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A MOLECULAR INVESTIGATION OF THE MAJOR HISTOCOMPATIBILITY COMPLEX OF SEVERAL DOMESTIC ANIMAL SPECIES

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John Adam Gerlach

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A MOLECULAR INVESTIGATION OF THE MAJOR HISTOCOMPATIBILITY COMPLEX OF SEVERAL DOMESTIC ANIMAL SPECIES

By

John Adam Gerlach

A DISSERTATION

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ABSTRACT

A MOLECULAR INVESTIGATION OF THE MAJOR HISTOCOMPATIBILITY COMPLEX OF SEVERAL DOMESTIC ANIMAL SPECIES

By

John Adam Gerlach

The major histocompatibility complex (MHC) of the dog (DLA) and sheep (OLA) were investigated using serologic, cellular and molecular techniques. DLA specific alloantisera were used to serotype animals according to International Workshop guidelines. Mixed lymphocyte reactions (MLR) were performed with both species. Human and murine derived complementary deoxyribonucleic acid probes were used to generate restriction fragment length polymorphism (RFLP) patterns detecting class II and class I regions of the MHC respectively. There were a total of twenty dogs evaluated in this study. These dogs were divided into four groups and included two full-sibling families that included the sire and dam. One of the groups was composed of three littermates. A group of juvenile onset insulin-dependant diabetic dogs and controls were evaluated by the above techniques and also by epitope specific oligonucleotide probing of amplified regions of the DQB gene. These DQB regions were defined using primers derived from human sequence data and amplified using the polymerase chain reaction. These amplified regions were also digested with several restriction endonucleases and examined by polyacrylamide gel electrophoresis to identify banding patterns unique to diabetic animals. A group of seven sheep that included three generations was evaluated. The results of the serotyping, MLR assays and RFLP mapping were used to establish the existence of analogs to human and murine class I and class II genes

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present in the dog and sheep. There was also molecular evidence in dogs that the previously defined second class I locus, DLA-B may in fact be a class II locus. Furthermore, evidence was collected that indicates that these methods are useful to determine transplantation candidacy, substantiate pedigrees and perform disease association work in the dog and sheep. The disease association work with the diabetic dogs resulted in discovery of a banding pattern unique to the diabetic animals when the 240 base pair amplified DQB fragment was digested with the restriction endonuclease Alu I. Preliminary work using minisatellite fingerprinting for pedigree substantiation in the dog and its possible uses are presented.

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INTRODUCTION

Within the genome of vertebrates there resides a syntenic cluster of genes called the major histocompatibility complex (MHC) (1). It is the genes of the MHC that control the immune response by their involvement in the self/non-self recognition of antigen presentation (1,2). These functions ultimately play a role in disease susceptibility and resistance (3). This dissertation will compare and contrast the MHC systems of man and mouse to those of selected domestic animal species. Results from serologic, cellular and molecular investigations of the MHC of dogs and sheep will be given to document the homology of these systems with those of other species. The utility of these methods for transplantation immunology, pedigree substantiation and disease association work will be discussed.

It is not the intent of this dissertation to be an in-depth review of the MHC nor a primer for molecular biologic techniques. Readers needing more information on the MHC are directed to references (3,4,5,6,7). References (8,9) offer detailed information on molecular biology techniques.

The genes of the MHC system are divided into three classes; I, II and III (3). The class III genes encode various complement components and only their physical linkage to the other MHC genes will be discussed. Included in this syntenic group are also the two genes encoding the 21-hydroxylase enzymes that are involved in steroid

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hormone anabolism (10). The genes encoding tumor necrosis factor are also located in this linkage group (3,11). As with the class III genes, only the linkage relationship of 21-Hydroxylase and tumor necrosis genes to MHC genes will be the limit of their discussion. The discussion of the structure, proposed function and gene organization has been based on the human and murine MHC systems unless specifically stated otherwise. It was the assumption of this author and the premise of the work that the basic tenets of structure and function are homogeneous between species.

Class I MHC genes encode a protein with a molecular weight (MW) of 45 kilodaltons (kd) (12). This protein is an alpha heavy chain and has an associated with a light chain, beta 2 microglobulin (B_2M), MW 12.5kd (12,13). The gene encoding the B_2M protein is not syntenic with the other MHC genes and resides on a separate chromosome (12). The heavy chain is divided into five regions; the cytoplasmic, transmembrane, and three extracellular domains (5,12). The carboxyl end of the protein is cytoplasmic while the N terminus is extracellular. The extracellular domains are a folding of the protein back upon itself forming a loop that is held in place by an intrachain disulfide bond (5). It is the high degree of homology of these domains to those of the immunoglobulin proteins that has supported their inclusion in the immunoglobulin super gene family (14,15,16). B_2M is also domain like in structure but is non-covalently associated with the heavy chain and cell membrane (5,12). Class I proteins are present on the surface of virtually all nucleated cells with the exception of some endocrine and neural tissue cells (12). The homogeneity of heavy chains vary within and between species (5,17). The extent of homology also varies with the region

compared (5). The cytoplasmic and third extracellular domain regions are extremely non-variant (18,19). The degree of homogeneity of the rest of the chain varies in relationship to the domain's proximity to the membrane. The first or most distal extracellular domain is the most variant and thus the most antigenic (5). Exploitation of this variation permits serologic testing of cells for antigen assignment within a species (5).

Class I molecules are noted for their involvement in antigen presentation to T lymphocytes bearing CD8 markers (cytotoxic) (12). It is this requirement of a class I molecule in antigen presentation that renders this interaction to be MHC restricted and ultimately results in the self/non-self recognition seen in immune responses. The classic example of this restriction has been seen in experiments where virally infected cells of two distinct MHC lineages are mixed with T cells that are cytolytic for the virus used and share MHC epitopes with one of the cell lines but not the other (20). The outcome of those experiment was the lysis of the virally infected cells having MHC antigens in common with the T cells. The non-sharing of MHC antigens prevented the lysis of the cells from the other virally infected MHC disparate cell line. Class I MHC genes have been associated with acute, infectious diseases as opposed to those that are chronic and auto-immune in origin (21).

The three dimensional structure of a class I molecule, elucidated by crystallography, is consonant with the molecules' function (5). The two distal domains are alpha helices that lie parallel to each other forming a cleft. These helices are supported by resting on the third extracellur domain. The T cell receptor is thought to interact with

the upper surfaces of the alpha helices with the antigen being cradled in the cleft (5).

In the serum of several species (man. mouse, horse, sheep, cow and dog) there is soluble class I antigens (22). There are three groups of soluble molecules with differing MW's; 44kd, 40kd and 35-37kd (24,25,26,27). The 44kd molecule is thought to be intact shed expressed antigen. The 35-37kd molecule is thought to be proteolytic cleavage fragments of cellular antigens that lack cytoplasmic and transmembrane regions. The 40kd molecule is thought to be the product of an alternate splicing event as it lacks the transmembrane region. The functional role that circulating class I molecules may play is currently not known.

Class II MHC genes are divided into three groups; alpha (A), beta (B) and gamma (6,28). Class II A genes encode a 32kd protein while the class II B genes encode a 28kd protein (12). Gamma genes encode a cytoplasmic protein with a MW of 31kd (28). This protein is involved with both A and B proteins during maturation and their transport to the membrane. The gamma gene is not a part of the MHC syntenic group for it resides on a separate chromosome (29). Both A and B class II proteins are arranged with cytoplasmic, transmembrane and extracellular domain regions similar to those of the class I proteins (6,7). Class II proteins are oriented in/on the cell the same as class I (6,7). The two main differences between the proteins of the two classes are that class II proteins non-covalently associate with each other rather than B_2M and they only have two extracellular domains (6). These domains are also immunoglobulin like and the genes are considered to also be a part of the immunoglobulin super gene family (30). As with the class I

proteins, it is the most distal extracellular domain of the protein that is most variant and antigenic (7). This fact has also been exploited to enable the serologic typing of cells for some class II antigens within a species (31).

Expression of the class II MHC antigens are mainly restricted to certain cells of the immune system; antigen presenting cells and B lymphocytes (6,7). While not expressed on the surface of resting T lymphocytes class II antigens, expression can be induced *in-vitro* using various plant lectins and *in-vivo* via stimulation by other cells (6,7,12). The induction of class II antigen expression on resting T cells and others can also be attained by cultivation of the cells with interferon and other interleukins and leukokines. The class II antigens present on monocytes and macrophages, the major antigen presenting cells, are responsible for the presenting of antigen to T lymphocytes bearing CD4 markers (helper) (6,12). Again, this MHC restriction drives the self/non-self control of the immune system. Disease associations with these antigens are generally of an autoimmune nature (21).

Three other features of the MHC system bear mentioning at this point. The first is the mode of inheritance. Due to extremely low genetic recombination, associated with the MHC chromosomal region. The genes of the MHC are inherited as haplotypes, ie, the cluster of MHC genes present on one chromosome are passed to the next generation as a unit (32). This phenomenon has lead to the second unique feature of the MHC, linkage disequilibrium (32). Linkage disequilibrium is the phenomenon of two antigens of different loci occurring together at a frequency higher than would be predicted by their separate frequencies

(32). It has been the exploitation of this haplotypic passage of the MHC complex to offspring that has resulted in the use of the MHC for pedigree substantiation (33). The third unique feature of the MHC is the extreme polymorphism of the extracellular domain of the molecules (34,35,36). Most allelic protein systems are the result of single point mutations (35). The polymorphism of the MHC allelic system are the result of multiple mutations throughout the protein (34,35). Arguments have proposed that the high degree of polymorphism has been the result of the accumulation of neutral mutations while others have proposed that the high degree of polymorphism aids in the function of the MHC (34,35,36).

Currently the MHC system of man, the human leukocyte antigen (HLA) system, contains three closely linked groups of genes residing on the short arm of chromosome 6 at 6p21.3 (7). There are about 18 class I gene loci present in the human HLA (7). Most of these do not produce a detectable product. Currently there are four recognized loci for genes with serologically detectable products (HLA-A, -B, -C and -E) (7). The class II gene loci are subdivided into four subregions with each region having at least one A/B pair of genes (6,7). These subregions are HLA-DP,- DN/-DO, -DQ and -DR. The third block of genes is composed of the class III complement genes C2, factor B and C4 and the 21-hydroxylase (21OH) genes (7). Tumor necrosis factor (TNF) genes are located between class III and I gene clusters (37,38). The overall organizational map of the MHC region on the chromosome is:

Centromere--Class II--21OH--C4--Bf--C2--TNF--Class I--

(38). The overall size of the HLA complex is thought to be 3000 kilobase pairs (kbp) (7). There are still some gaps in the above map

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and while it is possible that there maybe some new class I and II genes discovered another attractive possibility would be the discovery of novel genes that truly account for the association of MHC region with disease (7).

All three classes of MHC genes have undergone expansion and contraction throughout evolution (7). Some of the duplicated genes have lost their function and are psuedogenes (7). Class II genes DPA2 and DPB2 of the DP locus have a defective sequence that prohibit their expression as normal class II dimers and have been labelled as psuedogenes (7). There are other genes with intact sequences but which have an undefined status and are termed pseudogenes (7). DNA and DOB are examples of pseudogenes that contain no defects but their corresponding protein products have not been identified (7). The currently known human class II pseudogenes are HLA-DPA2, -DPB2, -DNA, -DOB, -DXA, -DXB, and -DRB (7).

Class II alpha genes are generally non-polymorphic with the exception of DQA which is highly polymorphic having eight alleles (7). The beta genes of all of the class II subregions are extremely polymorphic and readily cross-hybridize with other of class II beta genes (39). This is due to a homologous sequence encoded for in the first domain of beta genes (39).

There are 15 alleles of DRB, 10 DQB alleles and at least 6 DPB alleles (7). Sequence analysis of the highly polymorphic distal extracellular domain of the class II beta chain has identified a number of hypervariable regions inserted between highly conserved regions (7). These variable blocks are at the amino acid residues 9-13, 26-33 and 67-74 of the molecule (7). Amino acid substitution at one or a

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combination of the three region results in novel combinations (7). The changes seen are often substitution of amino acids with opposite properties (7).

The nomenclature committee of the Tenth International HLA Histocompatibility Testing Workshop has accepted the following number of alleles for the HLA class I and class II loci: HLA-A(24), -B(52) - C(11), -DR(24), -DQ(9) and - DP(6) (40). Genes representing all classes of these HLA products have been cloned and sequenced.

The H-2 system is the MHC of the mouse. This system is very similar to the HLA system of man with some major, unique features (41). One of these differences is the order of the class I and II loci. In the mouse the class II genes bracket the class I genes rather than a sequential order on the chromosome. It has been postulated that this was the result of an ancient chromosomal translocation within the mouse (41). The H-2 syntenic group of the mouse is on chromosome 17 (41). Another unique H-2 feature is that the mouse does not have a HLA-DP homolog (41). Murine class II genes, I-E and I-A, appear to be homologs of HLA-DR and -DQ respectively. The class I genes of H-2 system are also more abundant with estimates that there are up to 30 class I gene loci (42,43). The serologically defined class I loci are H-2-K, -D and -L (41). The remaining class I genes are assigned to the largely not expressed Qa/Tla region (41). It has been estimated that there may be up to one hundred alleles for some class I loci in certain murine strains (41). Despite these obvious differences there is a great deal of homology between the H-2 and HLA systems in both organization and function (44). Much of the early work that elucidated

the MHC system was done in the mouse and then found to be applicable to man and the converse has also been true (41,42,42,44).

The MHC systems of mouse and man are the most extensively studied of all species due to: 1) the availability of inbred strains of mice; and 2) the monetary and humanitarian application in man.

The cattle MHC system has recently been detailed and found to have features similar to the H-2 and HLA systems (45). The bovine leukocyte antigen (BoLA) system is the cattle equivalent of the MHC (45). The BoLA system has numerous class I genes that can be detected serologically and visualized by restriction fragment length polymorphism (RFLP) mapping (45,46). The RFLP patterns generated are similar to other species studied (46). These class I genes are in close linkage with the cattle red cell blood group M, class II genes and the class III gene C4 (47,48,49). This syntenic group has been localized to bands q13-23 on cattle chromosome 23 (50). The class II genes in cattle have homologs to the human HLA-DRA, -DRB, -DQA, -DQB, -DNA and -DOB genes (51,52,53,54). There is currently no firm evidence for a DP subregion of cattle class II (51). There are two unique class II genes in cattle, defined as BoLA-DYA and -DYB (55). It is not currently known if BoLA-DYA and -DYB associate together or not (55). The BoLA DR and DQ RFLP patterns do correlate with the mixed lymphocyte reaction (MLR) results (55).

Serologically, there has been three International Workshops to identify and define alloantisera for use in class I cellular typing (56,57,58). International agreement has been reached for 33 unique class I specificities, the resultant products of one and tentatively two class I loci (58). BoLA class II serology has not developed

sufficiently to serve as a useful tool (59). Cloning attempts in cattle has Identified a BoLA-DRB-like pseudogene and two Class I genes (60,61). The cloned class I genes show greater sequence similarities to HLA molecules than to H-2 molecules (60,61).

The horse equivalent of the MHC, the equine leukocyte antigen (ELA) system, has been mapped to equine chromosome 20 bands q14-22 (62). Included in the ELA sytenic group are the two class I loci and genes for 21OH, C4 and the equine red cell blood group A (63,64). Hybridization studies using probes of human and mouse origin has shown that there is a class II region in the horse (64,65). There is preliminary evidence that the horse potentially has 20 class I gene loci (65). The Fourth International ELA Workshop established 21 class I ELA specificities (66). There have been no reports of cloned ELA genes.

Investigations of the feline leukocyte antigen (FLA) have shown that the cat has both class I and a class II MHC equivalents (67). These genes have been shown to be linked and have been localized to feline chromosome B2 (67). Attempts at cloning FLA genes has yielded a class II alpha gene thought to be the equivalent of a HLA-DPA pseudogene ((68). Due to the apparent inability of cats to produce FLA antibodies as a result of pregnancy and the weak cytotoxicity of the deliberately produced antisera, serologic evaluation of the FLA is lacking (67).

The MHC system of sheep is the ovine leukocyte antigen (OLA) system (69). Using molecular techniques with human probes it has been shown that sheep have class I and class II homologs of the human MHC genes (69,70). There are representative genes for HLA-DR and -DQ but

an apparent lack of HLA-DP genes (69,70). These results are contrary to work of others using murine monoclonal antibodies to HLA-DR, DQ and DP molecules. These studies determined that a DP-like epitopes were expressed on the surface of sheep B lymphocytes (71,72). There are five European defined class I OLA specificities (73). Four of these specificities agree with four Australian defined specificities (74). OLA gene cloning attempts have resulted in the production of nine class II OLA clones (70). These clones appeared to be two DRB-like, three DQA-like and four DQB-like genes (70).

The dog leukocyte antigen (DLA) system has been the focus of three International Workshops (75,76). These efforts established three serologically defined class I loci; DLA-A, DLA-B and DLA-C (76); and a class II homolog, DLA-D, defined by homozygous typing cells using the MLR (75). The class I loci nomenclature may not be analogous with HLA nomenclature. There were eight A locus, four B locus and 3 C locus specificities recognized in this workshop (76). Recently there has been mounting evidence that the DLA-B locus may be a class II antigen expressed on resting, circulating T lymphocytes (77,78,79). Evidence for this statement comes from flow cytometry assays using monoclonal antibodies, immunoprecipatation studies, blocking experiments and competition assays (77,78,79). These results indicate that DLA-B is a class II loci (77,78,79). Molecular studies have established the existence of homologs for HLA-DR, DQ and DP (80,81). The RFLP patterns generated with specific restriction enzymes correlate with the DLA-Dw homozygous cell types established by the Third International Workshop (80,81). Linkage of the DLA system genes with C4 genes has been

established by somatic cell hybrids (82). There have been no reports of cloned DLA genes.

The swine leukocyte antigen (SLA) has been mapped to swine chromosome 7 of the pig and localized to bands p1.4-q1.2 (83). It is interesting to note that there are also two red cell blood group genes (J and C) present on chromosome seven (83). Several other species; such as man, cattle, horse and rabbit; also have a red cell blood group within their MHC syntenic group (83). The swine MHC linkage includes class I, class II, C2, Bf and C4 class III genes (83,84). Class II genes have been cloned from the novel miniature swine genome (85). The class I and Class II regions of the swine MHC have been studied by serologic, cellular and molecular genetic techniques (86,87,88).

The MHC system of the goat is the caprine leukocyte antigen system (CLA) (89). The extent of definition is only serologic and cellular with no reported molecular work to date (89,90). There are 24 class I specificities that are assumed to be the product of one loci (89). Responses seen in mixed lymphocyte reaction assays are attributed to the presence of a yet to be defined class II homolog (89).

The rabbit leukocyte antigen (RLA) system includes class I, class II genes and the red cell blood group He genes in its MHC linkage group (83). Both class I and class II genes have been cloned and sequenced from the rabbit (91,92).

Anecdotally, the MHC systems of rare and exotic breeds and species have been examined to differing extents and by differing techniques (93,94,95). An example of this is a herd of Icelandic ponies (93). These investigations have documented the existence of MHC systems in

the rare and exotic breeds and species that are analogous with the more domesticated species and man.

The MHC system has often been associated with acute and chronic disease conditions (96). These associations are often only seen in conjunction with only certain products of the MHC. There are numerous possible mechanisms for this association (12,20,21,96). It has been proposed that pathogens may bind to certain MHC epitopes and not others. Another mechanism may be the alteration of MHC epitopes by pathogens. As stated earlier, there may also be non-MHC disease associated genes present or associated with the MHC group and are there by inherited with the MHC region due to linkage disequilibrium.

There are also diseases directly linked with the MHC. The difference between linkage and association is the identification of a particular gene and the identification of that gene's involvement in the disease versus a higher incidence of a disease when a particular antigen is present (96,97). Examples of MHC linked diseases would be 21 hydroxylase deficiency and hemochromatosis in man (96).

There are numerous examples of diseases associated but not linked with the MHC of most of the species studied. In man, ankylosing spondylitis and the HLA-B27 antigen are an excellent example with a relative risk (RR) value of 100-150 (32).

Type I insulin dependant diabetes mellitus (IDDM) has been associated with class I and class II antigens in humans (98). This association with both classes of antigens indicates a haplotype association with the disease associated alleles being included by linkage disequilibrium with the MHC (99). Initial studies associated IDDM with HLA-B8 and the complement alleles; Bf*F1, C2*2 and C4B*2

(21). The haplotype was later extended to include -DR3 and -DR4 alleles (21). There is also evidence that there are unique RFLP patterns in IDDM individuals that are the result of a cross-hybridization of an HLA-DQA probe with HLA-DXA genes after digestion with the restriction endonuclease Taq I (100). Recent studies have localized the loci associated with Type I IDDM to the 57^{th} codon of the DQB gene (101,102). It has been reported that an aspartate residue at that position confers resistance to the disease while substitution with a neutral amino acid results in a higher risk of disease (RR=100) (101,102). The mechanism for this is not understood. All diseases that have been associated with HLA antigens are too numerous to detail, there are several excellent texts and reviews on the subject (96,103).

There are examples of disease associations and the MHC in many of the domestic animal species. In cattle the progression of bovine leukemia virus can be assigned to particular BoLA haplotypes within a given herd (104,105,106). The number of parasitic eggs (*Cooperia* spp. and *Haemonchus placei)* shed can be associated with certain BoLA antigens (107). The BoLA system also has been associated with mastitis and bovine theileriosis (108,109). Equine sarcoid has been followed in a pedigree via ELA specificities (110). The susceptibility of caprine arthritis-encephalitis virus has been associated with certain CLA epitopes (89). Scrapies susceptibility in sheep has also been tracked in a pedigree by following the passage OLA haplotypes (111).

The above compilation of observations encourages the conclusion that the MHC of one species system is biologically very similar to those of other species systems. This is an incorrect conclusion. While there is mimicry of the molecular organization and the species do

share a common functional goal, the genes and resultant antigen products are not identical. Serologic reagents defining a unique epitope in one species are not likely to recognize the same or similar epitope in another species (59,112). There are some monomorphic monoclonal antibodies that can be used pan-species, to define similar gene products, but these are exceptions (71,72,112). In the third BoLA workshop it was found that antisera that clustered together to define an epitope in one breed of *Bos taurus* cattle did not consistently maintain their serological correlation in a different breed of *Bos taurus* and additionally behaved totally different in *Bos indicus* animals (58).

This inability to use serologic reagents that well defined one species MHC to also define the MHC of other species has been a continuous scientific hardship. The lack of well defined monospecific sera for domestic animals and the associated difficulty in the procurement of good serological reagents for the various species has prompted the search for alternate techniques to characterize the MHC of domestic animal species There is a continuing need to be able to perform disease association work, pedigree analysis and transplant candidacy evaluations in domestic animal species at a level beyond the available class I serology.

The two species evaluated in the context of this dissertation were the dog and sheep. The dog was selected because of its frequent use as an animal model for transplantation research and because the dog has many diseases that are pathophysiologically similar to diseases of humans. The sheep were selected for preliminary studies to establish a method for pedigree substantiation and disease association

investigations. Well defined and characterized human and mouse MHC probes were used to probe genomic blots of dogs and sheep rather than initially isolating dog and sheep specific probes. The RFLP patterns observed in the dog and sheep, using the human and murine probes, were correlated with existing canine or ovine serologic and cellular techniques that define the MHC.

As this project progressed, so did molecular technology. Fortunately, technology was on a different time line and new techniques were able to be introduced as they became available complimenting the technologies already in place. These were the amplification of portions of genes that allowed for epitope specific oligonucleotide probing, a useful tool in disease association work in humans (113). This technique was used to investigate IDDM associations with DLA based on recent human data (101,102). A second useful advance germane to the goals of this project was the advent of DNA fingerprinting using minisatellite probes (114,115,116,117). The application of this technique for canine pedigree substantiation was initiated to fulfill one of the goals of this project.

MATERIALS

Animals:

Dogs were either obtained from the Veterinary Clinical Center (VCC) on the campus of Michigan State University or from samples submitted to the Immunohematology and Serology Laboratory of Michigan State University. Four groups of dogs were examined. Sheep were obtained from the flock maintained by the Department of Animal Sciences at Michigan State University.

Group 1. The first group of dogs was composed of three litter mates. They were selected from a litter initially evaluated for a pancreas transplantation study. It was recognized early on that one of the animals was DLA disparate to the others and that two of them were similar. It was on this basis that they were selected.

Group 2. The encouraging group 1 investigations prompted the selection of family that included the sire and dam for the second canine group. Selection of such a group allowed the recognition and passage of DLA haplotypes within a family. There were six dogs in this family.

Group 3. Group 3 was a family that included both the sire, dam and two offspring. This family was a part of bone marrow transplantation study at the University of California (UC) at Davis. The four animals were sent to evaluate their DLA types to select suitable transplantation pairs.

Group 4. Based on the homology between the HLA and the DLA seen in previous canine experiments and the availability of the diabetic dog colony here on campus group 4 animals were selected to initiate disease association investigations. Group 4 included 7 dogs with juvenile onset IDDM and 7 non-IDDM control animals

Group 5. The seven sheep were selected to create a pedigree of several generations.

Humans:

There were six humans selected to serve as controls in the amplification and oligonucleotide probing experiments. They were selected from the HLA-typed cell panel used by the Immunohematology and Serology Laboratory. These individuals were selected because they had at least one DQw-3 allele. They also represented known IDDM individuals and normal controls.

Minisatellite Pedigree Analysis:

There were a group of 32 randomly selected dogs probed with the M13 minisatellite probe. These dogs were unrelated and used in other research investigations at the VCC. In addition, there were two pedigree analyses performed. One of the families was purposefully submitted for this testing (dogs 29, 30 and 31). The second family (32, 33, 34 and 35) where the group 3 dogs submitted for immunogenetic analyses by UC-Davis.

Class I Serologic Reagents:

DLA class I serological typing reagents were used that recognized the specificities in accordance with the Third International Workshop on Canine Immunogenetics. These reagents were either prepared in-house or were gifts from other laboratories.

OLA class I serological typing sera were obtained as a gift from Dr. P. R. Cullen, formerly at the Laboratoire de Genetique Biochimique, INRA-CNRZ, Jouy-en-Josas, France. Prior to the importation of this sera into the United States it was necessary to irradiate the sera in the frozen state, to assure sterility in accordance with United States Department of Agriculture guidelines.

HLA class I serological typing sera were commercially obtained from the American Red Cross (ARC), Washington, D.C. and One Lambda, Los Angles, California.

Class II Serologic Reagents:

HLA class II serological typing sere were commercially obtained from the ARC, Washington, D.C. and One Lambda, Los Angeles, California.

Microlymphocytotoxicity Trays :

Seventy two well microlymphocytotoxicity trays were purchased from Robbins Scientific, Mountain View, California.

Rabbit Complement:

Rabbit complement obtained from Pel-Freez,Brown Deer, Wisconsin, was screened to identify lots that were cytotoxic negative to canine and ovine isolated peripheral blood lymphocyte.

Mitomycin C:

Mitomycin C, used to generate stimulator cells for the mixed lymphocyte reaction assays, was purchased from Sigma, St. Louis, Missouri.

Tritiated Thymidine:

Tritiated thymidine, used in the mixed lymphocyte reaction assays, was purchased from the Amersham Corporation, Arlington Heights, Illinois.

Phenol:

Crystalized phenol was purchased from Sigma, St. Louis, MIssouri. Prior to use it was melted and extracted with salt buffers.

Proteinase K:

Proteinase K was purchased from Boehringer Mannheim Biochemicals (BMB), Indianapolis, Indiana or Bethesda Research Laboratories (BRL), Bethesda, Maryland.

Ribonuclease A:

Ribonuclease A (RNase), from bovine pancreas, was obtained from Sigma, St. Louis, Missouri. This RNase was boiled to remove any DNase activity and stored for future use.

Restriction Endonucleases:

The restriction endonuclease enzymes used in this project were purchased from commercial vendors, ie., BRL or BMB. The reason for selection of these sources was trial and error.

The restriction endonucleases used were: Bgl II, Eco R I, Hae III, Hind III and Taq I. The enzymes Bgl II, Eco R I and Hind III were initially selected for the investigations of group 1 dogs based on work done in other species. The enzyme Taq I was used for the investigations of group 2, 3, 4 and 5 dogs and sheep based on studies performed in human HLA investigations. The restriction endonucleases; Alu I, Hae III, Hga I, Hha I and Taq I were used to do RFLP investigations on the DQB amplified products. Hae III was used for

all DNA fingerprinting work based on previous work using the M13 probe (117).

Agarose:

GTG grade agarose was purchased from FMC Bioproducts, Rockland, Maine.

Membrane:

Charged nylon membrane was obtained from the Plasco Corporation, Woburn, Massachusetts.

Probes:

The class I murine complementary deoxyribonucleic acid (cDNA) probe used was a gift from the laboratory of L.R. Hood at the California Institute of Technology, Pasadena, California. The probe, pH-2IIa, was from a cDNA library constructed from poly(A)$^+$ ribonucleic acid (RNA)isolated from a murine cell line (42). This probe is complementary to a 3' fragment of the H-2 gene and as such is representative of a conserved region.

The class II probes used were purchased from the Cetus Corporation, Emeryville, California. These probes are a construct involving the full-length antisense strand of human class II genes inserted in a double stranded M13 bacteriophage that has been biotinylated. The advantage of using these constructs was that increasing hybridization times generated stronger signals. This was because the probe would only hybridize to target and itself. The class II probes used were HLA-DRA, -DRB, -DQA, -DQB, -DPA and -DPB.

Nick Translation Kits:

Kits containing the reagents necessary to perform nick translation reactions to incorporate deoxycytidine triphosphate (dCTP) radio-labelled with $_{32}P$ into pH-2IIa probes were obtained from BRL.

Random Prime Labelling Kits:

Kits containing the reagents necessary to perform random primed labelling of pH-2IIa probes with $_{32}P$ radiolabelled dCTP were obtained from BMB.

Oligonucleotide End-Labelling Kits:

Kits containing the reagents necessary to perform the end-labelling of the epitope specific oligonucleotides were obtained from BMB.

Radio-labelled dCTP and dATP:

Radio-labelled ($_{32}P$) alpha dCTP and gamma dATP were obtained from the Anersham Corporation, Arlington Heights, Illinois.

Gene Amplification Kits:

Kits containing Taq I polymerase and the other reagents necessary to amplify portions of genes using the polymerase chain reaction were purchased from the Perkin-Elmer/Cetus Corporation, Norwalk, Connecticut.

Epitope Specific Oligonucleotides:

Epitope specific oligonucleotides were custom made by the Macro-Molecular Structural Facility in the Department of Biochemistry on the campus of Michigan State University.

Formamide:

Formamide from Sigma, St. Louis, Missouri was used in hybridization mixtures. The formamide was mixed with mixed bed resin

beads to remove any formic acid prior to use. The purified formamide was stored frozen.

M13mp8 RF I DNA:

The bacteriophage M13mp8 RF I was purchased from Sigma, St. Louis, Missouri.

Hybridization Bags:

Hybridization bags manufactured to be used with biotinylated probes were obtained from BRL.

Films:

X-O-Mat X-Ray and Technical-Pan films were obtained from Eastman Kodak, Rochester, New York.

Type 667 black and white film was obtained from the Polaroid Corporation, Cambridge, Massachusetts.

Intensifying Screens:

Lightening plus intensifying screens used in autoradiography were obtained from DuPont

Miscellaneous Chemicals:

Unless stated otherwise, all salts, detergents, organic solvents and other chemicals were purchased from Sigma, St. Louis, Missouri or other laboratory supply houses.

METHODS

Reagent Preparation:

Composition and special preparation instructions for reagents used in the methods section can be found in the appendix section.

Venipuncture:

Whole blood was obtained from the animals by standard phlebotomy techniques. Twenty milliliters (mls) of heparinized blood was obtained for serologic assays. Forty mls of heparinized blood was obtained for mixed lymphocyte reaction assays and 20 mls of blood collected in ethylenediamine tetra-acetic acid (EDTA) was obtained for deoxyribonucleic acid (DNA) extraction. When possible assays were run simultaneously to minimize the amount of blood collected.

Class I Serology:

Cell Isolation. Heparinized whole blood diluted 1:3 with phosphate buffered saline (PBS) pH 7.4 was layered over a ficoll/hypaque mixture with a refractive index of 1.3570. The blood/ficoll/hypaque density gradient was spun at 200g for 18 minutes. The isolated lymphocytes were aspirated, transferred to another tube, and washed with PBS and pelleted by centrifugation at 150g for 12 minutes. The supernatant was discarded. If the mononuclear cell pellet was contaminated with red blood cells they were removed by a lysis step. The pellet is gently resuspend in a volume distilled water and allowed to stand for 15 seconds to hypotonically lyse the red blood

cells. The lysis was stopped by adding an equal volume of double strength PBS with EDTA (EPS) to re-establish an isotonic solution. The red blood cell decontaminated lymphocyte isolate was then pelleted at 50g for 10 minutes, the supernatant was discarded and the cells were washed with PBS, and pelleted at 50g for 10 minutes. The supernatant was discarded and the cells resuspended to a count of 2-2.5 x 10^6 per ml of McCoys media supplemented with 5% non-cytotoxic normal sera of the same species.

Two Stage Complement Dependent Lymphocytoxicity Assay. The standardized microlymphocytotoxicity test used was that previously described for HLA typing. Briefly, the first stage of the test consisted of combining 1 microliter (ul) of the peripheral blood lymphocytes isolated as described above with 1 ul of the antisera under 5 ul of non-toxic mineral oil in standard tissue-typing trays. The cells and antisera were incubated at approximately 22°C for 30 minutes. The second stage consisted of the addition of thawed fresh frozen rabbit serum (Dogs 1 ul, Sheep and Humans 5 ul) as a source of complement. The rabbit serum had been previously screened and determined not to be nonspecifically cytotoxic for isolated lymphocytes of the species tested. The incubation for the second stage was 60 minutes at approximately 22°C. At the end of the second incubation, 2-3 ul of 5% eosin was added, which was followed 5 minutes later with 5-8 ul 37% buffered formalin, pH 7.4.

By inverted phase microscopy, the test reactions were read according to the following code: 0-19% cytotoxicity = 1, 20-29% = 2, 30-49% = 4, 50-79% = 6, 80-100% = 8 and not readable = 0. The background cytotoxicity readings, those observed in the negative

control serum, were subtracted from the test values before coding. Positive reactions (6's or 8's) in the majority of the wells that contained antisera recognizing a given antigen would result in the assignment of that antigen to the individual (76).

Dogs in groups 1 and 2 were DLA typed while the dogs of group 3 and 4 were not. The group of sheep were OLA typed using the irradiated sera from Europe. The six humans used as controls in the amplification and oligonucleotide probing experiments were HLA typed for A, B and C loci and class II serologically typed as described below.

Class II Serology:

Cell Isolation. Human peripheral blood lymphocytes were isolated as described earlier. The B-cells were separated from the T-cells by a rosetteing technique. An anti-B-cell monoclonal antibody was coupled to ox cell using chromium chloride. These coupled ox cells were incubated with the isolated peripheral lymphocytes to form rosettes with B-cells. The rosettes are separated from the unrosetted cells using a ficoll-hypaque gradient and the red cells lysed. The B-cells were washed with PBS and used in a two stage lymphocyte microcytotoxicity assay (118). The microcytotoxicity test was basically the same assay as described earlier with the exceptions of incubation times and temperatures.

Mixed Lymphocyte Reaction Assay:

Lymphocytes were isolated using a sterile technique in the same manner as those used for class I serology. Half of these isolated lymphocytes were suspended to a count of 1.0×10^6/ml in RPMI nutrient media supplemented with antibiotics, fungizone and 10% non-cytotoxic sera of the species being. These cells are considered the responder

cells in the MLR. The other half of the isolated cells were treated with mitomycin C to render them incapable of responding. Mitomycin C does not alter surface epitopes but does block blastogenesis, this allows the cells to be used as antigen stimulator cells. These cells were also suspended in nutrient media at a concentration of 1.0 x 10^6/ml. Responder and stimulator cell populations were combined (1.0 X 10^5 each) in all possible reciprocal combinations for the one-way and two-way MLR. By performing one-way and two-way MLR's it was possible to evaluate the response of each animal to each other and their combined response to each other. It is by this dissection of the response that the number of haplotypes shared by each animal could be determined.. The one-way and two-way MLR were incubated at 37°C with 5% CO_2 and high humidity. The cultures were pulsed with tritiated thymidine at 96 hours and harvested at 114-120 hours. Cells were harvested on filter paper disks and added to solulene and scintillation cocktail in scintillation vials and counted by beta-scintillation counting. The counts per minute (cpm) of each responder/stimulation combination were determined for use in calculating stimulation indices. A low stimulation index denotes that the respondor and stimulator cells shared class II MHC epitopes. A high stimulation index denotes that the two animals had disparate class II epitopes. The MLR was performed with groups 1, 2, 3 and 5 animals (119).

DNA Isolation:

Whole blood collected in EDTA was spun at 200g for 20 minutes. The resultant buffy coat was transferred to a clean tube and two red cell washes were performed. Lysis buffer (1 ml of buffer for every 4mls of whole blood) and proteinase K (400 microgram (ug)/ml of lysis

buffer) were added and the tubes incubated overnight at 55C. Rnase was added (100 micrograms (ugs)/ ml of lysis buffer) and incubated at 37C for 60 minutes. An equal volume of a phenol/ chloroform/isoamyl alcohol (50/48/2) was used to extract the protein from the mixture. This extraction was repeated on the recovered aqueous phase. An equal volume of chloroform/isoamyl alcohol (96/4) was used to extract the aqueous phase twice. One tenth volume of 3 molar (M) sodium acetate was added to the final aqueous phase from the above extractions. An equal volume of -20C absolute ethanol was added and mixed by inversion. The DNA was allowed to precipitate and transferred to a snap top tube. The DNA pellet was washed twice with 70% ethanol and allowed to air dry for 30-60 minutes. The DNA was dissolved in 100mM Tris·hydrochloride (HCl) to a concentration of 1-2 ug/ul. Dissolved DNA was evaluated by spectrophotometry to determine the purity and concentration. Samples were evaluated at wavelengths of 280 nanometers (nm), 260nm, 250nm and 230nm. Ratios between the absorbances at the 260/280, 260/250 and 230/260 wavelengths were calculated. The 260/280 ratio is a measure of the amount of protein and phenol contamination of the preparation, an acceptable range is 1.8 - 2.0. The 260/250 ratio is a measure of the carbohydrate contamination of the preparation, an acceptable range is 1.0 - 1.5. The 230/260 ratio is also an assessment of protein contamination, an acceptable range is 0.4 - 0.5. Samples outside of these values were re-extracted with phenol and chloroform or a new sample was collected (8,9).

DNA Digestion:

Twenty ugs of DNA for each animal were digested with selected restriction endonucleases according to the manufacturers temperature

and buffer directives. Four units of enzyme/ ug of DNA was used and the mixtures incubated overnight. Digestions were stopped by heating the reaction mixture to 65C and incubated for 10 minutes. The exception to this routine were samples digested with the Taq I enzyme. This enzyme remains active at 65C. The Taq I digested samples were cooled to end the reaction. An appropriate amount of 5X gel loading buffer was added to each sample. The dogs of group 1 were digested with the restriction endonucleases EcoR I, Bgl II and Hind III. The dogs of groups 2, 3 and 4 were only digested with the restriction endonuclease Taq I. The amplified DQB gene products of the group 4 dogs and the human controls were digested with Alu I, Hae III, Hga I, Hha I and Taq I. The sheep group was only digested with the restriction endonuclease Taq I. The dogs used for minisatellite pedigree analyses were only digested with Hae III.

Electrophoresis:

Agarose was dissolved in Tris·HCl/acetic acid/EDTA (TAE) electrophoresis buffer to achieve a concentration of 0.75%. Molten agarose was poured into a 10 centimeter (cm) X 15 cm frame for class II gels or a 15 cm X 25 cm frame for class I gels. One hundred mls of the mixture was used for class II gels while 250 mls were used for class I. The mixture was allowed to harden and cure for one hour prior to the running of the gels. The digested DNA was loaded into the wells formed by combs and current was applied (8,9).

Standards of known base pair (bp) size were also run with each gel. Lambda DNA digested with Hind III was used for class I runs. Biotinylated lambda DNA digested with BstE II was used for class II runs.

Class I gels were electrophoresed overnight with 25 milliamps (mA) of current applied. Class II gels were electrophoresed for 4 hours at 50 mA. Gels were run in a submerged mode with recirculation of the electrode buffers. Electrophoresis was stopped when the two dye components of the gel loading buffer trisected the gel. Gels were then removed from the electrophoresis chamber and slid off their frames into distilled water with ethidium bromide at a concentration of 0.5 ug/ml. Gels were rotated and allowed to stain in this mixture for 30 minutes and subsequently they were washed twice with distilled water for 15-30 minutes while rotating. Gels were photographed with transillumination by ultraviolet (UV) using a Polaroid MP-4 camera and type 667 film. A wratten filter was used to block stray UV light. A scale was placed next to the gel. The photography documented the migration distance of the standards for generation of a scale of size and migration distance of the standard fragments used to determine the size of unknown fragments (8,9).

Transfer of Electrophoresed DNA to Membranes:

The method used to transfer the electrophoresed DNA from the agarose gel to nylon membrane was that of Southern (8,9,119,120). Briefly, gels were soaked in a solution of 1.5 M sodium chloride (NaCl) and 0.5 M sodium hydroxide (NaOH). for one hour. Gels were then transferred to a solution composed of 1.0 M Tris base and 1.5 M NaCl at pH 8.0. Gels were rinsed briefly in a 10X SSPE and inverted onto a solid support covered with filter paper with tails that served as wicks. This support was over a reservoir of 10X SSPE. A nylon membrane was prewetted with 10X SSPE and placed on top of the gel. Two pre-wetted filter papers were placed on top and covered with several

inches of paper towels. This stack was covered with a support and a 9 centimeter (cm) X 20 cm X 6 cm, 264 gram (gm) weight was set on top. The wicks allowed the passage of 10X SSPE from the reservoir through the gel and into the paper towels by capillary action. The liquid carried the DNA from the gel to the membrane. Transfers were allowed to go 16-20 hours. Post-transfer, the origins of the lanes were marked with pencil on the membrane. The membrane was rinsed with 6X SSPE for 1-2 minutes and air dried for one hour. The DNA was then fixed to the membrane by baking at 80C under vacuum for 2 hours.

Pre-Hybridization:

Membranes were pre-wetted with water and transferred to a hybridization bag. Hybridization buffer was added to bag, it was heat sealed and incubated at 42C with rocking 16-20 hours (8,9,120). Hybridization buffer volumes were predicated by the size of the membranes.

Hybridization:

Either a radio-labelled or biotinylated probe was added to the hybridization buffer and the mixture was returned to 42C and rocked (8,9,120). Class I membranes were hybridized for 48 hrs while class II membranes were allowed to hybridize for 5 days. One million cpm of radio-labelled pH-2IIa probe/ml of hybridization buffer was added to class I membranes and 1 ug of biotinylated HLA-DRA, -DRB, -DQA, -DQB, -DPA or -DPB probe was added to class II membranes. Only group 1 dogs were examined with the class I probe. The canine groups 1, 2 and 3 were evaluated with all six class II probes. Canine group 4 was only hybridized with the HLA-DQA, -DQB and -DRB probes The sheep were hybridized with only the six of the class II probes and not the class I

probe. The group of dogs used for the minisatellite pedigree analysis were hybridized with the M13 bacteriophage minisatellite probe.

Stringency Washes:

All membranes were washed with 2X SSPE/0.5% Tween 20 at room temperature for 15 minutes for a total of 3 washes. Membranes were washed at stringency temperature with 0.2X SSPE/0.5% Tween 20 for a total of three washes also. Class I membranes were washed at 60C. Class II membranes were washed at 55C. Membranes were air dried (8,9,120).

Autoradiography:

Class I membranes were placed in a casette with X-Ray film and an intensifying screen and stored at -70C. Films were exposed for a period of 5-14 days prior to development. Films were developed using GBX-2 developer and fixing reagents according to manufacturers directions (8,9,120).

Non-Radioactive Development:

Class II membranes were rinsed with PBS/5% Triton X-100 for five minutes prior to incubation with strep avidin horse radish peroxidase conjugate diluted in the solution to a concentration of 0.29 ugs/ml. Membranes were allowed to incubate for one hour. Membranes were washed for five minutes in a PBS/5% Triton X-100 buffer that included 1M urea and 1% dextran sulfate for a total of five washes. Membranes were then developed in a 10mM sodium citrate/10mM EDTA solution at pH 5.0 that included 0.4mM tetramethylbenzidine and 0.0014% hydrogen peroxide. Color development was allowed to progress for 60-90 minutes. Membranes were then rinsed with distilled water and photographed for documentation (120). Membranes were photographed using Kodak Technical

Pan film that was developed using Tech Ethol developer and Rapid Fix fixer and printed on medium contrast film.

Radiolabelling of Probes:

Deoxycytidine triphosphate tagged with radioactive phosphorus was incorporated into the pH-2IIa murine derived probe by nick translation or random priming methods (8,9,121,122). Both methods were performed using commercially obtained kits. The amount of radiolabel incorporation was 2.0 X 10^6 degradation particles/minute (dpm) /ug for nick translation reactions. The random primed reaction achieved 3 X 10^7 dpm/ug using kits. One ug of pH-2IIa probe unremoved from the vector plasmid was used for nick translation reactions. Unincorporated radiolabelled dCTP was removed from nick translation reactions by ethanol precipitations. One hundred and twenty five nanograms (ng) of plasmid removed pH-2IIa probe was used for random prime reactions. Unincorporated radiolabelled dCTP was not removed from the random prime reaction mixture prior to hybridization.

Preparation of Murine Probes:

The pH-2IIa probe is a 400 bp fragment inserted in the pBR325 that was removed by double digestion with SalI and Hha I restriction endonucleases. Large quantities of plasmid was prepared by growing the host bacteria (*Escherichia coli* K-803) containing the plasmid and pH-2IIa insert in Luria-Bertani medium at 37C with vigorous shaking. When the optical density (wavelength of 600nm) of the culture reached 0.4-0.5, 85 milligrams of chloramphenicol was added to amplify plasmid growth. Growth was continued overnight. The culture was then centrifuged at 5000g to pellet the bacteria and the supernatant was discarded. The pellet was resuspended in a solution containing 50mM glucose/25mM Tris·HCl (pH 8.0)/10mM EDTA and 2mg/ml lysozyme. This mixture was incubated at room temperature for 10 minutes before the

addition of NaOH and sodium dodecyl sulfate (SDS) to a final concentration of 130mM and 0.6% respectively. This mixture was allowed to stand on ice for 10 minutes. Sodium acetate was added to a final concentration of 1.5 M, and the mixture was allowed to stand for 10 minutes. This mixture was centrifuged at 15,000g at 4C and the supernatant transferred to a clean tube. Fifty ugs of Dnase Free Rnase was added for each ml of supernatant and incubated at 37C for one hour. The supernatant was extracted twice with an equal volume of a phenol/chloroform/isoamyl alcohol (50/48/2) saving the aqueous phase each time. Six tenths volume of isopropanol was added to the aqueous phase and incubated for thirty minutes. This mixture was centrifuged at 10,000g for thirty minutes at room temperature. The supernatant was discarded and the pellet dissolve in distilled water. Polyethylene glycol and NaCl were added to make final concentrations of 6.5% and 1 M respectively. This mixture was incubated on ice for one hour and then centrifuged at 10,000g for ten minutes. The resultant pellet was dissolved in distilled water (123). The concentration and purity of the DNA was determined as described under the isolation of genomic DNA. Four units of Sal I restriction endonuclease was added for each ug of plasmid DNA digested and allowed to digest overnight. Four units of Hha I restriction endonuclease enzyme was added for each ug of DNA in the Sal I digest and allowed to incubate overnight. The reaction was stopped by heating it to 65C for 10 minutes. The digested DNA was loaded on a 3% agarose gel and electrophoresed to allow separation of the pH-2IIa fragment from the digested plasmid DNA. The gel was stained and washed as described earlier and the 422 bp fragment was excised from the gel while being transilluminated by UV light. The

probe was electroeluted from the gel slice if it was to used for nick translation and left in the gel for random priming (8,9).

Calculation of Fragment Length of Unknowns:

The lengths of the fragments of the RFLP's generated from the above techniques were calculated using a least sum of squares computer program (124). The length of unknown fragments was determined by comparing the migration distance of the fragments with those of the standards run in parallel with each gel.

Amplification of Selected Portions of DQB Genes:

Amplification of selected portions of HLA-DQB genes of human and dog IDDM and controls was accomplished using a Perkin-Elmer/Cetus Thermocycler, Perkin-Elmer/Cetus, Norwalk, Connecticut. One ug quantities of selected genomic DNA was mixed with appropriate amounts of all four dNTP's, selected primers, reaction buffer and Taq I polymerase enzyme. This reaction mixture was subjected to 30 cycles of amplification. These cycles consisted of a period at 95C to allow dissociation of the DNA chains, a period at 57C to allow attachment of primers and a subsequent incubation at 72C to allow chain extension (125). The primers used were based on human sequence data (101,102). They were GLPDQB1 {5'GATTTCGTGTACCAGTTTAAGGGC 3'}(recognizes amino acid positions 6-13) and GAMPDQXB2 {5'CCACCTCGTAGTTGTGTCTGCA 3'}(recognizes amino acid positions 79-86) (101). These primers defined a 240 bp section of the HLA-DQB gene. This encompasses the area that encodes the 57^{th} codon region of interest. One tenth of the amplified product was run on an acrylamide gel to determine the size and purity of the product prior to dot blot analysis (9).

Portions of the amplified products of human and canine origin were digested with several restriction endonucleases and electrophoresed in a polyacrylamide gel to evaluate the fragments patterns generated. Restriction enzymes that have a four base pair recognition sequence were chosen. These were; Alu I, Hae II, Hha I and Taq I. The enzyme Hga I was also chosen based on human sequence data (101,102). Examination of the sequence of HLA-DQB amplified by the above process indicates that digestion of this product with Hga I would yield 2 fragments if aspartate is encoded at the 57^{th} codon and 3 if it is not. The purpose of this exercise was to identify unique RFLP's for diabetic humans and/or dogs.

End-Labelling of Oligospecific Probes:

Oligonucleotide probes were purified by passage over a high pressure liquid chromatography [Dupont Oligo-Column] system to isolate the specific oligonucleotides to be used as probes from the failure sequences and concatamers present as a result of synthesis. These probes were then desalted and end-labelled with gamma-$_{32}$P dATP. The probes used were HLA-DQw3.1-57 a 17-mer recognizing aspartate at the 57^{th} codon of the HLA-DQw3 gene and DQw3.2-57 a 17-mer recognizing non-aspartate at the 57^{th} codon of the HLA-DQw3 gene (101,102).

Dot Blotting:

One fourth of the amplified product from the polymerase chain reaction was heated to 95C for 10 minutes and then immediately iced. Once this DNA was chilled it was dot blotted to nylon membrane, washed with 10X SSC and baked. The baked membrane was prehybridized for 18 hours in a solution containing 40% formamide, 6X SSC, 5X Denhardt's reagent, 5 mM EDTA, 0.5% SDS and 1% non-fat dried milk. The end-

labelled probes were added to this mixture and hybridized for 36 hours. The blots were washed at stringencies that required completely identical base pair matching using 6X SSC. The blots were then autoradiographed overnight with 2 intensifying screens (9,101,102).

Minisatellite DNA Fingerprinting:

Blood from the animals used for M13 probing were processed in the fashion as previously stated for MHC analyses. Whole blood was collected and genomic DNA isolated from the leukocytes by extraction of cellular lysates with organic solvents and final precipitation with absolute ethanol. The concentration and purity of the DNA was determined and 15 ug quantities of acceptable samples were digested to completion with 60 units of Hae III restriction endonuclease. The digested DNA was electrophoresed, along with molecular weight markers, in a 1.0% agarose gel overnight at a power of 1 mA/cm of length. The migration distances of the molecular markers were documented by photographing the ethidium bromide stained gel. The gel was denatured, neutralized and the DNA transferred to a nylon membrane by capillary action. The membrane was rinsed, air dried and baked at 80C to assure binding of the DNA. The prehybridization period was 18 hours at 42oC and used the same solution as that used in the dot blots described previously. Forty five ng quantities of the M13 probe were random prime labelled and added to the reaction mixture. The probe was allowed to hybridize for 16-20 hours at 42oC. The membrane is washed twice at room temperature for fifteen minutes in a 2X SSC/0.5%SDS buffer. Four fifteen minutes washes at 65C with 2X SSC/0.5% SDS were followed by two 30 minute washes with 1X SSC at 65C complete the

stringency washes (116,117). The membrane is the dried and autoradiographed for 5-10 days.

M13 Minisatellite Probe:

The bacteriophage M13mp8 RF I was digested with 3 units of Hae III restriction endonuclease (116,117). The digest was electrophoresed in a 1.2% agarose gel. The 309 bp fragment generated by the digest was excised and placed in a pre-weighed tube. Water was added at a concentration of 3 mls/ gram of agarose. The mixture was boiled for 10 minutes, mixed and stored at -20C for future use. The DNA fragment when used was labelled without the removal of the agarose (121,122).

Analysis of DNA Fingerprint RFLP's:

The sizes of the fragments generated by the minisatellite technique were determined using the least sum of squares computer algorithm described earlier. The individual RFLP's were cross-compared to determine the mean number of bands in each major molecular weight region for each animal. The frequency of fragment repetition in each region was also calculated. On the basis of that sharing the paternity index was calculated which is the basis for inclusion or exclusion of a putative sire (33,126,127).

RESULTS

The results of the different immunogenetic assays are organized and will be presented by each group of animals. There are four groups of dogs and one group of sheep. Also presented are preliminary results from initial DNA fingerprinting using minisatellites probing unrelated dogs and various canine families.

Dogs Group 1:

The animals of this group were selected because they were three litter-mates. Knowing that the genes for MHC are passed as haplotypes it was assumed that there would be detectable differences.

Class I Serology. The DLA types of the three group 1 dogs are in listed in Table 1. Animals X, Y and Z had detectable class I DLA-A, -B and -C antigens. The dogs were identical except for the DLA-B loci. Dog Y and Z possessed a B-5 antigen that their littermate dog X lacked.

Mixed Lymphocyte Reaction. The stimulation indices of the one-way and two-way MLR performed between the dogs of group 1 are listed in Table 2. These results make it apparent that there was stimulation of some animals and tolerance by others when the reciprocal mixes were made. When cells of dogs Y and Z are co-cultured in the one-way and two-way MLR they were non-stimulatory meaning that Y and Z were MHC identical. Conversely, co-culturing of dog X cells with cells from dog Y or Z resulted increased stimulation indices confirming that X is not MHC identical to Y and Z.

Table 1. Class I DLA Serotypes of Group 1 Dogs.

Animal	DLA-A	DLA-B	DLA-C
X	(3,9)	(-,-)	(2,3)
Y	(3,9)	(5,-)	(2,3)
Z	(3,9)	(5,-)	(2,3)

Table 2. MLR Stimulation Indices of Group 1 Dogs.

ONE-WAY MLR			TWO-WAY MLR		
Dog (R)	vs. Dog (S)	Stimulation Index	Dog (R)	vs. Dog (R)	Stimulation Index
X	Y	8.5	X	Y	6.3
X	Z	5.0	X	Z	12.2
X	C1*	12.9	X	C1	4.7
X	C2*	16.2	X	C2	12.6
Y	X	2.1			
Y	Z	0.7	Y	Z	0.4
Y	C1	20.8	Y	C1	5.6
Y	C2	15.5	Y	C2	9.6
Z	X	8.0			
Z	Y	3.0			
Z	C1	145.0	Z	C1	6.1
Z	C2	71.4	Z	C2	18.8

R-RESPONDER S-STIMULATOR

*- C1 AND C2 were unrelated controls.

This table lists the stimulation indices from the MLR performed with cells from group 1 animals. Values greater than (>) 4.0 in the one-way and values > 2.0 in the two-way MLR were considered stimulatory. Animals Y and Z are non-stimulatory and animal X is mutually stimulatory with Y and Z.

RFLP with Murine Class I Probe. The hybridization of group 1 dogs digested by EcoR I, Bgl II and Hind III restriction endonucleases with the murine derived class I probe pH-2IIa resulted in the RFLP shown in Figure 1. The use of different enzymes for digestion resulted in different fragment patterns as was expected. The sizes of the fragments are given in Table 3. There were no detectable differences between the three animals using the murine probe, even though the class I serology denoted a difference at the DLA-B locus in animal X versus (vs) animals Y and Z.

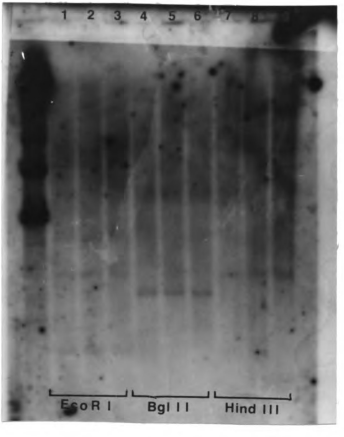

Figure 1. RFLP of Group 1 Dogs Probed with pH-2IIa. This RFLP was generated by hybridization of group 1 with the class I murine probe pH-2IIa. Molecular weight markers were loaded in the far left lane. Lanes 1, 4 and 7 are dog X. Lanes 2, 5, and 8 are dog Y. Lanes 3, 6, and 9 are dog Z.

Table 3. Calculated Fragment Lengths of Figure 1.

ENZYME	ANIMAL		
	X	Y	Z
EcoR I	7820*	7820	7820
	6815	6815	6815
	6250	6250	6250
	3550	3550	3550
	2690	2690	2690
	1500	1500	1500
Bgl II	9025	9025	9025
	5575	5575	5575
	5355	5355	5355
	5100	5100	5100
	3850	3850	3850
	2390	2390	2390
Hind III	14490	14490	14490
	9955	9955	9955
	8620	8620	8620
	7160	7160	7160
	2858	2858	2858

*- Size in base pairs, rounded off. These sizes were calculated using a least sum of squares algorithm based on the migration distances standard fragments compared to the unknowns.

This table lists the fragment sizes generated by probing digested DNA from group 1 with a murine class I probe. Lanes 1 - 3 are animals X, Y and Z digested with Eco R I respectively. Lanes 4 - 6 are animals X, Y and Z digested With Bgl II respectively. Lanes 7 - 9 are animals X, Y and Z digested with Hind III respectively.

Photography of the autoradiograph depicted in Figure 1 does not allow the visualization of the details present upon visual inspection. The line drawing presented in Figure 2 represents the fragment patterns visually seen with this autoradiograph. The sizes of the fragments are the same as those given for Figure 1 in Table 3.

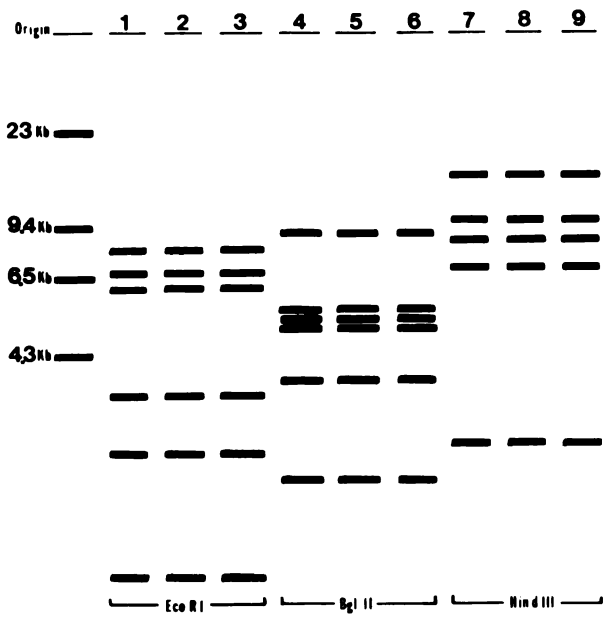

Figure 2. Line Drawing of RFLP of Group 1 Probed with pH -2IIa.
This RFLp was generated by hybridization of group 1 with the class I murine probe pH-2IIa. Molecular weight markers were loaded in the far left lane. Lanes 1, 4 and 7 are dog X. Lanes 2, 5, and 8 are dog Y. Lanes 3, 6, and 9 are dog Z.

RFLP of Unrelated Dogs to Murine Class I Probe. Hybridization of pH-2IIa to the Hind III digested DNA of six unrelated animals resulted in the RFLP shown in Figure 3. The rationale behind this experiment was to show that the murine class I probe, pH-2IIa, was capable of detecting the polymorphism inherent to the MHC. While the fragment patterns of the six dogs possess some commonalties none of the animals have identical patterns. This individuality was consistent with expectations. The sizes of the fragments in Figure 3 are not given.

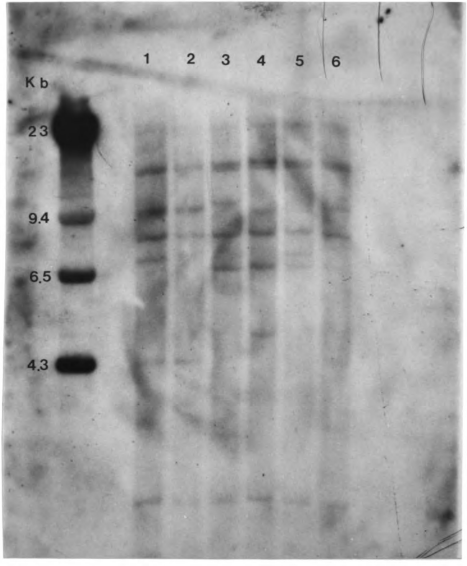

Figure 3. RFLP of Random Animals Probed with pH-2IIa. Molecular markers are on the left. This RFLP depicts the polymorphism inherent to the MHC. Each animal has a unique banding Pattern.

RFLP of Group I Dogs with HLA-DRA Probe. The hybridization of restriction endonuclease digested DNA of group 1 dogs with the human HLA-DRA probe gave the RFLP seen in Figure 4. The size of the fragments differed between enzymes but all three enzymes only yielded one fragment with each animal. The sizes of the fragments generated are given in Table 4.

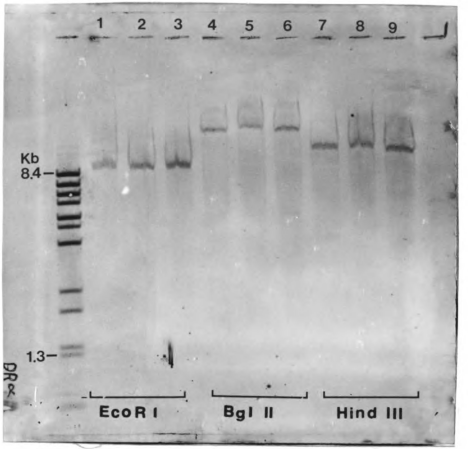

Figure 4. RFLP of Group 1 Dogs Probed with HLA-DRA. This RFLP was generated by hybridizing Eco R I, Bgl II and Hind III enzymatic digests of group 1 dogs with the human HLA-DRA class II probe. The order of the animals in each enzymes series are X, Y and Z.

Table 4. Calculated Fragment Lengths of Figure 4.

ENZYME	ANIMAL		
	X	Y	Z
EcoR I	9260*	9260	9260
Bgl II	20820	20820	20820
Hind III	13305	13395	13395

*-Size in base pairs, rounded off.

This table lists the fragment sizes generated by probing digested DNA of group 1 dogs with and HLA -DRA probe. Lanes 1 - 3 are animals X, Y and Z digested with Eco R I respectively. Lanes 4 - 6 are animals X, Y and Z digested With Bgl II respectively. Lanes 7 - 9 are animals X, Y and Z digested with Hind III respectively.

RFLP of Group 1 with HLA-DRB Probe. The patterns of hybridization using the human HLA-DRB probe are the most remarkable and are given in Figure 5. The patterns seen with this probe are highly polymorphic and variable not only with the enzyme used for digestion but also the animal hybridized. Animal X presents a different pattern from the other two littermates. The differences are indicated by arrows. The sizes of the fragments of the patterns seen in Figure 5 are given in Table 5. The patterns generated with the Bgl II enzyme and the HLA-DRB probe support the MLR results. Dogs Y and Z had identical banding patterns and are non-stimulatory and dog X had a pattern disparate from its siblings and is stimulatory.

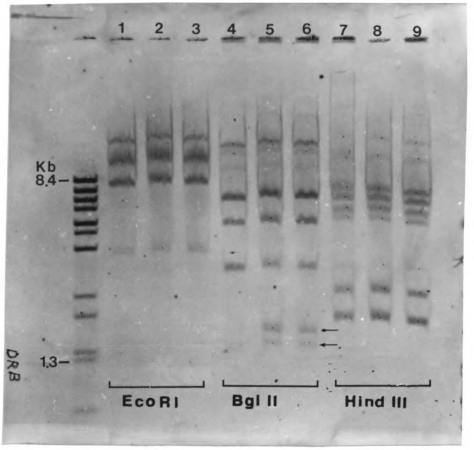

Figure 5. RFLP of Group 1 Dogs Probed with HLA-DRB. This RFLP was generated by hybridizing Eco R I, Bgl II and Hind III enzymatic digests of group 1 dogs with the human HLA-DRB class II probe. The order of the animals in each enzymes series are X, Y and Z. Arrows indicate the fragments shared by animals Y and Z causing them to be disparate from animal X, but identical to each other.

Table 5. Calculated Fragment Lengths of Figure 5.

ENZYME	ANIMAL		
	X	Y	Z
EcoR I	19170*	19170	19170
	12070	12070	12070
	8520	8520	8520
	3795	3795	3795
Bgl II	16390	16390	16390
	13650	13650	13650
	6825	6825	6825
	5220	5220	5220
	3140	3140	3140
		1755	1755
		1490	1490
Hind III	17230	17230	17230
	11610	11610	11610
	7135	7135	7135
	5620	5620	5620
	4975	4975	4975
	3075	3075	3075
	2410	2410	2410
	1755	1755	1755

*- Sizes in base pairs, rounded off.

This table lists the fragment sizes from probing digested DNA from group 1 dogs with an HLA-DRB probe. Lanes 1 - 3 are animals X, Y and Z digested with Eco R I respectively. Lanes 4 - 6 are animals X, Y and Z digested With Bgl II respectively. Lanes 7 - 9 are animals X, Y and Z digested with Hind III respectively.

RFLP of Group 1 with HLA-DQA Probe. Hybridization of digested group 1 dogs DNA with the human HLA-DQA probe resulted in the RFLP shown in Figure 6. All animals gave the same pattern of hybridization with a given enzyme but the pattern differed for each enzyme. The sizes of the fragments seen in Figure 6 are given in Table 6.

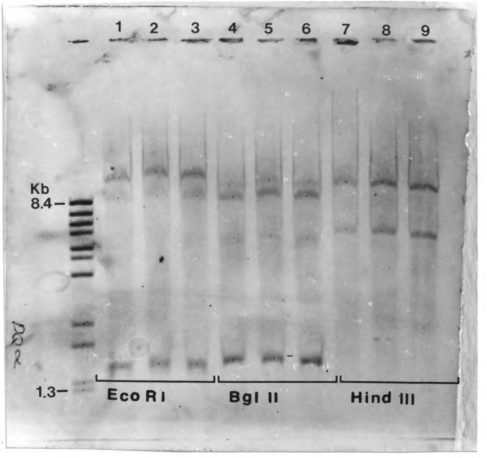

Figure 6 RFLP of Group 1 Dogs Probed with HLA-DQA. This RFLP was generated by hybridizing Eco R I, Bgl II and Hind III enzymatic digests of group 1 dogs with the human HLA-DQA class II probe. The order of the animals in each enzymes series are X, Y and Z.

Table 6. Calculated Fragment Lengths of Figure 6.

ENZYME	ANIMAL		
	X	Y	Z
EcoR I	13630*	13630	13630
	9100	9100	9100
	1640	1640	1640
Bgl II	11610	11610	11610
	9100	9100	9100
	5400	5400	5400
	1705	1705	1705
Hind III	10785	10785	10785
	5672	5672	5672

*- Size in base pairs, rounded off.

This table lists the fragment sizes from probing digested DNA from group 1 dogs with an HLA-DQA probe. Lanes 1 - 3 are animals X, Y and Z digested with Eco R I respectively. Lanes 4 - 6 are animals X, Y and Z digested With Bgl II respectively. Lanes 7 - 9 are animals X, Y and Z digested with Hind III respectively.

RFLP of Group 1 with HLA-DQB Probe. Figure 7 represents the hybridization pattern that resulted from the use of the human HLA-DQB probe and the digested DNA of group 1 dogs. There are multiple fragments present for each animal and enzyme used. As seen previously the patterns are different with each enzyme. The animals gave the identical banding patterns for each enzyme with the exception of the Bgl II generated patterns. Animal X lacks a 1500 bp fragment present in animals Y and Z. These results support the MLR results and are consonant with the patterns seen when Bgl II digests of the animals were probed with HLA-DRB. The sizes of all the fragments seen in Figure 7 are given in Table 7.

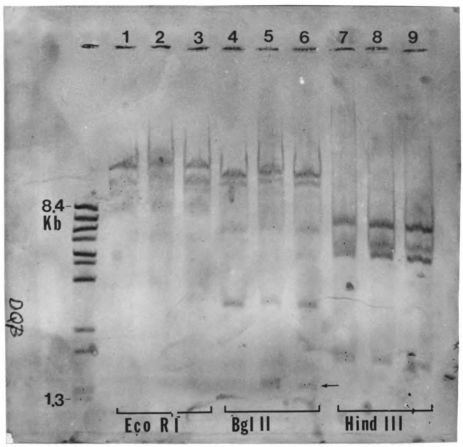

Figure 7. RFLP of Group 1 Dogs Probed with DQB. This RFLP was generated by hybridizing Eco R I, Bgl II and Hind III enzymatic digests of group 1 dogs with the human HLA-DQB class II probe. The order of the animals in each enzymes series are X, Y and Z. An arrow identifies the 1500bp fragment shared by Y and Z that is lacking in Z.

Table 7. Calculated Fragment Lengths of Figure 7.

ENZYME	ANIMAL		
	X	Y	Z
EcoR I	24550*	24550	24550
	15400	15400	15400
	12335	12335	12335
	9520	9520	9520
	8525	8525	8525
Bgl II	18580	18580	18580
	14410	14410	14410
	6945	6945	6945
	3015	3015	3015
		1500	1500
Hind III	7020	7020	7020
	5380	5380	5380
	4725	4725	4725
	1855	1855	1855

*- Size in base pairs, rounded off.

This table lists the fragment sizes from probing digested DNA from group 1 dogs with an HLA-DQB probe. Lanes 1 - 3 are animals X, Y and Z digested with Eco R I respectively. Lanes 4 - 6 are animals X, Y and Z digested With Bgl II respectively. Lanes 7 - 9 are animals X, Y and Z digested with Hind III respectively. Note the presence of a 1500 bp fragment in animals Y and Z when digested with Bgl II that is not present in animal X treated the same.

RFLP of Group 1 with HLA-DPA Probe. The RFLP generated by hybridizing a human HLA-DPA probe with the digested DNA of group 1 dogs are shown in Figure 8. Each animal has multiple bands common to all members of group 1, there was no polymorphism between the animals detected using this probe. The pattern changes with the use of a different enzyme. The sizes of the fragments seen in Figure 8 are given in Table 8.

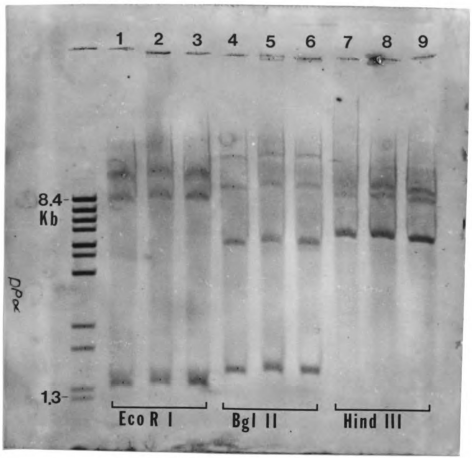

Figure 8. RFLP of Group 1 Dogs Probed with HLA-DPA. This RFLP was generated by hybridizing Eco R I, Bgl II and Hind III enzymatic digests of group 1 dogs with the human HLA-DPA class II probe. The order of the animals in each enzymes series are X, Y and Z.

Table 8. Calculated Fragment Lengths of Figure 8.

ENZYME	ANIMAL		
	X	Y	Z
EcoR I	12260*	12260	12260
	5000	5000	5000
	1463	1463	1463
Bgl II	30830	30830	30830
	12790	12790	12790
	5630	5630	5630
	1775	1775	1775
Hind III	12790	12790	12790
	10335	10335	10335
	6230	6230	6230

*- Size in base pairs, rounded off.

This table lists the fragments sizes from probing digested DNA from groupl dogs with an HLA-DPA probe. Lanes 1 - 3 are animals X, Y and Z digested with Eco R I respectively. Lanes 4 - 6 are animals X, Y and Z digested With Bgl II respectively. Lanes 7 - 9 are animals X, Y and Z digested with Hind III respectively.

RFLP of Group 1 with HLA-DPB Probe. Figure 9 represents the last of the hybridization experiments using group 1 animals. It was generated using digested DNA and a human HLA-DPB probe. The pattern seen with this probe follows the established trends. There are multiple fragments for each animal that vary with the enzyme used for digestion. The animals give identical banding patterns with the exception of those generated by using the Bgl II enzyme for digestion. Animals Y and Z have a common 1495 bp fragment not seen in animal X. This is consistent with the results seen with the HLA-drb and -DQb probes and supports the MLR results. The sizes of all the fragments generated by this experiment are given in Table 9.

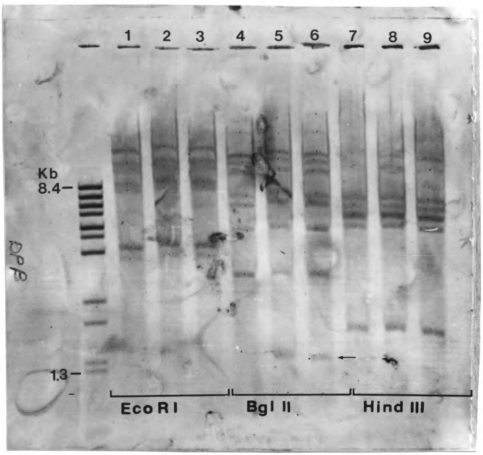

Figure 9. RFLP of Group 1 Dogs Probed with HLA-DPB. This RFLP was generated by hybridizing Eco R I, Bgl II and Hind III enzymatic digests of group 1 dogs with the human HLA-DPB class II probe. The order of the animals in each enzymes series are X, Y and Z. An arrow identifies the 1495bp fragment common to animals Y and Z that is not seen in animal X.

Table 9. Calculated Fragment Lengths of Figure 9.

ENZYME	ANIMAL		
	X	Y	Z
EcoR I	23755*	23755	23755
	15655	15655	15655
	13155	13155	13155
	9860	9860	9860
	8525	8525	8525
	4165	4165	4165
	3590	3590	3590
Bgl II	46565	46565	46565
	20925	20925	20925
	15840	15840	15840
	11715	11715	11715
	7835	7835	7835
	6920	6920	6920
	4935	4935	4935
	3075	3075	3075
		1495	1495
Hind III	17040	17040	17040
	12045	12045	12045
	7330	7330	7330
	6315	6315	6315
	5540	5540	5540
	4935	4935	4935
	1885	1885	1885

*- Size in base pairs, rounded off.

This table lists the fragment sizes generated when digested DNA from groupl dogs were probed with an HLA-DPB. Lanes 1 - 3 are animals X, Y and Z digested with Eco R I respectively. Lanes 4 - 6 are animals X, Y and Z digested With Bgl II respectively. Lanes 7 - 9 are animals X, Y and Z digested with Hind III respectively. Note the absence of the 1495bp fragment in animal X when digested with Bgl II.

Dog Group 2:

The results of group 1 showed that molecular evaluation of DLA system using murine and human probes had promise. In addition to the correlation of the serology and cellular assays with the molecular genetic results it was necessary to document the passage of MHC haplotypes within a pedigree. The ability to do this would document that the DLA genes were being detected, that they could be used to substantiate a pedigree and that these genes had characteristics homologous to previously studied species. A full pedigree of sire, dam and offspring was needed. Group 2 fulfilled these requirements.

Class I Serology. The DLA types of the dogs in group 2 and their familial relationships are given in Table 10. Epitopes representing all three serologically defined DLA class I loci could be detected in the animals of this family. Siblings A and C; and siblings D and F are serologically identical. These pairs were one haplotype different. The sire and dam were also one haplotype different from each grouping of pups.

Mixed Lymphocyte Reaction. The stimulation indices from the MLR performed using the dogs of group 2 are given in Table 11. The sire in these experiments was consistently stimulatory or stimulated when cultured with cells of the members of the pedigree. The offspring and the dam are non-stimulatory when their cells are co-cultured. Even though A and C were class I identical, but 1 haplotype dissimilar from D and F (MHC identical), none of these four animals were stimulatory to each other in this MLR. The dam which would also be 1 haplotype dissimilar to her offspring was also MLR nonstimulatory when co-cultured with these offspring

Table 10. Class I DLA Serotypes of Group 2 Dogs and Their Pedigree Relationship.

Animal	DLA-A	DLA-B	DLA-C	Relationship
A	(9,-)	(6,-)	(11,12)	Sibling
B	(9,-)	(6,13)	(12,-)	Sire
C	(9,-)	(6,-)	(11,12)	Sibling
D	(2,9)	(6,-)	(11,12)	Sibling
E	(2,9)	(6,-)	(11,-)	Dam
F	(2,9)	(6,-)	(11,12)	Sibling

This table lists the results of the class I DLA serotyping of the dogs from group 2. The familial relationship of the animals are also given. Animals A and C; and D and F are class I DLA identical.

Table 11. MLR Stimulation Indices of Group 2 Dogs.

ONE-WAY MLR			TWO-WAY MLR		
Dog (R)	vs. Dog (S)	Stimulation Index	Dog (R)	vs. Dog (R)	Stimulation Index
A	B	1.5	A	B	12.9
A	C	1.1	A	C	0.8
A	D	1.3	A	D	0.6
A	E	1.1	A	E	1.0
A	F	1.2	A	F	0.9
A	C1*	1.1	A	C1	34.1
A	C2*	1.2	A	C2	42.5
B	A	2.6			
B	C	1.2	B	C	11.8
B	D	2.8	B	D	11.4
B	E	2.3	B	E	9.5
B	F	4.9	B	F	7.0
B	C1	2.4	B	C1	17.9
B	C2	1.6	B	C2	23.2
C	A	0.9			
C	B	1.0			
C	D	1.0	C	D	1.0
C	E	0.9	C	E	1.4
C	F	0.9	C	F	0.7
C	C1	0.9	C	C1	35.7
C	C2	1.0	C	C2	39.0
D	A	0.9			
D	B	1.4			
D	C	0.9			
D	E	1.0	D	E	1.5
D	F	1.3	D	F	0.8
D	C1	2.6	D	C1	32.6
D	C2	1.3	D	C2	37.1
E	A	1.6			
E	B	1.6			
E	C	1.3			
E	D	1.4			
E	F	1.3	E	F	1.2
E	C1	1.2	E	C1	23.9
E	C2	1.2	E	C2	37.3
F	A	0.9			
F	B	1.1			
F	C	1.0			
F	D	1.0			
F	E	0.9			
F	C1	1.1	F	C1	40.4
F	C2	1.2	F	C2	29.8

*- C1 and C2 were unrelated controls

This table lists the stimulation indices from the MLR performed using cells from the group 2 family. Animal B (sire) was stimulatory and stimulated by all other members of the family.

The rest of the animals were non-stimulatory to each other. These judgements are based on the two-way indices. Any index > 2.0 was considered stimulatory.

RFLP of Group 2 with HLA-DRA probe. The animals of group 2 were digested with a single enzyme, Taq I, and hybridized with a human HLA-DRA probe. The results of that experiment are expressed by the RFLP seen in Figure 10 This was a non-polymorphic pattern whose fragment sizes are given in Table 12.

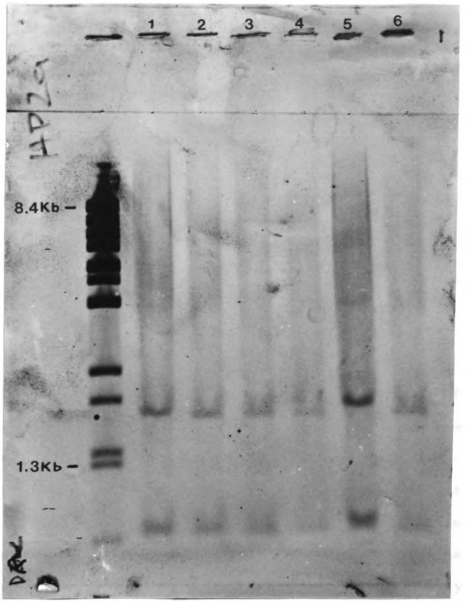

Figure 10. RFLP of Group 2 Dogs Probed with HLA-DRA. This RFLP was generated by hybridizing Taq I digested DNA of group 2 dogs with the human HLA-DRA class II probe. Molecular weight markers are in the far left lane. Animals A, B, C, D, E and F are in lanes 1, 2, 3, 4, 5 and 6 respectively.

Table 12. Calculated Fragment Lengths of Figure 10

ANIMAL					
A	B	C	D	E	F
1800*	1800	1800	1800	1800	1800
830	830	830	830	830	830

*- size in base pairs, rounded off.

This table lists the fragment sizes generated when Taq I digested DNA from group 2 dogs were probed with an HLA-DRA probe.

RFLP of Group 2 with HLA-DRB Probe. The RFLP generated by probing the Taq I digested DNA of group 2 dogs with a human HLA-DRB is shown in Figure 11. The pattern seen here does display the polymorphism associated with this system. The sizes of the fragments of this RFLP are given in Table 13. Passage of particular fragments with specific serologically defined epitopes can be seen and are indicated by arrows. Two fragments, 4560 bp and 1600 bp, follow the expression of DLA-C12. A 1970 bp fragment is present in animals expressing the DLA-C11 epitope. A 2820 bp fragment correlates with the expression of DLA-A2. A 2130 bp fragment can be seen only in the sire who coincidental is the only animal to express the DLA-B13 epitope. It should be noted that the sire is the only animal stimulatory in the MLR. The 1690 bp fragment in the dam is not correlated with any DLA specificity.

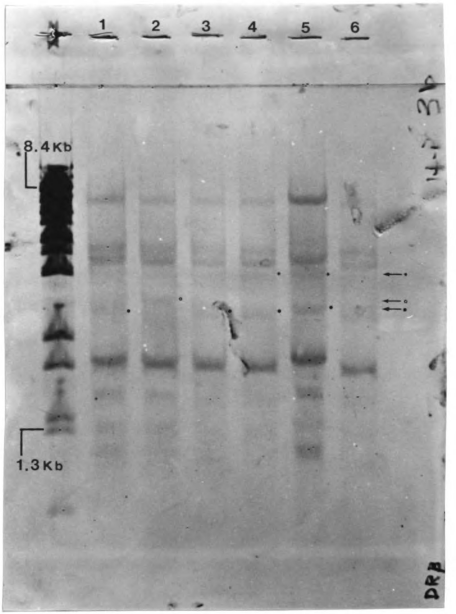

Figure 11. RFLP of Group 2 Dogs Probed with HLA-DRB. This RFLP was generated by hybridizing Taq I digested DNA of group 2 dogs with the human HLA-DRB class II probe. Molecular weight markers are in the far left lane. Animals A, B, C, D, E and F are in lanes 1, 2, 3, 4, 5 and 6 respectively. Arrows indicate fragments that follow serologic epitope passage.

Table 13. Calculated Fragment Lengths of Figure 11.

			ANIMAL		
A	B	C	D	E	F
7255*	7255	7255	7255	7255	7255
4560	4560	4560	4560		4560
3935	3935	3935	3935	3935	3935
			2820	2820	2820
	2130				
1970		1970	1970	1970	1970
				1690	
1600	1600	1600	1600		1600
1275	1275	1275	1275	1275	1275
900	900	900	900	900	900

*- Sizes given in base pairs, rounded off.

This table lists the sizes of the fragments generated when Taq I digested DNA from group 2 dogs was hybridized with an HLA-DRB probe.

RFLP of Group 2 with HLA-DQA. The RFLP generated from hybridizing Taq I digested DNA from group 2 dogs with a human HLA-DQA probe is shown in Figure 12. The patterns seen are not polymorphic between animals. The sizes of the fragments generated are given in Table 14.

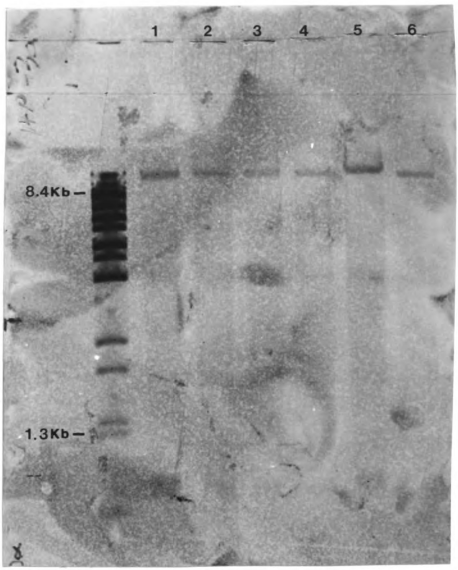

Figure 12. RFLP of Group 2 Dogs Hybridized with HLA-DQA. This RFLP was generated by hybridizing Taq I digested DNA of group 2 dogs with the human HLA-DQA class II probe. Molecular weight markers are in the far left lane. Animals A, B, C, D, E and F are in lanes 1, 2, 3, 4, 5 and 6 respectively.

Table 14. Calculated Fragment Lengths of Figure 12.

ANIMAL					
A	B	C	D	E	F
11300*	11300	11300	11300	11300	11300
945	945	945	945	945	945

*- Sizes in base pairs, rounded off.

This table lists the fragment sizes generated by probing Taq I digested DNA from group 2 dogs with an HLA-DQA probe.

RFLP of Group 2 with HLA-DQB. The hybridization experiment using the human HLA-DQB probe and Taq I digested DNA of the group 2 dogs resulted in the generation of the RFLP seen in Figure 13. This pattern reflects the non-polymorphism between animals seen with the DQA probe shown in Figure 12. The sizes of the fragments in Figure 13 are given in Table 15.

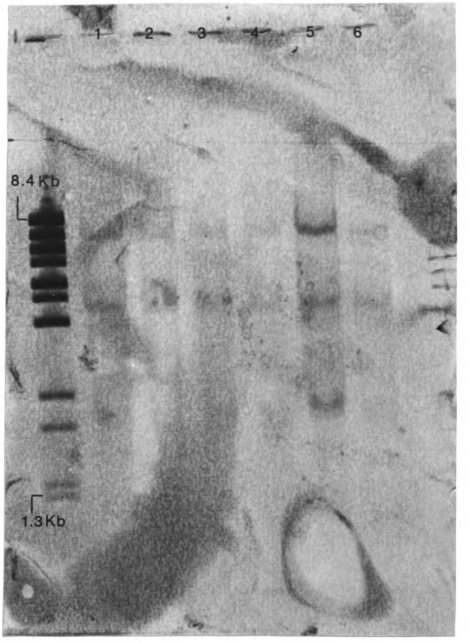

Figure 13. RFLP of Group 2 Dogs Probed with HLA-DQB. This RFLP was generated by hybridizing Taq I digested DNA of group 2 dogs with the human HLA-DQB class II probe. Molecular weight markers are in the far left lane. Animals A, B, C, D, E and F are in lanes 1, 2, 3, 4, 5 and 6 respectively.

Table 15. Calculated Fragment Lengths of Figure 13.

ANIMAL					
A	B	C	D	E	F
7300*	7300	7300	7300	7300	7300
4385	4385	4385	4385	4385	4385
1565	1565	1565	1565	1565	1565

*- Size in base pairs, rounded off.

This table lists the fragment sizes generated by probing Taq I digested DNA from group 2 dogs with an HLA-DQB probe.

RFLP of Group 2 with HLA-DPA Probe, The RFLP generated by hybridizing Taq I digested DNA from the family representing group 2 and the human HLA-DPA probe is depicted in Figure 14. Arrows in Figure 14 indicate a 2710 bp fragment that follows the expression of DLA-A2 epitope expression on the lymphocytes of three animals. The sizes of all the fragments generated by this hybridization reaction are given in Table 16.

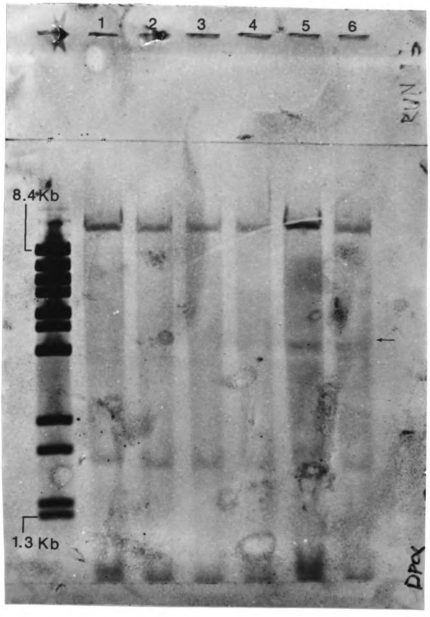

Figure 14. RFLP of Group 2 Dogs Probed with HLA-DPA. This RFLP was generated by hybridizing Taq I digested DNA of group 2 dogs with the human HLA-DPA class II probe. Molecular weight markers are in the far left lane. Animals A, B, C, D, E and F are in lanes 1, 2, 3, 4, 5 and 6 respectively.

Table 16. Calculated Fragments Lengths of Figure 14.

ANIMAL					
A	B	C	D	E	F
10365*	10365	10365	10365	10365	10365
			2710	2710	2710
1835	1835	1835	1835	1835	1835
485	485	485	485	485	485

*- Size in base pairs, rounded off.

This table lists the fragment sizes generated by probing Taq I digested DNA from group 2 dogs with an HLA-DPA probe.

RFLP of Group 2 with HLA-DPB Probe. Figure 15 shows the RFLP generated by hybridizing Taq I digested DNA from group 2 dogs and a human HLA-DPB probe. This RFLP is highly polymorphic. Arrows indicate specific fragments that correlate with the presence of certain serologically defined DLA epitopes. A 4790bp and a 2635 fragment are present when DLA-C12 is expressed. A 2010 bp fragment is present in the sire (B) who is the only family member to express DLA-B13. He was also the only stimulatory animal. The presence of a 3120bp fragment in the mother is uncorrelated with any epitopes. The sizes of all the fragments in Figure 15 are presented in Table 17.

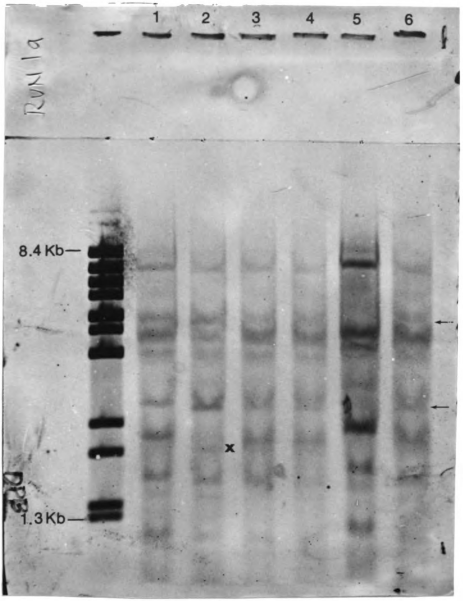

Figure 15. RFLP of Group 2 Dogs Probed with HLA-DPB. This RFLP was generated by hybridizing Taq I digested DNA of group 2 dogs with the human HLA-DPB class II probe. Molecular weight markers are in the far left lane. Animals A, B, C, D, E and F are in lanes 1, 2, 3, 4, 5 and 6 respectively.

Table 17. Calculated Fragment Lengths of Figure 15.

ANIMALS					
A	B	C	D	E	F
9000*	9000	9000	9000	9000	9000
7105	7105	7105	7105	7105	7105
4790	4790	4790	4790		4790
4210	4210	4210	4210	4210	4210
3720	3720	3720	3720	3720	3720
				3120	
2635	2635	2635	2635		2635
2200		2200	2200	2200	2200
	2010				
1655	1655	1655	1655	1655	1655
1155	1155	1155	1155	1155	1155
850	850	850	850	850	850

*-Size in base pairs, rounded off.

This table lists the fragment sizes generated by probing Taq I digested DNA from group 2 animals with an HLA-DPB probe.

Dogs Group 3:

Group 3 animals were submitted for transplantation candidacy evaluation. These animals comprise a full sibling family, including the sire and dam. They were a part of a bone marrow transplantation study being performed by UC-Davis. The relationship of the animals comprising group 3 are given in Table 18. Class I DLA serology and class I molecular evaluations were not performed.

Mixed Lymphocyte Reaction. The stimulation indices from the MLR performed with cells from group 3 dogs are given in Table 19. The results indicate that the animals are non-stimulatory to each other.

Table 18. The Familial Relationship of Group 2 Dogs.

DOG	RELATIONSHIP
G	DAM
H	SIBLING
I	SIBLING
J	SIRE

Table 19. MLR Stimulation Indices of Group 3 Dogs.

ONE-WAY MLR			TWO-WAY MLR		
Dog vs. Dog (R)	(S)	Stimulation Index	Dog vs. Dog (R)	(R)	Stimulation Index
G	H	1.6	G	H	0.3
G	I	0.2	G	I	0.4
G	J	0.2	G	J	0.6
G	C1*	0.2	G	C1	1.1
G	C2*	0.6	G	C2	2.1
H	G	1.7			
H	I	1.8	H	I	0.9
H	J	3.3	H	J	3.0
H	C1	1.7	H	C1	4.3
H	C2	2.1	H	C2	2.8
I	G	1.0			
I	H	1.2			
I	J	0.6	I	J	0.8
I	C1	0.8	I	C1	4.3
I	C2	1.7	I	C2	7.3
J	G	3.6			
J	H	2.3			
J	J	0.5			
J	C1	0.48	J	C1	2.3
J	C2	1.1	J	C2	8.2

*- C1 and C2 are unrelated controls.

This table lists the stimulation indices from the MLR performed with cells from group 3 animals. The two-way indices were used to evaluate the test. Values > 2.0 were considered stimulatory. While H and J appeared stimulatory in the two-way MLR is was discounted because this trend was not supported in the corresponding one-way tests.

RFLP's of Group 3 with HLA-DRA and -DRB Probes. The results from hybridizing Taq I digested DNA from group 3 dogs with human HLA-DRA and -DRB probes shown in Figure 16 by RFLP's A and B respectively. The RFLP's generated by this hybridization showed no differences between animals. This was consistent with the MLR non-responsiveness seen between family members. The sizes of the fragments generated are given in Table 20.

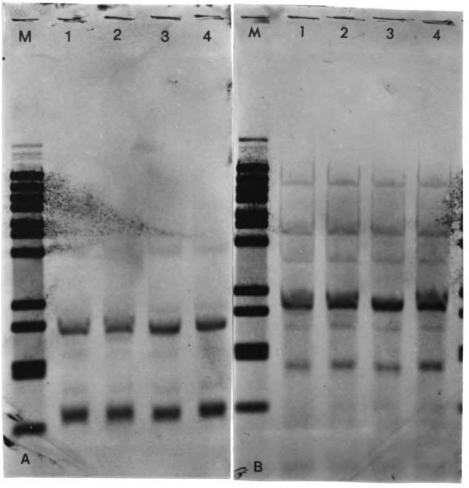

Figure 16. RFLP's of Group 3 Dogs with HLA-DR Probes. The RFLP's A and B were generated by hybridizing Taq I DNA digests of group 3 dogs with the human DRA and DRB class II probes respectively. Molecular weight markers are on the left of each gel. Lanes 1, 2, 3 and 4 are dogs G, H, I and J respectively.

Table 20. Calculated Fragment Lengths of Figure 16.

RFLP	ANIMAL			
	G	H	I	J
A	1845*	1845	1845	1845
	865	865	865	865
B	7050	7050	7050	7050
	4230	4230	4230	4230
	3200	3200	3200	3200
	2060	2060	2060	2060
	1735	1735	1735	1735
	1135	1135	1135	1135

*- Size in base pairs, rounded off.

This table lists the fragment sizes generated by probing Taq I digested DNA from group 3 dogs with HLA-DRA and -DRB probes.

RFLP's of Group 3 with HLA-DQA and -DQB Probes. The RFLP's (A and B) generated by hybridizing Taq I digested DNA from animals of group 3 and human HLA-DQA and -DQB are shown in Figure 17. These RFLP's display the generation of multiple fragments for each animal tested. The animals have identical RFLP's. This reflects the non-stimulatory response found in the MLR performed between family members. The sizes of the fragments are given in Table 21.

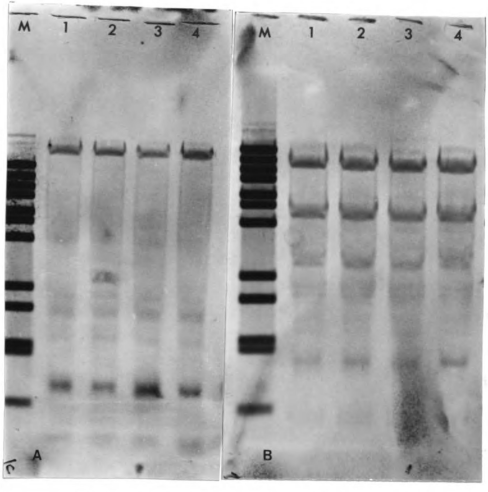

Figure 17. RFLP's of Group 3 Dogs with HLA-DQ Probes. The RFLP's A and B were generated by hybridizing Taq I DNA digests from the dogs of group 3 with the human DQA and DQB class II probes respectively. Lanes 1, 2, 3 and 4 are dogs G, H, I and J respectively.

Table 21. Calculated Fragment Lengths of Figure 17.

	ANIMAL			
RFLP	G	H	I	J
A	11025*	11025	11025	11025
	880	880	880	880
B	6975	6975	6975	6975
	4000	4000	4000	4000
	2505	2505	2505	2505
	2120	2120	2120	2120
	1975	1975	1975	1975
	1115	1115	1115	1115

*- Size in base pairs, rounded off.

This table lists the fragment sizes generated by probing the Taq I digests of the group 3 dogs with HLA-DQA and -DQB probes.

RFLP's of Group 3 with HLA-DPA and -DPB. Figure 18 gives the RFLP's, A and B, generated by hybridizing Taq I digested DNA from group 3 dogs and the human HLA-DPA and -DPB probes respectively. Each animal has multiple fragments generating the RFLP's seen while the RFLP of each animal with a given probe is identical with that of the other family members with that same probe. This correlates with the non-stimulatory MLR. The sizes of the fragments generated by these hybridizations are given in Table 22.

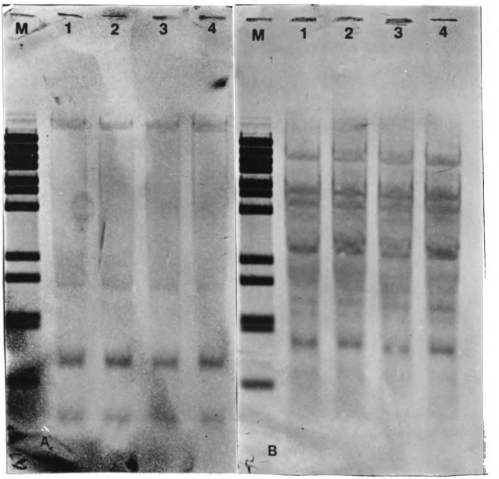

Figure 18. RFLP's of Group 3 Dogs with HLA-DP Probes. The RFLP's A and B were generated by hybridizing Taq I digested DNA from dogs of group 3 with the human HLA-DPA and -DPB class II probes respectively. Lanes 1, 2, 3 and 4 are dogs G, H, I and J respectively.

Table 22. Calculated Fragment Lengths of Figure 18.

	ANIMALS			
RFLP	G	H	I	J
A	10050*	10050	10050	10050
	1800	1800	1800	1800
	905	905	905	905
B	6435	6435	6435	6435
	4395	4395	4395	4395
	4035	4035	4035	4035
	2525	2525	2525	2525
	1115	1115	1115	1115

*- Size in base pairs, rounded off.

This table lists the fragment sizes generated by probing Taq I digested DNA from group 3 dogs with HLA-DPA and -DPB probes.

Dogs Group 4:

It was at this point in the project that it was realized that complementary molecular work was a useful tool in evaluating the DLA system. The high degree of homology between the human and canine systems were also realized. Great strides in localizing the HLA association with IDDM were also being made at this point and it seemed a natural shift to evaluate the DLA of IDDM dogs for homologous properties.

Group 4 animals were selected to represent a population of juvenile onset IDDM dogs and a population of non-diabetic controls. There were seven animals in each group. The IDDM dogs were selected from the diabetic animal model colony, while the controls were selected from the conditioned animals present in the Veterinary Clinical Center here at Michigan State University. Class I serology and an MLR were not indicated nor performed on group 4 animals. The enzyme Taq I and the HLA-DQA probe were chosen based a polymorphism of the DXA gene

seen in human diabetics. The HLA-DRB and -DQB were also included in this study based on the human HLA map. In man, the HLA-DRB and DQB surround the DQA gene.

RFLP of Group 4 with HLA-DRB Probe. The RFLP that was generated by hybridizing the Taq I digested DNA of this group with a human HLA-DRB probe is shown in Figure 19. The RFLP generated is highly polymorphic. There are no patterns unique to either population. The sizes of the fragments generated are given in Table 23.

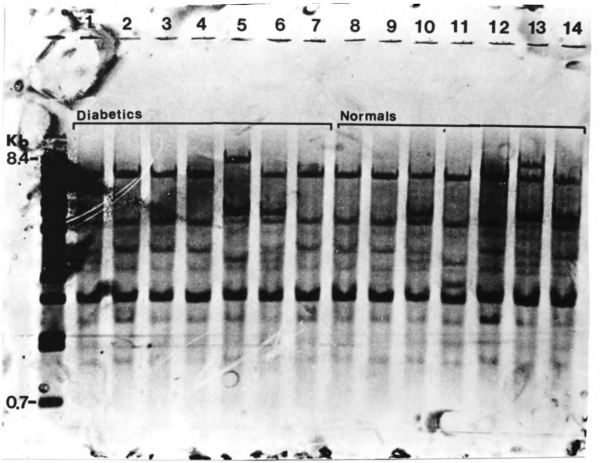

Figure 19. RFLP of Group 4 Dogs Probed with HLA-DRB. This RFLP was generated by hybridizing Taq I digested DNA from group 4 dogs with the human HLA-DRB class II probe. Molecular weight markers are on the far left. The grouping of IDDM animals and normal controls are indicated on the gel.

Table 23. Calculated Fragment Lengths for Figure 19.

				ANIMAL			
IDDM	1	2	3	4	5	6	7
					9000*		
	6995	6995	6995	6995		6995	6995
	4625				4625	4625	
	4040	4040	4040	4040		4040	4040
	3075	3075	3075	3075		3075	3075
	2900				2900	2900	
	2580	2580	2580	2580	2580	2580	2580
	1985	1985	1985	1985	1985	1985	1985
	1680	1680	1680	1680	1680	1680	1680
	8	9	10	11	12	13	14
CONTROL	6995	6995	6995	6995	6995	6995	6995
			4625			4625	4625
	4040	4040	4040	4040	4040		4040
	3075	3075	3075		3075	3075	3075
			2900	2900		2900	2900
	2580	2580	2580	2580	2580	2580	2580
				2300			
	1985	1985	1985	1985	1985	1985	1985
	1680	1680	1680	1680	1680	1680	1680

*- Size in base pairs, rounded off.

This table lists the fragment sizes generated by probing Taq I digested DNA from group 4 dogs with as HLA-DRB probe.

RFLP of Group 4 with HLA-DQA Probe. Hybridization of Taq I digested DNA from group 4 dogs and a human HLA-DQA probe generated the RFLP seen in FIgure 20. This RFLP has few fragments per animal but the animals are polymorphic if compared to each other. There are no unique patterns assignable to either population. The sizes of the fragment in Figure 20 are given in Table 24.

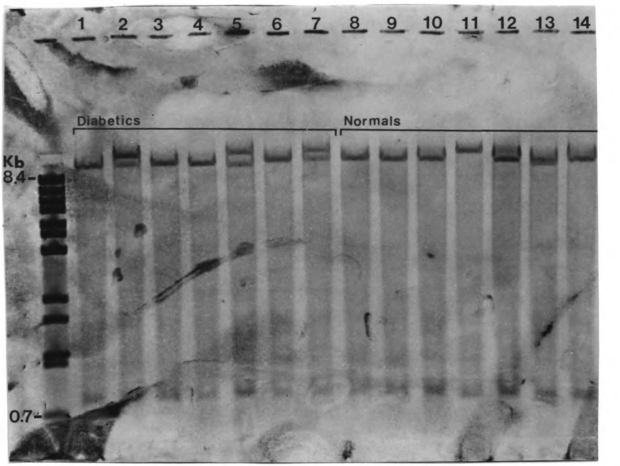

Figure 20. RFLP of Group 4 Dogs Probed with HLA-DQA. This RFLP was generated by hybridizing Taq I digested DNA from group 4 dogs with the human DQA class II probe. Molecular weight markers are on the far left. The grouping of IDDM animals and normal controls are indicated on the gel.

TABLE 24. Calculated Fragment Lengths of Figure 20.

				ANIMAL			
IDDM	1	2	3	4	5	6	7
		11235*			11235		11235
	9645	9645	9645	9645		9645	9645
					9155		
	810	810	810	810	810	810	810
	8	9	10	11	12	13	14
CONTROL				11235			
	9645	9645	9645		9645	9645	9645
						9154	
	810	810	810	810	810	810	810

*- Size in base pairs, rounded off.

This table lists the fragment sizes generated by probing the Taq I digested DNA of group 4 dogs with an HLA-DQA probe.

RFLP of Group 4 with HLA-DQB. Taq I digested DNA from group 4 dogs was hybridized with a human HLA-DQB probe and the resultant RFLP is seen in Figure 21. This RFLP is highly polymorphic with no unique patterns present in either population. The sizes of the fragments generated are given in Table 25.

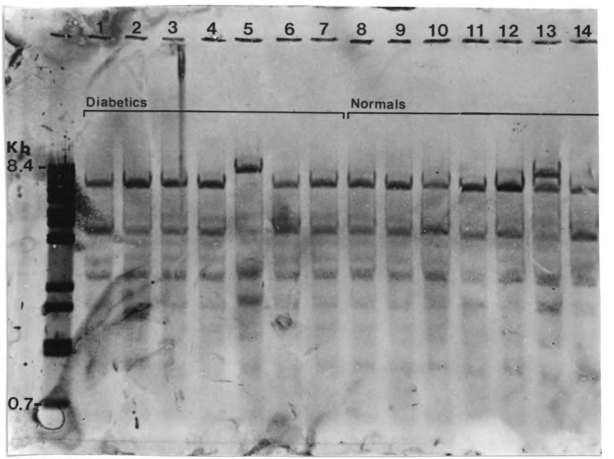

Figure 21. RFLP of Group 4 Dogs Probed with HLA-DQB. This RFLP was generated by hybridizing Taq I digested DNA from group 4 dogs with the human HLA-DQB class II probe. Molecular weight markers are on the far left. The grouping of IDDM animals and normal controls are indicated on the gel.

Table 25. Calculated Fragment Lengths of Figure 21.

	ANIMAL						
IDDM	1	2	3	4	5	6	7
					9332		
	7160	7160	7160	7160		7160	7160
	4755	4755	4755	4755	4755	4755	4755
	4115	4115	4115	4115	4115	4115	4115
	3670	3670	3670	3670	3670	3670	3670
	3080	3080	3080	3080		3080	3080
					2715		
	2605	2605	2605	2605	2605	2605	2605
	2045	2045	2045	2045	2045	2045	2045
		1860					
					1510		
	8	9	10	11	12	13	14
CONTROL						9330	
	7160	7160	7160	7160	7160	7160	7160
	4755	4755	4755	4755	4755	4755	4755
	4115	4115	4115	4115	4115	4115	4115
	3670	3670	3670	3670	3670	3670	3670
	3080	3080	3080		3080		3080
					2715	2715	2715
	2605	2605	2605	2605	2605	2605	
							2450
	2045	2045	2045	2045		2045	2045
					1605		
						1420	
		1205					
	1100		1100		1100		

*- Size in base pairs, rounded off.

This table lists the fragment sizes generated by the Taq I digested DNA from group 4 animals with an HLA-DQB probe.

HLA-DQB Gene Amplification. While the analysis of group 4 dogs did not yield an RFLP unique to IDDM dogs, the strong association of this region (DQ-like) with IDDM in humans and the IDDM disease mimicry between man and dog warranted further investigation. These investigations were modelled after the studies done in man.

The results from the electrophoresis of amplified product from thirty cycles of the polymerase chain reaction (PCR) using human primers are shown in Figure 22.. Both human and canine samples yielded

the expected product of approximately 240 bp. It is assumed that the fragment amplified from the dog represents the same region of the DQB chain that is amplified in humans using the same primers. The amplified products were bound to nylon membranes (dot blots) for use in hybridization with epitope specific oligonucleotides recognizing the presence or absence of Aspartate encodement at the 57^{th} codon. Figure 23 is a map of the dot blot to aid in interpreting hybridization results seen in Figure 24. Juvenile onset IDDM dogs and humans are indicated by a "*" next to their map position in Figure 23. Table 26 gives the HLA-DR and -DQ serotypes of the human controls used in these assays.

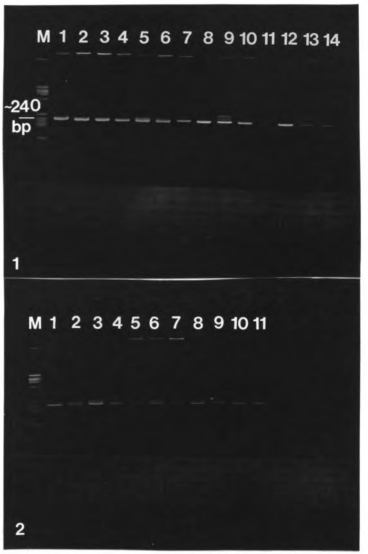

Figure 22. Results of Electrophoresis of PCR Products. The left lanes of each gel are molecular weight markers. Gel 1, lanes 1-6 are from amplified human genomic DNA. Lanes 7-14 of gel 1 and lanes 1-11 of gel 2 are the amplified products of canine genomic DNA

O H-1* O H-2* O H-3

O H-4 O H-5 O H-6

O C-1 O C-2 O C-3

O C-4 O C-5 O C-6

O C-7 O C-8 O C-9

O C-10 O C-11 O C-12

O C-13 O C-14 O C-15

O C-16 O C-17 O C-18 O C-19

Figure 23. Dot Blot Map.
Juvenile on-set IDDM individuals indicated by "*". Samples with an "H" prefix are human. Samples with a "C" prefix are canine.

Table 26. The Serotypes of Humans used in Dot Blots.

INDIVIDUAL	HLA SEROTYPE				
	A	B	C	DR	DQ
H1	(1,26)	(16, -)	(-, -)	(4, -)	(3, -)
H2	(2,31)	(7, -)	(2, -)	(4,11)	(3, -)
H3	(2,23)	(44, -)	(4, -)	(7, 9)	(2, 3)
H4	(2,31)	(44,60)	(3, -)	(4, 5)	(3, -)
H5	(2,30)	(60,63)	(2, 3)	(4, -)	(3, -)
H6	(3,30)	(60,62)	(3, -)	(4, 6)	(1, 3)

This table lists the HLA class I and II serotypes of the human samples used as controls in the amplification and oligonucleotide probing.

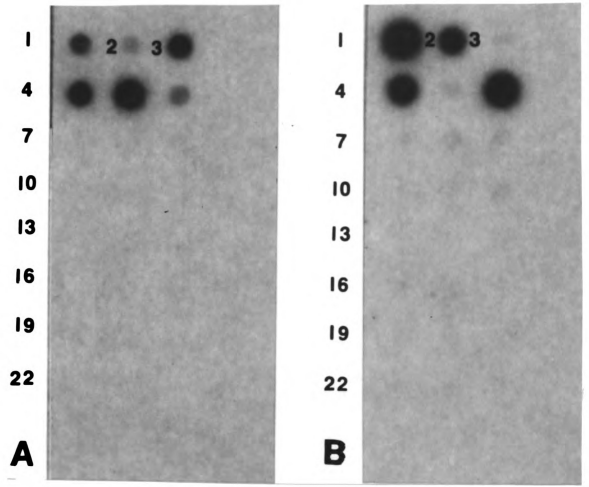

FIGURE 24. Results of Epitope Specific Probing. Blot A is the results of using epitope specific oligonucleotide probe DQw3.1-57. Bot B is the results of using epitope specific oligonucleotide probe DQw3.2-57. The organization of the dot blot is explained in Figure 23. The only hybridization of either probe was with the human samples. The probe DQw3.2-57 that recognizes non-aspartate encodement hybridized with both known human diabetics (position 1 and 2).

The results of oligospecific probing of the human controls were as anticipated. Homozygotes and heterozygotes for two of the HLA-DQ splits were recognized. Individuals with IDDM or a family history of IDDM hybridized with the DQw3.2 probe while others did not. Since all six individuals were serologically typed as HLA-DQ3 positive it was not surprising that at least one of the probes reacted with each individual.

Encouraged by the successful amplification of the canine equivalent of the HLA-DQB gene using human primers, portions of the amplified products were digested with several restriction endonucleases. The selection of enzymes with short (4 base pair) recognition sequences give a higher degree of polymorphism because of an increased number of recognition sites by random chance alone. Similarities in the amplified products of the human and canine diabetics should be visualized by this approach. The "four hitters" selected were; Alu I, Hae III, Hha I and Taq I. The enzyme Hga I was also chosen based on the sequence data of humans around the 57^{th} codon of the HLA-DQB gene. The enzyme Hga I should give different RFLP patterns for individuals with aspartate encodement at the 57^{TH} codon than do non-aspartate individuals when digested. The PCR products digested with the various enzymes were run out on a 10% polyacrylamide gel, stained with ethidium bromide and photographed using ultraviolet light. The resultant RFLP's are given in Figure 25. There were no common RFLP patterns unique to diabetic dogs and humans. The canine amplified products gave RFLP patterns different than their human counterparts with any given enzyme. Amplified diabetic canine DNA digested with Alu I did give a four band RFLP pattern unique from the

non-diabetic canine controls. The four bands are indicated by an "X" in panel F of Figure 25. These four bands are present in 8 of the 9 diabetics and all four bands are not present in the 6 control animals.

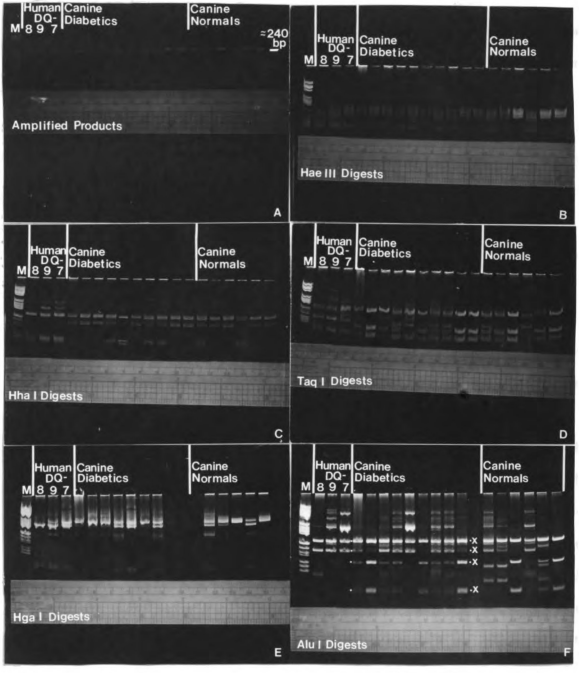

Figure 25. PCR Product RFLP's Generated by Multiple Enzymes. This figure gives the RFLP's generated from the digestion of PCR products with multiple enzymes. Panel A shows the undigested PCR products used. Panels B, C, D, E, and F represent the RFLP's generated by Hae II, Hha *I Taq I Hga I and Alu I respectively. The positions of human and canine diabetics and controls are indicated on each panel. The four band Alu I generated RFLP unique to canine diabetics is indicated by "X"'s on panel F.

Sheep Group 5:

The successful use of human HLA probes with the DLA system encouraged the inclusion of another species. Due to interests in disease association work and pedigree analysis the sheep was chosen. Based on the canine work it was felt that the initial work should involve a family. In order to trace haplotypic passage of OLA within a pedigree a family with multiple generations was chosen. The group 5 animals represent three generations. The relationship of the sheep selected for group 5 are best illustrated by the pedigree seen in Figure 26.

Class I Serology. The results of the OLA class I serotyping are not given because the OLA typing sera used was either non-specific or non-reactive. The reasons for this will be presented in the discussion.

Mixed Lymphocyte Reaction. The stimulation indices from the MLR performed with the cells of group 5 sheep are given in Table 27. All combinations of animals in the MLR resulted in stimulation. The two-way indices were used to reach this decision. None of the parings gave a stimulation index below 5.0. This value was empirically chosen based on previous experience evaluating MLR responses. None of the indices from the one-way pairings supported any of the two-way pairings that were marginally stimulatory. Correlation of the MLR results with the CLass II generated RFLP's was not possible. None of the animals had identical banding patterns which supports the interpretation.

Figure 26. Pedigree of Group 5 Sheep.

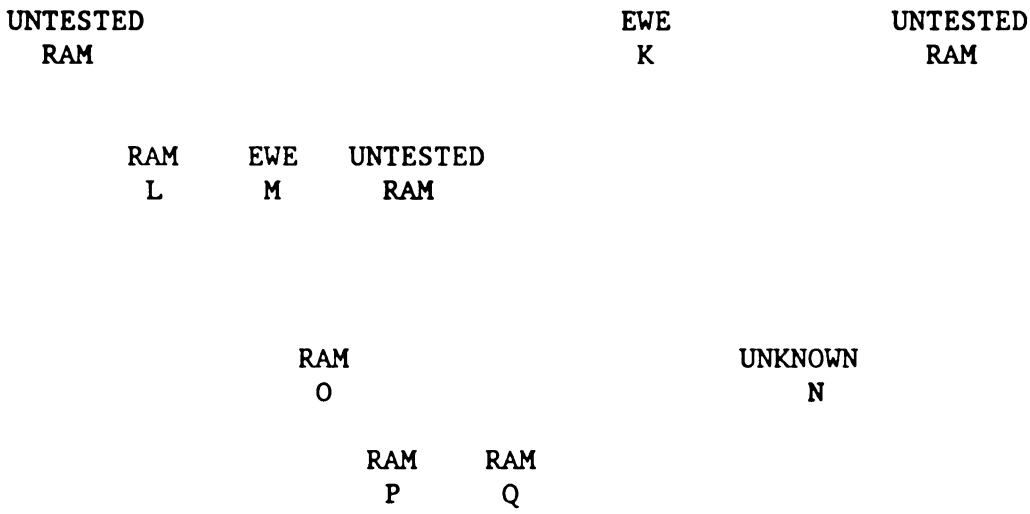

Table 27. MLR Stimulations of Group 5 Sheep.

ONE-WAY MLR			TWO-WAY MLR		
Sheep (R)	vs. Sheep (S)	Stimulation Index	Sheep (R)	vs. Sheep (R)	Stimulation Index
K	L	4.3	K	L	61.0
K	M	26.3	K	M	21.3
K	N	3.2	K	N	27.3
K	O	25.1	K	O	68.7
K	P	3.3	K	P	36.8
K	Q	4.4	K	Q	35.3
K	C1*	72.2	K	C1	84.1
L	K	6.4			
L	M	12.6	L	M	9.7
L	N	6.1	L	N	26.2
L	O	2.7	L	O	36.2
L	P	4.5	L	P	6.5
L	Q	1.3	L	Q	45.0
L	C1	19.3	L	C1	33.9
M	K	5.1			
M	L	4.2			
M	N	3.5	M	N	5.4
M	O	0.9	M	O	10.7
M	P	2.2	M	P	12.6
M	Q	1.7	M	Q	22.0
M	C1	7.0	M	C1	11.9
N	K	4.2			
N	L	2.8			
N	M	3.0			
N	O	1.6	N	O	14.7
N	P	1.3	N	P	23.5
N	Q	2.4	N	Q	12.5
N	C1	5.4	N	C1	6.0
O	K	21.8			
O	L	7.1			
O	M	2.7			
O	N	6.2			
O	P	1.4	O	P	34.0
O	Q	3.0	O	Q	29.9
O	C1	36.8	O	C1	21.6
P	K	1.9			
P	L	0.6			
P	M	4.3			
P	N	2.2			
P	O	0.6			
P	Q	1.6	P	Q	34.5
P	C1	4.3	P	C1	34.7
Q	K	3.1			
Q	L	4.3			
Q	M	25.5			
Q	N	7.6			
Q	O	1.8			
Q	P	1.0			
Q	C1	7.3	Q	C1	16.3

*- C1 is an unrelated control.

This table lists the stimulation indices generated from the MLR performed on group 5 sheep. None of the animals were considered non-stimulatory.

RFLP of Group 5 with HLA-DRA and -DRB probes. The RFLP's generated from hybridization of Taq I digested sheep DNA with human HLA-DRA and -DRB probes can be seen in Figure 27. These RFLP's are highly polymorphic. Passage of bands in a haplotypic fashion can be seen. An example of this is easily seen in RFLP B when the DRB probe was used. Two fragments, a 12875 and a 9785 bp, are seen in the ram O that are not present in the ewe K. Passage of the 12875 bp fragment can be seen in offspring P. It should also be noted that the fragment was introduced into the pedigree by the untested ram that was mated with ewe K to produce the ewe M, mother of ram O. The 9785 bp fragment is passed to offspring Q. This fragment probably came from the ram mated to ewe M that produced ram O. The sizes of the rest of the fragments of Figure 27 are given in Table 28.

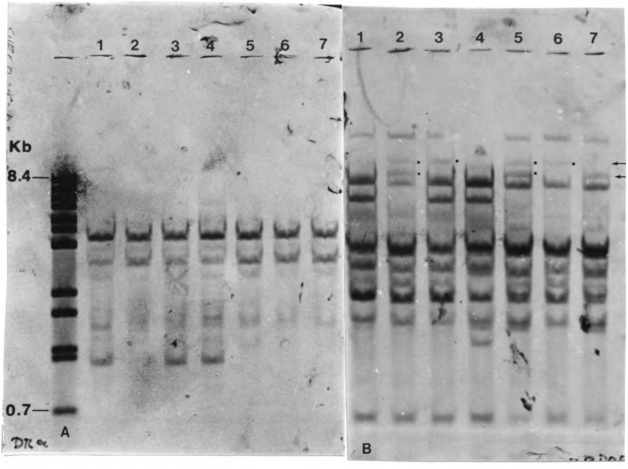

Figure 27. RFLP of Group 5 Sheep with HLA-DR Probes. The RFLP's A and B were generated by hybridizing the Taq I digested DNA of sheep from group 5 with the human HLA-DRA and -DRB class II probes respectively. Molecular weight markers are on the far left. Lanes 1, 2, 3, 4, 5, 6 and 7 are sheep K, L, M, N, O, P and Q respectively. Two fragments whose haplotypic passage can be traced from ram O to offspring P and Q are indicated by arrows

TABLE 28. Calculated Fragment Lengths of Figure 27.

	ANIMALS						
GEL	K	L	M	N	O	P	Q
A	3835*	3835	3835	3835	3835	3835	3835
	2990	2990	2990	2990	2990	2990	2990
	1630	1630	1630	1630	1630	1630	1630
	1125		1125	1125			
GEL							
B							
K	L	M	N	O	P	Q	
27315	27315	27315		27315	27315	27315	
	12875	12875		12875	12875		
	9785			9785		9785	
8370	8370	8370	8370	8370	8370	8370	
6390	6390		6390				
			4675	4675		4675	
3575	3575	3575	3575	3575	3575	3575	
2990	2990	2990	2990	2990	2990	2990	
2575	2575	2575		2575	2575		
2235	2235	2235	2235	2235	2235	2235	
1795	1795	1795	1795	1795	1795	1795	
			1730	1730		1730	
			1510				
740	740	740	740	740	740	740	

*- Size in base pairs, rounded off.

This table lists the fragment sizes generated by probing Taq I digested DNA of group 5 sheep with HLA-DRA and -DRB probes.

RFLP's of Group 5 with HLA-DQA and -DQB Probes. The results of hybridizing human HLA-DQA and -DQB probes with the Taq I digested DNA of sheep from group 5 can be seen in Figure 28. These experiments resulted in highly polymorphic patterns for both probes. A good example of haplotypic passage of fragments can be seen in RFLP B of Figure 28. Bands of 8380, 5950, 2780 and 2235 bp are passed from the sire O to the offspring Q as a block. The sizes of the rest of the bands are given in Table 29.

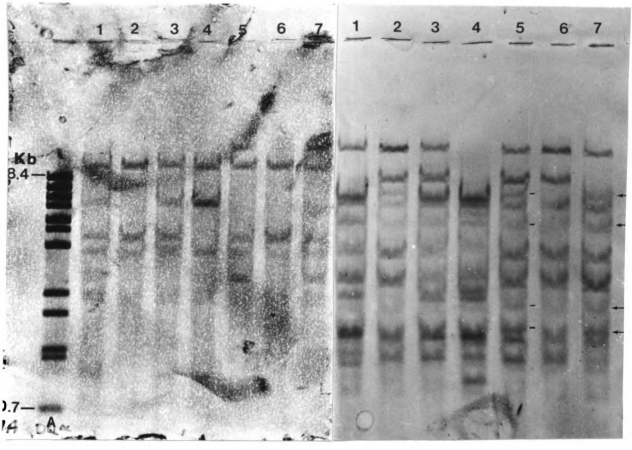

Figure 28. RFLP of Group 5 Sheep with HLA-DQ Probes. The RFLP's A and B were generated by hybridizing the Taq I digested DNA from sheep of group 5 with the human HLA-DQA and -DQB class II probes. Molecular weight markers are on the far left. Lanes 1, 2, 3, 4, 5, 6 and 7 are sheep K, L, M, N, O, P and Q respectively. The haplotypic block of fragments that can be traced from sire O to offspring Q are indicated by arrows.

Table 29. Calculated Fragment Lengths of Figure 28.

	ANIMAL						
GEL	K	L	M	N	O	P	Q
A					14820*		14820
	9900	9900	9900	9900	9900	9900	9900
		7421					
	5950		5950	5950			
	4060	4060	4060		4060	4060	4060
	3415	3415	3415	3415	3415	3415	3415
					2620		2620
GEL							
B							
18150	18150	18150			18150	18150	18150
	10260	10260			10260	10260	
	8380				8380		8380
7880		7880	7880				
	7420	7420	7420		7420	7420	7420
			6625				
5950	5950	5950	5950		5950		5950
			4650				
4245	4245	4245			4245	4245	4245
			3720				
3415	3415	3415	3415		3415	3415	3415
		3015	3015				
2895		2895	2895				
					2780		2780
					2235		2235
2105	2105	2105	2105		2105	2105	2105
1800	1800	1800	1800		1800	1800	1800
			1420				

*- Size in base pairs, rounded off.

This table lists the fragment sizes generated by probing Taq I digested DNA from group 5 sheep with HLA-DQA and -DQB probes.

RFLP's of Group 5 with HLA-DPA and -DPB Probes. The RFLP generated by hybridizing HLA-DPA and -DPB human probes with Taq I digested DNA from group 5 sheep is shown in Figure 29. There is a great deal of cross-hybridization of both the DPA and DPB probes with the alpha and beta genes of the other class II sub groups. The 2305 bp fragment of the RFLP A and the 4430 bp RFLP B are fragments that strongly hybridize with their respective probes. These fragments are indicated by arrows and represent strong candidates for HLA-DP-like genes in the sheep. Calculated fragment sizes for Figure 29 fragments are given in Table 30.

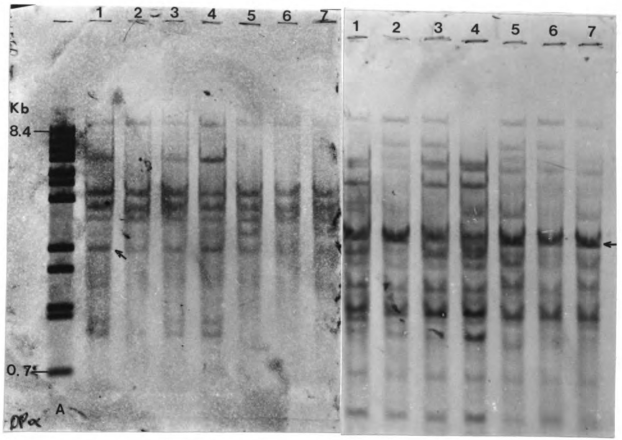

Figure 29. RFLP's of Group 5 Sheep with HLA-DP probes. The RFLP's A and B were generated by hybridizing the Taq I digested DNA from sheep of group 5 with the human HLA-DPA and -DPB class II probes. Molecular weight markers are on the far left. Lanes 1, 2, 3, 4, 5, 6 and 7 are sheep K, L, M, N, O, P and Q respectively. Arrows indicate the fragments thought to represent ovine HLA -DPA and -DPB homologs.

Table 30. Calculated Fragment Lengths of Figure 29.

	ANIMAL						
GEL	K	L	M	N	O	P	Q
A	5800*		5800	5800			
	3960	3960	3960	3960	3960	3960	3960
	3415	3415	3415	3415	3415	3415	3415
	3020	3020	3020	3020	3020	3020	3020
					2735		2735
	2305	2305	2305	2305	2305	2305	2305
GEL							
B							
16580	16580	16580			16580	16580	16580
	10130	10130			10130	10130	
	8790				8790		8790
7965		7965	7965				
7480	7480	7480	7480		7480	7480	7480
5800		5800	5800				
4755		4755	4755				
4430	4430	4430	4430		4430	4430	4430
3340	3340	3340	3340		3340	3340	3340
			2960				
2900	2900	2900	2900		2900	2900	2900
2630	2630	2630	2630		2630	2630	2630
2180	2180	2180	2180		2180	2180	2180
1775	1775	1775	1775		1775	1775	1775
1710	1710	1710	1710		1710	1710	1710
1650	1650	1650	1650		1650	1650	1650
1420	1420	1420	1420		1420	1420	1420
1030	1030	1030	1030		1030	1030	1030
710	710	710	710		710	710	710

*- Size in base pairs, rounded off.

This table lists the fragment sizes generated by probing Taq I digested DNA from group 5 sheep with HLA-DPA and -DPB probes.

Minisatellite Pedigree Analysis:

Immunogenetic analysis of group 3 dogs (an MHC identical family) indicated that detecting the passage of MHC haplotypes within a pedigree would not solve all disputed paternities. At this point in the project, DNA fingerprinting using minisatellite probes became technologically feasible and attractive. The results of initial fingerprinting work are presented here. A representative RFLP

generated by hybridizing the M13 minisatellite probe with the Hae III digested DNA of 14 randomly selected dogs is shown in Figure 30. There are approximately 15-30 fragments generated for each animal. This figure documents the high degree of polymorphism detectable in unrelated animals by this technology.

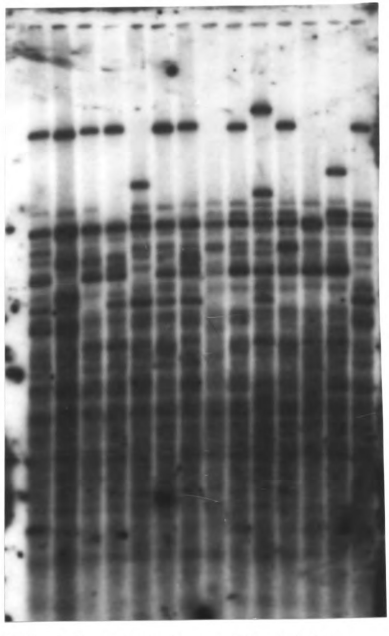

Figure 30. RFLP Generated by Probing with M13. Molecular sizes are indicate on the left. These are the results of probing 14 randomly selected dogs. Note the high degree of polymorphism. It should also be noted that there are less bands and band sharing in the higher molecular weight regions than lower weight areas.

A line drawing depicting the RFLP's generated by the probing of 32 randomly selected dogs is provided in Figure 31. This exemplifies the utility and power of minisatellite probing. There are relatively few unique bands in the higher molecular weight range and many common bands in the lower range. Despite this sharing there are no identical dogs.

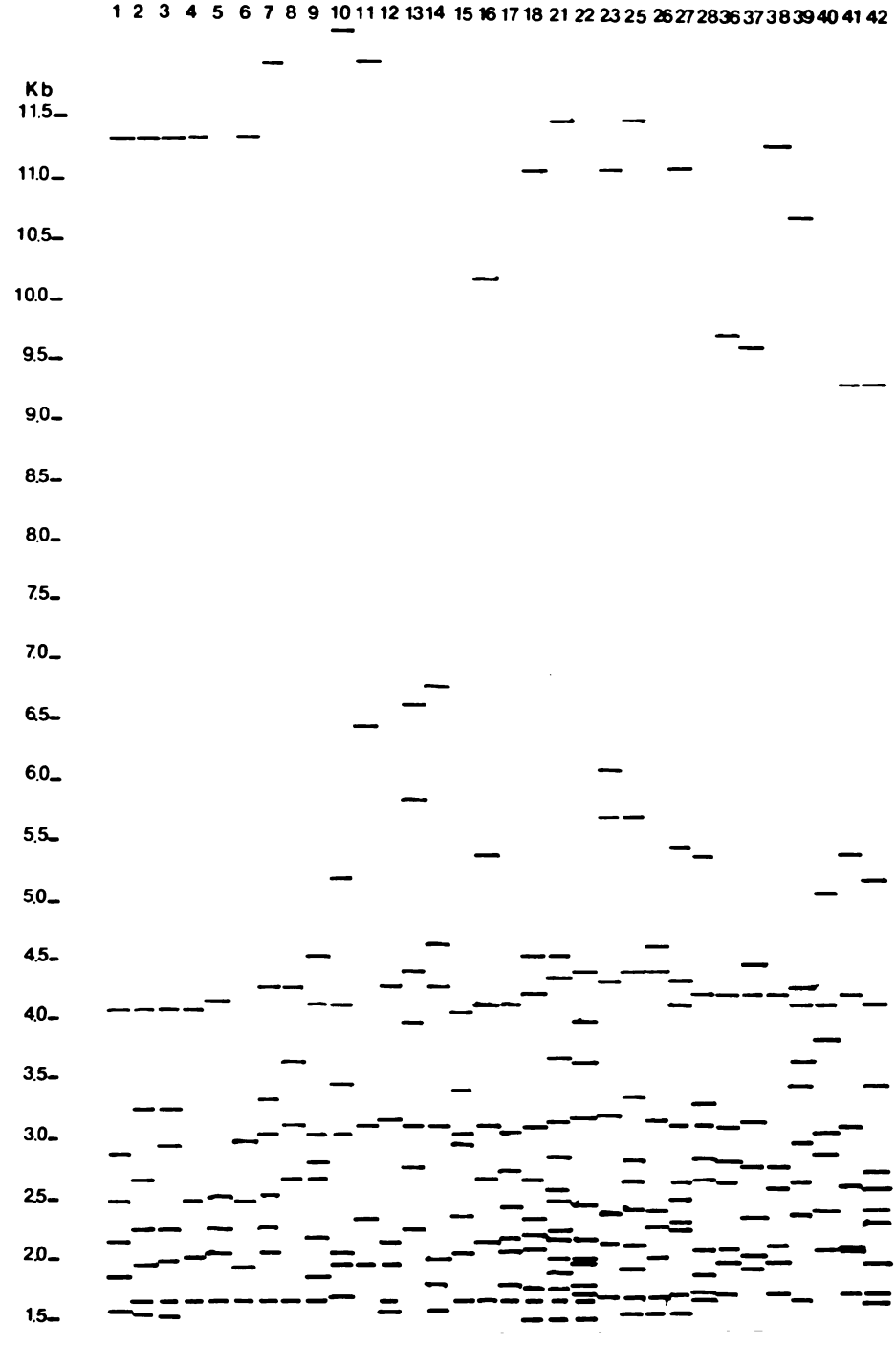

Figure 31. RFLP's of 32 Dogs Generated by M13. This is a line drawing depicting the RFLP's generated by the M13 minisatellite probing of 32 randomly selected dogs. Note the lesser number of bands and lower degree of band sharing present in the higher molecular weight regions than in the lower regions.

The RFLP's of two full canine pedigrees are represented in Figure 32. These RFLP's were generated by hybridizing Hae III digested DNA of the family members with the M13 minisatellite probe. Visual inspection of the RFLP's allow the assignment of the offsprings fragments to one or the other of the parents. There are no fragments present in offspring that are not present in the sire or dam. Animals 29, 30 and 31 were a family submitted specifically for pedigree substantiation. Dog 31 was the supposed littermate of dog 29. As indicated by the patterns animal 29 and 31 were nearly identical. This high degree of genetic similarity only allowed a non-exclusion interpretation of the results. No comment on inclusion probabilities could be made. Animals 32, 33, 34 and 35 are the previously studied group 3 dogs. While these animals were identical with all six HLA class II probes they are all unique using the M13 probe. The offspring 33 and 34 are each unique yet their identity in the pedigree can be traced. This is exemplary of the use of minisatellite probes for pedigree analysis.

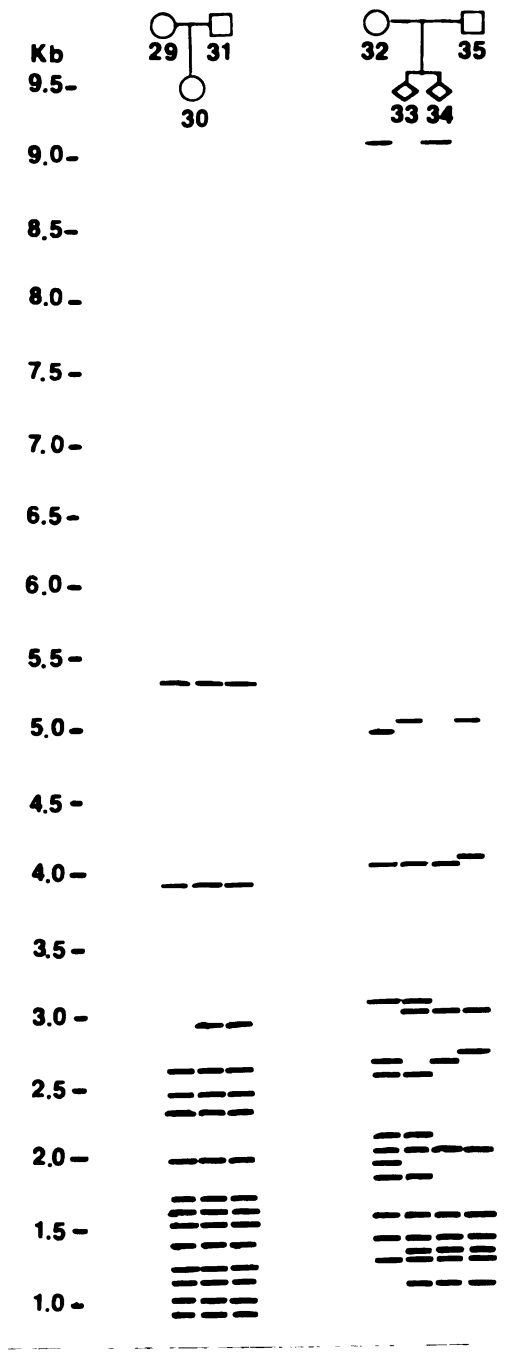

Figure 32. RFLP's of Pedigrees Probed with M13. This figure is a line drawing representing two families of dogs probed with the M13 probe. Note that animals 32, 33, 34 and 35 represent the animals from group 3 dogs.

DISCUSSION

The format for the discussion of the results will follow the same pattern as the previous sections. Each group of animals will be discussed separately. An overall discussion of the groups will follow in the conclusions section.

Group 1:

This is the group of dogs composed of three littermates.

Class I Serology. Review of the analyses performed on group 1 dogs produces conclusive evidence that there were two DLA identical siblings and one that was disparate. Antigens representing all three class I loci (DLA-A, -B and -C) were detected. The results of the DLA serotyping performed shows (Table 1) that dog X lacks the DLA-B5 epitopes on its lymphocytes while dogs Y and Z possess it. The dogs were identical at the DLA-A and -C loci.

Mixed Lymphocyte Reaction. This same pattern of identity and non-identity was also seen in the MLR responses (Table 2) of the animals. Dog X was stimulatory to both dogs Y and Z. Dogs Y and Z were also stimulatory to dog X. Dogs Y and Z were non-stimulatory to each other. Stimulation indices of 2.0 or below for the two-way response were considered non-stimulatory. These results reflected the serologic results of; animals Y and Z being DLA identical and animal X being DLA non-identical to it's siblings. The stimulation of dog X by dogs Y and Z reflect the presence of the DLA-B5 haplotype.

Class I RFLP. Analysis of the molecular work involving these siblings offers some surprises. The class I RFLP (Figure 1) generated with the different enzymes was consistent with those generated in other species. There appears to be a number of class I gene loci in the dog when the pH-2IIa probe was used. There are not as many copies present in the dog as has been seen in other species. The surprising facet of this RFLP was its failure to detect the serotyping differences. The RFLP generated with non-related animals (Figure 3) displayed a moderate degree of polymorphism between animals. It was from this result that this technique was used to detect the serologic differences at a molecular level The patterns of all three dogs are the same for each enzyme. The differences in DLA-B allotypes were missed. This could be accounted for if the DLA-B loci were a class II gene rather than a class I gene. The animals were DLA-A and -C identical and the class I generated RFLP's reflect that identity.

Class II RFLP's. The class II RFLP's generated are consistent with those generated by other investigators working with dogs and other species. The DRA-like homolog of the dog appears to be non-polymorphic with a single fragment generated by each animal with each enzyme (Figure 4). The DRB-like gene was highly polymorphic with each enzyme used on the animals (Figure 5). As in other species, DQA-like genes in the dog gave polymorphic patterns (Figure 6). The DQB-like genes of the dog were also polymorphic (Figure 7). The DPA and DPB analogs were present in the dog as evidenced by some unique fragments present in those RFLP's (Figures 8 and 9). These facts have also been documented by another group. Another feature of canine class II analogs were their cross-reactivity with each other. When alpha genes were used

lighter, less intense, hybridization fragments were present along with stronger hybridizing fragments. This phenomena was even more pronounced when beta gene probes are used. The ~1500 bp fragment present in both dogs Y and Z when digested with Bgl II and hybridized with beta probes (Figures 5, 7, and 9) was an example of this phenomena. The 1500 bp fragment gave the strongest signal when a DRB probe was used and was weaker with DQB and DPB. It is therefore interpreted as being a DRB-like gene. This cross-hybridization will be the focus of future work. Figure 33 is included as another example of the cross-hybridization phenomena. The RFLP generated was the result of hybridizing both an HLA-DRB and -DQB probe to separate lanes of a single gel. This gel was prepared by performing Taq I digests of a single animal and running multiple lanes. It can be seen (indicated by arrows) that there was cross-hybridization of the DRB probe (light fragments on the left lane) with DQB gene fragments (darker fragments on the right lane). The converse situation was also evident.

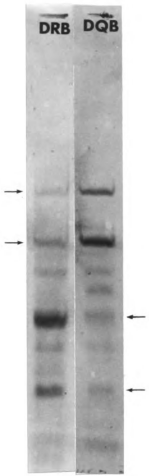

Figure 33. An Example of Beta Chain Cross-Hybridization. The left and right hand lanes are the DNA same dog digested with Tag I, electrophoresed on the same gel, separated and hybridized separately with either an HLA-DRB or -DQB probe. Arrows indicate examples of the cross-hybridization seen with beta chains. The probes were considered to be recognizing homologs when the bands were more intense.

It is noteworthy that the identity and non-identity of the siblings in group 1 was detected by the class II RFLP analyses. The presence of two fragments present in animals Y and Z that were absent in animal X correlates with the serologic and cellular work. These fragments were 1755 bp and 1490 bp in size and detected in the RFLP's generated by Bgl II digested DNA from dogs Y and Z that were probed with DRB. These fragments are indicated with arrows in Figure 5. As discussed earlier there were other fragments seen in Figures 7 and 9 that are thought to be cross-hybridization of the 1500 bp fragment. The presence of these fragments was only seen in the animals that had the DLA-B5 epitope serologically. These were animals Y and Z.

It was puzzling that of the 63 individual RFLP's generated by hybridizing the three enzymatic digests of the three dogs with the seven murine and human probes did not yield more evidence supporting the identity and non-identity seen in the siblings.

The lack of detection of the serologic discrepancy with the class I probe may be the first molecular evidence that DLA-B is class II and may in fact be DR-related. The differences in the DRB generated RFLP (Figure 5) correlate with the serotypes and MLR responses. These RFLP differences could be due to haplotypic transmission of class II genes and class I in concert and the detection of class II differences was coincidental. If this were true then there was no explanation for the lack of class I RFLP differences when DLA dissimilar animals were probed.

Group 2:

Group 2 was the full family composed of four offspring, the sire and dam.

Class I Serology. Review of the analyses performed with dogs from group 2 followed those seen with group I. The DLA serotypes given in Table 10 identified two pairs of DLA identical siblings. Dogs A and C were identical and so were dogs D and F. The dam, dog E is homozygous for DLA-B6 and -C11. Her haplotypes would be {A2/B6/C11} and {A9/B6/C11}. The sire, dog B, appears to have passed only one haplotype to the four offspring. That haplotype was {A9/B6/C12}. It was not possible to determine if the sire was homozygous for the A9 or C12 gens. He was heterozygous at the B locus having both B6 and B13 genes. The results clearly indicated haplotypic passage of the DLA genes and it was hoped that this would also be evidenced in the subsequent MLR and RFLP. The passage of the haplotypes would have been useful for pedigree substantiation due to the passage of the DLA-C12 epitope to all the offspring.

Mixed Lymphocyte Reaction. The MLR responses of group 2 dogs (Table 11) indicate that the sire was stimulatory and stimulated by all the other animals involved in the assay. A stimulation index below 2.0 for the two-way response was considered non-stimulatory for this assay. It should be noted that the haplotype dissimilarity of the littermates and the dam was not reflected in this assay as would have been expected. The presence of a DLA-A or -C dissimilar epitope did not elicit a stimulatory response. The sire had a DLA-B13 gene and was stimulatory and stimulated when co-cultured with the other family members. This may be further evidence that DLA-B is actually a class II gene.

Class II RFLP's. The class II RFLP's for this group were generated using DNA digested with only one enzyme (Taq I) and the six

human class II probes (HLA-DRA, -DRB, -DQA, -DQB, -DPA and -DPB). These RFLP's supported the previous statements about the DLA make-up of the group 2 family. Figure 10 was unremarkable and reflected the non-polymorphic trait of DRA-like genes. Certain fragments from the HLA-DRB generated RFLP (Figure 11) could be assigned as be maternal or paternal in origin. The 4560 bp and the 1600 bp fragments followed the passage of the C12 gene from the sire. The 2820 bp fragment follows the passage of the A2 gene from the mother. It should be noted that passage of this fragment did not affect the MLR responses. The passage of a 1970 bp fragment followed the passage of the C11 gene from the mother. A 2130 bp fragment was present only in the sire who was also the only B13 positive animal and the only MLR stimulatory animal. The HLA-DQA generated RFLP was unremarkable and non-polymorphic between animals (Figure 12). The HLA-DQB generated RFLP was also unremarkable (Figure 13). This was surprising. Passage of the A2 allege from the dam was evident with the presence of a 2710 bp fragment in the DPA generated RFLP (Figure 14). The DPB generated RFLP (Figure 15) also reflected the passage of many of the serologically defined alleles. The presence of 4790 and 2635 bp coincided with the expression of the C12 epitope. A 2200 bp fragment followed the passage of C11. The presence of a 2010 bp fragment in the sire reflected his lone expression of B13. The 3120 bp fragment present in the dam did not correlate with serology nor the MLR responses and was unable to be explained. The results of this pedigree documents the utility of RFLP's generated with MHC probes as useful tools for pedigree analysis as well as correlation of MHC haplotypes.

Group 3:

The members of the third group of dogs were another full-sibling family that included the sire and dam (Table 18). This was the family submitted for an immunogenetic work-up for a bone marrow transplant study.

Mixed Lymphocyte Reaction. The MLR responses (Table 19) of these animals indicated that they were mutually non-responsive and were thought to be possibly DLA identical. The MLR was difficult to interpret due to low responses even with unrelated control animals. The response of animal H and J in the two-way MLR was discounted because it was not supported by the responses in the individual one-way responses with these animals. A value of 2.0 was used as a break-point for stimulation and non-stimulation in this assay. This would be consistent with a DLA family identical at the class II loci.

Class II RFLP's. The RFLP's generated with the class II probes (HLA-DRA, -DRB, -DQA, -DQB, -DPA and -DPB) and Taq I digested DNA from the family of group 3 animals (Figures 16, 17 and 18) were unremarkable. They upheld the traits established for the different genes previously discussed. The animals yielded identical RFLP's for each probe used and the animal were interpreted to be DLA identical. This was consonant with the MLR results. No estimation of parentage could have been drawn from these analyses. The sire contributed no detectable alleles that were unique from the dam.

Group 4:

Group 4 was made up of seven juvenile on-set IDDM dogs and seven non-diabetic conditioned animals.

RFLP's. The RFLP generated by hybridizing Taq I digested DNA from juvenile on-set IDDM dogs and control dogs with the human HLA-DRB, -DQA

and -DQB probes were highly polymorphic (Figures 19, 20 and 21). While these probes would cover the map area associated with IDDM resistance and susceptibility in man there were no unique fragments nor were there unique patterns that could be associated with the susceptibility or resistance in the dog. A notable finding from these hybridizations was that a one probe/one enzyme combination cannot reflect the immunogenetic constitution of an animal. Four animals in Figure 19 have identical fragment patterns when HLA-DRB is employed. These were IDDM animals 2, 3, 4 and 7. When the RFLP's of these same animals using HLA-DQA were compared animals 2 and 7 were identical and dogs 3 and 4 are the same (Figure 20). When probed with HLA-DQB, Figure 21, dog 2 was unique while animals 3, 4 and 7 were identical. This documented that DLA identity can not be assessed using only one probe. This was also evidence that different DQ-like genes were associated with the DR-like genes as is seen in man and other species.

Amplification of DQB-like Genes. Pursuit of an association between IDDM and DLA led to the amplification of a 240bp fragment of the human and dog DQB genes. These were defined using primers based on human sequence data. Figure 22 shows the success of this experiment. There is a 240 bp fragment present in the human and canine IDDM and control amplified products. The ability to amplify genomic DNA using primers of a different species was considered a triumph. That fact will allow for rapid accumulation of knowledge concerning the MHC of domestic animals by comparing and contrasting the different species.

Oligonucleotide Probing. Epitope specific oligonucleotide probing of dot blots prepared with amplified DQB-like products failed to indicate analogous disease associated epitopes in the dog. Appropriate

reactions with the human IDDM and control subjects did validate the assay. This work is presented in Figures 23 and 24. The fact that the canine IDDM dogs and controls did not react with the human probes was attributed to the probable lack of homology present around the 57^{th} codon. The stringency washes would not allow any mis-match of sequence. The fact that the area could amplified (there was enough homology for that) encourage further investigations.

RFLP Mapping of Amplified DQB-like Products. RFLP mapping of the DQB-like amplification products using selected enzymes did uncover a similarity in IDDM dogs. The RFLP's generated (Figure 25) when Hae III, Hha I, Taq I (all 4 hitters) and Hga I (selected on human sequence data) did not show any patterns unique to IDDM animals. The RFLP's generated by these enzymes did differ between humans and dogs. Dogs gave different RFLP's than seen with humans and patterns of fragments were randomly dispersed throughout control and IDDM dogs. The RFLP generated (Figure 25) using the 4 hitter, Alu I, revealed a unique four band pattern present in 8 out of 9 diabetic animals. This four band pattern was not present in any of the six control animals. This encourages further work among these animals. It seems apparent that the DQB-like gene of the IDDM dog may play a role in disease susceptibility/resistance as its counterpart does in humans. Sequence data from these animals may lead to a canine specific IDDM marker.

Group 5:

The sheep selected for analyses represented three generations (Figure 26). These animals were selected to see if passage of OLA haplotypes could be seen and also the basis of preliminary work to address disease association work.

Class I Serology. An attempt to perform OLA serotyping was abysmal (data not shown). The OLA specific antisera obtained from Europe was either non-reactive or non-specific if reactive. This may have been due to the gamma irradiation performed to allow entry into this country. Consequently there were no serotypes assigned to the animals in group 5.

Mixed Lymphocyte Reaction. The MLR performed with the cells from the sheep of group 5 did not yield any non-stimulatory grouping of the animals (Table 27). The lower stimulation indices of the two-way MLR were not supported by the mutual one-way responses. Animals M and N are such a pair. Their two-way stimulation index was 5.4, the lowest of all the pairs. M's response to N was 3.5, the range was 0.9 to 7.0. N's response to M was 3.0, the range was 1.3 to 5.4. Review of these facts resulted in the conclusion that M and N were stimulatory. This form of comparison when used on the data of Table 27 supported the finding of no non-stimulatory pairs of animals. Extrapolation from this would lead one to expect that there would be no identical RFLP patterns among the animals.

Class II RFLP's. The RFLP's generated by the hybridization of Taq I digested DNA of sheep from group 5 with HLA-DRA and -DRB probes are depicted in Figure 27. The pattern of hybridization seen with the HLA-DRA was consistent with previous work in sheep and other species. DRA-like genes in sheep are not highly polymorphic. The DRB-like genes in the sheep were polymorphic. This observation was consistent with previous ovine work and other species studied. Haplotypic passage of fragments could be documented when the pedigree and the HLA-DRB generated RFLP were examined. There was a paternally derived 12875 bp

fragment present in ewe M whose passage to ram O and subsequently offspring P could be traced. A 9785 bp fragment also from ram O can be seen in offspring Q. That was the type of phenomena needing to be documented to enable this technique to be used for pedigree substantiation.

Figure 28 is the RFLP's generated by probing Taq I digested DNA from group 5 with HLA-DQA and -DQB probes. These results were also consistent with the systems of other species and information already published concerning OLA. The DQA- and DQB-like genes in the sheep were also polymorphic. DQB-like gene was more polymorphic than the DQA-like. Further documentation of haplotypic passage of fragments in a pedigree was available from Figure 28. A block of fragments, 8300, 5950, 2780 and 2235 bp, are passed in a haplotypic fashion from ram O to offspring Q. It was assumed that they were of paternal origin with respect to ram O. This haplotypic passage of fragments could be used to substantiate pedigree and determine disease associations.

The RFLP's generated with HLA-DPA and -DPB probes and the Taq I digested DNA of group 5 sheep was atypical. Many of the bands present in the RFLP's could be attributed to the cross-hybridization of HLA-DP probes to other subregion alpha and beta genes. However, there was a HLA-DPA generated fragment (2305 bp) and a HLA-DPB generated fragment (4430 bp) seen in the RFLP's A and B (Figure 29) respectively that were unique. These were strong hybridizing fragments that seem to indicate the presence of DP-like homologs in sheep. There is serologic evidence for their presence, but previous molecular work discounted their existence. This work indicates the presence of DP-like homologs in the sheep.

Careful examination of the RFLP's generated failed to disclose two animals with identical banding patterns. This was consonant with the MLR, where there were no non-stimulatory pairs. The two techniques complimented each other.

Minisatellite DNA FIngerprinting:

An RFLP generated by hybridizing an M13 derived minisatellite probe to Hae III digested canine DNA is given in Figure 30. There were highly polymorphic patterns generated for each of the non-related dogs used. This represent typical results. There was lower fragment sharing with unrelated animals in the higher molecular weight regions than compared with lower molecular weight regions. Preliminary work has demonstrated a probability of band sharing of 16% for fragments in the 20-4.5 kbp ranges compared to a 28% probability for 1.5-0.5 kbp fragments. This work was based on the RFLP's generated by 32 dogs probed with the M13 derived minisatellite probe. A line drawing depicting these RFLP's is shown in Figure 31. Based, on this data the probability that all fragments detected by the M13 probe in one individual are present in another is calculated to be 3×10^{-8}. This was consistent with work presented by others using different probes. The real power of this assay can be seen when the DLA identical dogs of group 3 were probed with the M13 probe. Results of this hybridization and that of another pedigree can be seen in Figure 32. Clearly the fragments present in the offspring can be assigned to their respective parents. There are no fragments seen in the offspring that were not of maternal or paternal origin. This work documents the applicability of minisatellite probing in the dog (DNA fingerprinting [paw -printing]) for pedigree substantiation.

CONCLUSIONS

In the genome of the dog there are MHC genes analogous to those of humans and mice. The RFLP's generated by probing these analogous genetic regions with human probes could be correlated with existing serologic and cellular techniques and offer the first molecular evidence that DLA-B may be a class II locus. While some of the molecular work may pre-empt the serology the complementation of cellular techniques meld their co-existence. This is to say that molecular genetic evaluation of an individual will compliment the serologic and cellular assays and not negate their use. The high degree of homology of the genes present in different species allows the exploitation of information from one to the other. It was from such transfer of information that it was found that portions of the canine DQB-like genes can be amplified, when defined with human primers, by the polymerase chain reaction. This finding is best described in the parlance of the times by one word; AWESOME! The potential for quantum leaps in the molecular knowledge of the immunogenetics of most species is available. It was with these techniques that it can be concluded that the IDDM susceptibility and resistance conferring factors related to the MHC differ in man an dog. It is apparent from the RFLP mapping done on the amplified DQB-like genes that juvenile on-set IDDM dogs have a commonalty in their DQB-like gene that is not shared with non-

diabetic animals. This indicates the desperate need for sequencing of dog genes and the probable finding of a canine specific IDDM marker.

An important conclusion drawn from the canine work is that while the use of xenospecific probes are a useful tool to identify and trace homologous genes in a species, detailed investigation of the genes will require the generation of species specific probes.

Sheep also possess genes analogous to human MHC genes. Conclusions drawn from the work with sheep in this study are that sheep do have DPA and DPB-like genes. It was unfortunate that correlation of serotyping and MLR work with the molecular investigations could not be made.

It is interesting to note that the passage of fragments could be documented in both species. These techniques could be used to substantiate pedigrees. This would be possible due to haplotype passage of these genes. It is the lack of this attribute that makes minisatellite probing so powerful a pedigree substantiation tool. The hypervariable regions are so numerous and disperse within a genome that the statistical power of DNA fingerprinting does not suffer due to linkage disequilibrium. The conclusion from preliminary work in the dog indicates that this will be a useful technique for pedigree substantiation.

FUTURE DIRECTIONS

Future investigations based on the knowledge and skills learned from this project will be in a directed effort to elucidate the MHC of the dog. In the near future the sequencing of amplified products is attainable. These techniques can be used for disease association work applicable to any species. Cloning of the DLA genes is also a real possibility using amplified products as probes. The mapping of the DLA region by clamped homogeneous electric field electrophoresis should be an attainable goal using xenogeneic probes. This would be a start to map the entire canine genome currently hindered by chromosomes technically difficult karyotype.

APPENDIX

APPENDIX (REAGENTS)

Phosphate buffered saline (PBS) pH 7.4:
2.7 mM KCl
137 mM NaCl
1.5 mM KH2PO4
8.0 mM Na2HPO4.

Ficoll-Hypaque:
54g Ficoll type 400 FS
qs 500 mls

113.65 ml 75% Hypaque

Combine together. Adjust refractive index to 1.3570 with distilled water.

Ethylenediamine tetraacetic acid (EDTA) buffered PBS double strength (2X):

McCoys Media:
Combine:
Powder for 4 liters
50 mls of 0.5% phenol red solution
16.4 g HEPES
3500 mls distilled water
Adjust to 7.4 qs 4 liters.
Sterile filter.

Prior to use add:
25cc sterile non-cytotoxic species specific sera.
Per 500mls 5ml Penicillin and Streptomycin (10000 U and ug/ml), 1 ml Fungizone (250 ug/ml) and 0.375 ml gentamycin sulfate (50mg/ml).

Eosin 5%:
5 grams eosin Y / 100 mls distilled water. Adjust pH to 7.2. Filter through a #4 Whatman and a 0.45 micron filter.

Formalin:
Adjust the pH of stock (37%) Formalin to exactly 7.4.

Saline:
150 mM NaCl

Chromium Chloride (CrCl3):10 mg CrCl3 / 50ml saline.
Prepare fresh.

Mitomycin C: 2 mg of mitomycin C / 40 ml saline.

DNA Lysis Buffer: 10 mM Tris pH 7.4
100mM NaCl
100mM EDTA
2% SDS

Sodium Acetate (3M):408.2 g NaAcetate / 100 mls
distilled water.

Tris Acetate EDTA (TAE) Electrophoresis Buffer:
242 g Tris-base
57.1 mls glacial acetic acid
200 mls 0.5M EDTA pH 8.0
qs 1 liter.

Ethidium Bromide: 1mg/ml stock solution.

Denature Solution: 1.5M NaCl
0.5M NaOH.

Neutralizing Solution:1.0M Tris-HCl
1.5M NaCl.
Adjust pH to 8.0.

SSPE Buffer (20X): 3.6 M NaCL
200 mM NaH2PO4
20 mM EDTA
pH 7.4.

SSC Buffer (20X): 3.0 M NaCl
0.3 M Na3 Citrate 2H2O
pH 7.4.

Transfer buffer MHC work:
10X SSPE
DNA fingerprinting:
10X SSC.

Denhardt's Solution (100X):
2% w/v ficoll
2% w/v polyvinylpyrrolidone
2% w/v Bovine serum albumin.

Dextran Sulfate (50X):50 g dextran sulfate / 100ml
distilled water.

Sheared Salmon Sperm DNA:
5 mg / ml salmon sperm DNA

dissolved in water. Sheared by passage through a 25 gauge needle 3 times. Prior to use boil for 10 minutes and immediately immersed in ice water.

Hybridization Buffer MHC work:
50 % Formamide
5X SSPE
5X Denhardt's
5% Dextran Sulfate
0.5% SDS
150ug / ml Sheared Salmon Sperm DNA

DNA fingerprinting work:
40% Formamide
6X SSC
5X Denhardt's
0.5% SDS
5 mM EDTA
1% Non-Fat Dried Milk.

Stringency Wash Buffers MHC work:
2X SSPE - 0.5% tween 20
wash twice at room temperature.
0.2X SSPE - 0.5% tween 20
wash twice at stringency temperature.

DNA fingerprinting:
2X SSC - 0.5% SDS
was twice at room temp, four times at stringency temperature.
1X SSC was twice at stringency temperature.

Color Development Buffers:
A: PBS - 5% Triton X-100.
B: Buffer A - 1M Urea - 1% Dextran Sulfate
C: 10 mM $Na_3C_6H_5O_7$ - 10 mM EDTA pH 5.0.

Hydrogen Peroxide: 3% H_2O_2.

Tetramethyl benzidine: 2 mg/ml in 100% ethanol.

LIST OF REFERENCES

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1. Ronne, H., Widmark, E., Rask, L. and Peterson, P.: Intron sequences reveal evolutionary relationships among major histocompatibility complex class I genes. Proc Natl Acad Sci 82:5860-4, 1985.

2. Benacerral, B.: Role of MHC gene products in immune regulation. Science 212:1229-38, 1981.

3. Trowsdale, J.: Still more genes in the MHC. Immunology Today 8:35-6,1987.

4. Bach, F. and Sachs, D.: Current concepts: Immunology; transplantation immunology. N Engl J Med 317:489-92, 1987.

5. Parham, P.: Function and polymorphism of human leukocyte antigen-A,B,C molecules. Am J Med 85(suppl 6A):1-5, 1988.

6. Schwartz, B.: Diversity and regulation of expression of human leukocyte antigen class II molecules. Am J Med 85(suppl6A):6-8, 1988.

7. Trowsdale, J.: The human MHC: an overview. Animal Genetics 19(suppl 1):5-9, 1988.

8. Maniatis, T., Fritsch, E. and Sambrook, J.: Molecular Cloning; a laboratory manual. Cold Spring Harbor, 1982.

9. Davis,L., Dibner, M. and Battey, J.: Basic methods in molecular biology. Elsevier New York, Amsterdam and London 1986.

10. Gillet, G., Mornet, E., Rocca, A., Degos, L., Cohen, D. and Pla, M.: Extensive genomic polymorphism in mouse 21-hydroxylase region. Immunogenetics 27:133-6, 1988.

11. Ragoussis, J., Bloemer, K., Weiss, E. and Ziegler, A.: Localization of the genes for tumor necrosis factor and lymphotoxin between the HLA class I and III regions by field inversion gel electrophoresis. Immunogenetics 27:66-9, 1988.

12. Male, D., Champion, B. and Cooke, A.: Advanced Immunology J.B. Lippincot Ca. Philadelphia 1987.

13. Gussow, D., Rein, R., Ginjaar, I., Hochstenbach, F., Seemann, G., Kotman, and Ploegh, H.: The human B2-microglobulin gene; Primary

structure and definition of the transcriptional unit. J Immunol 139:3132-8, 1987.

14. Dustin, M., Staunton, D. and Springer, T.: Supergene families meet in the immune system. Immunology Today 9:213-5, 1988.

15. Probing the parts of the immune network. Science Focus 2:1,14-5, 1988.

16. Williams, A.: The immunoglobulin superfamily takes shape. Nature 308:12-3, 1984.

17. Klein, J. and Figueroa, F.: The evolution of class I MHC genes. Immunology Today 7:41-4, 1986.

18. Steinmetz, M., Moore, K., Frelinger, J., Sher, B., Shen, F., Boyse, E. and Hood, L.: A pseudogene homologous to mouse transplantation antigens: Transplantation antigens are encoded by eight exons that correlate with protein domains. Cell 25:683-92, 1981.

19. Steinmetz, M., Winoto, A., Minard, K. and Hood, L.: Clusters of genes encoding mouse transplantation antigens. Cell 28:489-98, 1982.

20. Roitt, I., Brostoff, J. and Male, D.: Immunology. Gower Medical Publishing London and New York, 1985.

21. Albert, E., Baur, M. and Mayr, W.: Histocompatibility testing 1984. Springer-Verlag Berlin, Heidelberg, New York and Tokyo, 1984.

22. Lew, A., Valas, R., Maloy, W. and Coligan, J.: A soluble Class I molecule analogous to mouse Q10 in the horse and related species. Immunogenetics 23:277-83, 1986.

23. Lew, A., Bailey, E., Valas, R and Coligan, J.: The gene encoding the equine soluble class I molecule is linked to the horse MHC. Immunogenetics 24:128-30, 1986.

24. Lew, A., Maloy, W. and Coligan, J.: Characteristics of the murine soluble class I molecule (Q10). J Immunol 136:254-8, 1986.

25. Dobbe, L., Stam, N., Neefjes, J. and Giphart, M.: Biochemical complexity of serum HLA class I molecules. Immunogenetics @7:203-10, 1988.

26. Krangel, M.: Two forms of HLA class I molecules in human plasma. Hum Immunol 20:155-65, 1987.

27. Lew, A., Margulies, D., Maloy, W., Lillehoj, E., McCluskey, J. and Coligan, J.: Alternative protein products with different carboxyl termini from a single class I gene, H-2K^{b}. Proc Natl Acad Sci 83:6084-8, 1986.

28. Kudo, J., Chao, L., Narni, F. and Saunders, G.: Structure of the human gene encoding the invariant gamma chain of class II histocompatibility antigens. Nucleic Acids Res 13:8827-41, 1985.

29. Genuardi, M. and Saunders, G.: Localization of the HLA class II-associated invariant chain gene to human chromosome band 5q32. Immunogenetics 28:53-6, 1988.

30. Anderson, P., Morimoto, C., Breitmeyer, J. and Schlossman, S.: Regulatory interactions between members of the immunoglobulin superfamily. Immunology Today 9:199-203, 1988.

31. Joysey, V.: HLA-A, -B and -C antigens, their serology and cross-reaction. British Med J 34:217-22, 1978.

32. Arnett, F.: HLA genes and predisposition to rheumatic diseases. Hospital Practice 12:89-100, 1986.

33. Walker, R.: Inclusion probabilities in parentage testing. American Association of Blood Banks, Arlington, Virginia, 1983.

34. Klein, J.: Origin of major histocompatibility complex polymorphism: The trans-species hypothesis. Hum Immunol 19:155-62, 1987.

35. Andersson, L., Paabo, S. and Rask, L.: Is allograft rejection a clue to the mechanism promoting MHC polymorphism? Immunology Today 8:206-9, 1987.

36. Flaherty, L.: Major histocompatibility complex polymorphism: A nonimmune theory for selection. Hum Immunol 21:3-13, 1988.

37. Ragoussis, J., Bloemer, K., Weiss, E. and Ziegler, A.: Localization of the genes for tumor necrosis factor and lymphotoxin between the HLA class I and III regions by field inversion gel electrophoresis. Immunogenetics 27:66-9, 1988.

38. Carroll, M., Katzman, P., Alicot, E., Koller, B., Geraghty, D., Orr, H., Strominger, J. and Spies, T.: Linkage map of the human major histocompatibility complex including the tumor necrosis factor genes. Proc Natl Acad Sci 84:8535-9, 1987.

39. Turco, E., Fritsch, R. and Trucco, M.: First domain encoding sequence mediates human beta-chain gene cross-hybridization. Immunogenetics 28:193-204, 1988.

40. Nomenclature for factors of the HLA system, 1987. Immunogenetics 28:391-8, 1988.

41. Hood, L., Steinmetz, M. and Malissen, B.: Genes of the major histocompatibility complex of the mouse. Ann Rev Immunol 1:529-68, 1983.

42. Steinmetz, M., Frelinger, J., Fisher, D., Hunkapiller, T., Pereira, D., Weissman, S., Uehara, H., Nathenson, S. and Hood, L.: Three cDNA clones encoding mouse transplantation antigens: Homology to immunoglobulin genes. Cell 24:125-34, 1981.

43. Melief, C.: Remodelling the H-2 map. Immunology Today Circa 1982-3. Unable to locate volume and page numbers.

44. Steinmetz, M. and Hood, L.: Genes of the major histocompatibility complex in mouse and man. Science 222:727-33, 1983.

45. Teale, A.: Introduction to the BoLA system. Animal Genetics 19(suppl 1):1-4, 1988.

46. Lindberg, P. and Andersson, L.: Close association between DNA polymorphism of bovine major histocompatibility complex class I genes and serological BoLA-A specificities. Animal Genetics 19:245-55, 1988.

47. Hines, H. and Ross, M.: Serological relationships among antigens of the BoLA and the bovine M blood group systems. Animal Genetics 18:361-9, 1987.

48. Bensaid, A., Young, J., Kaushal, A. and Teale, A.: Genomic organization of the bovine class II MHC studied with field inversion gel electrophoresis. Animal Genetics 19(suppl 1):24-3, 1988.

49. Andersson, L., Lunden, A., Sigurdardottir, S., Davies, C. and Rask, L.: Linkage relationships in the bovine MHC region. High recombination frequency between class II subregions. Immunogenetics 27:273-80, 1988.

50. Fries, R., Hediger, R. and Stranzinger, G.: Tentative chromosomal localization of the bovine major histocompatibility complex by in situ hybridization. Animal Genetics 17:287-94, 1986.

51. Andersson, L. and Rask, L.: Characterization of the MHC Class II region in cattle. The number of DQ genes varies between haplotypes. Immunogenetics 27:110-20, 1988.

52. Andersson, L., Bohme, J., Rask, L. and Peterson, P.: Genomic hybridization of bovine class II major histocompatibility genes: 1. Extensive polymorphism of DQA and DQB genes. Animal Genetics 17:95-112, 1986.

53. Andersson, L., Bohme, J., Peterson, P. and Rask, L.: Genomic hybridization of bovine class II major histocompatibility genes: 2. Polymorphism of DR gens and linkage disequilibrium in the DQ-DR region. Animal Genetics 17:295-304, 1986.

54. Sigurdardottir, S., Lunden, A. and Andersson, L.: Restriction fragment length polymorphism of DQ and DR class II genes of the bovine major histocompatibility complex. Animal Genetics 19:133-50, 1988.

55. Andersson, L.: Organization of the bovine MHC class II region as revealed by genomic hybridizations. Animal Genetics 19(suppl 1):32-34, 1988.

56. Spooner R., Oliver, R., Sales, D., McCoubrey, C., Millar, P., Morgan, A., Amorena, B., Bailey, E., Bernoco, D., Brandon, M., Bull, R., Caldwell, J., Cwik, S., van Dam, R., Dodd, J., Gahne, B., Grosclaude, F., Hall, J., Hines, H., Leveziel, H., Newman, M., Stear, M., Stone, W. and Vaiman, M.: Analysis of alloantisera against bovine lymphocytes. Joint report of the 1st International Bovine Lymphocyte Antigen (BoLA) Workshop. Anim Blood Grps biochem Genet 10:63-86, 1979.

57. Anonymous: Proceedings of the Second International Bovine Lymphocyte Antigen (BoLA) Workshop. Anim Blood Grps biochem Genet 13:33-53, 1982.

58. Bull, R., Lewin, H., Wu, M., Peterbaugh, K., Antczak, D., Bernoco, D., Cwik, S., Dam, L., Davies, C., Dawkins, R., Dufty, J., Gerlach, J., Hines, H., Lazary, S., Leibold, W., Leveziel, H., Lie, O., Lindberg, P., Meggiolaro, D., Meyer, E., Oliver, R., Moss, M., Simon, M., Spooner, R., Stear, M., Teale, A. and Templeton, J.: Joint report of the Third International Bovine Lymphocyte Antigen (BoLA) Workshop,. Helsinki, Finland, 27 July 1986. Animal Genetics 20:109-32, 1989.

59. Spooner, R and Morgan, L.: Serological definition of MHC class II products in cattle. Animal Genetics 19(suppl 1):12-3, 1988.

60. Muggli, N., Stone, R. and Stear, M.: Identification of a bovine DRB-like pseudogene and the construction of probes for the definition of RFLP's of cattle. Animal Genetics 19(suppl 1):35-6, 1988.

61. Ennis, P., Jackson, A. and Parham, P.: Molecular cloning of bovine class I MHC cDNA. J Immunol 141:642-51, 1988.

62. Ansari, H., Hediger, R., Fries. and Stranzinger, G.: Chromosomal localization of the major histocompatibility complex of the horse (ELA) by in situ hybridization. Immunogenetics 28:362-4, 1988.

63. Bernoco, D. and Byrns, G.: Evidence of a second polymorphic ELA class I (ELA-B) locus and gene order for three loci of the equine major histocompatibility complex. Animal Genetics 18:103-18, 1987.

64. Guerin, G., Bertaud, M., Chardon, P., Geffrotin, C., Vaiman, M. and Cohen, D.: Molecular genetic analysis of the major histocompatibility complex in an ELA typed horse family. Animal Genetics 18:323-36, 1987.

65. Alexander, A., Bailey, E. and Woodward, J.: Analysis of the equine lymphocyte antigen system by southern blot hybridization. Immunogenetics 25:47-54, 1987.

66. Bernoco, D., Anczak, D., Bailey, E., Bell, K., Bull, R., Byrns, G., Guerin, G., Lazary, S., McLure J., Templeton, J. and Varewyck, H.: Joint report of the Fourth International Workshop on lymphocyte alloantigens of the horse, Lexington, Kentucky, 12-22 October 1985. Animal Genetics 18:81-94, 9187.

67. Yuhki, N. and O'Brien, S.: Molecular characterization and genetic mapping of class I and class II MHC genes of the domestic cat. Immunogenetics 27:414-425, 1988.

68. Verhoeven, A., Roos, M. and Ploegh, H.: A feline class II A gene with striking similarity to the HLA_DPA pseudogene. Immunogenetics 28:406-411, 1988.

69. Chardon, P., Kirszenbaum, M., Cullen, P., Geffrotin, C., Auffray, C., Strominger, J., Cohen, D. and Vaiman, M.: Analysis of the sheep MHC using HLA class I, II, and C4 cDNA probes. Immunogenetics 22:349-358, 1985.

70. Scott, P., Choi, C. and Brandon, M.: Genetic organization of the ovine MHC class II region. Immunogenetics 25:116-22, 1987.

71. Puri, N., deKrester, T. and Brandon, M.: Monoclonal antibodies to sheep MHC class II molecules recognize all HLA-D or subsets of HLA-D region products. Hum Immunol 20:195-207, 1987.

72. Puri, N., Walker, I. and Brandon, M.: N-terminal amino acid sequence analyses of the A and B polypeptides from four distinct subsets of sheep major histocompatibility complex class II molecules. J Immunol 139:2996-3002, 1987.

73. Cullen, P., Millot, P. and Nguyen, T.: Sheep histocompatibility antigens: a population level comparison between lymphocyte antigens previously defined in France, England and Scotland, and sheep red cell groups. Anim Blood Grps biochem Genet 16:19-34, 1985.

74. Outerridge, P., Cullen, P., Windon, R. and Brown, S.: Ovine lymphocyte antigens: a comparison of Australian and European antisera. Animal Genetics 19:159-69, 1988.

75. Deeg, H., Raff, R., Grosse-Wilde, H., Bijma, A., Buurman, W., Doxiadis, I., Kolb, H., Krumbacher, K., Ladiges, W., Losslein, K., Schoch, G., Westerbroek, D., Bull, R. and Storb, R.: Joint report of the Third International Workshop on canine immunogenetics. I. Analysis of homozygous typing cells. Transplantation 41:111-17, 1986.

76. Bull, R., Vriesendorp, H., Cech, R., Grosee-Wilde, H., Bijma, A., Ladiges, W., Krumbacher, K., Doxiadis, I., Ejima, H., Templeton, J., Albert, E., Storb, R. and Deeg, J.: Joint report of the Third International Workshop on canine immunogenetics. II. Analysis of the serological typing cells. Transplantation 43:154-61, 1987.

77. Dovern, R., Buurman, W., Schutte, B., Groenwegen, G. and van der Linden, C.: Class II antigens on canine T lymphocytes. Tissue Antigen 25:255-65, 1985.

78. Krumbacher, K., van der Feltz, M., Happel, M., Gerlach, C., Losslein, L. and Grosse-Wilde, H.: Revised classification fo the DLA loci by serological studies. Tissue Antigens 27:262-68, 1986.

79. Doxiadis, I., Krumbacher, K., Neefjes, J., Ploegh, H. and Grosse-Wilde, H.: Biochemical evidence that the DLA-B locus codes for a class II determinant expressed on all canine peripheral blood lymphocytes. Exp Clin Immunogenet (in press).

80. Sarmiento, U. and Storb, R.: Restriction fragment length polymorphism of the major histocompatibility complex of the dog. Immunogenetics 28:117-24, 1988.

81. Sarmiento, U. and Storb, R.: Characterization of class II alpha genes and DLA-D region allelic associations in the dog. Tissue Antigens 32:224-34, 1988.

82. Grosse-Wilde, H., Doxiadis, G., Krumbahcer, K., Dekkers-Bijma, A. and Kolb, H.: Polymorphism of the fourth complement component in the dog and linkage to the DLA system. Immunogenetics 18:537-40, 1983.

83. O'Brien, S.: Genetic Maps, Volume 4. Cold Spring Harbor, 1987.

84. Lie, W., Rothschild, M. and Warner, C.: Mapping of C2, Bf, and C4 genes to the swine major histocompatibility complex (swine leukocyte antigen). J Immunol 139:3388-95, 1987.

85. Sachs, D., Germana, S., El-Gamil, M., Gustafsson, K., Hirsch, F. and Pratt.: Class II genes of miniature swine I. Class II gene characterization by RFLP and by isolation from a genomic library. Immunogenetics 28:22-9, 1988.

86. Thistlehwaite, J., Auchincloss, H., Pescovitz, M. and Sachs, D. J Immunogenet 11:9-19, 1984.

87.Flanagan, M., Jung, Y., Rothschild, M. and Warner, C.: RFLP analysis of SLA class I genotypes in Duroc swine. Immunogenetics 27:465-9, 1988.

88. Chardon, P., Renard, C., Kirszenbaum, M., Geffrotin, C., Cohen, D. and Vaiman, M.: Molecular genetic analyses of the major histocompatibility complex in pig families and recombinants. J Immunogenet 12:139-49, 1985.

89. Ruff, G. and Lazary, S.: Evidence for linkage between the caprine leucocyte antigen (CLA) system and susceptibility to CAE virus-induced arthritis in goats. Immunogenetics 28:303-9, 1988.

90. Nesse, L. and Larsen H.: Lymphocyte antigens in Norwegian goats: serological and genetic studies. Animal Genetics 18:261-8, 1987.

91. Tykocinski, M., Marche, P., Max, E. and Kindt, T.: Rabbit class I MHC genes: cDNA clones define full-length transcripts of an expressed gene and a putative pseudogene. J Immunol 133:2261-9, 1984.

92. Sittisombut, N., Mordacq, J. Knight, K.: Rabbit MHC II. Sequence analysis of the R-DP A and B genes. J Immunol 140:3237-43, 1988.

93. Halldorsdottir, S. and Davies, B.: Restriction fragment length polymorphism (RFLP) in Icelandic horses observed by using human MHC class II probes. 18(suppl 1):29, 1987.

94. Nizetic, D., Stevanovic, M., Soldatovic, B., Savic, I. and crkvenjakov, R.: Limited polymorphism of both classes of MHC genes in four different species of the Balkan mole rat. Immunogenetics 28:91-8, 1988.

95. Jarvi, S. and Briles, W.: Identification of the major histocompatibility complex in the Ring-necked pheasant. p. 179 in The molecular biology of the major histocompatibility complex of domestic animal species Iowa State University Press Ames, 1988.

96. Tiwari, J. and Terasaki, P.: HLA and disease associations. Springer-Verlag New York, Berlin Heidelberg and Tokyo, 1985.

97. Dixon, F. and Fisher, D.: The biology of immunologic disease. Sinauer Associates, Inc. Sunderland, Massachusets, 1983.

98. Aparicio, J., Wakisaka, A., Takada, A., Matsuura, N. and Aizawa, M.: HLA-DQ system and insulin-dependent diabetes mellitus in Japanese: does it contribute to the development of IDDM as it does in Caucasians? Immunogenetics 28:240-246, 1988.

99. Thomsen, M., Molvig, J., Zerbib, A., de Preval, C., Abbal, M., Dugoujon, J., Ohayon, E., Svejgaard, A., Combon-Thomsen, A. and Nerup, J.: The susceptibility to insulin-dependent diabetes mellitus is associated with C4 allotypes independently of the association with HLA-DQ alleles in HLA-DR3,4 heterozygotes. Immunogenetics 28:320-7, 1988.

100. Hitman, G., Sachs, J., Cassell, P., Awad, J., Bottazzo, G., Tarn, A., Schwartz, G., Monson, J. and Festenstein, H.: A DR3-related DXA gene polymorphism strongly associates with insulin-dependent diabetes mellitus. Immunogenetics 23:47-51, 1986.

101. Todd, J., Bell, J. and McDevitt, H.: HLA-DQB gene contributes to susceptibility and resistance to insulin-dependent diabetes mellitus. Nature 329:599-604, 1987.

102. Morel, P., Dorman, J., Todd, J. and McDevitt, H.: Aspartic acid at position 57 of the DQ beta chain protects against type I diabetes: A family study. Proc Natl Acad Sci 85:8111-6, 1988.

103. Schwartz, B.: Workshop on the immunogenetics of the rheumatic diseases. Am J Med 85, 1988.

104. Lewin, H., Wu, M., Stewart, J. and Nolan, T.: Association between BoLA and subclinical bovine leukemia virus infection in a herd of Holstein-Friesian cows. Immunogenetics 27:338-44, 1988.

105. Stear, M., Dimmock, C., Newman, M. and Nicholas, F.: BoLA antigens are associated with increased frequency of persistent lymphocytosis in bovine leukemia virus infected cattle and with increased incidence of antibodies to bovine leukaemia virus. Animal Genetics 19:151-8, 1988.

106. Lewin, H. and Bernoco, D.: Evidence for BoLA-linked resistance and susceptibility to subclinical progression of bovine leukaemia virus infection. Animal Genetics 17:197-207, 1986.

107. Stear, M., Tierney, T., Baldock, F., Brown, S., Nicholas, F. and Rudder, T.: Class I antigens of the bovine major histocompatibility system are weakly associated with variation in faecal worm egg counts in naturally infected cattle. Animal Genetics 19:115-22, 1988.

108. Spooner, R., Morgan, A., Sales, D., Simpson, P., Solbu, H. and Lie, O.: MHC associations with mastitis. Animal Genetics 19(suppl 1):57-8, 1988.

109. Morrison, I., Goddeeris, B. and Teale, A.: The MHC in bovine Theileriosis. Animal Genetics 19(suppl 1):63-7, 1988.

110. Ruff, G. and Lazary, S.: Influence of the MHC on susceptibility to sarcoid affection in the horse and virus-induced arthritis in goats. Animal Genetics 19(suppl 1):58-9, 1988.

111. Millot, P., Chatelain, J., Dautheville, C., Salmon, D. and Cathala, F.: Sheep major histocompatibility (OLA) complex: linkage between a scrapie susceptibility/resistance locus and the OLA complex in Ile-de-France sheep progenies. Immunogenetics 27:1-11, 1988.

112. Brodsky, F. and Parham, P.: Evolution of HLA antigenic determinants: Species cross-reactions of monoclonal antibodies. Immunogenetics 15:151-66, 1982.

113. Mullis, K. and Fallona, F.: Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. Methods Enzymology 155:335-50, 1987.

114. Jeffreys, A., Wilson, V. and Thein, S.: Hypervariable "minisatellite" regions in human DNA. Nature 314:67-73, 1985.

115. Jeffreys, A. and Morton, D.: DNA fingerprints of dogs and cats.: Animal Genetics 18:1-15 1987.

116. Devor, E., Ivanovich, A., Hickok, J. and Todd, R.: A rapid method for confirming cell line identity: DNA "fingerprinting" with a minisatellite probe from M13 bacteriophage. Biotechniques 6:200-2, 1988.

117. Vassart, G., Georges, M., Monsieur, R., Brocas, H., Lequarre, A. and Christophe, D.: A sequence in M13 detects hypervariable minisatellites in human and animal DNA. Science 235:683-4, 1987.

118. Cicciarelli, J.,Ayoub, G., Terasaki, P. and Billing, R.: Improved HLA-DR typing of dialysis patients using monoclonal antibodies. Transplantation 33:558-60, 1982.

119. Southern, E.: Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:503-17, 1975.

120. SEE-QUENCE HLA-D™ product insert. Cetus Corporation, Emeryville, California, 1985.

121. Feinberg, A. and Vogelstein, B.: A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Analytic Biochemistry 132:6-13, 1983.

122. ibid Addendum. Analytic Biochemistry 137:266-7, 1984.

123. Quick large scale plasmid preparation provides transcript qualified DNA. Promega Notes, Promega Biotec, Madison, Wisconsin.

124. Schaffer, H. and Sederoff, R.: Improved estimation of DNA fragment lengths from agarose gels. Analytic Biochemistry 115:113-22, 1981.

125. GneAmp™ DNA amplification reagent kit product circular. Perkin Elmer Cetus, Norwalk, Connecticut.

126. Jeffreys, A., Brookfield, J. and Semeonoff, R.: Positive identification of an immigration test-case using human DNA fingerprints. Nature 317:818-9, 1985.

127. Helminen, P., Ehnholm, C., Lokki, M., Jeffreys, A. Peltonen, L.: Application of DNA "fingerprints" to paternity determinations. The Lancet March 12:574-6, 1988.