GENETIC MARKERS OF RHEUMATOID ARTHRITIS IN A WESTERN CAPE BLACK AND COLOURED POPULATION

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I declare that this thesis is my own work. It is being submitted for the Master's Degree in Medical Technology, to the Cape Technikon, Cape Town. It has not been submitted before for any diploma, degree 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 here are not necessarily those of the Cape Technikon.

Ljubica Pokorny

22.5.1996.

Date

SUMMARY

Intensive investigations in many different populations over the last decade, have indicated a failure to understand the inheritance of rheumatoid arthritis (RA). It was hoped that genes within the class II region of the major histocompatibility complex (MHC) could shed some light on the inheritance of this autoimmune disease and which are now known without doubt, to confer susceptibility to the disease. Genetic studies of RA have concentrated primarily on its autoimmune nature and several investigations of MHC class II molecules, have demonstrated an association between specific HLA alleles and susceptibility to RA, in particular the DRB1*04 and DRB1*01 alleles.

The HLA system is known to be associated with many diseases involving an immune aetiology. The structural features of specific DR and DQ genes give clues to the molecular mechanisms by which these alleles are associated with RA. It has been found by many investigators that there is more than one susceptibility allele for RA at the DRB1 locus.

Questions arise whether the DRB1 molecule itself directly contributes to the pathogenesis of RA and why some DRB1 genes carrying DRB1*04 alleles, are not associated with RA.

Animal studies have emphasised the critical importance of T-cells in the pathogenesis of RA. Immune responsiveness is thought to be controlled by specific allelic variation by determining the ability of specific T-cell receptors (TCRs) to be triggered by recognition of class II molecules during the induction of the immune response.

In a disease such as RA, however, where multiple alleles are thought to confer risk, it is not yet known whether each of these alleles shares some common structural feature triggering a single T-cell pathway or whether each allele represents an alternative recognition site which triggers different T-cell clones, all of which lead to a similar clinical syndrome.

This study was undertaken to ascertain whether a genetic susceptibility to RA exists in two Western Cape populations and whether the DRB1*04 allele is significantly associated with RA. The association of DRB1*04 with DQB1 and DQA1 alleles was compared to that reported for other populations.

The HLA frequencies were calculated and postulated haplotypes were determined in Western Cape black (Xhosa) and mixed ancestry (Cape coloured) RA affected patients and in ethnically-matched, healthy, comparison groups.

DNA-based HLA typing was performed on individual blood specimens after extracted DNA had been amplified by the polymerase chain reaction (PCR) method with DRB1*-generic-, DRB1*-group specific-, DQB1*- and DQA1*-primer pairs. Amplified samples were dot-blotted onto a nylon membrane and hybridized with sequence-specific oligonucleotide probes (SSOPs)

obtained from the XIth International HLA Workshop (IHW) and detected using a Digoxigenin system. Alleles were assigned, based on the reaction pattern observed.

The frequencies of HLA-class II alleles were calculated by the direct counting method and the results were expressed as a percentage of total alleles. Statistical analysis using chi-square (X^2), p values and the odds ratio (OR) with 95% confidence intervals (C.I.) were calculated in order to determine the strength of association of alleles with either the Xhosa, Cape coloured RA affected patients or with healthy, comparison groups.

The most frequently occurring allele in both Xhosa and Cape coloured RA affected patients was DRB1*0401. A significant association of this allele with RA was observed in both Xhosa and Cape coloured RA affected patients.

There was a significant increase in the frequency of DQB1*0501 and DQB1*0302 alleles in both Xhosa and Cape coloured RA affected patients. A strong, positive association was found for the DQB1*0501 allele in Cape coloured RA affected patients and with the DQB1*0302 allele in both Cape coloured and Xhosa RA affected patients. These alleles are known to be in linkage disequilibrium with DRB1 alleles in both populations. A high frequency of DQA1*0301 in both RA affected Xhosa and Cape coloured patients was observed and a significant association with RA, was observed with this allele.

The work presented in this thesis provides evidence for the association of DRB1*0401 alleles with RA and is consistent with other studies. In addition, the work is consistent with the recently reported finding of Zanelli *et al* (1995), that DQ alleles may be responsible for RA susceptibility.

OPSOMMING

Intensiewe ondersoeke die afgelope dekade in verskillende bevolkings het misluk om die oorerwing van rumatoïede artritis (RA) te verstaan. Daar is gehoop dat gene geleë in die Klas II area van die hoof histo-verenigbaarheidskompleks (MHK) lig sou kon werp op die oorerwing van hierdie outosomale siekte. Wat nou sonder twyfel bekend is, is dat dit 'n vatbaarheid verleen aan die siekte. Genetiese studies met betrekking tot RA, het hoofsaaklik op die outo-immune aspek gekonsentreer en verskeie ondersoeke van die Klas II MHK molekules het 'n assosiasie tussen spesifieke HLA-allele en vatbaarheid vir RA aangedui, in besonder die DRB1*04 en DRB1*01 allele.

Die HLA sisteem is bekend daarvoor dat dit met talle siektes van 'n immunologiese aard geassosieer kan word. Die strukturele kenmerke van spesifieke DR en DQ verskaf gee leidrade tot die molekulere meganismes waartoe hierdie allele met RA geassosieer word. Dit is deur talle ondersoekers bevind dat daar meer as een vatbaarheidsallel vir RA by die DRB1 lokus bestaan.

Vrae ontstaan egter of die DRB1 molekule self bydra tot die patogenese van RA asook waarom sekere DRB1*04 allele nie met RA geassosieer word nie.

Dierestudies het die kritiese belangrikhied van T-selle in die patogenese van RA beklemtoon. Daar word gespekuleer dat immuun-responsiwiteit deur spesifieke alleliese variasie beheer word as gevolg van die vermoë van spesifieke T-sel-reseptore om geaktiveer te word deur die herkenning van klas II molekules tydens die induksie van die immuunrespons.

In 'n siekte soos RA, waar talle allele verdink word om risiko te verwek, is dit onbekend of elk van hierdie allele 'n gemeenskaplike strukturele kenmerk deel wat 'n enkele T-sel roete aktiveer, en of elkeen 'n verskillende herkennings-struktuur voorstel wat dan almal lei tot aktivering van verskillende T-sel-klone en dus 'n gemeenskaplike kliniese sindroom openbaar.

Die studie is onderneem om te vas te stel of daar 'n genetiese vatbaarheid vir RA in twee Wes-Kaaplandse bevolkingsgroepe bestaan, en of die DRB1*04 allele in besonder, met RA geassosieer kan word. Die verwantskap tussen die DRB1*04 en tussen DQA1- en DQB1-allele is ook vergelyk met dié van ander bevolkingsgroepe.

HLA-allel-frekwensies is bepaal en gepostuleerde HLA-haplotipes van persone met RA in die Wes-Kaapse swartmense (Xhosas) en Kaapse kleurlinge asook onder etniese-vergelykbare gesonde, groepe, is vasgestel.

DNA-gebaseerde HLA-tiperings is op individue se bloedmonsters gedoen nadat geïsoleerde DNA in die polimerase kettingreaksie met DRB1*-generiese-, DQB1*-groep-spesifieke-, DQB1*- en DQA1*-primere pare geamplifiseer is. Monsters is gekol-klad op 'n nylon membraan en gehibridiseer met orde-spesifieke oligonukleotied-peilers wat vanaf die 11de Internasionale

HLA Werkswinkel verkry en gemerk is met die Digoxigenin sisteem. Allele is toegeken volgens die reaksiepatrone.

Die HLA-klas II allelfrekwensies is bepaal met die direkte telmetode en die resultate is uitgedruk as 'n persentasie van totale allele. Die Chi-vierkant (X^2), p waardes en kanse verhoudings met sekerheids-intervalle van 95% is bereken om die sterkte van assosiasie van die allele in of die Xhosa, Kaapse kleurling met RA, asook die gesonde groepe, te vergelyk.

Die allel wat die mees algemeen in beide die Xhosa en die Kaapse kleurlinge gevind was, is DRB1*0401.

Daar was 'n merkbare toename in die voorkomssyfer van die DRB1*0501 en DRB1*0302 allele in beide Xhosa en kleurlinge met RA. Daar was 'n sterk, positiewe assosiasie tussen die DQB1*0501 allel in die Kaapse Kleuring en die DQB1*0302 allel in beide die Kaapse kleurlinge en die Xhosa. Beide allele staan in sterk koppelings-disekwilibrium tot DRB1 in beide bevolkingsgroepe. Daar was 'n hoë frekwensie van DQA1*0301 in beide Xhosa en Kaapse kleurlinge met RA, sowel as 'n merkbare positiewe assosiasie van die allel en RA.

Die werk in hierdie tesis uiteengeset, dui op 'n assosiasie tussen die DRB1*0401 allele en persone met RA, en is in ooreenstemming met ander studies. Verder is die bevindings ook in ooreenstemming met die onlangse gerapporteerde bevindings van Zanelli *et al* (1995), dat die DQ allele verantwoordelik vir vatbaarheid vir RA mag wees.

Dedicated to my husband, Robert and our sons, Morgan and Wyatt

"Hold fast the truths the deep visions brings -

The mind and heart and spirit, freed from things

Of sense and sight and sound that lie outside

Know well the values that through life abide.

The things that really count

Are things we cannot count"

- Reigner

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INTRODUCTION

Rheumatoid arthritis (RA) is a complex, multifactorial disease in which genetic and non-genetic factors are involved. RA presents with a spectrum of clinical severity ranging from mild arthritis which may require little treatment, through to crippling, destructive joint disease with a high titre of rheumatoid factor (RF) autoantibody and life-threatening involvement of other visceral organs. Much of the joint destruction that occurs in RA appears to take place during the first few years of disease (Wicks *et al*, 1994). Early studies have established that RA is a human leukocyte antigen (HLA)-associated disease (Stastny, 1978).

Intense investigations were performed over the last decade, in order to understand the inheritance of RA. It was of major importance to identify genes within the HLA-class II region of the MHC, that confer susceptibility to the disease.

Many of these studies were largely based on finding an association between HLA-class II serologic specificities and the disease. The DNA technology which is widely used today as a tool for investigating the various genes associated with different diseases, has helped to expand our knowledge of this system (Gregersen *et al*, 1987).

RA is a common autoimmune disorder in which there is a demonstrable genetic contribution (Wordsworth and Bell, 1991; Rigby *et al*, 1991). Numerous population and family studies have demonstrated that an important element of the genetic component of RA arises from within the MHC, (Payami *et al*, 1986; Wordsworth and Bell, 1991; Wordsworth *et al*, 1992). The MHC, also known as the HLA system is situated on the short arm of chromosome six and is extremely polymorphic. The HLA region contains class I (A, B and C) and class II (DR, DQ and DP) genes, among others, that code for cell surface glycoproteins called DR, DQ and DP molecules which are expressed on macrophages and B lymphocytes and are responsible for presenting processed antigen to the TCR of CD4+ helper T-cells. Several alleles of the HLA-class II genes have been implicated in many autoimmune diseases (Tiwari and Terasaki, 1985).

Alleles at different loci show strong linkage disequilibrium between different populations (du Toit *et al*, 1988). It is possible that the HLA-class II gene itself is responsible for the disease predisposition, rather than a separate "disease gene" which may be in close linkage with HLA (du Toit *et al*, 1988). RA has been associated with DRB1*04 allele in many, but not all populations studied (Tiwari and Terasaki, 1985; Brautbar *et al*, 1986). The strongest associations are with alleles at the DRB1 locus, particularly with DRB1*04, but also, to a lesser extent, with DRB1*01 and DRB1*10 (Sanchez *et al*, 1990; Wordsworth *et al*, 1991; Ollier *et al*, 1991; Wordsworth and Bell, 1991). None of these associations, however, have been clearly demonstrated and they could result from linkage disequilibrium with other loci. DRB1*04 has been reported to be in association with RA in many populations while DRB1*01 association with RA has been reported only in a few select populations (Nelson and Hansen, 1990). The results of

serological studies which formed part of the XIth International Histocompatibility Workshop (IHW), confirmed DR4 (DRB1*04) association with RA in different populations including Canadian Caucasians, Asian-Indians, Japanese, Han Northern Chinese, Russian-Siberian Caucasians, Thais and Thai-Chinese (Nelson *et al*, 1992). In the majority of populations, the presence of DR4 (DRB1*04) is associated with disease risk (Nelson *et al*, 1992), while the association of DR1 (DRB1*01) with a disease has been reported in some populations to be associated with RA (Nichols and Woodrow, 1981). Studies of HLA-DQ, immunoglobulin genes, complement alleles and T cell receptor polymorphism in RA, have generated interest in a role for each in RA susceptibility or in disease expression. DQB1*0301 has been reported to be in association with severe autoimmune disease (Sansom *et al*, 1989), with IgM RF (Stephens *et al*, 1989) and with Felty's syndrome (Sansom *et al*, 1987) and in some instances to be increased in RA (Sansom *et al*, 1987) and in other cases not increased (Lanchbury *et al*, 1989). There have been reports of complement alleles in RA (Thomson *et al*, 1986) and of T cell receptor polymorphism (Duby *et al*, 1989). More than one autoimmune disease can occur in an individual and clustering of autoimmune diseases within families has been well described (Nelson and Hansen, 1990). The analysis of the overlap of RA with other autoimmune diseases could be particularly informative in the evaluation of HLA and disease. The frequency of both thyroid antibodies and thyroid disease has been reported to be increased in patients with RA (Grennan *et al*, 1983).

It is hoped that the molecular basis of arthritis would become firmly established in the near future and that the diversity of clinical expression and the pattern of disease in some families could be explained. In addition, questions why many individuals carrying HLA molecules associated with certain diseases, do not develop that disease; how an identical disease can occur in the absence of HLA molecules with which they are commonly associated and why an individual is usually susceptible at a particular time, remain unanswered. The time at which one individual is most susceptible may be determined by age, sex, stress, nutrition, neurogenic mechanisms, hormones, the immune system, the vascular endothelium and other factors.

If the nature of an arthritic disease and its intrinsic clinical details are mainly determined by the host (Brewerton, 1976) and HLA molecules have the ability to bind thousands of different peptides, many commonplace agents may prove to have a role in the aetiology of RA. Instead of a single specific agent causing a particular type of arthritis in one individual, diverse extraneous factors may be involved.

The agents which cause arthritis may vary in different parts of the world, social circumstances, individuals and in different types of arthritis. In the South African black population, the occurrence of RA is reported to be low in rural areas (0.87%), whereas in similar populations living in urban areas, the occurrence is comparable with that in other industrialised countries (3,3% ; Beighton *et al*, 1975; Solomon *et al*, 1975).

These observations suggest the possibility of urban environmental factors which have not been identified and which might be removable by preventive measures, having a profound influence on the frequency and severity of arthritis. The pathogenesis of RA and the mode of action of some of the effective anti-rheumatic drugs are unknown. It has been proposed that haemopoetic growth factors (colony stimulating factors) have an important role in RA of inflammatory leucocytes. Slow-acting anti-rheumatic drugs may work by inhibiting myelopoiesis. The cellular and biochemical events occurring during inflammation and tissue destruction in RA are complex and the critical control mechanisms remain to be defined. Several locally generated polypeptide mediators (cytokines) have been detected in rheumatoid lesions and could be important for disease progression (Lipsky *et al*, 1989). The efficacy of slow-acting anti-rheumatic drugs, such as gold salts and D-penicillinase, remains to be established.

This study was undertaken to investigate the genetic association of RA and HLA in randomly selected healthy individuals belonging to two populations: namely Western Cape black (Xhosa) and individuals of mixed ancestry (Cape coloured) as well as in patients seropositive for RA of the same ethnical composition.

The objective of this study was to determine whether there is a possible genetic susceptibility to RA and whether there is a possible association of RA with DRB1*04 subtypes and the association of DQA1 and DQB1 alleles with RA.

The present study was also undertaken to determine the frequency of HLA-DRB1, -DQB1 and -DQA1 alleles in these two populations and in ethnically-matched comparison populations. The linkage disequilibrium between HLA-DR, -DQA1 and -DQB1 obtained for the two local populations was compared with data published for other populations.

The statistical analysis of data included the determination of gene frequencies of HLA-class II alleles by the direct counting method, X^2, p values and OR with 95% C.I. The strength of association of HLA-class II alleles with RA was determined with the RA patients of both populations and with ethnically matched comparison groups.

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ABBREVIATIONS

AIDS ..... Acquired immunodeficiency syndrome

APC ..... Antigen presenting cell

bp ..... Base pair

°C ..... Degree Celsius

C 2 ..... Complement gene 2

C 4 ..... Complement gene 4

C.I. ..... Confidence interval

cM ..... CentiMorgan

Dig-ddUTP ..... Digoxigenin-dideoxy uridine triphosphate

DNA ..... Deoxyribonucleic acid

dNTP ..... Deoxy-nucleotide triphosphate

DTT ..... Dithiothreitol

EDTA ..... Ethylenediamine tetra-acetic acid

g ..... Gram

HLA ..... Human leucocyte antigen

HTC ..... Homozygous typing cell

HTLV ..... Human T cell leukaemia leucocyte virus

IDDM ..... Insulin dependent diabetes mellitus

IL-2 ..... Interleukin-2

Ir ..... Immune response

Kb ..... Kilobase

Kd ..... Kilodalton

M ..... Molar

mg ..... Milligram

MHC ..... Major histocompatibility complex

ml ..... Millilitre

MLC ..... Mixed lymphocyte culture

mM ..... Millimolar

MS ..... Multiple sclerosis

ng ..... Nanogram

OR ..... Odds ratio

p ..... Probability

PBS ..... Phosphate buffered saline

PCR ..... Polymerase chain reaction

RA ..... Rheumatoid arthritis

RF ..... Rheumatoid factor

RFLP ..... Restriction fragment length polymorphism

rpm ..... Revolution per minute

SDS ..... Sodium dodecyl sulphate

SSOP ..... Sequence-specific oligonucleotide probe

SSPE ..... Sodium sulphate phosphate ethylenediamino-tetra-acetic acid

TAE ..... Tris-acetate

TCR ..... T-cell receptor

TE ..... Tris-EDTA

TMAC ..... Tetramethylammonium chloride

μg ..... Microgram

μl ..... Microlitre

IHW ..... International histocompatibility workshop

X^2 ..... Chi-square

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CHAPTER 1

LITERATURE REVIEW

1.1. RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) remains one of the most common, puzzling and poorly treated diseases in humans. It was a rare disease until the Industrial Revolution, which led to changes in living conditions, diet and exposure to infections. The apparent increase in RA in recent times therefore, may reflect an imbalance between evolution and environment and mortality due to early childhood infections. RA still appears to be a rare disease in rural black communities, but its incidence increases with urbanization.

An interest in the biology of chronic inflammation and in the design of more potent and specific anti-inflammatory drugs, however, heralds an optimistic era for the treatment of RA. RA is a common chronic inflammatory disease involving the sinovial membranes of multiple joints and other non-articular tissues. The annual incidence is 2-4/10,000 adult population (Cush and Lipsky, 1991). RA may present clinically in several ways, the most common being an insidious onset with symptoms developing over months. A few patients present with an acute onset of polyarthritis or disease that develops over a few weeks (Harris Jr, 1990). Most patients have progressive disease with substantial morbidity and mortality. The diminished survival of patients is similar to that seen in diabetes, Hodgkin's disease and three-vessel coronary artery disease. The median life expectancy is reduced by seven years for men and three years for women (Panayi *et al*, 1992). Although RA is considered as a prototypical autoimmune disease, its aetiology is still unknown. Over time, inflammatory agents released within the joint lead to varying degrees of destruction (Cush and Lipsky, 1991). The frequently abrupt onset, prominence of the inflammatory lesion and HLA-class II association, suggest that a single or limited set of antigens is the trigger for this autoimmune process. A likely candidate is an infectious agent such as a bacterium, mycoplasma or virus.

It was believed in the 1940s, that non-group A Haemolytic streptococci were associated with RA in a similar manner to that demonstrated for group A streptococci in rheumatic fever. This possibility was supported by the observation that sera of RA patients could agglutinate these streptococci (Wicks *et al*, 1994). This phenomenon was later shown, however, to be due to the agglutination of streptococcal-bound IgG by RF.

Laboratory work on animals eg. the development of an inflammatory arthropathy in mice transgenic for HTLV-I, favours a retroviral agent (Breedveld and Trentham, 1987; Sewel and Trentham, 1993). Human studies implicate the Epstein-Barr virus (Roudier *et al*, 1989). The formation of collagen and adjuvant arthritis is induced in rats sensitized against epitopes of

native cartilage collagen (type II) or mycobacteria. In both of these models, there is sustained proliferative synovitis with cartilage and bone destruction. These features make up the classic morphological triad of RA (Trentham *et al*, 1978). The role of collagenous or mycobacterial stimuli is unknown; these experimentally inducible conditions do have several parallels with the human disease.

Beside the various factors mentioned above which are thought to contribute to disease susceptibility, unequivocal evidence indicates that susceptibility to an autoimmune disease is largely genetically determined. Genetic studies in humans and experimental models have clearly documented the primary contribution of the MHC genes to autoimmune disease. Immune responsiveness is determined by these polymorphic HLA-class II gene products which contribute to over 50 different autoimmune diseases, including RA, insulin dependent diabetes mellitus (IDDM) and multiple sclerosis (MS; Todd *et al*, 1988). Some of the HLA-class II molecules are present in affected individuals with certain diseases more frequently than in normal, healthy individuals, showing a strong indication that susceptibility to a particular autoimmune disease is at least in part a genetic trait mapping to the MHC (Todd *et al*, 1988).

The association between the HLA-class II alleles and disease is often maintained in different ethnic groups. The association between RA and HLA-class II genes has been intensely investigated by many researchers over the last decade and their subsequent findings are directed at the DRB1*04 allele at the DRB1 locus in most of the diseased populations investigated and to a lesser extent to DRB1*0101 and DRB1*1001 alleles at the same locus, in selected populations. The *DRB1*04 allele is believed to be the main causative agent in RA and disease predisposition* in the majority of the RA affected populations studied.

1.2. THE MAJOR HISTOCOMPATIBILITY COMPLEX (MHC)

The MHC which is also known as the HLA system, is located on the short arm of chromosome 6 and encompasses a region of approximately 3,500 kilobases (Kb). It consists of three major gene clusters, class I, class II and class III, which include a large number of genes important for the immune system. The class I region contains the 3 loci currently known as HLA-A, -B and -C, while the class II region contains the HLA-D locus, which itself contains a number of individual sub regions (Figure 1.1.). The class III region contains a heterologous collection of genes such as the complement genes C 2, C 4 and factor-B, the tumour-necrosis factor α (TNF α) and -ß (TNF ß) genes, as well as the major heat-shock protein 70 gene HSP 70 (Trowsdale *et al*, 1991).

A large number of new genes, which also are believed to be of immunological importance, have been discovered in the MHC class II and III regions within the last few years (Trowsdale *et al*, 1991; Robertson, 1991).

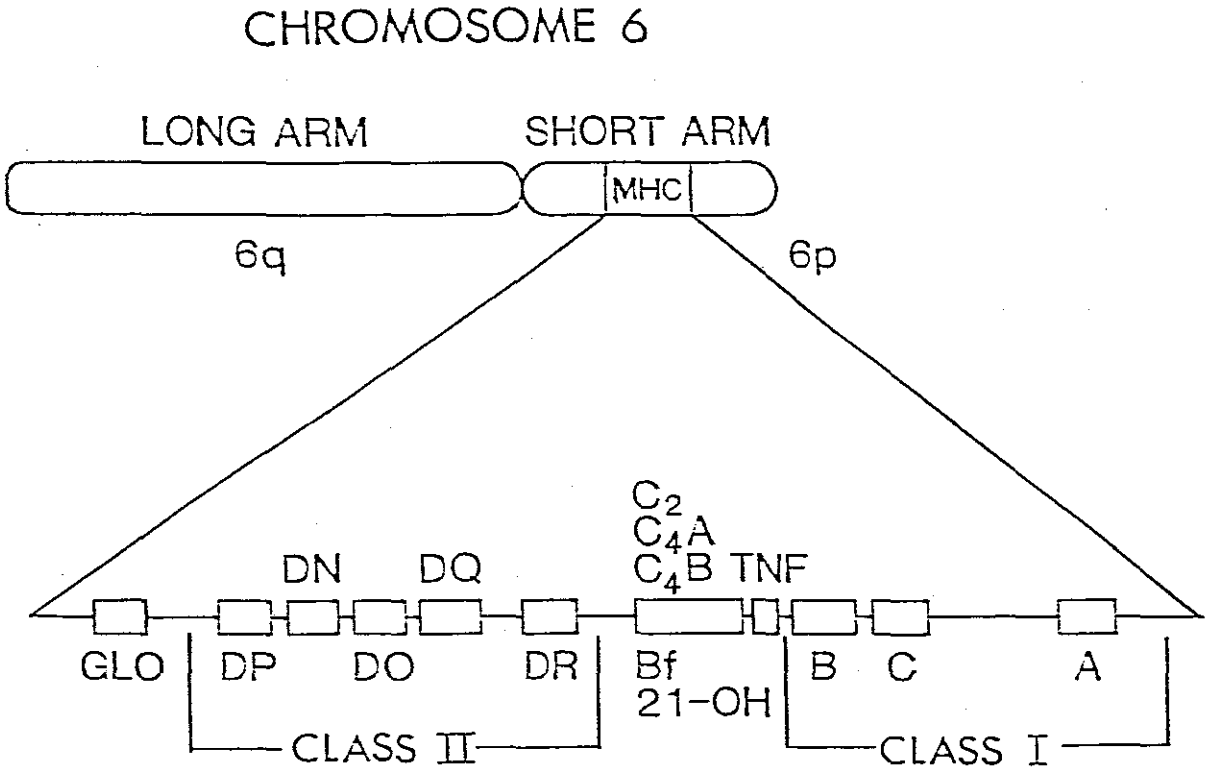

Figure 1.1. **Schematic representation of a map of the short arm of chromosome 6 of the MHC region known in man as the HLA region.**

1.2.1. Structure and function of MHC molecules

1.2.1.1. MHC class I molecules

HLA-class I spans approximately 200 Kb on the distal end of the MHC (Kendall *et al*, 1991). HLA-A, -B and -C gene products are expressed on the surface of all cells except red blood cells (Kara and Glimcher, 1991). The class I molecule consists of three independently folded extracellular domains, a transmembrane region and a cytoplasmic tail. The α1, α2 and α3 domains are linked in one chain, while the fourth domain, ß2-microglobulin, is a separate molecule and is bound preferentially to the third extracellular domain. Sequence analyses of both polymorphic and non-polymorphic class I HLA heavy chains revealed that the third domain is more conserved than the first and second domains (Kendal *et al*, 1991). The α chain has a molecular weight of ± 44 Kilodaltons (Kd), represents the class I gene product and is glycosylated. The ß2-microglobulin chain has a molecular weight of about 12 Kd and is encoded on chromosome 15 (Goodfellow *et al*, 1975).

HLA-class I molecules are involved in the recognition and interaction of cytotoxic T-lymphocytes with their target cells. This has been demonstrated in studies using cytotoxic lymphocytes from donors that have been sensitized against minor histocompatibility antigens (Goulmy *et al*, 1977), viral antigens (Mc Michael, 1978; Kreth *et al*, 1979; Sethi *et al*, 1980) and haptens (Dickmeiss, 1977; Figure 1.2.).

1.2.1.2. 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). It comprises nearly 1,000,000 base pairs (bp) and includes at least 14 different genes, of which 9 are expressed (Grey and Chesnut, 1985). With the exception of DOß and DZα, these genes are generally found in one of the three major subregions: DR, DQ or DP. Each subregion contains at least one functional α and ß chain pair gene in addition to a variable number of nonfunctional genes or pseudogenes. Gene products of the HLA-D region, eg. HLA-DR, -DQ and -DP are expressed on the cell surface of all B-lymphocytes, macrophages, Langerhans cells, dendritic cells and activated T-cells (Kara and Glimcher, 1991). Before expression on the cell surface they are found intracellularly in association with a transmembrane glycoprotein of 31 Kd known as the gamma, or invariant chain which is known to be non-polymorphic (Charron and Mc Devitt, 1979; Kvist *et al*, 1982). The invariant chain may facilitate assembly and/or transport of the class II α and ß chains to the cell surface (Cresswell, 1987).

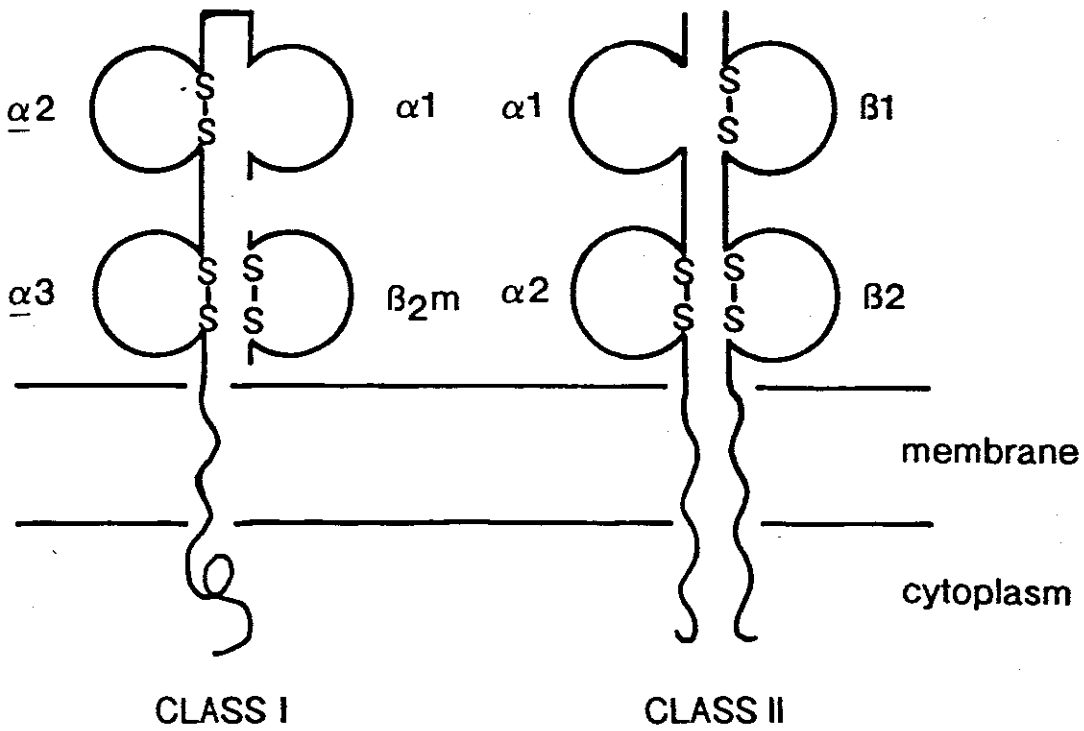

Figure 1.2. **Schematic representation of MHC class I and class II molecules. S-S indicates disulphide bridges. α_1, α_2, α_3 and $ß_1$ and $ß_2$ indicate domains corresponding to the exon of the genes and $ß_2$ indicates the $ß_2$-microglobulin chain.**

The class II molecule is composed of two different polypeptide chains: a heavy α chain with a molecular weight of ± 32-34 Kd and a light, ß chain with a molecular weight of ± 29-32 Kd (Jones, 1978; Figure 1.2.).

The MHC class I and class II molecules are structurally similar, differing mainly in the connectivity of the extracellular domains. In HLA-class II molecules, both α and ß chains are inserted into the membrane and associate with each other in a non-covalent fashion to form an α/ß heterodimer. The HLA-class II molecule contains two immunoglobulin-like external domains similar to the HLA-class I α2 and α3 domains. The first, or N-terminal domain is the site of most of the variability found in HLA-class II molecules and contains regions of variability that alternate with invariant regions (Cresswell, 1987).

MHC class II molecules have an immunoregulatory role. These cell-surface glycoproteins present fragments of protein antigens (or peptides) to thymus-derived lymphocytes (T-cells; Nagy *et al*, 1981). Nucleotide sequence polymorphism in the genes that encode the MHC class II products, determines the specificity of the immune response and is correlated with the development of autoimmune diseases (Todd *et al*, 1988). Helper TCRs recognize peptide fragments of foreign proteins only when they are bound to a self MHC class II molecule (Grey and Chestnut, 1985). The T-cell is both peptide-specific and MHC-restricted in that it recognises peptide only when bound to a particular class II molecule (Goronzy *et al*, 1986). Immune responsiveness is determined by the array of these highly polymorphic class II gene products.

1.2.2. Polymorphism at the MHC loci

The HLA system is extremely polymorphic - more than any other currently known human polymorphism (Gorski *et al*, 1984; Kappes *et al*, 1984). The level of heterozygosity at each locus is very high and is approximately 90% for both the A and B loci (Baur and Danilovs, 1980). The number of nucleotide differences between alleles is unusually high. Polymorphic alleles persist in the population for tens of millions of years (Erlich and Gyllensten, 1991).

In the most recent HLA nomenclature report, 59 HLA alleles have been defined for the HLA-A locus, 117 for the -B locus, 36 for the -C locus and 155 for the -DR locus (Bodmer *et al*, 1995) and new alleles are constantly being discovered. There is no single very commonly occurring allele - rather there are several alleles which occur frequently. All the alleles of the HLA loci have not as yet been determined. There are a number of substantial differences in allele frequencies for the HLA loci in the major population groups (Terasaki, 1980).

The HLA polymorphism also extends to the genes coding for 4 different complement proteins where different alleles have been identified for C 2, Bf, C 4A and C 4B. The mechanism(s) for such extensive polymorphism within the MHC system is unknown, but two main hypotheses to explain this phenomenon have been proposed (Alper *et al*, 1986).

One theory, the "random drift hypothesis", explains that polymorphism is the outcome of random variations of basic gene structure that has a certain frequency of distribution by chance alone within the population.

The other hypothesis, "natural selection", proposes selection among genes for mono- or polymorphism.

The "recombinant fraction", which is the measure of the homologous section exchanged by crossing over and creating new combinations of alleles in the products of meiosis between HLA-DR and -B loci is 0.010, between HLA-B and -C loci, 0.001 and between the HLA-C and -A loci, 0.007 (Robson and Lamm, 1983). Some of the alleles of the HLA loci tend to occur together on the same chromosome more often than is expected by chance alone. Such non-random association is termed "linkage disequilibrium" and its extent is measured by the difference under random association between the observed haplotype frequency and the expected haplotype frequency (Baur and Danilovs, 1980). The expected haplotype frequency is the product of the separate allele frequencies of the antigens. Each population tends to have its characteristic group of alleles which show significant "pairwise" linkage disequilibrium.

The tight linkage disequilibrium (Section 1.9.) between the DR and DQ subregions of class II, decreases the observed polymorphism of the DRB-DQA-DQB haplotype.

Linkage disequilibrium between class I loci is less marked and exists with certain combinations of A and B alleles which appear to be characteristic of certain populations (Riley and Olerup, 1992).

Genetic distance analysis revealed that a large number of the HLA alleles in contemporary populations were extremely ancient and predate speciation. It has been found that a considerable degree of DRB1 polymorphism almost certainly predates the separation of the lineages leading to humans and chimpanzees (Klein *et al*, 1990). This implies that the transition from one species to the next must have occurred *via* populations of some considerable size and also that the human species must have maintained a minimum population size (± 10 individuals) at all stages subsequent to its emergence (Klein *et al*, 1990).

The human population contains a considerably restricted set of possible allele combinations at the DRB1, DQB1 and DQA1 loci. It has been established that polymorphism at the DQA1 locus reflects the maintenance over 5 million years of ancient allelic types - eg. α1, α3, α4 or DQA1*01, *03 and *04 - *06, with minimal changes in the protein sequence. Only 1 amino-acid residue has changed on average, in the evolution of the DQA1*04 - *06 alleles in humans, chimpanzees and gorillas from an inferred ancestral DQA4 sequence (Erlich and Gyllensten, 1991). The DQA1*0201 allele is found only in humans and is situated on the DRB1*07 haplotype (Erlich and Gyllentein, 1991). The strong linkage disequilibrium exhibited by DR and DQ could be due to either one or a combination of three mechanisms:

1. Molecular barriers to recombination between some haplotypes.

2. Selective constraints on the possible combinations of class II alleles based on the peptide-binding and presentation and/or on the MHC determined repertoire of expressed TCRs.

3. A small number of haplotype combinations during human evolution (Erlich and Gyllensten, 1991).

1.2.3. HLA-D region

The HLA-class II genes are closely distinguished from those controlled by the HLA-A, -B and -C loci, are clustered within sub-regions and are arranged with increasing distance from the centromere, in the order DP, DQ and DR (Carrol *et al*, 1987). Products of the HLA-D region are highly polymorphic, although the degree of polymorphism in the subsets of expressed HLA-class II alleles is quite different between subregions (Carrol *et al*, 1987). The α and ß chain gene products from the DQ subregion are polymorphic - i.e. there are multiple alleles at each locus in the population. These gene products code for the DQ alleles DQA1*0101 - *0601 and DQB1*0201 - *0609. The DR subregion contains four functional ß chain genes, designated DRB1, DRB3, DRB4 and DRB5 which are polymorphic. There is extensive polymorphism in the DRB1 gene which encodes the DRB1*0101 - *1418 alleles, the DRB3 gene encodes the DRB3*0101 - *0301 alleles, the DRB4 gene encodes DRB4*0101 - *0103 alleles and DRB5 gene encodes DRB5*0101 - *0203 alleles. The DRA1 gene is non-polymorphic. There is also apparent conservation of the other expressed DRB genes, with limited polymorphism of the DRB3 gene.

The genes within the DR and DQ subregion are very closely linked and are almost always inherited together as a unit. DRB1, DQB1 and DQA1 alleles form stable haplotypes in the population (Figure 1.3.).

There are three major hypervariable regions in the DRB chain. These are designated HV1, HV2 and HV3. The third hypervariable region is the major site of sequence difference that distinguishes the various DRB1*04 subtypes.

Polymorphism exists in both expressed genes of the DQ subregion and there is a high degree of polymorphism in both expressed genes of the DP subregion (Auffray *et al*, 1984).

The DRB1*04 allele, encoded by the DRB1 gene, is always found in association with DRB4*01 - encoded by the DRB4 gene, DQA1 and DQB1*03 alleles - encoded by the DQA1 and DQB1 genes, forming the typical DRB1*04, DRB4*01, DQA1*03 and DQB1*03 haplotypes. The variability between different DRB1*04 haplotypes is located in the DRB1, DRB4, DQA1 and DQB1 gene (Gregersen *et al*, 1986; Lechler, 1986).

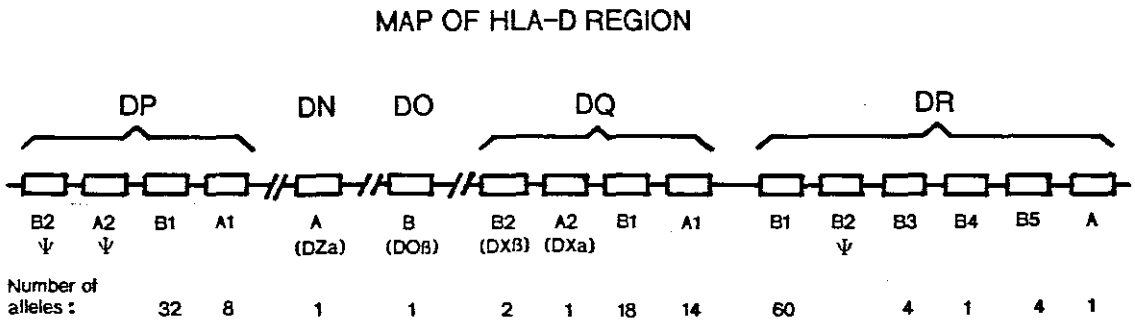

Figure 1.3. **Schematic representation of the HLA-class II region and the number of known alleles at different loci (Mach *et al*, 1995).**

1.2.4. Genomic organisation of DR genes

DR genes are encoded on the short arm of chromosome 6, in the HLA-class II region of the human MHC (Trowsdale *et al*, 1991). The number of DRB genes varies from 2 to 5 between haplotypes. The DRA gene which codes for the α chain of the DR molecule is invariant. The DRB gene codes for the ß chain of the DR molecule and exists in multiple copies, probably having arisen by gene duplication and/or recombination events. Translated DRA and DRB mRNA generate α and ß chains that form glycosylated α/ß cell surface heterodimers. Each chromosome expresses 2 DRB genes and their products can heterodimerize with the DRα chain. This subregion is unique amongst the class II genes in that the organization of the DRB genes is not identical for all haplotypes. The DRB genes have been classified into 9 different loci designated DRB1, DRB2, DRB3, DRB4, DRB5, DRB6, DRB7, DRB8, DRB9 and a single DRA1 gene. The DRB2, DRB6, DRB7, DRB8 and DRB9 are pseudogenes (Gorski *et al*, 1985). Some human chromosomes carry only 1, 2 or 3 DRB loci. The DRB3 and DRB4 genes show marked differences in the number and relative arrangement of their alleles (Anderson *et al*, 1987; Bodmer *et al*, 1987; Figure 1.4.).

1.2.4.1. The DRB1 locus

The DRB1 is the main functional locus of the DRB region. In some haplotypes it is the only functional DRB locus and is located at the centromeric end of the DR region, with its single DRA gene at the telomeric, or distal end. Approximately 60 alleles have been identified at the DRB1 locus. These alleles can be divided into 2 major lineages or DRB1 polymorphism, on the basis of sequence similarity with alleles belonging to the same lineage being more similar to each other than to alleles of different lineages. The representatives of the 2 major lineages are DRB1*03 and *04 alleles. The DRB1*03 lineage consists of DRB1*01, *08, *10, *11, *12, *13, *14, *15 and *16 alleles, in addition to the DRB1*03 allele. Although it occupies a separate locus, the DRB3 gene is part of this lineage.

Within this lineage, DRB1*03, *11, *12, *13, *14 and *08 alleles are more closely related to one another than they are to other members of the lineage (Klein *et al*, 1991).

The DRB1*04 allelic lineage consists of the DRB1*07 and DRB1*09 alleles in addition to DRB1*04 alleles. These 3 groups of alleles are united in a single lineage in exon 6, where most substitution occurs which are shared by all DRB1*04, DRB1*07 and DRB1*09 sequences, but are absent in all other sequences. In exon 3, 4 and 5 there appears to exist a weaker clustering of the three groups of sequences. In exon 2, the sequences of DRB1*04, DRB1*07 and DRB1*09 are divergent from one another. The exon 2 sequences of the DRB1*09 alleles are closely related

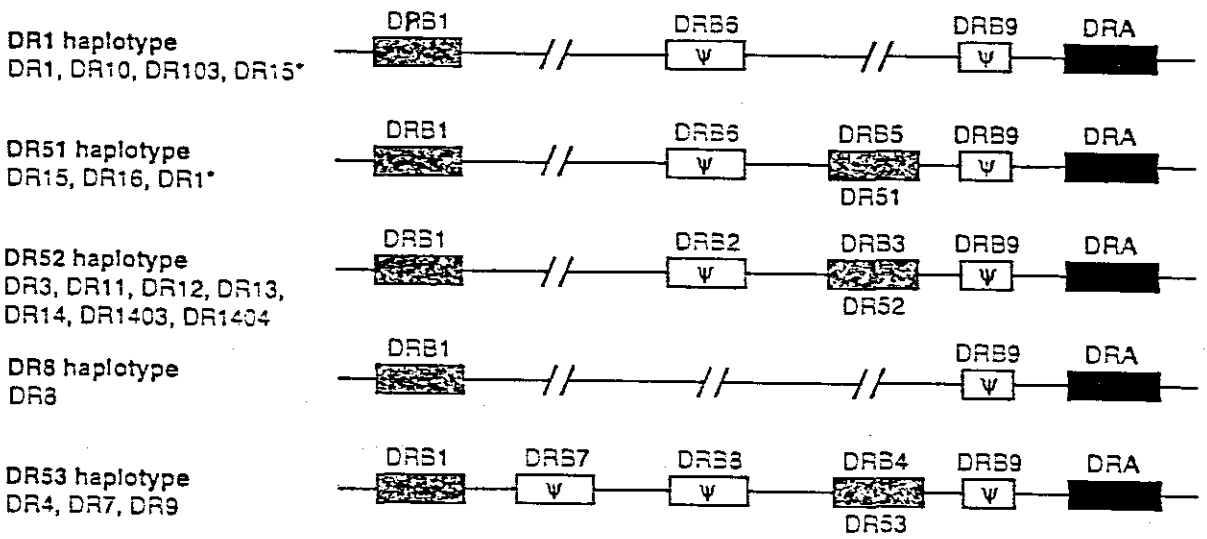

Figure 1.4. **Schematic representation of the 5 main DR haplotypic groups. Organization of the DR1 and DR8 haplotypes are inferred from the known genomic organization of the DR51, DR52 and DR53 haplotypes. The haplotypic designations are indicated to the left. DRA and DRB genes are shown as boxes. The expressed serological specificities are indicated below the haplotypic group. Presumed allelic genes are shown in identical shades. Brackets in the lines are introduced in order to vertically align putative allelic genes. Ψ denotes DRB pseudogene. * denotes rarely observed serological specificities (Anderson *et al*, 1994).**

to exon 2 sequences of alleles at the DRB5 locus, which suggest that either one or the other of the two loci is the product of recombination. However, the exon 6 sequences of the DRB5 alleles are very different from those of the DRB1*09 alleles (Klein *et al*, 1991).

1.2.4.2. The DRB2 and DRB6 loci

The DRB2 gene is a pseudogene of the DRB1*03 haplotype (Rollini *et al*, 1985). This pseudogene lacks exon 2 and has a characteristic 20 nucleotide deletion in exon 3 that destroys the correct reading frame (Anderson *et al*, 1987).

The DRB6 gene is a pseudogene of the DRB1*01, DRB1*15, DRB1*16 and DRB1*1001 haplotypes and lacks exon 1 (Klein *et al*, 1991).

1.2.4.3. The DRB3 locus

The DRB3 locus is present on DRB1*03, *11, *12, *13 and *14 haplotypes (Rollini *et al*, 1985). A fragment of the DRB3 gene is also present in the DRB1*08 haplotype (Klein *et al*, 1991). Four alleles have been identified at this locus. The closest relatives among the DRB sequences are the genes of the DRB1*03 lineage. The DRB1*03 allele and DRB3 gene apparently arose by duplication from a common ancestral gene (Rollini *et al*, 1985).

1.2.4.4. The DRB4 locus

The DRB4 locus is present in DRB1*04, *07 and *09 haplotypes and is virtually monomorphic, i.e. one common allele and a few rare variants (Klein *et al*, 1991). The DRB4 locus is closely related to DRB1 genes DRB1*15, *16 and *13 with which it has apparently shared a common ancestor.

1.2.4.5. The DRB5 locus

The DRB5 locus is present in DRB1*15 and *16 haplotypes (Klein *et al*, 1991). There are only 4 alleles identified at this locus which show decreased allelic polymorphism to that of the DRB1 locus (Andersson *et al*, 1991).

1.2.4.6. The DRB7 locus

The human DRB7 locus comprises a pseudogene containing defective splice junctions, premature termination signals and a frameshift mutation (Anderson *et al*, 1987). The DRB7 pseudogene is present in the haplotypes DRB1*04, *07 and *09.

1.2.4.7. The DRB8 locus

The DRB8 locus comprises a pseudogene without exons 1 and 2 (Anderson *et al*, 1987). There is a relatively large distance from DRB8 gene to other DRB genes on the chromosome. DRB8 locus is present on DRB1*04, *07 and *09 haplotypes.

1.2.4.8. The DRB9 locus

The DRB9 locus seems to be present in all haplotypes and constitutes a free DRB exon 2. This locus is located centromeric to the DRA gene. The DRB5 gene is also located centromeric to DRB9 gene (Andersson *et al*, 1991).

1.2.5. The DQ genes

The DQA1 and DQB1 genes are 11 Kb apart in a tail to tail fashion and code for the DQA and DQB polypeptide chains. The products of both DQA and DQB genes show sequence variation at their 3'ends some of which are due to alternate splicing and the use of alternate poly-A addition sites (Moriuchi *et al*, 1985). Differently sized mRNA are produced which vary significantly between alleles, but no functional role has yet been attributed to any of this variation (Kappes and Strominger, 1988).

There is no evidence for the expression of DQA2 and DQB2 genes (Korman *et al*, 1985). In contrast to DR, both DQA and DQB chains have been shown to be polymorphic (Auffray *et al*, 1984).

1.2.6. The DP genes

The DP subregion is composed of two α genes, DPA1 and DPA2 and two ß genes, DPB1 and DPB2 (Trowsdale *et al*, 1984; Servenius *et al*, 1984).

The DPA1 and DPB1 genes are expressed while DPA2 and DPB2 are pseudogenes (Gorski *et al*, 1984; Kappes *et al*, 1984; Servenius *et al*, 1984, Trowsdale *et al*, 1984). Polymorphism has been detected in the DP α chain and DP ß chain (Gorski *et al*, 1984, Kappes *et al*, 1984; Ando *et al*, 1986).

1.2.7. DO and DN genes

DOB and DNA genes have been mapped in the HLA-D region between DP and DQ (Tonnelle *et al*, 1985; Trowsdale and Kelly, 1985). There is no evidence that these two genes are composed of an α/β pair (Tonnelle *et al*, 1985).

1.3. INHERITANCE OF HLA MOLECULES

The HLA genes are inherited as a co-dominant trait. A haplotype consist of a set of HLA genes and is inherited from one parent (Ceppellini *et al*, 1967). Each parent contributes one haplotype, which are usually passed unaltered on to the offspring. During meiotic division, however, exchange of chromosomal segments, "crossing-over", may occur resulting in recombination between genes which are far apart. The closer 2 genes are on any particular chromosome, the less likelihood there is that crossing-over will occur (Hardy *et al*, 1986). The distance between two genes on a chromosome can be measured using recombination frequency, usually expressed as centiMorgans (cM; 1% recombination frequency = 1 cM; Ragoussis *et al*, 1986).

1.4. DISTRIBUTION OF HLA ALLELES IN DIFFERENT POPULATIONS

The HLA system is extremely polymorphic and the majority of alleles occur with different frequencies in any given population (Bodmer and Bodmer, 1978). For example, DRB1*15, DRB1*16, DQB1*05 and DQB1*06 are found in all populations with high frequencies, while DRB1*03 and DRB1*13 are almost absent from Orientals.

At the XIth International Histocompatibility Workshop (IHW; DNA component) in 1991, 1,300 subjects were typed for HLA-DR, -DQ and -DP alleles using DNA based methods. Results presented at the Workshop indicated differences in allele frequencies between different populations and even between the subjects living in the same geographical area, although their ethnic origin was the same. The differences in allele frequencies were earlier attributed to possible genetic drift, natural selection and founder effects (Ryder *et al*, 1981). The frequencies of HLA haplotypes among different populations had previously been shown to be highly diverse (Bodmer and Bodmer, 1978).

1.5. HLA AND DISEASE ASSOCIATION

HLA association with disease was first demonstrated in studies with mice when it was shown that virally induced leukemia was associated with the mouse MHC H-2 type (Lilly, 1966).

An association between spondylotropathies and HLA-B27 has been described (Ryder *et al*, 1981) and more than 50 diseases have been reported recently to have an association with one or more HLA alleles (Buus, 1986).

It has been known for some time that the HLA region of chromosome 6 is associated with certain diseases. The nature of the mechanism underlying the observed association between HLA alleles and disease has been widely discussed. Some of the data available on HLA and disease association shows increased frequencies of certain HLA alleles in groups of patients as compared with a sample of normal healthy individuals.

The strongest association reported is that of ankylosing spondylitis with HLA-B27 (relative risk of approximately 70) which was estimated from pooled data on Caucasian patients (Ryder *et al*, 1981).

It has been observed that the diseases that are associated with HLA alleles do not show simple Mendelian segregation in families, have a very weak or no effect on reproducing and are of unknown etiology. Heterogeneity is another important characteristic of these diseases (Tiwari and Terasaki, 1985).

Environmental factors such as exposure to viruses, determine whether individuals with disease susceptibility will contract the disease. If the association is due to an undetected allele at the closely linked locus, then this allele may occur at a much lower frequency then the one detected at the presently known HLA-class I and -class II loci (Mc Devitt and Bodmer, 1974; Bodmer and Bodmer, 1978).

Two possibilities exist to explain HLA disease associations. Disease susceptibility is the direct result of the presence of a particular HLA allele(s). Alternatively, the association of a particular allele(s) with a disease is the result of linkage disequilibrium between the allele(s) and the allele at a nearby locus which confers susceptibility to the disease. The most likely candidate(s) for these "disease" loci are "immune response" genes (Ir) which are well documented in mouse studies (Breedveld and Trentham, 1987). While a large number of individuals with a disease may all possess the same HLA allele, the majority of individuals with this allele do not have one of the HLA associated diseases (Thomson *et al*, 1976). This observation does not rule out the possibility that it is the allele itself which predisposes the disease. The allele may represent a heterogeneous class. The observation of cases of multiple allele association with a disease, which are in linkage disequilibrium in the general population is basically compatible with both predisposition due to the allele itself and predisposition due to a linked "disease" gene (Thomson *et al*, 1976; Thomson, 1977).

It is sometimes observed that along with significant increases in the frequency of one or more alleles with a disease, there are significant decreases in frequency of some other alleles. The HLA alleles A5, B7 and DRB1*15 and *16 for example, are significantly decreased in

Caucasoid individuals with IDDM (Dauset and Svejgaard, 1977; Svejgaard *et al*, 1980) and the alleles A2 and B12 are decreased in Caucasoids with MS.

Diseases associated with HLA alleles involve a person's genetic as well as environmental factors that together might influence the susceptibility of a person to a specific disease. The term "association" is used to make a distinction between association and linkage. "Association" refers to the relationship between two separate traits or findings, whereas "linkage" can only be identified by family studies in more than one generation to determine if certain traits are transmitted together. Most diseases studied by MHC are associated and no linkage has been demonstrated.

1.5.1. Involvement of the HLA molecule as a receptor for pathogens

Haspel *et al* (1977), reported the interaction of HLA alleles with measles virus antigens. A large amount of virus antigens inserted into the membranes remained physically independent from HLA alleles; the HLA alleles neither served as the receptors for measles virus nor were incorporated into the maturing virus particle.

The HLA molecule may act as a receptor for pathogenic organisms (eg. viruses). It is believed that the HLA-B27 molecules on the surface of lymphocytes may be the receptor for some organisms responsible for ankylosing spondylitis, Reiter's syndrome and other B27-associated diseases (Tiwari and Terasaki, 1985).

1.5.2. Involvement of genes closely linked with the HLA complex

Tiwari and Terasaki (1985) suggested that HLA alleles are not involved in the causation of the "disease susceptibility genes" that are very closely linked with the HLA complex. They assumed that association of a disease with HLA alleles in a population might be caused by linkage disequilibrium between the HLA alleles and the alleles for the disease susceptibility genes (Tiwari and Terasaki, 1985).

DRB1*15 and *16 are associated with MS where an MS susceptibility gene is very closely linked with a DR allele. The disease susceptibility allele at this locus is in linkage disequilibrium with the DR allele of the neutral marker for an MS susceptibility allele (Tiwari and Terasaki, 1985).

It has been postulated that genetic factors not linked with HLA may be associated with disease susceptibility and that the disease may be heterogeneous and have more than one etiology (Bodmer and Bodmer, 1978).

1.5.3. Role of T-cell in disease association

Evidence to support the role of T-cells in the progression of joint destruction is well documented:

i) CD4 cells are the dominant T-cell found in pannus (synovial thickening; Panayi *et al,* 1992).

ii) T-cells express several activation markers (Panayi *et al,* 1992).

iii) The disease improves with T-cell targeted intervention such as thoracic duct drainage, total lymphoid irradiation and cyclosporin (Panayi *et al,* 1992).

iv) Active arthritis is less severe in acquired immunodeficiency syndrome (AIDS) patients who have CD4 cytopenia (Solinger and Hess, 1990).

Various studies in animal models have emphasised the critical importance of T-cells in the pathogenesis of RA (Trentham *et al,* 1978; Holoshitz *et al,* 1983, 1984; Holmdahl *et al,* 1985; Helfgott *et al,* 1985). Activated T-cells in peripheral blood and synovial fluid in RA have been detected by the presence of HLA-class II alleles on their surface (Brumester *et al,* 1981; Searles *et al,* 1988; Miyasaka *et al,* 1988; Kirkham *et al,* 1989; Hirose *et al,* 1990).

Despite the presence of HLA-class II alleles, however, the proliferative capacity of RA associated T-cells is low (Kirkham *et al,* 1989; Hirose *et al,* 1990). T-cells from these patients respond poorly to mitogenic lectins, antibodies against TCR-CD3 antigen structure, soluble antigens and in the autogenous mixed lymphocyte reaction (Silverman *et al,* 1976; Pope *et al,* 1984, 1989; Lotz *et al,* 1986; Hovdenes *et al,* 1989).

Activation of T-cells in these patients produces lower levels of interleukin 2 (IL-2) and the addition of exogenous IL-2 only partially restores the cell proliferation (Pope *et al,* 1984; Lotz *et al,* 1986). The mechanism responsible for the suppressed activation of T-cell from these patients, is not known.

1.6. MECHANISM OF DISEASE PREDISPOSITION

The HLA associated diseases all have an autoimmune aetiology that has been either demonstrated or suggested. The mechanisms of HLA disease associations are largely unknown. Apart from C 2 deficiency and congenital adrenal hyperplasia (Steroid 21-monooxygenase deficiency) there is no evidence to date that any of the HLA-class II genes is the absolute cause of an autoimmune disease (Dupont *et al,* 1980).

Specific autoantibodies have been demonstrated in a number of diseases, eg. IDDM and Grave's disease and an autoimmune mechanism is suspected in a number of other HLA-associated

diseases, eg. MS and RA (Ryder *et al*, 1981; Svejgaard *et al*, 1980). There is a distinction between the role of HLA-class I determinants (-A, -B and -C locus products) and HLA-class II determinants (-DR, -DQ and -DP products). Class I molecules present antigens on target cells to cytotoxic T-cells. The function of T-lymphocytes is to recognize an incoming antigen with self HLA molecules of the antigen presenting cell (APC). This combined presentation is termed a "restriction phenomenon" (Svejgaard *et al*, 1980; Ryder *et al*, 1981; Figure 1.5.). HLA-class II molecules are primarily involved in presentation of antigens by macrophages and B-lymphocytes to helper T-cells (Ryder *et al*, 1981; Svejgaard *et al*, 1980).

A major locus affecting the Ir genes has been mapped to the MHC of a number of animal species.

Different class II alleles of the MHC have been mapped to the same position as the Ir genes (Ryder *et al*, 1981). It is possible that in some cases the HLA-class II DR allele may be directly involved in disease predisposition in humans, or at least that genes mapping closely to this locus (loci) are involved in cases showing strong HLA-DR association with a disease (Ryder *et al*, 1981). An HLA-associated disease does not show simple Mendelian segregation in predisposing individuals, but it may possess one inherited component with incomplete penetrance to disease in predisposing individuals, due to certain environmental factors.

Two methods - that of allele genotype frequencies among the diseased (Thomson and Bodmer, 1977) and the affected sib pair method (Cudworth and Woodrow, 1975; Bobrow *et al*, 1975; Thomson, 1983), have been described for some of the HLA-associated diseases (Thomson, 1981).

The above two methods assume that disease predisposition is due to a closely linked "disease gene" and not to the HLA alleles themselves. The basis of the "allele genotype frequencies among diseased" test is as follows:

If a "disease allele" is recessive, then a strong association of the disease with a particular allele implies a higher frequency of individuals homozygous for the allele amongst the diseased group, whereas for a dominant "disease allele", the majority of diseased individuals with this allele will be heterozygotes for the "diseased allele" (Thomson and Bodmer, 1977).

1.7. HLA-CLASS II ASSOCIATION WITH RA

A number of DRB genes of the HLA-class II region have been implicated in increasing susceptibility to RA. Analysis of families with multiple cases of RA demonstrated genetic susceptibility to RA. Haplotypic sharing in affected pairs of siblings directly demonstrates the linkage of a susceptibility gene to the HLA region (Khan *et al*, 1983; Nunez *et al*, 1984; Payami

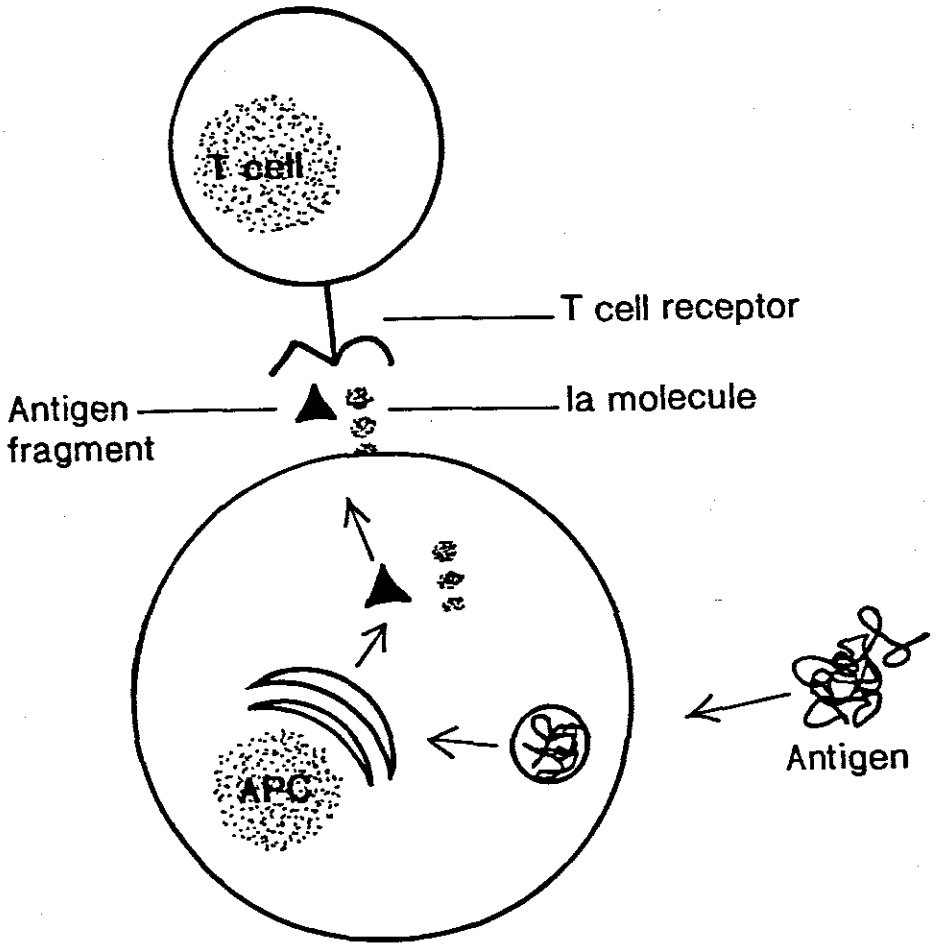

Figure 1.5. **Figure showing one of several possible models for the process of direct physical interaction between class II molecules and the TCR and/or antigen. Processed antigen fragments are presented to T-cell on the surface of an APC in association with Ia molecule. This structure may interact with TCR and result in T-cell activation (Gregersen *et al*, 1987).**

et al, 1986) There is an increased incidence of RA among monozygotic twins compared to dizygotic twins with an affected sibling (Lawrence, 1970). The precise genetic contribution of the MHC for susceptibility to RA has, however, remained obscure, despite these analysis.

1.7.1. DRB1*04

The DRB1*0401 - *0422 alleles have been defined by molecular typing of the DRB1 gene and these alleles can be expressed on at least 6 distinctly different haplotypes. Each allele corresponds to a particular epitope(s) which is present on molecules encoded within the HLA-class II region.

Allelic variation of HLA genes forms the genetic basis for the polymorphism crucial for self-recognition in the immune system and which controls several aspects of the immune response, as well as for allo-recognition. The DRB1*04 haplotypes differ from each other either in their DR genes, in DQ genes, or both (Nepom *et al*, 1987).

The DRB1*04 allele is also known as the DR4 antigen defined by HLA serological typing using standard National Institute of Health microlymphocytotoxicity technique (Terasaki and Clelland, 1964). The concept of DRB1*04 has been used throughout this study so as not to create a confusion with different terminologies, when referring to investigations of DR4 antigens and RA association, prior to molecular analysis of DNA. It should be noted that the subtypes of DR4 antigen cannot be defined by serological typing methods. The term DR4 thus refers to the broad typing of DRB1*04 allele and could represent any one of the DRB1*0401 - *0422 alleles that can be detected by molecular typing.

1.7.2. DRB1*04 and RA

The association between RA and DRB1*04 alleles has been well described in a number of populations studies in multiple ethnic groups. This consistent association observed in widely diverse populations strongly suggests that the DRB1*04 allele is at least one of the susceptibility allele (Tiwari and Terasaki, 1985). Tiwari and Terasaki (1985) showed a strong association between RA and DRB1*04 in particular. Analysis of individual DR and DQ alleles in RA showed that some DRB1*04 alleles have a strong correlation with disease susceptibility while others do not. Molecular analysis of MHC genes expressed on haplotypes in association with DRB1*04 reveals that at least 6 different alleles of the DRB1 gene and at least 6 different alleles of the DQB1 gene, occur on different DRB1*04 haplotypes.

Association of DRB1*0401 and RA has been well documented by numerous researchers (Mc Michael *et al*, 1977; Stastny, 1978; Thomsen *et al*, 1979; Young *et al*, 1984; Tiwari and

Terasaki, 1985; Martell *et al*, 1989, 1990; Cutbush *et al*, 1993). The DRB1*0101 and *1001 alleles have also been linked to RA, but to a lesser extent (Zoschke and Segall, 1986).

Winchester (1989) reported that disease susceptibility can be explained by the presence of a critical sequence of residues along the rim of the α-helix of the DRB1 gene from position 67-74 that is found in all of the susceptibility haplotypes. This association between the DRB1*04 allele and RA stands in favour of the concept that genes in the MHC are important factors in disease susceptibility (Tiwari and Terasaki, 1985).

Among the DRB1*04 subtypes, *0401, *0404 and *0405 alleles are found in the third variable region of the DRB1 chain (Gregersen *et al*, 1986). This finding was based on the hypothesis that conformations specified by this region, rather than other elements encoded by the haplotype, determine the molecular basis of disease susceptibility in RA. This is the so called "shared epitope hypothesis" (Gregersen *et al*, 1986).

The DRB1*0401 and *0404 alleles are prevalent in RA (Nepom *et al*, 1984; Tiwari and Terasaki, 1985). Patients positive for serum RF and patients with more severe polyarticular disease, appear in some studies to have increased expression of these two markers (Stastny, 1978; Young *et al*, 1984; Christiansen *et al*, 1984). One quarter to one third of affected individuals with RA, do not carry the DRB1*04 allele (Christiansen *et al*, 1984). By analysing so-called "extended" haplotypes linked to DRB1*04, large numbers of associated HLA-B locus alleles, Bf, C2 and C4 complement markers have been identified, suggesting that several different combinations of MHC alleles can associate with DRB1*04 in RA patients (Christiansen *et al*, 1984).

The DRB1 locus has more than 1 susceptibility allele for RA. It has been noted that the frequency of DRB1*0101 is markedly increased among the subset of RA patients who are not DRB1*04 positive (Winchester, 1986).

It seems that the DRB1*0401 and *0404 alleles in particular confer disease risk in RA, while other alleles like DRB1*0402 may represent DRB1 genes altered during evolution which may have lost their importance to RA susceptibility (Nepom *et al*, 1987). The DQB1*0301 and *0302 alleles, which are present in DRB1*04 haplotypes, are not directly implicated in RA, but may account for DRB1*04 association with other diseases, such as the association of DQB1*0302 with IDDM (Nepom *et al*, 1987).

1.8. DQ ASSOCIATION WITH AUTOIMMUNE DISEASE

DR, DQ and DP gene products can all present antigen to human CD4 positive T-cells although the DR restriction is much more marked (Altman *et al*, 1991). The DQ molecule appears to be inefficient as an antigen restriction molecule. DQ-restricted T-cell clones are rare and might have

a minor role in human immune responses, although disease association studies frequently implicate DQ, rather than DR alleles in predisposition to autoimmune disease (Altman *et al*, 1991, Zanelli *et al*, 1995). There is a strong linkage disequilibrium between particular DR and DQ alleles and poor understanding of the extent of DQA1 and DQB1 sequence diversity existed prior to molecular analysis of DNA being made possible. By making use of these relatively new techniques, however, DQ molecules can be analyzed with DQA1 and DQB1 restriction fragment length polymorphisms (RFLP), sequence-specific oligonucleotide probes (SSOPs) and sequencing. It has, however, become possible to analyse DQ more accurately.

Various examples of primary association of particular DQ alleles with diseases have been proposed (Table 1.1.). Although DQ and DR molecules are in strong linkage disequilibrium, higher relative risks occur for a particular DQ allele than for the linked DR genes, due to some DRB alleles occurring with alternative DQ alleles (Qvigstad *et al*, 1984). Some DQ alleles are associated with more than one DR allele and some ethnic groups show different DR and DQ alleles in linkage disequilibrium compared with others (Todd *et al*, 1988).

1.9. LINKAGE DISEQUILIBRIUM

An important characteristic of HLA alleles is the existence of linkage disequilibrium between the alleles of the loci.

In a randomly mating population, the Hardy-Weinberg equation predicts that the joint frequency of 2 alleles from two different loci will be the product of their individual gene frequencies (Baur and Danilovs, 1980). If the observed value of the joint frequency is significantly different from the expected frequency (the product of the individual allele frequencies) then the 2 alleles are said to be in "linkage disequilibrium". The estimated measure of linkage disequilibrium is the value of the observed minus expected haplotype frequency. In population genetics, the linkage disequilibrium is also denoted by delta.

1.9.1. Linkage disequilibrium between DQB1 and DQA1

There are 4 major allelic lineages at the DQA1 locus (*01, *0201, *0301 and *0401 - *0406) and five at the DQB1 locus (*02, *03, *04, *05 and *06). Strong linkage disequilibrium is found between the alleles at these loci in humans, with only 62% of the potential allelic combinations observed in Caucasian populations (Kwok *et al*, 1989).

DQA1*0101 is found exclusively with DQB1*0501 or *0503, indicating that this particular haplotype combination may have been conserved over millions of years. It has been observed that the protein encoded by DQA1*01 may not be able to pair with ß chains encoded by the DQB1*02 - *04 alleles (Kwok *et al*, 1989).

Table 1.1. DQ disease association.

Disease	Associated DQ allele	Resistance (R) / Susceptibility (S)
Narcolepsy	DQ1 serotype	S
Multiple sclerosis	DQA1*0102/DQB1*0602	S
Pemphigus vulgaris	DQA1*0101/DQB1*0503	S
Type I diabetes	DQA1*0301/DQB1*0302	S
	DQA1*0102/DQB1*0502	S
	DQA1*0501/DQB1*0201	S
	DQA1*0101/DQB1*0501	S
	DQA1*0102/DQB1*0604	S
	DQA1*0102/DQB1*0602	R
	DQA1*0103/DQB1*0603	R
Coeliac disease	DQA1*0501/DQB1*0201	S
Rheumatoid arthritis	DQA1*0301/DQB1*0301	S
Idiopatic nephropathy	Taq1 4.5 Kb DQA RFLP	S

(Altman *et al*, 1991)

CHAPTER 2

MATERIALS AND METHODS

2.1. THE POPULATIONS STUDIED

The socio-cultural history of South Africa is remarkable in that a large diversity of ethnic groups have settled in all parts of the country over several centuries and have managed to retain their cultural heritage and languages to the present day.

There are several predominant population groups living in South Africa at the present time: *viz.* South African blacks who can be further grouped by language and geography, mixed ancestry (coloured), Caucasoids, Asians and Indians. Language, culture and physical appearance possibly allow one to distinguish between these groups.

2.1.1. The western Cape black (Xhosa) population

It is believed that the Xhosa people residing in the western Cape today are descendants from black people in east Africa (Dart, 1937). Archeological and anthropological evidence indicates that these people moved south in a series of migrations between 500 and 1500 A.D., down through east Africa, across the great Zambezi river and into South Africa, *via* the Zimbabwean highlands or along the coast of Mozambique (Goodwin, 1937). The name "Xhosa" is derived from their chiefdoms and is similar to the custom employed in other black groups, e.g. "Zulu", "Tswana" and "Sotho" chiefdoms, which are used to describe their specific subunits in the South African black population. All these ethnic subgroups belong to the south-eastern linguistic division of Bantu (Jenkins *et al*, 1970). Of these chiefdoms, the Xhosa were selected to represent the black population in this study since they constitute the majority of black people living in the western Cape. They also form one the largest and most homogenous black chiefdoms in South Africa, accounting for approximately 26% of the South African blacks (Jenkins *et al*, 1970).

Of the Bantu-speaking chiefdoms, the Xhosa are the southernmost and belong to the Cape Nguni linguistic division of the Bantu. They reside primarily in the regions formally designated as the homelands of Ciskei and Transkei. They are presumed to have had the greatest contact with the Khoikhoi and San population groups.

The Xhosa selected for this study as a comparison group included normal, healthy volunteers employed at the Provincial Laboratory for Tissue Immunology, Cape Town, as well as random individuals working locally in the Cape peninsula.

The RA affected Xhosa population group comprised patients attending Rheumatoid Clinics at the Groote Schuur and Princess Alice Orthopaedic Hospitals. Patients were screened for RA during routine clinical assessments. Blood specimens from Xhosa RA affected patients were kindly supplied with permission of Professor O L Meyers, Rheumatoid Diseases Unit, Department of Medicine, Groote Schuur Hospital and the University of Cape Town.

2.1.2. The mixed ancestry (Cape coloured) population

The people of mixed ancestry in the Cape (Cape coloured) are an anthropologically distinct population. Their origin was comparatively recent and includes Southern African, Eastern and European elements (Botha, 1972). The gene-mixing between the Caucasoids and the Khoikhoi at the Cape and with the San, although to a lesser extent, commenced when the first Caucasians settled in the Cape (Jenkins *et al*, 1970; Botha, 1972).

The South-East Asian genetic mix commenced when the first slaves from Indonesia and old India, mainly Coromandel, Malabar and Bengal, arrived in the Cape. Slaves were also imported from Ceylon (Sri-Lanka) and from many islands of the Dutch East Indies (Indonesia; Botha *et al*, 1975). The Cape coloured population has approximately 34% Western European, 36% Southern African and 30% Asian genes (Botha, 1972).

The sample which constituted the comparison population for this study was made up of people of mixed ancestry (Cape coloured) and was obtained from random, healthy individuals employed in the Provincial Laboratory for Tissue Immunology, Cape Town and from workers employed locally in the Cape peninsula.

The Cape coloured RA affected population group comprised patients attending the Rheumatoid Clinic at Groote Schuur and Princess Alice Orthopaedic Hospitals. Patients were screened for RA during routine clinical assessments. Blood specimens from the Cape coloured RA patients were kindly supplied with permission of Professor O L Meyers, Rheumatic Diseases Unit, Department of Medicine, Groote Schuur Hospital and the University of Cape Town.

2.2 SPECIMEN PREPARATION

2.2.1. Specimen collection from RA affected and comparison populations

Blood specimens were collected from 25 RA affected Xhosa and 65 Cape coloured patients, all of whom were seropositive for RA. Blood specimens were collected from 94 healthy Xhosa and 114 Cape coloured individuals. Fresh whole blood was used throughout the present study. DNA extraction (Section 2.2.1.1.) proceeded as soon as specimens were received from the hospitals and specimens were kept on ice throughout transportation as well as during the DNA extraction

procedure. If a blood sample could not be processed within 48 hours, it was frozen at -20°C in an equal volume of cold lysis buffer (Appendix 6.1.1).

2.2.1.1. Extraction of DNA from whole blood

Blood specimens (± 10 ml) were collected into glass Vacutainer test tubes (Vac-U-test, Sterilab) containing 2 ml 5% EDTA. The EDTA whole blood solution was mixed with an equal volume (10 ml) of cold lysis buffer and any visible clots were disrupted with a glass homogenizer. The lysis buffer lyses red and white blood cells (RBC and WBC), respectively and releases cellular debris and nuclei into solution.

The solution was transferred to 50 ml polypropylene tubes and centrifuged at 4,500 rpm for 20 minutes at 4°C to pellet the nuclei. The supernatant was discarded and the nuclear pellet washed in 5 ml lysis buffer until the supernatant was clear and no haemolysis was observed.

The pellet was vortexed and suspended in 500 μl Saline-EDTA (Appendix 6.1.3.) and 50 μl 10% SDS (Appendix 6.1.4.). Twenty μl Proteinase K (Appendix 6.1.5.) was added, the tubes vortexed and incubated at 56°C overnight to release the nucleic acid into solution. The viscous, homogeneous solution was transferred into an Eppendorf tube. A salting-out method (Miller *et al*, 1988) was used to purify the DNA. One hundred and twenty five μl saturated sodium acetate was added to the tube and vortexed for 15 seconds to precipitate the protein which was pelleted by centrifugation at 10,000 rpm for 10 minutes at 4°C. The supernatant was decanted into an Eppendorf tube containing an equal volume (695 μl) of cold isopropanol to precipitate the DNA. The tube was vortexed and centrifuged at 10,000 rpm for 10 minutes at 4°C to remove excess salt. The DNA was washed twice in 1 ml 70% ethanol for 10 minutes at 4°C. The DNA pellet was dried briefly under vacuum in a speedvac concentrator (Savant) and dissolved overnight at 4°C in 1 ml sterile distilled H_2O.

A 1:20 dilution of an aliquot of the DNA solution was made and scanned from 220 nm to 300 nm on a Beckman DU 60 spectrophotometer to determine the concentration of DNA. A DNA solution of 1 ng/ml has an absorbance of 20 OD units at 260 nm. The DNA solution was diluted to a concentration of 100 ng/ml with sterile distilled H_2O and an aliquot was used for the PCR amplification (Section 2.3.1.).

2.2.2. Homozygous typing cells

Homozygous typing cells (HTCs) were used as positive and negative controls in each PCR amplification (Section 2.3.1.) and were obtained from cells grown in culture media from lymphocytes isolated from blood samples of individuals found by tissue typing techniques to be homozygous for specific alleles, namely DR and DQ. The majority of HTCs at the Provincial

Laboratory for Tissue Immunology were obtained while routinely screening families of patients requiring a genetically related donor for a bone marrow or renal transplant as well as from normal, healthy families which were tissue typed for the IHW. HTC phenotypes defined at the Provincial Laboratory for Tissue Immunology are listed in Table 2.1. and those obtained by exchange with other laboratories are listed in Table 2.2. Amplified DNA prepared from HTCs (Section 2.2.2.1.) was used as positive and negative controls in the dot-blot hybridization procedure (Section 2.3.3.).

2.2.2.1. Extraction of DNA from HTCs

The procedure for DNA extraction from HTCs was the same as that for whole blood collected into EDTA (Section 2.2.1.1.) except that lysis buffer was not used to lyse the cells. The cells were instead washed twice in phosphate buffered saline (PBS; Appendix 6.1.2.) to remove culture media.

2.3. DNA TYPING

2.3.1. DNA amplification

DNA samples isolated from whole blood (Section 2.2.1.1.) and from HTCs (Section 2.2.2.1.) were left briefly at room temperature and vortexed. Specific HTCs required for DRB generic, DR1 (DRB1) gene specific, DR2 (DRB1) gene specific, DR4 (DRB1) gene specific, DR52 associated group (DRB1), DR52 group (DRB3), DQA1 gene specific and DQB1 gene specific typing as indicated in Tables 2.3., 2.4, 2.5., 2.6., 2.7., 2.8., 2.9. and 2.10., were selected for PCR amplification. Strict attention to prevent sample contamination was paid at each stage of sample preparation for PCR. Disposable plastic gloves were worn and pipette tips (Gilson) were changed after the addition of each reagent or specimen.

PCR mixtures were constituted as follows: to appropriately labelled 0.5 ml Eppendorf tubes containing 45 μl standard PCR reaction mixture, [5 μl 10 x PCR buffer (Appendix 6.2.1.), 5 μl dNTP mix (2 mM of each deoxynucleotide triphosphate; Appendix 6.2.2.), 2 μl of each of the 2 primers (25 pmol), 0.2 μl (one unit) Taq DNA polymerase (Appendix 6.2.3.) per sample and 30.8 μl sterile distilled H_2O], 5 μl (0.1 μg) of DNA (extracted from whole blood or specific HTCs as required; Sections 2.2.1.1. and 2.2.2.1.) was added giving a total reaction volume of 50 μl. The Eppendorf tubes were briefly vortexed and centrifuged for 10 seconds at 10,000 rpm.

Mineral oil (50 μl) was layered on top of each sample to prevent evaporation. Amplification of the samples was performed in a DNA Thermal Cycler (thermocycler; Hybaid-Omni Gene) using a program specified by the protocol of the XIth IHW (DNA component) and appropriate to the specifications of the various primers.

Table 2.1. **HLA phenotypes of HTCs defined at the Provincial Laboratory for Tissue Immunology.**

Cell identification	Workshop number	Population group	HLA					
			A	B	C	DR	DQ	Dw
JGA		C	1,2	37,56	w1,w6	1	5	1
ZSI		C	2	7	w7	2	6	2
COX	10W90022	C	1	8	w7	17	2	3
BSMI		CC	2,28	7,70	w3,w7	4	8	4
MIS		CC	43	14	w8	4	8	4
RDA		CC	28,33	14,44	w7,w8	11	7	5
CLIMD		X	2,3	7,57	w4,w6	14	5	9
MST		AJ	3,26	38	w-	4	8	10
ARU		CC	2,28	14,47	w6,w8	7	9	11
RED		AJ	2	63	w7	4	8	13
PITOUT	10W9051	C	29	44	w-	7	2	17
OMW	10W9058	X	2	45	w-	13	6	18
NSI		X	28,34	70	w4	13	6	19
RSH	10W9021	Z	30,68	42	w2	w18	w4	RSH
BTI		X	30	42	w2	w18	w4	RSH
PJOH		CC	2,24	35,60	w4,w7	w12,x6	w7	PJOH
FJOH		CC	2,24	35,60	w4,w7	w12,x6	w7	JOH
BME		X	28,30	58	w6	w12,x6	w5	BME

C = SA Caucasoid

CC = Cape coloured

X = Xhosa

AJ = Ashkenazi Jew

Z = Zulu

Table 2.2. HLA phenotypes of HTCs obtained from laboratories other than the Provincial Laboratory for Tissue Immunology.

Cell identification	Workshop Number	Origin	HLA					
			A	B	C	DR	DQ	DW
QBL	10W9020	Van Rood	26	18	w5	17	2	3
LOO	10W9018	Dawkins	3,24	18	w5	17	2	3
BSM	10W9032	Van Rood	2	62	w3	4	3	4
EK	10W9054	Thorsby	2	44	w5	14	1	9
BGE	9W1201	Brautbar	1,2	52	w-	2	1	12
CRI	10W9064	Layrisse	2	62	w9	14	7	16
FJO		Betuel	2,3	7,39	w7	16	5	21
REM	9W1803	Layrisse	2	51	w-	16	7	22
HAG	9W1802	Wernet	2	41	w7	13	7	HAG
HERLUF	9W1503	Svejgaard	2	35,44	w4,w5	12	7	DBG

(Oudshoorn, 1989)

Table 2.3. HTC controls used for DRB generic typing.

DRB SSO	Positive (+) or negative (-) control			
	JGA DRB1*0101	CONSMAZ DRB1*0102	COX DRB1*0301 DRB3*0101	BTI DRB1*0302 DRB3*0101
1001	+	+	-	-
2801	+	+	-	-
	RDA DRB1*1101 DRB3*0101;*0202	JVM DRB1*1102 DRB3*0202	JGA DRB1*0101	CONSMAZ DRB1*0102
1003	+	+	-	-
5703	+	+	-	-
	ARU DR7 DRB4*0101	DBB DR7 DRB4*0101	JGA DRB1*0101	CONSMAZ DRB1*0102
1006	+	+	-	-
2803	+	+	-	-
	DKB DR9 DRB4*0101	DKB DR9 DRB4*0101	JGA DRB1*0101	CONSMAZ DRB1*0102
1007	+	+	-	-
2804	+	+	-	-
	HERLUF DRB1*1201 DRB3*0202	MAB DRB1*1201 DRB3*0202	JGA DRB1*0101	CONSMAZ DRB1*0102
2802	+	+	-	-
1005	+	+	-	-
	COX DRB1*0301 DRB3*0101	BTI DRB1*0302 DRB3*0101	CONSMAZ DRB1*0102	MST DRB1*0402 DRB4*0101
1010	+	+	-	-
8601	+	+	-	-
	NSI DRB1*1302 DRB3*0301	NSI DRB1*1302 DRB3*0301	JGA DRB1*0101	BTI DRB1*0302 DRB3*0101
1011	+	+	-	-
8603	+	+	-	-
	MIS DRB1*0401 DRB4*0101	MST DRB1*0402 DRB4*0101	JGA DRB1*0101	CONSMAZ DRB1*0102
3708	+	+	-	-
2810	+	+	-	-

Table 2.3. (*continued*)

DRB SSO	Positive (+) or negative (-) control			
	HERAN DRB1*0405;*1602(2HER) DRB4*0101 DRB5*0101	ZSI DRB1*1501 DRB5*0101	JGA DRB1*0101	CONSMAZ DRB1*0102
2805	+	+	-	-
3709	+	+	-	-
	FJO2 DRB1*1601 DRB5*02	RML DRB1*1602 DRB5*02	JGA DRB1*0101	CONSMAZ DRB1*0102
1009	+	+	-	-
7011	+	+	-	-
	FJO2 DRB1*1601 DRB5*02	RML DRB1*1602 DRB5*02	JGA DRB1*0101	JKO DRB1*0101
3707	+	+	-	-
8602	+	+	-	-
	SHARKEMP DR10 DRB1*1601	MIS DRB1*0401 DRB4*0101	JGA DRB1*0101	CONSMAZ DRB1*0102
1008	+	-	-	-
1004	-	+	-	-

Table 2.4. HTC controls used for DR1 (DRB1) gene specific typing.

DRB SSO	Positive (+) or negative (-) control			
	JGA DRB1*0101	CONSMAZ DRB1*0102	COX DRB1*0301 DRB3*0101	LOO DRB1*0301 DRB3*0202
2801	+	+	-	-
	JGA DRB1*0101	CONSMAZ DRB1*0102	COX DRB1*0301 DRB3*0101	LOO DRB1*0301 DRB3*0202
3701	+	+	-	-
	JGA DRB1*0101	CONSMAZ DRB1*0102	COX DRB1*0301 DRB3*0101	LOO DRB1*0301 DRB3*0202
5701	+	+	-	-
	JGA DRB1*0101	CONSMAZ DRB1*0102	COX DRB1*0301 DRB3*0101	LOO DRB1*0301 DRB3*0202
7001	+	+	-	-
	JGA DRB1*0101	TERND DRB1*0103	COX DRB1*0301 DRB3*0101	LOO DRB1*0301 DRB3*0202
8601	+	+	-	-
	CONSMAZ DRB1*0102	CONSMAZ DRB1*0102	COX DRB1*0301 DRB3*0101	LOO DRB1*0301 DRB3*0202
8602	+	+	-	-
	TERND DRB1*0103	TERND DRB1*0103	COX DRB1*0301 DRB3*0101	LOO DRB1*0301 DRB3*0202
7007	+	+	-	-

Table 2.5. HTC controls used for DR2 (DRB1) gene specific typing.

DRB SSO	Positive (+) or negative (-) control			
	ZSI DRB1*1501 DRB5*0101	BGE DRB1*1502 DRB5*0102	JGA DRB1*0101	COX DRB1*0301 DRB3*0101
3702	+	+	-	-
	ZSI DRB1*1501 DRB5*0101	BGE DRB1*1502 DRB5*0102	JGA DRB1*0101	COX DRB1*0301 DRB3*0101
5706	+	+	-	-
	ZSI DRB1*1501 DRB5*0101	BGE DRB1*1502 DRB5*0102	JGA DRB1*0101	COX DRB1*0301 DRB3*0101
7011	+	+	-	-
	ZSI DRB1*1501 DRB5*0101	ZSI DRB1*1501 DRB5*0101	JGA DRB1*0101	COX DRB1*0301 DRB3*0101
8603	+	+	-	-
	FJO2 DRB1*1601 DRB5*02	RML DRB1*1602 DRB5*02	JGA DRB1*0101	COX DRB1*0301 DRB3*0101
8601	+	+	-	-
	FJO2 DRB1*1601 DRB5*02	FJO2 DRB1*1601 DRB5*02	JGA DRB1*0101	COX DRB1*0301 DRB3*0101
7002	+	+	-	-
	RML DRB1*1602 DRB5*02	RML DRB1*1602 DRB5*02	JGA DRB1*0101	COX DRB1*0301 DRB3*0101
7003	+	+	-	-

Table 2.6. HTC controls used for DR4 (DRB1) gene specific typing.

DRB SSO	Positive (+) or negative (-) control			
	BSM DRB1*0401 DRB4*0101	MIS DRB1*0401 DRB4*0101	MST DRB1*0402 DRB4*0101	RED DRB1*0403 DRB4*0101
7005	+	+	-	-
	BSM DRB1*0401 DRB4*0101	MIS DRB1*0401 DRB4*0101	MST DRB1*0402 DRB4*0101	RED DRB1*0403 DRB4*0101
8601	+	+	-	-
	BSM DRB1*0401 DRB4*0101	MIS DRB1*0401 DRB4*0101	HAS15 DRB1*0405 DRB4*0101	HAS15 DRB1*0405 DRB4*0101
5701	+	+	-	-
	BSM DRB1*0401 DRB4*0101	MIS DRB1*0401 DRB4*0101	KT2 DRB1*0406 DRB4*0101	KT2 DRB1*0406 DRB4*0101
3704	+	+	-	-
	MST DRB1*0402 DRB4*0101	MST DRB1*0402 DRB4*0101	BSM DRB1*0401 DRB4*0101	RED DRB1*0403 DRB4*0101
7007	+	+	-	-
	MST DRB1*0402 DRB4*0101	MST DRB1*0402 DRB4*0101	BSM DRB1*0401 DRB4*0101	HAS15 DRB1*0405 DRB4*0101
8603	+	+	-	-
	RED DRB1*0403 DRB4*0101	RED DRB1*0403 DRB4*0101	BSM DRB1*0401 DRB4*0101	MST DRB1*0402 DRB4*0101
7006	+	+	-	-
	RED DRB1*0403 DRB4*0101	RED DRB1*0403 DRB4*0101	BSM DRB1*0401 DRB4*0101	MST DRB1*0402 DRB4*0101
7001	+	+	-	-
	HAS15 DRB1*0405 DRB4*0101	HAS15 DRB1*0405 DRB4*0101	BSM DRB1*0401 DRB4*0101	MST DRB1*0402 DRB4*0101
5702	+	+	-	-
	KT2 DRB1*0406 DRB4*0101	KT2 DRB1*0406 DRB4*0101	BSM DRB1*0401 DRB4*0101	MST DRB1*0402 DRB4*0101
3701	+	+	-	-

Table 2.7. HTC controls used for DR52 associated group (DRB1) gene typing.

DRB SSO	Positive (+) or negative (-) control			
	COX DRB1*0301 DRB3*0101	LOO DRB1*0301 DRB3*0202	JGA DRB1*0101	BSM DRB1*0401 DRB4*0101
2807	+	+	-	-
3703	+	+	-	-
	BTI DRB1*0302 DRB3*0101	RSH DRB1*0302 DRB3*0101	JGA DRB1*0101	BSM DRB1*0401 DRB4*0101
2809	+	+	-	-
7004	+	+	-	-
	MAD DRB1*0801	MAD DRB1*0801	JGA DRB1*0101	BSM DRB1*0401 DRB4*0101
5702	+	+	-	-
7002	+	+	-	-
	JVM DRB1*1102 DRB3*0202	JVM DRB1*1102 DRB3*0202	JGA DRB1*0101	BSM DRB1*0401 DRB4*0101
5703	+	+	-	-
7007	+	+	-	-
	HERLUF DRB1*1201 DRB3*0202	MAB DRB1*1201 DRB3*0202	JGA DRB1*0101	BSM DRB1*0401 DRB4*0101
2802	+	+	-	-
8602	+	+	-	-
	HERLUF DRB1*1201 DRB3*0202	MAB DRB1*1201 DRB3*0202	JGA DRB1*0101	BSM DRB1*0401 DRB4*0101
5705	+	+	-	-
7010	+	+	-	-
	CLIMD DRB1*1401 DRB3*0202	EK DRB1*1401 DRB3*0202	JGA DRB1*0101	BSM DRB1*0401 DRB4*0101
5704	+	+	-	-
7009	+	+	-	-

Table 2.7. (*continued*)

DRB SSO	Positive (+) or negative (-) control			
	CLIMD DRB1*1401 DRB3*0202	EK DRB1*1401 DRB3*0202	JGA DRB1*0101	BSM DRB1*0401 DRB4*0101
3712	+	+	-	-
8603	+	+	-	-
	CRI DRB1*1402 DRB3*0101	CRI DRB1*1402 DRB3*0101	JGA DRB1*0101	BSM DRB1*0401 DRB4*0101
7001	+	+	-	-
8601	+	+	-	-
	COX DRB1*0301 DRB3*0101	LOO DRB1*0301 DRB3*0202	JGA DRB1*0101	BSM DRB1*0401 DRB4*0101
5701	+	+	-	-
7003	-	-	-	-
	COX DRB1*0301 DRB3*0101	LOO DRB1*0301 DRB3*0202	JGA DRB1*0101	BSM DRB1*0401 DRB4*0101
7008	-	-	-	-

Table 2.8. HTC controls used for DR52 (DRB3) gene typing.

DRB SSO	Positive (+) or negative (-) control			
	COX	BTI	LOO	QBL
	DRB1*0301	DRB1*0302	DRB1*0301	DRB1*0301
	DRB3*0101	DRB3*0101	DRB3*0202	DRB3*0202
2807	+	+	-	-
	COX	BTI	LOO	QBL
	DRB1*0301	DRB1*0302	DRB1*0301	DRB1*0301
	DRB3*0101	DRB3*0101	DRB3*0202	DRB3*0202
3710	+	+	-	-
	COX	BTI	NSI	NSI
	DRB1*0301	DRB1*0302	DRB1*1302	DRB1*1302
	DRB3*0101	DRB3*0101	DRB3*0301	DRB3*0301
8601	+	+	-	-
	COX	BTI	LOO	QBL
	DRB1*0301	DRB1*0302	DRB1*0301	DRB1*0301
	DRB3*0101	DRB3*0101	DRB3*0202	DRB3*0202
5705	+	+	-	-
	LOO	QBL	COX	BTI
	DRB1*0301	DRB1*0301	DRB1*0301	DRB1*0302
	DRB3*0202	DRB3*0202	DRB3*0101	DRB3*0101
2808	+	+	-	-
	LOO	QBL	COX	BTI
	DRB1*0301	DRB1*0301	DRB1*0301	DRB1*0302
	DRB3*0202	DRB3*0202	DRB3*0101	DRB3*0101
3711	+	+	-	-
	LOO	QBL	COX	BTI
	DRB1*0301	DRB1*0301	DRB1*0301	DRB1*0302
	DRB3*0202	DRB3*0202	DRB3*0101	DRB3*0101
5701	+	+	-	-
	NSI	NSI	COX	LOO
	DRB1*1302	DRB1*1302	DRB1*0301	DRB1*0301
	DRB3*0301	DRB3*0301	DRB3*0101	DRB3*0202
8603	+	+	-	-
	NSI	NSI	COX	LOO
	DRB1*1302	DRB1*1302	DRB1*0301	DRB1*0301
	DRB3*0301	DRB3*0301	DRB3*0101	DRB3*0202
2809	+	+	-	-
	NSI	NSI	COX	LOO
	DRB1*1302	DRB1*1302	DRB1*0301	DRB1*0301
	DRB3*0301	DRB3*0301	DRB3*0101	DRB3*0202
3712	+	+	-	-

Table 2.9. HTC controls used for DQA1 gene specific typing.

DQA1 SSO	Positive (+) or negative (-) control			
	CLIMD DQA1*0101	MAB DQA1*0101	NSI DQA1*0102	HERAN DQA1*0102;*0103
3401	+	+	-	-
	CLIMD DQA1*0101	NSI DQA1*0102	DBB DQA1*0201	RED DQA1*0301
5501	+	+	-	-
6901	+	+	-	-
	HERAN DQA1*0102;0103	HERAN DQA1*0102;0103	CLIMD DQA1*0101	DBB DQA1*0201
3402	+	+	-	-
4102	+	+	-	-
	DBB DQA1*0201	PITOUT DQA1*0201	CLIMD DQA1*0101	NSI DQA1*0102
5502	+	+	-	-
6902	+	+	-	-
	DBB DQA1*0201	FJOH DQA1*0601	CLIMD DQA1*0101	NSI DQA1*0102
7502	+	+	-	-
2502	+	+	-	-
	RED DQA1*0301	DKB DQA1*0301	CLIMD DQA1*0101	NSI DQA1*0102
2503	+	+	-	-
5503	+	+	-	-
	BTI DQA1*0401	FJOH DQA1*0601	CLIMD DQA1*0101	NSI DQA1*0102
3403	+	+	-	-
6904	+	+	-	-
	BTI DQA1*0401	JVM DQA1*0501	DBB DQA1*0201	DKB DQA1*0301
5504	+	+	-	-
2501	+	+	-	-
	CRI DQA1*0501	JVM DQA1*0501	CLIMD DQA1*0101	NSI DQA1*0102
7504	+	+	-	-
6903	+	+	-	-

Table 2.10. HTC controls used for DQB1 gene specific typing.

DQB1 SSO	Positive (+) or negative (-) control			
	ARU DQB1*0201	COX DQB1*0201	CRI DQB1*0301	BSM DQB1*0302
5705	+	+	-	-
7005	+	+	-	-
	CRI DQB1*0301	FJOH DQB1*0301	ARU DQB1*0201	HAS15 DQB1*0401
4501	+	+	-	-
5706	+	+	-	-
	BSM DQB1*0302	DBB DQB1*0303	ARU DQB1*0201	CRI DQB1*0301
5707	+	-	-	-
2302	-	+	-	-
	HAS15 DQB1*0401	HAS15 DQB1*0401	ARU DQB1*0201	CRI DQB1*0301
2301	+	+	-	-
5708	+	+	-	-
	BME DQB1*0501	JGA DQB1*0501	ARU DQB1*0201	CRI DQB1*0301
4901	+	+	-	-
3701	+	+	-	-
	FJO2 DQB1*0502	HERAN DQB1*0502;*0503	ARU DQB1*0201	CRI DQB1*0301
5702	+	+	-	-
2601	+	+	-	-
	BGE DQB1*0601	TAB DQB1*0601	ARU DQB1*0201	CRI DQB1*0301
3702	+	+	-	-
2602	+	+	-	-
	BGE DQB1*0601	TAB DQB1*0601	ARU DQB1*0201	CRI DQB1*0301
7002	+	+	-	-
5703	+	+	-	-
	ZSI DQB1*0602	ZSI DQB1*0602	ARU DQB1*0201	CRI DQB1*0301
7003	+	+	-	-
2603	+	+	-	-
	HHK DQB1*0603	OMW DQB1*0603	ARU DQB1*0201	CRI DQB1*0301
2604	+	+	-	-
5704	+	+	-	-
	NSI DQB1*0605	NSI DQB1*0605	ARU DQB1*0201	CRI DQB1*0301
2606	+	+	-	-
5701	+	+	-	-

In general, the samples were heated rapidly to 94-96°C and incubated for 30 seconds to denature the DNA and cooled to an annealing temperature (depending on the primers used). The samples were left for 30 seconds to anneal, heated to 72°C and then incubated for an additional 2 minutes for DNA extension to occur. The heating-cooling-anealing cycle was repeated 30 times after which the samples were incubated at 72°C for an additional 10 minutes to complete the final extension step after the last cycle (Figure 2.1.). Primers used for DRB, DQB1 and DQA1 gene amplification were synthesized on request by the Department of Medical Biochemistry, University of Cape Town according to supplied nucleotide sequences specified in the protocol of the DNA component of the IHW. The specific details of these primers for DRB1, DQB1 and DQA1 gene amplification are listed in Tables 2.11., 2.12. and 2.13.

2.3.2. Gel electrophoresis of amplified DNA

After completion of the heating-cooling-anealing cycles in the thermocycler, samples were allowed to cool to room temperature and the mineral oil was removed. An aliquot (3 μl) of the amplified sample was removed from the tube and electrophoresed in a 1% agarose gel (Appendix 6.3.3.) containing 0.5 μg/ml ethidium bromide (Appendix 6.3.4.) to visualize the amplified DNA. The agarose gel was immersed into an electrophoresis tank containing 0.5 x TAE buffer (Appendix 6.3.2.) and electrophoresed at 120 volts for ± 15 minutes. DNA fragments ranging between 210 and 330 bp (depending on the primer used), were visible when the gel was placed on a UV transilluminator light box (UVP Incorporated, San Gabriel, California). The amplified DNA samples were stored at 4°C.

2.3.3. Dot-blot hybridization of amplified DNA

Positively charged nylon membranes (Boehringer Mannheim) labelled with the date and the SSOP on the lower border and left to soak in a glass dish of distilled H_2O, were placed into a dot-blot apparatus (Bio Rad). Distilled H_2O (100 μl) pipetted into each well was pulled through the nylon membrane by means of a vacuum. Amplified DNA specimens (Section 2.3.1.) were denatured with 50 μl 0.4 N NaOH / 25 mM EDTA, the mixtures were incubated at room temperature for 10 minutes and then transferred on ice. Fifty μl of each of these mixtures were loaded into the wells of the dot-blot apparatus containing a nylon membrane and the vacuum was again applied. Amplified DNA samples from HTCs (Section 2.3.1.) chosen to serve as positive and negative controls were added to wells in the upper left and/or lower right positions of the manifold. One hundred μl of TE (Appendix 6.2.4.) were added to each well to wash the DNA sample. The nylon membrane was then removed from the manifold and heat-sealed in a plastic sleeve. The DNA thus applied to a nylon membrane was fixed by illuminating the membrane with UV light at 254 nm in a Spectrolinker XL-1500 UV Crosslinker (Spectronics Corporation) and the nylon membranes stored at 4°C.

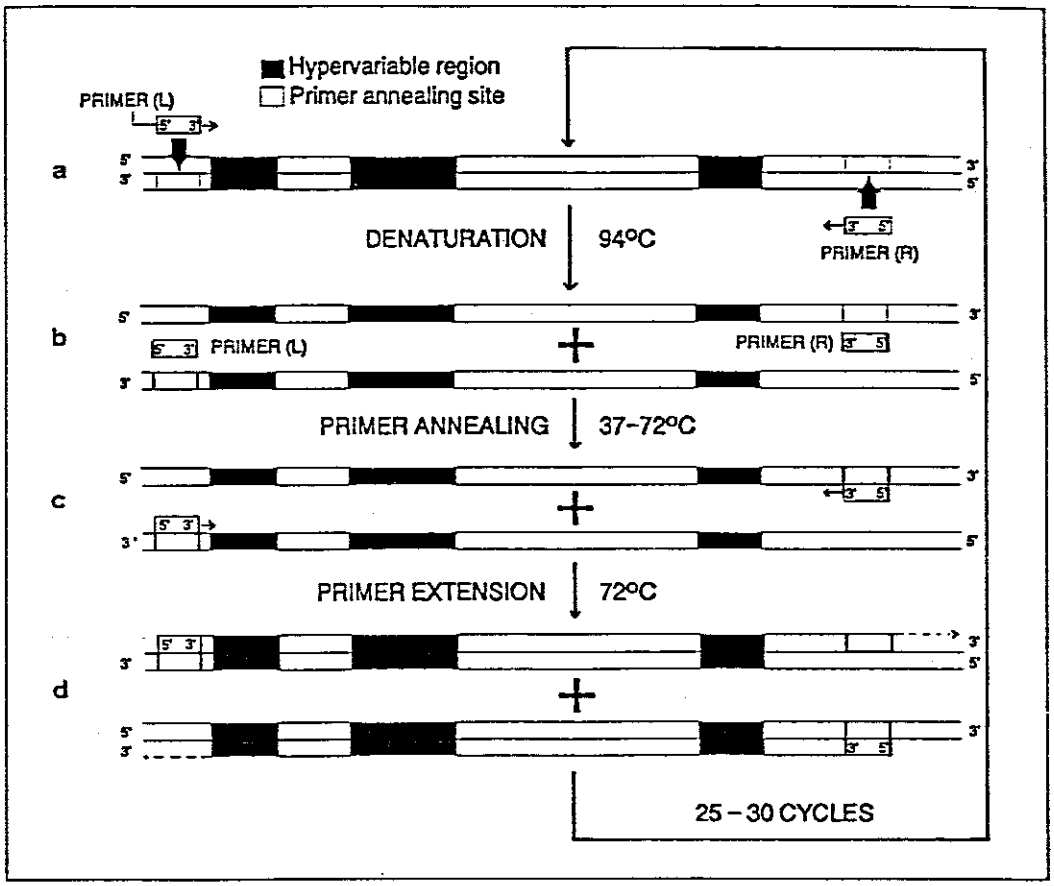

Figure 2.1. **Figure showing amplification of DNA by PCR. a) Genomic target DNA of the DRB1 gene second exon showing conserved sequences (white), hypervariable regions (black), primer annealing sites and left (L) and right (R) PCR primers. b) Heat-denaturation of DNA separates coding (uppermost) and non-coding (lowermost) strands. c) Cooling permits the annealing of PCR primers to their respective complementary sequences on the target DNA. d) Extension of both PCR primers mediated by heat-stable Taq DNA polymerase. From cycle 2 onward, newly synthesized sequences also become targets for replication (Bidwell, 1992.).**

Table 2.11. Primers used for DRB gene amplification.

Name	Codons	DNA nucleotide sequence	Orientation
DRB generic	Exon-2		
DRBAMP-A	2-8	CCCCACAGC A CGT TTC TTG	5' codon
DRBAMP-B	87-94	CCG CTG CAC TGT GAA GCT CT A	complementary
DR1 - DRB1	Exon-2		
DRBAMP-1	8-14	TTC TTG TGG CAG CTT AAG TT	5' codon
DRBAMP-B	87-94	CCG CTG CAC TGT GAA GCT CT A	complementary
DR2 - DRB1	Exon-2		
DRBAMP-2	7-13	TTC CTG TGG CAG CCT AAG AGG	5' codon
DRBAMP-B	87-94	CCG CTG CAC TGT GAA GCT CT A	complementary
DR4 - DRB1	Exon-2		
DRBAMP-4	6-13	GT TTC TTG GAG CAG GTT AAA C	5' codon
DRBAMP-B	87-94	CCG CTG CAC TGT GAA GCT CT A	complementary
DR52 - DRB1	Exon-2		
DRBAMP-3	5-12	CA CGT TTC TTG GAG TAC TCT AC	5' codon
DRBAMP-B	87-94	CCG CTG CAC TGT GAA GCT CT A	complementary
DR52 - DRB3	Exon-2		
DRBAMP-52	2-10	CC CAG CA CGT TTC TTG GAG CT	5' codon
DRBAMP-B	87-94	CCG CTG CAC TGT GAA GCT CT A	complementary

Table 2.12. Primers used for DQB1 gene amplification.

Name	Codons	DNA nucleotide sequence	Orientation
	Exon-2		
DQBAMP-A	13-20	C ATG TGC TAC TTC ACC AAC GG	5' codon
DQBAMP-B	78-84	CTG GTA GTT GTG TCT GCA CAC	complementary

Table 2.13. Primers used for DQA1 gene amplification.

Name	Codons	DNA nucleotide sequence	Orientation
	Exon-2		
DQAAMP-A	11-18	AT GGT GTA AAC TTG TAC CAG T	5' codon
DQAAMP-B	80-87	TT GGT AGC AGC GGT AGA GTT G	complementary

2.3.3.1. 3'-Labelling of SSOP with digoxigenin-ddUTP (dig-ddUTP)

SSOPs received from the XI[th] IHW and Conference, were 3'- labelled according to a protocol from the DNA component of the same Workshop and Conference (Kimura *et al*, 1992). The oligonucleotide probes used were: SSO DRß, SSO DQß and SSO DQα.

The 3'-labelling of SSOPs was performed as follows: 2.5 μl 10 x tailing Buffer (Appendix 6.4.1.), 1.0 μl 1 mM dig-ddUTP (Appendix 6.4.2.), 1.0 μl 1 mM DTT (Appendix 6.4.3.), 1.0 μl (20 units) terminal deoxynucleotidyl transferase (Appendix 6.4.4.) and 13.5 μl sterile distilled H_2O were added to 0.5 ml Eppendorf tubes and briefly vortexed.

Various SSO probes (6 μl; 30 pmol; Appendix 6.4.5.) were added (giving a final volume of 25 μl) and incubated for two hours at 37°C. If the probe was not used immediately, it was stored at -20°C. Each 3'-labelled SSOP was added to 10 ml hybridization buffer (Appendix 6.5.2.) before the probe could be used in hybridization (Section 2.3.3.2.). Nucleotide sequences and allelic specificities of the various SSOPs used for DRB generic, DR1 (DRB1) gene specific, DR2 (DRB1) gene specific, DR4 (DRB1) gene specific, DR52 associated group (DRB1), DR52 group (DRB3), DQB1 and DQA1 gene typing are listed in Tables 2.14., 2.15., 2.16., 2.17., 2.18., 2.19., 2.20. and 2.21.

2.3.3.2. Hybridization of DNA with SSOPs

2.3.3.2.1. Prehybridization of nylon membranes

The nylon membranes were removed from the sealed plastic sleeves and incubated at 42°C for 1 hour in 10 ml of hybridization buffer (Appendix 6.5.2.) by continuous rotation in a Hybaid dual hybridization oven (Teddington, Middlesex, UK). The hybridization solution was stored at -20°C and was suitable for reuse at least twice.

2.3.3.2.2. Hybridization of nylon membranes

Each 3'-labelled SSO probe (0.5 pmol/ml; Section 2.3.3.1.) was added to the appropriate nylon membrane and incubated at 43°C for 2 hours with constant agitation in a Hybaid dual hybridization oven.

Table 2.14. SSOPs used for DRB generic typing.

DRB SSO	Nucleotide sequence	Locus	Allelic specificities.
1001	TGG CAG CTT AAG TTT GAA	DRB1	*0101;*0102;*0103;*0104
1003	G TAC TCT ACG TCT GAG TG	DRB1	*03011;*03012;*0302;*0303;*11011; *11012;*1102;*1103;*11041;*11042; *1106;*1107;*1301;*1302;*1303;*1304; *1305;*1306;*1307;*1308;*1401;*1402; *1403;*1405;*1406;*1407;*1408;*1409
1004	GAG CAG GTT AAA CAT GAG	DRB1	*0401;*0402;*0403;*0404;*0405;*0406; *0407;*0408;*0409;*0410;*0411;*0412; *0413;*0414;*0415;*0416;*0417;*0410
1005	GAG TAC TCT ACG GGT GAG	DRB1	*0801;*08021;*08022;*08031;*08032; *08041;*08042;*08051;*0806;*1105; *1201;*1202;*1404;*1411
1006	TGG CAG GGT AAG TAT AAG	DRB1	*0701
1007	G AAG CAG GAT AAG TTT GA	DRB1	*09011;*09012
1008	GAG GAG GTT AAG TTT GAG	DRB1	*1001
1009	GAG GAG GTT AAG TTT GAG	DRB5	*0101;*0102;*0201;*0202;*0203
1010	GAG CTG CGT AAG TCT GAG	DRB3	*0101
1011	GAG CTG CTT AAG TCT GAG	DRB3	*0201;*0202;*0301
2801	CGG TTG CTG GAA AGA TGC	DRB1	*0101;*0102;*0103;*0104
2802	C GG TTA CTG GAG AGA CAC	DRB1	*1201;*1202
2803	CAG TTC CTG GAA AGA CTC	DRB1	*0701
2804	G TAT CTG CAC AGA GGC AT	DRB1	*09011;*09012
2805	G TTC CTG CAC AGA GAC AT	DRB5	*0101
2810	G CGA GTG TGG AAC CTG AT	DRB4	*0101;*0102
3707	AAC CAA GAG GAG AAC GTG	DRB1	*09011;*09012
		DRB5	*0102;*0201*0202;*0203

Table 2.14. (*continued*)

DRB SSO	Nucleotide sequence	Locus	Allelic specificities
3708	G CGC GTA CTC CTC TTG GT	DRB1	*1001
		DRB4	*0101;*0102
3709	G GAG GAC TTG CGC TTC GA	DRB5	*0101
5703	G CCT GAT GAG GAG TAC TG	DRB1	*1102;*1103;*11041;*0415;*11011; *11012;*11042;*1105;*1106;*1107; *1411
7011	GAC ATC CTG GAG CAG GCG	DRB1	*1501;*1502;*1503
		DRB5	*0201;*0202;*0203
8601	AAC TAC GGG GTT GGT GAG	DRB1	*0101;*0103;*0302;*0401;*0405;*0407; *0408;*0409;*0414,*0416;*0417;*0701; *0801;*08021;*08022;*08031;*08032; *0805;*09011;*09012;*1001;*11011; *11012;*1105;*1302;*1303;*1305;*1307; *1402;*1403;*1407;*1409;*1502;*1601; *1602;*1603
		DRB3	*0101;*0202
		DRB5	*0101;*0102;*0203
8602	AAC TAC GGG GCT GTG GAG	DRB1	*0102;*1106;*1201;*1202
		DRB5	*0201;*0202
8603	AAC TAC GGG GTT GTG GAG	DRB1	*0104;*03011;*03012;*0303;*0402;*0403; *0404;*0406;*0410;*0411;*0412;*0413; *08041;*0806;1102;*1103;*11041;*11042; *1107;*13011;*1304;*1306;*1308;*1401; *1404;*1405;*1406;*1410;*1411;*1501; *1603
		DRB3	*0201;*0301
		DRB4	*0101;*0102

Table 2.15. SSOPs used for DR1 (DRB1) gene specific typing.

DRB SSO	Nucleotide sequence	DRB1 allelic specificities
2801	CGG TTG CTG GAA AGA TGC	*0101;*0102;*0103;*0104
3701	C CAA GAG GAG TCC GTG CG	*0101;*0102;*0103;*0104
5701	G CCT GAT GCC GAG TAC TG	*0101;*0102;*0103;*0104
7001	TC CTG GAG CAG AGG CGG G	*0101;*0102;*0104
7007	AC ATC CTG GAA GAC GAG C	*0103
8601	AAC TAC GGG GTT GGT GAG	*0101;*0103
8602	AAC TAC GGG GCT GTG GAG	*0102

Table 2.16. SSOPs used for DR1 (DRB2) gene specific typing.

DRB SSO	Nucleotide sequence	DRB2 allelic specificities
3702	AAC CAG GAG GAG TCC GTG	*1501;*1502;*1503;*1601;*1602;*1603
5706	G CCT GAC CGT GAG TAC TG	*1501;*1502;*1503;*1601;*1602;*1603
7002	GAC TTC CTG GAA GAC AGG	*1601;*1603
7003	GAC CTC CTG GAA GAC AGG	*1602
7011	GAC ATC CTG GAG CAG GCG	*1501;*1502;*1503
8601	AAC TAC GGG GTT GGT GAG	*1502;*1601;*1602;*1603
8603	AAC TAC GGG GTT GTG GAG	*1501;*1503

Table 2.17. SSOPs used for DR4 (DRB1) gene specific typing.

DRB SSO	Nucleotide sequence	DRB1 allelic specificities
3701	C CAA GAG GAG TCC GTG CG	*0406
3704	AT CAC CAA GAG GAG TAC G	*0401;*0402;*0403;*0404;*0405;*0407; *0408;*0409;*0410;*0411;*0412;*0413; *0414;*0415;*0416;*0417
5701	G CCT GAT GCC GAG TAC TG	*0401;*0402;*0403;*0404;*0406;*0407; *0408;*0413;*0414
5702	G CCT AGC GCC GAG TAC TG	*0405;*0409;*0410;*0411;*0412;*0417
7001	TC CTG GAG CAG AGG CGG G	*0403;*0404;*0405;*0406;*0407;*0408; *0410;*0411;*0417
7005	AC CGC GGC CCG CTT CTG C	*0401;*0409;*0413;*0416
7006	G CAG AGG CGG GCC GAG GT	*0403;*0406;*0407;*0411;*0417
7007	AC ATC CTG GAA GAC GAG C	*0402
8601	AAC TAC GGG GTT GGT GAG	*0401;*0405;*0407;*0408;*0409;*0414; *0416;*0417
8603	AAC TAC GGG GTT GTG GAG	*0402;*0403;*0404;*0406;*0410;*0411; *0412;*0413;*1410

Table 2.18. SSOPs used for DR52 associated group (DRB1) gene typing.

DRB SSO	Nucleotide sequence	DRB1 allelic specificities
2802	CGG TTA CTG GAG AGA CAC	*1201;*1202
2807	G CGG TAC CTG GAC AGA TA	*03011;*03012
2809	TTC CTG GAG AGA TAC TTC C	*0302;*0303;*1402;*1403;*1406
3703	AC CAG GAG GAG AAC GTG C	*03011;*03012;*0302;*0303;*1301;*1302; *1305*1306;*1402;*1403;*1406;*1409
3712	CAG GAG GAG TTC GTG CGC	*1308;*1401;*1404;*1405;*1407;*1408; *1411
5701	G CCT GAT GCC GAG TAC TG	*03011;*03012;*0302;*0303;*08021; *08022;*08041;*08042;*1301;*1302; *1305;*1306;*1307;*1308;*1402;*1403; *1406;*1409
5702	G CCT AGC GCC GAG TAC TG	*0801;*08031;*08032;*0805;*0806;*1303; *1304
5703	G CCT GAT GAG GAG TAC TG	*11011;*11012;1102;*1103;*11041; *11042;*1105;*1106;*1107;*1411
5704	G CCT GCT GCG GAG CAC TG	*1401;*1404;*1407
5705	G CCT GTC GCC GAG TCC TG	*1201;*1202
7001	TC CTG GAG CAG AGG CGG G	*1402;*1406;*1409
7002	GAC TTC CTG GAA GAC AGG	*0801;*08021;*08022;*08041;*08042; *0805;*0806;*11011;*11012;*11041; *11042;*1105;*1106;1202;*1305;*1307
7003	GAC CTC CTG GAA GAC AGG	*1403
7004	GGC CGG GTG GAC AAC TAC	*03011;*0302;*0303;*1107

Table 2.18. (*continued*)

DRB SSO	Nucleotide sequence	DRB1 allelic specificities
7007	AC ATC CTG GAA GAC GAG C	*1102;*1301;*1302;*1304;*1308
7008	AC TTC CTG GAA GAC GAG C	*1103
7009	AG CGG AGG CGG GCC GAG G	*1401;*1404;*1405;*1407;*1408;*1411
7010	C ATC CTG GAA GAC AGG CG	*08031;*08032;*1201;*1306
8601	AAC TAC GGG GTT GGT GAG	*0302;*0801;*08021;*08022;*08031; *08032;*0805;*11011;*11012;*1105; *1302;*1303;*1305;*1307;*1402;*1403; *1407;*1409
8602	AAC TAC GGG GCT GTG GAG	*1106;*1201;*1202
8603	AAC TAC GGG GTT GTG GAG	*03011;*03012;*0303;*08041;*0806; *1102;*1103;*11041;*11042;*1107; *1301;1304;*1306;*1308;*1401;*1404; *1405;*1406;*1408;*1411

Table 2.19. SSOPs used for DR52 group (DRB3) gene typing.

DRB SSO	Nucleotide sequence	DRB3 allelic specificities
2807	G CGG TAC CTG GAC AGA TA	*0101
2808	G TTC CTG GAG AGA CAC TT	*0201;*0202
2809	TTC CTG GAG AGA TAC TTC C	*0301
3710	CAG GAG GAG TTC CTG CGC	*0101
3711	AG GAG GAG TAC GCG CGC T	*0201;*0202
3712	CAG GAG GAG TTC GTG CGC	*0301
5701	G CCT GAT GCC GAG TAC TG	*0201;*0202
5705	G CCT GTC GCC GAG TCC TG	*0101;*0301
8601	AAC TAC GGG GTT GGT GAG	*0101;*0202
8603	AAC TAC GGG GTT GTG GAG	*0201;*0301

Table 2.20. SSOPs used for DQA1 gene typing.

DQA1 SSO	Nucleotide sequence	DQA1 allelic specificities
2501	T GGC CAG TAC ACC CAT GA	*0101;*0102;*0104;*0401;*05011;*05012; *05013
2502	T GGC CAG TTC ACC CAT GA	*0103;*0201;*0601
2503	T GGG CAG TAC AGC CAT GA	*03011;*03012;*0302
3401	GA GAT GAG GAG TTC TAC G	*0101;*0104
3402	GA GAT GAG CAG TTC TAC G	*0102;*0103;*05011;*05012;*05013
3403	GA GAC GAG CAG TTC TAC G	*0401;*0601
4102	AC CTG GAG AAG AAG GAG A	*0103
5501	TC AGC AAA TTT GGA GGT T	*0101;*0102;*0103;*0104
5502	TC CAC AGA CTT AGA TTT G	*0201
5503	TC CGC AGA TTT AGA AGA T	*03011;*03012;0302
5504	TC AGA CAA TTT AGA TTT G	*0401;*0601;*05011;*05012;*05013
6901	ATG GCT GTG GCA AAA CAC	*0101;*0102;*0103;*0104
6902	ATC GCT GTG CTA AAA CAT	*0201;*03011;*0302
6903	ATC GCT GTC CTA AAA CAT	*03012;*05011;*05012;*05013
6904	ATC GCT GTG ACA AAA CAC	*0401;*0601
7502	C TTG AAC ATC CTG ATT AA	*0201;*0401;*0601
7504	C TTG AAC AGT CTG ATT AA	*05011;*05012;*05013

Table 2.21. SSOPs used for DQB1 gene typing.

DQB1 SSO	Nucleotide sequence	DQB1 allelic specificities
2301	G ACC GAG CTC GTG CGG GG	*0401
2302	AAC GGG ACC GAG CGC GTG	*0402;*03031;*0305
2601	CGG GGT GTG ACC AGA CAC	*0501;*0502;*05031;*05032
2602	CGT TAT GTG ACC AGA TAC	*0301;*0304;*06011;*06012
2603	CGT CTT GTG ACC AGA TAC	*0302;*03031;*03032;*0602
2604	CGT CTT GTA ACC AGA CAC	*0603;*0604;*0607;*0608
2606	CGT CTT GTA ACC AGA TAC	*0605;*0606
3701	AG GAG TAC GTG CGC TTC G	*0501;*0502;*05031;*05032
3702	AG GAG GAG GTG CGC TTC G	*06011;*06012
4501	GAC GTG GAG GTG TAC CGG	*0302;*0304
4901	G GTG TAC CGG GCA GTG AC	*0501
5701	G CGG CCT GTT GCC GAG TA	*0501;*0604;*0605;*0606;*0608
5702	G CGG CCT AGC GCC GAG TA	*0502;*0504
5703	G CGG CCT GAC GCC GAG TA	*05031;*06011;*06012
5704	G CGG CCT GAT GCC GAG TA	*05032;*0602;*0603;*0607
5705	GG CTG CCT GCC GCC GAG T	*0201
5706	GG CCG CCT GAC GCC GAG T	*0301;*03031;*03032
5707	TG GGG CCG CCT GCC GCC G	*0302;*0304;*0305
5708	G CGG CTT GAC GCC GAG TA	*0401;0402
7002	G ACC CGA GCG GAG TTG GA	*06011;*06012
7003	GAG GGG ACC CGG GCG GAG	*0602;*0603;*0608
7005	G AAA CGG GCG GCG GTG GA	*0201

2.3.3.2.3. Washing excess labelled SSOP from nylon membranes

Excess probe bound to nylon membranes was removed by washing the nylon membranes twice for 10 minutes at room temperature in 100 ml Washing Buffer 1 (Appendix 6.6.2.1.) with constant, gentle agitation on a bench shaker. The nylon membranes were then washed with constant agitation in 100 ml TMAC solution (Appendix 6.6.2.2.) for 10 minutes at room temperature and then twice at 58°C in a heated waterbath. Several nylon membranes (up to 4; depending on a number of SSO probes) were washed simultaneously. The nylon membranes were then washed in 100 ml 2 x SSPE (Appendix 6.6.2.3.) for 10 minutes at room temperature. The temperature used for hybridization and washing depended on the length of oligonucleotides. Since the SSO probes were 18 mer, the nylon membranes were washed at 58-60°C. Nylon membranes were stored wet in heat-sealed plastic sleeves.

2.3.3.2.4. Detection of SSOPs with dig-ddUTP

The nylon membranes were removed from the sealed plastic sleeves, washed in 100 ml Buffer 1 (Appendix 6.6.3.1.) in plastic containers for 15 minutes and incubated in 190 ml Buffer 2 (Appendix 6.6.3.2.) for 30 minutes. The volume of the solutions were based on a typical nylon membrane size of ± 100 cm^3. Nylon membranes were incubated for 30 minutes in 10 ml/blot anti-dig-AP conjugate solution (diluted 1:1,000 in Buffer 2; Appendix 6.6.3.4.) and washed twice in 100 ml Buffer 1 for 15 minutes followed by equillibration in 100 ml Buffer 3 (Appendix 6.6.3.3.) for 5 minutes.

A 10 mg/ml stock solution of Luminogen PPD (LMPPD; Appendix 6.6.3.5.) was diluted 1:100 in Buffer 3. The nylon membranes in heat-sealed plastic sleeves were incubated for 5 minutes in 10 ml 1:100 LMPPD solution with gentle agitation on a bench shaker. The sealed plastic sleeves, containing the nylon membranes were protected from light by covering them with a sheet of aluminium foil, since the LMPPD solution is light sensitive.

LMPPD solution was recovered from the plastic sleeves and was reused for a further 2 or 3 applications provided it was stored in the dark at 4°C. The excess LMPPD liquid was allowed to drip from the nylon membranes which were then heat-sealed in plastic sleeves (Figure 2.2.).

2.3.3.2.5. Autoradiography of the nylon membranes

The nylon membranes were incubated without removal from their plastic sleeves for 5 to 15 minutes at 37°C and exposed for 15 to 20 minutes at room temperature to black and white X-ray autoradiographic film (Kodak XAR-5 Kodak, Rochester, NY, USA) or Fuji (Fuji Photo film Co. Ltd, Japan) between 2 intensifying screens (Dupont Cronex lightning plus, Dupont, Boston MA,

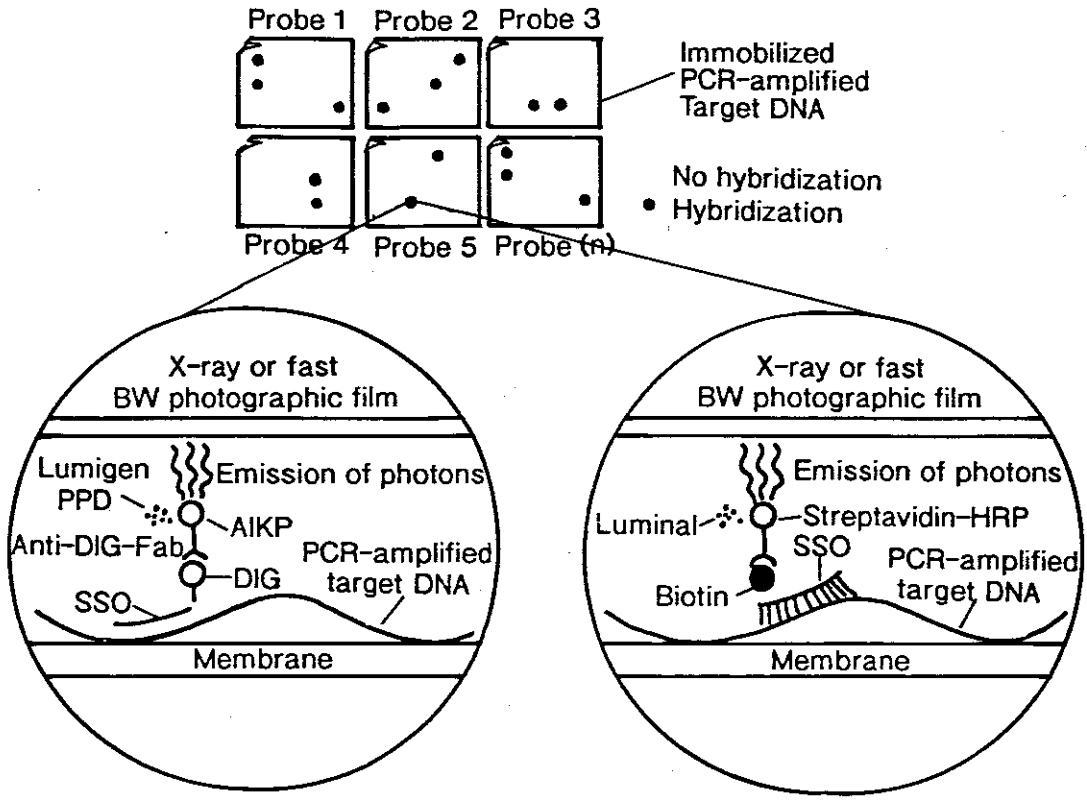

Figure 2.2. **Figure showing PCR-based probe hybridization techniques for dot-blot PCR-SSO typing. 4-methoxy-4-(-phosphatephenyl)spiro-(1,2-dioxetane-adaman-tane) (LUMIGEN PPD; Appendix 6.6.3.5.) and 5-amino-2,3-dihydro-1,4-phthalazinedione (LUMINOL; Boehringer Mannheim) are the chemiluminescent substrates for the enzymes alkaline phosphatase (alkP) and horseradish peroxidase (HRP), respectively.**

In the LUMIGEN PPD-alkP system (left), SSOPs are 3'-labelled with digoxignein (dig) and anti-digoxigenin Fab fragment (anti-dig-Fab) conjugated with alkP is used as a reporter molecule. In the LUMINOL-streptavidin-HRP system (right), SSOPs are 5'-labelled with biotin. B/W indicates black and white photographic film (Bidwell, 1994).

USA), within a X-ray cassette. The X-ray autoradiographic film exposure time depended on the strength of signal and on the film background. Films were developed by processing through developer (AGFA; Champion Photochemistry), stop bath (± 10% dilution of glacial acetic acid) and fixer (AMFIX; Champion Photochemistry). They were subsequently rinsed in running tap water for ± 10 minutes and left to dry in air at room temperature. Multiple exposures of nylon membranes at different exposure times were made, since luminescence continued for at least 24 hours. Signal intensity appeared to accumulate with time. For every SSOP, HTCs were included as positive and negative controls in the upper left and lower right regions of the nylon membranes. A signal stronger than the negative control was considered positive. Weakly positive dots were evaluated by taking into consideration the amount of amplified DNA in the dot. This was achieved by comparison with the signal obtained with the SSOP. A SSOP which was positive with all allelic forms, represented a "constant" sequence. The signal obtained was the maximal signal for that spot and was compared to the signal obtained with a typing SSOP.

When a difference between positive and negative signal was very small, an additional stringent wash at a slightly increased temperature of 2-3°C was carried out in order to make the difference between the signal of the positive and negative controls more clearly visible.

For each SSOP, the exposure time of the nylon membrane to the autoradiographic film was chosen so that the negative control showed a very weak spot or no spot at all.

The intensity of the spots was graded as follows:

1 = negative (definite)

2 = negative (probably)

4 = indefinite

6 = positive (probably)

8 = positive (definite)

2.3.3.2.6. Removal of SSOPs from nylon membranes

Nylon membranes were able to be used repeatedly provided the probes were successfully removed. This was achieved by immersing the nylon membranes in 100 ml 0.4 N NaOH (Appendix 6.6.5.1.) at 42°C for 20 minutes. Excess fluid was blotted onto filter paper.

The nylon membranes were immersed in 100 ml 0.2 M Tris-HCl, pH 8.0; 0.1 x SSPE; 0.1% SDS (Appendix 6.6.5.2.) and incubated for 20 minutes at 42°C with constant agitation. The nylon membranes were subjected to autoradiography (Section 2.3.3.2.5.) to establish whether the probe had been successfully removed, sealed in a plastic sleeves and stored at 4°C.

2.4. STATISTICAL ANALYSIS OF DATA

2.4.1. Analysis of data for the determination of the association of HLA with disease

In a study of HLA association with disease, statistical analysis is performed on the data obtained from a group of unrelated patients who are typed for HLA alleles, as well as from a group of unrelated, healthy individuals matched as best as possible with the patients, for race, sex and age.

2.4.2. Allele frequencies

HLA allele frequencies are derived from matched patient and control groups as described below. It is possible from the data to determine whether there is a statistically significant difference between the frequency of specific alleles in patient and comparison groups.

Frequencies of HLA-class II alleles were estimated by directly counting the number of times a particular allele occurred ("direct counting method") and expressing this value as a percentage of the "total possible number of alleles". For a sample of size n specimens, the "total possible number of alleles" is equal to 2n.

A method for analyzing population data was originally proposed by Woolf (1955) and modified by Haldane (Haldane, 1956). The process of data analysis with respect to a particular allele, begins with the construction of a "2 x 2 contingency table". This table contains the number of allele-positive and allele-negative individuals in the patient and control group and is subsequently analysed with a X^2 test or with the Fisher's 2-tailed exact probability test if the expected number of occurrences of the allele is < 5. Computer programs such as Epi Info have been developed to analyze such data.

The extent of association can be estimated by calculating an OR value by using Woolf's formula (Woolf, 1955).

2.4.3. Chi-square test (X^2), p value and OR with 95% C.I.

The X^2 test is a measure of the significance of an observed set of data in comparison with an expected (or calculated) set of data and is applicable to many problems in genetics.

To calculate the X^2 value from a conventional 2 x 2 contingency table, the observed number of occurrences in each category (O) and the expected number of occurrences in each category (E) is calculated as described below. The sum of the square of the difference between O and E, divided by E, in each of the four categories, gives the X^2 value with one degree of freedom for a

conventional 2 x 2 contingency table. A value of X^2 associated with a probability (p) of less than 0.05 is considered to be significant and indicates a significant disagreement between the observed and expected data (Thompson *et al*, 1983).

2 x 2 Contingency tables were constructed from the allele frequency data (Section 3.2.), as shown in the following hypothetical example.

Four "cells" are defined at the intersections of vertical and horizontal rows and columns as indicated:

	Patient	Control	
Allele +	a	b	a + b
Allele -	c	d	c + d
	a+c	b+d	n=a+b+c+d

a, b, c and d refer to actual observed numbers of alleles+ or alleles- occurring in the respective patient or control (comparison) categories in each of the four "cells."

a+b and c+d are referred to as "row totals"; a+c and b+d are referred to as "column totals". The overall total, n = a+b+c+d.

The expected value (E) for each of the four "cells" was calculated by multiplying a respective row total by a respective column total and dividing the result by the overall total.

For example, an expected value (E), corresponding to "a" in the observed 2 x 2 table above is obtained as follows:

$$\frac{(a+b) \times (a+c)}{n}$$

Likewise, the expected values for each of the 3 remaining alleles were calculated accordingly.

From the values from the observed and expected tables, X^2 values were calculated using the formula:

$$X^2 = \Sigma \frac{(O - E)^2}{E}$$

where O represents the actual observed number of alleles and E, the expected (calculated) number of alleles.

It is customary to apply a Yate's correction for continuity when any one of the expected values are small, since otherwise X^2 values would tend to be overestimated. This correction is used because the X^2 distribution is discrete, whereas the values obtained by the formula result in a continuous probability model. When expected values are large, this correction makes very little difference to the X^2 value; as the X^2 values become small, Yate's correction should be applied (Downie and Heath, 1965). Some statisticians always use Yate's correction when degrees of freedom (df) equals 1 regardless of sample size. A Yate's corrected formula for X^2 is:

$$X^2 = \Sigma \frac{[\,|O-E| - 0.5]^2}{E}$$

where 0.5 represent the Yate's correction value. In the present study, X^2 was calculated without Yate's correction with no major significant effect on the results.

2.4.4. The level of significance and p values

The level of significance is reported in terms of a p value. The smaller the p value, the greater the likelihood that the estimated value of the OR (described below) is not different by chance, from unity.

It is generally accepted that for a difference to be considered significant, $p < 0.05$. The p value corresponding to the X^2 of 3.84 is 0.05, therefore the general rule is that the calculated value of X^2 must be > 3.84. A p value of < 0.001 is considered highly significant (Fleiss, 1973).

2.4.5. Odds ratio

In order to determine whether an association exists between an allele and a disease and what is the strength of that association, statistical analysis is performed on data obtained from analytical studies. The number of disease-free people with, or without specific alleles, who are newly diagnosed with a specific disease over a specific period of time (the incidence rate) is used to calculate a measure of association called the relative risk (RR). RR is defined as the ratio of the incidence rate for persons exposed to a factor (or possess an allele) to the incidence rate for those not exposed (or do not possess the allele). Prospective studies enable one to calculate the incidence rate and therefore the RR directly.

In cross-sectional (prevalence) studies such as the present, RR of "exposure" (possession of specific alleles), were estimated by OR. OR is conceptually an indication of the odds in favour of having the disease with the allele present or with the allele absent, respectively. The OR is an estimate of the RR and indicates how much more (or less) likely an individual with an allele is at risk for the disease compared to an individual who is negative for the allele.

If the number of individuals positive for a particular HLA allele is similar to that of the individuals negative for that same HLA allele, then the OR will be close to unity. The OR will be < 1 if the allele occurs less frequently in patients than in controls. The calculated value of OR, however, may not be significantly different from unity. Ninety five percent C.I. of the OR, embrace the spread of possible values the mean OR value could assume and indicate the variability of the data. Ninety five percent C.I. values which exclude unity are usually indicative of significant OR values.

The statistical significance of the difference of OR from unity is tested by using the principle of X^2 with 1 df. If $X^2 > 3.84$, then the OR is significantly different from 1 and the allele is associated with the disease: i.e. there is a significant difference between the frequency of the allele in the patient and control groups.

Occasionally, in studies of HLA alleles and disease association, data is obtained by testing for the presence of several alleles in each individual and the same data is also used to compare the frequencies of all typed alleles. When multiple comparisons such as these are made, allele frequencies found to be statistically increased or decreased ($p < 0.05$; $X^2 > 3.84$) may be due to chance alone and thus accepting $p < 0.05$ as the acceptable level of significance ($X^2 > 3.84$), is incorrect. This error can be compensated for by the Bonferoni inequality method (Dunn, 1958, 1961) where p values, corresponding to calculated X^2 values for each allele comparison, are "corrected" by multiplying the p value regarded as being acceptable for the statistical significance (0.05) by the total number of different alleles which are compared. If any resulting corrected p value for a specific allele is still < 0.05, then that allele is considered as being significantly associated with the disease.

The Bonferoni correction for multiple comparison was used when statistical analysis was performed on allele frequency data where there was little or no *a priori* reason - such as that described in the literature, for association. For example, no Bonferoni correction was made when DRB1*04 data was analysed, but the correction was used when allele data from the DQB1 and DQA1 loci was analysed for association with RA.

CHAPTER 3

RESULTS

3.1. HLA-CLASS II TYPING

HLA-class II typing was performed on DNA from RA affected Xhosa and Cape coloured patients and from a healthy, ethnically-matched comparison group as described in Section 2.3.

The second exon of HLA-DRB1, -DRB3, -DRB4, -DRB5, -DQB1 and -DQA1 genes was amplified by PCR as described in Section 2.3.1. The results of a typical DNA amplification are shown in Plates 3.1 and 3.2., respectively.

The amplified DNA was spotted onto a nylon membrane and hybridized with Digoxigenin-labelled allele-specific oligonucleotide probes as described in Section 2.3.3. A non-radioactive detection method was employed using Digoxigenin-labelled probes and a chemilluminescent detection assay as described in Section 2.3.3. The results of a typical dot-blot are shown in Plates 3.3. and 3.4.

The postulated HLA haplotypes at the DR and DQ loci from samples of RA affected Xhosa patients, healthy Xhosas, Cape coloured RA affected patients and healthy Cape coloureds are reported in Tables 3.1., 3.2., 3.3. and 3.4., respectively. These postulated haplotypes were assigned on assumed linkage disequilibria between different alleles occurring in different population groups since no family study was performed on any patient or control individual used in the present study.

3.2. ALLELE FREQUENCIES

3.2.1. DRB1 allele frequencies

By using a panel of 49 DRB probes at the DRB1 locus (Section 2.3.3.2.), 50 alleles were detected in RA affected Xhosa patients, 188 in ethnically-matched comparison samples, 130 in RA affected Cape coloured patients and 228 in ethnically-matched comparison samples. Frequencies of DRB1* alleles or RA affected Xhosa and Cape coloured patients and healthy, ethnically-matched comparison group are reported in Table 3.5.

DRB1*0401 was the most frequently occurring allele in both Xhosa and Cape coloured RA affected patients (22.0% and 24.6%, respectively; Table 3.5.). Likewise, the frequencies of all DRB1*04 alleles ("total 04") was highest among all other alleles in both Xhosa and Cape coloured RA affected patients (28.0% and 32.3%, respectively; Table 3.5.).

The DRB1*0302 allele occurred most frequently in the Xhosa comparison group and the DRB1*0701 allele occurred most frequently with Cape coloured comparison group (14.4% and 11.8%, respectively; Table 3.5.).

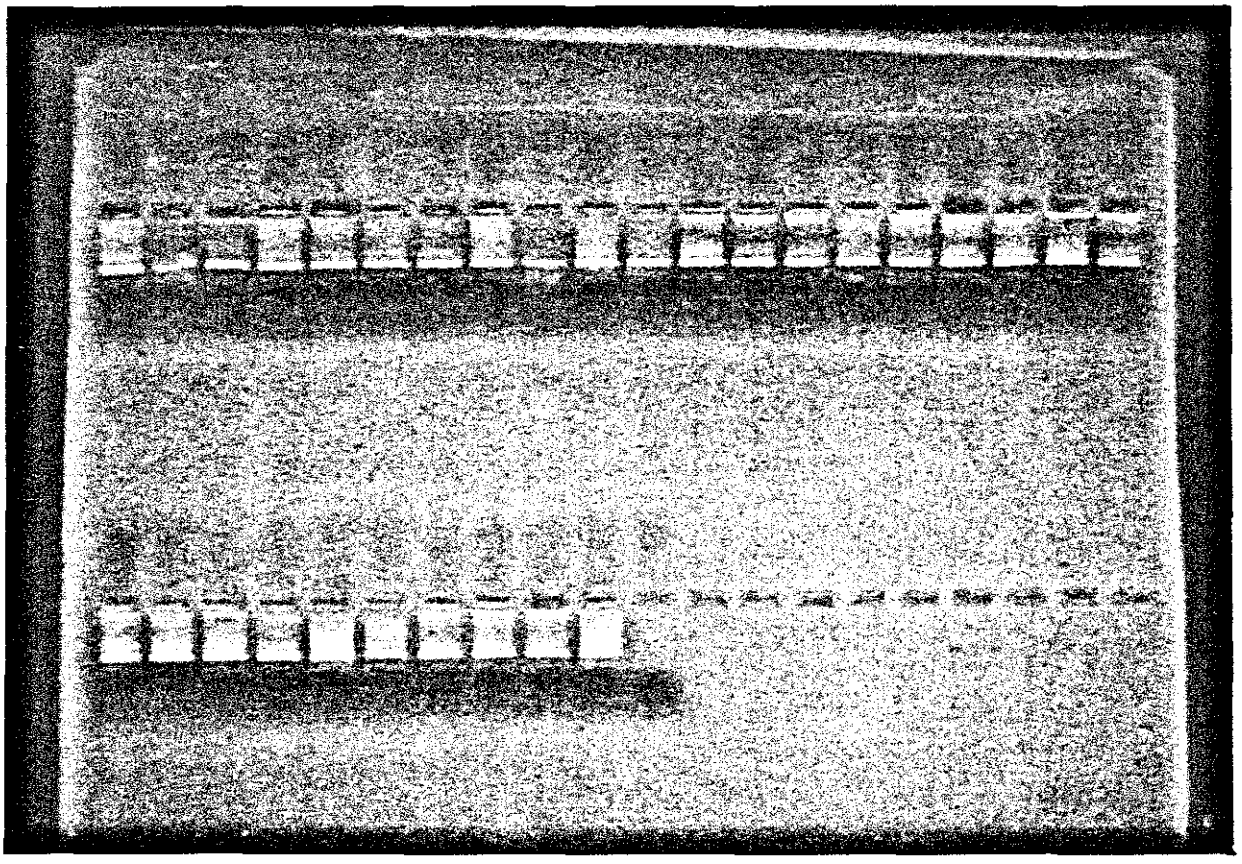

Plate 3.1. **Agarose (1%) gel electrophoresis of amplified DNA from RA affected Xhosa patients. The DNA was amplified with DRB generic primers, as described in Section 2.3.1.**

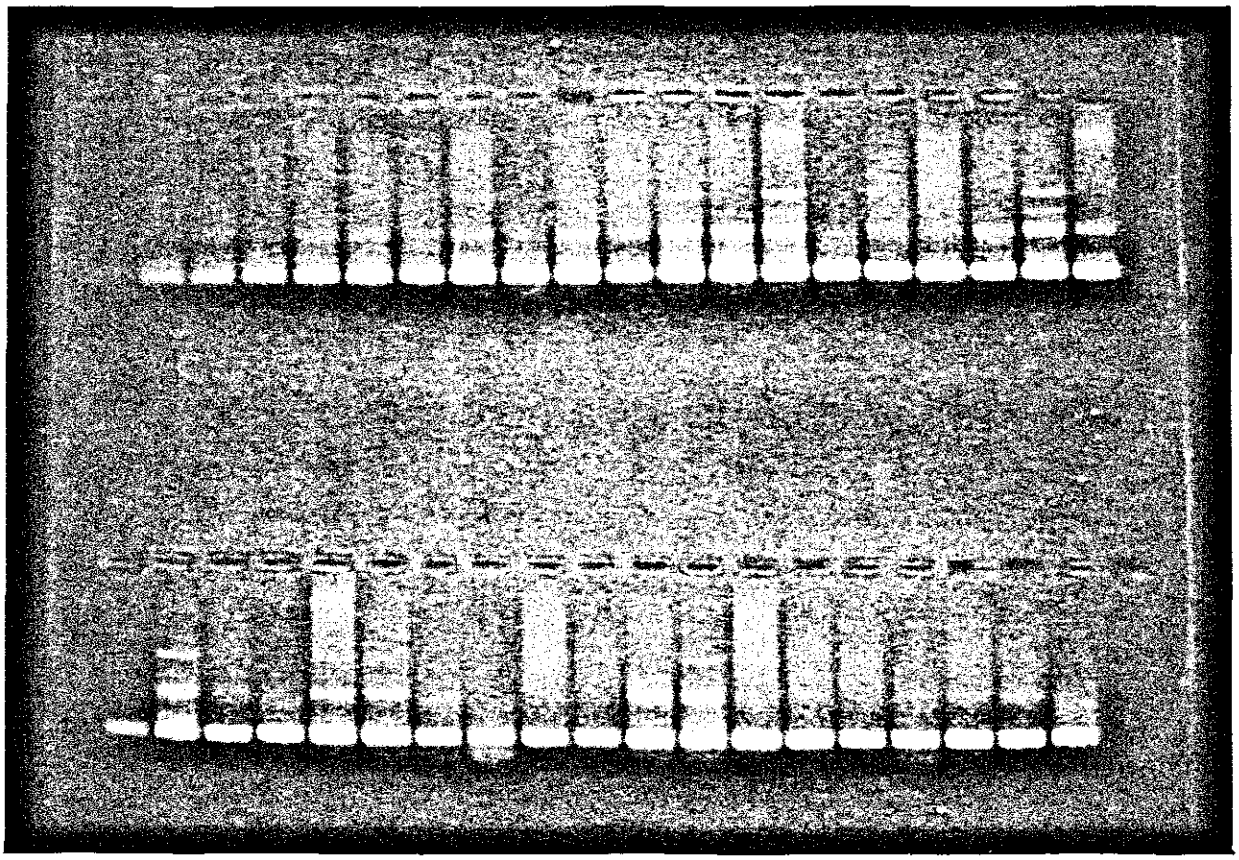

Plate 3.2. **Agarose (1%) gel electrophoresis of amplified DNA from RA affected Cape coloured patients. The DNA was amplified with DRB generic primers, as described in Section 2.3.1.**

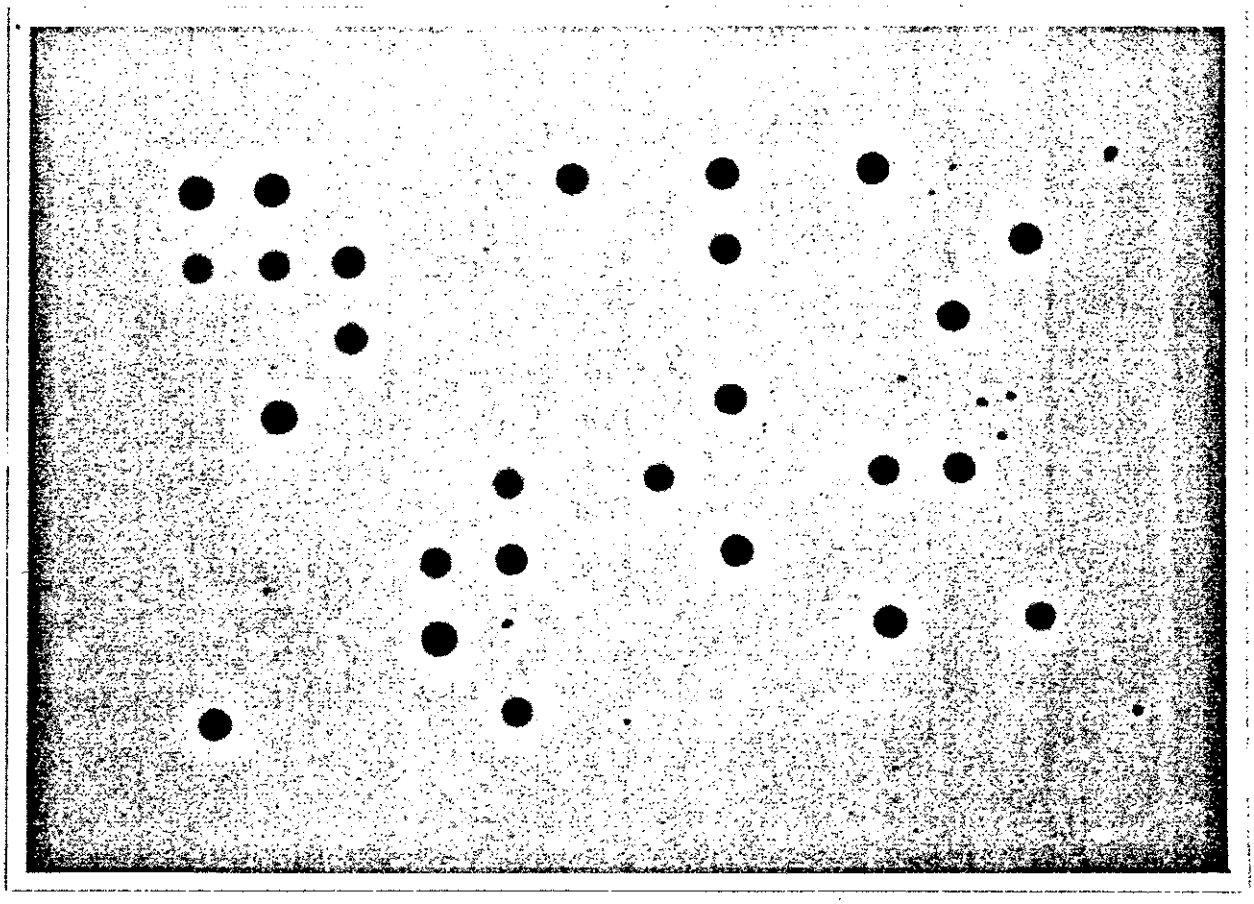

Plate 3.3. **A typical example of a dot-blot of amplified DNA from RA affected Xhosa patients, hybridized with a Digoxigenin-labelled allele-specific oligonucleotide probe.**

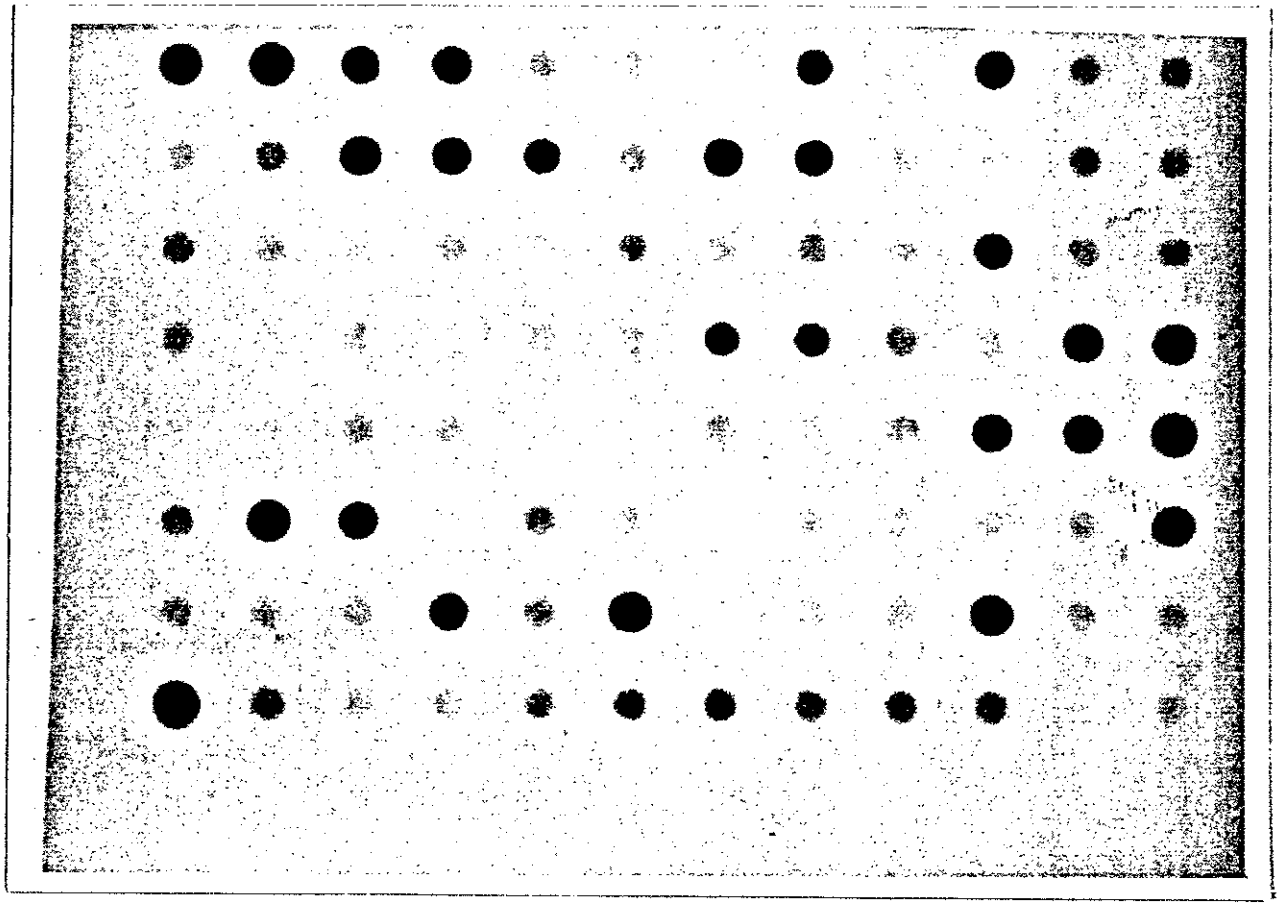

Plate 3.4. **A typical example of a dot-blot of amplified DNA from RA affected Cape coloured patients, hybridized with a Digoxigenin-labelled allele-specific oligonucleotide probe.**

Table 3.1.
Postulated HLA haplotypes of RA affected Western Cape Xhosa.

	ID	DRB1* a	DRB1* b	DRB3*	DRB3*	DRB4*	DRB4*	DRB5*	DRB5*	DQA1* a	DQA1* b		DQB1* a	DQB1* b	
1	005	0401	1001	—	—	0101	—	—	—	0301	01	a b	0302	0501	a b
2	006	0401	0901	—	—	0101	0101	—	—	0301	0301	a b	0302	0201	a b
3	009	1001	1102	—	0202	—	—	—	—	01	0501	a b	0501	0301	a b
4	010	0302	1101	0101	0202	—	—	—	—	0401	0102	a b	0402	0602	a b
5	013	0301	0401	0202	—	—	0101	—	—	0501	0301	a b	0201	0302	a b
6	014	1101	1101	0202	0202	—	—	—	—	0102	0102	a b	0602	0602	a b
7	026	0101	0401	—	—	—	0101	—	—	0101	0301	a b	0501	0302	a b
8	047	0401	1503	—	—	0101	—	—	0101	0301	0101	a b	0302	0501	a b
9	049	0401	0401	—	—	0101	0101	—	—	0301	0301	a b	0302	0302	a b
10	064	0302	1303	0101	0101	—	—	—	—	0401	0201	a b	0402	0201	a b
11	080	1001	1301	—	0101	—	—	—	—	01	0103	a b	0501	0603	a b
12	096	0404	1101	—	0202	0101	—	—	—	0301	0102	a b	0301	0602	a b
13	108	0405	1501	—	—	0101	—	—	0102	0301	0103	a b	0402	0601	a b
14	110	1102	1302	0202	0301	—	—	—	—	0501	0102	a b	0301	0605	a b
15	111	0101	1101	—	0202	—	—	—	—	0101	0501	a b	0501	0301	a b
16	122	0401	1101	—	0202	0101	—	—	—	0301	0102	a b	0302	0602	a b
17	129	0702	1101	—	0202	0101	—	—	—	0301	0102	a b	0201	0602	a b
18	141	0401	1302	—	0301	0101	—	—	—	0301	0102	a b	0302	0501	a b
19	151	0401	0701	—	—	0101	0101	—	—	0301	0201	a b	0302	0201	a b
20	156	0901	1202	—	0202	0101	—	—	—	0301	0101	a b	0201	0501	a b
21	157	0702	1101	—	0202	0101	—	—	—	0301	0102	a b	0201	0602	a b
22	158	0405	1201	—	0101	0101	—	—	—	0301	0101	a b	0402	0501	a b
23	159	0102	1001	—	—	—	—	—	—	0101	0101	a b	0501	0501	a b
24	160	0302	1101	0101	0202	—	—	—	—	0401	0501	a b	0402	0301	a b
25	161	0401	1202	—	0202	0101	—	—	—	0301	0101	a b	0302	0501	a b

KEY:

a, b	refer to alleles arbitrarily defined as 'a' and 'b'
a b	refers to the alleles 'a' and 'b' being in linkage disequilibrium

Table 3.2.
Postulated HLA haplotypes of Western Cape Xhosa comparison group.

	ID	DRB1* a	DRB1* b	DRB3*	DRB3*	DRB4*	DRB4*	DRB5*	DRB5*	DQA1* a	DQA1* b		DQB1* a	DQB1* b	
1	206	1301	1501	0101	———	———	———	———	0101	0103	0102	a b	0603	0602	a b
2	218	0702	1001	———	———	0101	———	———	———	0301	01	a b	0201	0501	a b
3	222	0901	1503	———	———	0101	———	———	0101	0301	0102	a b	0201	0602	a b
4	227	0301	0401	0202	———	———	0101	———	———	0501	0301	a b	0201	0302	a b
5	241	0302	1501	0101	———	———	———	———	0101	0401	0102	a b	0402	0602	a b
6	245	0301	0302	0202	0101	———	———	———	———	0501	0401	a b	0201	0402	a b
7	246	1102	1301	0202	0101	———	———	———	———	0501	0103	a b	0301	0603	a b
8	249	0102	0301	———	0202	———	———	———	———	0101	0501	a b	0501	0201	a b
9	250	0301	1501	0202	———	———	———	———	0101	0501	0102	a b	0201	0602	a b
10	251	1104	1401	0202	0201	———	———	———	———	0103	01	a b	0603	0602	a b
11	253	0302	1101	0101	0202	———	———	———	———	0401	0102	a b	0402	0602	a b
12	255	0302	1501	0101	———	———	———	———	0101	0401	0102	a b	0402	0602	a b
13	256	0701	1501	———	———	0101	———	———	0101	0201	0102	a b	0201	0602	a b
14	257	0302	0404	0101	———	———	0101	———	———	0401	0301	a b	0402	0301	a b
15	264	0702	1302	———	0301	0101	———	———	———	0301	0102	a b	0201	0604	a b
16	265	0302	1102	0101	0301	———	———	———	———	0401	0501	a b	0402	0301	a b
17	267	0302	1302	0101	0301	———	———	———	———	0401	0102	a b	0402	0604	a b
18	268	0301	1101	0202	0202	———	———	———	———	0501	0102	a b	0201	0602	a b
19	269	1101	1501	0202	———	———	———	———	0101	0102	0102	a b	0602	0602	a b
20	270	0301	0302	0202	0101	———	———	———	———	0501	0401	a b	0201	0402	a b
21	275	0401	1001	———	———	0101	———	———	———	0301	01	a b	0301	0501	a b
22	277	1503	1503	———	———	———	———	0101	0101	0101	0102	a b	0501	0602	a b
23	278	0702	1302	———	0301	0101	———	———	———	0301	0102	a b	0201	0604	a b
24	281	0101	0302	———	0101	———	———	———	———	0101	0401	a b	0501	0402	a b
25	283	1001	1301	———	0101	———	———	———	———	01	0103	a b	0501	0603	a b
26	287	0301	1101	0202	0202	———	———	———	———	0501	0102	a b	0201	0602	a b
27	288	0301	0302	0202	0101	———	———	———	———	0501	0401	a b	0301	0402	a b
28	289	0301	0401	0202	———	———	0101	———	———	0501	0301	a b	0201	0303	a !
29	290	0302	1001	0101	———	———	———	———	———	0401	01	a b	0402	0501	a b
30	291	0302	1001	0101	———	———	———	———	———	0401	01	a b	0402	0501	a b
31	292	1104	1302	0202	0301	———	———	———	———	0103	0102	a b	0603	0604	a b
32	293	0401	1302	———	0301	0101	———	———	———	0301	0102	a b	0302	0604	a b
33	295	0101	0401	———	———	———	0101	———	———	0101	0301	a b	0501	0302	a b
34	297	0302	0701	0101	———	———	0101	———	———	0401	0201	a b	0402	0201	a b
35	298	0302	1102	0101	0301	———	———	———	———	0401	0501	a b	0402	0301	a b
36	299	0302	1305	0101	0202	———	———	———	———	0401	0501	a b	0402	0301	a b
37	300	0401	1301	———	0101	0101	———	———	———	0301	0103	a b	0302	0603	a b
38	301	0302	1101	0101	0202	———	———	———	———	0401	0501	a b	0402	0301	a b
39	310	0701	1101	———	0202	0101	———	———	———	0201	0102	a b	0201	0602	a b
40	314	1101	1301	0202	0101	———	———	———	———	0102	0103	a b	0602	0603	a b
41	318	1101	1401	0202	0202	———	———	———	———	0102	01	a b	0602	0602	a b
42	319	1302	1501	0301	———	———	———	———	0101	0102	0102	a b	0605	0602	a b
43	320	0302	1201	0101	0101	———	———	———	———	0401	0101	a b	0402	0501	a b
44	323	0302	1401	0101	0202	———	———	———	———	0401	01	a b	0402	0602	a b
45	324	1201	1301	0101	0101	———	———	———	———	0101	0103	a b	0501	0603	a b
46	325	0301	1201	0202	0101	———	———	———	———	0501	0101	a b	0201	0501	a b
47	326	1104	1301	0202	0101	———	———	———	———	0103	0103	a b	0603	0603	a b
48	330	0301	1102	0202	0301	———	———	———	———	0501	0501	a b	0201	0301	a b
49	332	0302	1302	0101	0301	———	———	———	———	0401	0102	a b	0402	0605	a b
50	403	0101	1301	———	0101	———	———	———	———	0101	0103	a b	0501	0603	a b

Table 3.2. *(continued)*

		DRB1*	DRB1*	DRB3*	DRB3*	DRB4*	DRB4*	DRB5*	DRB5*	DQA1*	DQA1*		DQB1*	DQB1*	
51	208	1101	1303	0202	0101					0102	0201	a b	0602	0201	a b
52	221	0302	0901	0101			0101			0401	0301	a b	0402	0201	a b
53	225	1101	1503	0202					0101	0501	0102	a b	0301	0602	a b
54	226	0401	1501			0101			0101	0301	0102	a b	0302	0602	a b
55	230	1401	1401	0202	0202					01	01	a b	0602	0602	a b
56	233	0804	1101		0202					0401	0102	a b	0301	0602	a b
57	235	0301	1201	0202	0101					0501	0101	a b	0201	0501	a b
58	242	1101	1503	0202					0101	0102	0102	a b	0602	0602	a b
59	243	1101	14	0202	0101					0102	0501	a b	0602	0301	a b
60	260	0803	1104		0202					0103	0103	a b	0602	0603	a b
61	262	0405	1503			0101			0101	0301	0102	a b	0302	0602	a b
62	266	0802	1404		0101					0401	0501	a b	0402	0301	a b
63	272	1302	1503	0301					0101	0102	0101	a b	0605	0501	a b
64	273	1302	1503	0301					0101	0102	0102	a b	0605	0501	a b
65	282	1302	1501	0301					0101	0102	0102	a b	0501	0501	a b
66	284	0302	1104	0101	0202					0401	0103	a b	0402	0603	a b
67	285	0302	1104	0101	0202					0401	0103	a b	0402	0603	a b
68	294	1302	1501	0301					0101	0102	0102	a b	0501	0501	a b
69	307	0301	1101	0101	0202					0501	0102	a b	0301	0602	a b
70	309	0301	0701	0101			0101			0501	0201	a b	0201	0201	a b
71	311	1102	1104	0202	0202					0501	0103	a b	0301	0603	a b
72	333	1501	1503					0101	0101	0102	0101	a b	0602	0602	a b
73	PD	04	07			0101	0101						0302	0201	a b
74	HM	15	15					51	51				0602	0602	a b
75	JM	13	1302	0101	0301								0301	0609	a b
76	PK	12	1302	0202	0301								0501	0609	a b
77	SM	0302	1302	0101	0301								0402	0609	a !
78	OK	1101	1302	0202	0301								0602	0301	a b
79	CS	1301	1301	0101	0101								0303	0603	a b
80	PN	07	1101		0202	0101							02	0301	a b
81	MW	0302	1101	0101	0202								0402	0602	a b
82	NZ	0302	0701	0101			0101						0402	0201	a b
83	EM	0301	1501	0202					0101				0201	0602	a b
84	JT	0301	0701	0202			0101						0201	0201	a b
85	TK	04	0701			0101	0101						0302	0201	a b
86	AT	1001	13		52								0501	06	a b
87	SB	0302	13	52	52								0402	06	a b
88	NT	12	13	52	52								0501	06	a b
89	RM	1301	1301	0202	0202								0602	0602	! !
90	NM	04	11		52	0101							0302	0301	a b
91	JM	01	13		52								0501	0301	a b
92	VT	11	12	52	52								0301	0501	a b
93	JM	0302	13	0101	52								0402	06	a b
94	SLS	1001	1302		0301								0501	0501	a b

KEY:

a, b	refer to alleles arbitrarily defined as 'a' and 'b'
a b	refers to the alleles 'a' and 'b' being in linkage disequilibrium
!	indicates alleles not in an usual DR-DQ association

Table 3.3.
Postulated HLA haplotypes of RA affected Western Cape coloured.

	ID	DRB1* a	DRB1* b	DRB3*	DRB3*	DRB4*	DRB4*	DRB5*	DRB5*	DQA1* a	DQA1* b		DQB1* a	DQB1* b	
1	016	0401	1501	———	———	0101	———	———	0101	0301	0102	a b	0302	0601	a !
2	017	0301	0301	0101	0101	———	———	———	———	0501	0501	a b	0201	0201	a b
3	018	0102	0401	———	———	———	0101	———	———	0101	0301	a b	0501	0302	a b
4	022	0101	0103	———	———	———	———	———	———	0101	0501	a b	0501	0301	a b
5	024	1501	1501	———	———	———	———	0101	0101	0102	0102	a b	0501	0501	a b
6	025	0101	0401	———	———	———	0101	———	———	0101	0301	a b	0501	0301	a b
7	029	1201	1202	0101	0301	———	———	———	———	0501	0601	a b	0301	0301	a b
8	031	0401	1502	———	———	0101	———	———	0102	0301	0103	a b	0301	0601	a b
9	033	1501	1501	———	———	———	———	0101	0101	0102	0102	a b	0602	0602	a b
10	036	0101	0101	———	———	———	———	———	———	0101	0101	a b	0501	0501	a b
11	038	0101	0405	———	———	———	0101	———	———	0101	0301	a b	0501	0401	a b
12	040	1501	1501	———	———	———	———	0101	0101	0102	0102	a b	0501	0501	a b
13	041	1501	1501	———	———	———	———	0101	0101	0102	0102	a b	0501	0501	a b
14	042	0101	1501	———	———	———	———	———	0101	0101	0102	a b	0501	0501	a b
15	043	0404	1302	———	0301	0101	———	———	———	0301	0102	a b	0302	0501	a b
16	044	0401	1501	———	———	0101	———	———	0101	0301	0102	a b	0302	0501	a b
17	045	0101	0401	———	———	———	0101	———	———	0101	0301	a b	0501	0302	a b
18	050	1001	1101	———	0202	———	———	———	———	01	0501	a b	0501	0301	a b
19	051	0101	15	———	———	———	———	———	0101	0101	0102	a b	0501	0501	a b
20	053	0804	1101	———	0202	———	———	———	———	0401	0501	a b	0301	0301	a b
21	055	0401	0401	———	———	0101	0101	———	———	0301	0301	a b	0302	0302	a b
22	057	1501	1502	———	———	———	———	0101	0102	0102	0103	a b	0602	0601	a b
23	059	0401	0701	———	———	0101	0101	———	———	0301	0201	a b	0302	0201	a b
24	060	0803	1502	———	———	———	———	———	0102	0601	0101	a b	0301	0501	a b
25	061	0401	1502	———	———	0101	———	———	0102	0301	0101	a b	0302	0501	a b
26	065	0404	0901	———	———	0101	0101	———	———	0301	0301	a b	0302	0303	a b
27	066	0401	0401	———	———	0101	0101	———	———	0301	0301	a b	0301	0302	a b
28	067	0401	1001	———	———	0101	———	———	———	0301	0101	a b	0302	0501	a b
29	068	1001	1101	———	0202	———	———	———	———	0101	0501	a b	0501	0301	a b
30	070	0401	0401	———	———	0101	0101	———	———	0301	0301	a b	0301	0302	a b
31	071	0405	1404	———	0101	0101	———	———	———	0301	0501	a b	0402	0501	a !
32	072	0401	0901	———	———	0101	0101	———	———	0301	0301	a b	0302	0303	a b
33	073	0702	1101	———	0202	0101	———	———	———	0301	0302	a b	0201	0201	a b
34	076	0405	1101	———	0202	0101	———	———	———	0301	0501	a b	0401	0301	a b
35	077	0402	0402	———	———	0101	0101	———	———	0301	0301	a b	0302	0302	a b
36	078	0401	1101	———	0202	0101	———	———	———	0301	0302	a b	0302	0201	a b
37	081	0401	0402	———	———	0101	0101	———	———	0301	0301	a b	0301	0302	a b
38	082	0803	1401	———	0202	———	———	———	———	0103	01	a b	0601	0501	a b
39	084	0403	04	———	———	0101	0101	———	———	0301	0301	a b	0302	0501	a b
40	086	1001	1202	———	0202	———	———	———	———	01	0102	a b	0501	0301	a b
41	087	1001	1101	———	0202	———	———	———	———	01	0501	a b	0501	0301	a b
42	088	0401	1202	———	0202	0101	———	———	———	0301	0102	a b	0301	0301	a b
43	089	0101	1501	———	———	———	———	———	0101	0101	0102	a b	0501	0501	a b
44	092	1202	1303	0301	0101	———	———	———	———	0401	0201	a b	0301	0201	a b
45	093	0701	1202	———	0301	0101	———	———	———	0201	0601	a b	0201	0301	a b
46	094	1104	1502	0202	———	———	———	———	0102	0103	0101	a b	0603	0501	a b
47	100	0401	1502	———	———	0101	———	———	0102	0301	0103	a b	0302	0601	a b
48	106	0401	1502	———	———	0101	———	———	0102	0301	0103	a b	0302	0601	a b
49	109	0102	0405	———	———	———	0101	———	———	0101	0301	a b	0501	0401	a b
50	117	0401	1001	———	———	0101	———	———	———	0301	01	a b	0302	0501	a b

Table 3.3. *(continued)*

		DRB1*	DRB1*	DRB3*	DRB3*	DRB4*	DRB4*	DRB5*	DRB5*	DQA1*	DQA1*		DQB1*	DQB1*	
51	119	0801	1502	----	----	----	----	----	0102	0301	0101	a b	0302	0501	a b
52	120	1501	1502	----	----	----	----	0101	0102	0102	0103	a b	0602	0601	a b
53	123	0401	1001	----	----	0101	----	----	----	0301	01	a b	0302	0501	a b
54	124	0401	0401	----	----	0101	0101	----	----	0301	0301	a b	0302	0302	a b
55	126	1101	1302	0202	0301	----	----	----	----	0501	0102	a b	0301	0605	a b
56	127	0901	1104	----	0202	0101	----	----	----	0301	0103	a b	0201	0603	a b
57	130	1501	1502	----	----	----	----	0101	0102	0102	0101	a b	0501	0501	a b
58	132	0401	0401	----	----	0101	0101	----	----	0301	0301	a b	0301	0302	a b
59	135	0101	0701	----	----	----	0101	----	----	0101	0201	a b	0501	0201	a b
60	136	0401	0401	----	----	0101	0101	----	----	0301	0301	a b	0302	0302	a b
61	137	0401	0701	----	----	0101	0101	----	----	0301	0201	a b	0302	0201	a b
62	142	0401	1101	----	0202	0101	----	----	----	0301	0102	a b	0302	0502	a b
63	145	0302	1104	0101	0202	----	----	----	----	0401	0103	a b	0402	0603	a b
64	146	1101	1502	0202	----	----	----	----	0102	0501	0103	a b	0301	0601	a b
65	152	0401	1501	----	----	0101	----	----	0101	0301	0102	a b	0302	0502	a b

KEY:

a, b	refer to alleles arbitrarily defined as 'a' and 'b'
a b	refers to the alleles 'a' and 'b' being in linkage disequilibrium
!	indicates alleles not in an usual DR-DQ association

Table 3.4.
Postulated HLA haplotypes of Western Cape coloured comparison group.

	ID	DRB1* a	DRB1* b	DRB3*	DRB3*	DRB4*	DRB4*	DRB5*	DRB5*	DQA1* a	DQA1* b		DQB1* a	DQB1* b	
1	202	0408	0701	———	———	0101	0101	———	———	0301	0201	a b	0301	0201	a b
2	204	0103	1102	———	0202	———	———	———	———	0501	0501	a b	0501	0301	a b
3	210	1104	1202	0202	0101	———	———	———	———	0103	0501	a b	0603	0301	a b
4	213	1201	1502	0202	———	———	———	———	0102	0101	0103	a b	0501	0601	a b
5	214	0101	0405	———	———	———	0101	———	———	0101	0301	a b	0501	0402	a b
6	215	0301	0701	0202	———	———	0101	———	———	0501	0201	a b	0201	0201	a b
7	216	0701	0702	———	———	0101	0101	———	———	0201	0301	a b	0201	0201	a b
8	217	0701	1501	———	———	0101	———	———	0101	0201	0102	a b	0201	0502	a b
9	219	1101	1101	0202	0202	———	———	———	———	0501	0102	a b	0301	0602	a b
10	220	0101	0401	———	———	———	0101	———	———	0101	0301	a b	0501	0302	a b
11	223	0301	0401	0202	———	———	0101	———	———	0501	0301	a b	0201	0302	a b
12	224	1101	1101	0202	0202	———	———	———	———	0501	0102	a b	0301	0602	a b
13	229	0302	0701	0101	———	———	0101	———	———	0401	0201	a b	0402	0201	a b
14	234	0401	0702	———	———	0101	0101	———	———	0301	0301	a b	0301	0201	a b
15	240	0701	0701	———	———	0101	0101	———	———	0201	0201	a b	0201	0201	a b
16	259	0302	1202	0101	0301	———	———	———	———	0401	0601	a b	0402	0301	a b
17	280	0301	0901	0202	———	———	———	———	0101	0501	0301	a b	0201	0201	a b
18	312	0701	1101	———	0202	0101	———	———	———	0201	0501	a b	0303	0301	a b
19	313	0403	1401	———	0202	0101	———	———	———	0301	01	a b	0302	0602	a b
20	329	0301	1101	0101	0202	———	———	———	———	0501	0501	a b	0201	0301	a b
21	331	1101	1501	0202	———	———	———	———	0101	0501	0102	a b	0301	0602	a b
22	334	1201	1501	0101	———	———	———	———	0101	0101	0102	a b	0501	0602	a b
23	337	0401	1001	———	———	0101	———	———	———	0301	01	a b	0302	0501	a b
24	338	0401	1201	———	0101	0101	———	———	———	0301	0101	a b	0302	0501	a b
25	340	0701	1501	———	———	01	———	———	0101	0201	0102	a b	0201	0602	a b
26	341	1101	1501	0202	———	———	———	———	0101	0501	0102	a b	0301	0602	a b
27	343	0701	1501	———	———	0101	———	———	0101	0201	0102	a b	0303	0603	a !
28	344	1104	1401	0202	0202	———	———	———	———	0501	01	a b	0301	0602	a b
29	345	0301	1502	0202	———	———	———	———	0102	0501	0103	a b	0201	0601	a b
30	346	0701	1501	———	———	0101	———	———	0101	0201	0102	a b	0201	0502	a b
31	347	0301	0701	0202	———	———	0101	———	———	0501	0201	a b	0201	0201	a b
32	348	0401	1202	———	0301	0101	———	———	———	0301	0601	a b	0302	0301	a b
33	349	1202	1502	0101	———	———	———	———	0102	0501	0103	a b	0301	0601	a b
34	350	0301	1202	0202	0301	———	———	———	———	0501	0601	a b	0201	0301	a b
35	351	0701	1602	———	———	0101	———	———	0202	0201	0102	a b	0303	0502	a b
36	352	08	1501	———	———	———	———	———	0101	0401	0102	a b	0402	0502	a b
37	353	0701	1102	———	0301	0101	———	———	———	0201	0501	a b	0303	0301	a b
38	354	0302	1302	0101	0301	———	———	———	———	0401	0102	a b	0402	0605	a b
39	355	1101	1104	0202	0202	———	———	———	———	0501	0103	a b	0301	0603	a b
40	356	1101	1501	0202	———	———	———	———	0101	0501	0102	a b	0301	0602	a b
41	357	0101	1202	———	0301	———	———	———	———	0101	0601	a b	0501	0301	a b
42	358	1001	1101	———	0202	———	———	———	———	01	0501	a b	0501	0301	a b
43	359	0101	1101	———	0202	———	———	———	———	0101	0102	a b	0501	0602	a b
44	360	0401	1201	———	0101	0101	———	———	———	0301	0101	a b	0302	0501	a b
45	361	0302	1101	0101	0202	———	———	———	———	0401	0501	a b	0402	0301	a b
46	362	1101	1104	0202	0202	———	———	———	———	0501	0103	a b	0301	0603	a b
47	363	0401	0701	———	———	0101	0101	———	———	0301	0201	a b	0302	0302	a !
48	366	0401	1104	———	0202	0101	———	———	———	0301	0501	a b	0302	0301	a b
49	367	1101	1503	0202	0101	———	———	———	———	0501	0102	a b	0301	0602	a b
50	368	1202	1202	0101	0301	———	———	———	———	0501	0601	a b	0301	0301	a b

Table 3.4. *(continued)*

		DRB1*	DRB1*	DRB3*	DRB3*	DRB4*	DRB4*	DRB5*	DRB5*	DQA1*	DQA1*		DQB1*	DQB1*	
51	369	1401	1503	0202	———	———	———	———	0101	01	0101	a b	0501	0501	a b
52	370	0301	1501	0202	———	———	———	———	0101	0501	0102	a b	0201	0602	a b
53	371	0301	0701	0202	———	———	0101	———	———	0501	0201	a b	0201	0201	a b
54	372	0701	1202	———	0301	0101	———	———	———	0201	0601	a b	0301	0301	! b
55	373	0102	1503	———	———	———	———	———	0101	0101	0102	a b	0501	0501	a b
56	374	1102	1501	0202	———	———	———	———	0101	0501	0102	a b	0301	0602	a b
57	375	0401	0401	———	———	0101	0101	———	———	0301	0301	a b	0302	0302	a b
58	376	0301	0405	0202	———	———	0101	———	———	0501	0301	a b	0201	0402	a b
59	377	0301	0701	0202	———	———	0101	———	———	0501	0201	a b	0201	0201	a b
60	378	1101	1401	0202	0202	———	———	———	———	0501	01	a b	0301	0501	a b
61	379	0701	1101	———	0202	0101	———	———	———	0201	0102	a b	0201	0602	a b
62	381	1301	1501	0101	———	———	———	———	0101	0103	0102	a b	0603	0602	a b
63	382	0101	1101	———	0202	———	———	———	———	0101	0501	a b	0501	0301	a b
64	383	0405	1101	———	0202	0101	———	———	———	0301	0102	a b	0302	0502	a b
65	384	08	1101	———	0202	———	———	———	———	0401	0501	a b	0402	0301	a b
66	386	0102	1101	———	0202	———	———	———	———	0101	0501	a b	0501	0301	a b
67	387	0301	1501	0202	———	———	———	———	0101	0501	0102	a b	0201	0602	a b
68	390	0701	1101	———	0202	0101	———	———	———	0201	0501	a b	0201	0301	a b
69	393	0102	1001	———	———	———	———	———	———	0101	01	a b	0501	0501	a b
70	394	1001	1001	———	———	———	———	———	———	01	01	a b	0501	0501	a b
71	395	0701	1101	———	0202	0101	———	———	———	0201	0501	a b	0201	0301	a b
72	397	0101	0702	———	———	———	0101	———	———	0101	0301	a b	0501	0201	a b
73	398	0901	1501	———	———	0101	———	———	0101	0301	0102	a b	0201	0602	a b
74	400	0301	1303	0202	0101	———	———	———	———	0501	0501	a b	0201	0301	a b
75	402	0401	1101	———	0202	0101	———	———	———	0301	0501	a b	0302	0301	a b
76	DS	1302	15	0301	———	———	———	———	51				0604	0501	a b
77	MV	11	15	52	———	———	———	———	51				0301	0601	a b
78	FG	0102	13	———	0101	———	———	———	———				0604	0501	a b
79	AR	01	01	———	———	———	———	———	———				0501	0501	a b
80	GM	04	1301	———	0101	0101	———	———	———				0302	0603	a b
81	RA	11	16	02	———	———	———	———	0101				03	03	a b
82	HB	04	0701	———	———	0101	0101	———	———				0302	0303	a b
83	AJ	13	15	0101	———	———	———	———	0101				0603	0602	a b
84	ND	07	07	———	———	0101	0101	———	———				0302	0302	a b
85	KT	13	13	0202	0301	———	———	———	———				06	06	a b
86	SD	12	1502	52	———	———	———	———	51				0301	0601	a b
87	ZI	1502	1502	———	———	———	———	0102	0102						
88	HG	0701	1404	———	52	53	———	———	———						
89	LP	01	13	———	52	———	———	———	———				0501	06	a b
90	ME	04	14	———	52	53	———	———	———				0302	0503	a b
91	PJ	15	15	———	———	———	———	0101	0101	0102	0103	a b	0601	0602	a b
92	MAJ	01	13	———	0101	———	———	———	———	0101	0103	a b	0501	0603	a b
93	EG	0302	15	52	———	———	———	———	51				0402	0602	a b
94	MA	0701	14	———	52	53	———	———	———				0201	0503	a b
95	AP	12	13	52	52	———	———	———	———				0301	0604	a b
96	HJS	0301	12	52	52	———	———	———	———				0201	0301	a b
97	RP	11	16	52	———	———	———	———	51				0301	0502	a b
98	CB	0301	1302	0101	0301	———	———	———	———				0609	0201	a b
99	EC	0901	15	———	———	53	———	———	51				0303	0602	a b
100	HM	1503	1503	———	———	———	———	51	51				0602	0602	a b

Table 3.4. *(continued)*

		DRB1*	DRB1*	DRB3*	DRB3*	DRB4*	DRB4*	DRB5*	DRB5*	DQA1*	DQA1*	DQB1*	DQB1*	a b
101	JM	03	0404	0101	———	———	0101	———	———			0402	0302	a b
102	RG	0403	1001	———	———	0101	———	———	———			0302	0501	a b
103	AJ	09	11	———	02	0101	———	———	———			0303	0301	a b
104	DJ	03	0401	0202	———	———	0101	———	———			02	0302	a b
105	WD	1301	1308	0101	0202	———	———	———	———			0602	0603	a b
106	GD	15	15	———	———	———	———	0101	0101			0601	0602	a b
107	RL	12	1302	0202	0301	———	———	———	———			0301	06	a b
108	REF	0701	1301	———	0101	0103	———	———	———			02	0603	a b
109	RT	1404	1407	0101	0202	———	———	———	———			0503	0503	a b
110	AM	0701	1503	———	———	0101	———	———	0101			0302	0602	! b
111	MV	0701	15	53	———	———	———	———	51			0303	0502	a b
112	AA	0702	13	———	52	53	———	———	———			0201	0602	a b
113	CM	01	11	———	52	———	———	———	———					
114	JM	04	15	———	———	53	———	51	———					

KEY:

a, b	refer to alleles arbitrarily defined as 'a' and 'b'
a b	refers to the alleles 'a' and 'b' being in linkage disequilibrium
!	indicates alleles not in an usual DR-DQ association

Table 3.5. **Frequencies of HLA-class II alleles at the DRB1 locus of RA affected Xhosa (AB) and Cape coloured (AC) patients and healthy, ethnically-matched comparison groups (CB and CC, respectively). n_s = number of subjects; n_a = number of alleles.**

	(n_s=25)		(n_s=94)		(n_s=65)		(n_s=114)	
	n_a=50 alleles		n_a=188 alleles		n_a=130 alleles		n_a=228 alleles	
DRB1*	**AB**	**%**	**CB**	**%**	**AC**	**%**	**CC**	**%**
01			1	0.5			5	2.2
0101	2	4.0	3	1.6	10	7.7	6	2.6
0102	1	2.0	1	0.5	2	1.5	4	1.8
0103					1	0.8	1	0.4
Total 01	3	6.0	5	2.7	13	10.0	16	7.0
0301	1	2.0	16	8.5	2	1.5	16	7.0
0302	3	6.0	27	14.4	1	0.8	6	2.6
Total 03	4	8.0	43	22.9	3	2.3	22	9.6
04			3	1.6			4	1.8
0401	11	22.0	7	3.7	32	24.6	13	5.7
0402					3	2.3		
0403					1	0.8	2	0.9
0404	1	2.0	1	0.5	2	1.5	1	0.4
0405	2	4.0	1	0.5	4	3.1	3	1.3
0408							1	0.4
Total 04	14	28.0	12	6.5	42	32.3	24	10.5
07			2	1.1			2	0.9
0701	1	2.0	7	3.7	4	3.1	27	11.8
0702	2	4.0	3	1.6	1	0.8	4	1.8
Total 07	3	6.0	12	6.4	5	3.8	33	14.5
08					2	1.5	2	0.9

Table 3.5. (*continued*)

	(n_s=25)		(n_s=94)		(n_s=65)		(n_s=114)	
	n_a=50 alleles		n_a=188 alleles		n_a=130 alleles		n_a=228 alleles	
DRB1*	**AB**	**%**	**CB**	**%**	**AC**	**%**	**CC**	**%**
0803			1	0.5	1	0.8		
0804			1	0.5	1	0.8		
Total 08			2	1.1	4	3.1	2	0.9
09					1	0.8	1	0.4
0901	2	4.0	2	1.1	2	1.5	3	1.3
Total 09	2	4.0	2	1.1	3	2.3	4	1.8
1001	4	8.0	7	3.7	7	5.4	6	2.6
	2	1.1			5	2.2		
1101	9	18.0	17	9.0	10	7.7	24	10.5
1102	2	4.0	5	2.7			3	1.3
1104			7	3.7	3	2.3	5	2.2
Total 11	22.0	31	16.5	13	10	37	16.2	
12			3	1.6			4	1.8
1201	1	2.0	4	2.1	1	0.8	4	1.8
1202	2	4.0			5	3.8	9	3.9
Total 12	3	6.0	7	3.7	6	4.6	17	7.5
13			6	3.2			5	2.2
1301	1	2.0	12	6.4			6	2.6
1302	2	4.0	16	8.5	2	1.5	5	2.2
1303	1	2.0	1	0.5	1	0.8	1	0.4
1305			1	0.5				
1308							1	0.4
Total 13	4	8.0	36	19.1	3	2.3	18	7.9

Table 3.5. (*continued*)

	(n_s=25)		(n_s=94)		(n_s=65)		(n_s=114)	
	n_a=50 alleles		n_a=188 alleles		n_a=130 alleles		n_a=228 alleles	
DRB1*	**AB**	**%**	**CB**	**%**	**AC**	**%**	**CC**	**%**
14			1	0.5	1	0.8	2	0.9
1401			5	2.7			4	1.8
1404			1	0.5	1	0.8	2	0.9
1407							1	0.4
Total 14			7	3.7	2	1.5	9	3.9
15			2	1.1	1	0.8	8	3.5
1501	1	2.0	12	6.4	16	12.3	15	6.6
1502					11	8.5	8	3.5
1503	1	2.0	9	4.8			6	2.6
Total 15	2	4.0	23	12.2	28	21.5	37	16.2
16							2	0.9
1602							1	0.4
Total 16							3	1.3
TOTAL	50	100.0	188	100.0	130	100.0	228	100.0

3.2.2. DQB1 allele frequencies

By using a panel of 22 DQB probes at the DQB1 locus, 50 alleles were detected in RA affected Xhosa patients, 188 in ethnically-matched comparison samples, 130 in the RA affected Cape coloured patients and 220 in ethnically-matched comparison samples. Frequencies of DQB1* alleles are reported in Table 3.6.

DQB1*0501 was the most frequently occurring allele in both the Xhosa and Cape coloured RA affected patients (24.0% and 30.0%, respectively; Table 3.6.).

DQB1*0602 was the most frequently occurring allele in the Xhosa comparison group (21.3%; Table 3.6.) and DQB1*0301, the most frequently occurring allele in the Cape coloured comparison group (20.0%; Table 3.6.).

3.2.3. DQA1 allele frequencies

By using 17 DQA probes at the DQA1 locus, 50 alleles were detected in the RA affected Xhosa patients, 144 in ethnically-matched comparison samples, 130 in RA affected Cape coloured patients and 154 in ethnically-matched comparison samples. Frequencies of DQA1* alleles are reported in Table 3.7.

The DQA1*0301 was the most frequently occurring allele in both Xhosa and Cape coloured RA affected patients (36.0% and 35.4%, respectively; Table 3.7.).

The DQA1*0102 allele occurred most frequently in the Xhosa comparison group (27.8%; Table 3.7.) and the DQA1*0501 allele was the most frequently occurring allele in the Cape coloured comparison group (27.3%; Table 3.7.).

3.3. STATISTICAL ANALYSIS OF ALLELE FREQUENCY DATA

2 x 2 Contingency tables prepared from DRB1, DQB1 and DQA1 allele frequency data of RA affected Xhosa, ethnically-matched Xhosa comparison group, RA affected Cape coloured and ethnically-matched Cape coloured comparison group (Section 3.2.) with X^2, p, OR and 95% C.I. values, are reported in Tables 3.8., 3.9., 3.10., 3.11., 3.12. and 3.13.

p Values were obtained from standard X^2 tables assuming 1 degree of freedom for the case of a 2 x 2 contingency table. Where any one expected (calculated) value was < 5, p values were obtained using the Fisher exact method (Fisher, 1954) and are indicated in the Tables 3.8 - 3.13. with a superscripted star (*). Bonferoni corrected p values (Section 2.4.5.) are indicated with a superscripted dubble-star (**) and were obtained by multiplying the specific p value by 28 in Table 3.8; by 37 in Table 3.9; by 8 in Table 3.10; by 10 in Table 3.11; by 8 in Table 3.12 and by 9 in Table 3.13.

Table 3.6. **Frequencies of HLA-class II alleles at the DQB1 locus of RA affected Xhosa (AB) and Cape coloured (AC) patients and healthy, ethnically-matched comparison groups (CB and CC, respectively). n_s = number of subjects; n_a = number of alleles.**

	(n_s=25)		(n_s=94)		(n_s=65)		(n_s=110)	
	n_a=50 alleles		n_a=188 alleles		n_a=130 alleles		n_a=220 alleles	
DQB1*	**AB**	**%**	**CB**	**%**	**AC**	**%**	**CC**	**%**
02			1	0.5				
0201	7	14.0	28	14.9	11	8.5	39	17.7
03							2	0.9
0301	5	10.0	21	11.2	23	17.7	44	20.0
0302	11	22.0	9	4.8	32	24.6	23	10.5
0303			2	1.1	2	1.5	8	3.6
0401					3	2.3		
0402	5	10.0	28	14.9	2	1.5	10	4.5
0501	12	24.0	27	14.4	39	30.0	30	13.6
0502					2	1.5	7	3.2
0503							4	1.8
06			4	2.1			4	1.8
0601	1	2.0			8	6.2	7	3.2
0602	7	14.0	40	21.3	4	3.1	27	12.3
0603	1	2.0	16	8.5	3	2.3	10	4.5
0604			5	2.7			3	1.4
0605	1	2.0	4	2.1	1	0.8	1	0.5
0609			3	1.6			1	0.5
TOTAL	50	100.0	188	100.0	130	100.0	220	100.0

Table 3.7. **Frequencies of HLA-class II alleles at the DQA1 locus of RA affected Xhosa (AB) and Cape coloured (AC) patients and healthy, ethnically-matched comparison group (CB and CC, respectively). n_s = number of subjects; n_a = number of alleles.**

	(n_s=25)		(n_s=72)		(n_s=65)		(n_s=77)	
	n_a=50 alleles		n_a=144 alleles		n_a=130 alleles		n_a=154 alleles	
DQA1*	**AB**	**%**	**CB**	**%**	**AC**	**%**	**CC**	**%**
01	3	6.0	10	6.9	6	4.6	9	5.8
0101	8	16.0	11	7.6	19	14.6	15	9.7
0102	9	18.0	40	27.8	22	16.9	24	15.6
0103	2	4.0	16	11.1	10	7.7	9	5.8
0201	2	4.0	5	3.5	5	3.8	21	13.6
03					3	2.3		
0301	18	36.0	14	9.7	46	35.4	22	14.3
0302					1	0.8		
0401	3	6.0	24	16.7	3	2.3	6	3.9
0501	5	10.0	24	16.7	12	9.2	42	27.3
0601					3	2.3	6	3.9
TOTAL	50	100.0	144	100.0	130	100.0	154	100.0

Table 3.8. **2 x 2 Contingency tables of DRB1 allele frequency data with X^2, p, OR and 95% C.I. values. AB = RA affected Xhosa, CB = ethnically-matched Xhosa comparison group. * = Fisher's exact p value. ** = Bonferoni corrected p value.**

DRB1*		AB	CB		X^2	p	OR	95% C.I.
0101	+	2	3	5				
	-	48	185	233				
		50	188	238	0.25	0.28*	2.57	0.21 - 22.98
0102	+	1	1	2				
	-	49	187	236				
		50	188	238	0.02	0.38*	3.82	0.05 - 301.12
Total 01	+	3	5	8				
	-	47	183	230				
		50	188	238	0.52	0.37*	2.34	0.35 - 12.45
0301	+	1	16	17				
	-	49	172	221				
		50	188	238	1.64	0.13*	0.22	0.01 - 1.49
0302	+	3	27	30				
	-	47	161	208				
		50	188	238	1.81	0.18	0.38	0.07 - 1.33
Total 03	+	4	43	47				
	-	46	145	191				
		50	188	238	4.61	0.84**	0.29	0.07 - 0.88
0401	+	11	7	18				
	-	39	181	220				
		50	188	238	16.35	1.29×10^{-4}*	7.29	2.38 - 23.42
0404	+	1	1	2				
	-	49	187	236				
		50	188	238	0.02	0.38*	3.82	0.05 - 301.12
0405	+	2	1	3				
	-	48	187	235				
		50	188	238	1.54	0.11*	7.79	0.39 - 462.04

Table 3.8. (*continued*)

DRB1*		AB	CB		X^2	p	OR	95% C.I.
Total 04	+	14	12	26				
	-	36	176	212				
		50	188	238	16.81	4.13×10^{-5}	5.70	2.22 - 14.62
0701	+	1	7	8				
	-	49	181	230				
		50	188	238	0.03	1.0*	0.53	0.01 - 4.28
0702	+	2	3	5				
	-	48	185	233				
		50	188	238	0.25	0.28*	2.57	0.21 - 22.98
Total 07	+	3	12	15				
	-	47	176	223				
		50	188	238	0.05	1.0*	0.94	0.16 - 3.67
0901	+	2	2	4				
	-	48	186	234				
		50	188	238	0.67	0.20*	3.88	0.27 - 54.32
Total 09	+	2	2	4				
	-	48	186	234				
		50	188	238	0.67	0.20*	3.88	0.27 - 54.32
1001	+	4	7	11				
	-	46	181	227				
		50	188	238	0.81	0.25*	2.25	0.46 - 9.26
1101	+	9	17	26				
	-	41	171	212				
		50	188	238	2.40	0.12	2.21	0.80 - 5.67
1102	+	2	5	7				
	-	48	183	231				
		50	188	238	0.00	0.64*	1.52	0.14 - 9.65

Table 3.8. (*continued*)

DRB1*		AB	CB		X^2	p	OR	95% C.I.
Total 11	+	11	31	42				
	-	39	157	196				
		50	188	238	0.49	0.48	1.43	0.59 - 3.24
1201	+	1	4	5				
	-	49	184	233				
		50	188	238	0.25	1.0*	0.94	0.02 - 9.77
Total 12	+	3	7	10				
	-	47	181	228				
		50	188	238	0.10	0.44*	1.65	0.26 - 7.56
1301	+	1	12	13				
	-	49	176	225				
		50	188	238	0.74	0.31*	0.30	0.01 - 2.12
1302	+	2	16	18				
	-	48	172	220				
		50	188	238	0.59	0.38*	0.45	0.05 - 2.02
1303	+	1	1	2				
	-	49	187	236				
		50	188	238	0.02	0.38*	3.82	0.05 - 301.12
Total 13	+	4	36	40				
	-	46	152	198				
		50	188	238	2.76	0.10	0.37	0.09 - 1.11
1501	+	1	12	13				
	-	49	176	225				
		50	188	238	0.74	0.31*	0.30	0.01 - 2.12
1503	+	1	9	10				
	-	49	179	228				
		50	188	238	0.23	0.70*	0.41	0.01 - 3.06
Total 15	+	2	23	25				
	-	48	165	213				
		50	188	238	2.04	0.15	0.30	0.03 - 1.29

Table 3.9. **2 x 2 Contingency tables of DRB1 allele frequency data with X^2, p, OR and 95% C.I. values. AC = RA affected Cape coloured, CC = ethnically-matched Cape coloured comparison group. * = Fisher's exact p value. ** = Bonferoni corrected p value.**

DRB1*		AC	CC		X^2	p	OR	95% C.I.
0101	+	10	6	16				
	-	120	222	342				
		130	228	358	3.85	0.05	3.08	0.98 - 10.55
0102	+	2	4	6				
	-	128	224	352				
		130	228	358	0.08	1.0*	0.88	0.08 - 6.20
0103	+	1	1	2				
	-	129	227	356				
		130	228	358	0.11	1.0*	1.76	0.02 - 138.73
Total 01	+	13	16	29				
	-	117	212	329				
		130	228	358	0.63	0.43	1.47	0.63 - 3.39
0301	+	2	16	18				
	-	128	212	340				
		130	228	358	4.12	1.0**	0.21	0.02 - 0.91
0302	+	1	6	7				
	-	129	222	351				
		130	228	358	0.68	1.0**	0.29	0.01 - 2.41
Total 03	+	3	22	25				
	-	127	206	333				
		130	228	358	5.79	0.74**	0.22	0.04 - 0.76
0401	+	32	13	45				
	-	98	215	313				
		130	228	358	25.26	5.0×10^{-7}	5.40	2.61 - 11.67
0403	+	1	2	3				
	-	129	226	355				
		130	228	358	0.25	1.0*	0.88	0.02 - 16.98

Table 3.9. (*continued*)

DRB1*		AC	CC		X^2	p	OR	95% C.I.
0404	+	2	1	3				
	-	128	227	355				
		130	228	358	0.25	0.30*	3.55	0.18 - 210.16
0405	+	4	3	7				
	-	126	225	351				
		130	228	358	0.58	0.26*	2.38	0.39 - 16.47
Total 04	+	42	24	66				
	-	88	204	292				
		130	228	358	24.69	7.0×10^{-5}	4.06	2.24 - 7.43
0701	+	4	27	31				
	-	126	201	327				
		130	228	358	6.97	0.31**	0.24	0.06 - 0.70
0702	+	1	4	5				
	-	129	224	353				
		130	228	358	0.09	0.66*	0.43	0.01 - 4.46
Total 07	+	5	33	38				
	-	125	195	320				
		130	228	358	8.77	0.11**	0.24	0.07 - 0.63
08	+	2	2	4				
	-	128	226	354				
		130	228	358	0.00	0.62*	1.77	0.13 - 24.59
Total 08	+	4	2	6				
	-	126	226	352				
		130	228	358	1.28	0.20*	3.59	0.50 - 40.03
09	+	1	1	2				
	-	129	227	356				
		130	228	358	0.11	1.0*	1.76	0.02 - 138.73
0901	+	2	3	5				
	-	128	225	353				
		130	228	358	0.09	1.0*	1.17	0.10 - 10.37

Table 3.9. (*continued*)

DRB1*		AC	CC		X^2	p	OR	95% C.I.
Total 09	+	3	4	7				
	-	127	224	351				
		130	228	358	0.0	0.71*	1.32	0.19 - 7.95
1001	+	7	6	13				
	-	123	222	345				
		130	228	358	1.09	0.24*	2.11	0.59 - 7.75
1101	+	10	24	34				
	-	120	204	324				
		130	228	358	0.48	0.49	0.71	0.29 - 1.60
1104	+	3	5	8				
	-	127	223	350				
		130	228	358	0.09	1.0*	1.05	0.16 - 5.52
Total 11	+	13	37	50				
	-	117	191	308				
		130	228	358	2.18	0.14	0.57	0.27 - 1.16
1201	+	1	4	5				
	-	129	224	353				
		130	228	358	0.09	0.66*	0.43	0.01 - 4.46
1202	+	5	9	14				
	-	125	219	344				
		130	228	358	0.06	0.81	0.97	0.25 - 3.32
Total 12	+	6	17	23				
	-	124	211	335				
		130	228	358	0.59	0.41	0.60	0.19 - 1.65
1302	+	2	5	7				
	-	128	223	351				
		130	228	358	0.00	1.0*	0.70	0.07 - 4.34
1303	+	1	1	2				
	-	129	227	356				
		130	228	358	0.11	1.0*	1.76	0.02 - 138.73

Table 3.9. (*continued*)

DRB1*		AC	CC		X^2	p	OR	95% C.I.
Total 13	+	3	18	21				
	-	127	210	337				
		130	228	358	3.72	0.05	0.28	0.05 - 0.97
14	+	1	2	3				
	-	129	226	355				
		130	228	358	0.25	1.0*	0.88	0.02 - 16.98
1404	+	1	2	3				
	-	129	226	355				
		130	228	358	0.25	1.0*	0.88	0.02 - 16.98
Total 14	+	2	9	11				
	-	128	219	347				
		130	228	358	0.91	0.34*	0.38	0.04 - 1.88
15	+	1	8	9				
	-	129	220	349				
		130	228	358	1.54	0.16*	0.21	0.00 - 1.63
1501	+	16	15	31				
	-	114	213	327				
		130	228	358	2.75	0.10	1.99	0.88 - 4.49
1502	+	11	8	19				
	-	119	220	339				
		130	228	358	3.12	0.08	2.54	0.09 - 7.47
Total 15	+	28	37	65				
	-	102	191	293				
		130	228	358	1.23	0.27	1.42	0.79 - 2.53

Table 3.10. **2 x 2 Contingency tables of DQB1 allele frequency data with X^2, p, OR and 95% C.I. values. AB = RA affected Xhosa, CB = ethnically-matched Xhosa comparison group. * = Fisher's exact p value. ** = Bonferoni corrected p value.**

DQB1*		AB	CB		X^2	p	OR	95% C.I.
0201	+	7	28	35				
	-	43	160	203				
		50	188	238	0.00	0.95	0.93.	0.32 - 2.38
0301	+	5	21	26				
	-	45	167	212				
		50	188	238	0.00	0.98	0.88	0.25 - 2.59
0302	+	11	9	20				
	-	39	179	218				
		50	188	238	13.05	3.83×10^{-3}**	5.61	1.95 - 16.31
0402	+	5	28	33				
	-	45	160	205				
		50	188	238	0.44	0.51	0.63	0.18 - 1.80
0501	+	12	27	39				
	-	38	161	199				
		50	188	238	2.02	0.16	1.88	0.79 - 4.26
0602	+	7	40	47				
	-	43	148	191				
		50	188	238	0.90	0.34	0.60	0.21 - 1.49
0603	+	1	16	17				
	-	49	172	221				
		50	188	238	1.64	0.13	0.22	0.01 - 1.49
0605	+	1	4	5				
	-	49	184	233				
		50	188	238	0.25	1.0*	0.94	0.02 - 9.77

Table 3.11. **2 x 2 Contingency tables of DQB1 allele frequency data with X^2, p, OR and 95% C.I. values. AC = RA affected Cape coloured, CC = ethnically-matched Cape coloured comparison group. * = Fisher's exact p value. ** = Bonferoni corrected p value.**

DQB1*		AC	CC		X^2	p	OR	95% C.I.
0201	+	11	39	50				
	-	119	181	300				
		130	220	350	5.0	0.25**	0.43	0.19 - 0.90
0301	+	23	44	67				
	-	107	176	283				
		130	220	350	0.15	0.70	0.86	0.47 - 1.55
0302	+	32	23	55				
	-	98	197	295				
		130	220	350	11.33	7.65×10^{-3}**	2.80	1.49 - 5.28
0303	+	2	8	10				
	-	128	212	340				
		130	220	350	0.65	0.33*	0.41	0.04 - 2.12
0402	+	2	10	12				
	-	128	210	338				
		130	220	350	1.42	0.22*	0.33	0.03 - 1.58
0501	+	39	30	69				
	-	91	190	281				
		130	220	350	12.81	3.45×10^{-3}**	2.71	1.53 - 4.83
0502	+	2	7	9				
	-	128	213	341				
		130	220	350	0.35	0.49*	0.48	0.05 - 2.55
0601	+	8	7	15				
	-	122	213	335				
		130	220	350	1.11	0.29	2.00	0.61 - 6.62
0602	+	4	27	31				
	-	126	193	319				
		130	220	350	7.46	0.06**	0.23	0.06 - 0.68
0603	+	3	10	13				
	-	127	210	337				
		130	220	350	0.60	0.39*	0.50	0.09 - 1.98

Table 3.12. **2 x 2 Contingency tables of DQA1 allele frequency data with X^2, p, OR and 95% C.I. values. AB = RA affected Xhosa, CB = ethnically-matched Xhosa comparison group. * = Fisher's exact p value. ** = Bonferoni corrected p value.**

DQA1*		AB	CB		X^2	p	OR	95% C.I.
01	+	3	10	13				
	-	47	134	181				
		50	144	194	0.01	1.0*	0.86	0.14 - 3.52
0101	+	8	11	19				
	-	42	133	175				
		50	144	194	2.07	0.10*	2.30	0.75 - 6.74
0102	+	9	40	49				
	-	41	104	145				
		50	144	194	1.40	0.24	0.57	0.22 - 1.34
0103	+	2	16	18				
	-	48	128	176				
		50	144	194	1.46	0.16*	0.33	0.04 - 1.51
0201	+	2	5	7				
	-	48	139	187				
		50	144	194	0.07	1.0*	1.16	0.11 - 7.36
0301	+	18	14	32				
	-	32	130	162				
		50	144	194	16.75	3.41×10^{-4}**	5.22	2.17 - 12.57
0401	+	3	24	27				
	-	47	120	167				
		50	144	194	2.69	0.10	0.32	0.06 - 1.13
0501	+	5	24	29				
	-	45	120	165				
		50	144	194	0.83	0.36	0.56	0.16 - 1.61

Table 3.13. 2 x 2 Contingency tables of DQA1 allele frequency data with X^2, p, OR and 95% C.I. values. AC = RA affected Cape coloured, CC = ethnically-matched Cape coloured comparison group. * Fisher's exact p value. ** = Bonferoni corrected p value.

DQA1*		AC	CC		X^2	p	OR	95% C.I.
01	+	6	9	15				
	-	124	145	269				
		130	154	284	0.04	0.84	0.78	0.22 - 2.53
0101	+	19	15	34				
	-	111	139	250				
		130	154	284	1.16	0.28	1.59	0.72 - 3.52
0102	+	22	24	46				
	-	108	130	238				
		130	154	284	0.02	0.89	1.10	0.56 - 2.18
0103	+	10	9	19				
	-	120	145	265				
		130	154	284	0.18	0.70	1.34	0.47 - 3.86
0201	+	5	21	26				
	-	125	133	258				
		130	154	284	6.99	0.07**	0.25	0.07 - 0.72
0301	+	46	22	68				
	-	84	132	216				
		130	154	284	16.09	5.43×10^{-4}**	3.29	1.78 - 6.15
0401	+	3	6	9				
	-	127	148	275				
		130	154	284	0.18	0.51*	0.58	0.09 - 2.80
0501	+	12	42	54				
	-	118	112	230				
		130	154	284	13.75	1.87×10^{-3}**	0.27	0.12 - 0.56
0601	+	3	6	9				
	-	127	148	275				
		130	154	284	0.18	0.51*	0.58	0.09 - 2.80

Values of X^2 associated with a p value of < 0.05 were considered significant and indicated a significant disagreement between the observed data and the expected (calculated) data. Accordingly, the null hypothesis (H_0: no difference between observed and expected data) was rejected and the alternative hypothesis (H_A: observed and expected data are different from one another), was accepted.

OR and 95% C.I. values were calculated using the computer programme Epi-Info (ver 6.0).

The DRB1*0401 allele in the RA affected Xhosa patients compared to the ethnically-matched comparison group had a significant X^2 value of 16.75 [$p = 1.29 \times 10^{-4}$; OR = 7.29 (95% C.I.: 2.38 - 23.42); Table 3.8.].

The DRB1*04 ("total DRB1*04 allele") in the RA affected Xhosa patients compared to the ethnically-matched comparison group had a significant X^2 value of 16.81 [$p = 4.13 \times 10^{-5}$; OR = 5.70 (95% C.I.: 2.22 - 14.62); Table 3.8.].

The DRB1*0401 allele in the RA affected Cape coloured patients compared to the ethnically-matched comparison group had a significant X^2 value of 25.26 [$p = 5 \times 10^{-7}$; OR = 5.40 (95% C.I.: 2.61 - 11.67); Table 3.9.].

The DRB1*04 ("total DRB1*04 allele") in the RA affected Cape coloured patients compared to the ethnically-matched comparison group had a significant X^2 value of 24.69 [$p = 7 \times 10^{-5}$; OR = 4.06 (95% C.I.: 2.24 - 7.43); Table 3.9.].

The DQB1*0302 allele in the RA affected Xhosa patients compared to the ethnically-matched comparison group had a significant X^2 value of 13.05 [Bonferoni corrected $p = 3.83 \times 10^{-3}$; OR = 5.61 (95% C.I.: 1.95 - 16.31); Table 3.10.].

The DQB1*0302 allele in the RA affected Cape coloured patients compared to the ethnically-matched comparison group had a significant X^2 value of 11.33 [Bonferoni corrected $p = 7.65 \times 10^{-3}$; OR = 2.80 (95% C.I.: 1.49 - 5.28); Table 3.11.].

The DQB1*0501 allele in the RA affected Cape coloured patients compared to the ethnically-matched comparison group had a significant X^2 value of 12.81 [Bonferoni corrected $p = 3.45 \times 10^{-3}$; OR = 2.71 (95% C.I.: 1.53 - 4.83); Table 3.11.].

The DQA1*0301 allele in the RA affected Xhosa patients compared to the ethnically-matched comparison group had a significant X^2 value of 16.75 [Bonferoni corrected $p = 3.41 \times 10^{-4}$; OR = 5.22 (95% C.I.: 2.17 - 12.57); Table 3.12.].

The DQA1*0301 allele in the RA affected Cape coloured patients compared to the ethnically-matched comparison group had a significant X^2 value of 16.09 [Bonferoni corrected $p = 5.43 \times 10^{-4}$; OR = 3.29 (95% C.I.: 1.78 - 6.15); Table 3.13.].

The DQA1*0501 allele in the RA affected Cape coloured patients compared to the ethnically-matched comparison group had a significant X^2 value of 13.75 [Bonferoni corrected $p = 1.87 \times 10^{-3}$; OR = 0.27 (95% C.I.: 0.12 - 0.56)].

CHAPTER 4

DISCUSSION

The present study was conducted on two distinct populations, *viz*: Western Cape Xhosa and Cape coloured RA affected patients and on ethnically-matched, healthy, comparison groups. The data showed that the DRB1*0401, DQB1*0302 and DQA1*0301 alleles occurred in RA affected Xhosa and Cape coloured patients. Beside being significantly associated with RA in both RA affected populations, the DRB1*0401 allele was also the most frequently occurring allele in both the RA affected Xhosa and Cape coloured patients (22% and 24.6%, respectively). These results are in agreement with a study of RA affected South African Caucasoids, Xhosa and Cape coloured patients (Martell *et al*, 1989) and with a study of Zimbabwean RA affected black patients (Martell *et al*, 1990).

Martell *et al* (1989), reported an increase in the frequency of the DR4 (DRB1*04) allele in RA affected South African patients when compared to ethnically-matched comparison groups. A subsequent study by Martell *et al* (1990), reported a significant association ($p < 0.03$) of DR4 (DRB1*04) with RA in RA affected Zimbabwean black patients when compared with an ethnically-matched comparison group. Cutbush *et al* (1993) found a highly significant increase in the frequency of DRB1*04 subtype DRB1*0405 (29% *versus* 4%; $p < 0.001$) and DRB1*1001 (19% *versus* 2%; $p < 0.002$).

In a study by Gao *et al* (1990), DRB1*0401 was significantly increased ($p < 0.001$) in DR4 (DRB1*04) Caucasian RA affected patients when compared to a control group (86.5% *versus* 55.9%, respectively). The allele frequencies for RA affected patients and controls gave the highest RR (5.31) for the DRB1*0401 allele. The association of RA with DR4 (DRB1*04) was highly significant ($p < 0.00001$), while the differences in the frequencies for other DR4 alleles (DRB1*04) were not significant.

A study of RA in Israeli Jews by de Vries *et al* (1993), concluded that RA is associated with DRB1*01 - mainly the DRB1*0102 subtype, but not with DRB1*04 alleles. The allele frequency data obtained in the present study, however, does not agree with the above findings of de Vries *et al* (1993), since the DRB1*0102 allele was not significantly associated with RA in either the Xhosa or Cape coloured RA affected patients.

In the present study, DRB1*1101 occurred with a frequency of 18.0% in RA affected Xhosa patients, while the ethnically-matched control group had a frequency of 9.0%. There was a fairly high frequency (10.5%) of the same allele in the Cape coloured comparison group. Since the calculated X^2, p and OR values were not significant for this allele in either the Xhosa or Cape coloured RA affected patients, DRB1*1101 could not be considered a risk factor for predisposition to RA.

The DRB1*0302 allele had a frequency of 14.4% in the Xhosa comparison group when compared to RA affected Xhosa patients (6.0%). The X^2, p and OR values were not significant, therefore this allele could not be considered as a risk factor for predisposition to RA.

The DRB1*0701 allele had a frequency of 11.8% in the Cape coloured comparison group. This allele had a very low frequency in RA affected Cape coloured patients (3.1%). There was no association of this allele with a risk for RA, but it could be said that it occurred almost exclusively in the healthy, Cape coloured comparison group and with a very low frequency in RA affected patients of both populations. It is possible, as proposed by Zanelli *et al* (1995), that DRB1*0701 might be a protective DRB1 allele in the Cape coloured population which are not affected with RA. Zanelli *et al* (1995), in a study on an experimental mouse model of collagen-induced arthritis, suggested that the DRB1 locus is associated with a protective function in RA and that the DQB molecule is the actual arthrogenic peptide-presenting molecule. These workers proposed that the mechanism involved in DRB1 molecules protecting against autoimmunity is by providing peptides that are presented by DQ molecules. For an individual to actually develop RA, the susceptible DQ allele would be expressed together with the non-protective DRB1 alleles, along with the environmental factors involved in the autoimmune process.

The DRB1*1502 had a frequency of 8.5% in RA affected Cape coloured patients and a frequency of only 3.5% in the ethnically-matched control group. No significant association of this allele with RA was found with RA affected Cape coloured patients. It should be noted that DRB1*1502 allele was not found in the RA affected Xhosa patients or ethnically-matched comparison group and this could be attributed to possible genetic distribution of alleles in different populations.

At the DQB1 locus, the most frequently occurring allele was DQB1*0501 with a frequency of 24% in RA affected Xhosa and 30% in RA affected Cape coloured. A significant association of this allele with RA was found with RA affected Cape coloured patients. However, there was no significant association with RA of the same allele in the RA affected Xhosa patients. It is possible that with an increase in sample size of RA affected Xhosa patients, a significant association might have been obtained.

The second highest frequency in both RA affected Xhosa and Cape coloured patients was observed for DQB1*0302, where 22% of RA affected Xhosa and 24.6% of RA affected Cape coloured possessed this allele. A strong positive association with RA was found for this allele in both RA affected Xhosa and Cape coloured patients. The association of DQB1*0302 with RA affected patients from this study is in agreement with the proposed association of DQB1*0302 allele with DRB1 alleles in diseased populations (Zanelli *et al*, 1995).

The DQB1*0301 occurred more frequently in the Cape coloured comparison group when compared to RA affected Cape coloured patients (20.0% *versus* 17.7%, respectively). No significant association of this allele with RA was observed in either RA affected Xhosa or

ethnically-matched comparison groups. By comparison, a study by Rousseau *et al* (1991), employed an RFLP method on RA DR4 (DRB1*04) patients of mixed ancestry (Cape coloured) and on DR4 (DRB1*04) healthy controls of the same population group. These authors proposed an association of the DR4 (DRB1*04) haplotype in RA patients and an expression of DQBw7 (DQB1*0301) in Cape coloured RA affected patients of whom the incidence of DR4 (DRB1*04) allele in RA patients is 50% (Martell *et al*, 1989).

The data on DQB1*0301 allele obtained from the present study, however, differs from the data of Rousseau *et al* (1991), since the present study found no increase in the frequency of the DQB1*0301 allele associated with the DRB1*04 allele in RA affected Xhosa or Cape coloured patients. The data obtained from present study showed 6 RA affected Cape coloured patients possessing the DRB1*0401 and DQB1*0301 allele on the same postulated haplotype. Only 1 RA affected Cape coloured patient was observed with both the DRB1*0405 and DQB1*0301 allele on the same postulated haplotype. The DRB1*0401 and DQB1*0301 alleles were not observed among RA affected Xhosa patients on the same postulated haplotype. One RA affected Xhosa patient had DRB1*0405 and DQB1*0301 alleles on the same postulated haplotype. The DRB1*0404 and DQB1*0301 alleles were observed on the same postulated haplotype in 1 RA affected Xhosa patient.

The occurrence of DRB1*0401 and DQB1*0302 alleles in 8 out of 25 postulated haplotypes (30%) in RA affected Xhosa patients was considered high, possibly on account of the small sample size for this group, but also due to linkage disequilibrium between DRB1 and DQB1 alleles. In the RA affected Cape coloured patients, the frequency of DRB1*0401 and DQB1*0302 alleles on the same postulated haplotype was similarly high, with 17 out of 65 (31%) postulated haplotypes having the two alleles.

The DRB1*0402 and DQB1*0302 alleles occurred on the same postulated haplotype in only 1 RA affected Cape coloured patient. The DRB1*0405 and DQB1*0302 also occurred in 1 RA affected Cape coloured patient on the same postulated haplotype. From the above observations it is possible to suggest that the main association with RA is with DRB1*0401 and DQB1*0302 alleles in both RA affected Xhosa and Cape coloured patients.

If a disease is suspected to be attributed to a specific defective gene, use of appropriate DNA probes will identify the DNA segment of that particular gene (Mc Daniel *et al*, 1987). These genetic markers could then more accurately predict the risk for the occurrence of a disease and it is hoped, response to therapy and outcome. It is known from studies investigating HLA-linked RA that DQB1*0301 and DQB1*0302 are in linkage disequilibrium with most DRB1*04 haplotypes (Zanelli *et al*, 1995). In RA, however, the causative gene has not yet been defined and several genes may be involved in the pathogenesis of RA.

The distribution of DQB1 alleles in RA patients was also of interest. In the postulated haplotypes, the DRB1*04 alleles were associated with either DQB1*0301 or DQB1*0302

alleles. However, DRB1*04 allele association with DQB1*0302 was higher than expected in RA affected Xhosa or Cape coloured patients than in ethnically-matched comparison groups. No increase in the frequency of the DQB1*0301 allele was observed in either RA affected patients of both populations.

It is possible that each of the DRB1*04 alleles confers disease susceptibility. The DQB1*0301 and DQB1*0302 alleles are also present on some haplotypes that are not DRB1*04. The DQB1*0301 is commonly associated with DRB1*11 and DRB1*12 alleles and DQB1*0302 is associated with DRB1*07 haplotypes as well as some of the DRB1*04 subtypes which do not associate with RA, such as the DRB1*0402. This raises the question as to whether the DQB1 gene together with the DRB1 gene plays a role in RA disease predisposition. It is difficult to answer this question, since many DRB1*04 alleles are in strong linkage disequilibrium with alleles of the DQB1 locus. The linkage between a particular DQB1 allele and a particular DRB1 allele within the DR4 (DRB1*04) family of haplotypes, is not random (Nepom *et al*, 1987). The variant of the DR4 antigen, Dw4 (DRB1*0401) which is most prevalent in Caucasians, is found on two distinct haplotypes; one in association with the variant of DQ antigen, DQw3.1 (DQB1*0301) and the other in association with DQw3.2 (DQB1*0302; Nepom *et al*, 1987).

It has been suggested that despite linkage disequilibrium between DQ and DR alleles, higher RR occurs for a particular DQ allele than for the linked-DR genes because some DRB1 alleles can occur with DQ alleles not described to be in linkage disequilibrium and some DQ alleles are associated with more than one DR allele (Altman *et al*, 1991). Population groups show different DR and DQ allele associations (Altman *et al*, 1991).

It is important to note the association of DQB1 alleles with the DRB1*04 haplotype in all RA affected patients, despite the linkage disequilibrium between the two alleles. This poses the question whether these DQB1 alleles may be important in the recognition and development of RA. The DQ molecule has been implicated in other autoimmune diseases, e.g. Myasthenia Gravis (Bell *et al*, 1986) and in patients with IDDM (Nepom *et al*, 1986). It has been suggested by Zanelli *et al* (1995) that the DQ molecule presents the arthrogenic peptide that initiates the autoimmune response. No DQ allele has been clearly associated with a disease and different DQ alleles might be responsible for RA susceptibility in different population groups.

The DQB1*0602 allele was observed more frequently in the Xhosa comparison group (21.3%) when compared to RA affected Xhosa patients (14.0%) and similarly more frequently observed in the Cape coloured comparison group (12.3%) when compared to ethnically-matched RA affected patients (3.1%).

At the DQA1 locus, the most frequently occurring allele in the present study was DQA1*0301 in both RA affected Xhosa (36%) and Cape coloured patients (35.4%). A significant association for this allele with RA was found in both RA affected Xhosa and Cape coloured patients. The frequencies of DQA1*0301 from the present study were distinctly different to the frequencies

reported in other populations (Helmut *et al*, 1990). The relatively low frequency of this allele in US blacks (4.5%), US Caucasoids (8.5%), Hispanics (Mexican; 1.2%), Southeast Asians (0%), Indonesians (2.8%) and Nigerian blacks (4.2%) poses a question whether the DQA1 allele might be implicated together with DQB1*0302 and DRB1*0401 in RA and disease predisposition, although the three alleles, DQB1*0301, DQB1*0302 and DQA1*0301, are in strong linkage disequilibrium. The relatively low frequency of this allele observed in other populations and who are not affected with RA, could directly implicate DRB1*0401 as the causative disease allele in RA, since both DQA1*0301 and DRB1*0401 alleles are almost always found on the same haplotype. However, the only report of a high frequency of this allele was in a Japanese population (22.8%; Helmut *et al*, 1990), but which is still lower then the frequency obtained for the same allele in this study. This could be due to the large sample size used by Helmut *et al* (1990) compared to the relatively small sample size from the present study.

The DQA1*0501 had a high frequency of 27.3% in the Cape coloured comparison group, while in the RA affected Cape coloured patients, the frequency was only 9.2%. A strong negative association with RA (OR = 0.27; 95% C.I. = 0.12 - 0.56) was observed for this allele in the Cape coloured comparison group. This negative association of DQA1*0501 with RA in the Cape coloured comparison group suggests that this allele has a protective effect in RA or may be compensatory to the significant increase in the frequency of other alleles in patients with RA.

No significant increase in the frequency of DQA1*0501 allele was observed in the RA affected Xhosa patients or in the ethnically-matched comparison group.

The frequency of DQA1*0501 was also compared to results obtained from Helmut *et al* (1990). The highest frequency of DQA1*0501 was reported in Hispanics (Mexican; 36.7%) and US blacks (30.4%). The DQA1*0501 allele frequencies of US Caucasoids and Nigerian blacks (27.1% and 29.2%, respectively) was comparable to the frequency obtained in the present study for the Cape coloured comparison group.

The DQA1*0201 occurred fairly frequently (13.6%) in the Cape coloured comparison group. The frequency of DQA1*0201 in RA affected Cape coloured patients was only 3.8%. The Bonferoni corrected p value of DQA1*0201 was 0.07, just beyond the acceptable 0.05 significance level. The corresponding OR for DQA1*0201 in Cape coloured patients was 0.25 which indicates a tendency toward this allele being negatively associated with RA, thereby conferring a protective effect in RA patients.

The frequency of the DQA1*0201 allele was compared to that reported for other populations (Helmut *et al*, 1990). The highest frequency of DQA1*0201 was observed in Indonesians and Nigerian blacks (46.9% and 41.7%). The frequency of the same allele was also higher in US blacks and Caucasoids (26.3% and 19.1%) then the frequencies observed for this allele in the present study. The lowest frequency of DQA1*0201 was observed in a Japanese population (5.6%) which is higher than the frequency of this allele in the Cape coloured RA affected

population, but much lower than the frequency of the Cape coloured comparison group from the present study.

The above-mentioned differences in allele frequencies in different populations could result from differences in sample size in the populations studied. It could also be due to the genetic diversity (heterozygosity) of the HLA-DQA locus which has been reported to be fairly high (Helmut *et al*, 1990).

The differences in DQA1 allele frequencies in different populations including the present study, suggest that this diversity might be related to patterns of migration and the evolutionary history of human populations.

An interesting observation from the present study was the exclusive occurrence of DRB1*0401 with either the DQA1*0301 and DQB1*0302 alleles in RA affected Xhosa and Cape coloured patients. These alleles have been described to be in linkage disequilibrium with most DRB1*04 haplotypes in other populations (Zanelli *et al*, 1995).

A number of unusual associations of alleles at the DR and DQ locus, which have not been described previously, are reported in the present study. Several postulated haplotypes consisting of alleles not usually described to be in DR-DQ linkage disequilibrium, are reported in the present study; *viz.* DRB1*0401 - DQB1*0303 and DRB1*1301 - DQB1*0602 in the Xhosa comparison group; DRB1*0701 - DQB1*0301, DRB1*0701 - DQB1*0302 and DRB1*1503 - DQB1*0602 in the Cape coloured comparison group.

Since DRB1*0401 was the most frequently occurring allele in both RA affected Xhosa and Cape coloured patients, the present study suggests that the DRB1*0401 allele could be responsible for susceptibility to RA, since the frequency of this allele was very low in both comparison groups. Suggestions have been made which implicated DQB1 alleles as being responsible for RA susceptibility in different population groups. In this regard, the significant association of the DQB1*0302 allele with RA affected Xhosa patients and the DQB1*0302 and DQB1*0501 alleles in RA affected Cape coloured patients, indicates such a possibility as being feasible. In order to understand the predisposition to RA and other autoimmune diseases, with specific HLA haplotypes, a more extensive study that is restricted to DQ and DR genes, should be a goal for testing such an hypothesis.

CHAPTER 5

CONCLUSION

Based on the statistical data analysis from this study, it is possible that some HLA-class II alleles and RA can be associated, although no specific HLA allele was identified as being the cause of RA.

The data from this study showed an association of the DRB1*0401 allele with RA in both RA-affected Xhosa and Cape coloured patients. Statistical analysis indicated a strong association of the DRB1*0401 allele with RA which is in agreement with the published work of Stastny (1978) and Martell *et al* (1989, 1990).

The findings from this study are in agreement with other published data on RA and DRB1*0401 association.

The DRB1*0401 allele and occasionally other DRB1*04 alleles seemed to occur more frequently in almost all RA-affected individuals and this finding was similar to the findings reported in studies on RA in different populations from diverse ethnic backgrounds. It is possible that only one susceptibility allele in linkage disequilibrium with DR alleles, is the causative allele of RA and disease predisposition. Whether the DRB1 gene itself or the DRB1*0401 allele, as suggested by other researchers, including the present study, is the causative allele of RA and disease predisposition, still needs to be investigated.

5.1. DIRECTION FOR FUTURE STUDIES

RA is a disease which has not until recently, responded well to new treatments, eg. corticosteroids, non-steroidal anti-inflamatory drugs and disease-modifying agents such as gold and penicillamine. The progress of biotechnology, however, offers new hope in the treatment of RA, especially in preventing helper T-cells mobilizing the immune system to respond to "foreign" joint tissue. By suppressing the immune response using traditional therapy, the patient's general immunity is reduced, leaving the system open to opportunistic infection. The development of a monoclonal therapy for the treatment of RA could become a goal for many researchers. In this respect, the monoclonal antibody developed at the University of Cambridge Pathology Department (UK), has been reported to react to protein marker on human T-cells, B-cells and monocytes. This "therapeutic mAb" was administered every 10 days to 8 patients with severe RA who failed to respond to most of the usual anti-RA drugs. The treatment proved to be successful for up to 7 months, although the patients had some unwelcome side-effects which were caused by a reaction to the destruction of lymphocyte by antibody. The patient's immune system did not, however, react against the introduced mAb. Researchers are seeking to provide

longer symptom relief, by reducing rejection of the mAb over time. The possibility of treating RA patients with mAb, however, is still some years away.

Despite the enormous costs of research, a short-term treatment that provides long-term protection against further disease-reducing disability and the need for other therapies, could prove to be highly favourable from an economic perspective.

It has been known for some time that DRB1 and DQB1 alleles differ from one another in 1 or 2 amino acids and that some of the alleles with only 1 amino acid substitution could be responsible for disease predisposition. The ultimate goal for researchers in HLA and its association with disease appears to be gene-sequencing of diseased patients, so that class-II alleles occurring at a particular HLA locus could be compared with class II alleles from control subjects. The HLA-disease associations would thereby become better understood by researchers in the field of molecular biology and medical science.

CHAPTER 6

APPENDIX

BUFFERS AND REAGENTS

All reagents were of the highest grade commercially available.

6.1. DNA EXTRACTION

6.1.1. Lysis buffer

0.32 M Sucrose	109.5 g
10% (v/v) Triton-X-100	10 ml stock
50 mM $MgCl_2$	5 ml 1 M stock
10 mM Tris-HCl (pH 7.4)	10 ml 1 M stock

Make to one litre with filtered distilled H_2O.

6.1.2. Phosphate Buffered Saline (PBS; pH 7.5)

137 mM NaCl	8 g
2.7 mM KCl	2 g
1.5 mM KH_2PO_4	2 g
8.1 mM Na_2HPO_4	1.15 g

Dissolve in one litre of filtered distilled H_2O. Adjust the pH between 7.3 - 7.5.

6.1.3. Saline-EDTA

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

Make up to 200 ml with filtered distilled H_2O.

6.1.4. Sodium dodecyl sulphate (SDS)

10% (w/v) SDS	100 g (Sigma, St Louis, USA)

Add to 700 ml distilled H_2O. Heat to 65°C to dissolve, adjust volume to 1 litre with sterile distilled H_2O.

6.1.5. Proteinase K

10 mg/ml solution

6.1.6. Sodium acetate

Saturated solution

6.2. DNA AMPLIFICATION - PCR

6.2.1. 10 x PCR buffer C (final concentration 1.5 mM $MgCl_2$)

100 mM Tris-HCl (pH 8.4 at 24°C)	1 ml 1 M stock
500 mM KCl	2.5 ml 2 M stock
15 mM $MgCl_2$	150 μl 1 M stock
1 mg/ml Gelatine	0.5 ml 20 mg/ml stock
0.2 % (v/v) Nonidet P-40	20 μl stock (Sigma, St Louis, USA)

Make up with 5.85 ml sterile distilled H_2O to volume of 10 ml. Sterilize stock solution by autoclaving. Store at 4°C. Optimal concentration of $MgCl_2$ depends on the primers.

6.2.2. 10 x dNTP (deoxy-nucleotide triphosphate mixture)

2 mM dATP (Boehringer Mannheim)

2 mM dCTP (Boehringer Mannheim)

2 mM dGTP (Boehringer Mannheim)

2 mM dTTP (Boehringer Mannheim)

Add 2 μl of each dNTP (0.1 M) to 92 μl TE (3 mM Tris-HCl, pH 7.0 / 0.1 mM EDTA). Store at -20°C in a small aliquot.

6.2.3. TAQ-DNA Polymerase

Use 1 unit (0.2 μl) Taq DNA polymerase (Boehringer Mannheim) per sample to be amplified.

6.2.4. Tris-EDTA (TE; pH 7.4)

10 mM Tris base	1.21 g
0.1 mM $Na_2EDTA.2H_2O$	0.037 g

Add Tris and EDTA to 900 ml distilled H_2O and adjust pH to 7.5 with HCl. Adjust volume to one litre with sterile distilled H_2O.

6.3. GEL ELECTROPHORESIS

6.3.1. Loading buffer (5 x)

50 mM Tris-HCl (pH 7.6)	0.5 ml 1 M stock
50 mM EDTA	1 ml 0.5 M stock
0.5% (w/v) SDS	0.5 ml 10% (w/v) solution
0.1% (w/v) Bromophenol blue	0.01 g
40% (w/v) Sucrose	4 g

Make up to 10 ml with sterile distilled H_2O.

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

6.3.2. Tris-acetate (TAE Electrophoresis buffer; 50 x)

2 M Tris-base	242.2 g
1 M Glacial acetic acid	57.1 ml
0.1 M EDTA	100 ml 0.5 M stock

Make up to one litre with sterile distilled H_2O. Make two litres of a 0.5 x TAE solution for electrophoresis.

6.3.3. Agarose gel (1%)

Agarose	2 g (type II; Sigma, St Louis USA)
TAE	4 ml 50 x stock
Filtered distilled H_2O	up to 200 ml
Ethidium bromide	10 μl 10 mg/ml stock

Boil, cool and add 10 μl ethidium bromide (final concentration of 10 mg/ml).

6.3.4. Ethidium bromide

Stock solution: 10mg/ml in double distilled H_2O

Dilute 5 μl stock solution per 100 ml 1 x TAE for electrophoresis buffer.

6.4. LABELLING OF SSOPs

6.4.1. 10 x Tailing buffer

1.4 mM Na-cacodylate	0.27 mg
300 mM Tris-base	36.33 mg
10 mM $CoCl_2$	2.38 mg

Make up to 1 ml with sterile distilled H_2O and keep frozen at -20°C.

6.4.2. Digoxigenin-ddUTP (dig-ddUTP)

1 μl 1 mM stock digoxigen-ddUTP (Boehringer Mannheim)

6.4.3. Dithiothreitol (DTT)

1 μl 1 mM DTT

6.4.4. Terminal deoxynucleotidyl transferase

1 μl (25 units) terminal deoxynucleotidyl transferase (Boehringer Mannheim).

6.4.5. Sequence-specific oligonucleotide probes (SSOPs)

6 μl (30 pmol)

6.5. PREHYBRIDIZATION AND HYBRIDIZATION OF NYLON MEMBRANES

6.5.1. 30 x SSPE

NaCl	262.96 g
$NaH_2PO_4.2H_2O$	46.80 g
EDTA (0.4 M stock)	75 ml

Add sterile distilled H_2O up to 800 ml mark. Slowly add NaCl. Add 10 N NaOH, slowly to adjust pH of the solution. Dissolve all NaCl. Final pH is 7.7 adjusted with NaOH. Adjust the volume to one litre with sterile distilled H_2O and autoclave.

6.5.2. Hybridization buffer

6 x SSPE	8 ml 30 x stock solution
5 x Denhardt	4 ml 50 x stock solution
0.1% (v/v) Sodium sarcosine	400 μl 10% (v/v) stock solution
0.02% (w/v) SDS	80 μl 10% (w/v) solution
Distilled H_2O	27.52 ml

Make up to a final volume of 40 ml with sterile distilled H_2O.

6.5.3. 50 x Denhardt's solution

2% (w/v) Polyvinylpyrolidone 40	2 g (Sigma, St Louis, USA)
2% (w/v) Ficoll 400	2 g (Sigma, St Louis, USA)

Make up to 100 ml with distilled H_2O. Autoclave for 10 minutes at 120°C. Cool to room temperature. Add 2 g bovine serum albumin (BSA, Fraction V; Boehringer Mannheim) and

sterile distilled H_2O to make 200 ml of solution. Filter through a 0.45 μm filter and store in aliquots at -20°C until use.

Note: BSA is made separately in 100 ml of distilled H_2O by layering BSA on top of distilled H_2O and allowing to dissolve.

The two solutions are now added together.

6.5.4. Sodium sarcosine (10%)

10 g in 100 ml of sterile distilled H_2O. Autoclave and store at 4°C.

6.6. WASHING OF SSO PROBES FROM NYLON MEMBRANES

6.6.1. Stock solutions

6.6.1.1. 0.5 M EDTA (pH 7.0)

$Na_2EDTA.2H_2O$	186.1 g

Add 800 ml of sterile distilled H_2O. Stir vigorously on a magnetic stirrer. Adjust the pH to 8.0 with NaOH pellets (± 20 g). Make up to one litre with sterile distilled H_2O. Autoclave.

6.6.1.2. 2 M Tris (pH 8.0)

Tris base	242.2 g

Dissolve in 800 ml distilled H_2O. Adjust pH to 8.0 with concentrated HCl. Adjust volume to one litre with sterile distilled H_2O.

6.6.1.3. Tetramethylammonium chloride (TMAC)

50 mM Tris-HCl (pH 8.0)	50 ml 1 M stock
3.0 M TMAC	491.8 ml 0.1 M stock
2 mM EDTA	4 ml 0.5 M stock
0.1% (w/v) SDS	10 ml 10% (w/v) stock

Add 444 ml of sterile distilled H_2O to make one litre.

6.6.2. Washing buffers

6.6.2.1. Washing buffer 1

2 x SSPE	33 ml 30 x stock solution
0.1% (w/v) SDS	5 ml 10% (w/v) solution

Make up to 500 ml with sterile distilled H_2O.

6.6.2.2. TMAC buffer

50 mM Tris (pH 8.0)	12.5 ml 2 M stock solution
3 M TMAC	254 ml 5.9 M stock solution
2 mM EDTA	2 ml 0.5 M stock solution

0.1% (w/v) SDS	5 ml 10% (w/v) solution

Make up to 500 ml with sterile distilled H_2O.

6.6.2.3. 2 x SSPE

13.33 ml of 30 x stock solution

Male up to 200 ml with sterile distilled H_2O.

6.6.3. Detection of dig-labelled dUTP

6.6.3.1. Buffer 1

0.1 M Maleic Acid	50 ml 2 M stock
0.15 M NaCl	75 ml 2 M stock

Make up to one litre with sterile distilled H_2O and pH to 7.5 with NaOH. For detection of dig-labelled dUTP, use 5 ml of 30% (v/v) Tween in Buffer 1 and make up to 500 ml with Buffer 1.

6.6.3.2. Buffer 2

10% Blocking stock solution	24 ml

Make up to 240 ml with Buffer 1.

6.6.3.3. Buffer 3

0.1 M Tris	12.11 g
50 mM $MgCl_2.6H_2O$	10.166 g
0.1 M NaCl	5.844 g

Dissolve in 800 ml of sterile distilled H_2O. Adjust the pH to 9.5 with concentrated HCl.

6.6.3.4. Anti-Digoxigenin-Ap, Fab fragment

Add 5 μl Anti-Dig-Ap, Fab fragment (Boehringer Mannheim) to 50 ml of Buffer 2.

6.6.3.5. Lumigen-PPD [LMPPD; 4-Methoxy-4-(3-phosphatphenyl)spiro-(1,2-dioxeton-3,2-adamantan)]

Add 100 μl of LMPPD (Boehringer Mannheim) to 9.9 ml of Buffer 3.

6.6.4. Removal of SSO probes from nylon membranes

6.6.4.1. Stock solution (4 N NaOH)

NaOH	160 g

Add sodium hydroxide pellets slowly into a plastic container kept on ice. Stir constantly on a magnetic bench stirrer. Adjust to one litre with sterile distilled H_2O. Do not autoclave. Store in a plastic bottles.

6.6.5. Dehybridization buffers

6.6.5.1. 0.4 N NaOH

20 ml of 4 N stock solution

Make up to 200 ml with sterile distilled H_2O. Heat the solution to 42°C.

6.6.5.2. 0.2 M Tris - 0.1 x SSPE - 0.1 x SDS

0.2 M Tris, pH 8.0	20 ml 2 M stock solution
0.1 x SSPE	1.46 ml 30 x stock solution
0.1% (w/v) SDS	2 ml 10% (w/v) solution

Make up to 200 ml with sterile distilled H_2O. Heat the solution to 42°C.

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