THE UNIVERSITY OF CALGARY

GENETIC FACTORS PREDISPOSING TO RHEUMATOID ARTHRITIS IN MEXICAN MAYAN INDIANS

by

NORMA SCHMILL LOPEZ

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Genetic Factors Predisposing to Rheumatoid Arthritis in Mexican Mayan Indians" submitted by Norma Schmill Lopez in partial fulfillment of the requirements for the degree of Master of Science.

Dr. L.L. Field, Supervisor
Department of Pediatrics

Dr. M.J. Fritzler
Department of Medicine

Dr. A.T. Garber
Department of Pediatrics

Dr. R.B. Bell
Department of Clinical Neuroscience

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ABSTRACT

The aim of this study was to determine if HLA and immunoglobulin genes predispose to RA in Mexican Mayan Indians, and if so whether the predisposing genes are the same as those that predispose to RA in other ethnic groups.

The patient group consisted of 41 unrelated Mexican Mayan Indians with a diagnosis of RA as defined by the ARA. The control group consisted of 139 unrelated Mexican Mayan Indians, as well as 87 individuals from Mexico City.

The results of this study show that there was no statistical difference in frequencies of Gm haplotypes and Km allotypes between Mayan RA patients and the Mayan controls.

The frequency of individuals positive for HLA-DR4 was found to be very high in the control group (0.74). This group was highly homogeneous for the DR4 subtypes DRB1*0403/0407 (Dw13) or DQB1*0404/0408 (Dw14). In the RA patient group, the frequency for DR4 was significantly increased (0.92). The predominant subtype in the patient group was DRB1*0403/0407 (Dw13). The gene frequencies of Dw subtypes were not significantly different in RA patients and controls, suggesting that Dw subtypes per se do not predispose to RA independently of the DR4 effect.

ACKNOWLEDGMENT

I would like to thank my supervisor Dr.L.L. Field for her invaluable assistance and guidance and for providing me with the opportunity to perform this study in her laboratory. I would also like to thank the members of my supervisory committee Dr.M. Fritzler and Dr.A. Garber and also Dr.R.B. Bell for his contribution as an examiner.

Many thanks to all members in the laboratory, especially R.Tobias, E.Swiergala, D.Nolan and T.Magnus for their technical support and friendship.

I dedicate this thesis to my father for his unfailing love and support.

To my family with all my love.

To Bob and friends many thanks.

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amp	ampicillin
ATP	adenosine triphosphate
bp	base pair
BSA	bovine serum albumin
°C	Celsius
Ci	Curies
cm	centimetre
CsCl	cesium chloride
C	cytidine
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
cpm	counts per minute
DNA	deoxyribonucleic acid
dTTP	deoxythymidine triphosphate
EDTA	ethylene-diaminetetra-acetic acid
g	gram
G	guanosine
h	hour
high TE	100 mM Tris (pH 8.0) 40 mM EDTA
HLA	Human Lymphocyte Antigen
IMSS	Instituto Mexicano del Seguro Social
ISSSTE	Instituto de Seguridad y Servicios Sociales para los Trabajadores del Estado
Kb	Kilobase pairs
Klenow	large fragment of E.coli DNA polymerase I
l	litre
LB	Luria broth
low TE	10 mM Tris (pH 8.0) 1 mM EDTA
M	molar

µg	microgram
min	minute
µl	microlitre
ml	millilitre
mm	millimetre
mM	millimolar
Mw	molecular weight
n	number
ng	nanogram
nm	nanometre
OD	optical density
OLB	oligonucleotide labelling buffer
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pH	-log [H^+]
pM	picomolar
PNK	Polynucleotide Kinase
RA	Rheumatoid Arthritis
RFLP	restriction fragment length polymorphism
rpm	revolutions per minute
SDS	sodium dodecylsulfate
sec	second
SSOP	sequence specific oligo probe
TEMED	N,N,N',N'-Tetramethylethylenediamine
tRNA	transfer ribonucleic acid
V	Volt
v	volume
vs	versus
w/v	weight/volume

A. INTRODUCTION.

A 1. Overview.

The present study represents an effort to demonstrate the existence or non-existence of genetic characteristics differing between healthy controls and Rheumatoid Arthritis patients (RA) in the so-called Mayan linguistic family.

The ability to predict a predisposition to the development of a disease in a certain population could be of particular value in formulating treatments directed toward postponing early disease onset or preventing the disease itself. However, this is an ideal goal whose achievement is still very far from reach. The contribution of a variety of disciplines including genetics, epidemiology, and molecular genetics, in conjunction with clinical research, could help delineate the risk factors for the occurrence of RA and, possibly, could help predict the gene (or genes) involved in the etiology and pathogenesis of RA.

The etiology of RA is unknown, but both genetic and environmental factors appear to play very important roles in disease development. Genes within the HLA region are known to contribute to genetic susceptibility, and there is a well documented association between RA and genes of the HLA-D region (Goldstein and Arnett 1987; Puttick et al 1990a,b). Immunoglobulin heavy chain allotypes (Gm) have not been directly associated with RA, but an association between the G1m(2) allotype and HLA-DR4 in RA patients has been reported, suggesting that immunoglobulin heavy chain genes and HLA-DR4 or a gene in linkage disequilibrium with DR may be interacting in the pathogenesis of RA (Ollier et al 1988b; Thomson et al 1986).

Plasma and DNA samples from 139 healthy controls of Mayan background, and 41 RA patients with the same ethnic origins were submitted to a series of studies comparing the Gm and Km allotypes and HLA types. Eighty-seven plasma samples from healthy Mexico City (non-Mayan) controls were used only for immunoglobulin Gm and Km allotype frequency studies. Several different methodologies were used in order to determine the frequency of HLA-DRB and -DQB alleles in the control population and the RA patients group.

A 2. The Mayans.

The Maya Civilization arose around 2500 BC, in the area called Mesoamerica. The region in which the Maya civilization developed includes the present states of Yucatan, Campeche, Tabasco, the eastern half of Chiapas and the territory of Quintana Roo in the Republic of Mexico; the Department of Peten and the adjacent highlands to the south of Guatemala; the western section of the Republic of Honduras and all of British Honduras; at the east, the border of the Lempa River in EL Salvador (Comas 1966) (see Fig.1).

There have been several attempts to group the various Mayan tongues into larger groups. The linguistic family called "Mayan" contains a number of closely related but mutually unintelligible languages- the result of a long period of internal divergence (Coe 1984).

McQuown (1956) believes that the very first Maya were a small Indian tribe of North American origin, distantly affiliated with some peoples in southern Oregon and Northern California, and more closely related to the Totonacan and Zoquean speakers of Mexico. They might have settled in the highlands of Guatemala by the middle of the third millennium BC, and used the proto-Maya language.

Diebold (1960) has used the "Migration Theory" to determine the most probable homeland of proto-Maya language. He suggests that the Guatemala Highlands somewhere between the present city of Guatemala and the Chiapas border.

He also infers a later split between three ancestral groups of the contemporary peoples:

1)- The predecessors of the Huastec and Chicomuceltec.

2)- The predecessors of the Yucatec Maya.

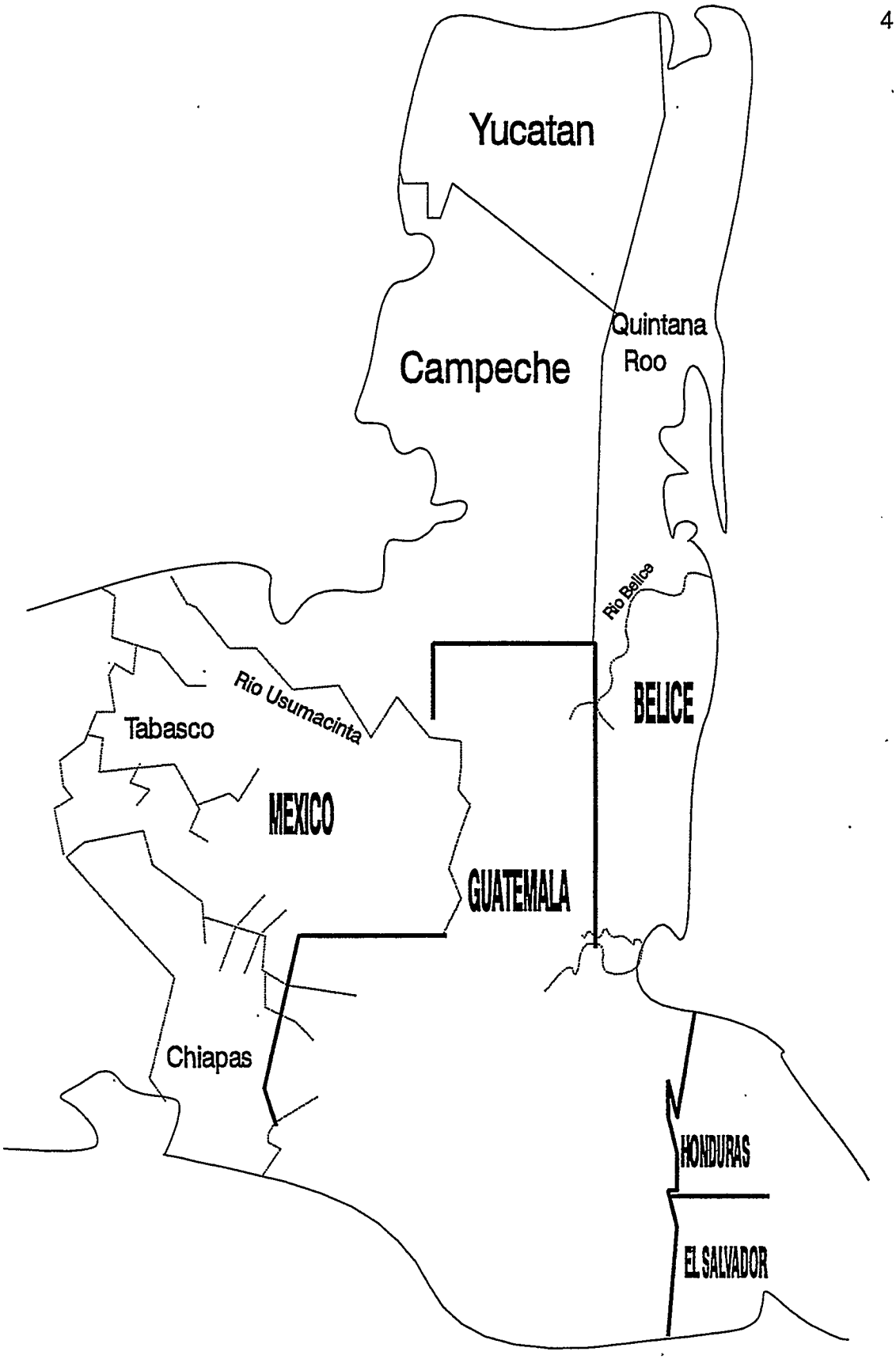
3)- The predecessors of all the other Mayans.

The modern Yucatan Maya are mesomorphic. They have rather long arms and small hands and feet, the average height of the men is 155.11 cm and of women 142.72 cm. They are one of the broadest-headed peoples in the world. Their cephalic index averages 85.01 for men and 87.11 for women, as compared with 79.30

Fig.1 MAP OF THE ACTUAL MAYA REGION.

In the Mexican Republic including the states of Yucatan, Campeche, Quintana Roo, Tabasco and Chiapas and almost all the Guatemala Republic and Belice, as well as small portions of Honduras and El Salvador.

Adapted from "Los Mayas el tiempo capturado". Demetrio Sodi, 1980.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for Caucasian men and 80.06 for Caucasian women (Steggerda 1932).

The Maya have a copper-brown skin colour, their hair is straight and is black to dark brown in colour. The Maya do not have a large amount of body hair.

Two other physical characteristics of the modern Yucatan Maya are an epicanthic eye fold (a fold at the inner corner of the eye) which is characteristic of eastern Asiatics, and a Mongolian spot (which is an almost universal physical characteristic of the peoples of the eastern Asia), is also very common among Mayan babies. This is a small bluish to purple spot at base of the spine, which is present at birth and fades or disappears before the tenth year (Morley 1956).

Some serological investigation of the Maya population has been done. Matson et al (Comas 1966) observed that in the ABO blood system, the frequency of the "O" group ranged from 86.5% to 100%, while in the Rh-hr system, all the Mayan groups were Rh(+) as other Amerindians. The presence of the series A1, A2, B and Rh(-) indicate some admixture from other populations (Comas 1966). Today there are approximately 5 million people of Mayan ancestry.

A 2.1 The presence of Africans in Mesoamerica.

The slave traffic between Spain and America started between 1502 and 1509 AD during the administration of Governor Ovando in Santo Domingo. The first Negro slaves, already speaking Spanish, were servants recruited among the many Negro slaves who at the time were kept in Spain as well as in Portugal (Rout 1976). Between the first years of the XVI century and the total abolition of slavery in Cuba (1886) and in Brazil (1888), it is estimated that around 9,200,000 Black slaves and 50,000 Black non-slaves arrived to the New World (Mintz 1977). The stimulus for direct and large scale slave trade between Africa and America was provided by the exploitation of mines and the establishment of plantations (Mörner 1967). Furthermore, African males also sought the sexual favours of the Indian women. The result was a rapid growth in the number of Zambos (Afro-Indians) and Mulattoes (Euro-Africans) (Rout 1976). In Mexico today, the many Blacks of a hundred years ago have been almost entirely absorbed into the local population.

A 2.2 The presence of Europeans in Mesoamerica.

The first contact of Europeans (Spanish) with the Mayan was in 1511, when the survivors of a shipwreck landed on the Yucatan Peninsula. Sixteen years later (1527) the Maya resisted the Spanish invasions led by Francisco de Montejo and his son Montejo El Mozo. By 1540 the eastern part of the Peninsula was completely under Spanish domination, and the first Spanish plantations began to flourish. Plantations became a centres specialized in the commercial production and processing of agricultural goods. Examples of this commercial production are the banana plantations on the Atlantic and Pacific coast of Mexico, and the henequen plantations in the south-east part of Mexico. The Spanish occupation lasted 275 years (Bartolome and Barabas 1977).

At the beginning, few unmarried European women came to the colonies, and Spanish males had access to the African and Indian females. The Spaniards obtained the Indian women by force or by peaceful means. Inter-racial marriages between Spanish males and Indian females as well as Spanish women and Indian men started to occur by 1525 (Mörner 1967). The consequence of the racial mixing was the creation of the so-called "Mestizos" race.

A 2.3 The presence of Asians in Mesoamerica.

After Black slavery was abolished, the demand for docile manpower on the plantations was satisfied by the use of immigrant contract labour, which was mostly of Asiatic origin. The first documentation of Oriental immigration to Mexico occurred in a colonial document dated about 1664-1673. It was written in the time of Antonio Sebastian de Toledo, marques de Mancera, and it was addressed to the Queen Maria of Austria who governed the Spanish Empire at that time. This document described the intensive traffic of Oriental slaves from the Philippines, using the "Galeon de Manila" or the "Nao de China" to transport people as well as other commercial trades. The document does not mention the number of slaves transported to Mexico (Gonzalez 1989).

Toward the middle of the last century a large number of Chinese (coolies) were imported by the Government of British Honduras as labourers on the land. A considerable number of these Chinese ran away as soon as they reached the colony. They sought refuge in the south-east part of Mexico.

Documentation shows that approximately 2,400 Koreans were imported to the Yucatan to work in the henequen plantations in 1905 (Nidia 1984). Suarez Molina (1977) indicates the importation of Asians was to supply labour for the henequen plantations. He also concludes that the importation of Koreans and Chinese was unsuccessful because they preferred to work in the vegetable fields or as cooks, laundrymen or in other domestic services.

The Orientals were readily accepted by the native Mayans. They were given wives and land, and, in fact, encouraged to settle in the country. The natural consequence of this immigration was that in a few years they had mixed and in the last years of the nineteenth century, had reached the second, and the third generation born in the Americas (Bartolome and Barabas 1977).

A 2.4 Genetic admixture into Mayan population.

From the above discussion, it can be seen that the modern Mayan population, while predominantly Indian, may contain an admixture from other populations such as Europeans (Spanish), African, and Oriental. The mixing of these four populations opens the doors for a very interesting genetic analysis.

A 3. The HLA system.

HLA is an abbreviation for "human leucocyte antigen". Although these cell surface glycoproteins were originally detected on leucocytes, antigens of the HLA-A,-B,-C loci are present on all nucleated cells. HLA-DR,-DQ, DP and -Dw antigens are present on B lymphocytes and some other cell types.

The HLA complex, located on the short arm of chromosome 6, contains several distinct loci that have been organized into different classes based on the structure and function of the products they encode. Genes at the HLA-A, -B, and

-C loci encode the class I molecules, genes at the HLA-DR, -DQ, and -DP loci encode the class II molecules, and genes at the C2, BF, C4A, C4B, 21-hydroxylase loci encode the class III molecules (McCarty and Koopman 1993; Roitt 1988).

Other genes within the class III region which have functions that could be associated with the immune system are the tumour necrosis factor A and B genes (TNF) and the heat-shock protein HSP70 family (located between C2 and TNF genes) (Trowsdale and Campbell 1992).

TAP1 and TAP2 are two major histocompatibility complex genes, located between HLA-DP and -DQ genes, whose products form a transporter molecule involved in the endogenous antigen processing (Powis et al 1993).

The HLA loci are tightly linked on chromosome 6 and therefore alleles at these loci are almost always inherited as a unit. This unit, composed of the combination of alleles at each locus, is called a haplotype. Particular alleles at the various loci show preferential association with one another, a phenomenon known as linkage disequilibrium. Thus, certain haplotypes are found with greater than expected frequency in the population.

The class I antigens are composed of a ≈44000 dalton heavy chain, termed α, noncovalently associated with a ≈12000 dalton light chain, termed β2 microglobulin. Although the α chain is encoded by an HLA locus on chromosome 6, the light chain is encoded on chromosome 15 (Schiffenbauer and Schwartz 1987).

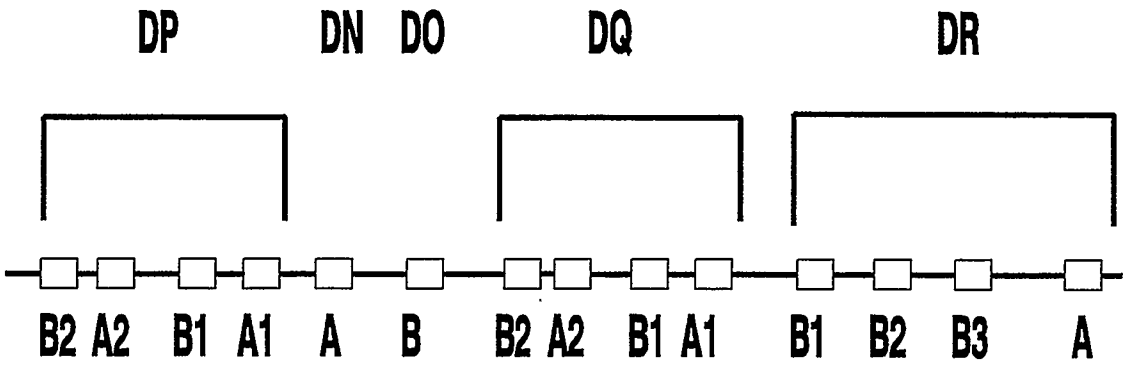
The general organization of the HLA-D region encoding class II molecules is shown in Figure 2. The class II molecules consist of a ≈34000 dalton heavy chain, termed α, noncovalently associated with a ≈29000 dalton light chain, termed β. Both chains are encoded by genes within the HLA complex. The region of chromosome 6 which encodes the class II molecules is designated the HLA-D region and is divided into three main subregions: DR, DQ, and DP. Each subregion contains at least one functional α chain gene and one functional β chain gene, which, when transcribed, form a functional αβ heterodimeric glycoprotein expressed on the cell surface (Mach et al 1988; Roitt 1988).

The HLA-DR subregion includes a single DRα chain gene termed DRA and five β chain genes termed DRB1-5. The DRA gene is not polymorphic, whereas

Fig.2 GENERAL ORGANIZATION OF HLA-D REGION.

The genes are linked on the short arm of chromosome 6. The DP region is closest to the centromere. DPA2, DPB2, DQA2, DQB2 and DRB2 are pseudogenes and not expressed. DN and DO are not actually known to be transcribed in vivo (DRB4 and DRB5 genes are not depicted in this figure). Adapted from McCarty and Koopman, 1993.

CHROMOSOME 6



the DRB1 gene is highly polymorphic. The α and β gene products are called antigens or epitopes. The DRB1 encoded DR antigens can be recognized by classic serology. The DRB4 genes encode the DRw53 serologic specificity, and the DRB3 gene encode the DRw52 serologic specificity. The DRB2 is a pseudogene which is not expressed and the DRB5 gene is only found on DR2 and rarely in DR1 (McCarty and Koopman 1993; Ollier and Thomson 1992; Male et al 1991).

HLA-DR types are serological specificities consisting of single epitopes which may be present on multiple allelic products of the DRB1 locus. For allelic products which carry the same -DR specificity, differences among them can often be distinguished in mixed lymphocyte culture. These allo-recognition differences between individuals expressing the same DR allele are referred to as HLA-D specificities or DR subtypes, such as the Dw4 subtype of DR4 (McCarty and Koopman 1993).

The HLA-DQ subregion has 2 pairs of α and β genes whose expression forms the DQ antigen. The DQα2 (DQA2) and the DQβ2 (DQB) pseudogenes are not expressed. The expression of the DQA1 and DQB1 genes form the DQαβ molecule which is in linkage disequilibrium with the DRB genes (McCarty and Koopman 1993).

The World Health Organization Nomenclature Committee for factors of the HLA system meets every 4 years to standardize and review the nomenclature of specificities defined by molecular and serological techniques. Genes encoding the serological specificities are called DR or DQ, followed by a letter (A or B) designating the gene coding the α or β chain followed by 4 digits. The first 2 digits refer to the broad DR or DQ specificities, and the last 2 digits refer to the particular variant type. Tables 1 and 2 show the recent nomenclature for some DRB, Dw and DQB specificities most relevant to this study.

A 4. The Gm and Km systems.

The basic antibody molecule is made up two identical heavy and two identical light chains held together by interchain disulphide bonds. Based upon the structure of their heavy chain constant regions, immunoglobulins are classed into major groups termed classes which may be further subdivided into subclasses. In the human, there are five classes: immunoglobulin G (or IgG), IgM, IgA, IgE, and IgD. They

Table 1. NOMENCLATURE OF HLA-DR4 ALLELES.

HLA allele	HLA-DR serological specificities	HLA-D-associated (T-cell-defined specificities)
DRB1*0401	DR4	Dw4
DRB1*0402	DR4	Dw10
DRB1*0403	DR4	Dw13
DRB1*0404	DR4	Dw14
DRB1*0405	DR4	Dw15
DRB1*0406	DR4	Dw KT2
DRB1*0407	DR4	Dw13
DRB1*0408	DR4	Dw14
DRB1*0409	DR4	----
DRB1*0410	DR4	----
DRB1*0411	DR4	----
DRB1*0412	DR4	----
DRB1*0101	DR1	Dw1

From the 11Th International Histocompatibility Workshop and Conference. Yokohama, Japan, 1991.

Table 2. DQB1 ALLELES ASSOCIATED WITH DR4 ALLELES.

HLA alleles	HLA-DQ serological specificities	HLA-D-associated (T-cell-defined specificities)	Previous equivalents
DQB1*0301	DQ7(3)	Dw4, Dw13	DQB 3.1
DQB1*0302	DQ8(3)	Dw4, w10, w13, w14	DQB 3.2
DQB1*03031	DQB9(3)	Dw23	DQB 3.3
DQB1*03032	DQB9(3)	Dw23, w11	DQB 3.3
DQB1*0402	DQ4	Dw15	DQB 4.1, Wa

From the 11Th International Histocompatibility Workshop and Conference Yokohama, Japan, 1991.

can be differentiated not only by their sequences, but also by their antigenic structures.

Since all the heavy chain constant region structure classes and subclasses are expressed together in the serum of a normal subject, they are termed isotypic variants. The light chain constant regions exist in isotypic forms known as κ and λ which are associated with all heavy chain isotypes. In a given antibody the light chains are identical, either k or λ, and are never mixed, for example IgG k or IgG λ (Roitt 1988).

The first Gm factors (or allotypes), which are detected as antigenic specificities on antibody molecules, were discovered by Grubb in 1956 (Cavalli-Sforza and Bodmer 1971). Antigen specificities of the Gm system are inherited in certain fixed combinations, known as Gm haplotypes, the type and frequencies of which differ between populations. The most common haplotypes in Native Americans are $Gm^{1,17;21}$, $Gm^{1,2,17;21}$, $Gm^{1,17;15,16}$, while the most common in Europeans are $Gm^{3;5,14}$, $Gm^{1,17;21}$, $Gm^{1,2,17;21}$. The Gm allotypes are found on the heavy chains of the immunoglobulin G subclasses IgG1, IgG2, and IgG3. The Km system represent allotypes of the Kappa light chains. Gm specificities are called individually by their subclass number with either an alphanumeric or numeric designations, for example IgG1 specificity G1m(a) or G1m(1) (Cavalli-Sforza and Bodmer 1971; Williams and Steinberg 1985). The WHO notation recognizes 4 G1m, 1 G2m, and 13 G3m markers (Grubb 1988).

A 5. HLA typing by Restriction Fragment Length Polymorphism (RFLP).

RFLP refers to the variation in length of specific DNA fragments between two cleavage sites of a restriction endonuclease (Nathan and Smith 1975). The specific fragments were originally detected with labelled DNA fragments (or probes) by hybridization using the method of Southern (1975). The high correlation between RFLP and serological specificity has enabled DNA genotyping by Southern blot analysis to be employed as a supplementary technique in assignment of cellular HLA-DR and -DQ types. The HLA-DRB and -DQB genes have been used as hybridization probes to detect RFLP patterns on the Taq I digested genomic DNA that correlate with HLA-DR and -DQ serologic specificities (Cox et al 1988; Rosenshine

et al 1986). Discrimination between certain DR alleles like DR3 and DR6, and DR7 and DR9 is not always possible using DRB probes alone. DQB and DQA probes can be used to help in DR typing, since these probes define DQ alleles which are in linkage disequilibrium with DR.

A 6. Polymerase Chain Reaction.

The discovery of specific restriction endonucleases made possible the isolation of discrete molecular fragments of naturally occurring DNA for the first time. This capability was crucial to the development of molecular cloning. The analysis of specific nucleotide sequences, like many analytic procedures, is often hampered by the presence of extraneous material or by the extremely small amounts available for examination. A recently described method, the polymerase chain reaction (PCR), overcomes these limitations (Mullis et al 1986; Saiki et al 1988). With this technique, a pair of oligonucleotide primers, complementary to sequences flanking a particular region of interest, is used to direct DNA synthesis in opposite and overlapping directions. With repeated cycles of DNA denaturation, primer reannealing, and DNA synthesis, human DNA sequences can be faithfully amplified exponentially several hundred thousand times. The capacity of a sample to be analyzed depends on the quantity and quality of DNA in the sample. The PCR offers significant advantages in this regard, since amplification is possible with very small amounts of DNA (Li et al 1988). This technique has been successfully used for the analysis of DNA polymorphisms in single human sperm (Li et al 1988), single diploid cells (Li et al 1988; Jeffreys et al 1988), and single hair roots (Higuchi et al 1988). Pieces of mitochondrial DNA from a 7000 year old human brain were amplified by PCR and sequenced (Pääbo et al 1988), and mitochondrial DNA from ancient bones was also amplified by PCR (Hagelberg and Sykes 1989).

A 6.1 HLA typing by PCR and Sequence Specific Oligonucleotide Analysis.

The class II antigens are characterized by a remarkable degree of allelic

polymorphism (Bell et al 1987). Different alleles at the class II loci have been shown to be associated with susceptibility to certain diseases like RA.

Certain HLA-D region haplotypes may escape detection by some current methodologies performed with antisera or by RFLP analysis of DNA (Cox et al 1988; Rosenshine et al 1986; Wordsworth et al 1989). Because of these limitations, the proposed alternative to HLA-RFLP-typing is the use of PCR and sequence specific oligonucleotide probes (PCR-SSOP) that correspond to specific regions of the polymorphic class II gene (Morel et al 1990; Vaugham et al 1990; Gao et al 1990; Reed et al 1992). These new methods permit the study of individual β-chain polymorphisms within the HLA-DR and -DQ subregions and the identification of specific alleles that differ by only a single nucleotide (Sharf et al 1990; Gyllensten and Allen 1991).

A 6.2 HLA typing by PCR and RFLP typing.

PCR-RFLP analysis is a technique for HLA Class II genotyping. The combination of PCR and RFLP is an efficient method that has some advantages over the PCR-SSOP method. PCR-RFLP method is based on digestion of PCR-amplified DNA with allele specific restriction endonucleases. This method is much simpler than PCR-SSOP because preparation of labeled probes is not necessary, and the demanding steps for careful hybridization reactions are not required in this method (Uryu et al 1990; Olerup 1990).

The PCR-RFLP method is more efficient than standard RFLP analysis since RFLP can not discriminate between certain alleles of the DR and DQ region. The DRB and DQB typing can be completed within 6-7 h after sample DNA is purified and amplified. The method is also very reliable and sensitive for HLA class II genotyping as it can distinguish a one-base-mismatch sequence easily and precisely. Several authors have recently used the PCR-RFLP method to successfully differentiate HLA-DR3 from -DR6, and -DR7 from -DR9, as well as to determine -DR4 subtypes (Ju et al 1991; Ota et al 1992).

In this thesis, I have used the three described methods to type the DRB (including Dw subtypes) and DQB genes of the HLA-Class II region: RFLP analysis,

PCR-SSOP, and PCR-RFLP.

A 7. Rheumatoid Arthritis.

A 7.1 Etiology of Rheumatoid Arthritis

Despite a great expenditure of time, resources and energy, the cause of RA has eluded detection. One impediment is that RA appears to be a uniquely human disease, and although experimental forms of arthritis exist, they are not satisfactory models for the disorder (Trentham 1987). Another problem is the fact that most observations about RA have been made on patients with established disease. Thus, it is difficult to decide whether the abnormalities found are the cause or the result of the rheumatoid process (Katz 1988).

Rheumatoid arthritis is a chronic and progressive inflammatory disease of joints. RA has a world-wide distribution and affects as many as 1% to 2% of almost all racial and ethnic groups (McCarty and Koopman 1993). The incidence of RA among Mayans is not known. Definite RA is two to three times more common in women than in men (McCarty and Koopman 1993). Although the etiology of RA remains unknown, there is increasing evidence that immunologic and genetic factors are involved in manifestations of the disease (McCarty and Koopman 1993). The inflammatory response is usually considered a biologic adaptation to protect the host from a hostile environment. A prerequisite is that this response occurs without significant injury to the host's own tissues. Thus, the inflammation in RA must be considered inappropriate. How this comes about is not know, but a number of factors predispose joints to injury and inflammation (McCarty and Koopman 1993).

A 7.2 Clinical Manifestations

There are two common kinds of degenerative joint diseases: rheumatoid arthritis (RA) and osteoarthritis (OA) (see Table 3). RA begins as pain , tenderness, and swelling of one or more joints in the extremities. The involvement is symmetrical,

Table 3. SIMILARITIES AND DIFFERENCES BETWEEN RA AND OA

	RA	OA
Stiffness	Lasts more than 30 min	Rarely exceeds 15-30 min
Swelling and/or fluid	Swelling consists of soft tissue thickening or fluid accumulation and not bony over-growth alone	Cartilage and bone proliferative spur formation synovitis with acute flares or after prolonged disease
Symmetry	Simultaneous involvement of the same joint on both sides of the body	May not be symmetrical
Clinical nodularity	Subcutaneous nodules must be observed	Nodal disease (patients have Heberden's nodes) and non-nodal disease
Rheumatoid factor	Any positive test for IgM rheumatoid factor is acceptable	Absence of rheumatoid factor

Table 3. (continued)

	RA	OA
Radiolologi-cal changes	X-ray changes typical of RA must include at least bony decalcify-cation localized to or greatest around the involved joints	Several grades for osteophytes and joint space narrowing, cartilage calcification
Inflama-tory synovial fluid	Decreased viscosity and/or polymorphonuclear leucocytosis	Deposit crystals of calcium pyrophosphate dihydrate or basic phosphate (Hydroxyapatite)

(MacCarty and Koopman 1993; Arnett et al 1988)

but is commonly more severe on the dominant side. The small joints of the hands and feet are involved; knees, elbows, ankles, wrist, and hips are also involved. When the disease involves the spinal cord, it is most frequently in the cervical region.

The disease is systemic. Extra-articular manifestations correlate with persistence of articular disease, and with a poor prognosis as evidently morbidity and for mortality (Samter et al 1988). Some of the classic criteria for RA are summarized in Table 3 as delineated by The American Rheumatism Association Criteria Committee (McCarty and Koopman 1993; Arnett et al 1988).

A 7.3 Non-genetic factors.

Polyarthritis occurs during many bacterial, spirochaetal, and viral infections of man and animals (McCarty and Koopman 1993). Infectious agents sometimes express constituents that share structural homology with host molecules, a phenomenon termed molecular mimicry.

All disease-associated DR4 alleles share epitope (a sequence homology) in the third hypervariable region (residues 70-74) of the DRB1 chain (Weyand et al 1992). Epitope sharing between certain DR4 alleles and certain viruses as well as certain bacteria is evidence for non-genetic factors in the etiology of RA.

The DRB chain gene coding sequence has a homologous sequence in the E-BV genome. Studies have shown that patients with RA have an increased number of circulating Epstein-Barr virus (E-BV) infected B lymphocytes (Petersen et al 1990). Antibody interactions with the E-BV antigen and the host-shared epitopes would support a possible role for the virus in the etiology of RA (Ilonen et al 1990).

The bacteria E.coli produces a 40 kd dnsJ heat shock protein (hsp) that shares an amino acid sequence with the third hypervariable region of the DRB1*0401 molecule (Albani et al 1992). Therefore it is possible that the immune response directed against the dnaJ hsp could elicit an autoreactive immune response directed against the shared epitope which is associated with susceptibility to rheumatoid arthritis (Koopman 1993).

A 7.4 Genetic factors.

A 7.4.1 Rheumatoid Factors.

Rheumatoid factors (RF) are IgM antibodies directed against the Fc region of IgG molecules. Although RF are found in a number of rheumatic disorders as well as in nonrheumatic conditions (McCarty and Koopman 1993), their role is limited to the clinic as an aid in the diagnosis of RA.

The synovial fluid of most RA patients contains abundant aggregates of IgG and RF produced and deposited in situ. The synovial fluid also contains depressed levels of complement components. These findings suggest that RF may contribute to immune complex formation, complement consumption, and chronic tissue damage in the rheumatoid synovium (McCarty and Koopman 1993).

IgM-RF from RA patients are polyclonal and multivalent and hence are efficient agglutinators of antigen coated cells. Human red cells coated with incomplete IgG anti-Rh antibodies are well suited for the detection of IgM-RF, particularly those with anti-Gm specificities (Grubb and Laurel 1956). Conventional tests for RF are designed to detect IgM-RF (and more rarely IgG-RF) in patient serum that reacts with normal human IgG or normal animal IgG. The majority of tests use particulate carriers like erythrocytes, latex and bentonite particles. RF are usually present at a higher titre in patients with RA than in the normal population. Patients who are RF positive are called seropositive (Bryant 1986).

In adults and children, the HLA-DR4 antigen has been found to be associated significantly with seropositive RA but not with seronegative RA (Husby and Gran 1988).

A 7.4.2 HLA and RA.

Progress has been made in the past few years in defining the genetic factors predisposing an individual to RA. It is now over a decade since the association of the HLA class II antigens and RA was first established. Stastny (1978) demonstrated an increased frequency of HLA-DR4 in classic seropositive RA patients.

The HLA-DR4 association with RA has been confirmed in many Caucasian populations, and also has been found in other ethnic and racial groups, such as American Blacks, Latin Americans, and Japanese. Moreover, only certain Dw cellular specificities have been associated with DR4 in RA patients, including DRB1*0401 (Dw4), DRB1*0404/08 (Dw14) and DRB1*0405 (Dw15) (Zoschke and Segal 1986; Ollier et al 1988a) (see Tables 1 and 2 for recent nomenclature). While the association has been observed repeatedly and in different populations, the strength of the association has been quite variable. Many normal people who never develop RA have DR4 and many RA patients are DR4 negative. There are many questions about the genetics of RA that remain unanswered.

HLA-DR4 was found in 66% of Caucasians patients with RA, and 26% of Caucasians normals in the combined studies of Tiwari and Terasaki (1985). Similarly, 40% of Black Americans with RA (as reported by Tiwari and Terasaki 1985) were DR4 positive as compared with 10% of Black controls. HLA-DR4 antigen was significantly increased in Zimbabwean Black RA patients at 23% as compared with 8% of the Black controls (Martell et al 1990) (see Table 4).

In Mexicans, Ueno et al (1981) found 77% of RA patients to be DR4 positive as compared to 38% in normal controls (see Table 4). Similar results were found in Mexicans by Tiwari and Terasaki (1985): 64% of DR4 in RA patients and 35% of DR4 in the controls (see Table 4). In the Spanish population, Sanchez et al (1990) found that both DR4 and DRw10 were increased in RA as compared to controls: 40% versus 26% and 20% versus 4.5% for DR4 and DRw10 respectively (see Table 4). However, the DRw10 specificity was more strongly associated with disease susceptibility than DR4 ($p= 0.001$ vs $p= 0.05$ respectively).

The epidemiology of rheumatic diseases in the Native American population is of special interest because of the extremely high prevalence rate of RA in many North American Indian groups (Bias et al 1981; Harvey et al 1983; Del Puente et al 1989; Atkins et al 1987). It has been postulated by medical historians and researchers that RA is a "New World" disease, first appearing in Native Americans centuries ago before contact with Europeans (McCarty and Koopman 1993; Alarcon-Segovia 1976; Schumacher and Alarcon-Segovia 1980).

The Mille Lacs band of Chippewa Indians in Central Minnesota has a high population

Table 4. FREQUENCIES OF HLA-DR4 IN RA PATIENTS AND CONTROLS OF VARIOUS POPULATIONS.

	CONTROL		RA PATIENTS		REFERENCE
		n		n	
U.S.A. Caucasian	39%	425	66%	142	Tiwari and Terasaki 1985
U.S.A. Black	10%	367	40%	109	Tiwari and Terasaki 1985
Zimbabwean Blacks	8%	104	23%	26	Martell et al 1990
Mexican	38%	180	77%	17	Ueno et al 1981
Mexican	35%	199	64%	58	Tiwari and Terasaki 1985
Spanish	26%	200	40%	90	Sanchez et al 1990
Native American Indians					
Chippewa Indians	68%	168	100%	12	Bias et al 1981
Yakima Indians	38%	52	41%	29	Willkens et al 1982
Tlingit Indians	21%	61	6%	32	Nelson et al 1992
Israeli Jews	2%	134	12%	131	Gao et al 1991
Chinese	6%	32	14%	49	Seglias et al 1992

frequency of DR4, 68%, and a high population prevalence of RA (5-7%) (Bias et al 1981; Harvey et al 1983). In addition DR4 was found in 100% of Chippewa RA patients (Bias et al 1981) (see Table 4). Among the Chippewa, there are at least five variants of DR4, called DR4.1 chip, DR4.2 chip, DR4.3 chip, DR9 chip, and DR(2x4) chip. However, none of the five DR4-chip variants correspond to any known HLA-Dw specificities (Bias et al 1981).

Unlike most ethnic groups, the Yakima Indians in Central Washington State showed no increased frequency of HLA-DR4 or DRB1*0401 (Dw4) in patients who have apparent RA as compared to the control groups: 41% vs 38%, and 7% vs 4% for DR4 and Dw4 respectively (Willkens et al 1982). However the DRB1*1402 allele (Dw16, not associated with DR4) was identified in 83% of Yakima RA patients as compared to 60% in the control population. The DRB1*1402 allele had previously been recognized in a Venezuelan Indian population (Willkens et al 1991).

The Alaskan Tlingit Indians have a high prevalence of RA (Boyer et al 1991). The Alaskan Tlingit Indians had a decreased frequency of HLA-DR4 in the RA group as compared to the controls, 6% vs 21% respectively. Like the Yakima Indians, this group also had a predominance of DRB1*1402 (Dw16) in RA patients. This allele is present in 91% of RA patients as compared to 80% of the controls. The predominant DR4 allele in this RA population was DRB1*0403/07 (Dw13) (Nelson et al 1992).

In Jews, Asian Indians and some Hispanics, DR1 has been reported as being associated with RA (Schiffenbauer and Schwartz 1987; Nelson et al 1992). The three groups also show some association of RA with DR4. In RA patients who are Azkenazi Jews or non-Azkenazi Jews, the DR4 associated variant DRB1*0405 (Dw15) was found to be the main allele associated with risk of developing RA, 12% in RA vs 2% in the controls (Gao et al 1991) (see table 4). The DRB1*0402 (Dw10) subtype of DR4 was not associated with the disease in this population (Merryman et al 1989; Gao et al 1991).

The Southern Chinese population are similar to the Askenazi Jews in that they have a high frequency of the DRB1*0405 (Dw15) allele in RA patients: 24.5% vs 9.4% in the control population. The DRB1*0404/08 (Dw14) allele is also increased in RA patients in this population, 14.3% in patients vs 6.3% in the controls (Seglias

et al 1992). The DRB1*0405 (Dw15) subtype of DR4 is also associated with RA in Japanese patients (Schiffenbauer and Schwartz 1987; Gao et al 1991).

Thus, there are 5 distinct DRB1 genes associated with different HLA-DR specificities that are increased in RA in different populations: DRB1*0401 (Dw4), DRB1*0404/08 (Dw14), DRB1*0405 (Dw15), DRB1*0101 (DR1.1, Dw1) and DRB1*1402 (DRw14.2, Dw16). Several investigators have suggested that the risk for RA associated with all these genes can be explained by a shared epitope involving amino acids 67 through 74, and amino acids 85 and 86 in the third hypervariable region (HVR) of the DR molecule (Seglias et al 1992; Gao et al 1991; Weyand et al 1992). DRB1*0404 (Dw14) and *0405 (Dw15) encode an identical sequence in this region, while *0401 (Dw4) encodes a sequence only differing from this with respect to a conservative arginine to lysine substitution at residue 71. The DRB1*0101(DR1) is identical to that encoded by DRB1*0404 and *0405 in the third HVR, but otherwise quite different. The DRB1*1402 (Dw16) allele shares sequence at the third HVR with DRB1*0404 (Dw14) allele (Willkens et al 1991). The DRB1*0402 (Dw10) and *0403 (Dw13) alleles encode sequences in this region differing with respect to non-conservative amino acids substitutions, which may lead to comformational changes in this region (Wordsworth and Bell 1992). This may result in lack of susceptibility to RA for individuals carrying DRB1*0402 or *0403.

Genotyping for HLA-DRB1 alleles can be used to categorize patients with RA into groups with different clinical presentations. Erosive RA is associated with DRB1*0401 and *0404/08 alleles (Weyand et al 1992). Homozygosity for the DRB1*0401 or *0404 alleles, and the combination DRB1*0401/04 or *0401/08 are frequent in patients with the most severe form of RA (Weyand et al 1992). Felty syndrome patients often have a heterozygous combination of Dw4/Dw14 (Wordsworth et al 1992). Seropositive juvenile RA (JRA) has been shown to be associated with both DRB1*0401 (Dw4) and DRB1*0404/*0408 (Dw14) alleles. The frequency of Dw14 (*0404/*0408) in JRA is especially high compared with the frequency of Dw14 in the normal population (36% vs 5%) (Nepom et al 1986b).

In summary, while DR4 occurs in varying frequencies within different populations, an association between DR4 and RA is usually seen. The usual Dw types associated with RA are DRB1*0401 (Dw4), DRB1*0404/08 (Dw14), and

DRB1*0405 (Dw15). However, there is a controversy regarding the association of HLA-DR4 in seronegative disease (Husby and Gran 1988; Gran et al 1983).

By using RFLPs, HLA-DQB related specificities have been split into two predominant specificities on DR4 haplotypes: DQB1*0301 (DQB3.1) and DQB1*0302 (DQB3.2) (Nepom et al 1989a; Perdriger et al 1990). However, these do not correlate with susceptibility to RA. In other words, there appeared to be no influence on the susceptible haplotype contributed by variation at DQB as supported by different authors (Nepom et al 1989a,b,1986a) (see Table 5).

These findings suggest that susceptibility to RA is primarily associated with DRB1 alleles and not with DQ alleles (Perdriger et al 1990; Gao et al 1991; Sanchez et al 1990). Ilonen et al (1990) suggest that HLA-DQ genes may have a role in protection from the disease. Stephens et al (1989) found that DQB1*0301 (DQB3.1) is associated with high serum levels of IgM-RF in RA patients, especially those with more severe forms of RA, suggesting that this allele may represent a marker for disease severity or may be related to clinical features associated with the disease (Lanchbury et al 1989).

A 7.4.3 HLA-Gm-Km and RA.

Several reports have explored whether an interaction between HLA-DR4, Gm-Km allotypes, and RA exists. It has been found that there was an increase of the Gm(1,2,3,17;23;5,21) phenotype and the G1m(2) allotype in DR4 positive RA patients compared with DR4 negative patients and controls (Loftus et al 1989; Puttick et al 1990(a); Collier et al 1985). Also, it was found that the uncommon Gm phenotype Gm(1,2;21) occurred significantly more often in DR4 positive patients than in controls (Zarnowski et al 1986). In another study, significant interaction was found between α1-antitrypsin and the Gm(1,2,17;21) phenotype in RA patients but no association between Gm allotypes and RA itself was observed (Ollier et al 1988b).

No studies have found significant association between Gm allotype and RA when considering RA patients as a whole. Genes linked to Gm may predispose to RA but only by interaction with HLA alleles and other non-HLA genetic markers (Thomson et al 1986).

Table 5. FREQUENCIES OF HLA-DQB1 IN RA PATIENTS AND CONTROLS IN CAUCASIANS.

HAPLOTYPE		RA	
DQB	DRB1	Patients %	Control %
DQB 3.1	Dw4 (DR4)	35	65
DQB 3.2	Dw4 (DR4)	40	60
DQB 3.2	Dw14 (DR4)	28	72

(Nepom et al 1989a)

Analysis of the DR and Gm data in RA susceptibility indicates that the best model involves looking for interactions between Gm and HLA extended haplotypes (Collier et al 1985; Puttick et al 1986).

Moxley (1989a,1989b,1992) found that the immunoglobulin kappa constant segment contributes to the risk of RA. The use of constant segment (Ck), Km, and variable segment V_{κ}(B3) immunoglobulin kappa genotypes have allowed the differentiation of DR4+ and DR4- RA patients (Moxley 1992). DR4- RA patients tend to be homozygous for either B3/Ck or B3/Km3 genotypes whereas DR4+ RA patients are not (Moxley 1992). The Km(1) allotype was found to be increased in DR4- patients (Sanders et al 1985).

A 8. Research Goals.

The goals of this research project are to search for possible association between RA and particular DR alleles and/or Gm/Km allotypes using Mayan RA patients and controls.

A 9. Hypothesis.

It is hypothesized that RA will be found to be associated with particular DR alleles, as well as particular Gm/Km allotypes.

B. MATERIALS AND METHODS.

B 1. Patients and Controls.

The blood from 41 Mayan RA patients was provided by the Rheumatic Disease Clinic at the Clinica T-1 Instituto Mexicano del Seguro Social (IMSS), and by the Blood Bank Service at the Regional Hospital Merida Instituto de Seguridad y Servicios Sociales para los Trabajadores del Estado (ISSSTE), both in Merida, Yucatan, Mexico. All the patients fulfilled the criteria for the classification of RA by the American Rheumatism Association (McCarty and Koopman 1993; Arnett et al 1988), and were positive for the IgM Rheumatoid Factor using the latex test.

The healthy controls were provided by the Blood Bank Services ISSSTE, and consisted of 92 controls from Cancun, Quintana Roo Mexico, and 47 controls from Merida, Yucatan Mexico. There were therefore a total of 136 controls of Mayan origin.

Blood from 87 healthy non-Mayan controls from Mexico City was also tested. The population from Mexico City was provided by The Clinic No 1 and by The General Hospital at Medical Center "La Raza" at the Mexican Institute of Social Security (IMSS). This population was more heterogeneous than the Mayan controls. The Mexico City controls were used to study the frequency of Gm only.

All patients donating blood for genetic studies of RA did so after giving their informed consent to participating physicians, in accordance with ethical guidelines established at the University of Calgary (see copy of consent form in Appendix 1).

B 2. Blood Samples.

Blood samples of 10-12 ml were obtained by venipuncture using vacutainer tubes with EDTA (ethylene diaminotetraacetic acid). The blood was centrifuged at 1500 rpm for 15 minutes at room temperature . The plasma fraction was removed, aliquoted and stored at -80 degrees Celsius (°C) until used in the Gm and Km allotyping tests.

After removing the plasma, the buffy coat was removed (approx.

1 ml) and placed in a 1.5 ml microfuge tube containing 300 µl of lysis solution (Tris 100 mM pH 8.0, EDTA 40 mM, SDS 0.2%, NaCl 500 mM). At this stage the lysate mixture was stable and stored at -80°C for several months, until the tubes were shipped to Calgary. The genomic DNA was then extracted as described below.

B 3. DNA Sources.

B 3.1 Genomic DNA.

The lysate was decanted to a 15 ml plastic tube and 5 ml of high TE buffer (Tris 100 mM pH 8.0, EDTA 40 mM) was added. The tube was then vortexed and stored overnight at 4°C prior to DNA extraction.

DNA extraction was performed as described by Maniatis et al 1982. An equal volume of high TE saturated phenol (0.1% 8 hydroxyquinoline is added to highly purified phenol [Fisher A 881] prior to saturation with high TE buffer) was added to the lysed cells and mixed on a rotator for 30 min. The mixture was centrifuged at 3000 rpm for 5 min in a IEC Centra-7R bench centrifuge to separate the aqueous and organic phases. The upper aqueous layer was recovered and the phenol extraction was repeated twice. The aqueous layer was extracted once with an equal volume of chloroform-isoamyl-alcohol (24:1) and recovered once again. The DNA was precipitated by the addition of 1/10 volume 4 M ammonium acetate and 1 volume of absolute isopropanol. The long strands of high molecular weight DNA were swirled together and removed from the solution with the end of a Pasteur pipette that had been heat sealed and bent to form a hook. The precipitate was rinsed with 70 % ethanol and allowed to air dry. The coated pipette tip was broken off into a 1.5 ml microfuge tube and 500 µl of low TE (Tris 10 mM pH 8.0, EDTA 1 mM) was added. The DNA was solubilized by rotating overnight at 4°C.

B 4. DNA Quantitation.

The concentration and purity of the DNA samples were determined by measuring the optical density of the samples at 260 nm and 280 nm with a Beckman

DU-65 spectrophotometer. An OD_{260}/OD_{280} ratio of approximately 2 was desired. An absorbance of one optical density unit at 260nm (in a 1 cm light path) is equivalent to approximately 50 µl/ml of double stranded DNA. DNA samples were stored at 4°C.

B 5. Probes.

B 5.1 Isolation of Probe: Large Scale Plasmid Cultures.

For a large scale preparation of ampicillin resistant plasmids, 1 ml of a 5-10 ml starter culture, from a single colony grown overnight in LB media (Peptone-Tryptone 10 g, yeast extract 5 g, NaCl 2 g, Dextrose 2 g in 1 l distilled water pH 7.0-7.5) containing 50 µl/ml ampicillin (amp) was inoculated into 500 ml of similar media containing 50 µl/ml amp and incubated at 37°C with vigorous mixing at 300 rpm for 18 h. The growing culture was boosted by the addition of 170 µl/ml chloramphenicol after the first 7-8 h of incubation. The cells grown in LB media were harvested by centrifugation at 5,000 rpm for 10 min at 4°C using GSA rotor in a Sorval RC-5B super speed centrifuge. The cellular pellet was resuspended in 4 ml of Sol I (Sucrose 15% w/v, Tris 0.5 M pH 8.0, EDTA 0.05 M) and 10 mg of lysozyme was added. The capped tubes were left in ice for 30 min. After that, 5 ml of Sol II (Triton X-100 0.5%, Tris 0.05 M pH 8.0, EDTA 0.05 M) was added to each tube and were further incubated on ice for 20 min. The samples were centrifuged at 19,000 rpm for 40 min at 4°C in a Sorval SS-34 rotor using a Sorval RC-5B super speed centrifuge. The supernatant was recovered (approx 8.5 ml), 9 g of $CsCl_2$, and 0.5 ml of ethidium bromide (5 mg/ml) were added to each 8.5 ml of supernatant and dissolved by gentle inversion. The solution was poured into a 10 ml Beckman Quick-Seal ultra centrifuge tube, any volume deficiencies in the tube was corrected with paraffin oil, heat sealed and spun at 44,000 rpm for 69 h in a Beckman rotor type 80 TI using a Beckman L8-M ultracentrifuge.

After first equilibrating the contents by piercing the tube with an 18 gauge needle, the upper genomic band was removed and discarded. The lower plasmid band was then removed with another 18 gauge needle and the volume noted.

The ethidium bromide was removed by extraction with water saturated

N-butanol. Low TE was then added to increase the volume to 3X the extracted amount previously noted and 1/10 volume ammonium acetate and 2.5 volumes of -20°C ethanol 95% (v/v) were added. The solution was frozen in liquid nitrogen and the nucleic acid collected by centrifugation at 15,000 rpm for 15 min at 4°C in a Sorval SS-34 rotor using a Sorval RC-5B superspeed centrifuge. The pellet was washed with 70% ethanol (v/v), air dried, and redissolved in low TE to approximately 0.5 µg/ml. Plasmid extracted by this method was used in restriction enzyme studies.

B 5.2 Isolation of Probe: Small Scale Plasmid Cultures.

Prior to large scale preparation, the plasmid identity was confirmed by restriction enzyme digestion of plasmid DNA isolated by the method suggested by Serghini et al 1989.

Small scale plasmid DNA was prepared in a 15 ml Falcon sterile tube containing 5 ml of LB media with 50 µl/ml amp inoculated with a single colony and grown overnight with vigorous shaking at 37°C. Then a 1.5 ml culture was transferred to a microfuge tube. The cells were pelleted by centrifugation for 5 min at 5,000 rpm in a Silencer H-31 centrifuge at room temperature. The pellet was resuspended in 50 µl of low TE-Saline (NaCl 100 mM), and 100 µl of mixed (v/v/v) phenol:chloroform-isoamyl-alcohol (1:24:1) was added. The mixture was vortexed for approximately 1 minute and centrifuged for 5 min at 10,000 rpm in a Silencer H-31 centrifuge at 4°C. Following this, 50 µl of the aqueous phase was transferred to a second microfuge tube, the DNA was precipitated with 1/10 volume of 4 M ammonium acetate and 2.5 vol of -20°C cold 95% ethanol for 15 min on dry ice. The precipitated DNA was collected by centrifugation for 15 min at 10,000 rpm at 4°C, washed with 75% ethanol, air dried and dissolved in 25 µl of low TE. Aliquots of 2 µl (containing approx 0.2-0.3 µg of DNA) were digested with the appropriate restriction enzymes. Any low molecular weight RNA present in the sample was hydrolysed by addition of DNAase-free RNase A (50 µl/ml) to restriction buffer. The sample was analyzed in 1% agarose gel to observe the expected fragment sizes.

B 5.3 Sequence Specific Oligoprobes (SSOP).

The SSO probes used for generic and specific oligotyping for DRB and DQB alleles are listed in Table 6. All probes are the ones used by the 11th International HLA Workshop. Reference Protocols Yokohama, Japan 1991.

The oligotyping procedure was done in collaboration with Dr. Massimo Trucco at the Department of Human Genetics, University of Pittsburgh. A visit was made to his laboratory to learn the protocol and use his SSO probes on the Mayan RA patient and control samples.

B 6. Gel Electrophoresis.

Digested DNA samples, digested plasmids, PCR products, and digested PCR products were routinely separated on both agarose or native polyacrylamide gels.

B 6.1 Agarose Gel Electrophoresis.

DNA samples were electrphoresed through 0.8-3% w/v (depending on the fragment sizes to be resolved) agarose gels or 2% Nu Sieve/1% Agarose gel. The agarose gels were made by boiling the appropriate amount of electrophoresis purity agarose in 1X TAE buffer (Tris-acetate 40 mM, EDTA 1 mM, glacial acetic acid 20 mM, pH 8.0) (Maniatis et al 1982.) containing ethidium bromide (0.5 µl/ml). The melted agarose was poured into gel molds with well forming combs and cooled until solidified. The gel was placed into an electrophoresis apparatus filled with 1X TAE. The DNA samples were loaded with 1/10 volume tracking dyes (glycerol 50% v/v, SDS 0.1% w/v, bromophenol blue 0.025% w/v, xylene cyanol FF 0.025% w/v; or glycerol 60% v/v, orange G 0.5%, SDS 0.1%) and electrophoresed for varying times and voltages (2-3 v/cm) depending on each case.

After electrophoresis, the DNA in the gel was visualized using a long wavelength ultraviolet (365 nm) transilluminator, and photographed with a Polaroid camera using type 52 film.

Table 6. OLIGOTYPING PROBES USED.

	DRB1 generic oligotyping probes.	Specificity
1001	TAA GTT TGA ATG TCA TTT	DR1
1003	GTA CTC TAC GTC TGA GTG	DR3,DRw11,DRw13,DRw14 (except 1404, 1410)
1004	GAG CAG GTT AAA CAT GAG	DR4,DR1410
1005	AGA AAT AAC ACT CAC CCG	DRw12,DRw8,DR1404
1006	TGG CAG GGT AAG TAT AAG	DR7
1007	GAA GCA GGA TAA GTT TGA	DR9
1008	GAG GAG GTT AAG TTT GAG	DRw10
1010	GAG CTG CGT AAG TCT GAG	DRw52a,DRB30101
2802	GGT TAC TGG AGA GAC ACT	DRw12
5703	GCC TGA TGA GGA GTA CTG	DRw11
7011	GAC ATC CTG GAG CAG GCG	DR2,DR15
	DQB generic oligotyping probes.	
2602	CGT TAT GTG ACC AGA TAC	DQB0301,0601
2603	CGT CTT GTG ACC AGA TAC	DQB0302,03,0602
5701	GCG GCC TGT TGC CGA GTA	DQB0501,0604
5702	GCG GCC TAG CGC CGA GTA	DQB0502
5703	GGC GGC CTG ACG CCG AGT	DQB0503,0601
5704	GCG GCC TGA TGC CGA GTA	DQB0602,03
5705	GGC TGC CTG CCG CCG AGT	DQB0201
5707	GGC CGC CTG CCG CCG AGT	DQB0302
5708	GCG GCT TGA CGC CGA GTA	DQB0401,02

Table 6. (continued)

	DRB1 specific oligotyping probes.	
	DR2 group	
7002	GAC TTC CTG GAA GAC AGG	DRB1601
7003	GAC CTC CTG GAA GAC AGG	DRB1602
7011	GAC ATC CTG GAG CAG GCG	DRB1501,02,03
8601	AAC TAC GGG GTT GGT GAG	DRB1502,1601,1602
8603	AAC TAC GGG GTT GTG GAG	DRB1501,03
	DRw52 associated group (DRB1 gene)	
3712	CAG GAG GAG TTC GTG CGC	DRB1404,05,07,08,10
5703	GCC TGA TGA GGA GTA CTG	DRB1101,02,03,04,05
5705	GCC TGT CGC CGA GTC CTG	DRB1201,02
7001	TCC TGG AGC AGA GGC GGG	DRB1402,06,09
7004	GGC CGG GTG GAC AAC TAC	DRB0301,02,03
7007	ACA TCC TGG AAG ACG AGC	DRB1102,1301,1302,1304
8601	AAC TAC GGG GTT GGT GAG	DRB0302,0801,0802,0803 0805,1105,1305,1405,1407 1409
8603	AAC TAC GGG GTT GTG GAG	DRB0301,0303,0801,1102 1103,1104,1301,1304,1401 1404,1405,1406,1409,1410

B 6.2 Non-Denaturing Polyacrylamide Gel Electrophoresis.

Non-denaturing polyacrylamide gels of 8% and 10% (w/v) were used. The gel solution was poured between glass plates with dimensions of 20 x 18 x 0.3 cm and 20 x 16 x 0.3 cm that were held together by sandwich screw clamps and separated by spacers 0.1 mm wide. A 50 ml solution of 8% polyacrylamide was made by combining 10 ml of acrylamid-bis acrylamide 40% w/v (acrylamide 360 g, bis-acrylamide 20 g, water to 1 l), 10 ml of 5X TBE buffer (tris-base 432 g, boric acid 220 g, EDTA 0.5 M 160 ml, water to 8 l), and 29.2 ml of water. A 50 ml solution of 10% polyacrylamide was made by combining 12.5 ml of acrylamide-bis-acrylamide 40%, 10 ml of 5X TBE buffer, and 26.97 ml of water. The acrylamide solution was polymerized by adding 30 µl of TEMED (N,N,N',N'-Tetramethylethylenediamine) and 500 µ of freshly made ammonium persulfate (10% w/v) was added. The solution was then poured between the glass plates. Prior to polymerization, a teflon comb was inserted between the plates to form wells. After polymerization the comb was removed and the plates were placed into the electrophoresis tank filled with 1X TBE buffer. A run of 3 h at 80 volts was required to separate the digested PCR products which ranged in size from 262 to 52 bp . At the end of the run, the plates were separated and the gel removed to be soaked in a staining solution of ethidium bromide (0.5 µg/ml) and 1X TBE buffer for 5 min. The gel was visualized and photographed as above.

B 7. DNA Transfers.

B 7.1 Vacuum Blot.

After the electrophoresis was completed, the agarose gel was vacuum transferred to a Hybond-N^+ (Amersham Inc.) nylon membrane. Vacuum transfers were performed using a vacuum blotting apparatus supplied by Tyler Research Instruments Corporation (Edmonton Alberta).

To facilitate transfer of large DNA fragments, acid depurination was performed by soaking the gel in 0.25 N HCl for 10 min. The gel was rinsed briefly

with water before proceeding with the transfer. The membrane was cut slightly larger than the gel, pre-wet with water and centred on the porous support. A plastic film with a hole in it (usually 5 mm smaller than dimensions of the gel) was positioned over the membrane and support. The lucite frame was positioned on the top of the plastic mask and fastened tight using fastening bolts ensuring a seal around the periphery of the plastic film and creating a reservoir over the transfer platform. The gel was placed on the top of the membrane and covered with 0.4 M NaOH. A vacuum of 25 mm Hg was applied for approximately 2 h. The remaining NaOH was then aspirated and the membrane removed and neutralized with 2X SSC (20X: NaCl 175.3 g, sodium citrate 88.2 g pH 7.0, water to 1l). The membrane was sealed in a plastic bag and used directly for prehybridization.

B 7.2 Dot-Blot of Amplified DNA.

Equal volumes of amplified DNA was transferred to a nylon membrane (Hybond N^+, Amersham Inc.) using the following procedure: the membrane was divided into 6 mm^2 squares and cut into strip of 3.5 x 7.2 cm long. In each square 1 µl of amplified DNA was spotted and allowed to dry at room temperature. To fix the DNA onto the membrane the strip was then treated with 0.4 M NaOH for 15 min and then neutralized in 2X SSC for 15 min. The membrane was sealed in a plastic bag and used directly for prehybridization.

B 8. Restriction Endonuclease Digestion.

The digestion of genomic DNA, plasmids, and PCR products was performed using 1-3 units of enzyme per µg of DNA and 1/10 volume of the manufacturer provided 10X reaction buffer (see Table 7). Three to five µl of genomic DNA, plasmid or 5 µl of PCR product was mixed with the appropriate volume of enzyme, buffer and water. In some cases nuclease free bovine serum albumin (BSA) was also added to a final concentration of 100 µl/ml. Incubations were done at the temperature required for maximal enzyme activity. The reaction was stopped by adding 1/10 volume of loading dye.

Table 7. ENZYMES USED.

NAME	SUPPLIER
1. Enzymes used in DNA-RFLP analyses.	
Taq I	Pharmacia-Bio-Bar
2. Enzymes used in PCR-RFLP analyses.	
BstU I	New England Bio-Labs
Hae II	"
Hinf I	"
Hph I	"
Mnl I	"
Sac II	"
3. Enzymes used to digest plasmids.	
Hae III	Pharmacia-Bio-Bar
Hpa II	"
Hind III	"

B 9. Probe Elution from Agarose Gels.

DNA fragments of interest were excised after overnight electrophoresis. The piece of agarose gel containing the desired fragment was cut from the gel and the resulting well was lined with a piece of Type I (6000-8000 MW cutoff) dialysis tubing. The gel slice was placed back in the same slot and the gel apparatus was filled with 1X TAE buffer. The DNA in the gel slice is electroeluted for 30 min onto the inner wall of the dialysis tube. The polarity was reversed for 1 min to release the DNA from the wall of the dialysis membrane. A hand held ultraviolet lamp was used to monitor the movement of the DNA from the gel slice onto the membrane. A Pasteur pipette was used to collect the DNA from the surface of the membrane. Ethidium bromide was removed from the sample using water saturated N-butanol. The sample was cleaned once by extracting with phenol:chloroform-isoamyl-alcohol (1:24:1). The DNA was precipitated by adding 1/10 volume 4 M ammonium acetate and 2 1/2 volumes ice cold 95% ethanol. The sample was centrifuged at 10,000 rpm for 30 min, the supernatant carefully discarded, and the pellet resuspended in low TE, to final concentration between 50 to 100 ng/µl. DNA concentration was determined as described in section B 4.

B 10. Radiolabeling of DNA Probes.

B 10.1 5' End labelling of SSO Probes.

The SSO was 5' end labelled using T4 Polynucleotide Kinase (T4 PNK) as described by Maniatis et al 1982, and by the DNA component of The 11th International HLA Workshop (Tsuji et al 1992). Five to 10 µM of SSO were combined with 100 µCi of [gamma ^{32}P] ATP, 20 units of T4 PNK, 2.5 µl of 10X Kinase buffer (Tris-HCl 0.5 M pH 7.6, $MgCl_2$ 0.1 M, dithiothreitol 50 mM, EDTA 1 mM, nuclease free spermidine HCl 1 mM) and water added for a total volume of 25 µl. The reaction mixture was incubated at 37°C for 45 min. The reaction was stopped by the addition of 1 µl of EDTA 0.5 M (pH 8.0).

The labelled SSOP was separated from unincorporated gamma ^{32}P by

column Sephadex-G50 (Pharmacia) gel filtration. 5 µl of labelled SSOP was counted in a Bioscan/QC 2000 bench top counter.

B 10.2 Radiolabelling of Probes by Hexanucleotide Primer Method.

The probe was isolated from a plasmid vector by restriction endonuclease digestion, gel purified (as described in section 1. above) and labelled by the hexanucleotide primer method of Feinberg and Vogelstein 1984. The isolated fragment (200-250 ng) was denatured by heating for 5 min in boiling water. It was cooled by placing on ice. The following reaction components were then added to the isolated fragment, 13 µl of OLB buffer (5X OLB consist of solution A:B:C in a 100:250:150 ratio: solution A: 1 ml of Tris 1.25 M pH 8.0, $MgCl_2$ 0.125 M, 18 µl of β-mercaptoethanol 14.4 M; solution B: HEPES 2 M pH 6.0; solution C: hexanucleotides at 4.5 µg/µl in Tris 3 mM pH 7.0, EDTA 0.2 mM), 50 µCi of alpha ^{32}P dCTP (3000 Ci/mM), 3 µl of Klenow fragment, and water up to 50 µl. The reaction mix was incubated for 2 h at room temperature. The reaction was stopped by the addition of 100 µl of 200 mg/ml tRNA in HENS buffer (Hepes 10 mM pH 7.2, NaCl 10 mM, EDTA 1 mM, SDS 0.1% w/v). The labelled probe was purified by Sephadex G-50 column filtration using HENS buffer. The first peak of radioactivity was monitored with a Ludlum Scintillation Counter and collected. Specific activities of 6-9 x 10^8 cpm/µg were routine.

B 11. Hybridization Conditions.

B 11.1 When probing with hexanucleotide primer labelled DNA fragments, the membranes were prehybridazed at 65°C in a water bath for 1 to 2 h in a bag containing 10 ml of prehybridization solution consisting of 2.5 ml dibasic sodium phosphate-EDTA (dibasic sodium phosphate 1 M, phosphoric acid 80 mM, EDTA 4 mM), 330 µl bovine serum albumin 30% w/v (BSA), 500 µl heat denatured sheared Salmon sperm DNA (5 mg/ml), 2.5 ml SDS 20% w/v, 3 ml 100% formamide, and 1.17 ml of water. The prehybridization solution was removed from the bag and replaced with 10 ml of hybridization solution similar to the one above except the

amount of Salmon sperm was reduced to 200 µl, 0.5 g of dextran sulphate was added, and the probe, then water up to 10 ml. Hybridization was performed overnight at 65°C. Following hybridization, the membranes were removed from the bags and washed. All the washes were performed at 65°C as follows: one 15 min wash was performed in 2X SSC:SDS 0.1%, and two 10 min washes were performed in 0.2X SSC:SDS 0.1%, after that membranes were blotted dry, wrapped in plastic wrap, and placed inside Wolf cassettes with Kodak X-OMAT XAR-5 X-ray film against a Quanta III (DuPont) intensifying screen. Exposures were performed at -70°C for 24 h to 1 week followed by development in a Kodak M35A X-OMAT processor.

B 11.2 When probing with SSO probes, the 3.5 x 7.2 cm strips were prehybridized at 42°C in a water bath with constant agitation for 30 to 40 min 3 ml of hybridization buffer (9 ml TMAC buffer: 500 ml tetramethylammonium chloride 5 M, 21 ml Tris 2 M pH 8.0, 3 ml EDTA 0.5 M, 8 ml SDS 10%, 100 ml water), 1 ml Denhardt's solution (50X: 100 ml PVP 2%, Ficoll 2%, BSA 2%), and 100 µl herring sperm DNA (100 µg/ml heat denatured) was added to the bags containing the strips. After prehybridization, the SSO probe was added and incubated for 1 to 3 h using the same conditions for prehybridization.

The washing conditions were with constant gentle agitation at 42°C, the membranes were washed twice for 10 min each time with 2X SSC:SDS 0.1%, once for 10 min with TMAC buffer, and once for 10 min with TMAC buffer at 56-58°C. Several membranes were washed at once. At the end of the washes the membranes were prepared for exposure, and exposed for 2-24 h as described above.

In the presence of TMAC, the hybridization and washing conditions were independent of the GC content of the probe (Wood et al 1985). The conditions described above were for 18 mer SSO probes. The sequence of the SSOPs are described in Table 6.

B 12. Restriction fragment length polymorphism analysis.

Genomic DNA (5µg) was digested with a restriction enzyme Taq I according to the manufacturer's specifications and electrophoretically separated on

0.7% agarose gel. The DNA fragments were transferred to nylon membrane Hybond N^+ under conditions described in section B 7.1. Hybridizations were carried out using one HLA-D region probe. The ~520 bp Pst I fragment of the exon-specific HLA-DRB cDNA clone PRTV1 was used to determine the HLA-DR type (Bidwell et al 1987, Long et al 1982 and 1983, Cox et al 1988).

B 13. DNA Amplification (PCR).

B 13.1 Preparation of Samples.

To a sterile 0.5 ml microfuge tube, the following ingredients were added 0.5-1 µg of genomic DNA for one reaction in a final volume of 50 µl, 10 µl 5X PCR buffer (Tris-HCl 1 M pH 8.0, KCl 1 M, BSA 170 µg/ml, $MgCl_2$ 1 to 10 mM, the optimal $MgCl_2$ concentration was primer dependant and ranged from 1 to 10 mM, Tween 20 12.5 µl, NP-40 12.5 µl, water up to 5 ml), 5 µl 10X dNTPs (10X dNTPs: dATP 2 mM, dCTP 2 mM, dGTP 2 mM, dTTP 2 mM), 1 µl of each primer see Table 8 for list of primers (25 pM to 100 pM of oligonucleotide primers), 1-2 Units Taq DNA polymerase (BRL or Perkin-Elmer Cetus), water up to 50 µl. The mixture was overlaid with 50 µl of mineral oil (Sigma) to prevent evaporation of the sample during repeated cycles of heating and cooling.

B 13.2 Thermal Cycling in a Programmable Heat Block.

The thermo-cycler (Perkin Elmer-Cetus Instruments) was programmed to set and maintain the typical temperature for denaturing, annealing and polymerization used in this work.

Table 8. PRIMERS USED FOR DRB AND DQB TYPING.

		Product
	DRB generic amplification.	
DRB AMP-A	CCC CAC AGC ACG TTT CTT G	247 bp
DRB AMP-B	CCG CTG CAC TGT GAA GCT CT	
	DQB generic amplification.	
DQB AMP-A	CAT GTG CTA CTT CAC CAA CGG	214 bp
DQB AMP-B	CTG GTA GTT GTG TCT GCA CAC	
	DRB group-specific amplification.	
	DR2-DRBI specific primer pair	
DRB AMP-2	TTC CTG TGG CAG CCT AAG AGG	259 bp
DRB AMP-B	same as in the generic amplification	
	DR4-DRBI specific primer pair	
DRB AMP-4	GTT TCT TGG AGC AGG TTA AAC	261 bp
DRB AMP-B	same as in the generic amplification	
	DRw52 associated group-DRBI specific primer pair	
DRB AMP-3	CAC GTT TCT TGG AGT ACT CTA C	263 bp
DRB AMP-B	same as in the generic amplification	

B 13.2.1 General conditions.

	10 min	94°C	denaturation
a)	30 sec	92-96°C	denaturation
b)	30 sec to 1 min	58-64°C	annealing (temperature depending on the primers)
c)	1 min to 2 min	72°C	extension

Steps a, b, and c were repeated for 30 cycles. After the last cycle the samples were incubated at 72°C for an additional 5 min to complete the final extension step.

In order to monitor the amplification of DNA, after completion of thermal cycles, an aliquot of 5 µl of the sample was taken and electrophoresed in a 2% Nu Sieve/1% agarose gel.

B 13.3 Primers and Conditions.

Primers	Denaturation	Annealing	Extension	$MgCl_2$ (mM)
DRB generic DQB generic	94°C 30 sec	55°C 1 min	72°C 1 min	1.5
DRB specific	94°C 30 sec	60°C 30 sec	72°C 1 min	2.5
DRw52-DRB1	94°C 30 sec	60°C 30 sec	72°C 1 min	1.5

The primer's sequence used in this work are listed in Table 8.

B 14. Gm and Km allotyping.

Gm and Km allotyping was performed by the technique of haemagglutination inhibition (Steinberg 1962) adapted for microtitre plates, using the reagents listed in Table 9.

B 14.1 Determination of Gm and Km allotypes by Haemagglutination-inhibition method.

Gm/Km allotyping is based on an agglutination-inhibition method wherein the presence of the antigen of interest in the test sample inhibits agglutination of the test system. The test system consists of red blood cells (RBC) coated with anti-Rh (D) antibodies carrying the Gm allotype of interest and an agglutinating serum containing antibodies toward the Gm allotype of interest. In the absence of test sample the agglutinator causes agglutination of the coated RBC. In the presence of test sample, the system will still agglutinate if the sample does not contain the Gm allotype of interest, but will not agglutinate if the test sample inhibits the agglutination (see Fig.3).

B 14.2 Coating of erythrocytes with anti-Rh(D) serum containing the desired Gm specificity.

a)-erythrocytes: whole human blood (blood group O, R2R2 or R1R2) was obtained in an appropriate anticoagulant.

b)- erythrocytes were washed 3 times with 1x phosphate-buffered-saline (PBS) and packed by centrifugation at 3000 rpm for 1 minute in a Clay-Adams Sero-Fuge II centrifuge [10x PBS consists of 2 g KCl, 2 g KH2PO4, 80 g NaCl, 21.6 g Na2HPO4x7H2O made up to litre, pH 7.2-7.4].

c)- one volume of packed washed erythrocytes was incubated with 2 volumes of anti-Rh(D) coat (see Table 9) and 3 volumes of PBS at 37°C during 60 minutes. The incubation reaction was mixed every 15 minutes by shaking gently. In the case of G3m(6), 4 volumes of anti-Rh serum and no PBS was used (like was indicated by the supplier).

Table 9. Gm AND Km ALLOTYPING REAGENTS.

	Anti-D coat	Anti-Gm agglutinator
G1m		
(1)	CB: Peters	NRC: 3950
	NRC: 3417	
(2)	NB: Peters	CB: Tyler
	NRC: 3545	NRC: 2135
(3)	NRC: 2447	NRC: 2871
	NRC: 3480	
(17)	NRC: 3417	NRC: MG 101-A1
		NRC: 3272
G3m		
(5)	NRC: 2127	NRC: 3908
(6)	NRC: 2781	NRC: 3200
(14)	CRPG: ADJ	CRPJ: BON
(15)	NRC: 3068	NRC: 2624
(16)	NRC: 3068	NRC: 2639
(21)	NRC: 3285	NRC: 4040
	NRC: 3359	
Km		
(1)	NRC: 2447	NRC: 3951
(3)	NRC: 3597	NRC: 3674

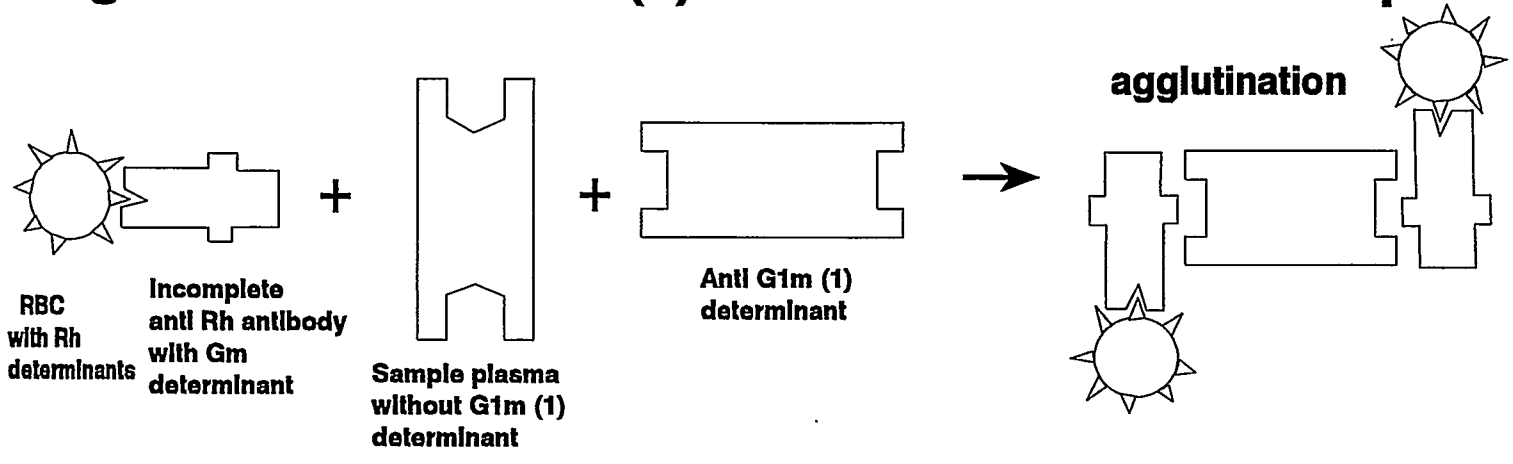
CB: Cleveland Biologicals, Cleveland, Ohio.

NRC: Netherlands Red Cross, Amsterdam, Netherlands.

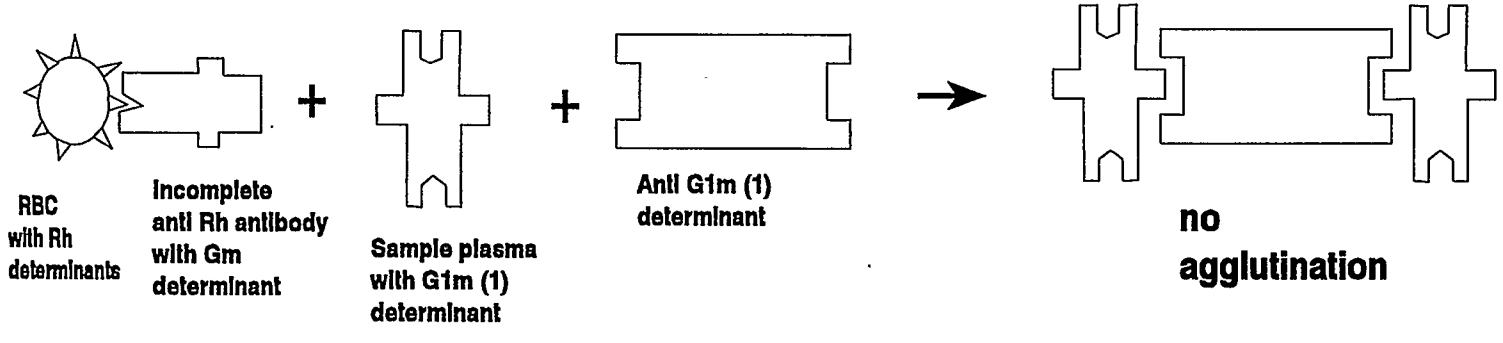
CRPG: Centre National De La Recherche Scientifique,Toulouse, France.

Fig.3 SCHEMATIC ILLUSTRATION OF Gm TYPING.
Negative and Positive test for G1m(1).

Negative Test for G1m (1) in Unknown Plasma Sample



Positive Test for G1m (1) in Unknown Plasma Sample



After the incubation, the coated erythrocytes (coated red blood cells:CRBC) were washed three times with PBS. After the last centrifugation a 0.2% suspension of CRBC was made with PBS and 2 drops of 30% w/v bovine serum albumin (Sigma A-3299) was added to each 5 ml of cell suspension.

B 14.3 Preparation of the Gm and Km agglutinators.

All the agglutinators used in this test were tittered with the appropriate CRBC in order to get their best working dilution for the conditions used in our laboratory (see Table 9). The starting dilution was the one suggested by the supplier. The optimal dilution of the agglutinator was determined by titration with negative and positive control samples.

B 14.4 Preparation of the plasma samples to be tested.

All the plasma samples were diluted 1:10 or 1:20 with PBS containing 0.1% w/v sodium azide to prevent bacterial growth in the samples. Those samples containing agglutinating antibodies were heat-inactivated for 10 minutes at 65°C and retested. Any specimens which could not be heat-inactivated were excluded.

B 14.5 Positive and Negative controls.

All the controls were also diluted at 1:10 or 1:20 with PBS containing 0.1% (w/v) sodium azide in order to prevent bacterial growth.

B 15. Procedure to Determine Gm and Km Allotyping.

The test was carried out in U-bottom microtiterplates (96-well Micro Test III Assay Plate Falcon 3910) using the standard-three-drop technique.

Mix successively in a microtiter plate equal volumes of:

a) Test sample:

25 µl of plasma to be tested in the appropriate dilution

25 µl of agglutinator in the optimal dilution

25 µl of antigen CRBC in a 0.2% suspension

The plate was mixed by hand, covered with parafilm to prevent evaporation, then incubated at room temperature for 60 min. The plate was then centrifuged using a microplate carrier at 1000 rpm for 30 sec in a IEC Centra-7R bench centrifuge. The microplate was placed at an angle of about 60° for 10 minutes, and the extent of agglutination was then determined. A second reading can be obtained by rotating the plate 180°.

b) Positive control:

25 µl of positive control diluted 1:10 or 1:20

25 µl of CRBC in a 0.2% suspension

25 µl of agglutinator in the optimal dilution

This should always show inhibition (no agglutination).

c) Negative control:

25 µl of negative control diluted 1:10 or 1:20

25 µl of CRBC in a 0.2% suspension

25 µl of agglutinator in the optimal dilution

This should always show agglutination.

d) System control:

25 µl of agglutinator in the optimal dilution

25 µl of CRBC in a 0.2% suspension

25 µl of PBS

This should always show agglutination.

e) Sample control:

25 µl of CRBC in a 0.2% suspension

25 µl of test plasma

25 µl of PBS

This should always show no agglutination. If agglutination occurs, this indicates agglutinating antibodies in the test sample. These must be

inactivated (section 13.4)

Interpretation of test results:

no agglutination = inhibition: allotype present (see Fig.3)

No agglutination is shown by a smooth vertical stream of coated RBC in the U-bottom microplate, and indicates that the allotype tested is present in the unknown test sample or in the positive control.

agglutination = no inhibition: allotype absent (see Fig.3)

Agglutination is shown by macroscopically visible round button in the middle of the U-bottom microplate, and indicates that the allotype tested is not present in the unknown sample nor in the negative control.

B 16. Summary of Sample Processing.

The processing of a patient or control sample was as follows: Blood was obtained by venipuncture, and was separated into buffy coat and plasma by centrifugation. The plasma fraction was frozen (-80°C) until required for Gm/Km allotyping as described in the experiments section B 14. The buffy coat was mixed with the lysis solution and stored and transported in this condition until genomic DNA was extracted as described in sections B 2. and B 3.

The plasma sample was used to determine the Gm and Km allotypes by the Haemagglutination-inhibition method. The complete procedure for allotyping is described in detail in sections B 14.1 to B 14.5.

DNA was extracted as described in section B 3. After extraction, the DNA was successfully used in several methodologies to complete the HLA analysis.

HLA determination by RFLP analysis was performed by digesting genomic DNA with the restriction endonuclease Taq 1 (section B 8), electrophoresing in .8% agarose gel (section B 6.1), transferring to a nylon membrane by vacuum blot (section B 7.1), and probing with the HLA-DRB chain probe as described in section B 12. The preparation of the probe is described in section B 10.2, and the hybridization conditions are described in section B 11.1.

HLA typing by the PCR method was used to overcome limitations of the HLA-RFLP-Analysis. In sections B 13 to B 13.3 the PCR method is described in

detail.

Molecular HLA typing by Dot-Blot and SSO Probes (PCR-SSOP) was done by dotting 1 µl of the amplified DNA onto a nylon membrane (section B 7.2). The membrane was probed with SSO probes as described in section B 11.2 for generic and specific oligotyping. The labelling of the probes is described in section B 10.1

DNA typing for subsets of HLA-DR4 was performed by digesting PCR-amplified DNA with allele specific restriction endonucleases (PCR-RFLP) using 5 µl of the amplified DNA and the appropriate restriction endonucleases as described in section B 8. After digestion, the samples were subjected to electrophoresis in an 8-10% polyacrylamide gel as described in section B 6.2.

B 17. Statistical Analysis.

Gm haplotype frequencies were estimated from the phenotype data by maximum-likelihood, using the program MAXLIK, kindly provided by T.E. Reed (Reed and Schull 1968). This program also tests the chi-squared goodness-of-fit of the observed phenotype frequencies to those expected at Hardy-Weinberg equilibrium given the estimated haplotype frequencies. The MAXLIK program was run on a Sun Sparc Workstation.

Significance of genetic differences between RA patients and controls was tested using the chi-square statistic, calculated with the assistance of a programmable calculator. P values for chi-square statistics were obtained from published tables (Pearson and Hartley 1966).

C. RESULTS.

C 1. Gm and Km typing.

The observed phenotype frequencies of the Gm and Km allotypes in Mexicans are presented in Table 10. Table 11 presents the probable genotype for each phenotype. One RA patient and 1 Cancun control could not be typed due to agglutinating antibodies that could not be inactivated.

Gm haplotype frequencies in each of the four populations were estimated from the phenotype data by the maximum-likelihood method. This program also tests the goodness-of-fit of the observed phenotype frequencies to those expected at Hardy-Weinberg equilibrium given the estimated haplotype frequencies.

The presence of the 14 phenotypes in the populations can be explained by the presence of the following haplotypes identified: $Gm^{1,17;21}$, $Gm^{1,2,17;21}$ and $Gm^{1,17;15,16}$ from Native American Indians (Williams et al 1985; Schanfield and Fudenberg 1978), $Gm^{1,17;5,6}$ and $Gm^{1,17;5,14}$ from Africans (Johnson et al 1977), and $Gm^{3;5,14}$ from Europeans (Johnson et al 1977).

The phenotype Gm(1,17;21) indicates the homozygous presence of the Native haplotype $Gm^{1,17;21}$, the phenotype Gm(1,2,17;21) can be explained by the presence of the Native haplotype $Gm^{1,2,17;21}$ in homozygous combination or the presence of the Native haplotypes $Gm^{1,17;21}$ and $Gm^{1,2,17;21}$ in heterozygous combination, and the phenotype Gm(1,17;15,16,21) is explained by the presence of the Native haplotypes $Gm^{1,17;15,16}$ and $Gm^{1,17;21}$ in heterozygous combination. The phenotypes Gm(1,3,17;5,14,21), Gm(1,2,3,17;5,14,21) and Gm(1,3,17;5,14,15,16) can be explained by the presence of the Caucasian haplotype $Gm^{3;5,14}$ and the Native haplotypes $Gm^{1,17;21}$, $Gm^{1,2,17;21}$ and $Gm^{1,17;15,16}$ in heterozygous combinations. The phenotypes Gm(1,17;5,14,21), Gm(1,17;5,14,15,16) and Gm(1,17;5,6,21) can be explained by the presence of the African haplotypes $Gm^{1,17;5,14}$ and $Gm^{1,17;5,6}$ and the Native haplotypes $Gm^{1,17;21}$ and $Gm^{1,17;15,16}$. In the case of the phenotypes Gm(1,3,17;5,14) and Gm(1,3,17;5,6,14), these can only be explained by the presence of the African haplotypes $Gm^{1,17;5,14}$ and $Gm^{1,17;5,6}$ and the Caucasian haplotype $Gm^{3;5,15}$, and phenotype

Table 10 OBSERVED PHENOTYPES IN MEXICAN POPULATION.

Phenotype	Frequency							
	Mexico City		Cancun		Merida		RA	
	n		n		n		n	
Gm(1,17;21)	33	(.38)	34	(.38)	22	(.47)	19	(.48)
Gm(1,2,17;21)	9	(.11)	16	(.18)	11	(.23)	10	(.25)
Gm(1,3,17;5,14,21)	27	(.31)	24	(.27)	5	(.11)	7	(.18)
Gm(1,17;5,14,21)	1	(.01)	2	(.02)	1	(.02)	0	
Gm(1,17;15,16,21)	0		2	(.02)	1	(.02)	0	
Gm(1,2,3,17;5,14,21)	3	(.03)	6	(.07)	3	(.06)	1	(.03)
Gm(3;5,14)	8	(.09)	0		3	(.06)	1	(.03)
Gm(1,3,17;5,14)	3	(.03)	0		0		0	
Gm(1,3,17;5,14,15,16)	1	(.01)	0		0		0	
Gm(1,17;5,14,15,16)	1	(.01)	0		0		0	
Gm(1,17;5,6,21)	1	(.01)	2	(.02)	1	(.02)	0	
Gm(1,3,17;5,6,14)	0		1	(.01)	0		0	
Gm(1,17;15,21)	0		3	(.03)	0		0	
Gm(1,2,17;15,21)	0		0		0		2	(.05)
Total	87		90		47		40	
Km(3)	47	(.52)	36	(.40)	23	(.49)	12	(.30)
Km(1,3)	37	(.43)	47	(.52)	22	(.47)	27	(.67)
Km(1)	5	(.06)	8	(.09)	2	(.04)	1	(.03)
Total	87		91		47		40	

Table 11. OBSERVED PHENOTYPES AND PROBABLE GENOTYPES.

Phenotype	Genotype
Gm(1,17;21)	$Gm^{1,17;21}/Gm^{1,17;21}$
Gm(1,2,17;21)	$Gm^{1,17;21}/Gm^{1,2,17;21}$ or
	$Gm^{1,2,17;21}/Gm^{1,2,17;21}$
Gm(1,3,17;5,14,21)	$Gm^{1,17;21}/Gm^{3;5,14}$
Gm(1,17;5,14,21)	$Gm^{1,17;21}/Gm^{1,17;5,14}$
Gm(1,17;15,16,21)	$Gm^{1,17;21}/Gm^{1,17;15,16}$
Gm(1,2,3,17;5,14,21)	$Gm^{1,2,17;21}/Gm^{3;5,14}$
Gm(3;5,14)	$Gm^{3;5,14}/Gm^{3;5,14}$
Gm(1,3,17;5,14)	$Gm^{1,17;5,14}/Gm^{3;5,14}$
Gm(1,3,17;5,14,15,16)	$Gm^{1,17;15,16}/Gm^{3;5,14}$
Gm(1,17;5,14,15,16)	$Gm^{1,17;5,14}/Gm^{1,17;15,16}$
Gm(1,17;5,6,21)	$Gm^{1,17;21}/Gm^{1,17;5,6}$
Gm(1,3,17;5,6,14)	$Gm^{1,17;5,6}/Gm^{3;5,14}$
Km(3)	Km^{3}/Km^{3}
Km(1,3)	Km^{1}/Km^{3}
Km(1)	Km^{1}/Km^{1}

Gm(3;5,14) is explained by the homozygous combination of the Caucasian haplotype $Gm^{3;5,14}$. The phenotypes Gm(1,17;15,21) and Gm(1,2,17;15,21) (see Table 10) cannot be explained on the basis of known polymorphic haplotypes in similar populations and are probably due to rare haplotypes.

Gm haplotype frequencies in the RA patients and controls (Merida, Cancun and Mexico City populations) were estimated assuming the presence of six haplotypes $Gm^{1,17;21}$, $Gm^{1,2,17;21}$, $Gm^{3;5,14}$, $Gm^{1,17;5,14}$, $Gm^{1,17;15,16}$ and $Gm^{1,17;5,6}$.

Table 12 shows the estimated Gm haplotype frequencies in each of the populations, as well as the Km^1 and Km^3 allele frequencies determined by simple gene counting. Merida and Cancun frequencies were combined since the haplotype frequencies were not different from each other, as shown in Table 12. The haplotype frequencies of $Gm^{1,17;21}$, $Gm^{1,2,17;21}$ and $Gm^{3;5,14}$ are not significantly different between the combined control group and the RA patients, p= 0.457 (see Table 13). However, there were significant haplotype frequency differences between the Mexico City population and the RA patients, p= 0.00867, as well as significant differences between the Mexico City population and the combined control group, p= 0.0015 (see Table 13). Therefore, the Mexico City population cannot be used as a control group when testing for significance of genetic differences between RA patients and healthy controls.

Table 14 shows Km^1 and Km^3 allele frequencies as determined by simple gene counting. Upon inspection, no differences in the allele frequencies can be observed between the four populations. When the combined control group was tested for significance with the RA patients, no significant difference was found, p= 0.42 (see Table 14).

C 2. HLA-DRB RFLP analysis.

Fig.4 shows a typical autoradiogram of a Southern transfer of DNA digested with Taq I and hybridized with the DRB probe. The banding pattern produced for each DR type is very consistent. For this reason, the bands were numbered from largest to smallest as shown in Fig.4 and these numbers were used as identifiers. The same band number is used for fragments that appear to be the same size, even when they are found in different DR specificities. Thirty-six RA patients,

Table 12. ESTIMATED Gm HAPLOTYPE FREQUENCIES (± STANDARD ERROR) AND Km FREQUENCIES IN MAYAN AND MEXICO CITY POPULATION.

	Mexico City	Merida	Cancun	RA	Merida + Cancun
$Gm^{1,17;21}$	0.59	0.66	0.65	0.71	0.66
	(±.04)	(±.05)	(±.04)	(±.05)	(±.09)
$Gm^{1.2.17;21}$	0.07	0.16	0.14	0.16	0.15
	(±.02)	(±.04)	(±.03)	(±.04)	(±.07)
$Gm^{3;5,14}$	0.29	0.15	0.18	0.13	0.17
	(±.03)	(±.04)	(±.03)	(±.04)	(±.07)
$Gm^{1,17;5,14}$	0.03	0.01	0.01	0	0.01
	(±.01)	(±.01)	(±.008)		(±.018)
$Gm^{1,17;15,16}$	0.01	0.01	0.01	0	0.01
	(±.008)	(±.01)	(±.008)		(±.018)
$Gm^{1,17;5,6}$	0.006	0.01	0.02	0	0.01
	(±.006)	(±.01)	(±.001)		(±.011)
	n= 87	n= 47	n= 87	n= 38	n= 134
Km^{1}	0.27	0.28	0.35	0.36	0.32
Km^{3}	0.73	0.72	0.65	0.64	0.68
	n= 174	n= 94	n= 182	n= 80	n= 276

Table 13. Gm HAPLOTYPE FREQUENCIES IN MAYAN AND MEXICO CITY POPULATION.

	Mexico City	RA	Merida+Cancun
$Gm^{1,17;21}$	103 (0.59)	51 (0.66)	176 (0.66)
$Gm^{1,2,17;21}$	12 (0.07)	12 (0.15)	40 (0.15)
$Gm^{3;5,14}$	50 (0.29)	13 (0.17)	43 (0.16)
$Gm^{1,17;5,14}$, $Gm^{1,17;15,16}$, $Gm^{1,17;5,6}$	9 (0.05)	0	9 (0.035)
	n=174	n=76	n=268

RA vs Mexico City

$X_3^2 = 11.67$

p= 0.00867

RA vs Merida+Cancun

$X_3^2 = 2.64$

p= 0.457

Mexico City vs Merida+Cancun

$X_3^2 = 15.4$ p= 0.0015

Table 14. Km ALLELE FREQUENCIES IN MAYAN POPULATION.

	Merida	Cancun	RA	Merida+Cancun
Km^1	26 (0.28)	63 (0.35)	29 (0.36)	89 (0.32)
Km^3	68 (0.72)	119 (0.65)	51 (0.64)	187 (0.68)
	n= 94	n= 182	n= 80	n= 276

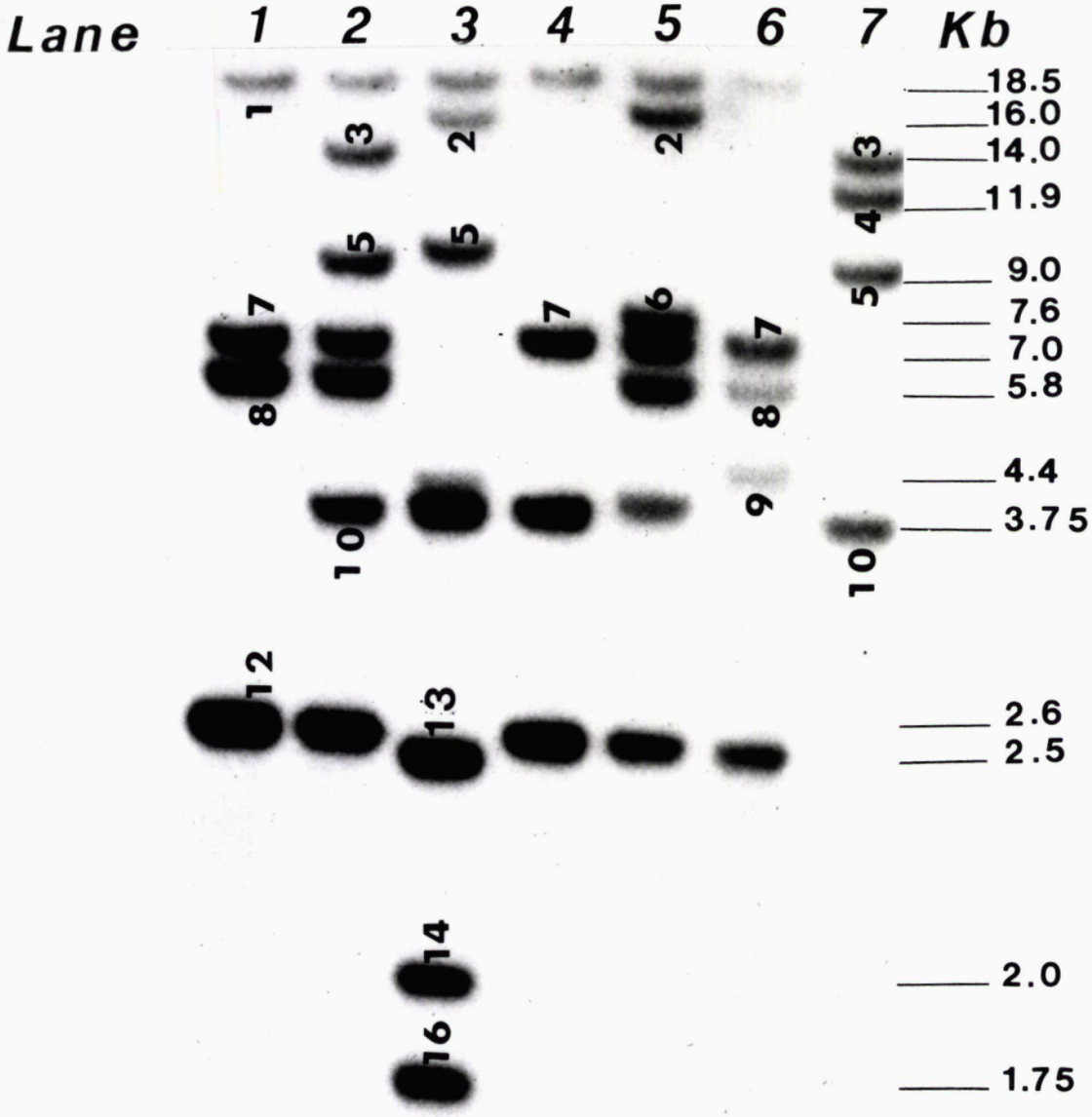
RA vs Merida+Cancun

$X_1^2 = 0.66$

p= 0.42

Fig.4 RESTRICTION PATTERNS OF Taq I DIGESTION.

Representative autoradiographic Taq I DNA band patterns detected with the DRB probe after Southern blotting. The number of each band is arranged in increasing sizes to indicate the band associated with each DR antigen. The sizes (in Kb) of the Taq I fragments (bands) are on the left. All lanes contain samples from control subjects.


Lane
1
2
3
4
5
6
7
Kb
18.5
16.0
14.0
11.9
9.0
7.6
7.0
5.8
4.4
3.75
2.6
2.5
2.0
1.75

44 controls from Merida, and 86 controls from Cancun were DRB typed using this method. Fig.4 shows samples DR typed by this method . The homozygous DR4 individual in lane 1 has bands 1,7,8 and 12. Lane 2 shows the pattern of a heterozygote for DR4 (bands 1,7,8 and 12) and DR3-DR6 (bands 3,5 and 10). Lane 3 shows the pattern of a heterozygote for DR2 (bands 2,10,14 and 16) and DR7 (bands 1,5,10 and 13). Lane 4 shows the pattern of a homozygote DR7 (bands 1,7,10 and 12). Lane 5 shows the pattern of a heterozygote DR4 (bands 1,7,8 and 12) and DR5 (bands 2,6 and 10). Lane 6 shows the pattern of a heterozygote DR1 (bands 7 and 9) and DR4 (bands 1,7,8 and 12). Lane 7 shows the pattern of a heterozygote DR3-DR6 (bands 3,5 and 10) and DR8 (band 4 only).

Four DR alleles do not produce distinctive band patterns when probed with the DRB probe after Taq I digestion. DR3 and DR6 cannot be differentiated since both can produce the 2,5,10 or 3,5,10 banding patterns while DR7 and DR9 cannot be differentiated since both can produce the 1,7,10,12 or 1,5,10,13 banding patterns. Because these four alleles cannot be distinguished by this method, PCR-SSOP and PCR-RFLP were used to complete the genotyping of the samples processed by this method.

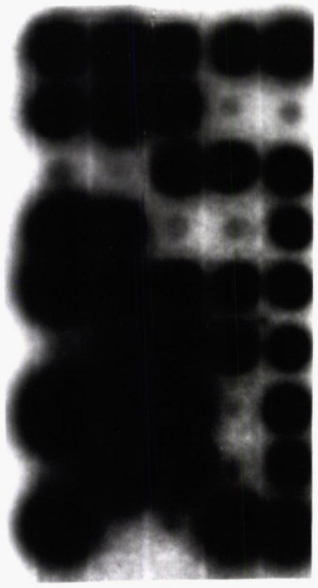
C 3. Molecular oligotyping analysis of the HLA class II alleles.

C 3.1 DRB oligotyping by PCR-SSOP.

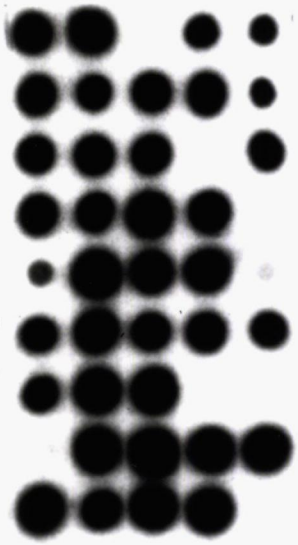
Following the genotyping by RFLP analysis, the 176 samples were hybridized with SSO probes (see Table 6) to DNA enzymatically amplified with generic amplification primers (see Table 8). Fig.5 shows a typical autoradiogram of dot-blot hybridization pattern obtained with the probe DRB 1004 (specific for DR4 alleles) (A- Cancun samples, B- Merida samples, C- RA patients). By this method the already known DR types (by RFLP analysis) were confirmed, and the HLA-DR3, -DR6, -DR7 and -DR9 types were clearly distinguished.

Table 15 shows the DR4 individual genotype frequencies in the Mayan controls and RA patients. Any group differences were tested for significance using the chi-square test. The Merida and Cancun control groups were combined since no

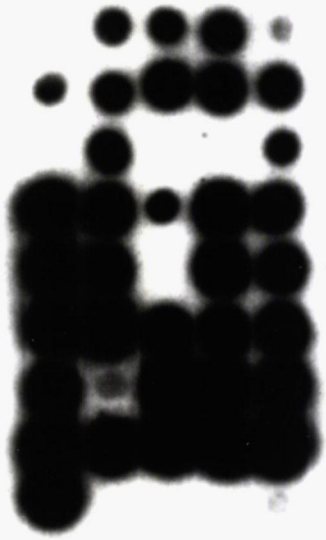
Fig.5 DNA TYPING FOR HLA-DR4 SPECIFIC BY PCR-SSOP.
Representative autoradiogram of PCR-amplified DNA, dot blotted and hybridized with SSOP (1004 probe-DR4 specific). (A) Cancun samples. (B) Merida samples. (C) RA patients.



A



B



C

Table 15. INDIVIDUAL GENOTYPE FREQUENCIES FOR DR4 IN MAYAN POPULATION.

DR	Merida	Cancun	RA	Merida+Cancun
4/4	12 (0.27)	27 (0.31)	23 (0.64)	39 (0.30)
4/X	24 (0.55)	33 (0.38)	10 (0.28)	57 (0.44)
X/X	8 (0.18)	26 (0.31)	3 (0.08)	34 (0.26)
	n= 44	n= 86	n= 36	n= 130

Merida vs Cancun
X_2^2= 3.5
p= 0.2

RA vs Merida+Cancun
X_2^2= 14.5
p= 0.00071

significant difference exists between them, p= 0.2. When the combined control group was compared with the RA patient group the difference was highly significant, p= 0.00071. Table 16 shows the Mayan controls and RA patient DR4 gene frequencies. Again, the Merida and Cancun controls were combined since no significant difference was found between them, p= 0.54. As with the genotype frequency comparison, the gene frequency comparison between the RA patient group and the combined control group was highly significant, p= 0.00008.

Because of the high frequency of DR4 found in the combined Mayan control group (0.74) and in the RA patients (0.92) (see Table 15), the samples were subtyped for DR4 Dw specificities.

C 3.2 DQB oligotyping by PCR-SSOP.

When the results were analyzed, it was found that the DQB1*0303 frequency was high in the RA patients and, since this allele is not known to be in linkage disequilibrium with the DR4 allele, the RA samples were sent back a second time to Dr. Trucco's laboratory in Pittsburgh to be retested by the reverse-dot-blot method (Rudert and Trucco 1992). The results after the second test were different than the ones obtained by the conventional dot-blot method. All the samples that were initially typed as positive for DQB1*0303 were subsequently subtyped as DQB1*0302 homozygotes by the reverse dot-blot method. The problem was that with conventional dot-blot, cross reaction of DQB1*0302 with the probe 5706 (DQB1*0301/03) is particularly strong so that when the sample is homozygous for DQB1*0302, it is easy to mistake the cross reaction with probe 5706 as positive for DQB1*0301/0303. Because there was also no hybridization with probe 2602 (DQB1*0301/0601), the typing then mistakenly becomes DQB1*0303 positive. When both DQB1*0301 and DQB1*0302 are present, the signals from probes 5706 (DQB1*0301/0303) and 5707 (DQB1*0302) should be about equal. Similarly, reactions with probes 2602 (DQB1*0301) and 2603 (DQB1*0302) should be about equal. With conventional dot-blot, however, it is very difficult to distinguish the intensity of signal which corresponds to a real signal rather than a cross reaction. At this point, it is not possible to know how many of the samples are truly homozygous for DQB1*0302 or

Table 16. GENE FREQUENCIES FOR DR4 IN MAYAN POPULATION.

DR	Merida	Cancun	RA	Merida+Cancun
DR4	48 (0.55)	87 (0.51)	56 (0.78)	135 (0.52)
DRX	40 (0.45)	85 (0.49)	16 (0.22)	125 (0.48)
	n= 88	n= 172	n= 72	n= 260

Merida vs Cancun
X_1^2= 0.37
p= 0.54

RA vs Merida+Cancun
X_1^2= 15.43
p= 0.00008

heterozygous for DQB1*0301/0302 in the control group. Therefore, no statistical analysis of DQB1 typing will be presented.

C 3.3 Determination of DR4 subtypes by PCR-RFLP analysis.

DR4-DRB1 group-specific primers (see Table 8) were used to amplify DNA by PCR. The use of the DRB AMP-4 primer complementary to the first hypervariable region of DRB1*04 between amino acid positions 6 and 13 allows a selective amplification of the DRB1 exon of DR4 sequences . The second primer DRB AMP-B is complementary to the DNA coding sequence between amino acid positions 87 and 96 for all of the HLA-DRB genes. Amplification with this primer pair generates a 262 bp product.

Figure 6 summarizes the different patterns generated when the PCR amplified DR4-DRB gene region is restricted with the five different enzymes used in this analysis. Fig.7 and 8 show the typical patterns of restriction fragments of DR4 subtypes obtained with digestion using five restriction enzymes after PCR amplification.

Table 17 shows the individual genotype frequencies for Dw13 and Dw14 subtypes of DR4 alleles in Mayan controls and RA patients who were DR4 positive. All other DR4 subtypes are subsumed under "other", since Dw13 and Dw14 were the most common subtypes among RA patients. Chi-square testing was not done due to the small sample sizes within cells (see Table 1 for description of the relation between DRB1 DR4 alleles and Dw subtypes).

Table 18 shows the individual genotype frequencies for DRB*0403 and 0407 alleles (both Dw13) in Mayan controls and RA patients who were positive for DR4. When comparing Merida and Cancun controls, no significant difference was found, p= 0.96. In addition, no significant difference was found between the combined controls and the RA patient group, p= 0.46.

Table 19 shows the individual genotype frequencies for DRB1*0404 and 0408 (both Dw14) in Mayan controls and RA patients who were positive for DR4. When comparing Merida and Cancun controls, no significant difference was found, p= 0.08. The difference between the RA patients and the combined controls also was not significant, p= 0.07.

Fig.6 FLOW CHART OF RESTRICTION FRAGMENT PATTERNS OF DR4 SUBTYPE ALLELES.

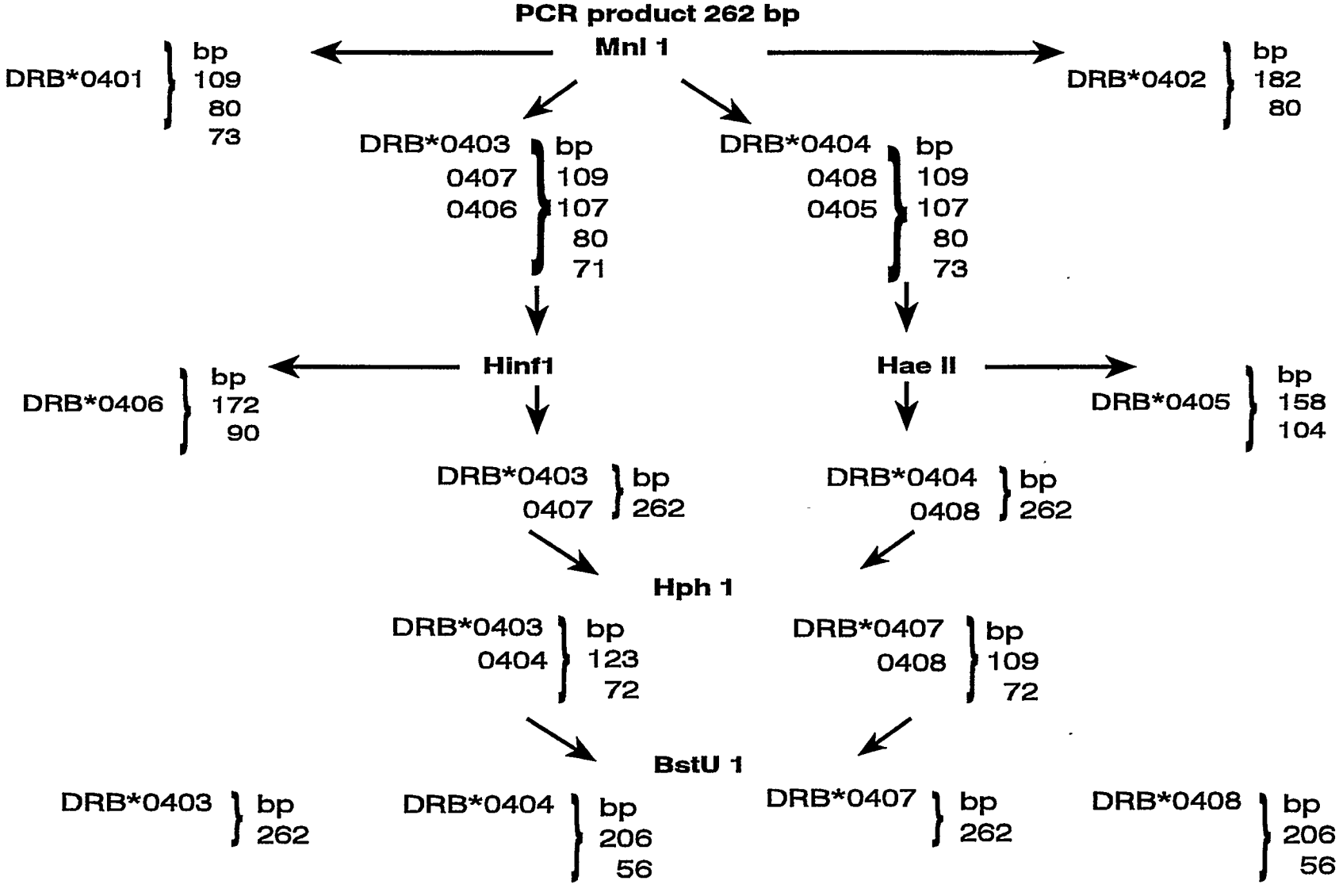
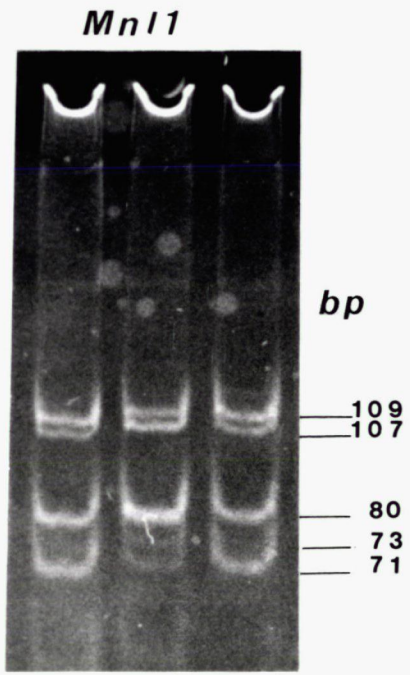
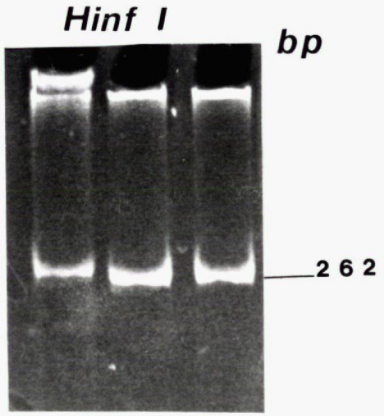

PCR product 262 bp
Mnl 1
DRB*0401 bp 109 80 73
DRB*0402 bp 182 80
DRB*0403 0407 0406 bp 109 107 80 71
DRB*0404 0408 0405 bp 109 107 80 73
Hinf1
Hae II
DRB*0406 bp 172 90
DRB*0405 bp 158 104
DRB*0403 0407 bp 262
DRB*0404 0408 bp 262
Hph 1
DRB*0403 0404 bp 123 72
DRB*0407 0408 bp 109 72
BstU 1
DRB*0403 bp 262
DRB*0404 bp 206 56
DRB*0407 bp 262
DRB*0408 bp 206 56

Fig.7 RESTRICTION FRAGMENT PATTERNS OF THE DR4 SUBTYPE ALLELES OBTAINED AFTER DIGESTION OF THE PCR PRODUCTS WITH 3 ENZYMES.

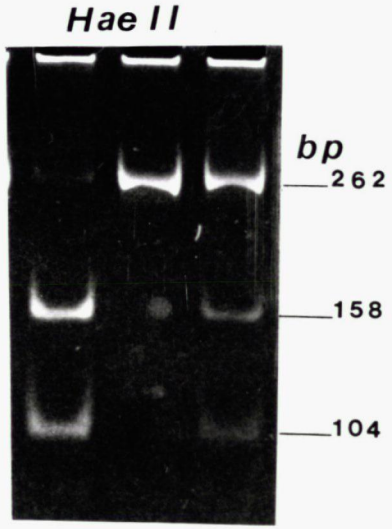
PCR products were separated in a 10% polyacrylamide gel after Mnl I digestion, and an 8% polyacrylamide gel for Hinf I, and Hae II. (A) Mnl I digestion yields fragments of 109, 107, 80, 73 bp with DRB*0404, 0405 and 0408 (lane 2), and fragments of 109, 107, 80, 71 bp with DRB*0403, 0406 and 0407 (lane 1-3). DRB1*0401 yields 109, 80, 73 and DRB1*0402 yields 182 and 80 bp fragments (examples not shown). (B) Hinf I digestion yield fragments of 172 and 90 bp only with DRB*0406 (example not shown), and does not digest the other DR4 alleles resulting in a 262 bp uncut fragment (lanes 1-3). (C) Hae II digestion yields fragments of 158 and 104 bp (lanes 1 and 3) with DRB*0405 and does not digest the other DR4 alleles resulting in a 262 bp uncut fragment (lanes 2 and 3).



A



B

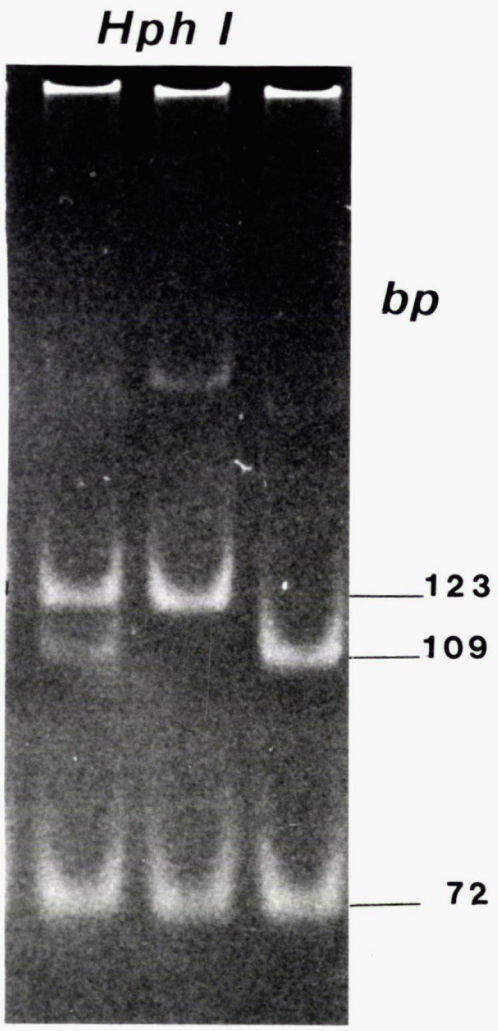


C

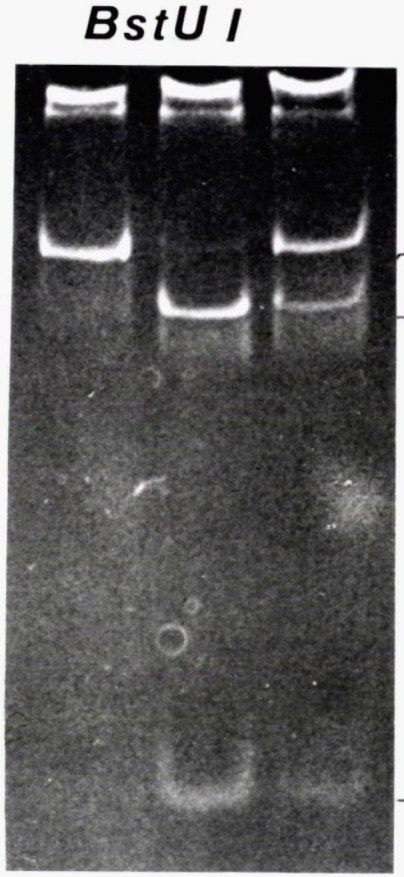
Fig.8 RESTRICTION FRAGMENT PATTERNS OF THE DR4 SUBTYPE ALLELES OBTAINED AFTER DIGESTION OF THE PCR PRODUCTS WITH 2 ENZYMES.

PCR products were run on 8% polyacrylamide gel. (A) Hph I digestion yields fragments of 123 and 72 bp with DRB*0403, 0404, (lanes 1 and 2) and fragments of 109 and 72 bp with DRB*0407, 0408 (lanes 1 and 3).

(B) BstU I yields fragments of 206 and 56 bp with DRB*0404, 0408 (lanes 2 and 3), and does not digest DRB1*0403 and DRB1*0407 resulting in a 262 bp uncut fragment (lanes 1 and 3).



A



B

Table 17. INDIVIDUAL GENOTYPE FREQUENCIES FOR DR4 SUBTYPES IN MAYAN POPULATION (DR4 POSITIVE INDIVIDUALS ONLY).

DRB1 DR4 alleles	Dw	Merida	Cancun	RA
0403/0403 0407/0407 0403/0407	13/13	5 (0.14)	9 (0.15)	7 (0.21)
0404/0404 0408/0408 0404/0408	14/14	3 (0.08)	0	2 (0.07)
0403/0404 0407/0408 0403/0408 0404/0407	13/14	2 (0.06)	4 (0.07)	8 (0.24)
0403/other 0407/other	13/X	17 (0.47)	26 (0.43)	5 (0.15)
0404/other 0408/other	14/X	7 (0.19)	12 (0.2)	7 (0.21)
other	X/X	2 (0.06)	9 (0.15)	4 (0.12)
		n= 36	n= 60	n= 33

Table 18. INDIVIDUAL GENOTYPE FREQUENCIES FOR DRB1*0403 AND 0407 ALLELES (Dw13) IN MAYAN POPULATION (DR4 POSITIVE INDIVIDUALS ONLY).

Dw	Merida	Cancun	RA	Merida+Cancun
13/13	5 (0.14)	9 (0.15)	7 (0.21)	14 (0.15)
13/X	19 (0.53)	30 (0.50)	13 (0.39)	49 (0.51)
X/X	12 (0.33)	21 (0.35)	13 (0.39)	33 (0.34)
	n= 36	n= 60	n= 33	n= 96

Merida vs Cancun
X^2_2= 0.07
p= 0.96

RA vs Merida+Cancun
X^2_2= 1.53
p= 0.46

Table 19. INDIVIDUAL GENOTYPE FREQUENCIES FOR DRB1*0404 AND 0408 ALLELES (Dw14) IN MAYAN POPULATION (DR4 POSITIVE INDIVIDUALS ONLY).

Dw	Merida	Cancun	RA	Merida+Cancun
14/14	3 (0.08)	0	2 (0.06)	3 (0.03)
14/X	9 (0.25)	16 (0.27)	15 (0.45)	25 (0.26)
X/X	24 (0.67)	44 (0.73)	16 (0.48)	68 (0.71)
	n= 36	n= 60	n= 33	n= 96

Merida/Cancun
X^2_2= 5.17
p= 0.08

RA vs Merida+Cancun
X_2^2= 5.4
p= 0.07

Table 20 shows the gene frequencies for Dw subtypes of DR4 positive alleles found in Mayan controls and RA patients. When Merida and Cancun controls were compared, no significant difference was found, p= 0.05. The Merida and Cancun controls were therefore combined and compared to RA patients, and the result was also not significant, p= 0.30.

Table 21 shows the gene frequencies for the Dw14 subtype versus all other DR4 subtypes occurring in patients and controls. When Merida and Cancun controls were compared, no significant difference was found, p= 0.14. When the combined controls were compared to the RA patient group, the difference was also not significant, p= 0.14.

C 3.4 HLA-DR4 haplotype and G1m(2)/Km(1) allotype analysis.

When the RA patients were analyzed for interaction of DR4 with G1m(2) and Km(1) allotypes, only 35 of the 36 RA patients were tested by chi-square, because a plasma sample was not obtained from one of these patients.

Table 22 shows the frequency of DR4(+) and (-) haplotype and G1m(2) (+) and (-) alleles in the RA patient group. No significant interaction between DR4 and G1m(2) was found, p= 0.22.

Table 23 shows the frequency of DR4(+) and (-) haplotypes and Km1(+) and (-) alleles in the RA patient group. No significant interaction between DR4 and Km1 was found, p= 0.22.

Table 20. GENE FREQUENCIES FOR DR4 SUBTYPES IN MAYAN POPULATION (DR4 POSITIVE ALLELES ONLY).

DR4 alleles	Dw	Merida	Cancun	RA	Merida+Cancun
0403 and 0407	13	29 (0.60)	48 (0.55)	26 (0.46)	77 (0.57)
0404 and 0408	14	14 (0.30)	16 (0.18)	18 (0.32)	30 (0.22)
other	other	5 (0.10)	24 (0.27)	12 (0.22)	29 (0.21)
		n= 48	n= 88	n= 56	n= 136

Merida vs Cancun
X_2^2= 6.0
p= 0.05

RA vs Merida+Cancun
X_2^2= 2.38
p= 0.30

Table 21. GENE FREQUENCY OF DRB1*0404 AND 0408 (Dw14) IN MAYAN POPULATION (DR4 POSITIVE ALLELES ONLY).

DR4 alleles	Merida	Cancun	RA	Merida+Cancun
Dw14	14 (0.29)	16 (0.18)	18 (0.32)	30 (0.22)
other	34 (0.71)	72 (0.82)	38 (0.68)	106 (0.78)
	n= 48	n= 88	n= 56	n= 136

Merida vs Cancun
X_1^2= 2.18
p= 0.14

RA vs Merida+Cancun
X_1^2= 2.15
p= 0.14

Table 22. FREQUENCIES OF DR4(+) DR4(-) AND G1m(2)+ G1m(2)- IN 35 RA PATIENTS.

	DR4 +	DR4 -	% DR4+
G1m(2) +	10	2	83%
G1m(2) -	22	1	96%
% G1m(2)	31%	67%	

$X_1^2 = 1.5$

$p = 0.22$

Table 23. FREQUENCIES OF DR4(+) DR4(-) AND Km1(+) Km1(-) IN 35 RA PATIENTS.

	DR4 +	DR4 -	% DR4+
Km1 +	21	3	91%
Km1 -	11	0	100%
% Km 1	66%	100%	

$X_1^2 = 1.5$

$p = 0.22$

D. DISCUSSION.

D 1. Gm typing.

The frequencies of the immunoglobulin haplotypes in the two Mayan controls (Merida and Cancun) and the RA patients were very similar and reflect the historical events that happened in that area during the past four centuries.

It has been suggested by some authors that the Oriental haplotype $Gm^{1,3;5}$ is present in Native American Indians. Steinberg et al (1967) found this haplotype in some Mexican populations, but Williams et al (1985) who studied 5392 Native American Indians did not find this haplotype. Similarly, Field et al (1988) did not find this haplotype in a study of Native people of British Columbia. Therefore in the data presented in this study (see Table 11 and 12), only the European haplotype $Gm^{3;5,14}$ is assumed to be present in individuals whose phenotype could be explained by the presence of either $Gm^{1,3;5}$ or $Gm^{3;5,14}$. The haplotype $Gm^{3;5,14}$ is polymorphic in European populations and its presence in this population can be considered a reflection of Caucasian migratory patterns. Merida, Cancun and RA patients share similar frequencies for this haplotype. The presence of the African haplotypes $Gm^{1,17;5,14}$ and $Gm^{1,17;5,6}$ in the Mayan and Mexico City populations reflects African gene flow into non-African populations, as suggested by historical data. Similarly the presence of the Native American haplotype $Gm^{1,17;15,16}$ (which is also found in Asia) reflects the Native American contribution to these Mayan populations, and may reflect migratory patterns into the different areas of Central America. The frequency of this haplotype in Mayan populations (0.01) was not different from the frequencies found in other Native American Indians. For example, Williams et al (1985) found a frequency of 0.01 in Navajo Indians and Zuni Indians, while Schanfield and Fudenberg (1978) found a frequency of 0.01 to 0.08 of this haplotype in two Tlaxcaltecan populations from Mexico. As stated previously in the results, Merida is not different for $Gm^{1,17;15,16}$ haplotype from Cancun, and RA patients are not different from the Mayan control group.

The Mexico City population is different from the Mayan control group due to a greater Caucasian admixture (as indicated by the frequency of the European

$Gm^{3;5,14}$ haplotype, 0.29 vs 0.17 respectively) and lesser Native American contribution. Decreased Native American contribution to the Mexico City population is indicated by decreased $Gm^{1,17;21}$ and $Gm^{1,2,17;21}$, both of which are high frequency Native haplotypes. Due to its greater ethnic heterogeneity, the Mexico City population is probably not an appropriate control group for the Mayan RA patient group. In other words, the Gm haplotype data indicate that the Mexico City population is ethnicly different from the Mayan control and patient groups.

There were 3 individuals from Cancun with the phenotype Gm(1,17;15,21) which could be explained by the presence of the haplotypes $Gm^{1,17;21}$ and $Gm^{1,17;15,16(-)}$. Similarly, 2 RA patients with phenotype Gm(1,2,17;15,21) could be explained by the presence of the haplotype $Gm^{1,2,17;21}$ and $Gm^{1,17;15,16(-)}$. This is the first report of the $Gm^{1,17;15,16(-)}$ phenotype in any population. It is not possible to resolve the question of whether this is a new haplotype because family data would be needed to confirm the exact nature of the unusual haplotype.

Sanders et al (1985) found the phenotype Gm(1,2,3,17;5,21,23) and the G1m(2) allotype increased in DR4(+) patients compared with DR4(-) patients and controls, as well as four patients with pulmonary fibrosis who were Km(1) positive suggesting a role for the κ chain genes in the pathogenesis of RA. Zarnowski et al (1986) also found an increased frequency of Gm(1,2;21) phenotype in DR4 (+) RA patients. In this study, the frequency of the phenotypes Gm(1,2,17;21) and Gm(1,2,3,17;5,14,21) were not increased in the RA patient group, (0.25 and 0.03, respectively) compared with the combined control group (0.20 and 0.06 respectively) (see Table 10). In addition, the frequency of $Gm^{1,2,17;21}$ in the RA patients (0.16) was not significantly different from the combined control group (0.15)(see Table 12). Finally, there was no evidence for interaction between DR4 and G1m(2) in the RA patients (see Table 22). The distribution of Km(1) and (3) alleles showed no difference between RA patients and control group (see Table 14). Similarly, there was no evidence for interaction between DR4 and Km(1) in RA patients (see Table 23).

D 2. HLA-DRB RFLP analysis.

One hundred and sixty-six DNA samples were DR typed using a cDNA

probe for the β-chain of HLA-DR. Most of the known DR antigens were easily determined by hybridization to Taq I digested and Southern transferred genomic DNA. All the DR designations using this method were confirmed by other methods used later in this study. The frequencies of the DR types were different from those observed in Caucasian populations. The preliminary results observed by this method indicated that these Mayan populations (both RA patients and controls) have a very high frequency of DR4. Subtyping DR4 allowed a determination of the DR4 allele frequency in these Mayan populations and in patients.

D 3. HLA-DRB typing by PCR-SSOP analysis.

The application of SSO probes for typing the highly polymorphic DRB alleles was used in this study. The primers amplify all the alleles tested causing no problems with allelic identification. It is a simple and effective way of typing DRB at the sequence level. The typing strategy used the generic DRB primers (see Table 8) to initially amplify the sample, followed by the use of specific DRB oligotyping probes which hybridize to unique DRB sequences and thereby identify the majority of the serological types (see Table 6). For samples that were positive for a particular DR type, additional amplification with the DRB1-specific pair of PCR primers (see Table 8) and probes (see Table 6) was sometimes required in order to split the alleles. By combining the results of the RFLP analysis and the probing from the two amplifications, it was possible to distinguish all the DRB1 alleles in this Mayan population. The DR4-Dw subtypes were not distinguished by these probes, and an additional subtyping method was required for the final analysis (PCR-RFLP).

The results obtained for DR typing by the RFLP method were confirmed by PCR-SSOP analysis. Table 15 shows the very high frequency of DR4 positive genotypes in the Mayan population generally. In the combined Mayan control group, the gene frequency of DR4 is 52% (see Table 16), while in most Caucasian populations it is in the range of 10-15% (Terasaki 1980).

Puttick et al (1990a) found very high frequencies of DR4 in Caucasian RA patient populations. When the Mayan RA patients were compared to the Mayan control group, the difference in the individual genotype frequency of DR4 was highly

significant (p= 0.00071) indicating that DR4 is indeed associated with RA in this population (see Table 15). The DR4 gene frequencies were also found to be significantly different (p= 0.00008) (see Table 16). However, the DR4 genotype frequencies in patients (see Table 15) are similar to those expected at Hardy-Weinberg equilibrium given the DR4 gene frequencies (see Table 16), so there does not appear to be any particular DR4 genotype (homozygous vs heterozygous) which is especially high risk for RA.

D 4. HLA-DR4 subtyping by PCR-RFLP analysis.

The PCR-RFLP method has been previously reported as an efficient and convenient typing technique for accurate subtyping of the HLA-DRB alleles. In this case, the method was used to subtype the Dw specificities of DR4 alleles. In order to perform allele-specific amplification a primer homologous to codons 6-13 of the first hypervariable region of DRB1 of DR4 (see table 8) was used. The pattern generated after restriction digestion was distinctive for each allele. The subtypes could be distinguished by restriction endonucleases Mnl 1, Hinf 1, Hae II, Hph 1 and BstU 1. The same patterns were observed by Ju et al (1991).

The PCR-RFLP study confirms the results obtained by RFLP analysis and PCR-SSOP. Of the 130 random Mayan controls, 96 were DR4 positive; of the 36 RA patients, 33 were DR4 positive. All but four DR4 positive patients carried either DRB1*0404/0408 (Dw14) or 0403/0407 (Dw13) alleles (see Table 17). No patients were found to have DRB1*0401, 0402, 0405 or 0406 alleles.

When analyzing individuals who were positive for DR4, genotype frequencies for Dw13 were not significantly different between the combined control group and the RA patients, p= 0.46 (see Table 18). Similarly, genotype frequencies for Dw14 subtypes of DR4 were not significantly different between patients and combined controls, p= 0.07 (see Table 19). The data was also analyzed by considering the Dw subtype frequencies of DR4 alleles in patients and controls, but again no significant differences were found, p= 0.30 (see Table 20). The frequency of Dw14 among DR4 alleles was not significantly different between patients and controls, p= 0.14 (see Table 21), although the low p value suggests that with larger sample

sizes, weakly significant differences might be found.

Stroncek et al (1990) studied 261 healthy Native American Indians from Minnesota (Chippewa, Sioux, and other) and found a DR4 genotype frequency of 51%. In 113 healthy Pima and Papago Indian controls from Arizona, Ensroth et al (1983) found a DR4 genotype frequency of 16%. In 45 healthy American Indians (origin not stated), Fernandez-Vina et al (1991) found 43% were positive for DR4, with DRB1*0407 (Dw13) the predominant subtype (33% of DR4 alleles) . Sanchez et al (1990) studied 200 healthy Spanish controls and found a DR4 genotype frequency of 26%. In the present study, the Mayan controls have a DR4 genotype frequency of 74%, with Dw13 the predominant subtype (57% of DR4 alleles) (see Tables 15 and 20). All the above differences in the frequency of DR4 alleles reflect ethnic variations in the distribution of the DR4-Dw subtypes.

In many different populations, DR4 is at raised frequency in RA patients. Among the DR4 alleles, Dw4 is the most prevalent in healthy Caucasian populations, whereas Dw14 is less common. The HLA-Dw4 gene is also highly associated with both adult and seropositive juvenile RA (Nepom et al 1986b). Nepom et al (1989b) suggested that Dw4 and Dw14 alleles are independent susceptibility genes, and are more specific markers for genetic susceptibility than is HLA-DR4. DR4 heterozygotes carrying DR4-Dw4 or DR4-Dw14 with a non-DR4 allele are at increased risk. Wordsworth et al (1992) suggest that the genotype Dw4/Dw14 may be particularly associated with more severe RA and in those with Felty syndrome. The Dw14 allele sequence is shared by a majority of the non-DR4 DRB alleles implicated in RA, which are predominantly DR1. Nelson et al (1991) found that among Caucasian RA patients heterozygous for DR4 (DR4/X), the frequencies of Dw4 and Dw14 were 69% and 15%, compared with 48% and 28% in DR4/X controls. They therefore concluded that Dw4, but not Dw14, was an independent risk factor for RA.

Only a few studies of DR4 distribution in RA patients and healthy controls in Mexican populations exist in the literature and all of them refer to Mexicans from U.S.A or Mexicans from Mexico City (Ueno et al 1981; Tiwari and Terasaki 1985). These populations are not the best to use for comparative purposes with Mayan RA patients.

This study demonstrates an increased frequency of DR4 in the Mayan

control group compared to other North American Native Indians, and an increased frequency of DR4 in the RA patient group compared to controls (see Table 16). Since the gene frequency of Dw14 in the DR4 alleles of the RA patient group was not significantly increased as compared with the combined control group (see Table 21), Dw14 cannot be considered an independent risk factor for RA. However, it is possible that with larger sample sizes, a weak predisposing effect of Dw14 might be detectable.

When comparing the Mayan RA patients with other RA patients of Native American ethnic origin, the Dw13 and Dw14 alleles have a higher frequency; i.e., the Chippewa RA patients from Minnesota are rich in DR4, but the Chippewa have DR4 variants such as DR4.1^{Chip}, DR4.2^{Chip}, DR4.3^{Chip} which are not found in other ethnic groups. The Tlingit and Yakima Indians RA patients have a decreased DR4 frequency, and the DR4 alleles observed in these populations were Dw13 in the Tlingit Indians, and Dw4 and Dw14 in the Yakima Indians.

D 5. HLA-Gm interaction.

There was no evidence for interaction between G1m(2) and DR4 (see Table 22) so the current study results do not support those of Sanders et al (1985) and Zarnowski et al (1986). In the DR4(-) RA patients, there was a higher frequency of Km(1) than in DR4(+) RA patients (see Table 23), however the difference was not significant, p= 0.22. These results are consistent with those of Sanders et al (1985) and Moxley (1989a,b) who reported an increase of the Km(1) haplotype in DR4(-) patients but are not statistically significant.

D 6. HLA-DQB1 typing by PCR-SSOP analysis.

Due to the conflicting results from conventional and reverse dot-blot analyses (mentioned in section C 3.2) and in order to complete the DQB1 typing it will be necessary to use other methods in addition to PCR-SSOP to ensure the accuracy of the results. The alternate methods are PCR-RFLP of DQB1 (Salazar et al 1992; Nomura et al 1991), reverse dot-blot (Rudert and Trucco 1992) and/or direct sequence analysis (Santamaria et al 1991).

By differentiating 14 out of 15 HLA-DQB alleles, the PCR-RFLP method has proved to be a viable alternative in other investigations (Salazar et al 1992). This method permits the characterization of the *0302 and *0303 alleles, which are difficult to distinguish with the PCR-SSOP method (Salazar et al 1992; Nomura et al 1991).

The reverse dot-blot method uses a more reliable and effective approach to bind the probes to the membrane. Long polymers of the specific nucleotide sequence probe are attached to the membrane at a high concentration, enabling the use of shorter hybridization times and of non-radioactive (biotin) labelled primers. Although cross-reactivity is still apparent between the allelic *0302 and *0303 sequences, the intensities of the cross reactions and the positive control signals can be distinguished (Rudert and Trucco 1992).

A precise alternative to current methods of HLA-DQB1 allele typing is direct sequencing (Santamaria et al 1991). This method would readily permit identification of known HLA-DQB1 alleles as well as discovery of any new Mayan allelic sequences.

E. CONCLUSIONS.

1. This research is based on comparative studies of the HLA-DR and Gm/Km genetic polymorphisms within the so-called Maya linguistic family in groups of RA patients and controls. The data obtained in this study emphasizes the importance of historical events which explain some of the observed Gm haplotypes in the populations studied.

2. We were unable to show any independent effect of immunoglobulin heavy chain genes or κ light chain genes on susceptibility to RA, as the Gm and Km allotype frequencies were similar in control and RA populations. The occurrence of G1m(2) + or - is independent of DR4, and therefore, G1m(2) does not predispose to RA through interaction with HLA.

3. The dual goals of DR typing at the DNA level and increasing the resolution of DR typing by identifying Dw subtypes have been met. On a practical level this means that it is possible to perform HLA-DR typing from DNA, even if cells or sera for conventional typing are not available.

The conflicting PCR-SSOP and reverse dot-blot DQB1 typing results can be explained by inappropriate hybridization of SSOP to sequences which differed by one base pair.

4. The present study clearly demonstrates that the gene frequency of DR4 is increased among Mayan RA patients compared with controls ($p= 0.00008$). Only a few of the 12 different DR4 alleles are found in the Mayan population and none of these is significantly increased in RA. DR4 itself is the most potent determinant of susceptibility to RA in Mayans. There do not appear to be any Dw subtypes of DR4 that are more predisposing to RA than any other Dw subtypes of DR4. If independent effects of Dw subtypes of DR4 do exist in the Mayan population, these effects must be much weaker than the DR4 effect.

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"Appendix 1 Consent Form"

ESTUDIO PARA ARTRITIS REUMATOIDE
INFORME PARA CONSENTIMIENTO DE INVESTIGACION

NOMBRE_____

Estoy de acuerdo en participar en el proyecto de investigacion para la deteccion de factores geneticos asociados a la Artritis Reumatoide.

La cantidad de sangre tomada sera de 10 ml, los resultados que se obtengan de este estudio seran para uso exclusivo de investigacion, por lo que la publicacion que esto derive no mencionara mi nombre, a menos que mi permiso sea dado.

Esta donacion no me dara ningun problema de tipo legal en ningun momento. Las preguntas que tenga al respecto seran contestadas en cualquier momento por la Dra. Leigh Field al telefono (403) 220-3051, Medical Genetics Clinic, Alberta Children's Hospital, 1820 Richmond Road S.W., Calgary, Alberta, T2T 5C7 Canada.

Firma_____

Fecha_____

Si la persona es un menor (menor de 18 años) o no puede firmar, complete lo siguiente. Edad de el menor_____

Firma del padre o tutor_____

Firma_____