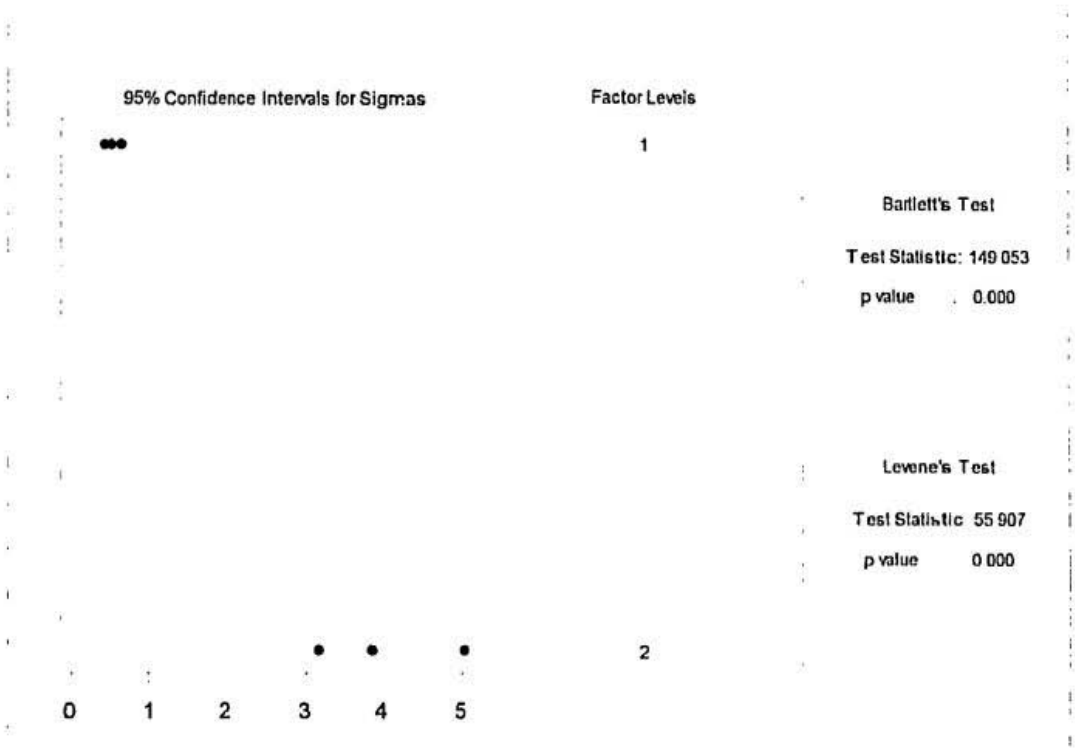

Figure E7

Bartlett's and Levene's test of homogeneity of variance to compare the variances of Vβs in group 1 with those Vβs in group 4 from total T cells of identical twins. Factor level 1 corresponds to the combined variances of all Vβs in group 1 and factor level 2 corresponds to the combined variances of all group 4 Vβs.

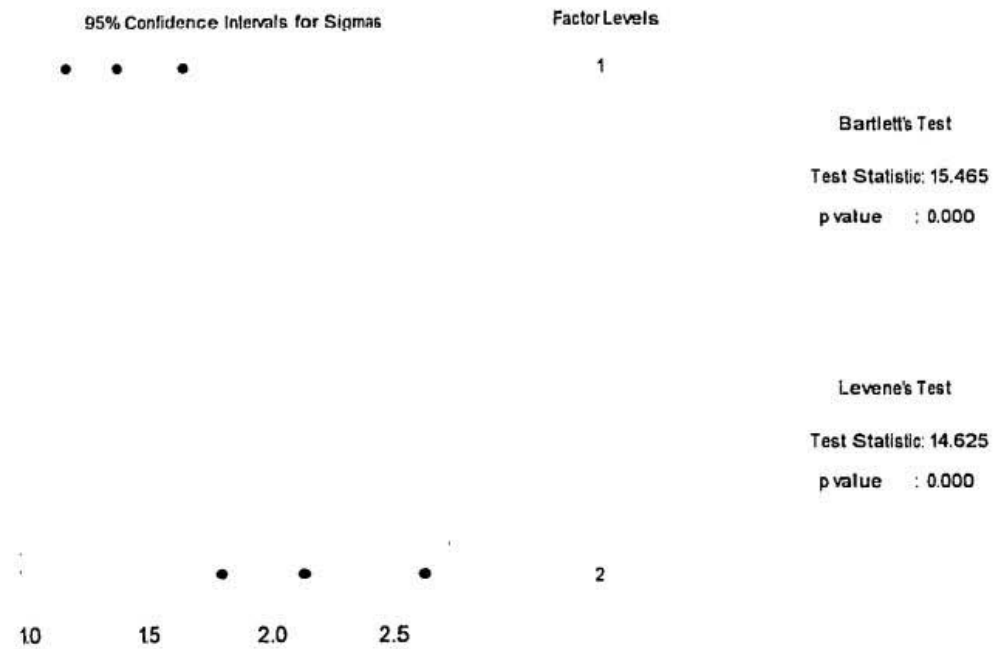

Figure E8

Bartlett's and Levene's test of homogeneity of variance to compare the variances of Vβs in group 2 with those Vβs in group 3 from total T cells of identical twins. Factor level 1 corresponds to the combined variances of all Vβs in group 2 and factor level 2 corresponds to the combined variances of all group 3 Vβs.

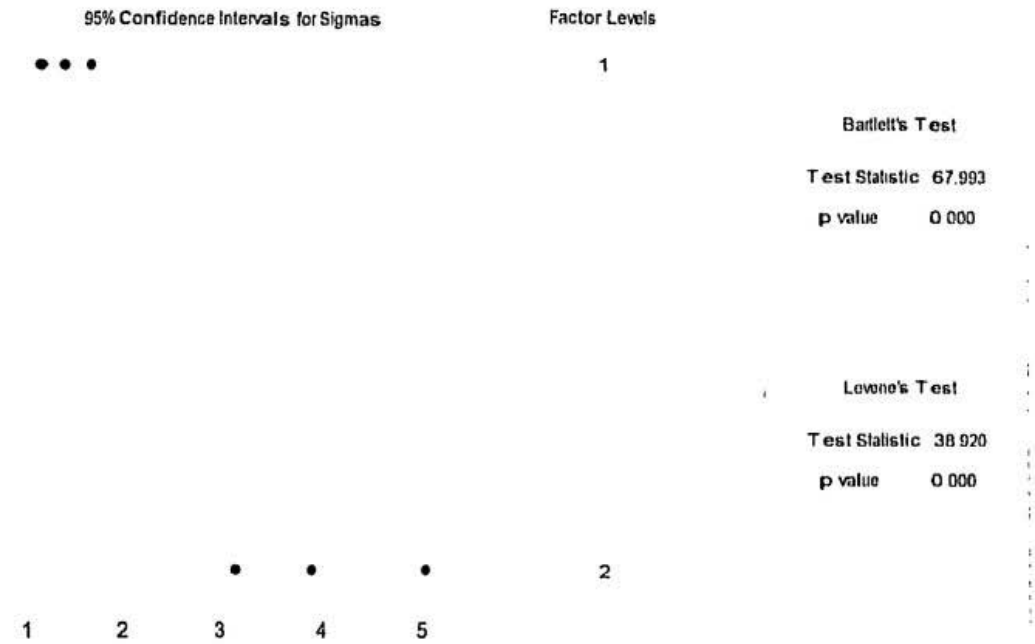

Figure E9

Bartlett's and Levene's test of homogeneity of variance to compare the variances of Vβs in group 2 with those Vβs in group 4 from total T cells of identical twins. Factor level 1 corresponds to the combined variances of all Vβs in group 2 and factor level 2 corresponds to the combined variances of all group 4 Vβs.

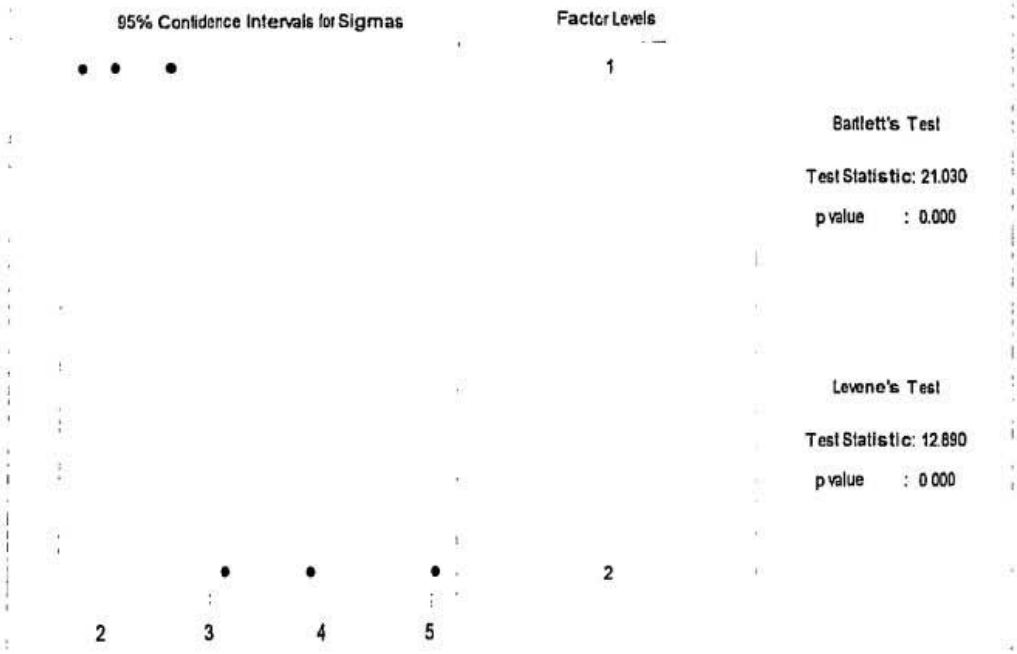

Figure E10

Bartlett's and Levene's test of homogeneity of variance to compare the variances of Vβs in group 3 with those Vβs in group 4 from total T cells of identical twins. Factor level 1 corresponds to the combined variances of all Vβs in group 3 and factor level 2 corresponds to the combined variances of all group 4 Vβs.