A STUDY TO DETERMINE GENETIC SUSCEPTIBILITY TO TUBERCULOSIS

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Thesis submitted in fulfilment of the requirements for the

Masters Diploma in Medical Technology

in the School of Life Sciences at the Cape Technikon.

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I declare that this thesis is my own work. It is being submitted for the Masters Diploma in Medical Technology, to the Cape Technikon, Cape Town. It has not been submitted before for any diploma or examination at any other Technikon or tertiary institution. The work was carried out at the Provincial Laboratory for Tissue Immunology, Cape Town. The opinions and conclusions drawn are not necessarily those of the Cape Technikon.

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Kathleen Meehan **Date**

ACKNOWLEDGEMENTS

I would like to thank Professor Ernette du Toit for permission to complete this work and in whose department it was performed.

I deeply appreciate the knowledge and guidance of Dr Jeanne Rousseau, without whose support, I could not have completed this study.

My thanks go to Dr Rob Martell for his valuable advice, helpful suggestions as well as his knowledge of the computer software used in this study.

I would also like to thank Dr Erwin Schurr of the Centre for Host Resistance, Mc Gill University, Montreal, Canada for affording this laboratory the opportunity to partake in this collaborative study and for coordinating and providing the guide-lines for it.

Special thanks to Mr Derek Taljaard and the staff of the Tissue Typing laboratory for the serological H L A typing of the patients.

My thanks are extended to Dr Pauline Creemers, Gadieja Martin and Carol-Ann Wyngaard for maintenance of the B cell lines.

Thank you to Montwedi Seane and Geneé Harms for the tedious task of initial DNA isolation and probe preparation.

I appreciate the contribution of Sister Eleanor Snyman and her staff for the collection of samples from the patients.

Mr Ernie Truter, my supervisor at the Technikon, has given me tremendous support and willingly shared his valuable experience in research with me, and to him I am indebted.

Finally, I could not complete this thesis without thanking my husband, Alan, my family, my colleagues and my friends, especially Dr Anwar Mall, for the moral support and encouragement that I, at times, needed during the course of my study. Thank you!

ABBREVIATIONS

AIDS	Acquired immune deficiency syndrome
APC	antigen presenting cells
BCG	Bacille Calmette Guerin
bp	base pairs
C	constant
cDNA	complementary DNA
Ci	Curie
CMI	cell mediated immunity
°C	degrees Celsius
D	diversity
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dpm	disintegrations per minute
EBV	Epstein Barr virus
EDTA	ethylenediaminetetra-acetic acid
FCS	foetal calf serum
g	gram
H_0	alternative hypothesis
H_1	hypothesis
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
Ir	immune response
J	joining

kb	kilobase
kD	kilodalton
MHC	major histocompatibility complex
ml	millilitre
mm	millimetre
mg	milligram
M	molar
mM	millimolar
ng	nanogram
OLB	oligomer labelling buffer
PBS	phosphate buffered saline
PHA	phytohaemagglutinin
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute (culture medium)
s.a.	specific activity
SDS	sodium dodecyl sulphate
TB	tuberculosis
TCR	T cell receptor
μg	microgram
μl	microlitre
μm	micrometre
V	variable

SUMMARY

Studies that document the higher incidence of tuberculosis as well as the variable efficacy of the BCG vaccine in Black, compared to White, populations have alluded to resistance or susceptibility to tuberculosis being genetically controlled.

The HLA system has been associated with many diseases involving an immune aetiology. It has been shown that T cell receptor genes have limited restriction fragment length polymorphisms, serving to create a variation in the repertoire of expressed T cell receptor genes. These repertoire differences may play a fundamental role in disease susceptibility.

A study was therefore undertaken to establish whether linkage exists between the HLA system or the T cell receptor genes and a putative susceptibility gene for tuberculosis.

Polymorphisms of these genetic markers were examined in three Cape Coloured multiplex families, affected individuals having culture-positive pulmonary tuberculosis.

HLA haplotypes were derived from serological typing of peripheral leucocytes from each individual. B-lymphoblastoid cell lines were established from each family member. DNA was then extracted and digested with a variety of restriction endonucleases. After gel electrophoresis and Southern blotting, the DNA fragments were probed with a panel of T cell receptor cDNA probes, revealing the allelic polymorphisms.

Linkage analysis was done using the Liped computer programme and Lod scores were determined for each marker locus using various genetic models. Haplotypes were also established for the T cell receptor genes and used in the linkage analysis.

Although most of the Lod scores fell within the indeterminate range, a cumulative Lod score of 1.79 was obtained from the allele generated by the EcoRV/α2 enzyme/probe combination under a recessive model with 50% penetrance. This represents odds of about 52:1 in favour of linkage between the T cell receptor α gene and a putative susceptibility gene to tuberculosis.

OPSOMMING

Studies wat die hoër insidensie van tuberkulose, sowel as die variërende effektiwiteit van die BCG vaksine in Swart, in vergeleke met Wit bevolkings dokumenteer, sinspeel op genetiese kontrole in die weerstand teen, of die vatbaarheid vir tuberkulose.

Die HLA-sisteem word met vele siektes van 'n immunologiese oorsprong geassosieer. Dit is aangetoon dat die T-sel reseptorgene beperkte restriksie-fragmentlengte polimorfisme het, vir die daarstelling van wisseling in die repertoire waarin die T-sel reseptorgeen uitdrukking gee. Hierdie verskille in die repertoire mag 'n fundamentele rol speel in siektevatbaarheid.

'n Studie was derhalwe onderneem om vas te stel of daar genetiese koppeling tussen die HLA-sisteem of die T-sel reseptorgene en 'n veronderstelde vatbaarheidsgeen vir tuberkulose, bestaan.

Polimorfisme van hierdie genetiese merkers is in drie Kaapse Kleurling veelvoudige families ondersoek; geaffekteerde individue synde kultuurpositief te wees vir pulmonale tuberkulose.

HLA-haplotipes is afgelei vanaf die serologiese tipering van perifere witbloedselle van elke individu. B-limfoblastoiede sellyne is van elke familielid opgestel. DNA is ge-ekstraheer en verteer met 'n aantal restriksie endonukleases. Na afloop van gel-elektroforese en Southern-klad, is die fragmente gepeil met 'n paneel van T-sel reseptor cDNA-peilers, wat die alleliese polimorfisme onthul het.

Genetiese koppelingsanalise is gedoen met die rekenaarprogram, Liped, en Lod-tellings is bepaal vir elke merker-lokus deur gebruik te maak van verskeie genetiese modelle. Haplotipes is ook afgelei vir die T-sel reseptorgene en in die genetiese koppelingsanalise gebruik.

Alhoewel die meeste van die Lod-tellings in die twyfelagtige gebied geval het, is die kumulatiewe Lod-telling van 1.79 behaal met die allele verkry met die EcoRV/α2 ensiem/peiler kombinasie, onderworpe aan 'n resessiewe model met 50% penetrasie. Dit verteenwoordig 'n kans van ongeveer 52:1 ten gunste van koppeling tussen die T-sel reseptor-α-geen en 'n veronderstelde vatbaarheidsgeen vir tuberkulose.

CONTENTS

CHAPTER 1

1.0 INTRODUCTION

The ability of an individual to respond to a foreign infectious antigen has been shown to be under the influence of the environment as well as disease resistance or susceptibility genes (Skamene and Pietrangeli, 1991).

The aim of identifying such genes is to determine their effect on the immune system and to isolate the gene products, so that the susceptible phenotype may be reversed. The understanding of such genetic control may have a tremendous effect on the development of vaccines synthesised from recombinant DNA, rather than viable attenuated organisms (Kaufmann, 1991).

Socio-economic and environmental conditions have been known to influence the epidemiology of tuberculosis for many years (Singh *et al*, 1983). However, the variable efficacy of the BCG vaccine, as well as studies documenting the higher incidence of tuberculosis in Black, compared to White populations, has indicated that resistance or susceptibility to tuberculosis may have a genetic component (Schurr *et al*, 1991 and Skamene and Pietrangeli, 1991).

In mice, a gene controlling the susceptibility to mycobacterial infections, Bcg, on chromosome 1, has been identified and a human equivalent has been proposed (Schurr *et al*, 1991 and Skamene and Pietrangeli, 1991).

The Major Histocompatibility Complex (MHC) genes on the short arm of chromosome six mediates essential interactions of cells in the immune system (Bodmer, 1987), thereby playing an important role in the regulation of the immune response. The search for linkage between the MHC genes and various diseases has been facilitated by the discovery of the high degree of polymorphism at the MHC gene complex (Garchon and Bach, 1991).

Polymorphisms at the MHC gene region are associated with many diseases with an immune aetiology eg. rheumatoid arthritis has been associated with HLA-DR4, ankylosing spondilitis with HLA-B27, insulin dependent diabetes mellitus with HLA-

DR3 and HLA-DR4 and coeliac disease with HLA-DR3 (See Garchon and Bach, 1991 for review). Haemachromatosis has been shown to be associated with HLA-A3 (Bodmer, 1987). Singh *et al* (1983) have shown that leprosy, a mycobacterial disease related to tuberculosis may be controlled by MHC-linked genes associated with HLA-DR2.

The MHC genes have also been reported to demonstrate an association to tuberculosis *per se* e.g. HLA-B8 (Selby *et al* 1979 and Takata *et al*, 1978) and HLA-B15 (Al Arif *et al*, 1979). On the contrary, Cox *et al* (1982) found no association between HLA and tuberculosis.

This study was therefore designed to investigate whether a resistance or susceptibility gene linked to the MHC, could be implicated in the aetiology of tuberculosis. As T cells, in conjunction with self-MHC molecules, recognise foreign antigens via the T cell receptors, the T cell receptor genes are candidate genes for determining susceptibility to diseases with MHC associations (Niven *et al*, 1990).

The T cell receptor genes have been implicated in diseases such as multiple sclerosis (Ocksenberg *et al*, 1990), polymyositis (Hohlfeld *et al*, 1991), leprosy (Modlin *et al*, 1989), measles and arthritis (Haas *et al*, 1990), systemic lupus erythematosis (Tebib *et al*, 1990), Graves' disease (Demaine *et al*, 1987), ataxia telangiectasia (Berliner *et al*, 1985), autoimmune thyroid disease (Mangklabruks *et al*, 1990), insulin dependent diabetes mellitus (Millward *et al*, 1987) and mycobacterial diseases (Kaufmann, 1991 and Scott and Kaufmann, 1991).

T lymphocytes play a central role in immune recognition by differentiating in the thymus and distinguishing self from non-self MHC molecules. The function of the T cell receptor is the dual recognition of processed foreign antigen fragments positioned in the groove of the MHC molecule and the MHC molecule itself (Garchon and Bach, 1991). Antigen presenting cells interact with the T cell receptors and initiate the cell-mediated response (Kaplan and Cohn, 1991). The T helper cells attract macrophages to the site where the macrophages become effective antimicrobial agents (Kaufmann, 1991).

The immunological defect in lepromatous leprosy, caused by *Mycobaterium leprae*, resides in the inability of a host to mount a cell-mediated response (Kaplan and Cohn, 1991) The role of T cells in mycobacterial infections is further supported by the fact that patients with the Human Immunodeficiency virus (HIV) are particularly susceptible to mycobacterial infections and HIV is characterised by a deficiency and dysfunction of T cells.

Germ-line polymorphisms may alter the response of an individual to a foreign antigen (Wright *et al*, 1991), and as the precise T lymphocytes in the pathogenesis of some diseases have not yet been identified, polymorphisms at this level may be useful as markers to locate the putative resistance or susceptibility genes (Stastny, 1987).

We therefore decided to investigate whether the putative resistance or susceptibility gene was linked to the T cell receptor genes.

The Lod score method of linkage analysis was used to analyse linkage between HLA haplotypes and tuberculosis and T cell receptor genes with tuberculosis as it provides the ability to include pedigrees of any form as well as the ability to add lod scores across pedigrees to produce a cumulative lod score (Risch, 1991).

OBJECTIVE OF THE STUDY

To determine whether genetic susceptibility to tuberculosis exists by performing linkage analysis in multiplex families with tuberculosis using HLA and the T cell receptor as genetic markers.

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 TUBERCULOSIS

Tuberculosis (TB) is one of the deadliest diseases in South Africa, manifesting itself mainly in the lungs, leading to possible permanent lung damage (Collins, 1991). Despite technological advances and an increase in knowledge about the disease, the causative agent, *Mycobacterium tuberculosis*, discovered by Robert Koch in 1882, presently claims, world wide, approximately three million lives annually and presents ten million new cases each year in the developing world (Schurr *et al*, 1991), making it the world's largest single infectious cause of death (Murray *et al*, 1991). The incidence of tuberculosis is expected to rise even more as a result of the present Human Immunodeficiency Virus (HIV) epidemic (Barnes *et al*, 1991). Although people of all ages are affected, 75% of people suffering from tuberculosis are adults between the ages of 15 and 59 i.e. the parents, workers and leaders of society. In South Africa, the mortality rate is at present estimated to be in excess of 20 per day (Collins, 1990).

Routes of infection are largely through aerosol inhalation of *Mycobacterium tuberculosis* during contact with individuals who shed large numbers of viable bacilli from the upper respiratory tract (Dockrell *et al*, 1991). Other routes of infection include trans-placental haemorrhage and carelessness of laboratory staff.

The incubation period of tuberculosis ranges from a few weeks to a lifetime. The majority of patients seem to combat the primary infection successfully by forming Ghon complexes in the lungs where most bacilli are destroyed, following the development of a competent immune response. During the immune response, macrophages and other antigen-presenting cells (APC) engulf the invading organisms, process them and present the cell-bound or soluble antigen to the T cells via the T cell receptors (TCR). The T cells are activated and T-helper cells assist the macrophages by secreting lymphokines, such as interleukin 1, interleukin 2 (which is necessary for the activation and the maintenance of CMI), and interferon gamma which enhance the macrophage's capacity to ingest the

bacteria. Macrophages that have been activated by interferon gamma become more effective antimicrobial agents due to an elevation of toxic oxygen radicals (Kaplan and Cohn, 1991). However, some bacilli resist being destroyed (Barnes *et al*, 1991) by remaining in a dormant state in sites such as the apical segments of the lungs, brain, vertebrae, kidneys and the ends of long bones.

The control of tuberculosis relies on the success of the Bacille Calmette Guerin (BCG) vaccine, a live attenuated tubercle bacillus (Stover *et al*, 1991), which has been used for more than 70 years. Although 2 million children's lives are saved annually by an immunisation programme, the variable efficacy of the BCG vaccine, as well as the higher incidence of TB in the Black, compared to the White population, has alluded to susceptibility or resistance to tuberculosis being genetically controlled (Skamene and Pietrangeli, 1991). The observation that not all individuals exposed to the disease become infected, has led investigators to believe that in the lepromatous form of tuberculosis, there appears to be an immunological defect accounting for the inability of the host to respond to the invading organisms in the form of a cell-mediated immune response.

An investigation that linked the incidence of Tay-Sachs disease to the epidemic of pulmonary tuberculosis in the major European epidemics in the 19th century, hypothesised that the defective enzyme in Tay-Sachs disease, beta-hexidosaminidase, could somehow be responsible for altering the dynamics of or efficiency of the tubercle bacillus. In this way, individuals that are heterozygous for the Tay-Sachs disease gene appear to be resistant to *Mycobacterium tuberculosis* as a result of evolutionary heterozygote advantage (O'Brien, 1991). It has been suggested that the reason for the racial difference in incidence in South Africa may be that South Africans of European origin are descendants of those who survived major TB epidemics in urban Europe in the 19th century, whereas the Coloured and Black populations seem to be experiencing their first epidemic (Collins, 1991).

It has also been suggested that the fundamental difference between susceptibility and resistance lies in the impaired functioning capacity of macrophages, in individuals who are susceptible to tuberculosis in that they have low or nonexistent oxidative potential, rendering them ineffective antimicrobial agents (Kaplan and Cohn, 1991). Protective immunity against mycobacterial infections is assumed to involve cell-mediated immune responses, seen by the increased populations of T cells in the tuberculoid form of leprosy, as opposed to the low populations of T cells found in the lepromatous form, suggesting defective T cell responses (Dockrell *et al*, 1991). The factors governing this phenomenon are, however, still under consideration (Kaplan and Cohn, 1991).

It has been shown that in mice, a single, dominant autosomal gene, BCG, on chromosome 1, is responsible for controlling resistance to murine mycobacterial infections. The hypothesis is that the Bcg gene regulates the level of macrophage activation and that macrophages in mice carrying the resistance gene, Bcg^r, are readily activated, whereas macrophages in mice carrying the susceptibility gene, Bcg^s, are less readily activated. As there is evidence that genetic factors play a role in determining human susceptibility or resistance to tuberculosis, it is hypothesised that a human equivalent of the mouse Bcg gene exists. The locus for this susceptibility gene is presently being sought (Schurr *et al*, 1991).

Different socio-economic conditions may also lead to variations in the prevalence of tuberculosis as was highlighted by a survey carried out in Black hostels in the Langa and Guguletu areas of the Western Cape in 1987-1988 (Rhampele, 1991). It was noted that the prevalence of the disease was higher where overcrowding and unemployment was more prominent and it epitomised the non-medical risk factors associated with tuberculosis.

2.1.1 TUBERCULOSIS AND HUMAN IMMUNUDEFICIENCY VIRUS

As the hallmark of Human Immunodeficiency Virus (HIV) is depleted T cells as well as impaired function of macrophages, HIV patients are continuously at risk for contracting

tuberculosis, especially as *Mycobacterium tuberculosis* is more virulent than any other HIV-associated pathogen. It has been shown that cities that have the largest number of HIV cases are also experiencing the greatest increases in the number of cases of tuberculosis (Barnes *et al*, 1991). In Sub-Saharan Africa there are 2.4 million patients with tuberculosis that are co-infected with HIV (Stanford *et al*, 1991).

2.2 THE IMMUNE SYSTEM

The function of the immune system is to resist infection (Ehrenstein and Isenberg, 1991). The ability of an individual to ignore non-infectious self, or syngenic, molecules and react to infectious non-self molecules, is the core of this function (Benoist and Mathis, 1991). When this function becomes impaired, autoimmunity occurs. The phenomenon of immunity was observed when individuals who recovered from infections appeared to be immune to them on repeated exposure.

During an immune response, foreign antigen is phagocytosed by the host's macrophages, processed intracellularly into peptide fragments of about 10-20 amino acids in length and presented to T cells between the two helices of class II MHC molecules (Ohno, 1991). It is these MHC-peptide complexes, rather than the whole protein, that the T cells recognise as foreign (Bloom and Oldstone, 1991). The same antigen may be trapped by B cells where immunoglobulin antibodies are secreted on the surface of the B cells to form complexes with the foreign antigen (Ohno, 1991).

Immunity is highly specific as almost any macromolecule, or antigen, can induce a response. Molecules that bind, but do not induce a response unless bound to a carrier molecule, are known as haptens.

Two types of immunity are recognised, viz. humoral and cell mediated which are mediated by B and T cells respectively. B cells may recognise proteins, polysaccharides and lipids, whereas T cells only recognise proteins, in the form of processed peptides. Humoral immunity generally results in the formation of immunoglobulin antibodies

directed at the invading extracellular foreign antigen, whereas cell-mediated immunity is an essential defence against intracellular pathogens eg. viruses and some fungi. It involves the production of specialised T cells that perform different functions when reacting with the foreign antigen on the surface of other cells. The reacting cell either eliminates the infected cell before it replicates, or may secrete chemical signals to activate macrophages to ingest them.

Three functionally distinct T cell subsets can be identified i.e. T-helper cells and T-supressor cells which are collectively known as regulatory T cells and cytotoxic (killer) T cells that together with B cells are collectively known as effector cells (Dembic *et al*, 1986). Two types of T helper cells have been identified viz. TH_1 and TH_2 . TH_1 has been identified in the tuberculoid form of tuberculosis and TH_2 has been identified in the lepromatous form (Bloom, 1991). CD4 and CD8 are cell surface glycoproteins that are expressed on the surface of mature T cells and serve as accessory molecules by facilitating interactions of T cells with antigen presenting cells (Abbas *et al*, 1991). CD4 is expressed on T helper cells and CD8 is expressed on cytolytic cells. It has been shown that CD8 may also be essential for protection against foreign antigen in all organs, except in saliva where $CD4^+$ cells appear to function in the same way (Scott and Kaufmann, 1991). Scott and Kauffman have shown that $CD8^+$ cells may play a role in immunity against mycobacterium infections such as *Mycobacterium tuberculosis*, as these cells recognise peptides derived from intracellular proteins and *Mycobacterium tuberculosis* resides in the host's cells. About 90% of all cells with alpha/beta T cell receptors express CD4 and CD8 on their surface, whereas those T cells bearing gamma/delta receptors express neither (Abbas *et al*, 1991).

An essential feature of cell mediated immunity is that T cells only recognise foreign protein antigens when they are presented on the surface of other cells, i.e. antigen presenting cells and only if they are in association with MHC (See Figure 2.1) molecules (Royer and Reinherz, 1987). This system is advantageous as it forces T cells to interact

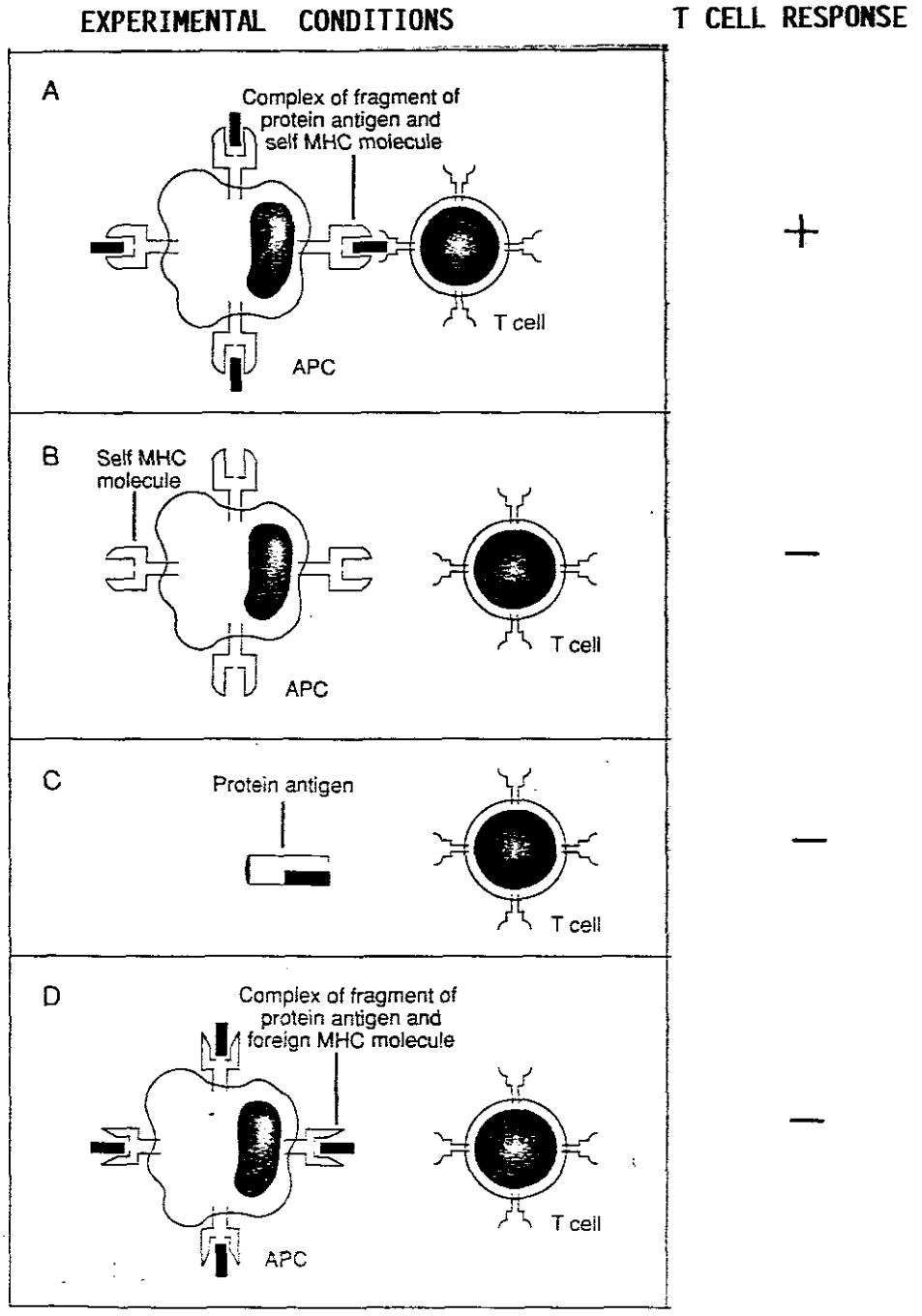


Figure 2.1

Figure showing that antigen presentation and T cell response occurs when an individual expresses MHC molecules that can bind peptides from processed antigen and T cells are present that recognise these complexes (A). MHC-restricted T cells do not recognise self MHC molecules alone or self MHC with self peptide (B), foreign antigens without MHC molecules (C), or complexes of foreign MHC molecules and peptide fragment of antigen. (Courtesy Abbass *et al*, Cellular and Molecular Immunology, 1991).

with cell-associated antigen and in this way allows efficient T cell function even in the presence of soluble native antigen that might otherwise occupy the TCR and prevent the cell from interacting with appropriate targets (Marack and Kappler, 1987).

A further requirement is that T cells must be self-MHC restricted (Marack and Kappler, 1987). MHC restriction is the phenomenon referring to the prerequisite that antigen presenting cells express MHC molecules that the T cell recognises as self. In this way, T cells recognise and respond to a foreign antigen that is presented to it. Molecules recognised as self are those encountered during the maturation process in the thymus (See Figure 2.2).

Cytotoxic T cells are specifically MHC class I (HLA-A, -B and -C) restricted, whereas Helper T cells are specifically MHC class II (HLA-DR, -DQ and -DP) restricted (Abbass *et al*, 1991). Each lymphocyte is committed to react with a particular antigen before being exposed to it. When that particular antigen binds to the T cell, only that T cell will proliferate, rendering immunity antigen-specific.

Immunoglobulins are found both membrane-bound on B cells as well as free in the plasma, therefore the ability to isolate them in the plasma has facilitated the study of humoral immunity. However, understanding CMI proves to be more complex as T cells are only activated through close contact with other cells and the T cell receptors are membrane bound, they are difficult to isolate. It was only in 1983 that the nature and the function of the specific T cell receptors was discovered (Clevers, 1988).

2.3 THE T CELL RECEPTORS

The T cell receptors (TCR), responsible for the highly specific recognition and response to a foreign antigen are composed of a number of integral plasma membrane proteins. Two types of TCR's have been identified viz. alpha/beta (α/ß) and gamma/delta (γ/δ) which have been classified according to their protein chains (Abass *et al*, 1991).

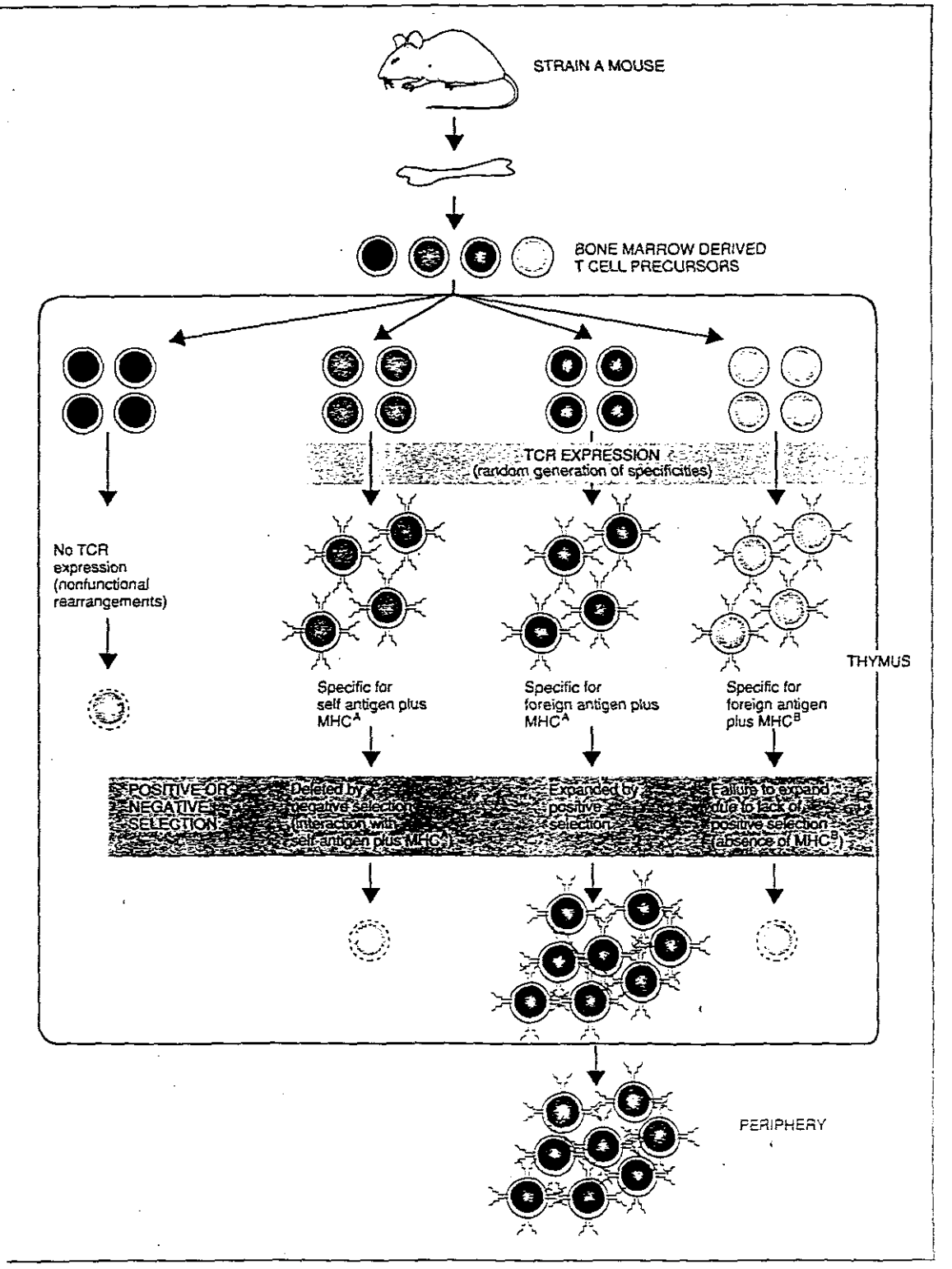


Figure 2.2

Figure showing how maturing thymocytes express TCR's with random specificities and only those clones specific for foreign antigen peptides bound to self-MHC molecules are selected to mature and leave the thymus. (Courtesy Abbass *et al*, Cellular and Molecular Biology, 1991).

2.3.1 ALPHA/BETA T CELL RECEPTORS

2.3.1.1 STRUCTURE

The alpha/beta TCR is a heterodimer consisting of an acidic (alpha) and a basic (beta) polypeptide chain covalently linked to each other by disulphide bonds and the cytoplasmic tails are embedded in the plasma membrane (See Figure 2.3) (Yoshikai *et al*, 1986).

The alpha chain is a 40-50 kD acidic glycoprotein whereas the beta chain is a 40-45 kD uncharged or basic glycoprotein. Both chains contain complex N-linked sugars with additional simple sugar side chains on the beta chains (Abbass *et al*, 1991).

The alpha and beta polypeptide chains have a variable (V) and a constant (C) polypeptide region, the junction of these two regions being encoded by a joining (J) region.

The C regions of the polypeptide chains vary from between 138 and 179 amino acids in length, each consisting of four functional domains, the first domain consisting of a loop, linked by the two cysteine residues, the second domain consisting of a short hinge region containing a cysteine residue, most likely involved in the forming of the disulphide bond between the two chains, the third part of the region containing the trans-membrane domain, with 20-24 hydrophobic amino acid residues and the fourth comprising of the carboxy terminal part of the C region which forms a 5-12 amino acid long cytoplasmic tail embedded in the membrane. There are at least three hypervariable regions in the alpha and beta chains which form the contact points for the binding of immune complexes (See Figure 2.3) (Abbass *et al*, 1991).

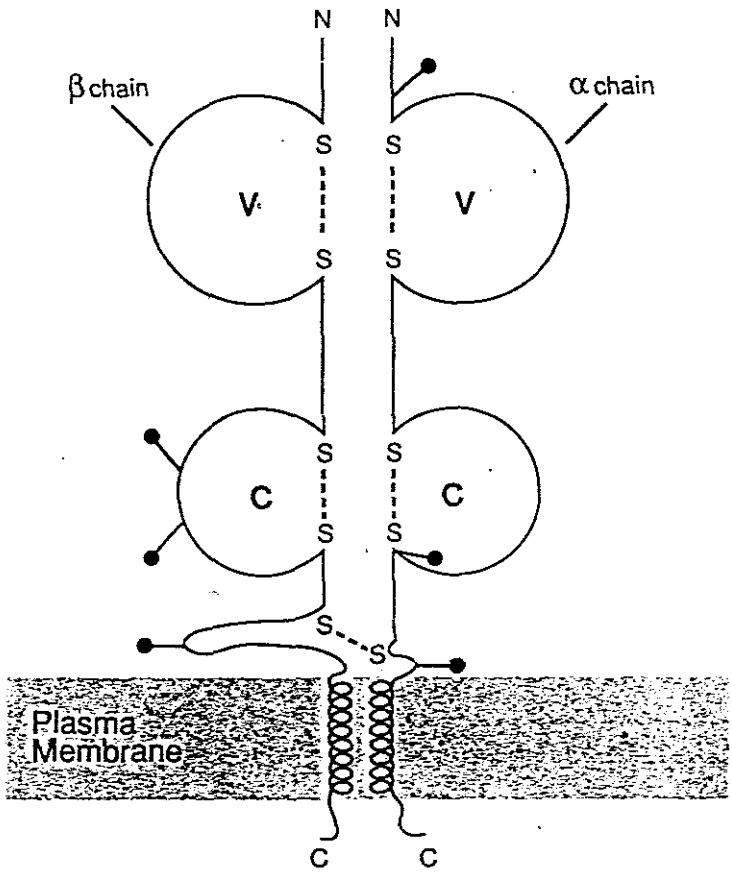


Figure 2.3

Figure showing the structure of a T cell receptor. V refers to a variable region, C refers to a constant region, N and C refer to amino and carboxy groups respectively, S--S refers to disulphide bridges and --- indicate approximate position of carbohydrate groups.

2.3.1.2 FUNCTION OF ALPHA/BETA T CELL RECEPTORS

The alpha/beta receptor recognises complexes of processed peptides, generated from foreign protein antigens, bound to self-MHC molecules. Both the alpha and beta chains are responsible for binding peptides i.e. they are not independently specific for antigen or MHC (Abbass *et al*, 1991).

There are three main functions of the alpha/beta TCR's viz. specific recognition of pathogen-infected host cells, regulation of the immune response via chemical signals and the initiation of specific antibody production by B cells (Royer and Reinherz, 1987).

The expression of the alpha/beta receptors and their function is also dependent on a group of proteins called the CD3 complex, consisting of at least five proteins which are physically associated with one another, and with the alpha/beta TCR (See Figure 2.4). It appears that when the antigen-MHC complex binds to the receptor, the CD3 complex transduces the signals to the cytoplasm of the T cells (Abbass *et al*, 1991).

2.3.2 THE GAMMA/DELTA T CELL RECEPTOR

2.3.2.1 STRUCTURE

The gamma/delta TCR is also a disulphide heterodimer expressed in a small number of alpha/beta negative peripheral T cells. The gamma polypeptide chain varies in length from 36-55kD and the delta chain from 40-60 kD. The gamma chain is similar to that of the beta chain and the delta chain similar to that of the alpha chain in that they also have a constant (C) region and a variable (V), the junction of these being coded by the joining (J) region. There are two types of C gamma chains, C gamma 1 which may form disulphide bridges with the delta chain, so the gamma/delta TCR may or may not be found in the disulphide bonded form. The gamma/delta T cell receptor structure appears similar to that of the alpha/beta T cell receptor in that it also contains the hinge regions,

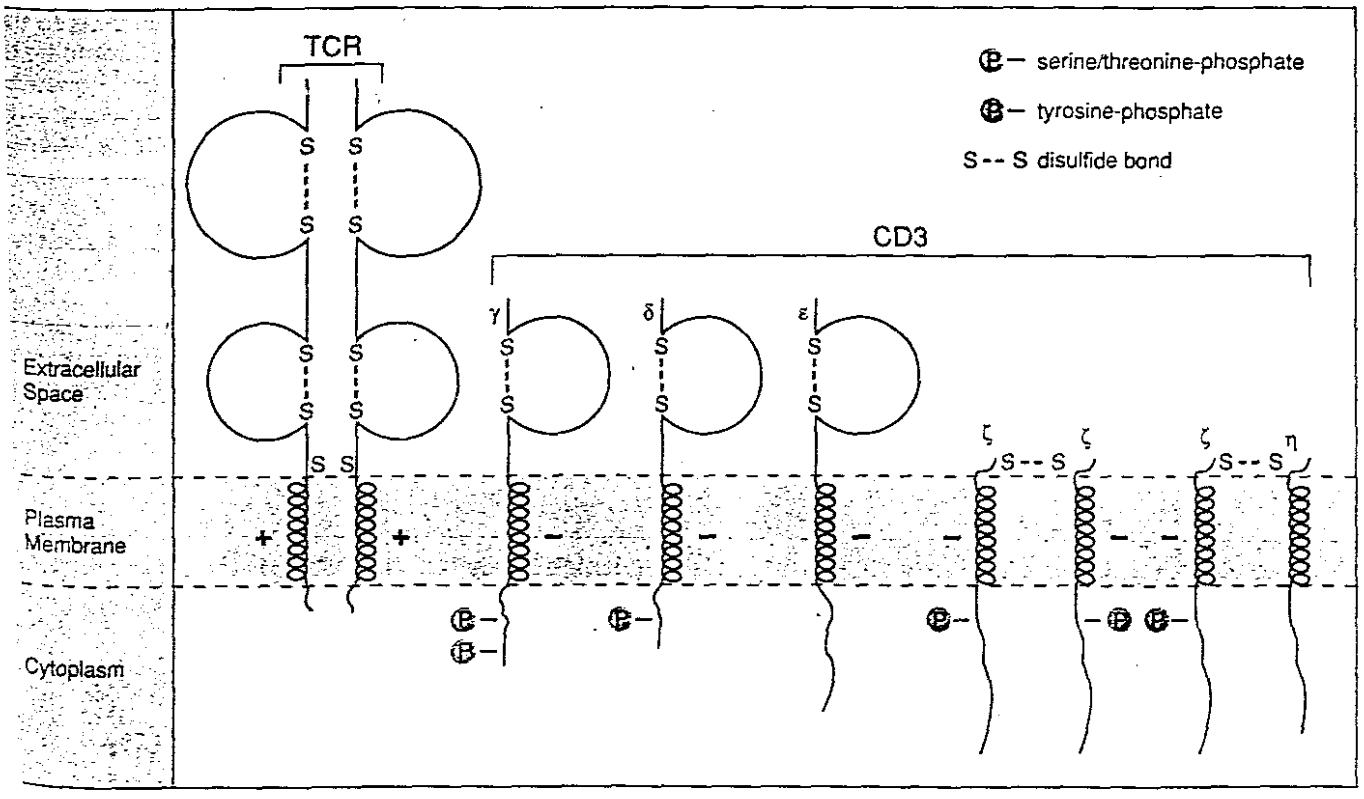


Figure 2.4

Figure showing the T cell receptor and its association with the CD3 complex. (Courtesy Abbass *et al*, Cellular and Molecular Biology, 1991).

the hydrophobic transmembrane segments and the cytoplasmic tails (See Figure 2.3) (Abbass *et al*, 1991). Most gamma/delta receptors are not MHC restricted (Haas *et al*, 1990).

2.3.2.2 FUNCTION OF GAMMA/DELTA T CELL RECEPTORS

There are questions as to whether the functions of the gamma/delta TCR's are the same as the alpha/beta TCR's. T cells bearing both the alpha/beta and the gamma/delta TCR produce interleukins and both types of T cells are cytotoxic (Kaufmann 1991). Gamma/Delta TCR's are also associated with the CD3 complex, which suggests that the events of activation are similar to that of the alpha/beta T cell receptors.

An argument in favour of the two types of T cell receptors having different functions is supported by the fact that the gamma/delta TCR have different features (Born *et al*, 1991) eg. the two types of receptors are encoded by different sets of genes, the gamma/delta genes are expressed earlier in ontogeny than the alpha/beta genes (Davis and Bjorkman, 1988), but they give rise to a smaller repertoire of functional receptor molecules and gamma/delta T cells appear to be strategically placed throughout the epithelial layers of the body (Lafaille *et al*, 1990) in contrast to the random appearance of alpha/beta T cells throughout the body. Epithelium represents the major port of entry for a number of pathogens, therefore the gamma/delta T cells may perform a surveillance function here (Kaufmann, 1991).

Another hypothesis is that gamma/delta TCR's may initiate immune responses to a small number of commonly encountered microbial agents, perhaps at epithelial boundaries (Born *et al*, 1991). Ligand recognition may be different in alpha/beta TCR's compared with gamma/delta TCR's, as antigens that stimulate alpha/beta T cells do not stimulate gamma/delta T cells, and it therefore appears that gamma delta T cells focus on different targets to alpha/beta T cells. It has also been shown that gamma/delta T cells fail to distinguish self from non-self (Born *et al*, 1991).

T cells bearing gamma/delta TCR's do not express CD4 or CD8. Cellular infiltrates in some diseases appear to contain a high number of these receptor-bearing cells, eg. untreated coeliac disease, localised cutaneous leishmanias (Modlin *et al*, 1989), tuberculous lymphadenitis and the peripheral blood of patients with measles (Haas *et al*, 1990). Although the role of gamma/delta T cells in disease may be to eliminate infectious agents, they are also prominent in immunological disorders eg. rheumatoid arthritis (Haas *et al*, 1990) and pulmonary sarcoidosis (Balbi *et al*, 1990). Kaufmann (1991) has shown evidence that they have been implicated in host response to certain bacterial infections eg. *Mycobacterium tuberculosis*. Some gamma/delta TCR bearing T cells appear to demonstrate non-specific cytolytic activity against tumour cells (Kaufmann, 1991).

2.3.3 THE T CELL RECEPTOR GENES

2.3.3.1 STRUCTURE OF ALPHA/BETA CHAIN GENES

Functional TCR genes are only present in cells of T lymphocyte lineage. The beta chain gene is located on chromosome 7 and the alpha chain gene is located on chromosome 14 (Royer and Reinherz, 1987). TCR genes in early T cell precursors are in the non-functional form and they become functional by somatic rearrangement of germ-line gene segments during the maturation process (See Figure 2.5). The beta chain gene is rearranged before the alpha chain gene and the rearrangement is mediated by recombinases. Only one gene is rearranged, the other is inhibited by the phenomenon known as allelic exclusion (Abbass *et al*, 1991).

Each TCR locus consists of variable (V), joining (J), and constant (C) region genes. The beta locus contains diversity (D) segments. There are two beta C genes, each being associated with six or seven J segments and one D segment. There are 20-30 Vbeta segments 5' of the C and J region (See Figure 2.6). When the gene is rearranged, one D segment joins with one J segment, which in turn joins with one V segment, hence forming

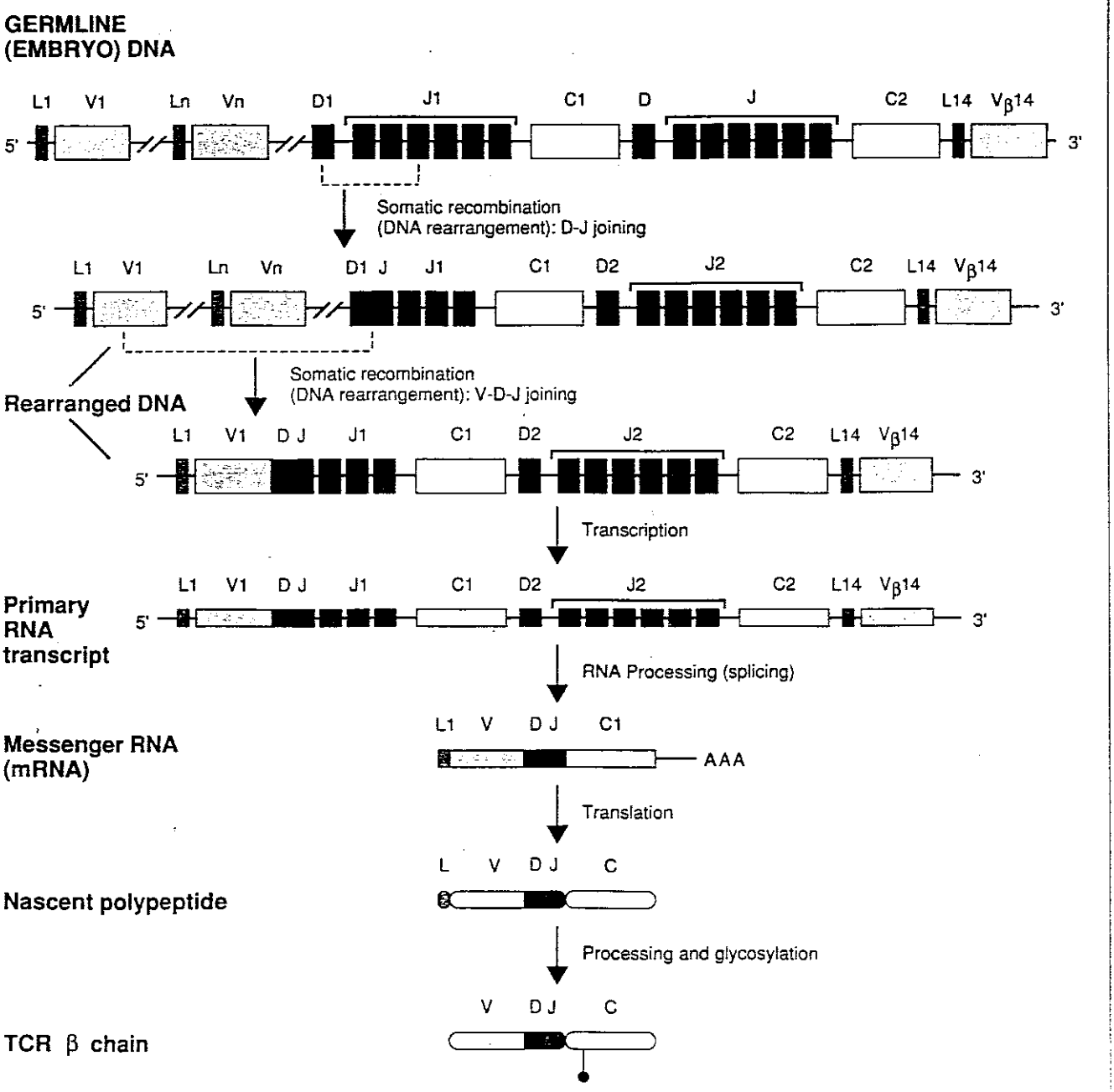


Figure 2.5

Figure showing how unrearranged T cell receptor gene segments rearrange during the maturation process and become functional T cell receptor genes (Courtesy Abbass *et al*, Cellular and Molecular Biology, 1991).

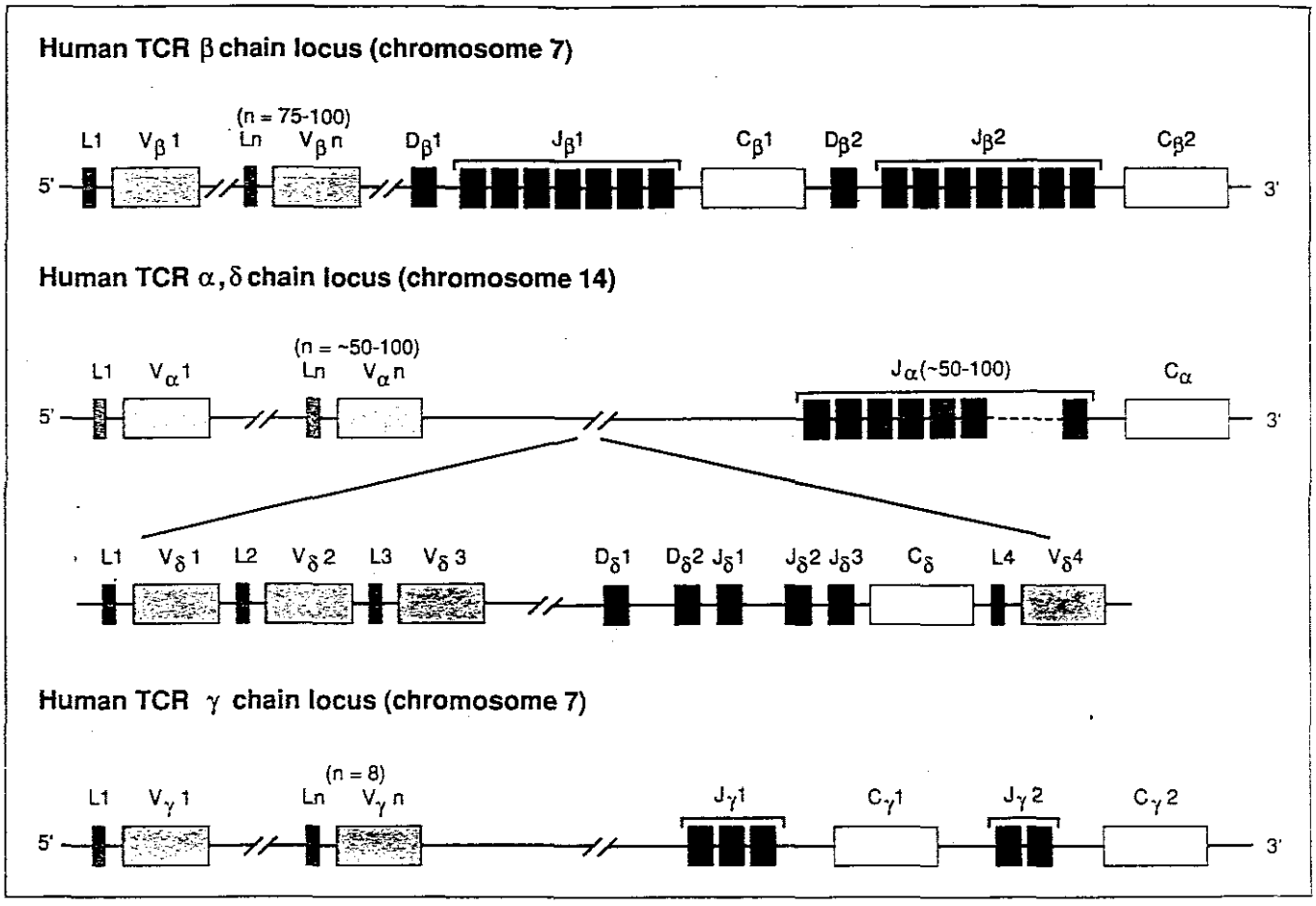


Figure 2.6

Figure showing organisation of human T cell receptor genes in the germ-line. (Courtesy Abbass *et al*, Cellular and Molecular Biology, 1991).

a VDJ gene. Introns are present between the VDJ and the C regions. These are spliced out and the VDJ complex associates with the C genes (See Figure 2.6) (Marrack and Kappler, 1987).

The rearrangement of the beta chain stimulates rearrangement of the alpha gene. As there are no D segments in an alpha chain gene, only one V and J segment join. There is only one C gene which associates with the complex. The C gene can associate with any one of up to 60 different J segments. There are about 75 different V segments located 5' of the J and C regions. Once a TCR has been rearranged, there will be no further genetic alterations (See Figure 2.6) (Abbass *et al*, 1991).

2.3.3.2 STRUCTURE OF THE GAMMA/DELTA CHAIN GENES

The gamma and delta chain genes are situated on the short arm of chromosome 7 and 14 respectively.

The gamma chain gene rearrangement is similar to that of the beta locus. The gamma locus consists of two constant region genes, Cγ1 and Cγ2. Two J-C complexes contain 5J and 2C segments in total. They are located 3' of up to 14 multiple V segments. No D segments have as yet been identified on the gamma chain gene (Moretta *et al*, 1991) (See Figure 2.6).

The delta locus is situated entirely within the alpha chain locus between the Valpha and JCalpha clusters. There is a single C region and six V regions have been identified. Five are located upstream of the C region and one downstream from it. They are associated with the three J and two D segments (Moretta *et al*, 1991) (See Figure 2.6).

2.3.4 T CELL RECEPTOR POLYMORPHISM

The rearrangement of germ-line TCR genes results in the production of a diverse functional repertoire of TCR's, made necessary by the wide spectrum of foreign antigen

that the T cells must be able to recognise. The variation may arise from germ-line differences in the number of V segments as well as allelic polymorphism (Kimura *et al*, 1986). Therefore, the ability of an individual to respond to a foreign antigen may be influenced by such a variation (Wright *et al*, 1991). The variation may also lead to an ineffective immune response.

Experiments on mice have indicated that these repertoire differences may play a fundamental role in disease susceptibility. To extend the concept to a human model, RFLP studies are carried out to establish genetic association with the TCR genes. The TCR genes may provide a unique opportunity to study heritable markers of T cell function in several human diseases.

2.4 THE MAJOR HISTOCOMPATIBILITY COMPLEX

In man, the Major Histocompatibility Complex (MHC), is known as the HLA system and spans a region of some 3.5 million bases of DNA (Wills, 1991). The genes coding for the HLA system are on the short arm of chromosome 6 (Wills, 1987) (See Figure 2.7).

The HLA system is highly polymorphic, i.e. the majority of individuals express a gene different to the homologous gene in the remaining members of the population. In 1970, it was discovered that antigen-specific T lymphocytes recognise protein antigen only when presented in conjunction with MHC molecules (Marrack and Kappler, 1987) and it was shown that the alleles confer the ability of an MHC molecule to respond to foreign antigens.

There are at least fifty loci in this region coding for subunits of HLA molecules, some of which are pseudogenes (Wills, 1991). Three classes of MHC molecules are recognised viz. class I, class II and class III. Class I and class II are the components of the immune system which recognise antigens and communicate amongst the cells during the immune

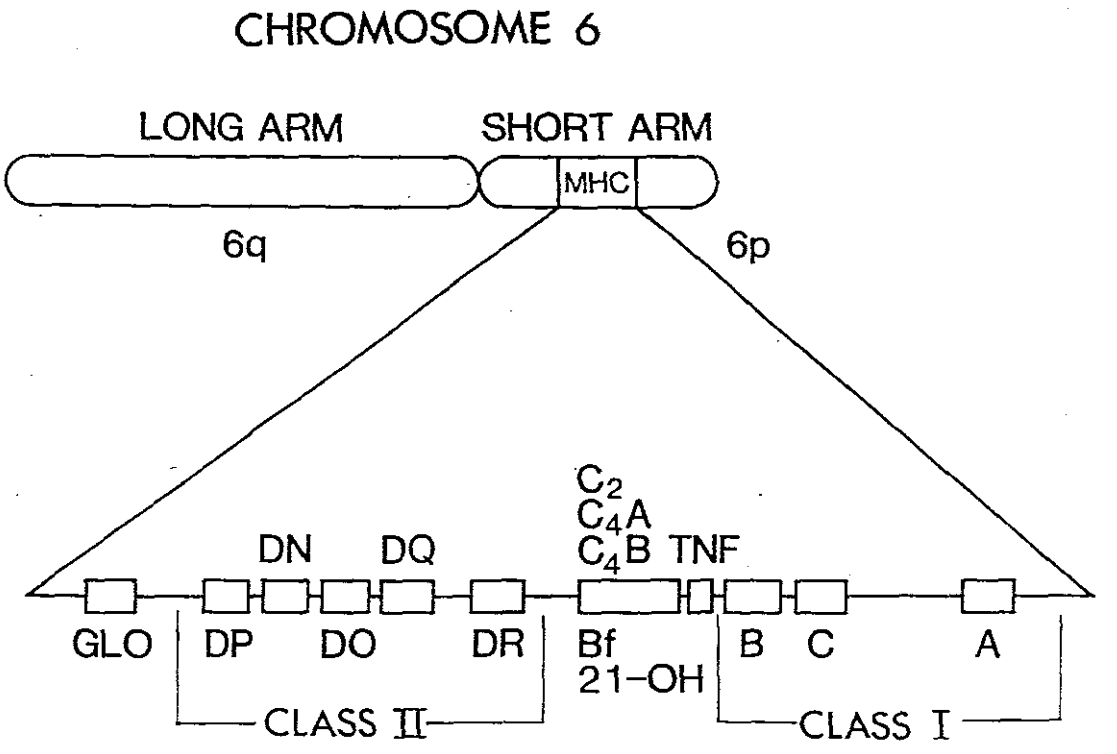


Figure 2.7

Figure showing a map of the MHC region, which is known in man as the HLA region, on the short arm of chromosome 6.

response. Class I MHC molecules are recognised as HLA-A, -B and -C, and Class II molecules are recognised as HLA-DP, -DQ and -DR. Although HLA-DZ and HLA-DO genes have been reported, their products have not been identified (Bell *et al*, 1986).

Class I and class II genes are separated by 1100 kb of DNA generally known as the HLA class III region (See Figure 2.7). Besides complement factors C2, factor B and C4, between 29 (Cross *et al*, 1991) and 36 (Kendall *et al*, 1991), other genes have been identified in this region.

2.4.1 STRUCTURE OF CLASS I MHC MOLECULES

Class I HLA spans approximately 200 kb on the telomeric end of the MHC complex (Kendall *et al*, 1991). HLA-A, -B and -C gene products are expressed on the surface of B lymphocytes, dendritic cells, thymic epithelial cells and activated T cells (Kara and Glimcher, 1991).

Each class I gene encodes a single trans-membrane chain, alpha, folded into three extracellular domains, alpha 1, alpha 2 and alpha 3 (See Figure 2.8). The alpha chain has a molecular weight of 44kD and each alpha domain is non-covalently associated with an extra-cellular small chain, Beta2 microglobulin, which has a molecular weight of 12kD and is coded for by a gene on chromosome 15. The two amino terminal domains of the alpha chain contain the polymorphic or variable residues which are recognised by the T cells receptors. A short hydrophobic region spans the membrane. The carboxy terminal is located in the cytoplasm. The beta chain (ß2 microglobulin) does not span the membrane.

2.4.2 STRUCTURE OF MHC CLASS II MOLECULES

The HLA class II gene region spans approximately 850 kb at the centromeric end of the MHC (Kendall *et al*, 1991).

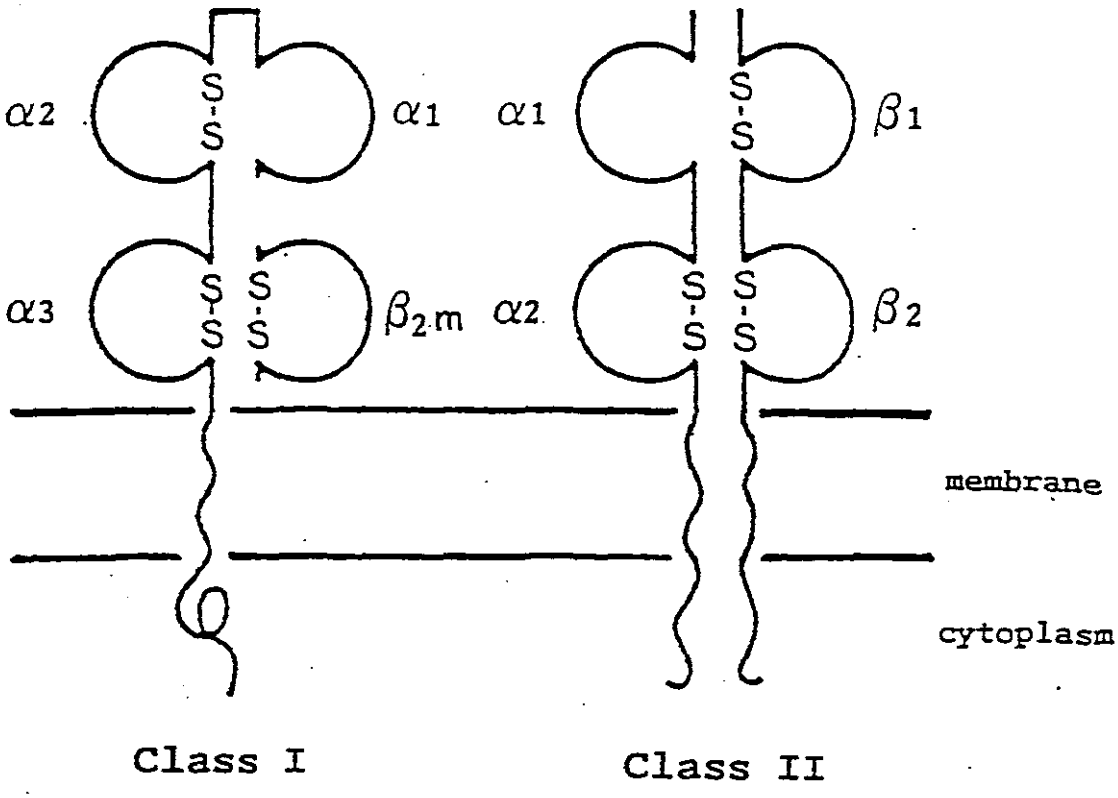


Figure 2.8

Schematic representation of MHC class I and class II molecules. S==S indicates disulphide bridges. α1, α2, α3 and ß1 and ß2 indicate domains corresponding to the exon of the genes and ß2 indicates the ß2 microglobulin chain.

Gene products of the HLA-D region i.e. HLA-DR, -DQ and -DP are expressed on the cell surface of all B-lymphocytes, macrophages, Langerhans cells, dendritic cells and activated T cells which are all involved in antigen presentation to T cells during an immune response (Kara and Glimcher, 1991).

The class II region consists of 14 loci, of which 9 are expressed. Class II MHC molecules are heterodimers and the structure of the two chains is similar (See Figure 2.8) as the alpha chain has a molecular weight of 32-34 kD and the Beta chain has a molecular weight of 29-32 kD. Both polypeptide chains have extracellular amino terminals, and intracellular carboxy terminals (Wills, 1991). The chains are polymorphic in both the alpha1 and beta1 regions, unlike HLA class I molecules where only the alpha chain is polymorphic (Kara and Glimcher, 1991).

2.4.3 FUNCTION OF MHC MOLECULES

The primary function of HLA class I antigens is to carry small intracellular peptides to the surface of the majority of cells in the body, whereas class II molecules bind to peptides resulting from partial digestion of proteins previously ingested by the cell. These peptides may be derived from invading organisms, or from native protein antigens ingested by the cell (Wills, 1991). When foreign antigens are presented by cells bearing class II molecules to the T cells, the T cells recognise both the foreign antigen as well as the self MHC class II molecule.

It has been shown that HLA-A and -B molecules are involved in antigen presentation whereas HLA-C has a much lower capacity (Wills, 1991). It has been suggested that particular alleles on the MHC may play a role in resistance to specific diseases.

Class III genes code for, among others, complement factors which play a fundamental role in immunity (Cross *et al*, 1991).

2.5 LINKAGE ANALYSIS

The advent of recombinant DNA technology has provided a number of polymorphic markers that are suitable for the study of genetic linkage in humans. Single gene traits are usually analysed simply by observation of the data, whereas various sophisticated efficient linkage software (Bryant, 1991) techniques e.g. Liped (Ott, 1985) have had to be developed to analyse the data where phenotypes have more complex modes of inheritance.

2.5.1 REVERSE GENETICS

There has been interest in locating single gene mutations on individual chromosomes that are responsible for specific diseases by reverse genetics. The first step is to map the disease to a chromosomal region. One may either map the gene directly when the chromosome concerned has physical aberrations, or indirectly by genetic linkage in multiplex families. Once the gene has been mapped, reverse genetics may be used to isolate and clone the causative gene. The linkage approach has been successful in mapping diseases such as heritable cancers (Bodmer, 1987), Ducchene muscular dystrophy (Mandel, 1989) and cystic fibrosis (Kerem *et al*, 1989). DNA polymorphisms and experimental techniques have aided researchers in tracing diseases as they segregate through families (Farral, 1991). Reverse genetics differs from forward genetics in that in forward genetics, the product of the gene causing the disease is known, eg. the lack of the enzyme, ß-hexoseaminidase in Tay-Sachs disease, whereas in reverse genetics, the gene is isolated although the product of the defective gene is not known.

2.5.2 DNA POLYMORPHISMS

Polymorphism is said to occur when at least 1% of the population express a gene different to the homologous gene in the remaining members of the population. As much as 99% of DNA has no known function and exists as non-coding DNA. Mutations occur within the

non-coding region. A single base difference which occurs roughly once in every 150 base pairs in the non-coding region and may give rise to restriction fragment length polymorphisms (RFLP's) (Lowe, 1986). The RFLP may occur at various sites, but a cytosine-guanine (CpG) dinucleotide is particularly susceptible. The cytosine derivative becomes methylated and the cytosine becomes converted to thymidine to form a thymidine-guanine dinucleotide (TpG). A restriction endonuclease specific for recognition of a CpG dinucleotide will no longer recognise that particular site. Thus, restriction enzyme digestion and hybridisation with an appropriate DNA probe will reveal the RFLP. The CpG hotspot, as well as the use of restriction endonucleases recognising this dinucleotide, has lead to the discovery of many RFLP's. RFLP's have alleles which segregate as co-dominant markers and limit the heterozygosity and informativity obtained with a single polymorphism (Farral, 1991).

2.5.3 LINKAGE DISEQUILIBRIUM

Alleles that map close to each other are usually associated with one another due to their tight genetic linkage. In a randomly mating population, the joint frequency of two alleles at different loci will be the product of their individual frequencies, according to the Hardy Weinberg equilibrium. When the observed joint frequency is significantly different to the expected joint frequency, the two alleles are said to be in linkage disequilibrium (Tiwari and Terasaki, 1985). Linkage disequilibrium will only occur when loci of two polymorphisms are close to each other. However, recombination may occur, resulting in the generation of a new combination of alleles. Recombination can thus generate new haplotypes and lead to a decrease of disequilibrium. In general, the stronger the linkage disequilibrium, the smaller the recombination fraction and hence the shorter the distance between the marker loci (Farral, 1991).

2.5.4 STATISTICS

2.5.4.1 HYPOTHESES

In linkage analysis, the null hypothesis (H_0) states that alleles at the disease locus and the polymorphic marker locus under examination segregate independently, i.e. the recombination fraction is 50%. The alternative hypothesis (H_1) states that the disease locus and the marker locus segregate together i.e. the recombination fraction is <50%.

2.5.4.2 LOD SCORES

Lod scores are a means of analysing human genetic data. Lod scores derived manually may be used in simple analysis but computer programmes have been developed to analyse complex studies eg. Liped (Ott, 1985). The mode of inheritance and the penetrance, i.e. the conditional probability that an individual with a particular genotype has of expressing that phenotype, are defined. Haplotypes may be used when the markers appear to show tight linkage to each other (Farrall 1991).

A lod score represents the $\log_{10}$ of the odds of the probability of linkage. Lod scores are calculated and reported at various recombination fractions, namely 0.000, 0.001; 0.05; 0.10; 0.20; 0.30 and 0.40. A lod score of >+3 suggests odds of more than 1000/1 in support of linkage where the recombination fraction is < 0.3 (Risch, 1991). This is generally accepted as adequate evidence to prove linkage between a disease and a marker locus. The raw odds of 1000/1 corresponds to a posterior probability of linkage. This calculation considers the probability of any two chosen loci being randomly linked. Similarly, a lod score of <-2 at a recombination fraction of < 0.3 is accepted as sufficient evidence to exclude linkage (Risch, 1991). Lod scores may be added across families to give a cumulative Lod score (Farrall, 1991), which is useful especially when analysing small families.

CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 SAMPLE SELECTION

Three multiaccess families with pulmonary tuberculosis were selected for the study. In each family, there were affected as well as unaffected individuals with at least 2 siblings being affected. Affected individuals presented with culture-positive pulmonary tuberculosis and unaffected individuals were determined by negative chest X-rays.

A recessive or dominant with incomplete penetrable mode of inheritance could be interpreted for Pedigree 1 as neither parent was affected with tuberculosis, Pedigree 2 could be interpreted as following a dominant mode of inheritance where both parents were affected and a recessive or dominant mode where only the one parent was affected and either a recessive or dominant mode could be interpreted in Pedigree 3 as only one parent was affected (See Figures 3.1, 3.2 and 3.3).

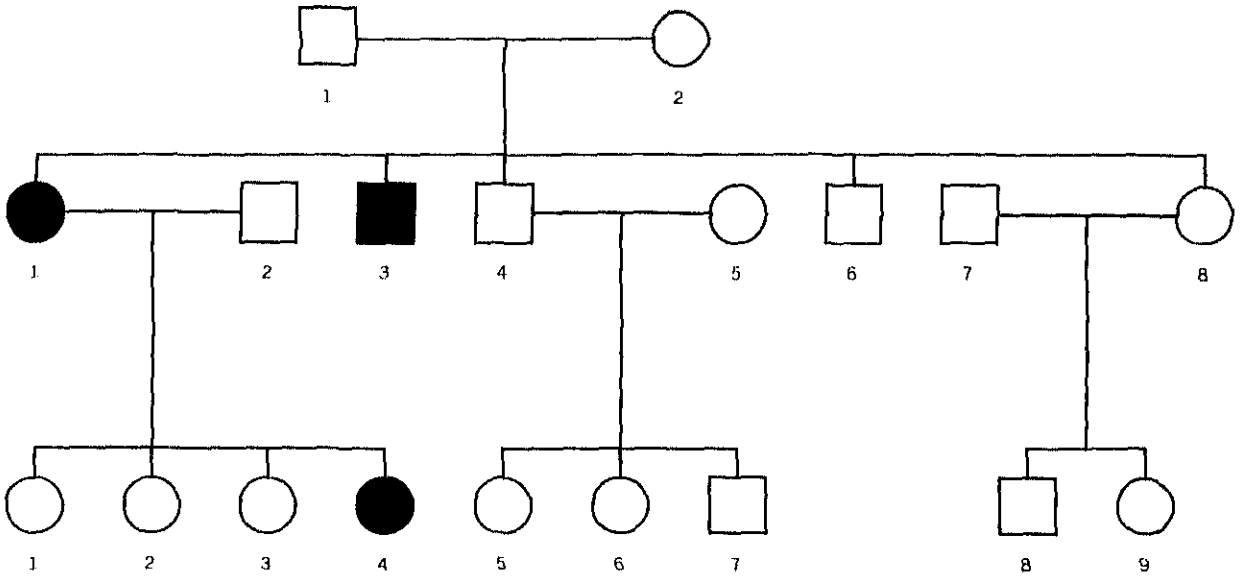
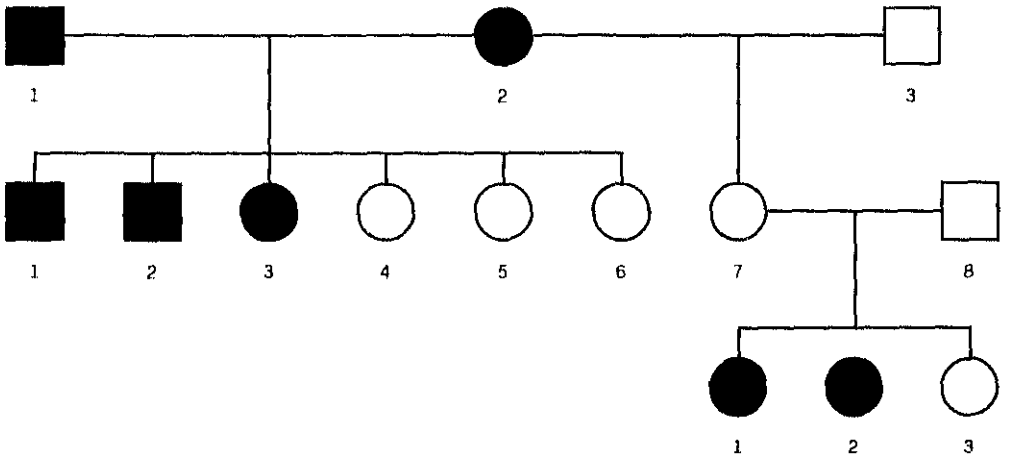


Figure 3.1

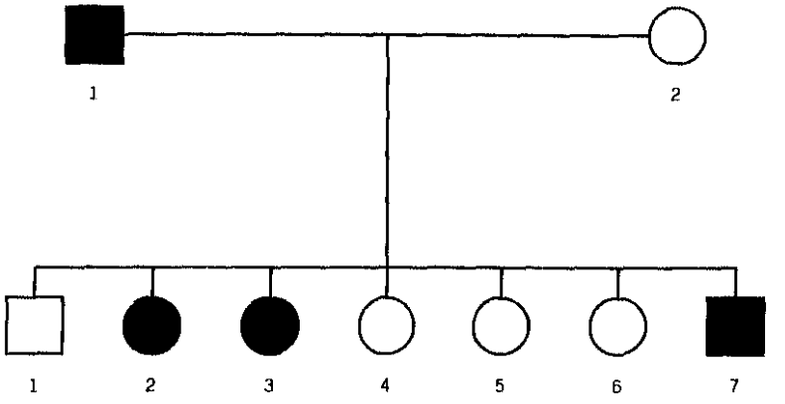
Diagramatic presentation of pedigree 1. Shaded symbols denote individuals with tuberculosis. Unshaded symbols denotes normal individuals.



PEDIGREE 2

Figure 3.2

Diagramatic presentation of pedigree 2. Shaded symbols denote individuals with tuberculosis. Unshaded symbols denotes normal individuals.



PEDIGREE 3

Figure 3.3

Diagramatic presentation of pedigree 3. Shaded symbols denote individuals with tuberculosis. Unshaded symbols denotes normal individuals.

3.2 SPECIMEN COLLECTION AND SAMPLE PREPARATION

3.2.1 HLA typing

Fresh whole blood (10 ml) was collected into heparin. As whole blood was used for obtaining lymphocytes, unwanted components had to be removed. Platelets and granulocytes were removed by adding 0.3 ml carbonyl iron/10 ml blood to the whole blood and incubated at 37°C for ten minutes. Carbonyl iron adhered to platelets and was ingested by the granulocytes. The red cells were removed by layering the blood on a high density Ficoll gradient. The sample was centrifuged at 2000 rpm for twenty minutes. The red cells formed rouleaux which enhanced their descent through the gradient.

The lymphocytes remained on the surface where they were harvested for testing.

3.2.2 DNA typing

The specimen of choice was 1 ml or more of fresh whole blood collected into EDTA. DNA extraction was started as soon as possible after collection of blood. The blood was kept cold on ice at all times. If the sample could not be processed within 48 hours, it was frozen at -20°C to -80°C.

3.3 HLA TYPING

3.3.1 Separation of B and T lymphocytes

Separation of B cells from T cells was performed using the nylon wool method (Bodmer, 1978). Briefly, the total lymphocyte population was incubated in a drinking straw, loosely packed with nylon wool. The straw was incubated at 37°C for 30 minutes. The non-adherent T cells were flushed from the straw with foetal calf serum (FCS), leaving the B

cells behind. The B cells were then harvested by squeezing the straw and the remainder of the B cells were flushed with 10% FCS. The B cells were then available for HLA typing.

3.3.2 The microlymphocytotoxicity test

3.3.2.1 HLA-A, -B and -C typing

The microlymphocytotoxicity test was carried out as described by Terasaki (1964). A volume of 0.5 μl aliquots of a T cell suspension was added to 0.5 μl of an antisera panel in a microtitre tray. The tray was incubated at 22°C for 45 minutes. Rabbit complement (2.5 μl) was added to the wells followed by incubation at 22°C for a further 60 minutes. If the lymphocytes carried the appropriate antigen, the antiserum combined and activated complement, damaging the cell membrane and lysing the cell. The tray was then flicked to remove excess protein. Membrane damage was detected by adding 1 μl of 1% Trypan Blue to each well in the tray. The tray was examined on an inverted microscope. The number of dead cells that took up the dye was expressed as a percentage of total cells (See Table 3.1).

Table 3.1.

Table showing the number of lysed lymphocytes that have taken up the dye expressed as a percentage of total cells for a positive reaction.

SCORE	INTERPRETATION	PERCENTAGE DEAD CELLS
1	Negative	0-10
2	Doubtful negative	11-20
4	Weak positive	21-50
6	Positive	51-75
8	Strong positive	76-100
0	Not readable or not done	

3.3.2.2 HLA-DR and -DQ typing

A volume of 0.5 μl of a B cell suspension was added to 0.5 μl aliquots of antisera in a microtitre tray and incubated at 22°C for 60 minutes. Rabbit complement (2.5 μl) was added to each well and the tray was incubated at 22°C for a further 120 minutes. The tray was flicked to remove excess protein. A volume of 1 μl of 1% Trypan Blue was added to each well. The amount of lysed cells that took up the dye was examined on an inverted microscope and expressed as a percentage of dead cells (See Table 3.1).

3.4 DNA ISOLATION AND PURIFICATION

3.4.1 Whole blood

EDTA whole blood (5-10 ml) was mixed with 30 ml cold lysis buffer on ice (See Appendix). Any visible clots were homogenised. The lysis buffer lysed the red and white blood cells and released the cellular debris and nuclei.

The solution was transferred to 50 ml polypropylene tubes and centrifuged at 4500 rpm for 20 minutes at 4°C to pellet the nuclei. The nuclear pellet was washed in 5 ml lysis buffer until the supernatant was clear and free of haemoglobin.

Saline EDTA (500 μl) (See Appendix) and 50 μl 10% SDS (See Appendix) was added to the pellet to solubilise the nuclear membrane. The samples were vortexed until the pellet was resuspended. Proteinase K (20 μl) was added to digest the nuclear membrane and nuclear proteins, including DNAases. The tubes were vortexed and incubated at 56°C overnight to release the nucleic acids into solution. A volume of 1 μl of a 5 mg/ml pancreatic RNA-ase solution was added and the solution incubated for a further hour at 56°C to digest the RNA.

A salting-out method as described by Miller *et al* (1988) and a phenol-chloroform method as described by Sambrook *et al* (1989) was used to further purify the DNA. Using the

salting out method, the viscous and homogenous solution was transferred into a labelled Eppendorf tube. 0.25% volumes (125 μl) of saturated sodium acetate was added and the solution vortexed for at least 15 seconds to salt out the proteins. The tube was centrifuged at 10 000 rpm for 10 minutes at 4°C to pellet the proteins. The supernatant was decanted into a labelled Eppendorf tube containing an equal volume (625 μl) of isopropanol which precipitated the DNA. The sample was mixed well and centrifuged at 10 000 rpm for ten minutes at 4°C to pellet the DNA. The DNA was washed twice in 500 μl of 70% ethanol by centrifugation at 10°C for ten minutes to remove excess salt. The DNA pellet was then dried briefly under vacuum in a speedvac concentrator. The DNA was dissolved in 1 ml of sterile distilled water overnight at 4°C. A 1:50 dilution of an aliquot of DNA was made and scanned on a Beckmann DU60 spectrophotometer from 220nm to 300nm to determine the concentration of the DNA. A 1 mg/ml solution of DNA yields 20 O.D. units at 260nm.

Using the phenol-chloroform extraction method, 0.25 volumes of 2 M sodium acetate was added to the sample after digestion with Proteinase K. The sample was transferred to a 15 ml polypropylene tube. An equal volume of phenol-chloroform (See Appendix) was added and the solution vortexed until well mixed. Ten volumes of absolute ethanol were added and the tube gently inverted until the DNA became visible. DNA was retrieved and washed twice in 70% ethanol. Excess ethanol was discarded and the sample was dried briefly under vacuum.

3.4.2 Transformation of B cells

3.4.2.1 Preparation of Epstein Barr Virus (EBV) supernatant

The Marmoset cell line B95.8 was used as a source of EBV. One ampoule of B95.8 cells was removed from liquid nitrogen, thawed, washed and collected into 20 ml RPMI 1640 medium supplemented with 10% FCS. The cells were then transferred to a 250 ml culture flask. The flask was laid horizontally, with the lid slightly loosened and incubated at 37°C

in a CO_2 incubator for 4-7 days. The cells were checked regularly for growth under the inverted microscope.

Once the cells were confluent, the medium was harvested into a 50 ml tube. Dead cells and debris were removed by centrifugation at 1500 rpm for 10 minutes. The supernatant was further purified by filtration through a Mellex-GS 0.22 μm filter unit. The cells that adhered to the flask were removed by manual agitation, transferred into a 15 ml polypropylene tube and centrifuged at 1000 rpm for 10 minutes. The supernatant was discarded and the cells washed twice in RPMI 1640 by centrifugation at 1000 rpm for 10 minutes. The cells were divided into two flasks. One was used to continue the culture and the other cells were frozen in 10% DMSO and stored in liquid nitrogen for use at a later stage. The cell lines were tested regularly for mycoplasma by means of a DNA fluorochrome stain, Hoechst stain #33258, which was purchased in kit form (Chen, 1977).

3.4.2.2 Transformation of B cells

Lymphocytes were collected by separation of whole blood over Ficoll or retrieved from liquid nitrogen storage. The cells were washed in phosphate-buffered saline (PBS) by centrifugation at 1700 rpm for 10 minutes. EBV supernatant was diluted 1:1 with 10% FCS-RPMI 1640 and 3-5 ml per sample was added to the B cells. The cells were resuspended and incubated at 37°C in a CO_2 incubator for 1-2 hours. The sample was shaken every 15 minutes. The supernatant was removed after centrifugation. Culture medium (2 ml) was added and the cell viability and concentration determined by Trypan blue exclusion. The cell concentration was adjusted to 1 x 10^6 viable cells/ml. One millilitre aliquots were added to each well of a 24 well flat bottom culture tray. A 1% PHA solution (1 ml) was then added to each well and the tray incubated for three days at 37°C in a 5% CO_2 incubator. After three days, 1 ml of medium was removed and 1 ml of fresh culture medium added to each well. The tray was incubated for a further three days. The wells were inspected under an inverted microscope and 1 ml of cells was resuspended and transferred to another well if the cells had grown sufficiently. Fresh medium (1 ml)

was added to these wells and the trays incubated for an additional three days. Four wells of each sample were pooled together, collected into a tube and centrifuged at 1200 rpm for 10 minutes. The cells were resuspended in 8 ml fresh medium and transferred to a 25 ml culture flask and incubated in an upright position for three days in a CO_2 incubator. Optimum growth was at a concentration of 0.3-1 x 10^6 cells/ml. Cells were passaged until sufficient cells were obtained for a DNA extraction, i.e. 6 x 10^6 cells.

3.5 PLASMIDS

The eleven probes were received as recombinant plasmids in HB101 *E. coli* cells in glycerol stocks and agar slopes from the Centre for the Study of Host Resistance at McGill University, Montreal, Canada.

3.5.1 Purification of plasmids

Plasmid purification was done using the Qiagen plasmid purification kit. A scraping of a glycerol stock was used to inoculate 10 ml lung/heart infusion broth and was incubated at 37°C overnight. Ten microlitres of this was plated onto a nutrient agar plate to obtain single colonies. A single colony was again inoculated into a further 200 ml lung/heart infusion culture medium and rotated vigorously at 37°C overnight.

The cloudy suspension was then centrifuged at 2000 rpm at 4°C for twenty minutes to pellet the bacteria. The bacterial pellet was resuspended in 10 ml of a 5 mg/ml lysosome solution, solution 1 (See Appendix) to break down the cell wall and the outer membrane. The suspension was transferred to a test tube and let to stand for 5 minutes at room temperature. A freshly prepared NaOH/SDS solution, solution 11 (See Appendix) (20 ml) was added and the contents mixed by gently inverting the tube several times. The suspension was left to stand on ice for ten minutes. An ice-cold solution (15 ml) of a potassium-acetate, pH 5.5 solution, solution 111 (See Appendix) was added. The solution was mixed by inverting the tube sharply several times and then placed on ice for ten

minutes. The tube was centrifuged at 20 000 rpm for 20 minutes at 4°C. The bacterial DNA and debris formed a pellet on the bottom of the tube.

Equal quantities of the supernatant were transferred to two tubes. The plasmid DNA was precipitated with the addition of 0.6 volumes of isopropanol to each tube, mixed well and

3.5.2.1.1 Alpha 2 probe

A 1.1 kb EcoRI full length cDNA fragment Vα1.2, containing V,D,J,C sequence of clone PY14, was subcloned into pUC8 (Yanagi *et al*, 1985; Mitchell *et al*, 1989).

3.5.2.1.2 Alpha 3 probe

A 492 bp EcoRI-AccI fragment, Vα12.1, containing the leader sequences and variable region of clone pGA5 (Yoshikai *et al*,1986; Hoover *et al*, 1985; Ball *et al*, 1987).

3.5.2.2 Beta chain genes

Polymorphisms at the Beta chain locus were identified using two beta chain probes with specificities as described below.

3.5.2.2.1 Beta 1 probe

A 320 bp Nco-PvuII fragment, Vß8, containing the variable region of clone YT35, was subcloned into Bluescribe (Kimura *et al*, 1986; Concannon *et al*, 1987).

3.5.2.2.2 Beta 2 probe

A 360 bp EcoRI-AvaII fragment, Vß11, containing the variable region of clone PL3.12 was subcloned into Bluescribe.

3.5.2.3 Gamma chain genes

Polymorphisms at the gamma chain locus were identified using three probes with specificities as described below.

3.5.2.3.1 Gamma 1 probe

A T cell receptor gamma variable region 1 probe, pV3S, a 1.2 kb SacI fragment containing the TRGV3 genes, was cloned into pUC8 (Ghanem *et al*, 1989).

3.5.2.3.2 Gamma 2 probe

A T cell receptor gamma variable region 4 probe, pV11SPRS, a 270bp SpHI-RsaI fragment containing the 5' region of the TRGV11 gene cloned into pUC19 (Ghanem *et al* 1989a; Ghanem *et al* 1989b).

3.5.2.3.3 Gamma 4 probe

A T cell receptor gamma constant region 1 probe, pCR10.940 bp EcoR1 fragment containing the TRGC1-exon 3 was cloned into pUC8 (Lefranc *et al*,1986).

3.5.2.4 Delta chain genes

Polymorphisms at the delta chain locus were identified using two probes with specificities as dscribed below.

3.5.2.4.1 Delta 1 probe

A T cell receptor delta variable region 2 probe, pδV2SP0.5, a genomic 500 bp, Sma1-Pst1 fragment was subcloned into pUC18 (Triebel *et al* 1989; Chuchana *et al*, 1989).

3.5.2.4.2 Delta 2 probe

A T cell receptor delta J2 probe, R21XH, a 2.8 kb Xho1-Hind111 fragment was subcloned into pUC8 (Chuchana *et al*, 1989a).

3.6 SOUTHERN BLOTTING

Restriction fragment length polymorphisms were detected using the Southern Blotting technique as described by Southern (1975) (See Figure 3.4).

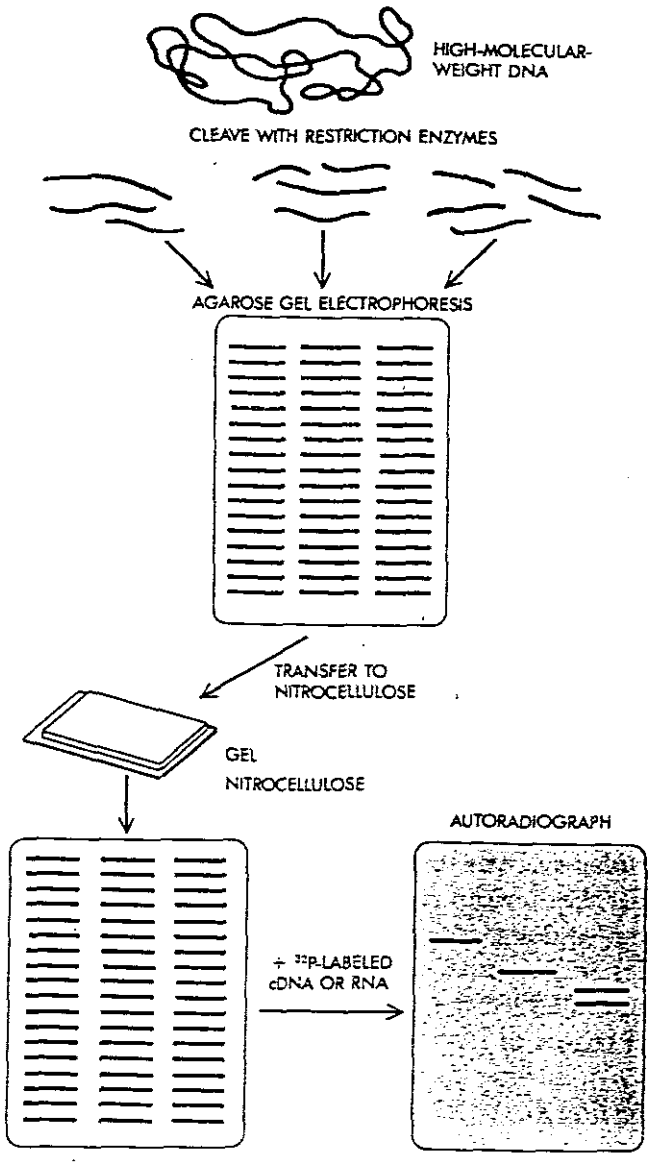


Figure 3.4

Schematic representation of Southern Blotting showing cleavage after digestion with restriction enzymes, separation of fragments by gel electrophoresis and hybridisation to a suitable labelled probe and specific DNA fragments that hybridise to the probe will give a signal following autoradiography. (Courtesy Watson *et al*, Recombinant DNA : A Short Course, 1983).

3.6.1 GENOMIC DNA DIGESTION

Restriction enzymes were used as directed by the manufacturers to digest DNA at a concentration of 3-5 units of enzyme/μg of DNA. Restriction enzymes used included HindIII; EcoRI; EcoRV; TaqI; SstI; BglII; BamHI; ScaI; PvuII and KpnI. Digestions proceeded at 37°C overnight except for TaqI which was incubated at 65°C overnight. The completeness of the digestions were checked by running 0.5 μg of DNA on a 0.8% agarose minigel for at least two hours at 50v.

3.6.2 GEL ELECTROPHORESIS

The restriction fragments were separated on 0.8% agarose gels by electrophoresis. On each gel, 500 ng Lambda HindIII electrophoresis marker was run. Ten nanograms of analytical molecular weight markers were also loaded viz. Lambda/HindIII and Lambda/EcoRI. Duration of electrophoresis and voltage was performed according to the size fragments required and ranged from 16 hours at 45 v to 24 hours at 40 v. The digested fragments were visualised by ultraviolet light after staining with ethidium bromide (Lowe, 1986) (See Plate 3.1).

3.6.3 DEPURINATION AND DENATURATION OF GELS

If large fragments (greater than 10 kb) were anticipated, the gel was depurinated by incubation with 0.15-0.25 M HCl for 10 minutes.

The gel was rinsed with distilled water and denatured with a solution of 1.5 M NaCl/0.5 M NaOH for a minimum of half an hour.

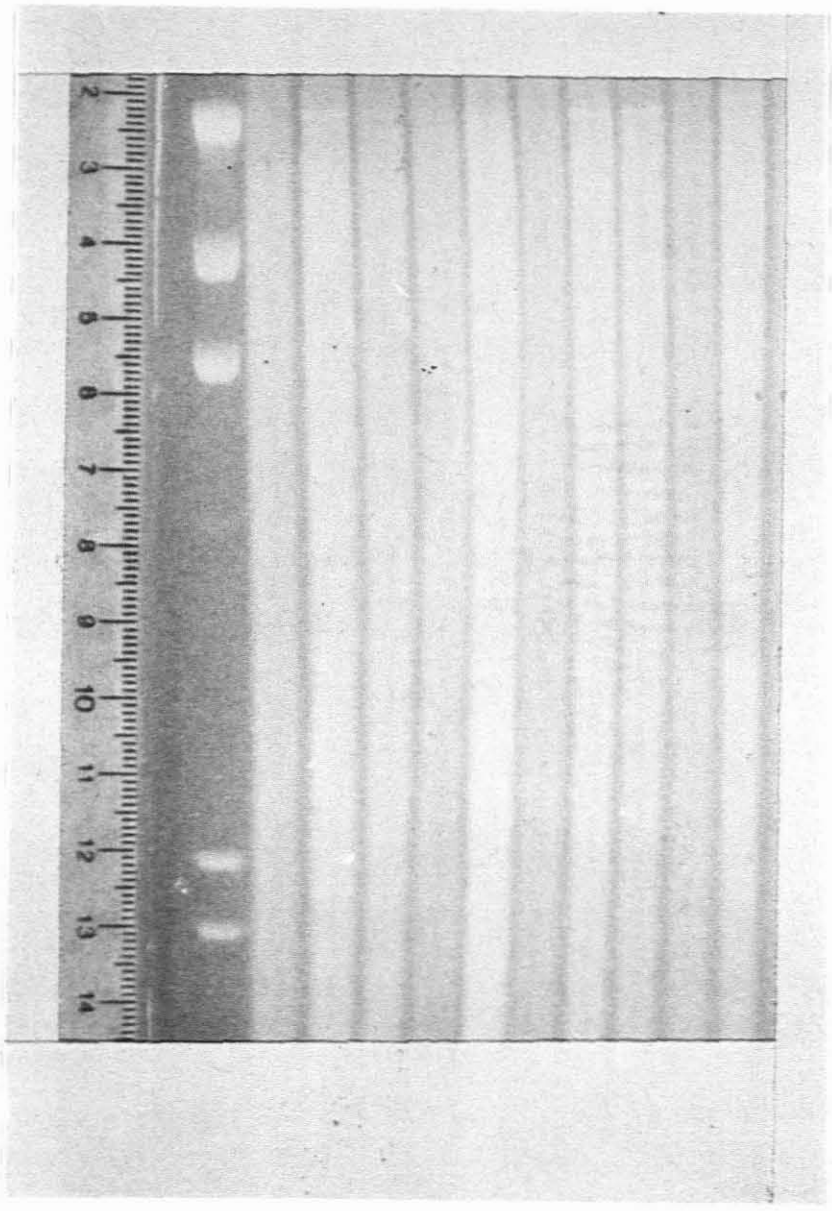


Plate 3.1

Ethidium bromide staining of high molecular weight DNA having been digested with a restriction endonuclease and viewed under UV light.

3.6.4 TRANSFER OF DNA FRAGMENTS TO NITROCELLULOSE MEMBRANES

The dry transfer method as described by Sambrook *et al* (1991) was used. Four sheets of Whatman 3mm filter paper were placed on a glass plate after being soaked in the NaCl/NaOH solution. The gel was placed on top of the filter paper, followed by Hybond N^+ nitrocellulose membrane. The edges of the gel were covered with clingwrap to prevent loss of transfer through the edges. Another four sheets of dry filter paper were placed on top of the membrane. A wad of roller towel was placed on top, followed by a glass plate. Two even weights of about 500 g each were equally distributed on top of the glass plate. The DNA was allowed to transfer overnight. The membranes were baked the following day for ten minutes at 80°C (Lowe, 1986).

3.6.5 NUCLEIC ACID LABELLING

Prehybridisation of the membranes was carried out for 4 to 24 hours at either 65°C or 55°C, depending on the protocol demanded by the probes being used.

3.6.5.1 LABELLING TECHNIQUES

The purified DNA inserts were labelled with ^{32}PdCTP using the random primer method as described by Feinberg and Vogelstein (1983).

This method utilises the ability of DNA polymerases to synthesise a new piece of DNA complementary to a template strand, starting from the 3'-hydroxyl end.

Hexanucleotides of random sequence as well as a cloned DNA polymerase (Klenow fragment) were used in the reaction. Most labelling reactions contained 50 ng of the DNA probe, 0.5 ng of analytical marker, 1 x oligomer labelling buffer (OLB) (See Appendix), 7 μl ^{32}PdCTP (specific activity 3000 Ci/mmol) and 5 units DNA polymerase. Labelling proceeded overnight at room temperature.

Unincorporated nucleotides were removed by centrifugation through Sephadex G50 columns. The percentage ^{32}P incorporated and the specific activity of the probe were calculated. Most reactions had a 50-70% incorporation and probes were only used when specific activities were at least 1 x 10^9 dpm/μg DNA.

The probes were denatured to single strands before use by boiling them for five minutes. They were then placed on ice immediately to prevent the single strands from re-anealing.

3.6.6 HYBRIDISATION

Hybridisation was carried out at 55°C or 65°C, depending on the protocol, in the same solution as prehybridisation (See Appendix), except in a smaller volume of 20 ml.

Membranes were hybridised in plastic bags from 24 to 48 hours in a shaking waterbath, depending on recommended protocols. The membranes were then washed twice for 45 minutes in 3x SSPE (See Appendix) and 1% SDS (See Appendix), followed by two stringency washes at reducing salt concentrations, depending on the homology of the probes to the DNA on the membrane.

Membranes were monitored throughout the washing process with a Geiger counter to determine the efficiency of the washes. Membranes were then sealed in plastic filing sleeves.

3.6.7 DETECTION OF POLYMORPHIC FRAGMENTS

Autoradiography was used to visualise ^{32}P-labelled hybridisation fragments. Sealed blots were exposed to Kodak XAR5 film (Kodak, Rochester NY USA) in a cassette with two Dupont lightning plus intensifying screens (Dupont, Boston MA, USA) at -70°C for 2-10 days. Two films were placed in the cassettes. One was used to determine the length of time required to visualise the fragments.

Films were developed by processing through developer, stop bath, fixer, washing and drying procedures.

3.6.8 ANALYSIS OF POLYMORPHIC FRAGMENTS

The autoradiographs were read and examined for the presence or absence of the following fragments as shown in the following Tables 3.2-3.5:

Table 3.2

Table showing polymorphic fragments detected at T cell receptor alpha chain genes.

LOCUS	PROBE	ENZYME	NO	ALLELE SIZE	FREQUENCY	LIPED CODE
TCR alpha	Vα1.2	EcoRV	1	9.5kb	0.08	E1a2
			2	2.5kb	0.92	
TCR alpha	Vα1.2	EcoRV	1	1.7kb	0.31	E2a2
			2	1.5kb	0.69	
TCR alpha	pY1.4	SstI	1	22kb	0.30	S1a2
			2	3.5kb	0.70	
TCR alpha	pY1.4	SstI	1	6.5kb	0.86	S2a2
			2	6.0kb	0.14	
TCR alpha	Vα1.2	TaqI	1	7.6kb	0.45	T1a2
			2	2.1kb	0.54	
			3	2.0kb	0.01	
TCR alpha	Vα1.2	TaqI	1	1.1kb	0.26	T2a2
			2	0.55kb	0.74	
TCR alpha	Vα12.1	BglII	1	10.5kb	0.05	Bga3
			2	4.9kb	0.95	
TCR alpha	Vα12.1	TaqI	1	4.0kb		Tqa3
			2	1.3kb		

Table 3.3

Table showing polymorphic fragments detected at T cell receptor beta chain genes.

LOCUS	PROBE	ENZYME	NO	ALLELE SIZE	FREQUENCY	LIPED CODE
TCR beta	Vß8	BamHI	1	23kb	0.53	Bab1
			2	2kb	0.47	
TCR beta	Vß11	BamHI	1	25kb	0.47	Bab2
			2	20kb	0.53	

Table 3.4

Table showing polymorphic fragments detected at T cell receptor gamma chain genes.

LOCUS	PROBE	ENZYME	NO	ALLELE SIZE	FREQUENCY	LIPED CODE
TCR gamma	pv3S	EcoRI	1	7.0kb		Ecg1
			2	2.4kb		
TCR gamma	pV3S	TaqI	1	16kb	0.21	Tqg1
			2	6kb	0.79	
TCR gamma	pV11SPS	SstI	1	13kb	0.91	Ssg2
			2	8kb	0.09	
TCR gamma	pV11SPS	TaqI	1	4.1kb	0.53	Tqg2
			2	3.7kb	0.47	
TCR gamma	pCIR0.9	PvuII	1	15.5kb	0.16	Pg4
			2	13.0kb	0.60	
			3	11.3kb	0.24	

Table 3.5

Table showing polymorphic fragments detected at T cell receptor delta chain genes.

LOCUS	PROBE	ENZYME	NO	ALLELE SIZE	FREQUENCY	LIPED CODE
TCR delta	pδV2SP0.5	BamHI	1	14.5kb	0.15	Bad1
			2	14.0kb	0.65	
			3	10.8kb	0.20	
TCR delta	R21XH	KpnI	1	20kb	0.15	Kpd2
			2	17.5kb	0.85	

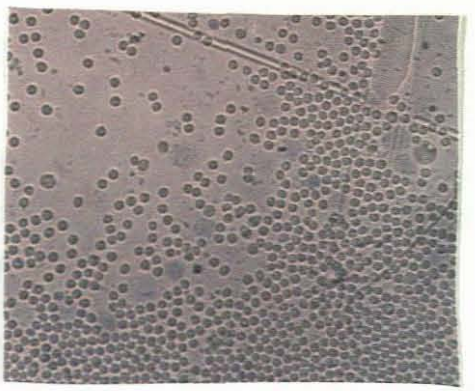
CHAPTER 4

4.0 RESULTS

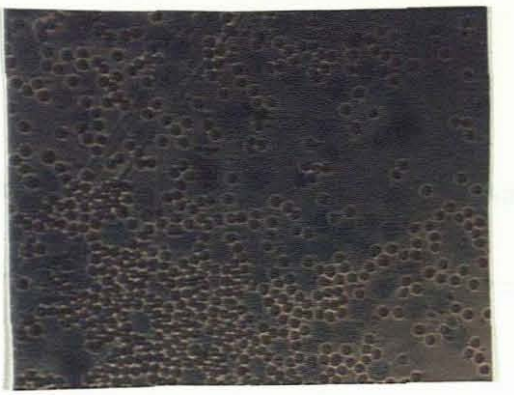
4.1 HLA

4.1.1 Serological HLA typing

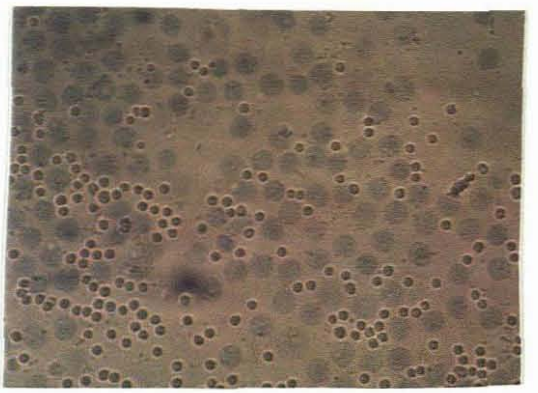
HLA typing was performed on all individuals using the microlymphocytotoxicity test as described by Terasaki (1976). The uptake of dye by lysed lymphocytes was graded as a percentage of total cells and interpreted as either a negative or a positive reaction as shown in Plate 4.1. Nineteen HLA-A antigens, 40 HLA-B antigens, 8 HLA-C antigens, 16 HLA-DR antigens and 5 HLA-DQ antigens were tested (See Table 4.1) and haplotypes were assigned for each individual (See Figures 4.1, 4.2 and 4.3). Haplotypes in brackets were inferred from offspring that were typed.



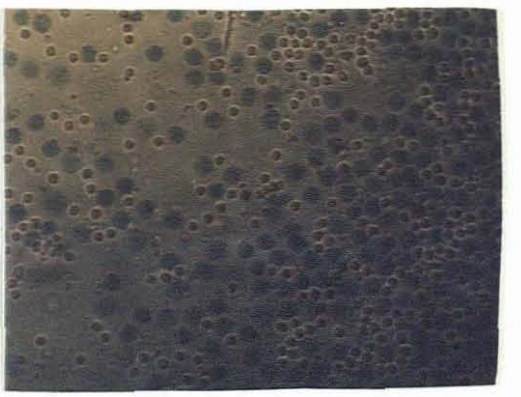
(i)



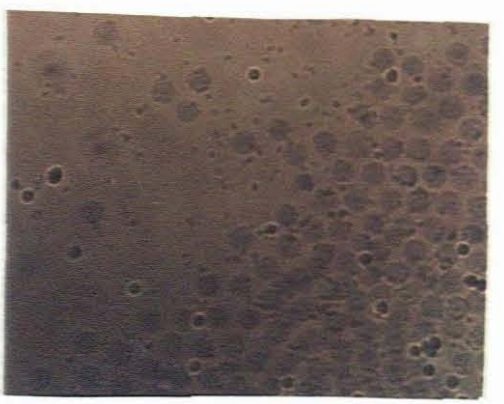
(ii)



(iii)



(iv)



(v)

Plate 4.1

Plate showing microlymphocytotoxicity reaction grading as (i) negative, (ii) weak positive, (iii) positive and (iv) strong positive.

Table 4.1

Table showing HLA antigens tested for using the microlymphocytotoxicity test. Antigens in brackets denote the broad antigen.

A	B	C	DR	DQ
A1	B7	Cw1	DR1	DQ1
A2	B8	Cw2	DR2	DQ2
A3	B13	Cw3	DR4	DQ4
A11	B22	Cw4	DR7	DQ7(3)
A23(9)	B27	Cw5	DR8	DQ8(3)
A24(9)	B35	Cw6	DR9	
A25(10)	B37	Cw7	DR10	
A26(10)	B38(16)	Cw8	DR11(5)	
A29(19)	B39(16)		DR12(5)	
A30(19)	B41		DR13(6)	
A31(19)	B42		DR14(6)	
A32(19)	B44(12)		DR17(3)	
A33(19)	B45(12)		DR18(3)	
A34(10)	B46		DR51	
A36	B47		DR52	
A43	B48		DR53	
A68(28)	B49(21)			
A69(28)	B50(21)			
A74(19)	B51(5)			
	B52(5)			
	B53			
	B55			
	B57(17)			
	B58(18)			
	B59			
	B60(40)			
	B61(40)			
	B62(15)			
	B63(15)			
	B64(14)			
	B65(14)			
	B67			
	B70			
	B73			
	B75(15)			
	B76(15)			
	B77(15)			
	B78			
	Bw4			
	Bw6			

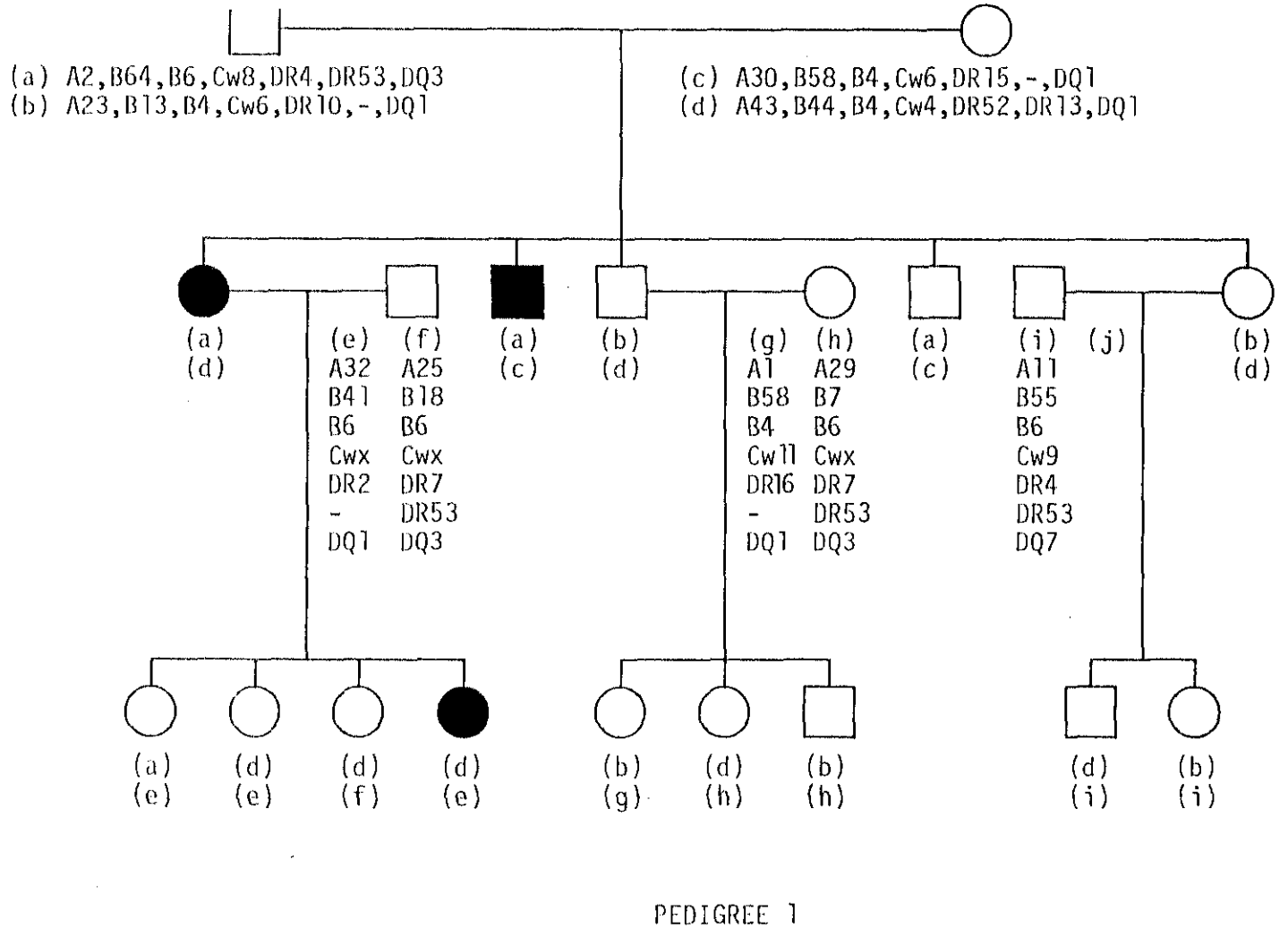


Figure 4.1

Figure showing results obtained from HLA typing for pedigree 1. Haplotypes in brackets were inferred from offspring.

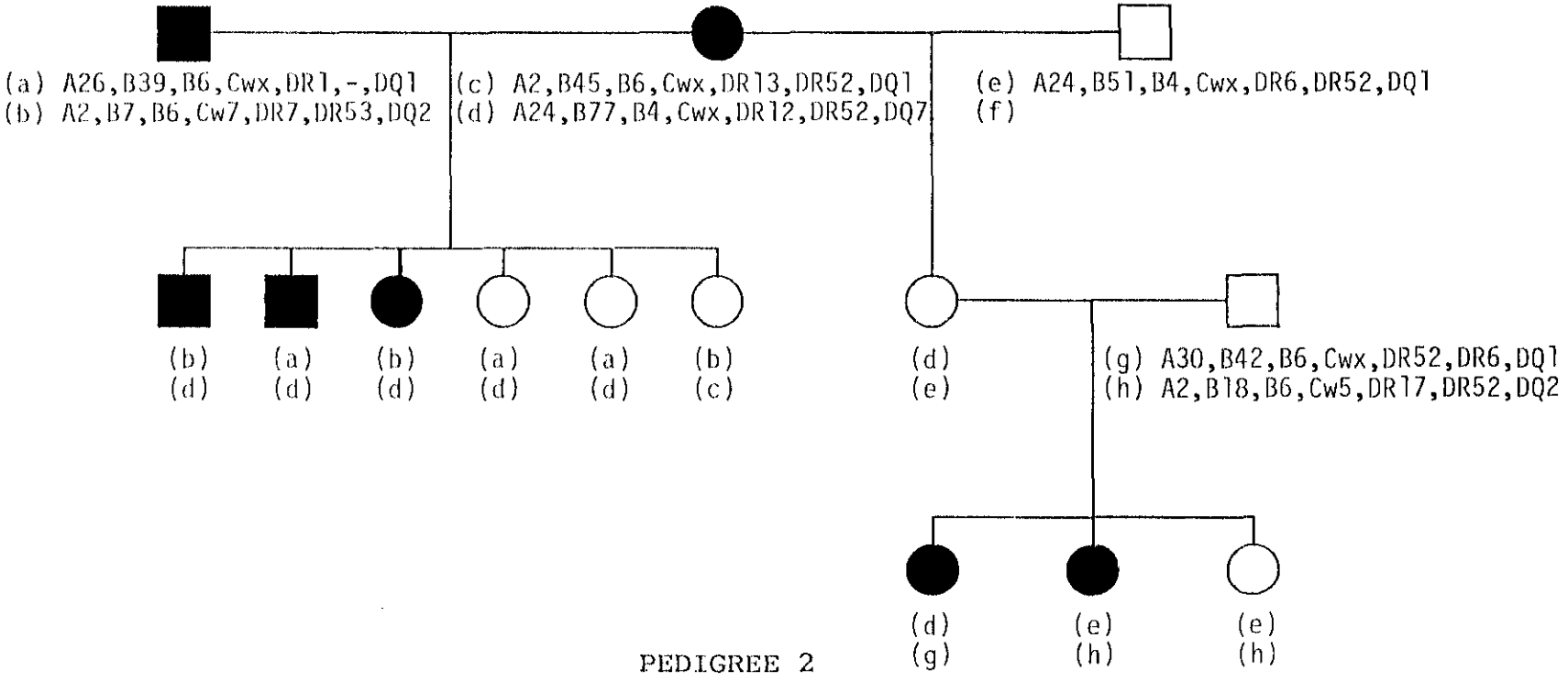


Figure 4.2

Figure showing results obtained from HLA typing for pedigree 2. Haplotypes in brackets were inferred from offspring.

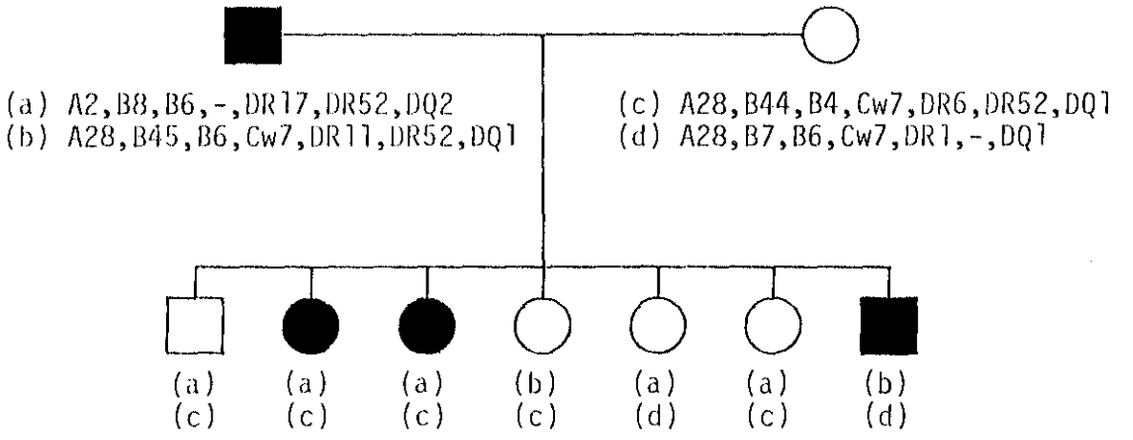


Figure 4.3

Figure showing results obtained from HLA typing for pedigree 3. Haplotypes in brackets were inferred from offspring.

4.1.2 Linkage analysis of HLA alleles and tuberculosis

By observation, no HLA allele appeared to be shared between affected individuals in each pedigree, suggesting that there is no preferential allele segregation to affected offspring.

4.1.3 Linkage analysis of HLA haplotypes and tuberculosis

4.1.3.1 Pedigree 1

At a recombination fraction of $\theta = 0$, a Lod score of -3.17 was obtained under a dominant model with 90% penetrance, -1.69 under a dominant model with 50% penetrance and -1.22 under the recessive model with 50% penetrance. However, as the recombination fraction increased to 0.5, the Lod scores tended to 0 (See Figure 4.4). Therefore, only tight linkage between susceptibility to tuberculosis and HLA may be excluded under a dominant model with 90% penetrance. However, the Lod scores fell within the indeterminate range as the recombination fraction increased to 0.001.

4.1.3.2 Pedigree 2

At a recombination fraction of $\theta = 0$, a Lod score of -3.07 was obtained under a dominant model with 90% penetrance, -1.50 under a dominant model with 50% penetrance and -3.95 under the recessive model with 50% penetrance. However, as the recombination fraction increased to 0.5, the Lod scores tended to 0 (See Figure 4.5). Therefore, only tight linkage between susceptibility to tuberculosis and HLA may be excluded under a dominant model with 90% penetrance and recessive with 50% penetrance as the Lod scores fell within the indeterminate range as the recombination fraction increased to 0.001.

4.1.3.3 Pedigree 3

At a recombination fraction of $\theta = 0$, a Lod score of -0.61 was obtained under a dominant model with 90% penetrance, 0.32 under a dominant model with 50% penetrance and -2.40 under the recessive model with 50% penetrance. However, as the recombination fraction increased to 0.5, the Lod scores tended to 0 (See Figure 4.6). Therefrore, only tight linkage between susceptibility to tuberculosis and HLA may be excluded under a recessive model with 50% penetrance. However, as the recombination fraction increased to 0.001, the Lod scores fell within the indeterminate range.

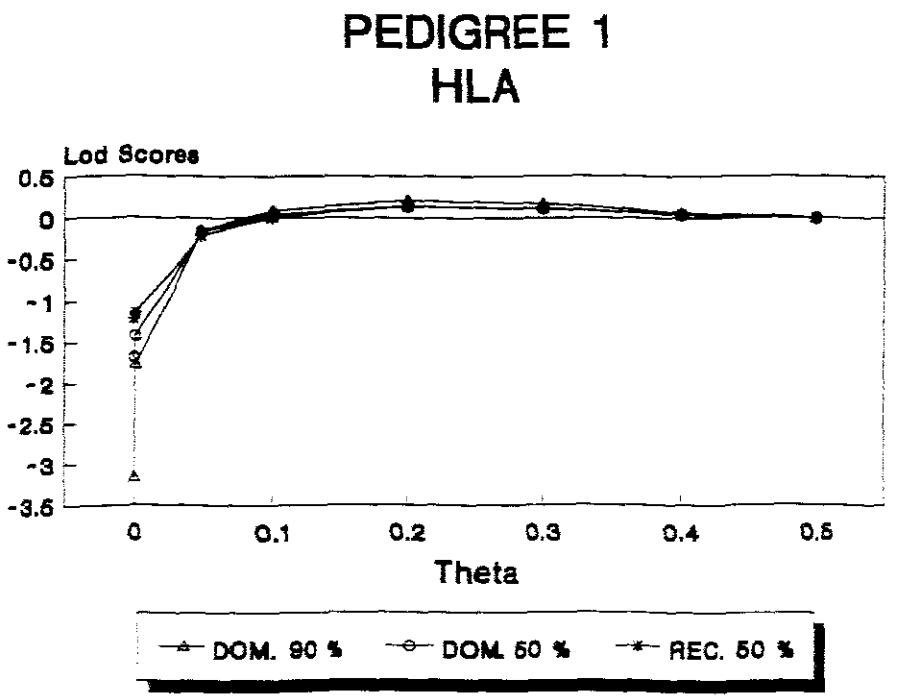


Figure 4.4

PEDIGREE 2
HLA

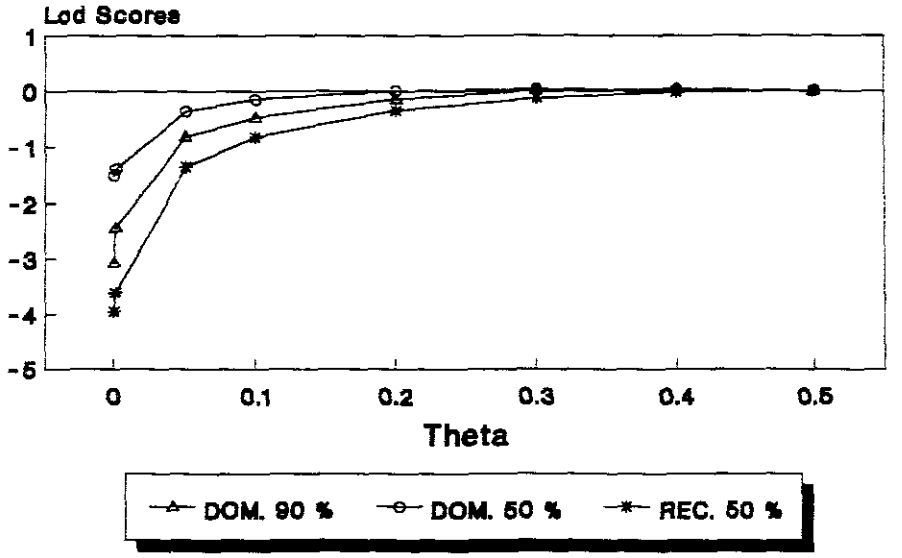


Figure 4.5

PEDIGREE 3
HLA

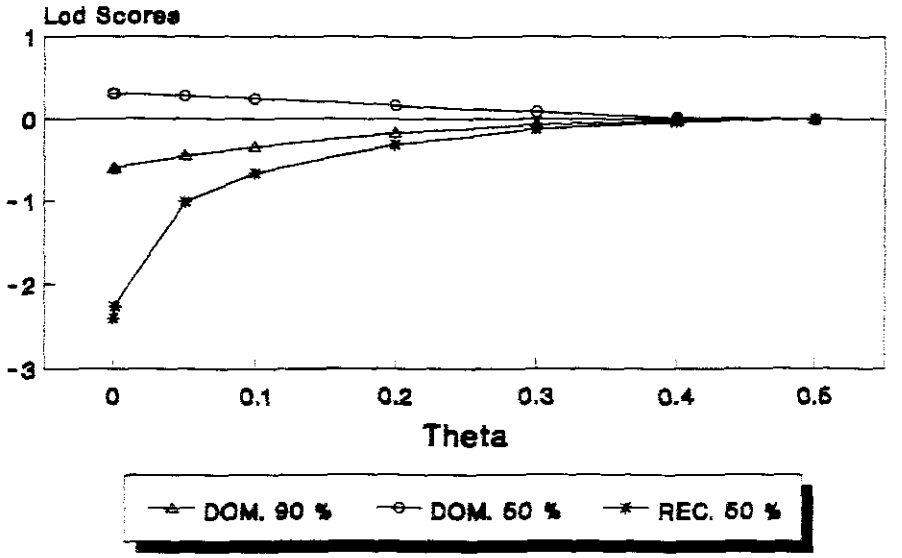


Figure 4.6

4.1.3.4 Cumulative Lod scores

Lod scores were added across the pedigrees to produce a cumulative result. At a recombination fraction of θ = 0, a Lod score of -6.85 was obtained under a dominant model with 90% penetrance, -2.87 under a dominant model with 50% penetrance and -7.57 under the recessive model with 50% penetrance. However, as the recombination fraction increased to 0.5, the Lod scores tended to 0. Therefore, only tight linkage between susceptibility to tuberculosis with HLA may be excluded under a dominant model with 90% and 50% penetrance as the Lod scores fell within the indeterminate range as the recombination fraction increased to 0.05 (See Table 4.2 and 4.3). Under the recessive model with 50% penetrance, linkage could be excluded at a recombination fraction of < 0.10 (See Table 4.4).

Table 4.2

Table showing addition of HLA Lod scores across families to obtain cumulative Lod scores under a dominant model with 90% penetrance.

GENETIC MODEL: DOMINANT, 90% PENETRANCE

HLA Lod scores at θ =

	0	0.001	0.05	0.10	0.20	0.30	0.40	0.5
1	-3.17	-1.79	-0.15	0.09	0.21	0.17	0.06	0
2	-3.07	-2.44	-0.81	-0.47	-0.15	-0.01	0.03	0
3	-0.61	-0.60	-0.45	-0.34	-0.17	-0.07	-0.02	0
SUM	-6.85	-4.83	-1.41	-0.72	-0.11	0.09	0.07	0

Table 4.3

Table showing addition of Lod scores of HLA across families to obtain cumulative Lod scores under a dominant model with 50% penetrance.

GENETIC MODEL: DOMINANT, 50% PENETRANCE

HLA Lod scores at θ =

	0	0.001	0.05	0.10	0.20	0.30	0.40	0.5
1	-1.69	-1.43	-0.16	0.04	0.14	0.11	0.04	0
2	-1.50	-1.39	-0.36	-0.15	-0.00	0.03	0.02	0
3	0.32	0.32	0.29	0.25	0.17	0.09	0.02	0
SUM	-2.87	-2.50	-0.23	0.14	0.39	0.23	0.08	0

Table 4.4

Table showing addition of Lod scores across families to obtain cumulative Lod scores under a recessive model with 50% penetrance.

GENETIC MODEL: RECESSIVE, 50% PENETRANCE

HLA Lod scores at θ =

	0	0.001	0.05	0.10	0.20	0.30	0.40	0.5
1	-1.22	-1.14	-0.21	0.01	0.14	0.12	0.04	0
2	-3.95	-3.61	-1.35	-0.82	-0.35	-0.13	-0.02	0
3	-2.40	-2.26	-1.01	-0.66	-0.31	-0.12	-0.03	0
SUM	-7.57	-7.01	-2.57	-1.47	-0.52	-0.13	-0.01	0

4.2 LINKAGE ANALYSIS OF T CELL RECEPTORS AND TUBERCULOSIS

4.2.1 Restriction Fragment Length Polymorphisms

The autoradiographs were examined for restriction fragment length polymorphisms as described. The sizes of the fragments were determined from a standard curve of the mobility (distance from the origin in cm of the Lambda Hind III molecular weight marker vs the sizes of the fragments in kb). Examples of the polymorphic restriction fragments obtained are shown in Plates 4.2-4.16.

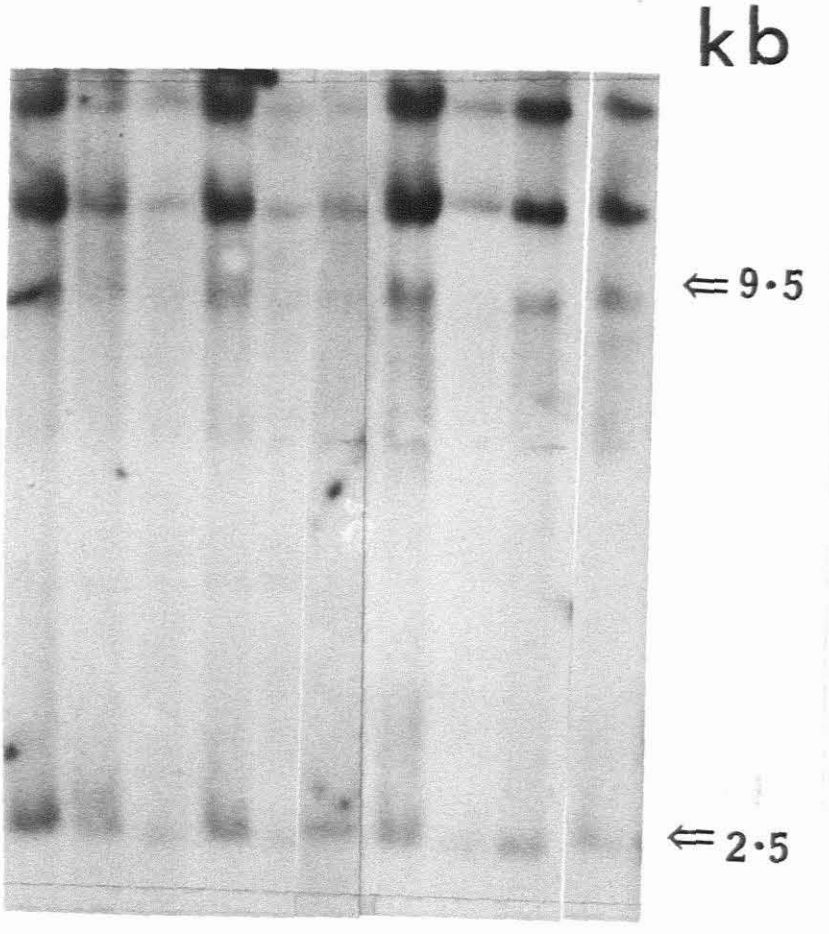


Plate 4.2

Southern Blot analysis of human DNA digested to completion with the restriction enzyme EcoRV and hybridised to the T cell receptor alpha-specific gene cDNA probe, Vα1.2.

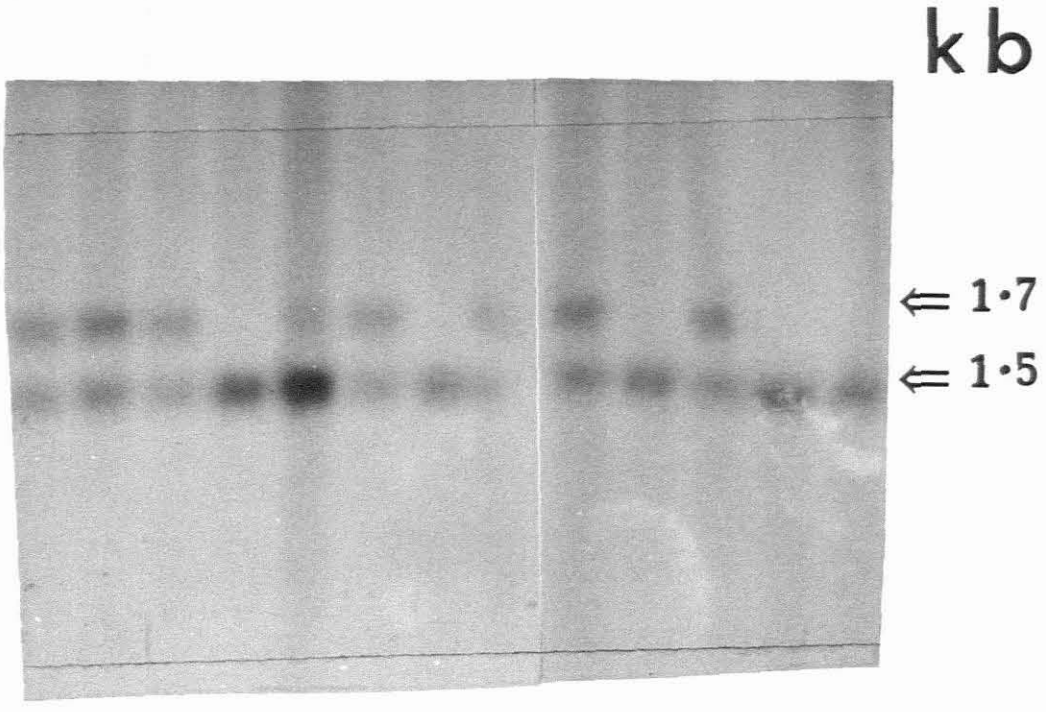


Plate 4.3

Southern Blot analysis of human DNA digested to completion with the restriction enzyme EcoRV and hybridised to the T cell receptor alpha-specific full length cDNA probe, Vα1.2.

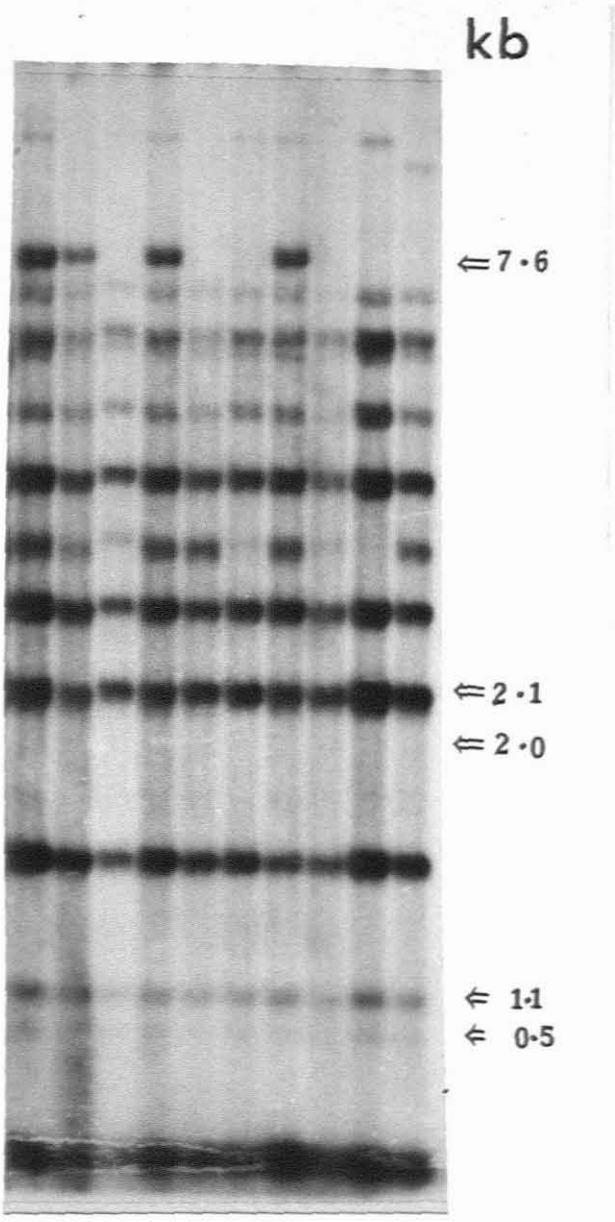


Plate 4.4

Southern Blot analysis of human DNA digested to completion with the restriction enzyme TaqI and hybridised to the T cell receptor alpha-specific full length cDNA probe, Vα1.2.

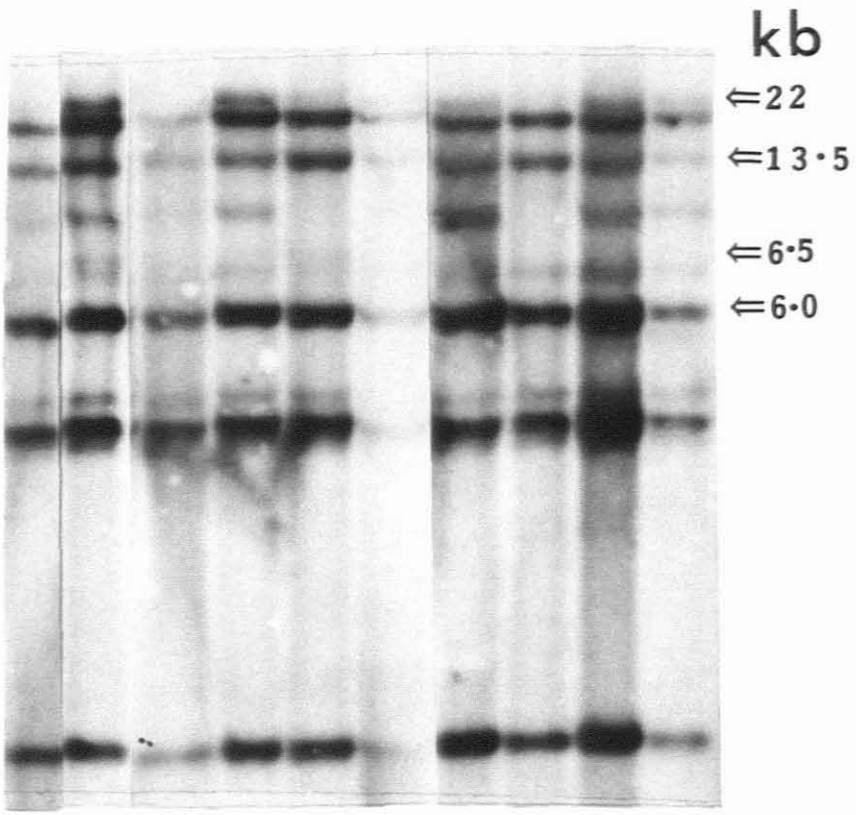


Plate 4.5

Southern Blot analysis of human DNA digested to completion with the restriction enzyme SstI and hybridised to the T cell receptor alpha-specific full length cDNA probe, Vα1.2.

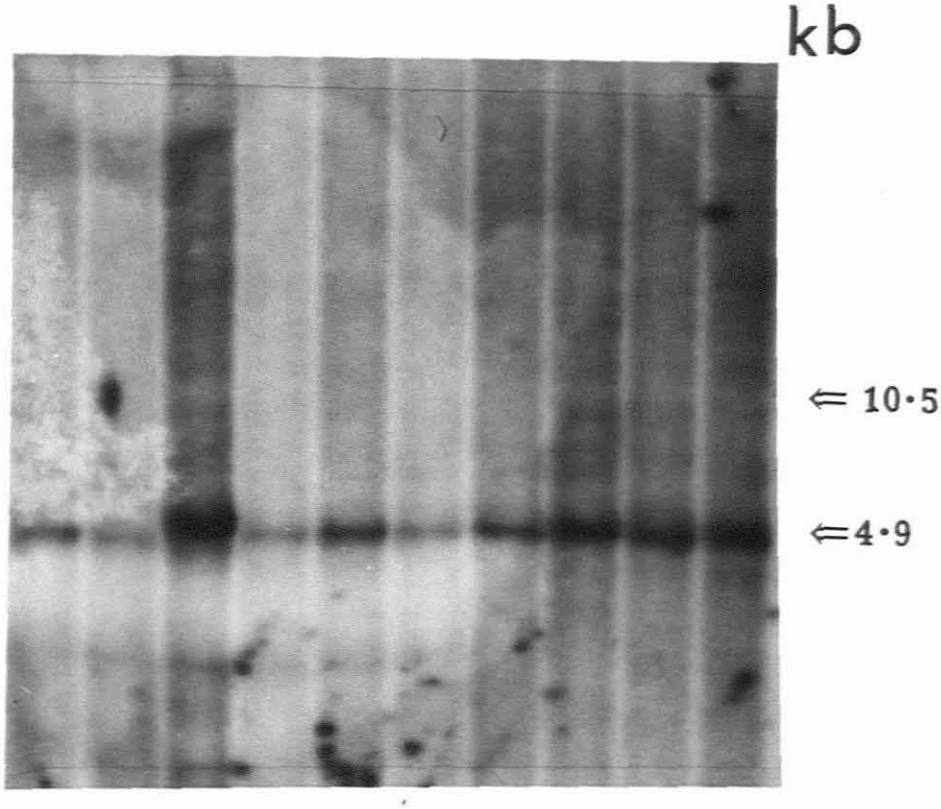


Plate 4.6

Southern Blot analysis of human DNA digested to completion with the restriction enzyme BglII and hybridised to the T cell receptor alpha-specific cDNA probe, Vα12.1.

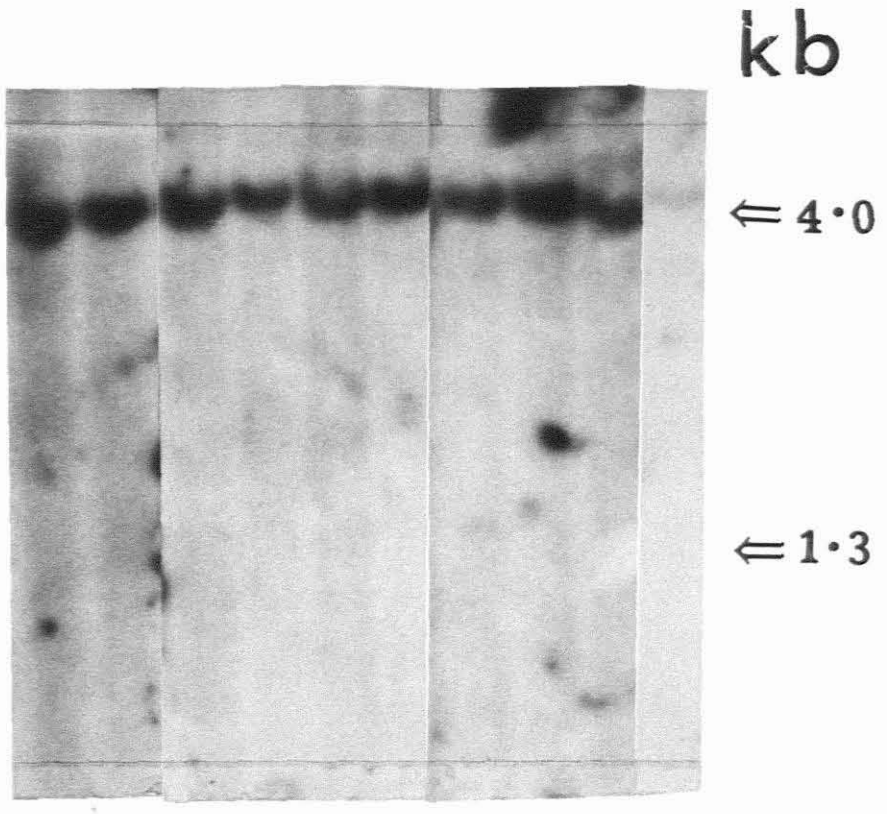


Plate 4.7

Southern Blot analysis of human DNA digested to completion with the restriction enzyme TaqI and hybridised to the T cell receptor alpha-specific cDNA probe, Vα12.1.

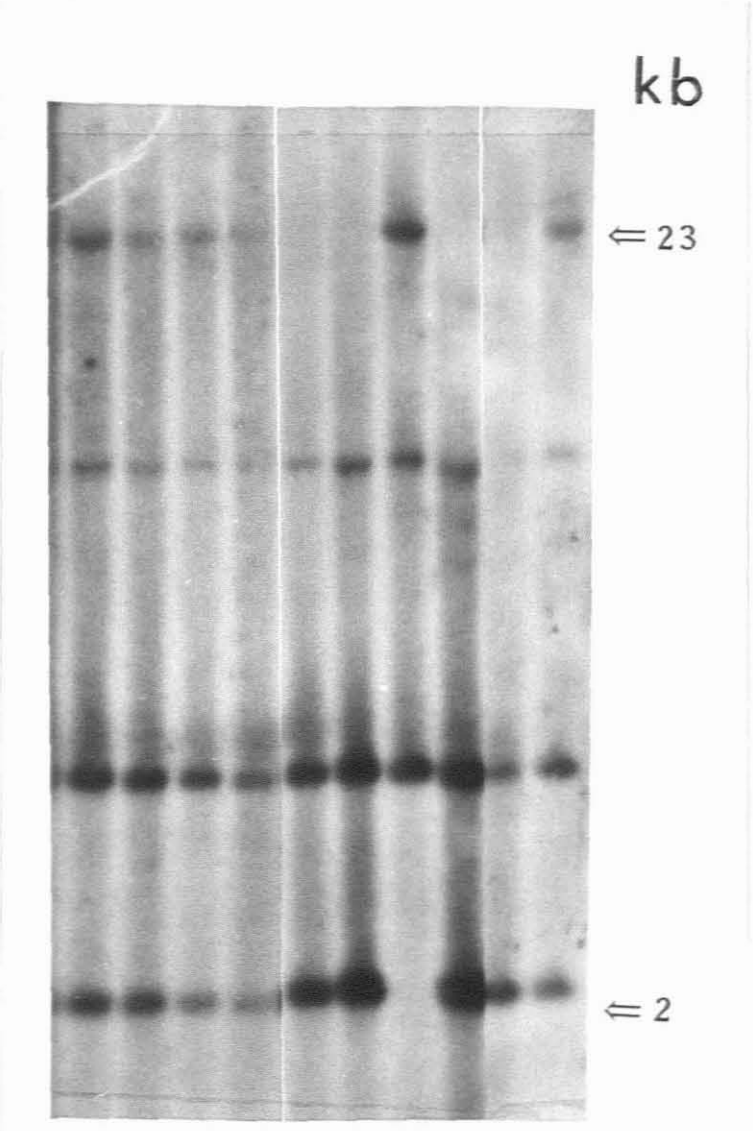


Plate 4.8

Southern Blot analysis of human DNA digested to completion with the restriction enzyme BamHI and hybridised to the T cell receptor beta-specific cDNA probe, Vß8.

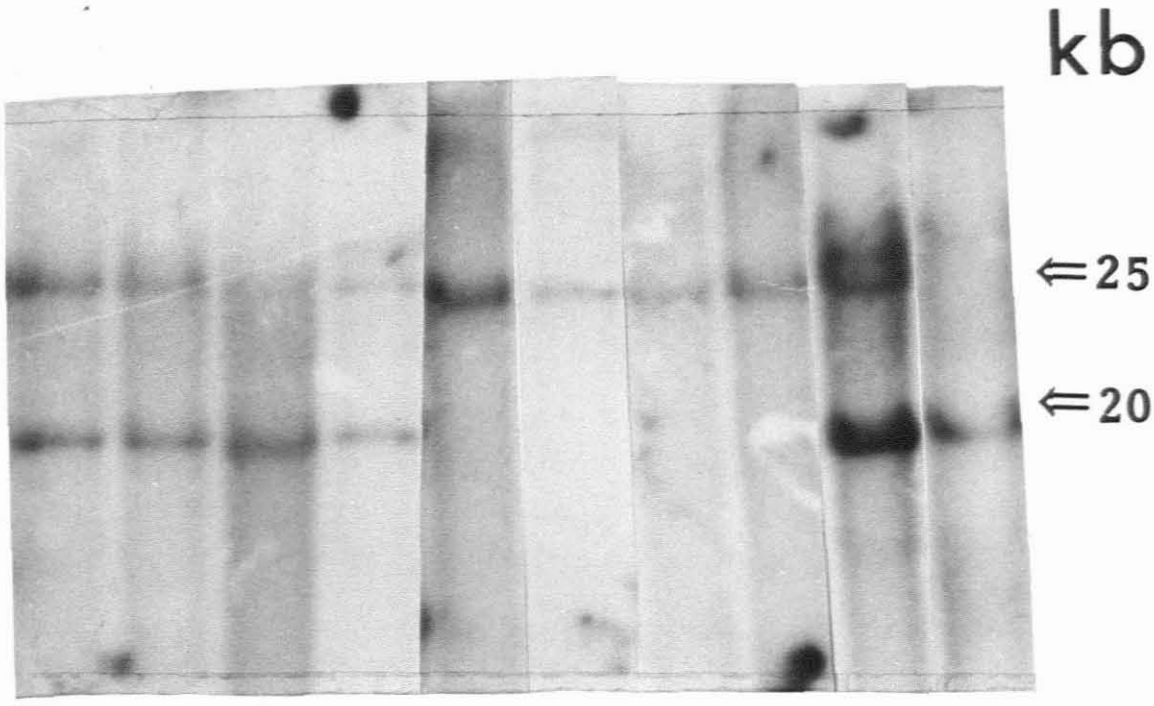


Plate 4.9

Southern Blot analysis of human DNA digested to completion with the restriction enzyme BamHI and hybridised to the T cell receptor beta-specific cDNA probe, Vß11.

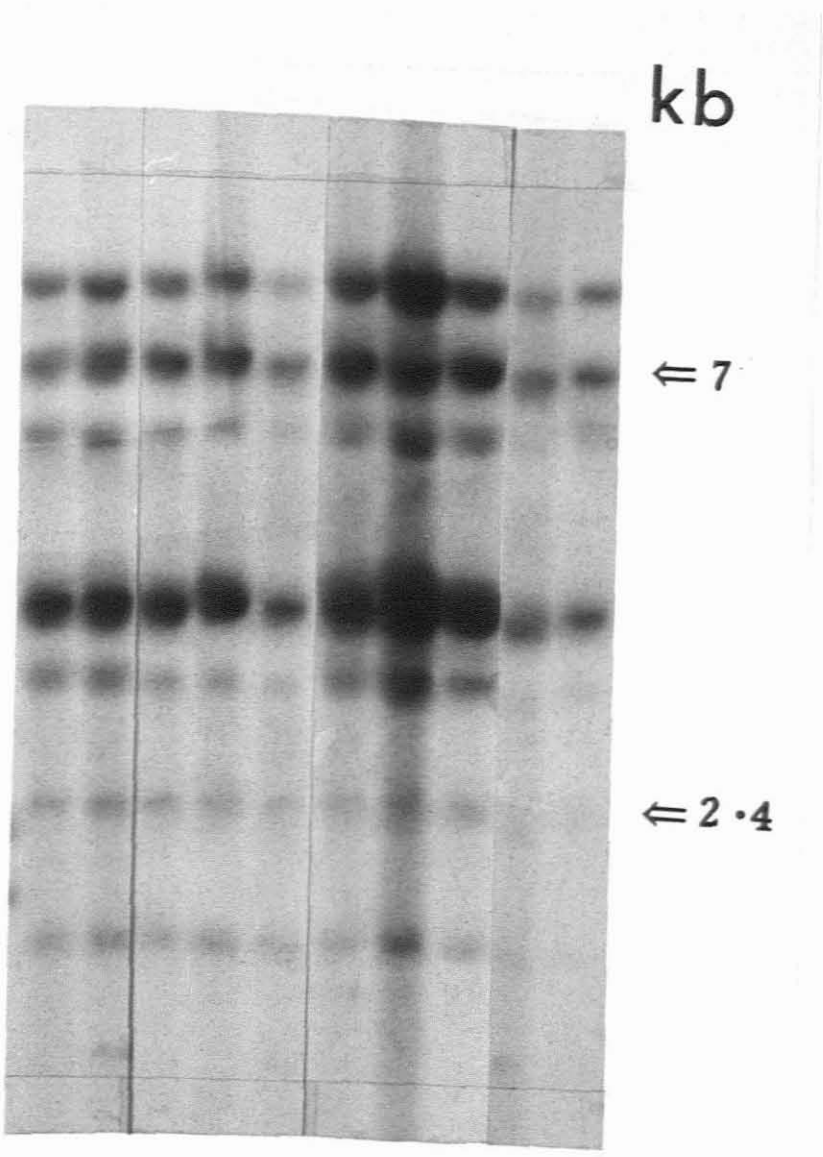


Plate 4.10

Southern Blot analysis of human DNA digested to completion with the restriction enzyme EcoRI and hybridised to the T cell receptor gamma-specific cDNA probe, pV3S.

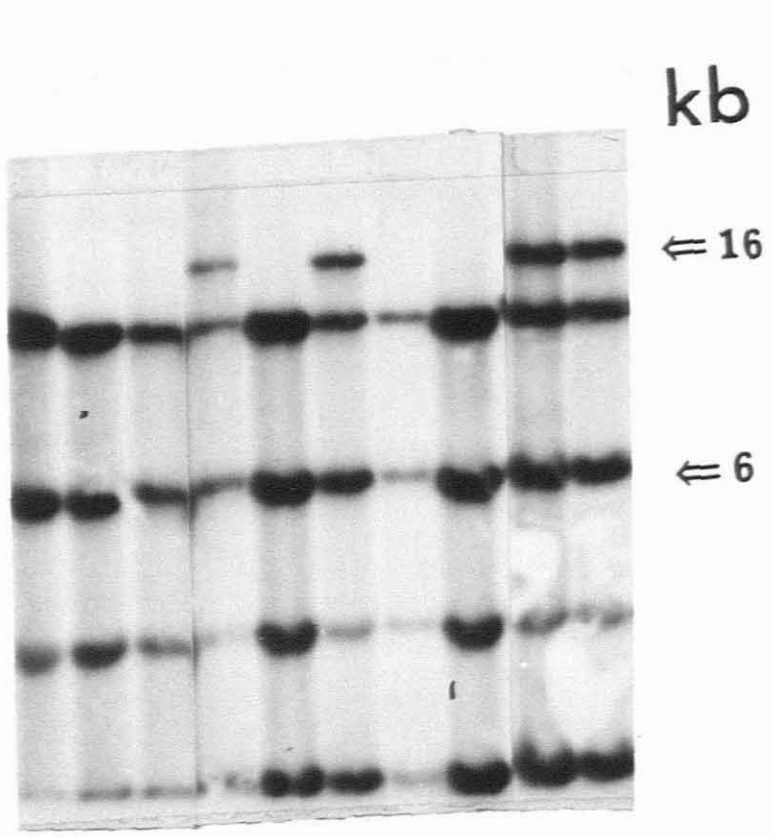


Plate 4.11

Southern Blot analysis of human DNA digested to completion with the restriction enzyme TaqI and hybridised to the T cell receptor gamma-specific cDNA probe, pV3S.

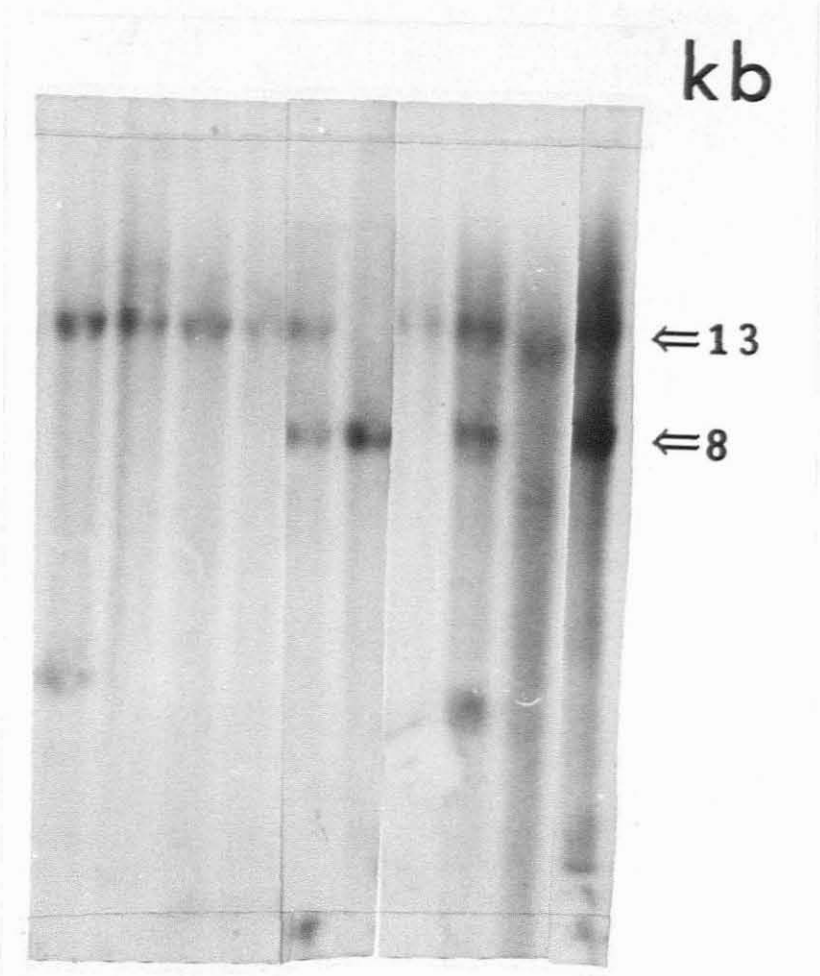


Plate 4.12

Southern Blot analysis of human DNA digested to completion with the restriction enzyme SstI and hybridised to the T cell receptor gamma-specific cDNA probe, pV11SPRS.

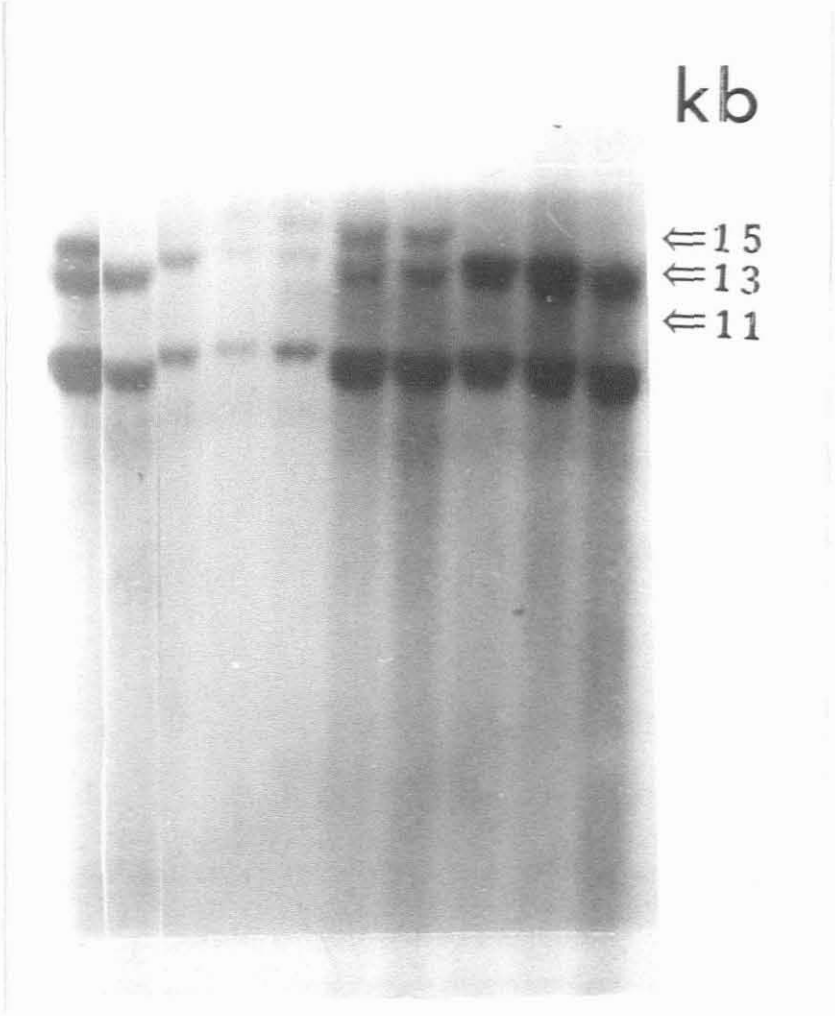


Plate 4.14

Southern Blot analysis of human DNA digested to completion with the restriction enzyme PvuII and hybridised to the T cell receptor gamma-specific cDNA probe, pCIR0.9.

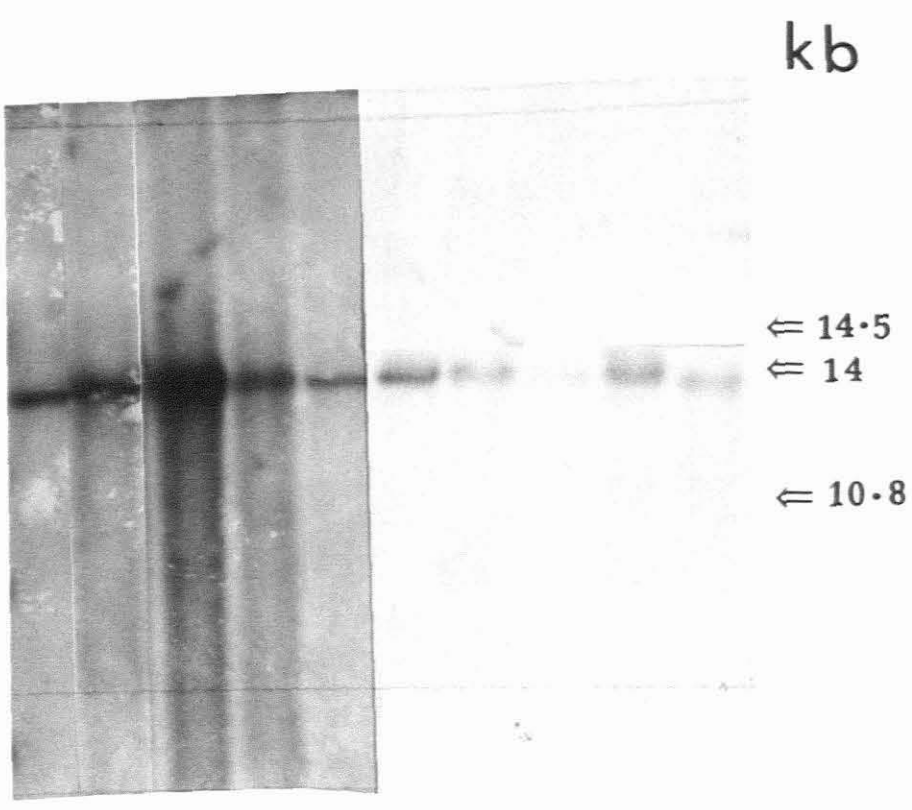


Plate 4.15

Southern Blot analysis of human DNA digested to completion with the restriction enzyme BamHI and hybridised to the T cell receptor delta-specific cDNA probe, Vδ V2SP0.5.

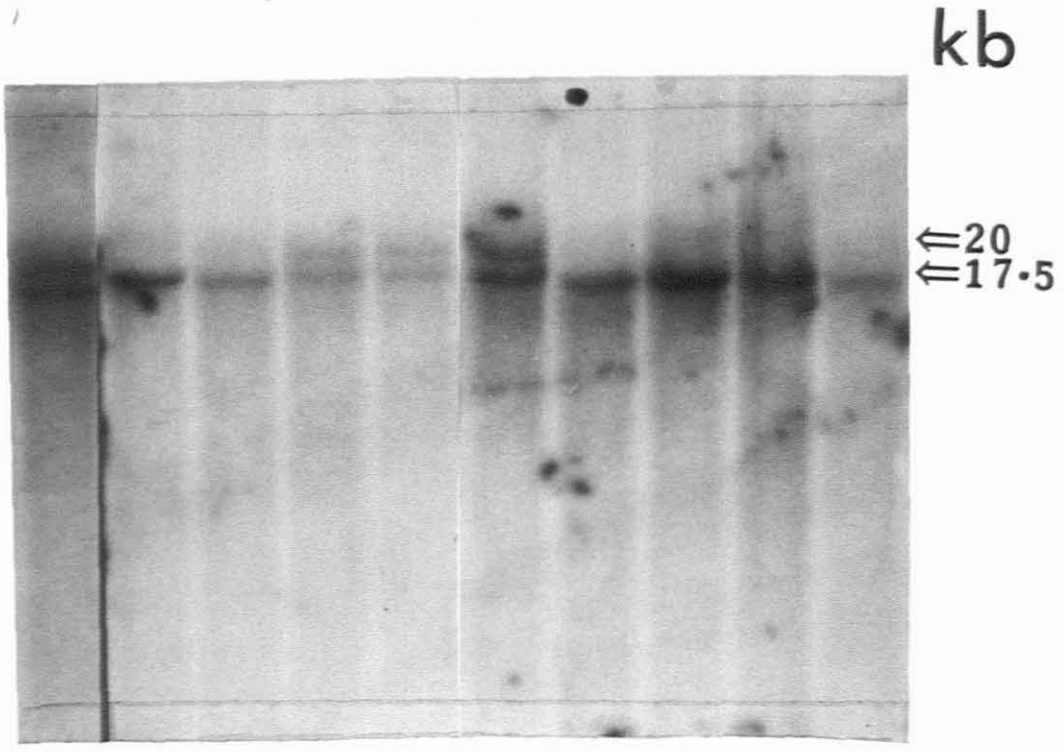


Plate 4.16

Southern Blot analysis of human DNA digested to completion with the restriction enzyme KpnI and hybridised to the T cell receptor delta-specific cDNA probe, R21XH.

4.2.2 Linkage analysis of T cell receptor genes and tuberculosis

Figures 4.7-4.34 show graphical representations of the results of linkage analysis of the T cell receptor genes to tuberculosis using the three pedigrees shown in Figures 3.1, 3.2 and 3.3. The restriction endonucleases and the probes used are noted on each figure. Lod scores are plotted versus the recombination frequency, theta. Linkage analysis was carried out by the likelihood method using the Liped computer software (Ott, 1985). Where Lod scores were equal to zero in all three models, no graphs are given.

Assumptions: The RFLP's studied were in linkage equilibrium and there was no sex or age difference in recombination fraction.

PEDIGREE 1
EcoRV/Va1.2 (ii)

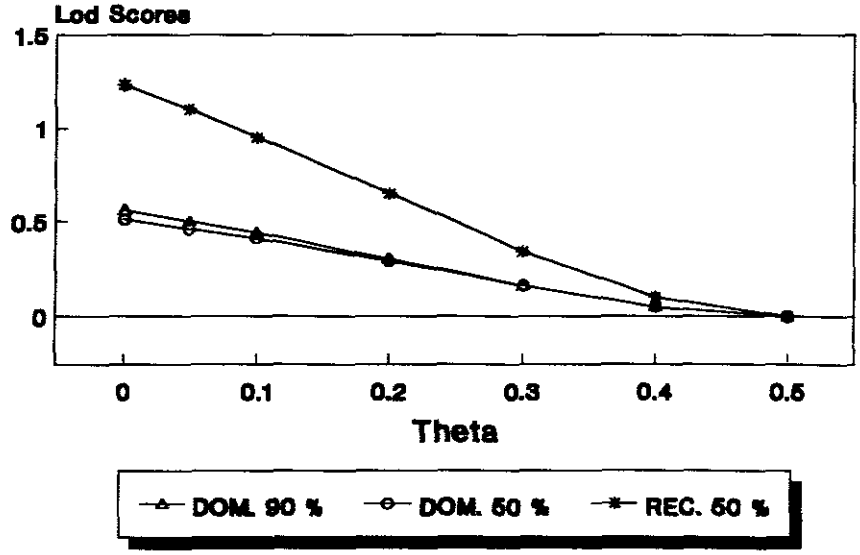


Figure 4.7

PEDIGREE 1
SstI/Va1.2 (ii)

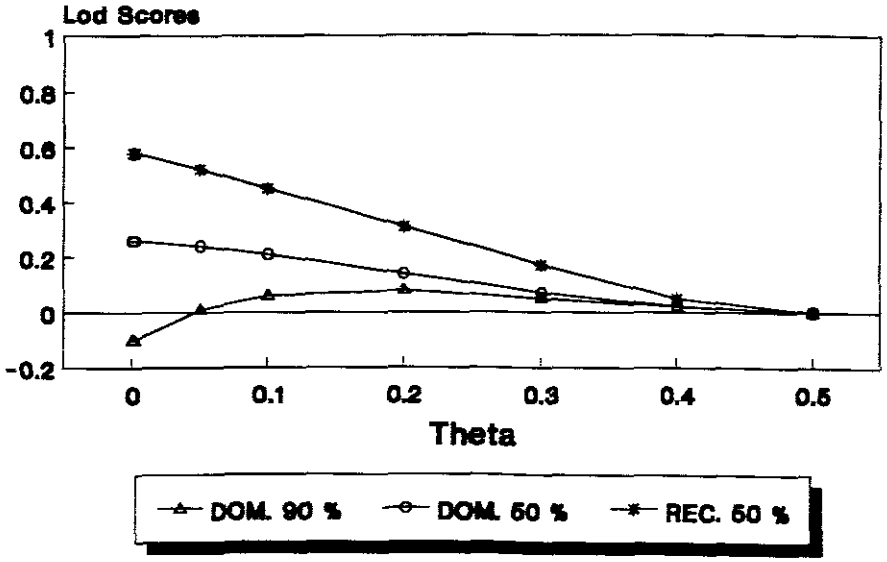


Figure 4.8

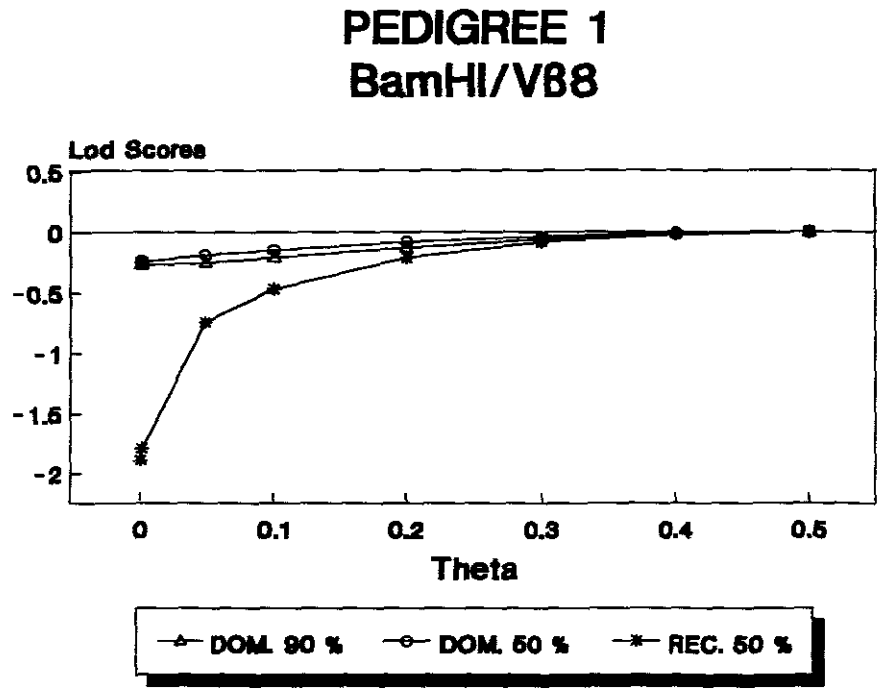


Figure 4.9

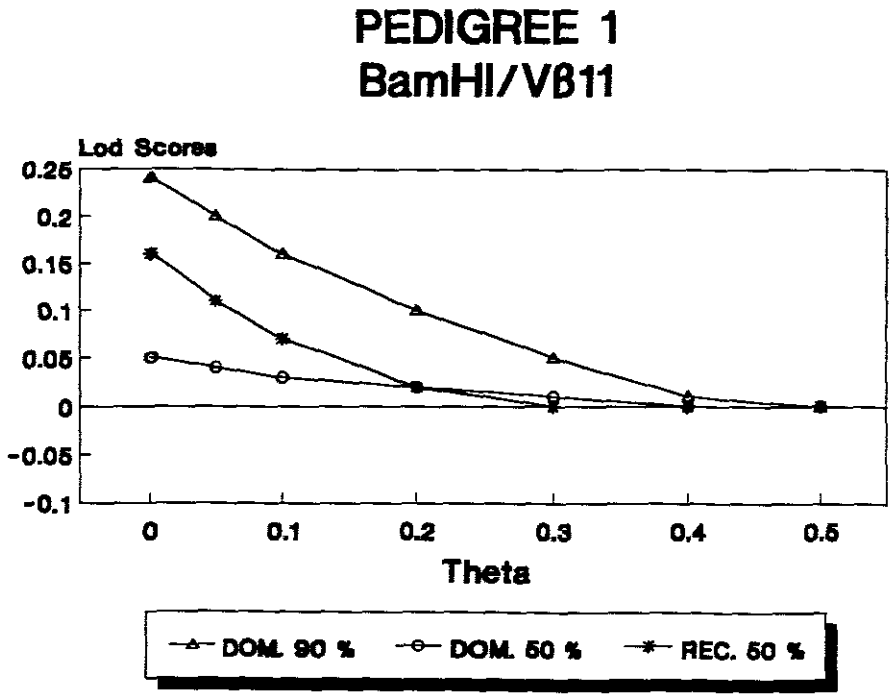


Figure 4.10

PEDIGREE 1
EcoRI/pV3S

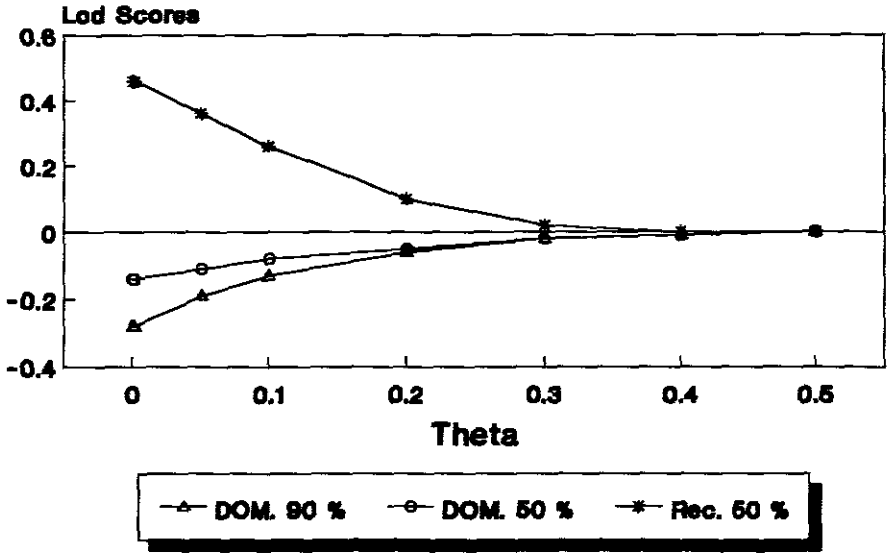


Figure 4.11

PEDIGREE 1
TaqI/pV3s

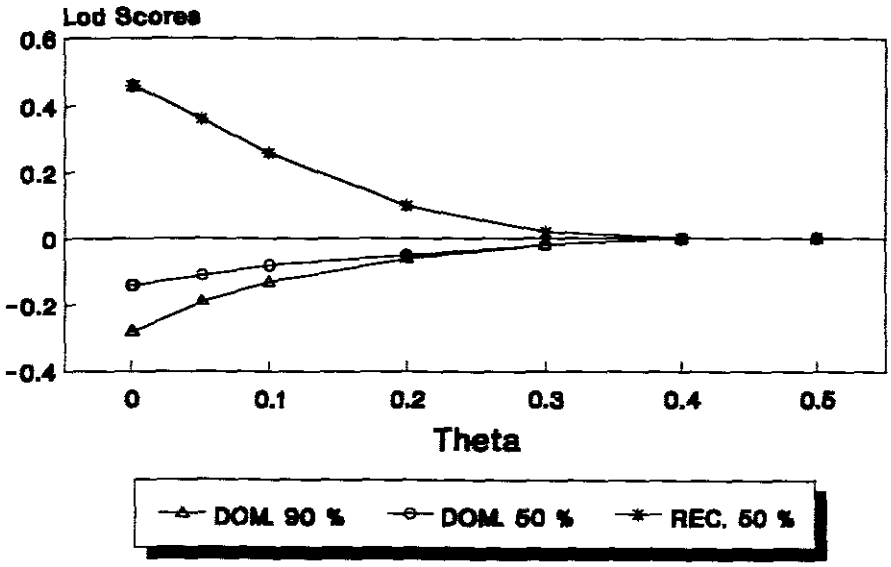


Figure 4.12

PEDIGREE 1
TaqI/pV11SPRS

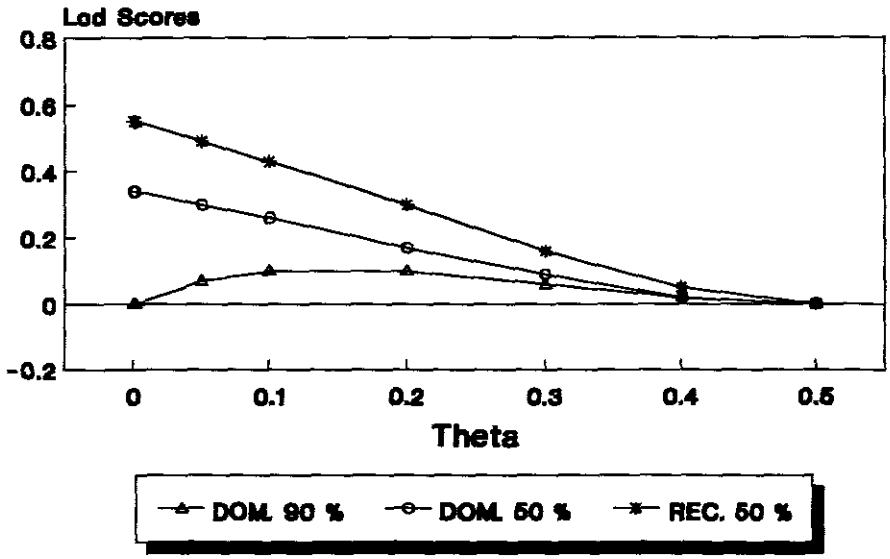


Figure 4.13

PEDIGREE 1
PvuII/pCIR0.9

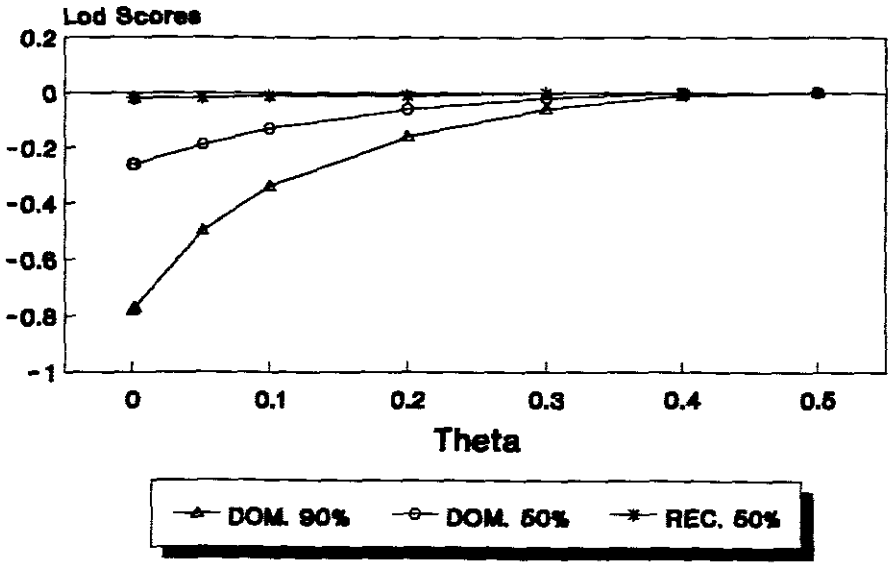


Figure 4.14

PEDIGREE 2
EcoRV/Va1.2 (ii)

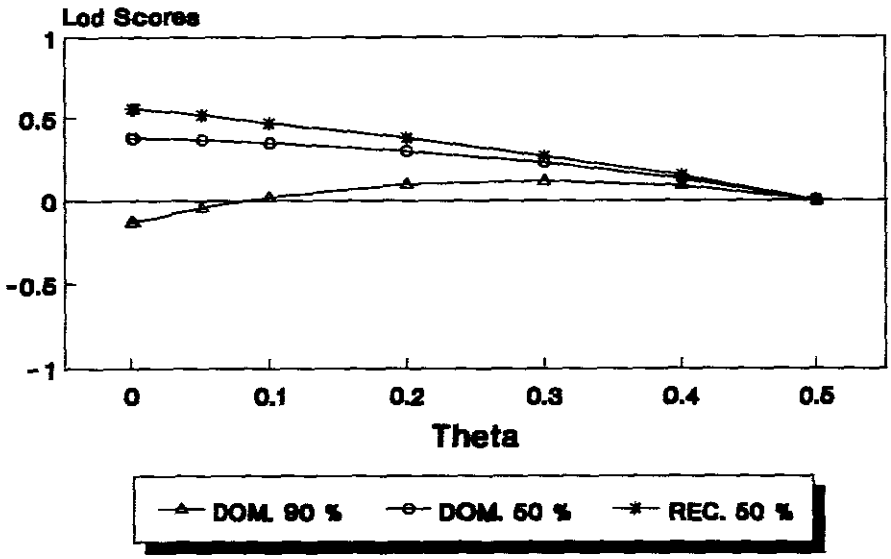


Figure 4.15

PEDIGREE 2
TaqI/Va1.2 (i)

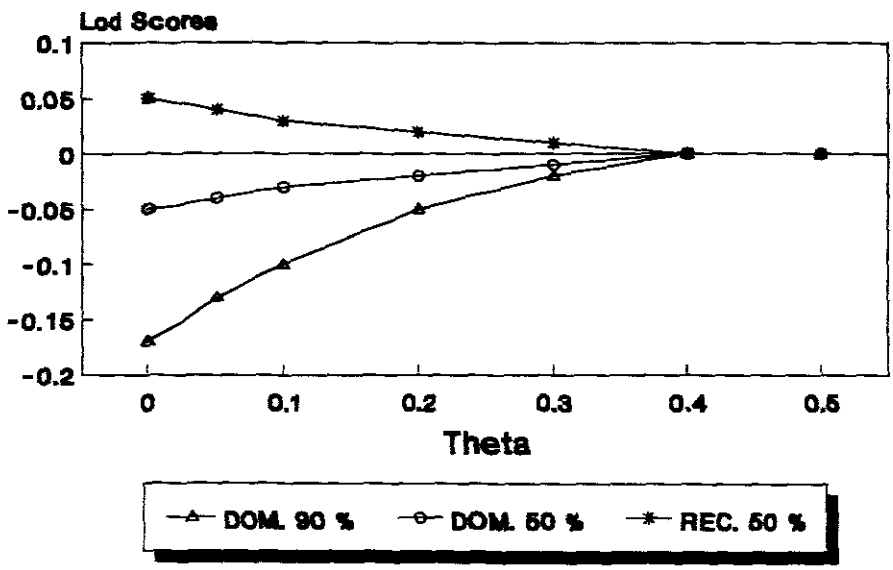


Figure 4.16

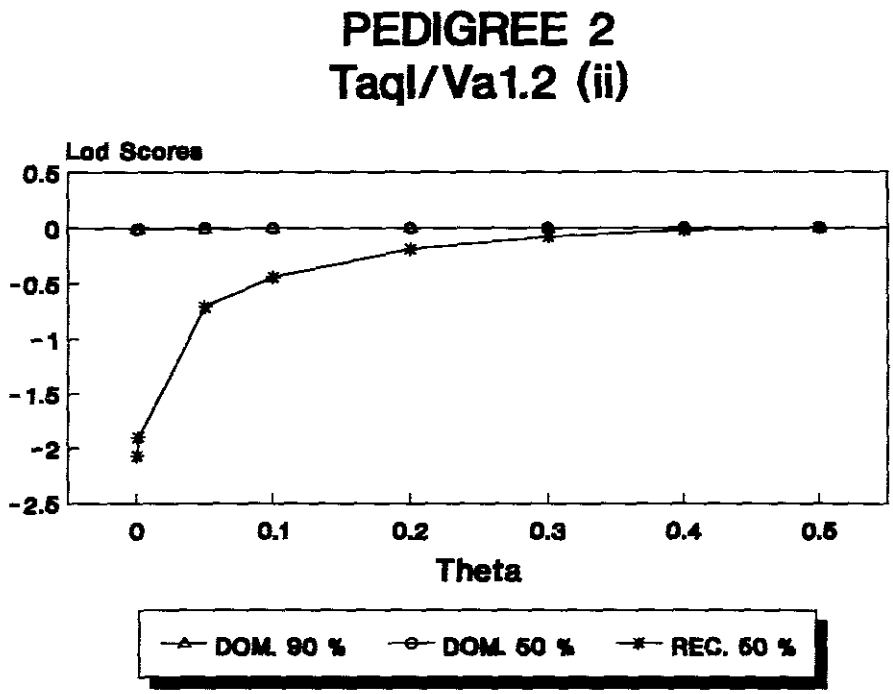


Figure 4.17

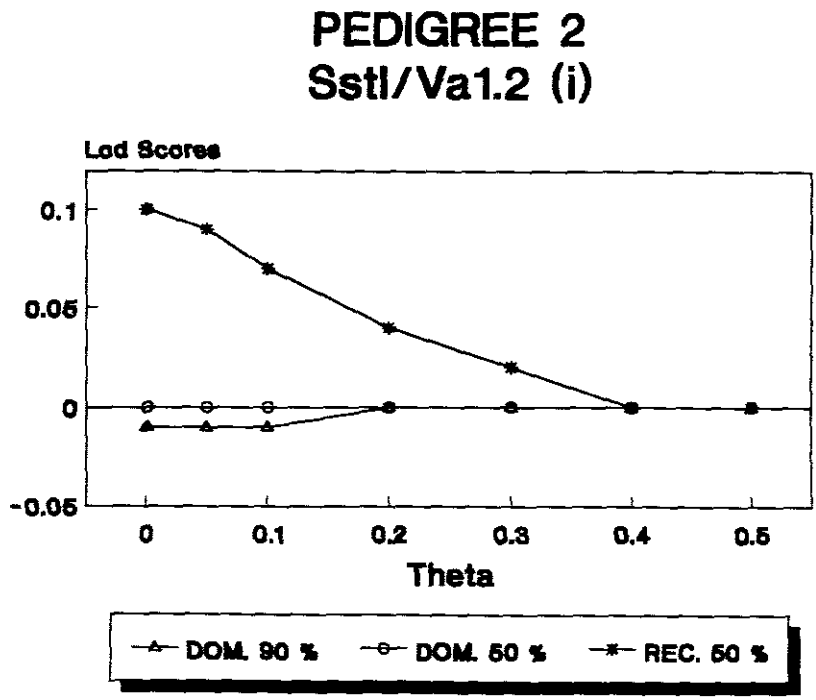


Figure 4.18

PEDIGREE 2
SstI/Va1.2 (ii)

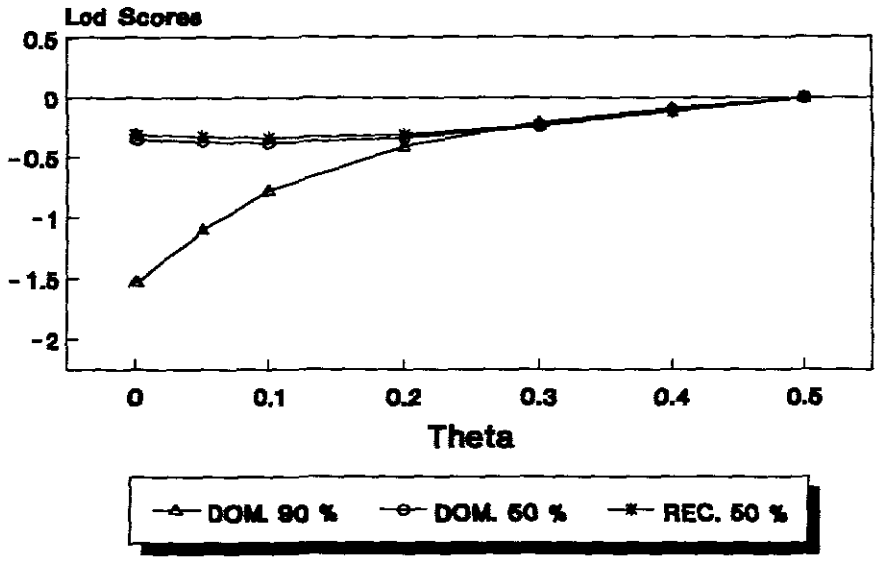


Figure 4.19

PEDIGREE 2
BglII/Va12.1

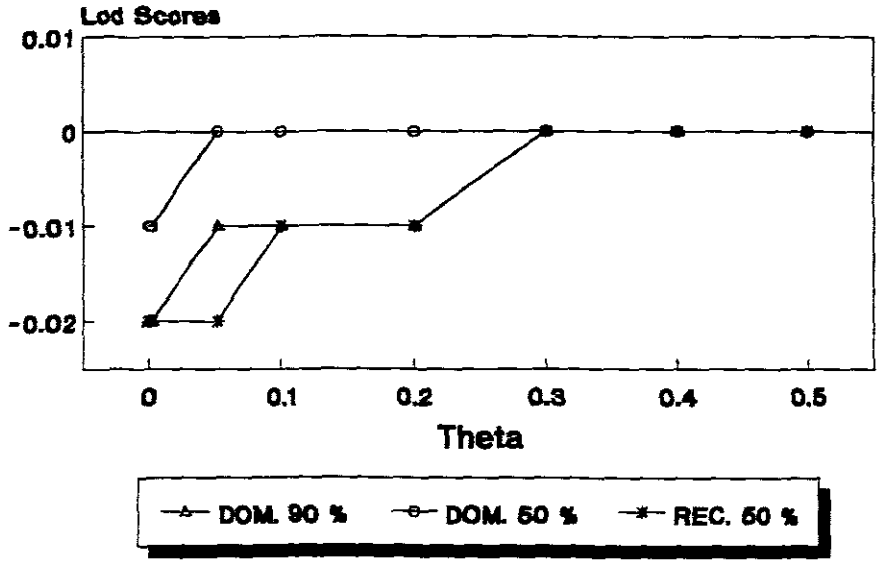


Figure 4.20

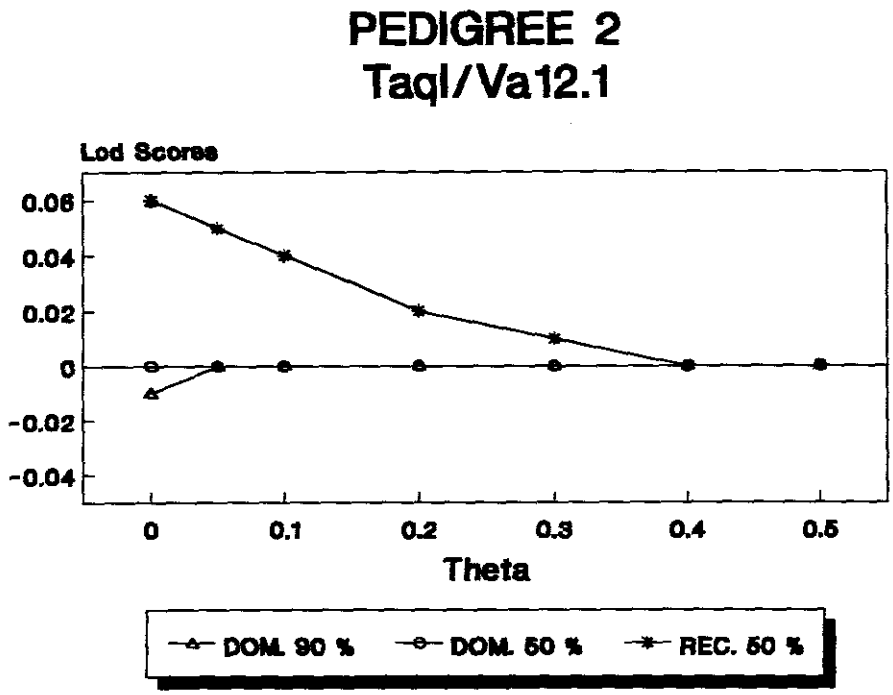


Figure 4.21

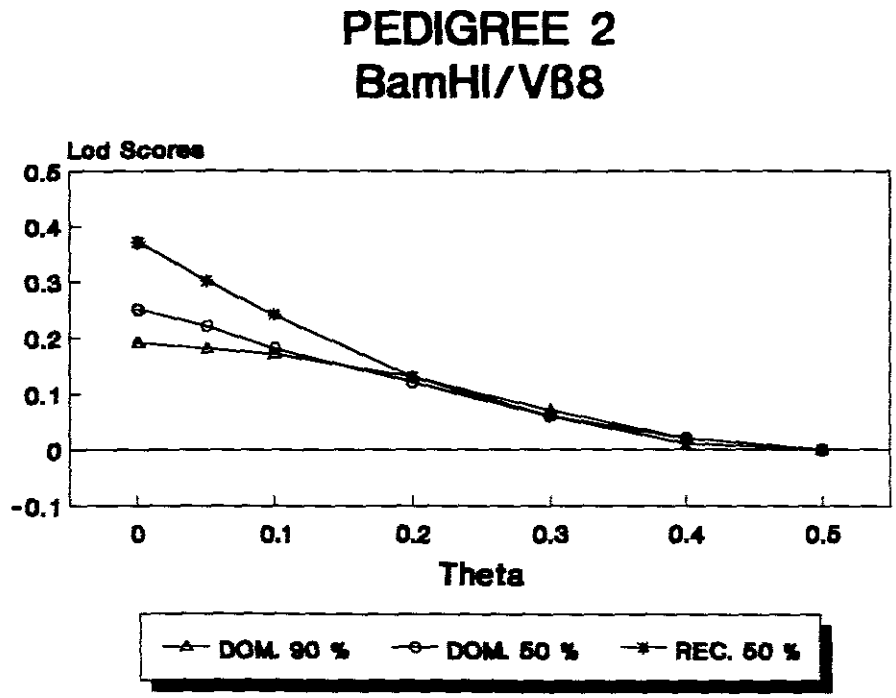


Figure 4.22

PEDIGREE 2
BamHI/Vβ11

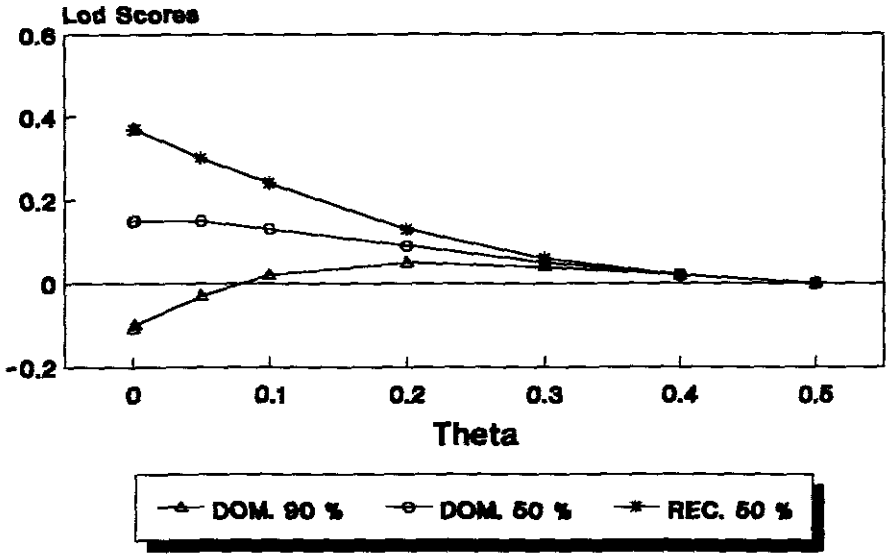


Figure 4.23

PEDIGREE 2
EcoRI/pV3s

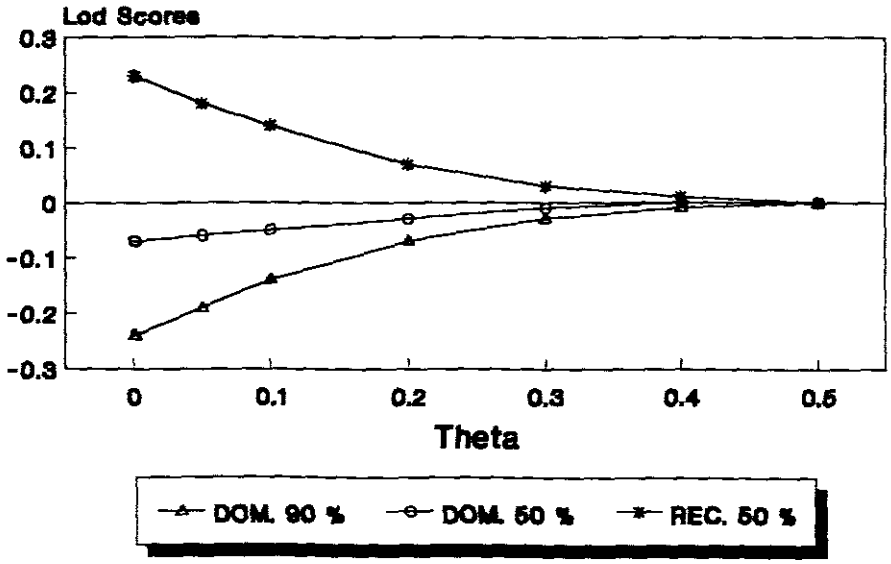


Figure 4.24

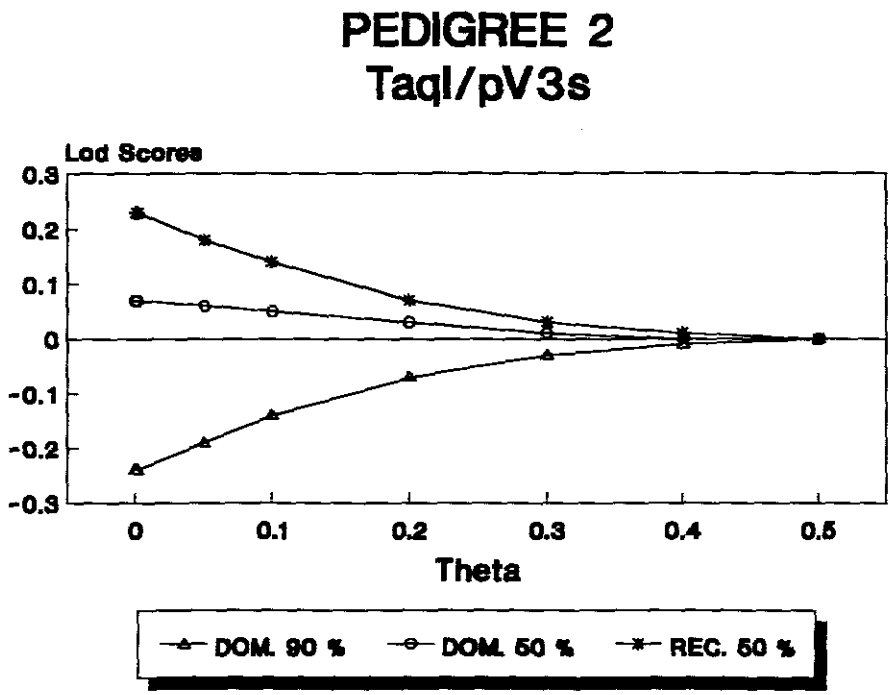


Figure 4.25

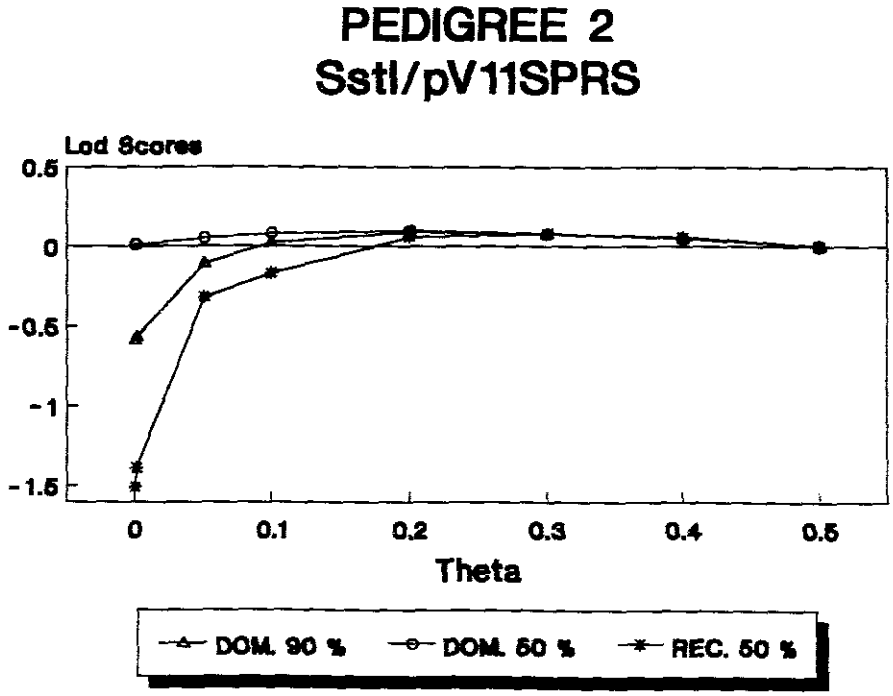


Figure 4.26

PEDIGREE 2
TaqI/pV11SPRS

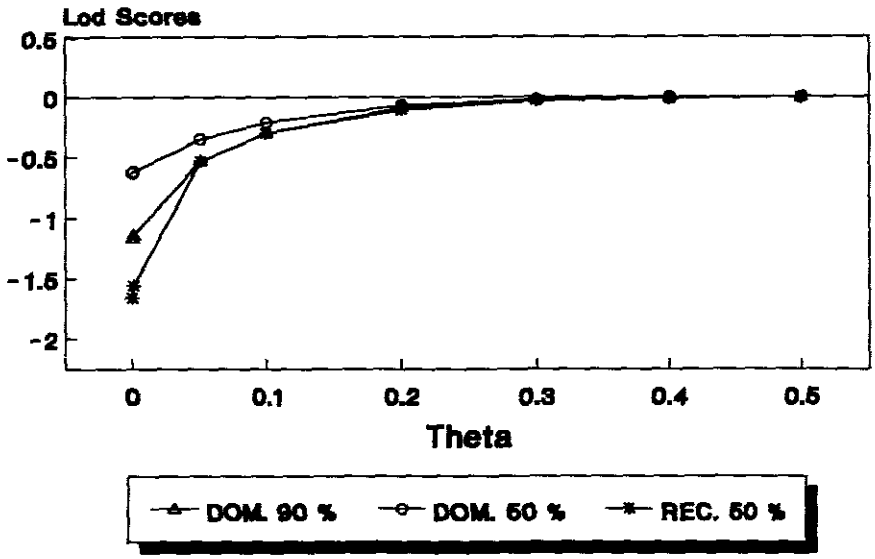


Figure 4.27

PEDIGREE 2
PvuII

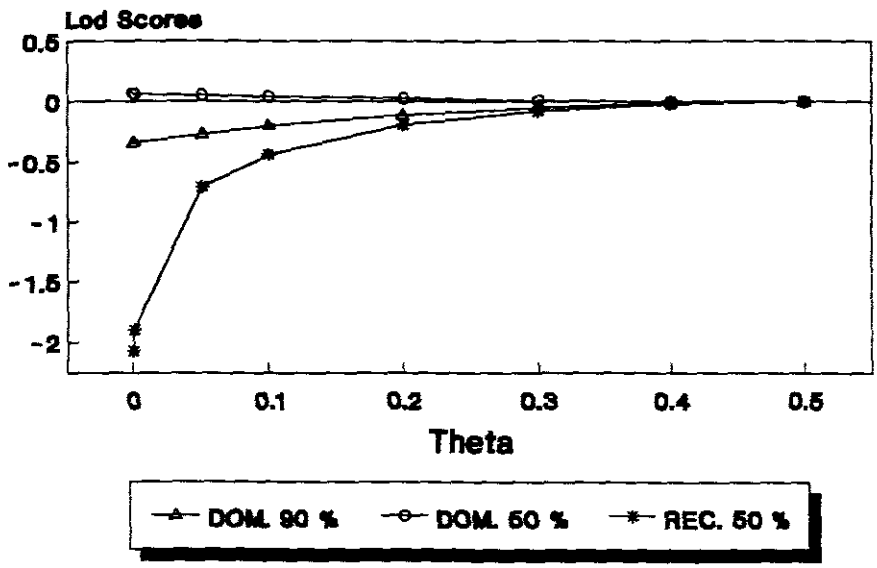


Figure 4.28

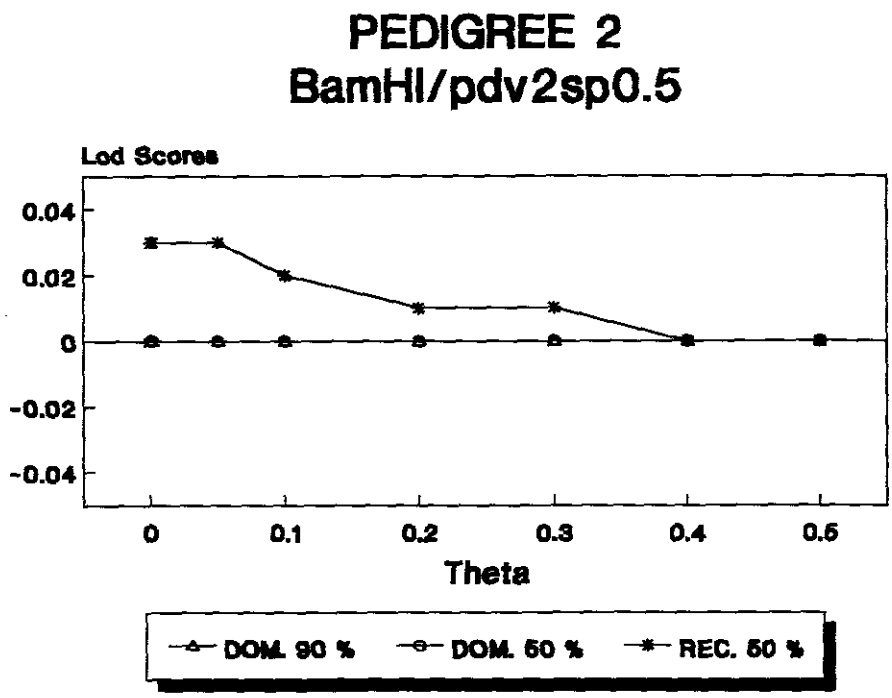


Figure 4.29

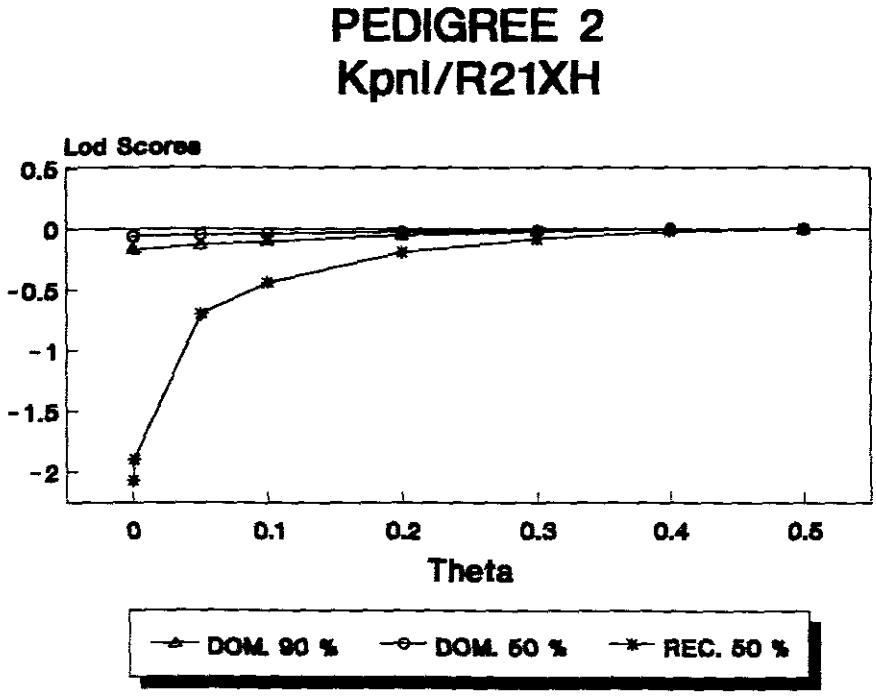


Figure 4.30

PEDIGREE 3
EcoRV/Va1.2 (ii)

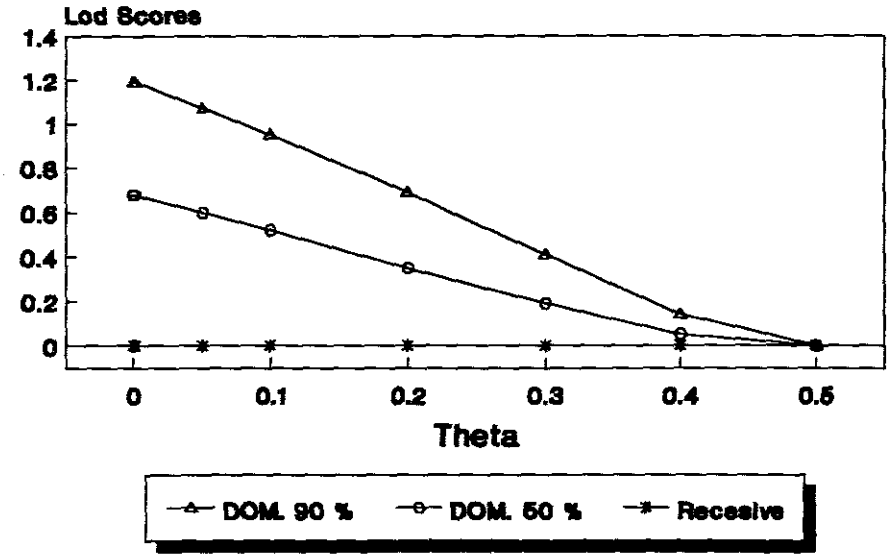


Figure 4.31

PEDIGREE 3
TaqI/Va1.2 (i)

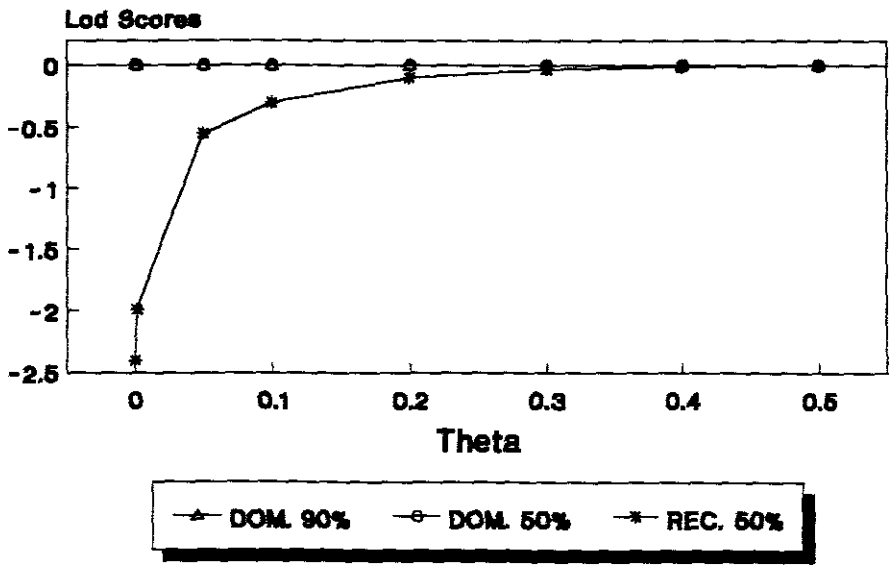


Figure 4.32

PEDIGREE 3
BamHI/vβII

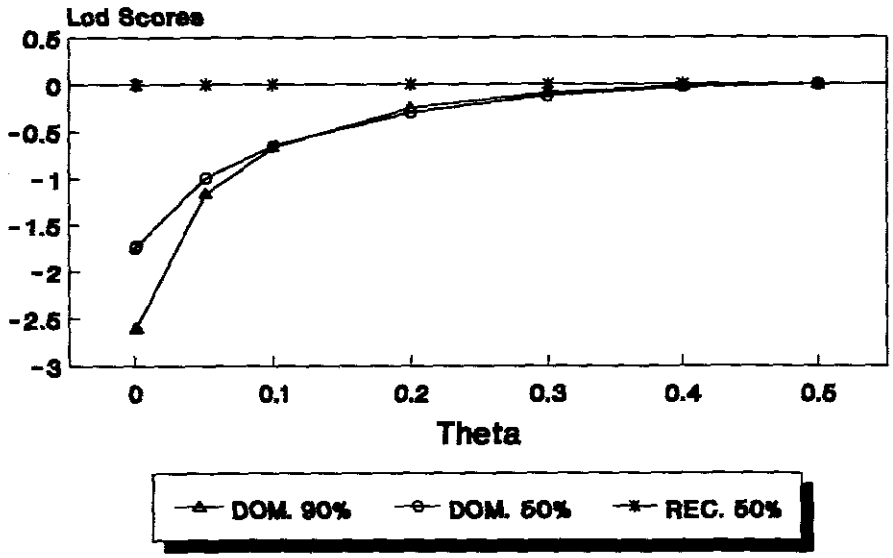


Figure 4.33

PEDIGREE 3
TaqI/pV11spRS

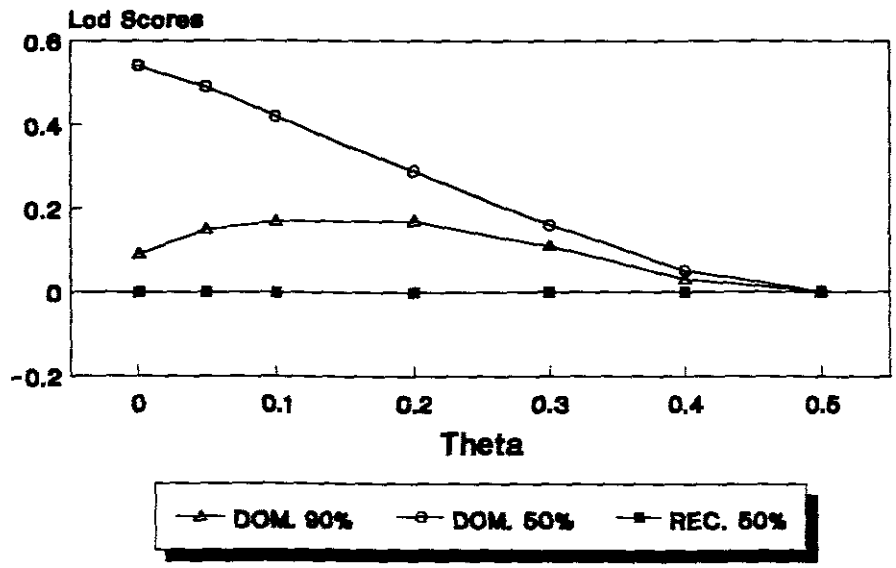


Figure 4.34

4.2.2.1 Pedigree 1

The Lod scores obtained from the linkage analysis of the T cell receptor alpha, beta, gamma and delta genes as described fell between -2 and +3 at a recombination fraction of 0 and tended to 0 at a recombination fraction of 0.5 using the inheritance models dominant with 90% penetrance, dominant with 50% penetrance and recessive with 50% penetrance (See Figures 4.7-4.14). Linkage of disease susceptibility to tuberculosis with T cell receptor genes could not therefore be accepted or excluded as all the Lod scores fell within the indeterminate range.

4.2.2.2 Pedigree 2

The Lod scores obtained from the linkage analysis of the T cell receptor genes in the alpha, beta, gamma and delta genes as described fell between -2 and +3 at a recombination fraction of 0 and tended to 0 at a recombination fraction of 0.5 using the inheritance models dominant with 90% penetrance and dominant with 50% penetrance. However, tight linkage of disease susceptibility to tuberculosis with T cell receptor genes could be excluded under the recessive model with 50% penetrance with the alleles generated using the TaqI/α2(ii), PvuII/γ4 and KpnI/δ2 enzyme/probe combinations as the Lod scores obtained were -2.07, -2.07 and -2.07 respectively at a recombination fraction of $\theta = 0$. However, the Lod scores fell within the indeterminate range as the recombination fraction increased to 0.001 (See Figures 4.15-4.30).

4.2.2.3 Pedigree 3

The Lod scores obtained from the linkage analysis of the T cell receptor genes in the alpha, beta, gamma and delta genes as described fell between -2 and +3 at a recombination fraction of 0 and tended to 0 as the recombination fraction increased to 0.5 using the dominant model with 50% penetrance. Tight linkage between susceptibility to tuberculosis with T cell receptor genes could be excluded under the dominant model with

90% penetrance with the alleles generated using the BamHI/ß2 enzyme/probe combination as the Lod score obtained was -2.61 and -2.60 at a recombination fraction of 0 and 0.001 respectively. Under the recessive model with 50% penetrance, tight linkage could be excluded with the alleles generated by the TaqI/α2(i) enzyme/probe combination as the Lod score was -2.4 at a recombination fraction of 0. However as the recombination fraction increased to 0.5, the Lod scores fell within the indeterminate range (See Figures 4.31-4.34).

4.2.2.4 Cumulative Lod scores

Lod scores were added across pedigrees to produce cumulative Lod scores for each T cell receptor gene and the Lod scores obtained under the dominant model with 50% penetrance fell between -2 and +3 at a recombination fraction of 0 and tended to 0 as the recombination fraction increased to 0.5 (See Tables 4.5, 4.6, 4.7 and 4.8). However, tight linkage could be excluded under the dominant model with 90% penetrance with the allele generated using the BamHI/ß2 enzyme/probe combination as the Lod scores obtained were -2.48 and -2.46 at a recombination fraction of 0 and 0.001 respectively. However as the recombination fraction increased to 0.5, the Lod scores fell within the indeterminate range (See Tables 4.9, 4.10, 4.11 and 4.12). Tight linkage could also be excluded under the recessive model with 50% penetrance with the alleles generated by the TaqI/α2(i), TaqI/α2(ii) and KpnI/δ2 enzyme/probe combination as the Lod scores were -2.38, -2.08 and -2.08 respectively at a recombination fraction of $\theta = 0$. However as the recombination fraction increased to 0.001, the Lod scores fell within the indeterminate range (See Tables 4.13, 4.14, 4.15 and 4.16). It was interesting to note that the Lod scores obtained for the allele generated by the EcoRV/α2 (ii) enzyme/probe combination were > 1.5 at recombination fractions of 0 and 0.001 for all three models of inheritance as shown in Tables 4.5, 4.9 and 4.13.

KEY FOR TABLES 4.5-4.16:

α2 = Vα1.2

α3 = Vα12.1

ß1 = Vß8

ß2 = Vß11

γ1 = pV3S

γ2 = pV11SPRS

γ4 = pCIR0.9

δ1 = pδV2sp0.5

δ2 = R21XH

Table 4.5

Table showing cumulative Lod scores obtained for the Alpha gene under the dominant model with 90% penetrance.

GENETIC MODEL: DOMINANT 90% PENETRANCE

Alpha gene Lod scores at θ =

	0.0	0.001	0.05	0.1	0.2	0.3	0.4	0.5
EcoRV/α2 (i)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
EcoRV/α2 (ii)	1.62	1.62	1.53	1.42	1.09	0.69	0.28	0.0
TaqI/α2 (i)	-0.17	-0.17	-0.13	-0.10	-0.05	-0.02	0.0	0.0
TaqI/α2 (ii)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
SstI/α2 (i)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
SstI/α2 (ii)	-1.63	-1.63	-1.09	-0.72	-0.33	-0.16	-0.07	0.0
BglII/α3	-0.03	-0.03	-0.02	-0.02	-0.01	0.0	0.0	0.0
TaqI/α3	-0.01	-0.01	0.0	0.0	0.0	0.0	0.0	0.0

Table 4.6

Table showing cumulative Lod scores obtained for the Beta gene under the dominant model with 90% penetrance.

GENETIC MODEL: DOMINANT 90% PENETRANCE

Beta gene Lod scores at θ =

	0.0	0.001	0.05	0.1	0.2	0.3	0.4	0.5
BamHI/ß1	-0.08	-0.08	-0.06	-0.04	0.0	0.01	0.01	0.0
BamHI/ß2	-2.48	-2.46	-0.99	-0.49	-0.01	0.0	0.18	0.0

Table 4.7

Table showing cumulative Lod scores obtained for the Gamma gene under the dominant model with 90% penetrance.

GENETIC MODEL: DOMINANT 90% PENETRANCE

Gamma gene Lod scores at θ =

	0.0	0.001	0.05	0.1	0.2	0.3	0.4	0.5
EcoRI/γ1	-0.52	-0.52	-0.37	-0.27	-0.13	-0.05	-0.02	0.0
TaqI/γ1	-0.52	-0.52	-0.37	-0.27	-0.13	-0.05	-0.02	0.0
SstI/γ2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
TaqI/γ2	-1.05	-1.05	-0.38	-0.03	0.13	0.15	0.05	0.0
PvuII/γ4	-1.13	-1.12	-0.78	-0.55	-0.28	-0.11	-0.02	0.0

Table 4.8

Table showing cumulative Lod scores obtained for the Delta gene under the dominant model with 90% penetrance.

GENETIC MODEL: DOMINANT 90% PENETRANCE

Delta gene Lod scores at θ =

	0.0	0.001	0.05	0.1	0.2	0.3	0.4	0.5
BamHI/δ1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
KpnI/δ2	-0.17	-0.17	-0.13	-0.1	-0.05	-0.02	0.0	0.0

Table 4.9

Table showing cumulative Lod scores obtained for the Alpha gene under the dominant model with 50% penetrance.

GENETIC MODEL: DOMINANT 50% PENETRANCE

Alpha gene Lod scores at θ =

	0.0	0.001	0.05	0.1	0.2	0.3	0.4	0.5
EcoRV/α2 (i)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
EcoRV/α2 (ii)	1.57	1.57	1.43	1.27	0.94	0.58	0.23	0.0
TaqI/α2 (i)	-0.05	-0.05	-0.04	-0.03	-0.02	-0.01	-0.0	0.0
TaqI/α2 (ii)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
SstI/α2 (i)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
SstI/α2 (ii)	-0.09	-0.09	-0.13	-0.17	-0.20	-0.17	-0.09	0.0
BglII/α3	0.0	0.0	0.01	0.01	0.0	0.0	0.0	0.0
TaqI/α3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Table 4.10

Table showing cumulative Lod scores obtained for the Beta gene under the dominant model with 50% penetrance.

GENETIC MODEL: DOMINANT 50% PENETRANCE

Beta gene Lod scores at θ =

	0.0	0.001	0.05	0.1	0.2	0.3	0.4	0.5
BamHI/ß1	0.0	0.01	0.02	0.03	0.04	0.02	0.01	0.0
BamHI/ß2	-1.55	-1.52	-0.80	-0.49	-0.19	-0.06	-0.01	0.0

Table 4.11

Table showing cumulative Lod scores obtained for the Gamma gene under the dominant model with 50% penetrance.

GENETIC MODEL: DOMINANT 50% PENETRANCE

Gamma gene Lod scores at θ =

	0.0	0.001	0.05	0.1	0.2	0.3	0.4	0.5
EcoRI/γ1	-0.07	-0.07	-0.04	-0.03	-0.02	-0.01	0.0	0.0
TaqI/γ1	-0.07	-0.07	-0.05	-0.03	-0.03	-0.01	0.0	0.0
SstI/γ2	0.01	0.01	0.05	0.08	0.10	0.09	0.05	0.0
TaqI/γ2	0.95	0.95	0.84	0.73	0.49	0.26	0.07	0.0
PvuII/γ4	-0.19	-0.19	-0.14	-0.09	-0.03	-0.01	0.0	0.0

Table 4.12

Table showing cumulative Lod scores obtained for the Delta gene under the dominant model with 50% penetrance.

GENETIC MODEL: DOMINANT 50% PENETRANCE

Delta gene Lod scores at θ =

	0.0	0.001	0.05	0.1	0.2	0.3	0.4	0.5
BamHI/δ1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
KpnI/δ2	-0.06	-0.06	-0.05	-0.04	-0.02	-0.01	0.0	0.0

Table 4.13

Table showing cumulative Lod scores obtained for the Alpha gene under the recessive model with 50% penetrance.

GENETIC MODEL: RECESSIVE 50% PENETRANCE

Alpha gene Lod scores at θ =

	0.0	0.001	0.05	0.1	0.2	0.3	0.4	0.5
EcoRV/α2 (i)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
EcoRV/α2 (ii)	1.79	1.79	1.62	1.42	1.03	0.61	0.25	0.0
TaqI/α2 (i)	-2.38	-1.97	-0.53	-0.29	-0.10	-0.02	-0.01	0.0
TaqI/α2 (ii)	-2.08	-1.91	-0.72	-0.45	-0.19	-0.07	-0.02	0.0
SstI/α2 (i)	0.08	0.08	0.07	0.06	0.03	0.02	0.0	0.0
SstI/α2 (ii)	0.27	0.27	0.19	0.11	0.0	-0.05	-0.06	0.0
BglII/α3	-0.06	-0.06	-0.05	-0.03	-0.02	-0.01	0.0	0.0
TaqI/α3	0.02	0.02	0.02	0.02	0.01	0.0	0.0	0.0

Table 4.14

Table showing cumulative Lod scores obtained for the Beta gene under the recessive model with 50% penetrance.

GENETIC MODEL: RECESSIVE 50% PENETRANCE

Beta gene Lod scores at θ =

	0.0	0.001	0.05	0.1	0.2	0.3	0.4	0.5
BamHI/ß1	-1.52	-1.43	-0.44	-0.23	-0.08	-0.02	0.0	0.0
BamHI/ß2	0.53	0.53	0.41	0.31	0.15	0.06	0.02	0.0

Table 4.15

Table showing cumulative Lod scores obtained for the Gamma gene under the recessive model with 50% penetrance.

GENETIC MODEL: RECESSIVE 50% PENETRANCE

Gamma gene Lod scores at θ =

	0.0	0.001	0.05	0.1	0.2	0.3	0.4	0.5
EcoRI/γ1	0.69	0.69	0.54	0.40	0.17	0.05	0.01	0.0
TaqI/γ1	0.69	0.69	0.54	0.40	0.17	0.05	0.01	0.0
SstI/γ2	-1.51	-1.39	-0.32	-0.10	0.06	0.08	0.06	0.0
TaqI/γ2	-1.11	-1.01	-0.04	0.13	0.19	0.13	0.04	0.0
PvuII/γ4	-1.32	-1.33	-0.26	-0.12	-0.06	-0.05	-0.02	0.0

Table 4.16

Table showing cumulative Lod scores obtained for the Delta gene under the recessive model with 50% penetrance.

GENETIC MODEL: RECESSIVE 50% PENETRANCE

Delta gene Lod scores at θ =

	0.0	0.001	0.05	0.1	0.2	0.3	0.4	0.5
BamHI/δ1	0.01	0.01	0.01	0.01	0.0	0.01	0.0	0.0
KpnI/δ2	-2.08	-1.91	-0.72	-0.44	-0.19	-0.08	-0.02	0.0

4.2.3 Analysis of T cell receptor haplotypes

Haplotypes of the T cell receptor loci were assigned using the alleles generated in the RFLP analysis. As the delta chain gene is situated within the alpha chain gene, haplotypes were assigned by combining the alleles from the alpha and delta loci. The alleles were phased using the minimum of ambiguous haplotypes for the alpha/delta, beta and gamma loci. One recombinat was noted in Pedigree 1 in the gamma locus as shown in Figure 4.37(a). The typing of these individuals were omitted as they were ambiguous. Haplotypes of individuals not tested were inferred from offspring.

The function of establishing haplotypes is to establish heterozygosity in individuals as opposed to relatively homozygous results from the data generated by the alleles. The haplotypes were then analysed using the Lod score method and the results are shown graphically in Figures 4.35-4.43.

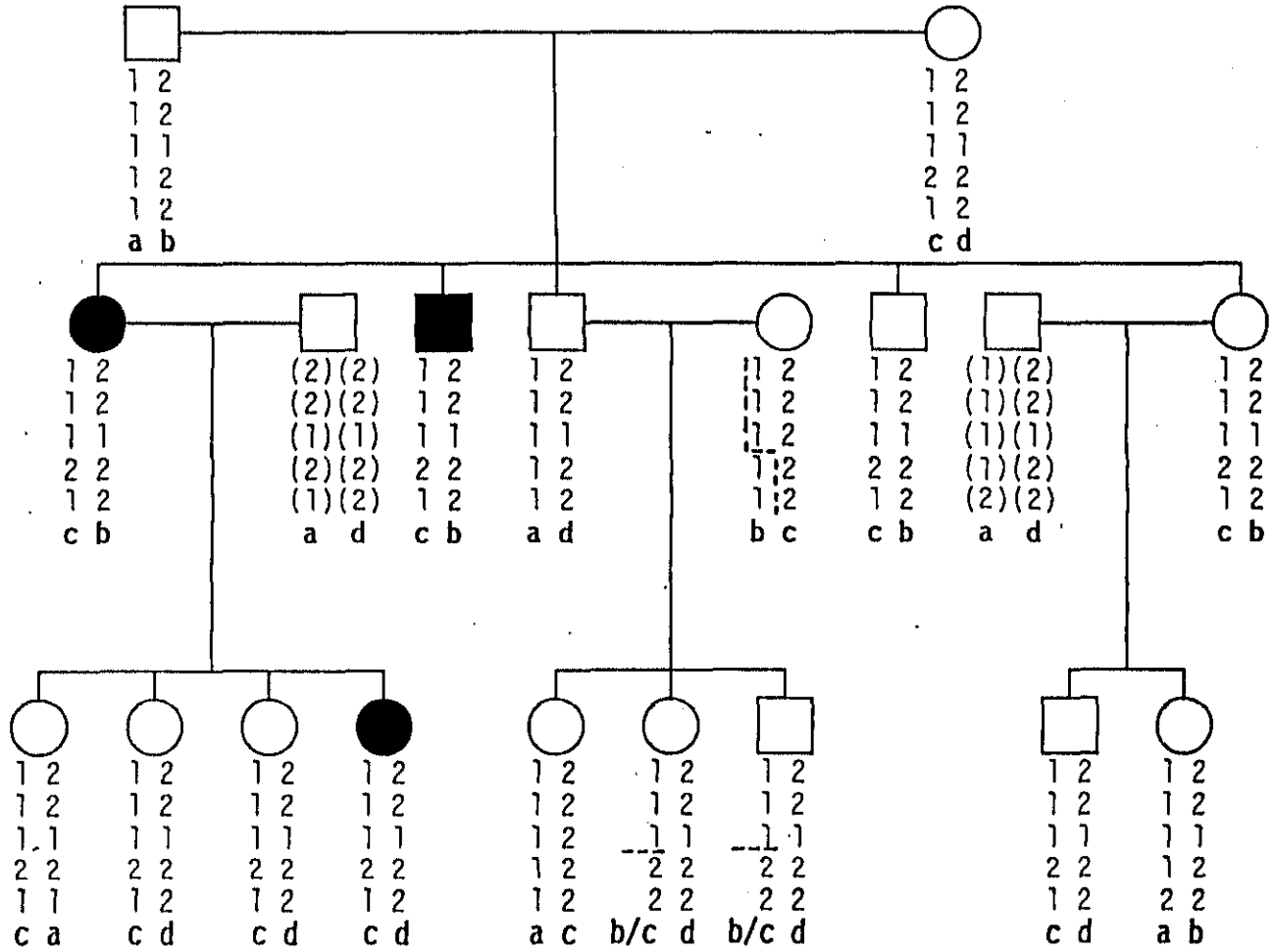


Figure 4.37 (a)

Diagrammatic presentation showing results at the gamma locus in pedigree 1. --- denotes location of crossover from generation 2 to generation 3. Results in brackets were inferred from offspring. (See also Figure 4.37).

PEDIGREE 1
TCR alpha/delta

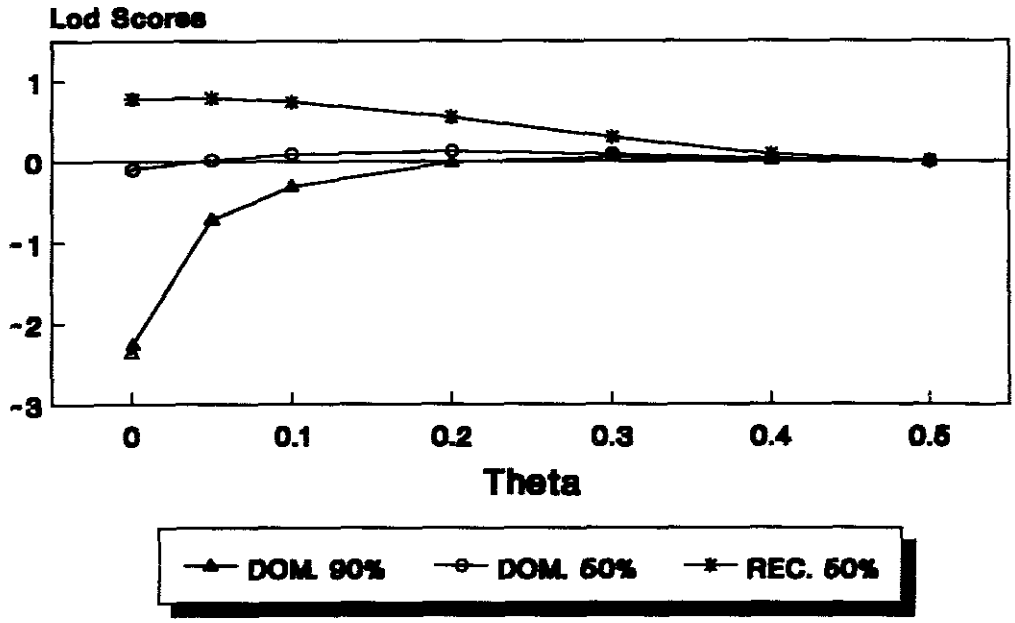


Figure 4.35

PEDIGREE 1
TCR beta

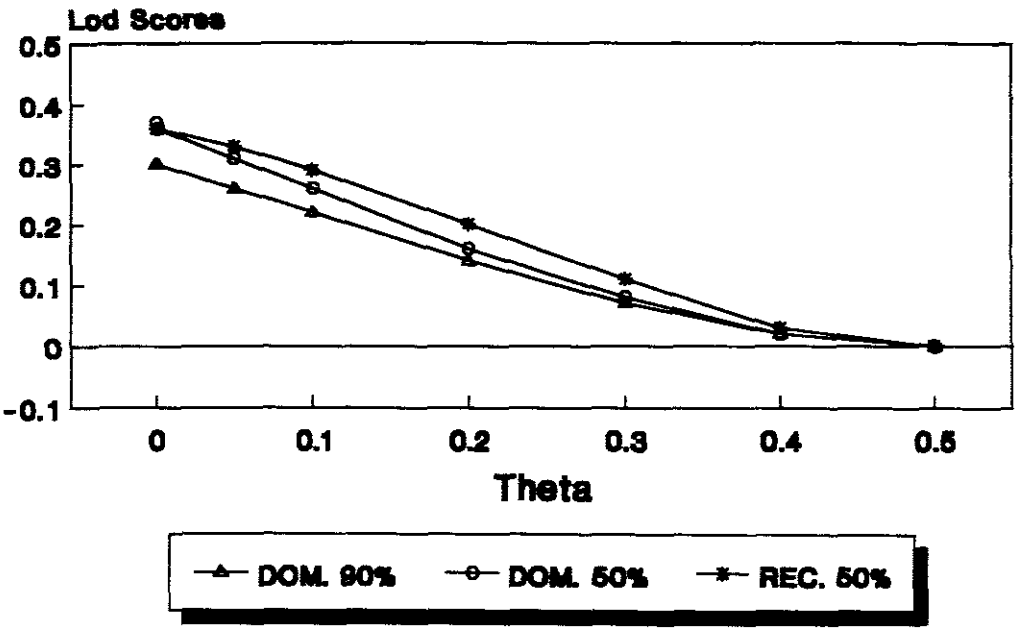


Figure 4.36

PEDIGREE 1
TCR gamma

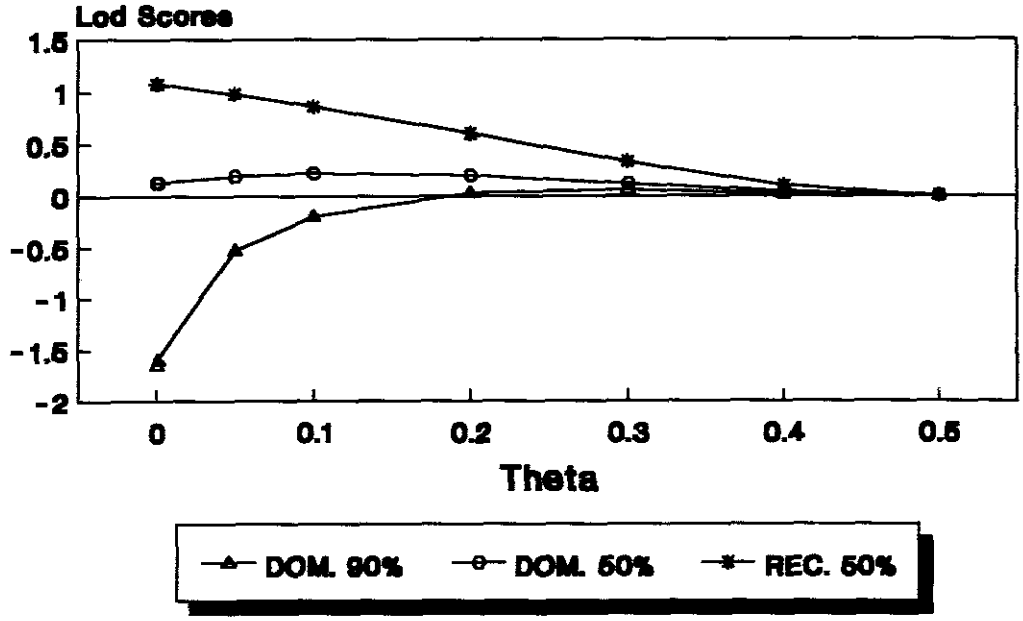


Figure 4.37

PEDIGREE 2
TCR alpha/delta

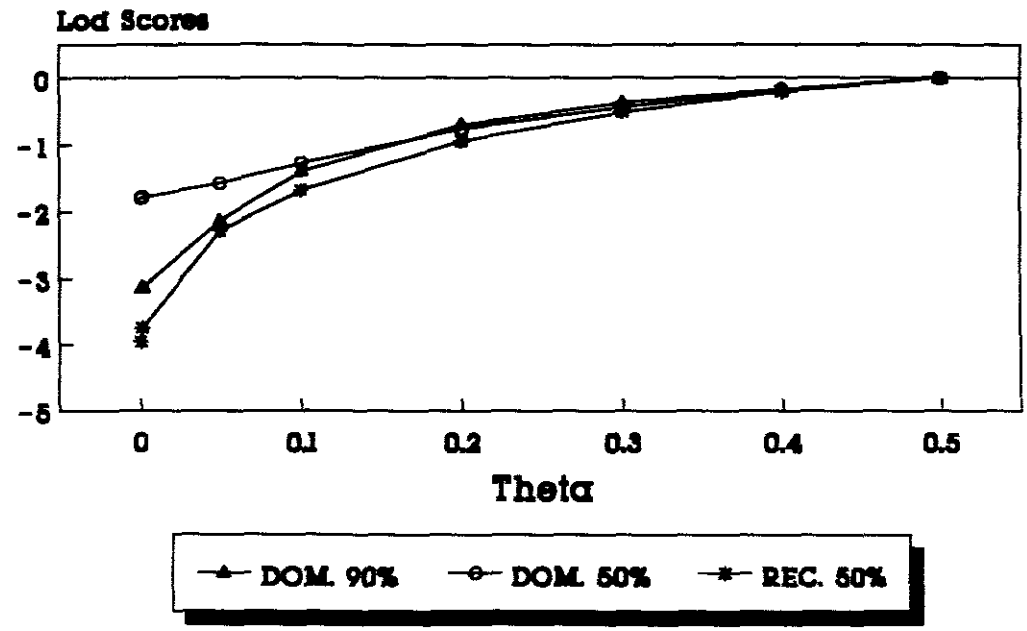


Figure 4.38

PEDIGREE 2
TCR beta

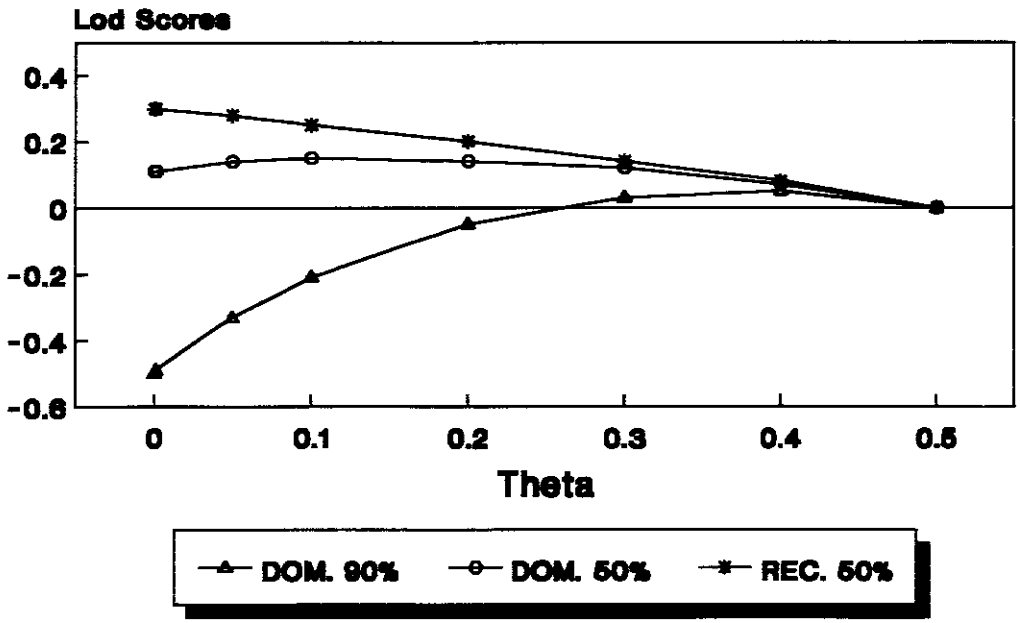


Figure 4.39

PEDIGREE 2
TCR gamma

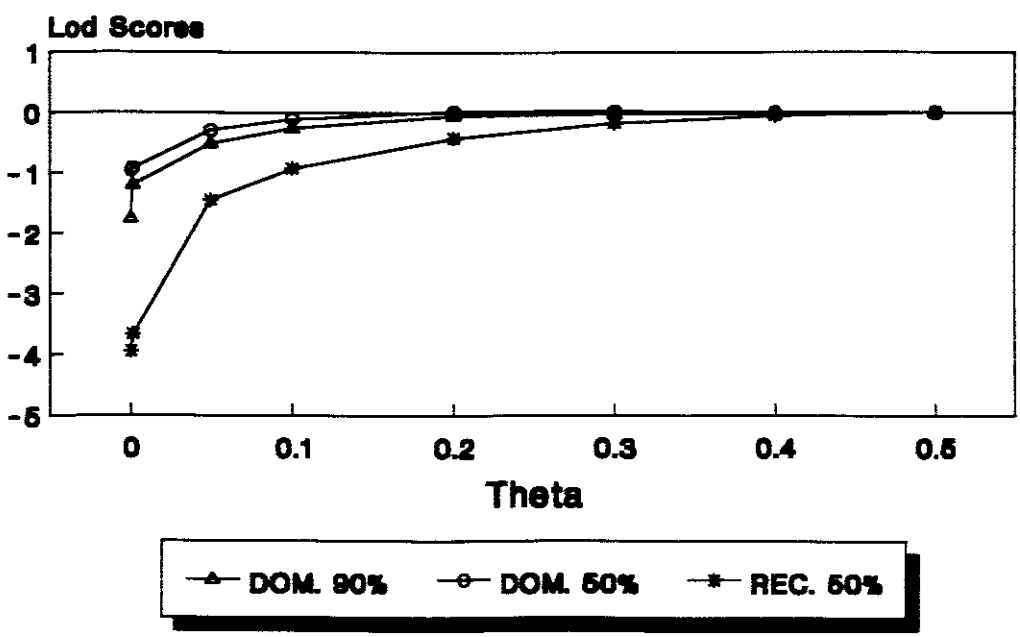


Figure 4.40

PEDIGREE 3
TCR alpha/delta

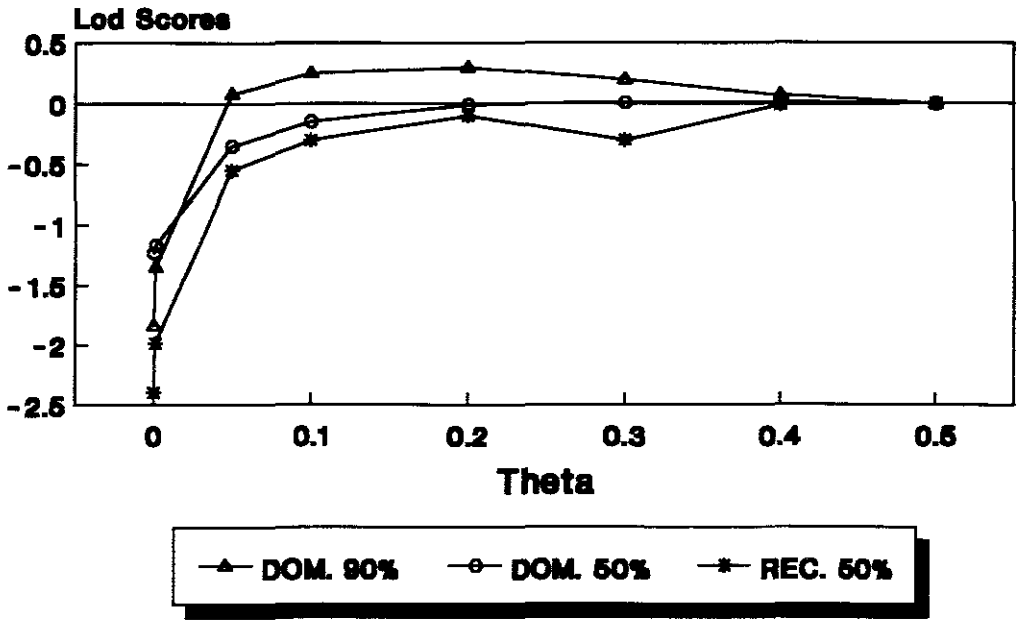


Figure 4.41

PEDIGREE 3
TCR beta

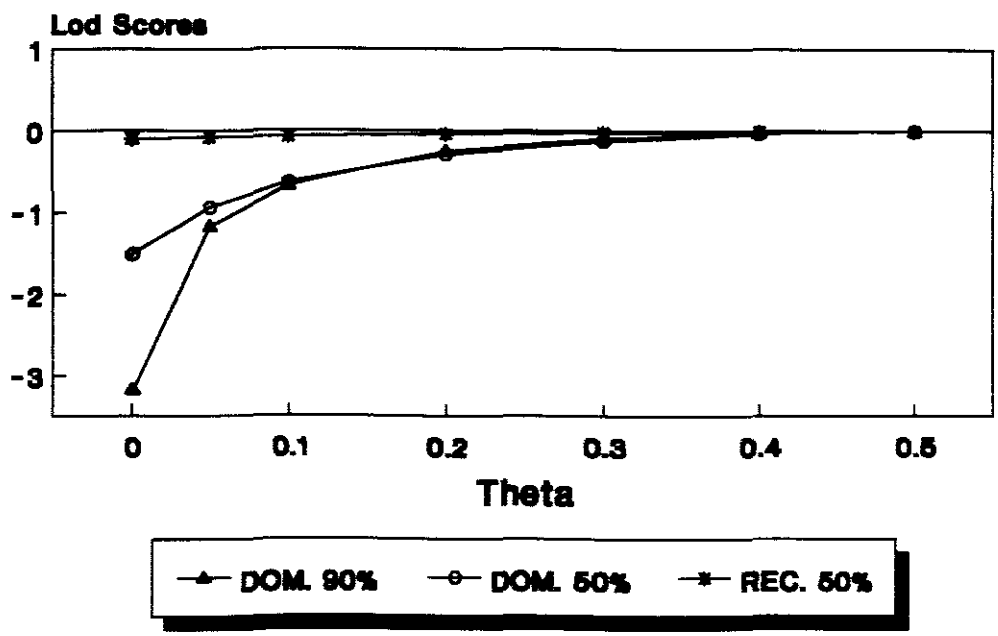


Figure 4.42

PEDIGREE 3
TCR gamma

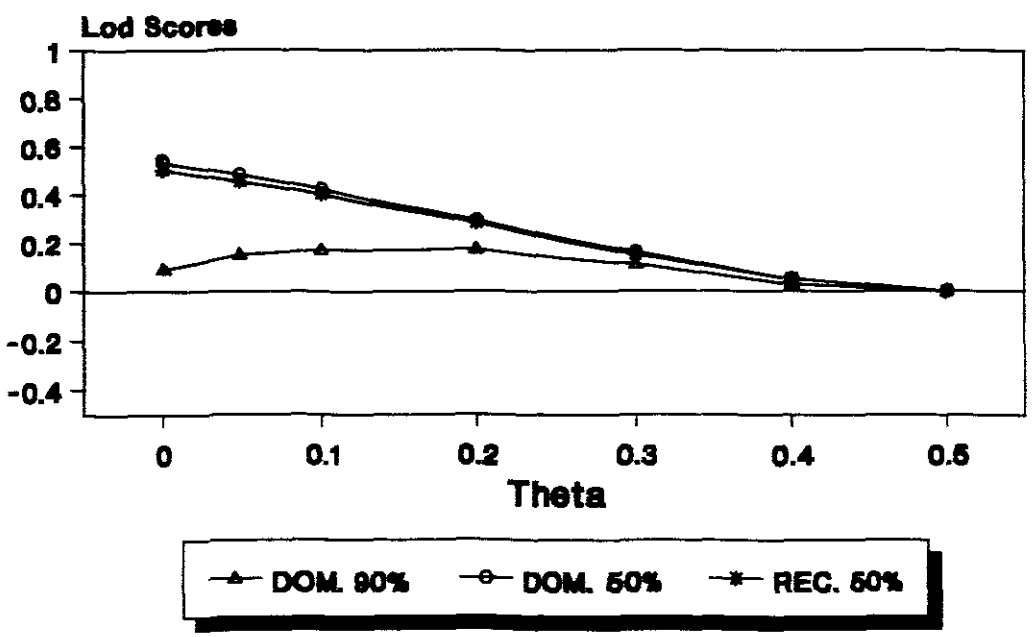


Figure 4.43

4.2.3.1 Pedigree 1

As shown in Figures 4.35-4.37, the Lod scores obtained from the linkage analysis of the T cell receptor haplotypes from the alpha/delta, beta and gamma loci as described fell between -2 and +3 under the dominant model with 50% penetrance and the recessive model with 50% penetrance at a recombination fraction of 0 and tended toward 0 as the recombination fraction increased to 0.5. Tight linkage between susceptibility to tuberculosis and T cell receptor haplotypes could be excluded under the dominant model with 90% penetrance at the alpha/delta locus as the Lod scores were -2.37 and -2.26 at a recombination fraction of 0 and 0.001 respectively. However, as the recombination fraction increased to 0.05, the Lod scores fell within the indeterminate range as shown in Figure 4.35.

4.2.3.2 Pedigree 2

As shown in Figures 4.38-4.40, the Lod scores obtained from the linkage analysis of the T cell receptor haplotypes from the alpha/delta, beta and gamma loci as described fell between -2 and +3 under the dominant model with 50% penetrance at a recombination fraction of 0 and tended toward 0 as the recombination fraction increased to 0.5. Tight linkage between susceptibility to tuberculosis and T cell receptor haplotypes could be excluded under the dominant model with 90% penetrance and the recessive model with 50% penetrance at the alpha locus as the Lod scores were -3.14 and -3.95 at a recombination fraction of 0, -3.13 and -2.28 at a recombination fraction of 0.001 and -2.13 and -2.28 at a recombination fraction of 0.05 respectively as shown in Figure 4.38. However, as the recombination fraction increased to 0.1, the Lod scores fell within the indeterminate range. Tight linkage could also be excluded at the gamma locus as the Lod scores were -3.95 and -3.67 at recombination fractions of 0 and 0.001 respectively. However, at recombination fractions of > 0.05, the Lod scores fell within the indeterminate range as shown in Figure 4.40.

4.2.3.3 Pedigree 3

As shown in Figures 4.41-4.43, the Lod scores obtained from the linkage analysis of the T cell receptor haplotypes from the alpha/delta, beta and gamma loci as described fell between -2 and +3 under the dominant model with 50% penetrance at a recombination fraction of 0 and tended toward 0 as the recombination fraction increased to 0.5. Tight linkage between susceptibility to tuberculosis and T cell receptor haplotypes could be excluded under the dominant model with 90% penetrance at the beta locus as the Lod scores were -3.16 and -3.12 at a recombination fraction of 0 and 0.001 respectively. However, as the recombination fraction increased to 0.05, the Lod scores fell within the indeterminate range as shown in Figure 4.42. Tight linkage could also be excluded under the recessive model with 50% penetrance at the alpha locus as the Lod score was -2.40 at a recombination fraction of 0. However as the recombination fraction increased to 0.001, the Lod scores fell within the indeterminate range as shown in Figure 4.41.

4.2.3.4 Cumulative Lod scores

Lod scores were added across pedigrees to produce cumulative Lod scores for T cell receptor haplotypes as shown in Tables 4.17-4.19.

Table 4.17

Table showing cumulative Lod scores obtained for the T cell receptor haplotypes under the dominant model with 90% penetrance.

GENETIC MODEL: DOMINANT 90% PENETRANCE

T cell receptor haplotype Lod scores at θ =

	0.0	0.001	0.05	0.1	0.2	0.3	0.4	0.5
Alpha/delta	-7.35	-6.74	-2.78	-1.47	-0.42	-0.12	-0.08	0.0
Beta	-3.37	-3.29	-1.24	-0.66	0.16	0.01	0.05	0.0
Gamma	-3.31	-3.12	-0.89	-0.28	0.14	0.16	0.03	0.0

Table 4.18

Table showing cumulative Lod scores obtained for the T cell receptor haplotypes under the dominant model with 50% penetrance.

GENETIC MODEL: DOMINANT 50% PENETRANCE

T cell receptor haplotype Lod scores at θ =

	0.0	0.001	0.05	0.1	0.2	0.3	0.4	0.5
Alpha/delta	-3.13	-3.06	-1.95	-1.35	-0.65	-0.33	-0.14	0.0
Beta	-1.04	-1.03	-0.49	-0.22	0.01	0.07	0.07	0.0
Gamma	-0.29	-0.26	-0.38	-0.34	0.51	0.31	0.09	0.0

Table 4.19

Table showing cumulative Lod scores obtained for the T cell receptor haplotypes under the recessive model with 50% penetrance.

GENETIC MODEL: RECESSIVE 50% PENETRANCE

T cell receptor haplotype Lod scores at θ =

	0.0	0.001	0.05	0.1	0.2	0.3	0.4	0.5
Alpha/delta	-5.57	-4.95	-2.04	-1.25	-0.31	-0.24	-0.14	0.0
Beta	0.56	0.56	0.53	0.47	0.36	0.23	0.10	0.0
Gamma	-2.37	-2.10	-0.02	0.34	0.46	0.31	0.09	0.0

As shown in Table 4.17, under the dominant model with 90% penetrance, tight linkage could be excluded with the alpha/delta, the beta and the gamma locus as the Lod scores were < -2 at a recombination fraction of 0.05 for the alpha locus, and 0.001 for the beta and gamma locus. However, as the recombination fraction increased to 0.1 for the alpha locus and 0.05 for the beta and gamma locus, the lod scores fell within the indeterminate range.

Under the dominant model with 50% penetrance, tight linkage could be excluded at the alpha locus as the Lod scores were -3.13 and -3.06 at a recombination fraction of 0 and 0.001 respectively. As the recombination fraction increased to 0.05, the Lod scores fell within the indeterminate range. Linkage could not be excluded or accepted at the beta or gamma locus as the Lod scores fell within the indeterminate range at all recombination fractions (See Table 4.18).

Under the recessive model with 50% penetrance, tight linkage at the alpha/delta locus could be excluded as the Lod scores were -5.57, -4.95 and -2.04 at recombination fractions of 0, 0.001 and 0.05 respectively. As the recombination fraction increased to 0.1,

however, the Lod scores fell within the indeterminate range. Linkage at the beta locus could not be excluded or accepted as the Lod scores fell within the indeterminate range at all recombination fractions. Tight linkage could also be excluded at the gamma locus as the Lod scores were -2.37 and -2.10 at recombination fractions of 0 and 0.001 respectively. However, as the recombination fraction increased to 0.05, the Lod scores fell within the indeterminate range as shown in Table 4.19.

CHAPTER 5

5.0 DISCUSSION

Evidence that genetic factors are crucial in determining the susceptibility to and the clinical spectrum of tuberculosis arises from the fact that most individuals exposed to *Mycobacterium tuberculosis* develop an effective immunity without evidence of the disease, whereas individuals who develop the disease present with a range of clinical manifestations (Schurr, 1991).

Although no studies have provided evidence that susceptibility *per se* to tuberculosis is controlled by HLA, there are reports that show an association, as discussed below.

Association and linkage to diseases may be analysed using various methods. The most widely used is that of a population based study whereby the disease population is matched with a healthy control population drawn from the same ethnic background. All individuals are typed for a genetic marker eg. HLA antigens and then the antigen frequencies of patients are compared with those of the normal healthy population. The data may then be analysed by the chi squared test and a relative risk calculated. This value is indicative of how many times more frequent the disease is in individuals positive for the relevant antigen than in individuals negative for that antigen (Ott, 1985). This method was used to analyse whether an association between tuberculosis and HLA existed by Selby *et al* (1978) and Takata *et al* (1978). Both found HLA-B8 to be associated with tuberculosis. On the contrary, Al-Arif *et al* (1979) found HLA-B15 to be associated with tuberculosis. These apparently discrepant findings may be attributed to either a chance finding, biased sampling or reflective of the different population groups studied.

Another way of analysis is that of sib pairing whereby pairs of sibs are taken at random from a series of families and analysed for a genetic marker. Three types of sib pairs should be apparent viz. those where both haplotypes are shared, those where one haplotype is shared and those where no haplotype is shared. Should the two genes segregate independently, the ratio of the above-mentioned types should be 0.25 : 0.5 : 0.25. However, if the two genes are linked, they will segregate together and the number of affected sibs sharing both haplotypes will be greater than expected. A chi squared test

may then be performed to compare the expected frequencies to those of the observed (Tiwari and Terasaki, 1985). Singh *et al* (1983) applied this method and established an association with HLA-DR2 and tuberculosis in 25 families.

Unlike the sib pair analysis, the Lod score method utilises information from the parents and other members of the pedigree to establish linkage between a disease locus and a marker locus. The Lod score method is the most efficient way of establishing linkage (Tiwari and Terasaki, 1985) as it can provide not only a test for the presence of linkage but also an estimate of the genetic distance (recombination) between the disease gene and the marker locus. Also, the Lod scores of new pedigrees may be added to existing data until a significant result of >+3 or <-2 is obtained.

Difficulty arises by applying this approach to the study of tuberculosis susceptibility as the disease has no clearly defined mode of inheritance. Linkage studies are most useful when the disease in question follows a straightforward Mendelian mode of inheritance. In an attempt to overcome this problem, we analysed the data under various modes of inheritance. However, it is clear from our results that no clear inheritance mode is apparent. Another difficulty lies in the fact that large numbers of families have to be studied before an unequivocal conclusion can be drawn.

As far as we are aware, this is the first study of this nature to be undertaken and therefore even though it is tempting to exclude linkage between susceptibility to tuberculosis and HLA on the basis of the results, perhaps this study has merely laid the foundation of a larger, more informative study.

A strong candidate for non-HLA genes associated with susceptibility to mycobacterial diseases is the T cell receptor genes. Studies in mice with experimentally induced autoimmune disease indicate that the T cell receptor repertoire differences may play a fundamental role in disease susceptibility (Niven *et al*, 1989).

It has been documented by Hillert and Olerup (1992) that the methods used in studies showing conflicting results with T cell receptor RFLP's have been population based. They feel that this method is unsuitable for the T cell receptor genes as the frequent recombination hotspots in the T cell receptor genes implies that it is unlikely that a given marker will be close enough to a biologically relevant allelic variation to reveal an association with a particular disease.

The difficulties described when analysing HLA were also experienced with the T cell receptor study. Although an unequivocal conclusion could not be reached between susceptibility to tuberculosis and T cell receptor genes, it was very interesting to note that the allele generated by the EcoRV/α2 enzyme/probe combination produced positive Lod scores under all three modes of inheritance used. The Lod score was as high as 1.79 at a recombination fraction of 0.001 under the recessive mode of inheriatnce with 50% penetrance. This indicates the odds to be approximately 52:1 in favour of linkage between the T cell receptor alpha gene and a putative disease susceptibility gene. Obviously, it is necessary to study more families in order to corroborate this finding.

CHAPTER 6

6.0 CONCLUSIONS

As no HLA allele appeared to be shared between affected individuals, no associations with any of the HLA antigens tested for were observed. A population based study to determine the effect of linkage disequilibrium of HLA alleles and a putative susceptibility gene to tuberculosis would be required to address this issue fully. Although this study was not designed to examine this aspect, a study of approximately 1000 unrelated individuals would probably prove to be more useful to determine whether an association of an HLA allele and tuberculosis susceptibility exists.

As the cumulative Lod scores obtained from the linkage analysis of HLA haplotypes to tuberculosis were <-2 at a recombination frequency of 0.001, linkage of disease susceptibility with HLA could be excluded under the dominant models with 50% and 90% penetrance, and the recessive model with 50% penetrance if the recombination frequency is low.

Therefore, although the literature published by Barnard *et al* (1976), Cox *et al* (1982), Takata *et al* (1978) Al-Arif *et al* (1979) and Singh *et al* (1983) describe an association of HLA and mycobacterial infections, our results do not support the existence of a major susceptibility gene located either within or in close proximity to the MHC.

Although the majority of the Lod scores for the T cell receptor genes fell within the indeterminate range, the recessive model with 50% pentrance excluded the alpha gene using the TaqI/α2(i) enzyme/probe combination and the delta gene using the KpnI/δ2 enzyme/probe combination. The dominant model with 90% penetrance excluded the delta gene using the BamHI/ß2 enzyme/probe combination. The results of the study show that no simple inheritance pattern for tuberculosis was apparent.

It was interesting to note that the odds in favour of linkage between a putative disease susceptibility gene at the T cell receptor alpha gene were approximately 52:1.

The results could therefore not confidently exclude the existence of a major susceptibility gene located within the T cell receptor genes.

Therefore, although these Lod scores are not conclusive, this study may have laid the foundation for future extended studies with many larger, selected multiplex families.

The Lod scores of the T cell receptor haplotypes obtained from the beta locus indicated that linkage could be excluded under the dominant model with 90% penetrance, but not under the dominant model with 50% penetrance or the recessive model.

The Lod scores obtained from the alpha/delta and the gamma locus indicated that the possibility of linkage could be confidently excluded under the dominant as well as the recessive models.

Therefore although an association of T cell receptor genes and mycobacterial diseases has been described (Kaufmann, 1991 and Kaufmann and Scott, 1991) the results of this study did not implicate a major susceptibility gene to tuberculosis linked to the T cell receptor genes.

6.1 DIRECTIONS FOR FUTURE STUDIES

Schurr *et al* (1991), have recently reported that they have identified a segment on chromosome 1 in the mouse that includes the Bcg gene (described on page 9.), and that it is precisely conserved on the telomeric end of the human chromosome 2. A number of polymorphic DNA probes to localise this gene segment have been generated and will be available for familial linkage analysis in the near future.

Once the human Bcg gene has been cloned, family studies similar to these used in this thesis, could elucidate the existence of a genetically linked susceptibility gene to tuberculosis.

APPENDIX

APPENDIX

1 DNA EXTRACTION

1.1 LYSIS BUFFER

0.32 M sucrose	109.5	g
10% Triton-X-100	10	ml concentrate
50 mM $MgCl_2$	5	ml of 1 M stock
10 mM Tris-HCl, pH 7.4	10	ml of 1 M stock

Make up to 1 litre with filtered distilled water.

1.2 SALINE EDTA

25 mM EDTA	50	ml of 100 mM stock
75 mM NaCl	3.75	ml of 4 M NaCl

Make up to 200 ml with filtered distilled water.

1.3 SODIUM DODECYL SULPHATE (SDS)

10% (w/v) SDS	100	g (Sigma)

Make up to 1 litre with filtered distilled water.

1.4 PROTEINASE K

10 mg/ml solution

1.5 SODIUM ACETATE

Saturated solution.

1.6 PHENOL CHLOROFORM

Mix 1 volume of melted phenol (Carlo Erba ref:451287) with 1 volume of chloroform-isoamyl alcohol (24/1) (PCI). Add 30 ml PCI to 20 ml 1 M Tris HCl, pH 8.0, in a 50 ml Falcon tube. Spin for 10 minutes at 1500 rpm. Remove the upper aqueous phase. Add 20

ml 0.1 M Tris HCl, pH 8.0, 0.2% mercaptoethanol and mix. Spin at 1500 rpm for 10 minutes. Eliminate the upper aqueous phase.

2 GEL ELECTROPHORESIS

2.1 LOADING BUFFER (5X)

50 mM Tris HCl, pH 7.6	0.5	ml of 1 M stock
50 mM EDTA	1	ml of 0.5 M stock
0.5% SDS	0.5	ml of 10% solution
0.1% (w/v) Bromophenol blue	1	g
40% (w/v) Sucrose	4	g

Use 4 μl of loading buffer 5x for 16 μg DNA.

2.2 ELECTROPHORESIS BUFFER (TAE-50X)

400 mM Tris	400	ml of 1 M stock
1 M Glacial acetic acid	57.2	ml
10 mM EDTA	10	mM of 0.5 M stock

Make up to 1 litre with sterile distilled water.
Make 2 litres of a 1 x TAE solution for electrophoresis.

2.3 AGAROSE GEL (0.8%)

Agarose (Sigma typeII)	3	g Sigma Type II
TAE	6	ml of 50x stock
Filtered distilled water up to 300 ml		
Ethidium bromide	18	μl of 8 mg/ml stock

Boil, cool and add 18 μl ethidium bromide (final concentration of 0.5 μg/ml).

3 NUCLEIC ACID LABELLING.

3.1 OLIGOMER LABELLING BUFFER (OLB)

Solution "O".

Tris	1.25	M pH 8.0
$MgCl_2$	0.125	M

Store at 4°C

Solution "A"		
Solution "O"	1	ml
ß2 mercaptoethanol (3 M)	18	µl
dATP (100 mM)	5	µl
dTTP (100 mM)	5	µl
dGTP (100 mM)	5	µl

Dissolve each triphosphate in 3 mM Tris, pH 7.0 and 0.2 mM EDTA. Store at -20°C.

Solution "B".		
Hepes pH 6.6	2	M
Store at 4°C		
Solution C.		
Hexadeoxynucleotides	90	OD units/ml

Resuspended in 3 mM Tris HCl pH 7.0 and 0.2 mM EDTA

5x OLB BUFFER.	
A;B;C mix ratio of	100:250:150.

Store at -20°C. May be used continuously.

4 PREHYBRIDISATION AND HYBRIDISATION OF MEMBRANES

$NaPO_4$ buffer		
Na_2HPO_4 1 M	70	ml
NaH_2PO_4 1 M	30	ml

4.1 HYBRIDISATION SOLUTION

$NaPO_4$ buffer 1 M	25	ml
SDS (25%)	14	ml
Dextran Sulphate (50%)	10	ml
Salmon Sperm DNA (10 µg/µl)	250	µl
H_2O	750	µl
	50	ml

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