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ANALYSIS OF TAP1, TAP2, AND LMP2 POLYMORPHISMS WITH INSULIN DEPENDENT DIABETES MELLITUS IN A PUERTO RICAN POPULATION

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By

Leann Michelle Hopkins

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ABSTRACT

ANALYSIS OF TAP1, TAP2, AND LMP2 POLYMORPHISMS WITH INSULIN DEPENDENT DIABETES MELLITUS IN A PUERTO RICAN POPULATION

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Insulin Dependent Diabetes Mellitus (IDDM) is an autoimmune disease destroying insulin producing cells in the pancreas. There have been a variety of proposed environmental and genetic factors leading to IDDM. The strongest genetic factor is mapped within the Major Histocompatibility Complex (MHC). Several genes (HLA-B, HLA-DR, and HLA-DQ) have been linked to IDDM in Caucasian populations, however, they do not show similar linkage across racial lineages. Therefore, other genes within the MHC region have been proposed to contribute to IDDM including Transporter Associated with Antigen Processing (TAP) and Low Molecular Mass Protease (LMP). This study's goal was to determine if there were any associations with TAP or LMP polymorphisms in a Puerto Rican population using an ARMS PCR typing method. There were no dominant or recessive susceptibility or protective effects found for polymorphisms at TAP1 position 333 or 637, TAP2 position 379, 565, or 665, or LMP2 position 60.

In dedication to all my friends, family, and mentors who have helped me through the difficult times of my life with encouragement, support, and understanding. In part, this thesis was made possible by you. May we all share the joy of this accomplishment.

A special dedication in memory of Tracy Anne Hammer, a friend who is deeply missed. May we all share her zest for life and be inspired by her accomplishments.

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LIST OF ABBREVIATIONS

Alanine	ala
Amplificaiton Refractory Mutation System	ARMS
Ankylosing Spondylitis	AS
Arginine	arg
Aspartic acid	asp
Class II-Associated Invariant-Chain Peptide	CLIP
Cluster Differentiation	CD
Cystine	cys
Deoxyribonucleic Acid	DNA
Glutamic Acid Decarboxylase	GAD
Glycine	gly
Histadine	his
Homozygous Typing Cell Line	HTCL
Human Lymphocyte Antigen	HLA
Immunoglobulin A Deficiency	IgAD
Insulin Dependent Diabetes Mellitus	IDDM
Interleukin	IL
Invariant Chain	Ii
Isoleucine	ile
Low Molecular Mass Protease	LMP
Leucine	leu
Major Histocompatibility Complex	MHC
Multiple Sclerosis	MS
Neonatal Alloimmune Thrombocytopenia	NAIT
Non-Insulin Dependent Diabetes Mellitus	NIDDM
Polymerase Chain Reaction	PCR
Rheumatoid Arthritis	RA
Systemic Lupus Erythematosis	SLE
T-cytolytic cell	Tc
T-helper cell	Th
T-helper 1 cell	Th1
T-helper 2 cell	Th2
Threonine	thr
Transporter Associated with Antigen Processing	TAP
Valine	val
World Health Organization	WHO

INTRODUCTION

Diabetes mellitus is a disease of abnormal glucose utilization leading to hyperglycemia, that can be categorized into two main types, Insulin Dependent Diabetes Mellitus (IDDM) and Non-Insulin Dependent Diabetes Mellitus (NIDDM). NIDDM, also referred to as adult-onset or Type II diabetes, differs from IDDM in that it is inherited as an autosomal dominant disease and its major defect is associated with insulin resistance or an alteration in insulin secretion. IDDM occurs when there is an autoimmune destruction of insulin producing cells within the pancreas. IDDM does not have as strong a genetic component as NIDDM, however, genes that map within the Major Histocompatibility Complex (MHC) which are involved in antigen processing and presentation are thought to contribute to the disease.

This research will focus on the genetic aspects of Insulin Dependent Diabetes Mellitus (IDDM). It will address a specific immunological process of antigen processing and presentation involving the Transporter Associated with Antigen Processing (TAP) and Low Molecular Mass Protease (LMP) genes that may be involved with this disease. To determine the specific genetic contribution to the disease, a Puerto Rican population has been selected to help eliminate common allelic associations found in the Caucasian population.

LITERATURE REVIEW

Puerto Rican Population

Puerto Rico is a small island in the Caribbean. It lies south of Florida and north of South America. It is one of four islands that is referred to as the Greater Antillies. The northern half of the island is wet while the southern half is dry. The samples used in the proposed research project came from Ponce, a city in the southern half of the island.

The first inhabitants of Puerto Rico were the Archaic Indians 20,000 to 5,000 years ago. The next group of inhabitants to this island were the Igneri Indians, 200 B.C. to 700 A.D. The Igneri Indians were from the Arawak language family, a group who originated from South America. The Taino Indians are thought to be the last group of Indians to have inhabited the island. The Tainos were also from the Arawak language family. The Taino Indians are thought to have lived in Puerto Rico 1,000 A.D. to the 1500's.

Christopher Columbus and the subsequent male Spanish settlers arrived in Puerto Rico on Columbus' second voyage to the New World in 1493. However, due to illness and other misfortunes, the island was not colonized by the Spanish until 1508 by Juan Ponce de Leon after permission was granted to him by the Spanish government to start a colony under Spanish rule. The Taino Indians treated the Spanish

settlers as gods. They were very impressed and overwhelmed with all their armor and weaponry. As the Spanish began to mistreat the Tainos and enslave them, the Tainos committed mass suicide or retreated into the mountains of Puerto Rico. The population of Taino Indians rapidly declined from 50,000 to about 5,000 in one year due to these mass suicides.

Soon after in 1509, South Africans were brought to Puerto Rico as slaves to mine gold. Not only was Puerto Rico rich in gold and spices, it was strategically located in the Atlantic Ocean between Europe and the Americas. It was referred to as the "gateway to the riches". Because of these factors, the island was frequently invaded by the British, French, and Dutch. Puerto Rico was constantly under attack, but Spain kept control until 1819 when the United States won the Spanish American War. The Puerto Rican island was awarded to the United States in the victory settlement (Perl).

Because of the diverse history of the Puerto Rican island and its many ethnic inhabitants, the mitochondrial lineage was studied to determine possible ethnic characteristics. The D region within the mitochondria was sequenced and compared among 50 Puerto Ricans. It was concluded, based on this sequence analysis, that 68% were from Amerindian lineage, 26% from South African lineage, and 6% European lineage (Abujoub). Because of the diverse background, this population may be interesting for studying genetic diseases and disease susceptibility.

For example, Puerto Rico, unlike other tropical regions, does not share the same incidence of IDDM as Mexico or other tropical islands (12/100,000 vs 1-5/100,000 respectively) (Frazer). Puerto Ricans, do have a similar incidence of IDDM as Spain (11-12/100,000) (Frazer), a historical inhabitant of the island. The other two predominant racial groups historically found in Puerto Rico have different incidences than that of Puerto Rico. The incidence of IDDM in the Native Indian populations is negligible (Tajima) while the incidence of IDDM in the Black American population is 7/100,000 (Tajima). Puerto Rican diabetics, because of their mixed Spanish, African, and Indian heritage contributions, may possibly have different susceptibility allele haplotypes. This unique mix of haplotypes may help isolate the gene responsible for susceptibility to IDDM, which is thought to be one of several contributing factors.

Insulin Dependent Diabetes Mellitus

The etiology of Insulin Dependent Diabetes Mellitus (IDDM) is unknown. Genetic as well as environmental factors are thought to contribute to this autoimmune disease. IDDM is an autoimmune process that involves destruction of the beta cells in the islets of Langerhan within the pancreas. The beta cells in the islets produce insulin, which when complexed with glucose, can be taken up by cells and used for energy metabolism. Without this uptake mechanism, cells

within the body are not able to utilize glucose as an energy source and glucose remains in the blood at elevated levels.

IDDM is a serious disease leading to mortality if not recognized and treated properly. There are several consequences relating to hyperglycemia. Ketoacidosis and diabetic coma are acute metabolic complications of IDDM. There are also several long term complications of IDDM which involve the kidney, blood vessels, eye, and nerves. About 40% of all Type I diabetics will develop end-stage renal disease (Dedeoglu). Retinopathy will eventually develop in 85% of diabetics due to microangiopathy and increased capillary permeability. Microangiopathy along with neuropathy results in diabetic foot ulcers.

Hyperglycemia is a direct consequence of the beta cell destruction in the pancreatic islets. The infiltrating cells in the islets are Cluster Differentiation (CD) 8+ T-cells as well as CD4+ T-cells (Todd). At the diagnosis of IDDM, about 80% of the islets contain no beta cells. The remaining beta cells hyperexpress MHC class I, begin to express MHC class II, and contain immunoreactive interferon alpha (Leslie).

Environmental Causes of IDDM

There is reason to believe that environmental factors also contribute to the cause of type I diabetes as monozygotic twins are only 36% to 48% concordant for the disease (Leslie). Viral causes, such as a retrovirus,

Rubella virus, and Coxsackie virus, as well as toxic agents have been implicated as environmental causes since Type I diabetes can be induced in animal models given these agents (Leslie). Analogous to an infectious disease, IDDM exhibits epidemic patterns. In the U.S. Virgin Islands, the incidence rate for children below 15 years increased from 7.5 per 100,000 in 1979-1988 to 28.4 per 100,000 in 1984 (Tajima).

There seems to be a trend with certain viral epidemics and increased IDDM incidences (Tajima). Activation of a retrovirus can produce an autoimmune process possibly leading to diabetes. Macrophages may ingest the viral protein and present it with the MHC class II molecule to CD4+ T cells. This process releases cytokines which then involve other T helper (Th) cells, B lymphocytes, and cytotoxic T cells (Tc). Cytotoxic T cells can then begin to lyse the islet cells in the pancreas (Yoon). Rubella virus is thought to induce diabetes by altering normal cell membrane antigens by budding through them (Yoon). Recently, homology was found between the Coxsackie B4 viral proteins and glutamic acid decarboxylase (GAD) an islet-specific 64 kDa protein. The presence of anti-GAD antibodies correlates diagnostically with the onset of IDDM. T cells may also recognize GAD on the pancreatic beta cells when infected with this virus causing CD4+ T-helper 1 (Th1) cell (cell mediated immunity) and CD4+ T-helper 2 (Th2) cell (humoral

immunity) proliferation (Yoon, Solimena, Kaufmann, Leslie, and Tisch).

Genetic Causes of IDDM

Environmental factors, however, can not explain the apparent inheritable aspects of Type I diabetes. A genomic search for an IDDM susceptibility gene located several possible candidates. IDDM1 (MHC genes on chromosome 6p21), IDDM2 (insulin and insulin-like growth factor gene on chromosome 11p15) and at least eighteen other genes were shown to have linkage to the disease (Davies). The proposed candidate gene for IDDM3 (chromosome 15q26) is the insulin-like growth factor 1 receptor gene (Field). The IDDM4 (chromosome 11q) candidate gene is the fibroblast growth factor 3 (FGF3) gene (Davies and Hashimoto). IDDM5 (chromosome 6q) maps close to the estrogen receptor gene (ESR) and the gene encoding superoxide dismutase (SOD2) which may make the B-cells sensitive to damage by oxygen free radicals (Davies). Other microsatelite markers such as D2S326 (chromosome 2q) and D10S193 (chromosome 10q) map near genes encoding glutamic acid decarboxylase (GAD1 and GAD2), the autoantigen in diabetes (Davies). Other studies have scanned individual chromosomes and found several markers linked to IDDM. One study scanned chromosome 7 which includes such genes as the T-cell receptor and the glucokinase gene. This study found the glukokinase gene to be associated with susceptibility to IDDM (Rowe).

Nonetheless, IDDM1 (MHC) has been the gene with the strongest linkage to diabetes. From family studies, a positive association within a region on chromosome 6 encoding the MHC class I molecules was found with B8 and B15 and IDDM (Leslie). Later, a stronger association was found with MHC class II molecules which are more centromeric than DR or class I (Leslie). Figure 1 illustrates a map of the MHC region. The most telomeric loci are the class I loci, A, B, and C. In between class I and class II, lies class III. Within the class II region, TAP and LMP map between the centromeric DP locus and the telomeric DR and DQ loci.

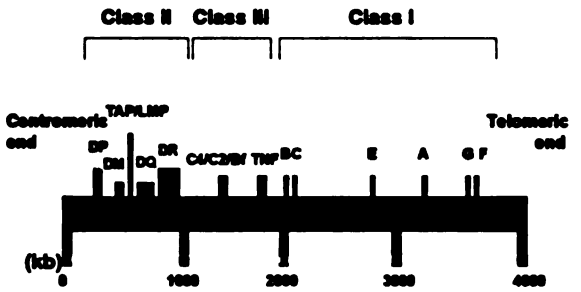

Figure 1. MHC Map
Illustrated is a map of the Major Histocompatibility Complex region located on chromosome 6. The class III region lies in-between class I and class II. The class II region(DR, DQ, TAP, LMP, and DP) is located the most centromeric. The class I loci (A, B, and C) are located on the telomeric end. DP is the most centromeric loci listed.

As the class II human lymphocyte antigen (HLA) genes are assembled as a heterodimer of alpha and beta subunits, they are designated as such with an A or B after the gene loci (EX: DQA and DQB). A number after the subunit designation indicates the gene origination. A 1 usually

designates the polymorphic genes while 3, 4, or 5 indicates public specificities (EX: DQB1). There are two basic types of nomenclature for HLA antigens. One revolves around the serological detection methods which names only the generic loci of the assumed beta chain (DQ) and generic allele (03) (EX: DQ3). The other nomenclature system is derived from molecular typing which names the gene (DQB1), the generic allele (03), and the subtype (02) of the allele (EX: DQB1*0302). The TAP genes have similar nomenclature, however, instead of alpha and beta, they are designated as TAP1 and TAP2. The LMP genes that map within the MHC region are designated LMP2 and LMP7 based on their proteasomal subunits.

HLA Associations with IDDM in Caucasians

The DRB1 gene, a class II MHC molecule, encoding the antigens DR3 and DR4 occur in 95% of Caucasian type I diabetics with a heterozygous DR3/4 conferring the greatest risk, however these alleles also occur in 50-60% of the normal population (Leslie and Lee). The DP locus, more centormeric than DR or DQ, was evaluated and certain haplotypes conferred risk, however, the risk was not as significant. This may suggest that the susceptibility to IDDM lies telomeric to the DP locus (Todd). An association with another MHC class II molecule, DQ, was found when 90% of the DR4 Caucasian patients had DQB1*0302 and only 10% had DQB1*0301 when they are distributed equally in a control

population (Leslie). Since the DQ molecule exists as a heterodimer of alpha and beta chains, an analysis of DQA alleles determined that DQA1*0301 and DQA1*0501 also added to the risk associated with IDDM in Caucasians (Cavan, 1993b).

After careful analysis of the DQ alpha and beta subunits, another association was determined. Certain amino acids in the antigen binding cleft of the MHC molecule were determined to contribute to the risk association. Not having the amino acid Asp at position 57 in DQB1 (Relative Risk=107, Heard), while having the amino acid Arginine (Arg) at position 52 in DQA1 was thought to denote maximal susceptibility in Caucasians (Penny, Trucco, Cavan, 1993b, and Leslie). Aspartic acid (Asp) at position 57 in the DQB chain forms a salt bridge with Asp in position 52 in the DQA chain. Absence of this salt bridge leads to an unstable DQ molecule (Janeway).

Therefore, the greatest risk for IDDM occurs with the Caucasian HLA haplotypes B8/DR3/DQB1*0201 (non Asp57)/DQA1*0501 (Arg52) and B15/DR4/DQB1*0302 (non Asp57)/DQA1*0301 (Arg52). However, some protective alleles, such as DR2, DR5, DR6 and DR7, also have portions of this risk haplotype. These protective DR alleles are present in some diabetics (Cavan, 1993b and Vicario). Because the susceptibility risk to IDDM increases the further centromeric the markers are located, other genetic markers within the class II region have been investigated. These

loci are located centromeric to DQ and include TAP and LMP genes involved with the processing of MHC class I peptides.

HLA Associations with IDDM in Other Races

The genes in the MHC region are in linkage disequilibrium, meaning they are inherited as a group. This makes the identity of the IDDM susceptibility gene difficult to determine because it is on the same haplotype and inherited almost indistinguishably from other genes on this haplotype. Disease association studies involving multiple races are beneficial because each race has uncommon or unique haplotype combinations and allele frequencies. This may allow the gene causing susceptibility to IDDM to appear on one haplotype in one race and another haplotype in other races. From this information, the identity of the predisposing gene may be isolated (She).

It has been determined that non-Caucasian diabetics have different class II alleles in addition to the traditional haplotypes that confer risk for IDDM. In the Black American population, risk has been associated with the traditional Caucasian haplotype as well as other haplotypes such as DR7\DQ9 and DR7/DQ2 (Vicario, Todd and Cavan, 1993a). In Ashkenazi Jewish and Hispanic population, there has not been an excess of DR3/DR4 heterozygotes found associated with IDDM (Rubinstein). In the Japanese population the incidence of IDDM is 2 in 100,000 (Tajima). The incidence of DR4 (a Caucasian risk allele) is increased

in the Japanese population (40.4%) (One Lambda) while the occurrence of IDDM is decreased. In the Japanese population, risk is associated with the DR4\DQ4 haplotype (Ronningen, 1991).

The Puerto Rican diabetic population associates with the same HLA DR/DQ markers identified in Caucasian populations, however, 10% of Puerto Rican IDDM population appear to have protective haplotypes (Frazer). The distribution of HLA alleles in Puerto Rico is more similar to the Spanish population (Frazer), which were historical inhabitants of the island. Also, the Puerto Rican population, like the Chinese and Black populations, has approximately 50% Asp/nonAsp heterozygosity at the DQB1 codon 57 loci; in the Caucasian population nonAsp homozygosity is thought to confer risk (Frazer and Lee). Having one Asp in position 57 of DQB1 is thought to confer protection in the Caucasian population. However, this is not seen in the Mexican American (Erlich) and Puerto Rican (Lee) populations. This suggests that the susceptibility gene may be associated with one haplotype in one racial group while it may be found on a similar, but different, haplotype in another racial group.

The Spanish inherit DR3 on a haplotype associated with B18 instead of B8 (Vicario). DR4 was also found to be inherited on a different haplotype than B15 in the Spanish (Morales). In Native Americans, the incidence of IDDM has been rare and populations that were Native American mixed

have protective haplotypes of Native American origin (DRB1*1402) and high risk haplotypes of European origin (Erlich).

Autoimmunity

Certain HLA haplotypes or parts of haplotypes are often observed with autoimmune disease. This may result from the right combination of alleles inherited on a haplotype leading to an aberrant immunological response. The MHC loci may also serve as a marker for a yet unidentified susceptibility gene to autoimmunity.

Autoimmunity has been characterized as a sustained and persistent T-cell dependent immunological response toward self tissues that produces long term tissue damage. Autoimmunity occurs when self tolerance has been lost. The cause of this loss of tolerance has been unknown. However, infectious agents are strongly associated with the autoimmune process. The only genetic marker strongly associated with autoimmunity has been the MHC molecules, however, their contributions were not the sole factor. Normal immunological recognition occurs when the MHC and T-cell receptor (TCR) interact. But, there has been no strong evidence associating autoimmunity with the TCR (Theofilopoulos). The complexity of the cause of autoimmunity can be attributed to its polygenic traits, phenotypic/genetic heterogeneity, incomplete penetrance, environmental factors, and chronic stimulation of common

pathogens. The combination of these factors plays a role in the abnormal immunological response against self (Theofilopoulos).

Antigen Processing and Presentation in Adaptive Immunity

Cell mediated immunity is a normal immunological response that relies on Tc cells. Tc cells are induced to mature by stimulation with interleukin-2 (IL-2) which is released by activated Th cells. The mature Tc cell, being CD8+, will associate via the TCR and the CD8 molecule with a target cell. The target cell presents antigen complexed with a MHC class I molecule. MHC class I molecules can be presented on all nucleated cells. Tc cells then release cytotoxic factors such as perforin, lymphotoxins, and serine dependent proteases that lyse their target cell (Janeway).

Antigen processing and presentation for MHC class I cells begins within the cytosol of cells as opposed to extracellularly. As virus reside within the cell and tumor and non-native cells present their unique proteins, immune recognition occurs. Therefore, this method of immunological response is directed toward virally infected cells, tumor cells, and determination of self vs. non-self cells. A protein within the cyotsol is degraded by a proteasome into oligopeptides of 8-10 amino acids in length. LMP2 and LMP7, when induced by γ-interferon, are a part of this proteasome that degrade proteins. These antigenic oligopeptides are

chaperoned by heat shock proteins (HSP). Within the cytosol, hsp70 and hsp90 bind and release these peptides in an ATP-dependent manner, to relay them to other heat shock proteins and ultimately to TAP. These oligopeptides are transported into the endoplasmic reticulum in an ATP-dependent manor by the TAP1 and TAP2 transmembrane heterodimer (Janeway and Srivastava).

Newly synthesized MHC class I alpha chains and β2 microglobulin are associated with a chaperone protein within the endoplasmic reticulum. This chaperone protein is an 88 kD membrane bound molecule called calnexin. HSP gp96 transfers the peptide to the MHC class I molecule (Srivastava). When the oligopeptide binds to the alpha chain of the MHC molecule, calnexin is released and the alpha chain, β2 microglobulin, and antigen complex can now make its way to the cell surface and be presented to CD8+ T-cells. The oligopeptide bound in the cleft of the MHC class I molecule, uses two anchor amino acid residues usually located toward the ends of the peptide. This leaves the rest of the peptide free to interact with the TCR. Self proteins as well as pathogenic cytosolic proteins are presented in this manor (Janeway).

Cell mediated immunity is also directed against intracellular bacteria and parasites found in intracellular vesicles. Acid proteases, such as cathepsin B and D, digest these proteins within these vesicles and complex them with

MHC class II. Inflammatory CD4+ Th1 cells with the TCR recognize an antigen complexed with MHC class II. This immune response typically takes place within and activates the macrophage, resulting in secretion of IL-1. The IL-1 activates Th cells and surrounding macrophages. IL-2 produced by the activated Th cell will induce maturation in the Tc cell (Janeway).

Humoral immunity is another type of immunological response which also occurs in IDDM as anti-GAD antibodies are a diagnostic indicator of IDDM. Humoral immunity involves the production of antibodies. This type of response is against extracellular proteins from bacteria and toxins. Extracellular proteins are endocytosed, degraded, and associated with MHC class II. Th2 CD4+ cells are activated by association of the CD4 and TCR molecules with the MHC class II and antigen complex and induced to secrete IL-4, IL-5, and IL-6. These cytokines induce proliferation of B-cells and differentiation into plasma cells (Janeway).

The MHC class II/CD4+ Th cell mechanism of antigen presentation is similar to the MHC class I method. In the endoplasmic reticulum, the MHC class II molecules associate with its chaperone molecule, the invariant chain (Ii). Ii is cleaved and 20-24 amino acids of the Ii (residue 81-104), called CLIP (class II-associated invariant-chain peptide), is left sitting in the cleft of the MHC class II molecule (Ghosh). The Ii/CLIP prevents MHC class II molecules from binding cytosolic proteins while it is being directed from

the golgi toward the endosomal compartments. Within these endosomal compartments, degradation of endocytosed proteins occurs. Endocytosed proteins are cleaved into oligopeptides of 13-18 amino acids (Monaco). In order for the MHC class II molecule to complex with the antigenic oligopeptide, DMA and DMB, genes that map within the MHC class II region on chromosome 6, are required to remove the CLIP molecule. The oligopeptide can now form bonds with the MHC class II molecule and go on to be presented on the surface of the cell (Janeway and Ghosh).

Origin of TAP and LMP Genes Involved in Antigen Processing

TAP and LMP are the genes associated with antigen processing and presentation in a MHC class I immunological response. LMP2 and TAP1 are believed to have evolved from LMP7 and TAP2 through a gene duplication event. The LMP7/TAP2 genes are believed to be the original genes because they have the least Alu repeats in the sequence (Beck). An Alu repeat unit consists of a 300 base pair repeating segment found widely distributed in 5% of the genome. Although the function of the Alu repeat is unknown, it is proposed that the purpose of these repeats is to act either as a control or regulatory sequence for nearby genes, as part of a replication origin, to facilitate chromosomal rearrangements, or is junk DNA (Voet).

Transporter Associated with Antigen Processing Genes

The TAP genes map within the MHC region (see Figure 1) and are thought to occur as a heterodimer with an alpha and beta subunit coded for by TAP1 and TAP2. TAP1 and TAP2 genes transverse the membrane of the endoplasmic reticulum and are thought to transport, by an ATP dependent mechanism, degraded oligopeptides into the endoplasmic reticulum. These peptides can then complex with the MHC class I molecule and β2 microglobulin and be expressed on the surface of the cell for detection by CD8+ T-cells (Powis, 1992a and 1992b, Spies). However, contrary to popular belief, class II molecules can also use this endogenous pathway to present antigen (Nuchtern). Certain TAP alleles have been found to alter the transport specificity of the degraded peptides into the endoplasmic reticulum of rats, varying the types of peptides that can complex with the MHC molecule (van Endert, 1992). It has also been shown that TAP proteins can transport peptides that are longer than the optimal class I 8-10 amino acid length and may also contain an ATP independent transport site (Androlewicz). Alterations in the TAP gene may contribute to an autoimmune process by preferentially altering the specificity or quantity of the peptides presented by the MHC. A predisposition to autoimmunity may be linked to a certain combination of TAP and MHC alleles.

Low Molecular Mass Protease Genes

Proteasomes, or multicatalytic proteinase complexes, are large molecular weight proteolytic complexes that are found in the cytosol and nucleus of all eukaryotic cells. The 20S proteasome is about 700kD and is composed of 28 subunits. The subunits are divided into two types, alpha and beta, which relates to the initial characterization and structure of the two *Thermoplasma acidophilum* proteasome subunits. The 20S proteasome is cylindrical in shape having a length of 15nm and a diameter of 11nm with a hollow core (Lupas). The proteasome structure is composed of four hollow rings arranged alpha ring, beta ring, beta ring, alpha ring. Each ring has seven subunits, The beta subunits are thought to have multiple enzymatic activities. The 26S proteasome is composed of the 20S proteasome with two 19S regulatory caps. These caps allow the degradation of ubiquitin conjugated proteins in an ATP dependent manner (Lupas).

LMP2 and LMP7 are two subunits of the 20S multicatalytic proteasome that map within the MHC region (see Figure 1). LMP2 is a γ-interferon induced gene translating a 219 amino acid protein (Kelly). The molecular mass of LMP2 is 23.3 kD unprocessed and 21.5 kD processed (Singal). LMP genes, when induced by γ-interferon, are part of the proteasome complex that cleaves cytosolic proteins which are favored for loading into the MHC class I molecule

(Howard and Driscoll). When not induced by γ-interferon, protein X and Y take their place (Akiyama). The function of LMP2 in the proteasome is thought to reduce cleavage of peptides after acidic residues, increase cleavage after basic, with no effect on hydrophobic residue cleavage activity (Gaczynska).

There is documented polymorphism of the LMP2 gene in humans (Kelly). The amino acid arginine or histidine is found at amino acid residue 60. This correlates with a guanosine or adenosine nucleotide substitution at the nucleotide position 3597 (van Endert, 1992). Because the LMP functions as a protease, certain alleles of the LMP genes may cut peptides differently or more efficiently, giving a different set of peptides to the TAP to transport and MHC to present. It can be hypothesized that this process may lead to an autoimmune state by degrading foreign or self peptides in an altered manner.

TAP and LMP known Mutations

Because the TAP and LMP genes are involved in antigen processing and presentation, mutations within these genes may decrease the efficiency of immune recognition, possibly leading to disease. Mutations within the TAP and LMP genes were searched for in several disease states.

Within TAP1, two original mutations were described at amino acid positions 333 and 637 (Powis, 1993 and Colonna).

See Table 1 for amino acid position, nucleotide position, amino acid substitution, and location in protein. The World Health Organization (WHO) recognizes these two mutations along with three others. They include amino acid substitutions at position 458 and 648 found in diabetic cells (Moins-Teisserenc and Jackson), and 661 found in a multiple sclerosis (MS) cell (Szafer). Four other mutations have been described in the literature which include amino acid position 254 and nucleotide position (nt) 2460 and 2512 in the 3' untranslated region found in a MS cell (Szafer), and a change in the amino acid at position 370 found in a colon cancer cell (Moins-Teisserenc),

Within TAP2, five original mutations were found at amino acid positions 379, 565, 665, 687, and nt 2091 (Powis, 1993 and Colonna). Positions 665, 687, and nt2091 were found to be in linkage disequilibrium. Therefore, in subsequent studies, only the 665 allele was typed for. The haplotype is 665Thr/687Stop/2091G or 665Ala/687Gln/2091T. See Table 2 for amino acid position, nucleotide position, amino acid substitution, and location in protein. WHO recognizes three of these five mutations in addition to five others. They include amino acid position 163, 387, and 436 (Moins-Teisserenc), 386 (Cano and Moins-Teisserenc), and 651 (Cano, Moins-Teisserenc, and Szafer). The mutations in amino acid 163 and 387 were found in a colon cancer cell line, the mutation at 651 was found in a MS cell, while the other cell origins were unavailable. The polymorphism at

Table 1. TAP1 Polymorphic Sites
Listed are the known polymorphic sites within the TAP1 gene. Included are the nucleotide and amino acid polymorphism and their corresponding location. As this data came from several sources with varying cDNAs used, the numbering systems may differ slightly.

TAP1 Polymorphic Sites				
Amino Acid Position	Nucleotide Position	Amino Acid Alleles	Nucleotide Alleles	Protein Location
254	834	Gly/Gly	C/T	trans-membrane
333	1069	Ile/Val	A/G	trans-membrane
370	1181	Ala/Val	C/T	trans-membrane
458	1582	Val/Leu	G/T	trans-membrane
637	1982	Asp/Gly	A/G	ATP binding
648	2015	Arg/Gly	A/G	ATP binding
661	2055	Pro/Pro	G/A	ATP binding
N/A	2460	N/A	C/T	3' untranslated region
N/A	2512	N/A	C/G	3' untranslated region

Table 2. TAP2 Polymorphic Sites
Listed are the known polymorphic sites within the TAP2 gene. Included are the nucleotide and amino acid polymorphism and their corresponding location. As this data came from several sources with varying cDNAs used, the numbering systems may differ slightly.

TAP2 Polymorphic Sites				
Amino Acid Position	Nucleotide Position	Amino Acid Alleles	Nucleotide Alleles	Protein Location
163	459	Val/Val	C/T	
379	1135	Ile/Val	G/A	
386	1158	Gly/Gly	G/T	ATP binding
387	1151	Val/Val	G/A	ATP binding
436	1288	Asn/Asn	C/T	ATP binding
565	1693	Thr/Ala	G/A	ATP binding
651	1951	Arg/Cys	C/T	ATP binding
665	1993	Ala/Thr	G/A	ATP binding
687	2059	Gln/Stop	T/C	ATP binding
697	2091	Val/Val	G/T	ATP binding

651 position may be of importance because the amino acid change is from Arg to Cys. The cystine allele may lead to altered disulfide bonds within the TAP2 protein. This cystine at amino acid position 651 may bind to other cystine residues found at amino acid positions 70, 197, 209, 353, 362, 394, 540, 571, or 641 (Cano).

Only polymorphisms in TAP1 position 333 and 637 and TAP2 position 379, 565, and 665 were included in the present study because at the time of selection, these were the only ones described in the literature. They have also previously been studied with regard to disease association and predisposition.

LMP2 polymorphisms have only been described in amino acid position 60 (Kelly). There is an Arg or His polymorphism which results in a guanosine or adenosine substitution.

TAP and LMP Associations with IDDM

It has been determined there is no association between the TAP1 gene and IDDM in Caucasians (Colonna, Ploski, van Endert, 1994, Jackson, and Caillat-Zucman, 1992 and 1993). It is believed that there is no linkage association between the TAP1 and TAP2 genes because a recombinational hot spot is thought to occur between these two genes (Caillat-Zucman, 1992 and 1993, Cullan and van Endert, 1994). A recombinational hot spot is a site on the chromosome where recombination occurs more frequently. A hot spot for

recombination has recently been mapped within the second intron of TAP2 spanning a 850 base pair region. This allows alleles of TAP2 to be found in linkage disequilibrium with MHC class II molecules, DR and DQ. However, TAP1, being more centromeric, is unlinked with DR and DQ molecules. This is thought to be the best characterized recombinational hot spot (Cullan).

It has been noted that linkage disequilibrium is present with DR, DQ, and TAP2 alleles (van Endert, 1992 and Tighe). TAP2*0101 (TAP2A) (See Table 3 for TAP nomenclature. All TAP alleles will show corresponding original nomenclature in parenthesis for ease of comparison), an allele that is increased in IDDM, is thought to be in linkage disequilibrium with the haplotypes DR4-DQ8 and DR3-DQ2 (Ronningen, 1993). However, other studies have demonstrated the susceptibility allele TAP2*0101 (TAP2A) and protective allele TAP2*0201 (TAP2B) in the absence of linkage disequilibrium in IDDM (Caillat-Zucman, 1993). If a TAP allele is increased in IDDM secondary to linkage disequilibrium, then it is assumed the TAP allele only serves as a marker for the true IDDM susceptibility gene.

TAP and LMP Genes and other Disease Associations

There has not been a strong association with TAP polymorphisms and other autoimmune diseases such as celiac disease, ankylosing spondylitis (Colonna), or multiple sclerosis (Kellar-Wood). Juvenile rheumatoid arthritis

Table 3. TAP Nomenclature
Listed below are several nomenclature systems used interchangably for the TAP1 and TAP2 loci and their corresponding types.

TAP1

				333	458	637	661
TAP1*0101	TAP1*0101	1A	A	ile	val	asp	G
TAP1*0102	TAP1*02011	1B	B	val	val	gly	G
TAP1*0102	TAP1*02012	1B	B	val	val	gly	A
TAP1*0103	TAP1*0301	-	C	val	val	asp	G
TAP1*0102	TAP1*0401	1B	B	val	leu	gly	G
-	-	1C	D	ile	-	gly	-

TAP2

				379	565	665	687
TAP2*0101	2A	A	A	val	ala	thr	stop
TAP2*0201	2B	B	B	val	ala	ala	gln
TAP2*0102	2C	C	C	ile	ala	thr	stop
TAP2*0102	2D	D	C	ile	thr	thr	stop
TAP2*0101	2E	E	A	val	thr	thr	stop
TAP2*0202	-	-	D	ile	thr	ala	gln
TAP2*0201	-	G	B	val	thr	ala	gln
TAP2*0202	2F	H	D	ile	ala	ala	gln

(DR8) has a weak association with a TAP allele polymorphism. Celiac disease may have a protective effect with a TAP allele (0% vs 22.5%) (Caillat-Zucman, 1993), however, there are conflicting results obtained from celiac disease studies.

Immunoglobulin A (IgA) deficiency (IgAD) is a condition where surface Ig positive B cells fail to undergo differentiation to plasma cells. It has been linked to HLA alleles A1, A2, A28, B8, B40, DR3, and DR7. The association between IgAD and TAP1 and TAP2 alleles was examined with no association being found (Powis, 1994). However, linkage disequilibrium was determined for the haplotype TAP2A/DQ2/DR3/B8/A1.

Neonatal alloimmune thrombocytopenia (NAIT) is a condition during pregnancy when the mother becomes sensitized to the platelet HPA-1a antigen. It was shown to be associated with DR3, DR13 and DRB3*0101 HLA antigens (Braud). Braud studied NAIT and TAP associations and found that TAP1*0102 (TAP1B) was only associated in DR13, DRB3*0101 positive patients. It is felt, however, that this is probably due to a yet unknown linkage within the HLA region and not with the TAP gene. This study confirmed the TAP2 (Thr665 Stop687) linkage.

Psoriasis is characterized by an infiltration of the dermis and epidermis by neutrophils, lymphocytes, macrophages, and mast cells causing a chronic hyperproliferative inflammatory disease. Psoriasis has been

linked with the HLA antigens DRB1*0701, DQA1*0201, DQB1*0303, B57, and Cw6. This disease is thought to be a T-cell mediated autoimmune disease as supported by the HLA predisposition. The association with psoriasis and TAP2 and DP genes was studied and no association was found with these genes (Falker).

Systemic Lupus Erythematosis (SLE) is an autoimmune disease process diagnostically characterized by antinuclear antibodies targeted against nucleic acids. SLE is associated with the HLA antigens DR2 and/or DR3 and more specifically with the haplotype DRB1*1501, DQA1*0102, DQB1*0602. The association of SLE with TAP1 and TAP2 in a Chinese population was determined. This study concluded that there was no TAP association found (Savage). It was also determined that the TAP allele frequencies in this Chinese population were similar to previously reported Caucasian frequencies (Savage).

The effects of matching at the TAP loci to try to reduce the incidence of kidney graft rejection was evaluated (Chevrier). A decrease in TAP2B in graft recipients because of the excess DR3 and DR4 was found in this population. DR3 and DR4 are thought to be in linkage disequilibrium with TAP2A, TAP2C, and TAP2D alleles. There was an increase in TAP1B in the recipient group as a whole (rejection and non-rejection) due to the assumed linkage with the initial disease, glomerulonephritis. Results from this study concluded there was no benefit to match at the TAP loci, but

it was important to match at the DR loci to reduce graft rejection, however, the exact mechanism of allorecognition is not clearly defined.

Celiac disease is defined as an enteropathy of the small bowel upon exposure to dietary gliadin proteins such as wheat, barley, rye, and oats. Celiac disease has been associated with DQA1*0501, DQB1*0201, and DR3 or DR7 with DR3 or DR5 (Tighe). In an Israel population, 20% lack the DQ association but are DR4 positive. There was no association found with TAP2 and Celiac disease in these populations (Tighe).

Ankylosing Spondylitis (AS) is a rheumatologic disease characterized by inflammation of axial skeletal structures, in particular, the sacroiliac joints and is associated with the HLA B27 antigen (Maksymowych, Westman, Burney, Ploski). LMP2, at amino acid position 60, arginine homozygosity has been shown in one study to be associated with the development of acute anterior uveitis and peripheral arthritis is AS patients (Maksymowych). Linkage disequilibrium has been demonstrated with HLA DR3 and arginine homozygosity in LMP2 (Makeymowych). Another study determined an increase in TAP1C and TAP2E alleles in AS patients. However, the increase in TAP2E was shown to be in linkage disequilibrium with B27. No association with LMP2 was detected (Barney and Westman). Linkage disequilibrium was also found with TAP1B and LMP2B (Ploski). Another study found no association with TAP1 or TAP2 and AS (Westman).

However, DRB1*08, DPB1*0301, and LMP2 Arg homozygosity had a relative risk of 14.8 in Juvenile AS. Although this increased risk was due to an increase in DRB1*08 and DPB1*0301 alleles, LMP2 homozygosity had an additive but independent susceptibility factor for juvenile AS. There was an increase in LMP2 homozygosity in patients without the DRB1*08 and DPB1*0301 alleles. The LMP2 Arg homozygosity was associated with acute anterior uveitis (Ploski).

Rheumatoid Arthritis (RA) is an autoimmune disorder with a chronic progressive inflammatory process involving multiple joints. This disease has been linked to the DR4 molecule of the MHC (Wordsworth and Maksymowych). There was no significant difference between TAP1 and RA, however, TAP2B was significantly reduced while TAP2D was significantly increased in RA patients (Wordsworth). However, when compared to DR4 positive controls, this association was not significant suggesting linkage disequilibrium between DR4 and TAP2D. There was not significant association with LMP2 residue position 60 and RA with either susceptibility or disease outcome (Maksymowych).

Purpose and Objectives

As DR3/4 susceptibility haplotypes do not hold true across all racial groups, the DR and/or DQ loci do not solely account for the inheritable aspects of IDDM. Since it is thought that the problem may be telomeric to DP, this study will focus on two novel groups of class II genes that map between DP and DQ. Since risk increases centromerically from HLA-B to DQ and TAP and LMP genes are centromeric to DQ but telomeric to DP, alterations in these TAP or LMP genes may be the true cause of altered peptide processing and presentation leading to an autoimmune process. A non-Caucasian population will be evaluated to minimize any Caucasian haplotypic inheritance so the susceptibility gene may become apparent when compared to other ethnic groups. This population will be evaluated for TAP1, TAP2, and LMP2. These genes are thought to be involved with the degradation and processing of endogenous proteins within the cell. The purpose of this study was to determine if there was an association of TAP or LMP alleles with IDDM and its susceptive or protective effects in a non-Caucasian population. From this study, extended MHC class II haplotypes to include A, B, DRB, DQB, TAP2, TAP1, and LMP2 in Puerto Ricans can be obtained.

MATERIALS AND METHODS

Samples were provided by Dr. Teresa Frazer at the Department of Pharmacology and Toxicology at the Ponce School of Medicine in Puerto Rico. One milliliter (mL) of frozen whole blood collected in ACD (acid citrate dextrose) or EDTA (ethylenediaminetetraacetic acid) anticoagulant blood collection tubes or isolated deoxyribonucleic acid (DNA) (at a concentration of 10 μg of DNA per 100 microliters (μL)) was supplied. Samples have been typed for HLA class I and II antigens by the Puerto Rican laboratory with most typings being confirmed by the Immunohematology and Serology Laboratory at Michigan State University.

Sample Population Characterization

Type I diabetic patients were defined as having a fasting plasma glucose level over 140 milligrams/deciliter (mg/dl) (>7.8 milliMolar (mM)) and a random plasma glucose level which exceeds 200 mg/dl (>11.1 mM) (Acevedo). Normal control Puerto Ricans were selected at random and did not have an immediate family member with IDDM. Samples included random insulin dependent diabetic Puerto Rican patients, random normal Puerto Rican controls, and four complete Puerto Rican families from a panel collected and maintained by the Puerto Rican laboratory. Samples were collected to fit into one of seven categories listed in Figure 2.

Group 1: Random Puerto Rican Diabetics with HLA DRB1*03XX and DRB1*04XX antigens (n=11).

Group 2: Random Puerto Rican Diabetics with an HLA DRB1*03XX or DRB1*04XX antigen and any other HLA antigen including blank (except DRB1*03XX and DRB1*04XX together)(n=28).

Group 3: Random Puerto Rican Diabetics with no DRB1*03XX or DRB1*04XX antigens (n=4).

Group 4: Random normal Puerto Rican controls with HLA DRB1*03XX and DRB1*04XX antigens (n=2).

Group 5: Random normal Puerto Rican controls with an HLA DRB1*03XX or DRB1*04XX and any other HLA antigen including blank (except DRB1*03XX and DRB1*04XX together) (n=6).

Group 6: Random normal Puerto Rican controls with no HLA DRB1*03XX or DRB1*04XX antigens (n=23).

Group 7: Four complete Puerto Rican families with one diabetic member to evaluate haplotype inheritance (families include both parents and 2-4 children).

Figure 2. Population Characterization
Random diabetic and control samples were characterized into one of six groups based on their disease state and HLA DR antigens. A set of four families were also included in this study.

DNA Isolation of Frozen Whole Blood Samples

Frozen whole blood samples were isolated with the QIAamp® Blood Kit (QIAGEN Incorporated, Chatsworth, California). A 200 µL aliquot of thawed, previously frozen, whole blood was placed into a 1.5 mL microfuge tube. 25 µL of QIAGEN protease (17.86 mg/mL stock concentration) and 200 µL of Buffer AL, a cell lysis buffer, were added. These samples were immediately vortexed and incubated at 70°C for ten minutes. Then 210 µL of isopropanol (J. T. Baker, Phillipsburg, New Jersey) was added to the samples and vortexed. The lysate was transferred to a QIAamp spin column and centrifuged at 9564 x gravity (g) for one minute. This was a higher g-force than the protocol suggested but was necessary to force all the cell debris through the column as the samples were somewhat old and lysed. The spin column was placed into a clean 2 mL microfuge tube, washed with 500 µL of Buffer AW, a wash buffer, and centrifuged at 9564 x g for one minute. Samples were washed again with 500 µL of Buffer AW and centrifuged at 9564 x g for three minutes. The spin column was placed into a clean UV-treated (90 seconds at 254 nanometers (nm)) (GS Gene Linker™ UV Chamber, Bio-Rad, Richmond, California) 1.5 mL microfuge tube and the DNA was incubated with 200 µL of sterile double

distilled water (ddHOH) at 70°C and the DNA was collected by centrifuging at 6000 x g for one minute.

The samples were vacuum dried (Savant Instruments Incorporated Speed Vac SC-100, Refrigerated Condensation Trap RT 100, Vacuum Gauge model VG-5, and High Vacuum Pump VP 100, Farmingdale, New York) and resuspended in 10 µL of sterile ddHOH or 10mM Tris (Tris[hydroxymethyl]-aminomethane hydrochloride) pH 9.0 (Sigma Chemical Company, St. Louis, Missouri). Using the GeneQuant RNA/DNA Calculator (Pharmacia LKB Biochrom Limited, Science Park, Cambridge, England), the optical density of a 1:100 dilution of the sample was spectrophotometrically measured using 260 nm, 280 nm, and 230 nm. Also measured and calculated was the protein contamination, purity, and 260/280 ratio. The formula for double stranded DNA, listed in Figure 3, was used to calculate DNA concentration. The concentration was adjusted to 10 µg of DNA per 100 µL of ddHOH. Samples isolated by the QIAGEN method were diluted 1:8 with ddHOH to give a final concentration of 0.0125 µg/µL. DNA was stored at -20°C prior to amplification.

Primer Preparation

Primers for the Polymerase Chain Reaction (PCR) were synthesized at the Macromolecular Facility at Michigan State

A. Formula for Double Stranded DNA

(Optical Density$_{260}$)(50μg/mL)(dilution factor)÷1000μL=X μg/μL of DNA

B. Formula for Single Stranded DNA

(Optical Density$_{260}$)(37μg/mL)(dilution factor)(mL resuspended)=Xμg DNA

C. Formula for Molecular Weight Calculation of DNA oligonucleotides

[(#dATP)(312.2)]+[(#dCTP)(288.2)]+[(#dGTP)(328.2)]+[(#dTTP)(303.2)] -61

Figure 3. Formulas for the Calculation of DNA Concentration and Primer Preparation

The formula for calculation of DNA concentration is based upon optical density. X is the calculated value of the concentration of DNA in μg/μl or μg . An optical density$_{260}$ of 1 corresponds to approximately 50 μg of DNA/mL for double stranded DNA (Maniatis, 1992). An optical density$_{260}$ of 1 corresponds to approximately 37 μg of DNA/mL for single stranded DNA (GeneQuant User Manual, 1993). In calculating the molecular weight of primers, 61 was subtracted for the absence of the terminal 5' phosphate and the addition of the terminal 3' hydrogen atom. The numbers in parenthesis are the molecular weights of the nucleotides.

University. Primers sequences for TAP1 and TAP2 were published sequences (Powis 1993). The 5' flanking and 3' flanking primer sequences for LMP2 were also published (Deng) and the two allele specific primers for LMP2 were developed specifically for this research project. Sequences for these primers are listed in Figure 4. Dried primers were reconstituted in ddHOH. The optical density at 260 nm was measured with a dilution of the primer using the GeneQuant RNA/DNA Calculator (Pharmacia LKB Biochrom Limited, Science Park, Cambridge, England). The primers were vacuum dried (Savant Instruments Incorporated Speed Vac SC-100, Refrigerated Condensation Trap RT 100, Vacuum Gauge model VG-5, and High Vacuum Pump VP 100 Farmingdale, New York). Using the calculated molecular weight, see Figure 3, and measured amount of recovered DNA, the amount of ddHOH was determined to resuspend the DNA at a concentration of 20 mM. A working solution of 20 microMolar (μM) was prepared from the stock diluting with ddHOH.

Typing TAP1, TAP2, and LMP2 Alleles

TAP1, TAP2, and LMP2 alleles were typed for using an Amplification Refractory Mutation System (ARMS) method. Typing included the following loci; TAP1 amino acid position 333 (ile/val), TAP1 amino acid position 637 (asp/gly), TAP2 amino acid position 379 (val/ile), TAP2 amino acid position 565 (ala/thr), TAP2 amino acid position 665 (ala/thr), and

TAP1	TAP1/ARMS1	5'-CCCTGCACTGAGATTTGCAGACCTCTGGAG-3'	5'flanking
Position 333	TAP1/ARMS2	5'-GATCAGTGTCCCTCACCATGGTCACCCGGA-3'	Ile-333
	TAP1/ARMS3	5'-GGGCAGAAGGAAAAGCAGAGGCAGGGTCAC-3'	Val-333
	TAP1/ARMS4	5'-ACCTGGGAACATGGACCACAGGGACAGGGT-3'	3' flanking
TAP1	TAP1/ARMS5	5'-CATCTTCCCAGAATCTCCCCTATCCAGCTA-3'	5' flanking
Position 637	TAP1/ARMS6	5'-CATCTTGGCCCTTTGCTCTGCAGAGGTACA-3'	Asp-637
	TAP1/ARMS7	5'-ACCCCCTGACAGCTGGCTCCCAGCCTCCC-3'	Gly-637
	TAP1/ARMS8	5'-TGGGGAGGCATCCAATGGAACTGGATTTGG-3'	3' flanking
TAP2	TAP2/ARMS1	5'-TTGGAGGGCTGCAGACCGTTCGCAGTTTTG-3'	5'flanking
Position 379	TAP2/ARMS2	5'-GAGACCTGGAACGCGCCTTGTACCTGCGCG-3'	Val-379
	TAP2/ARMS3	5'-ACAACCACTCTGGTATCTTACCCTCCTGAT-3'	Ile-379
	TAP2/ARMS4	5'-ACATAGCTCCCCACGCTCTCCTGGTAGATC-3'	3'flanking
TAP2	TAP2/ARMS5	5'-CTCACAGTATGAACACTGCTACCTGCACAG-3'	5'flanking
Position 565	TAP2/ARMS6	5'-TGTTCTCCGGTTCTGTGAGGAACAACAGTA-3'	Thr-565
	TAP2/ARMS7	5'-ATCATCTTCGCAGCTCTGCAGCCCATAAAC-3'	Ala-565
	TAP2/ARMS8	5'-GGAGCAAGCTTACAATTTGTAGAAGATACC-3'	3'flanking
TAP2	TAP2/ARMS9	5'-TTGGGGAATGGAATCCGGTGGTGTGAGGGC-3'	5'flanking
Position 665	TAP2/ARMS10	5'-CAGTGCTGGTGATTGCTCACAGGCTGCAAA-3'	Thr-665
	TAP2/ARMS11	5'-CACCAGGATCTGGTGGGCGCGCTGAACTAC-3'	Ala-665
	TAP2/ARMS12	5'-TCAGCCGCTGCTGCACCAGGCGGGAATAGA-3'	3'flanking
LMP2	LMP2-2	5'-GTGAACCGAGTGTTTGACAAGC-3'	5'flanking
Position 60	LMP2/ARG	5'-CTGTCCCCGCTGCACGATCG-3'	Arg-60
	LMP2/HIS	5'-AGAGAGTGCACAGTAGAAGT-3'	His-60
	LMP2-1	5'-GCCAGCAAGAGCCGAAACAAG-3'	3'flanking

Figure 4. PCR Primers
PCR primer sequences for TAP1 and TAP2 were obtained from Powis (1993). The LMP2 5' flanking and 3' flanking primer sequences were obtained from Deng. Underlined nucleotides are mismatched or possibly mismatched bases when compared to a consensus sequence.

LMP2 amino acid position 60 (arg/his).

TAP1 and TAP2 ARMS Polymerase Chain Reaction

The volume of the Polymerase Chain Reaction was 10 µL. The volume was doubled to 20 µL if AmpliWax™ PCR Gem 50 (Perkin Elmer, Foster City, California) was used to minimize nonspecific amplification or primer-dimer formation. A master mix containing all the PCR reagents except for the template was prepared for each typing system. These mixes included 0.01875 to 0.025 µg each of four primers (5' flanking, 3' flanking, and two internal allele specific primers listed in Figure 4 needed for a specific loci), 20 µM each of deoxyadenosine triphosphate (dATP), deoxycytosine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP), and deoxythymine triphosphate (dTTP) (Perkin Elmer, Foster City, California), 1X GeneAmp PCR Buffer (10mM Tris-HCl pH 8.3 [at 25°C]; 50 mM KCl; 1.5 mM $MgCl_2$; 0.001% weight/volume (w/v) gelatin; autoclaved) (Perkin Elmer, Foster City, California), and 0.2 Units of *Thermus aquaticus (Taq)* DNA polymerase (Perkin Elmer, Foster City, California) (modification of Powis, 1993). DNA template at a concentration of 0.0125 to 0.1 µg was added to the master mix.

PCR Reactions were carried out in a 9600 Thermo Cycler (Perkin Elmer, Foster City, California) with an initial

denaturation time of 5 minutes at 95 °C. Cycling parameters for TAP1 and TAP2 primers include 11 seconds of denaturation at 94 °C, anneal at 58 °C for 30 seconds, and extension at 72 °C for 30 seconds for 30-35 cycles. A final 72 °C extension for 10 minutes occurs at the end of cycling (modification of Powis, 1993).

LMP2 ARMS Polymerase Chain Reaction

The PCR conditions were optimized using the 9600 Thermo Cycler (Perkin Elmer, Foster City, California). A master mix containing all the PCR reagents except for the template was prepared for the LMP2 typing system. These mixes included 20 μM each of dATP, dCTP, dGTP, and dTTP (Perkin Elmer, Foster City, California), 1X GeneAmp PCR Buffer (10mM Tris-HCl pH 8.3 [at 25°C]; 50 mM KCl, 1.5mM $MgCl_2$, 0.001% (w/v) gelatin; autoclaved (Perkin Elmer, Foster City, California), 0.4 Units of *Thermus aquaticus (Taq)* DNA polymerase (Perkin Elmer, Foster City, California), 1uM each of LMP2-1, LMP2-2, LMP2-Arg, and 2uM of LMP2-His. DNA template at a concentration of 0.25 to 0.2 ug was added to this 20 uL master mix. Primer sequences are listed in Figure 4. Initial denaturation was at 95C° for 5 minutes followed by 35 cycles of 94C° for 30 seconds, 56C° for 30 seconds, 72C° for 60 seconds, and then a final extension at 72C° for 10 minutes.

PCR Controls

A negative control has been run with every TAP and LMP PCR reaction. As a negative control, one sample included ddHOH instead of DNA. Samples were typed in duplicate.

Controls for TAP1 and TAP2 were obtained from well characterized homozygous typing cell lines (HTCL) of the 10th and 11th International Histocompatibility Workshop purchased from the American Society of Histocompatibility and Immunogenetics cell repository (Powis, 1993 and Carrington). Cell lines are Epstein-Barr virus transformed B-cells for which DNA had been previously harvested by a salt extraction method at the Immunohematology and Serology Laboratory at Michigan State University. One positive homozygous cell was run for each allele with every reaction. A heterozygous sample was also run with each reaction. Because the HTCL are almost always homozygous for every loci in the HLA region, heterozygous control samples were made by making a 1:1 mixture of the appropriate homozygous cell types. Table 4 lists the controls used.

As no known types were available at the LMP2 loci, HTCLs were screened with the preliminary ARMS PCR typing method and then sequenced to determine their type. They were then used as controls in the ARMS PCR reaction and are listed in Table 4.

Table 4. Control Cell Lines
Listed are the control cell lines used for TAP1, TAP2, and LMP2 typing. Included are their types at each poymorphic site.

TAP1

Cell Line	Position 333	Position 637
9080	val,val	asp,asp
Madura	ile,ile	gly,gly
Mix	val,ile	asp,gly
Blank	Blank	Blank

TAP2

Cell Line	Position 379	Position 565	Position 665
9080	val,val	ala,ala	ala,ala
Madura	ile,ile	thr,thr	thr,thr
Mix	val,ile	ala,thr	ala,thr
Blank	Blank	Blank	Blank

LMP2

Cell Line	Position 60
9080	arg,arg
SPO010	his,his
RAJI	arg,his
Blank	Blank

Sequencing the LMP2 Controls

DNA previously amplified with the 5' flanking and the 3' flanking primers (LMP2-1 and LMP2-2) was diluted 1:10 and 6uL were used in the 20uL fluorescent dye terminator di-deoxy sequencing PCR reaction. The template was sequenced in both directions using 3.2 pmole of the primers individually with 8.0 uL of the ABI PRISM™ DNA Ready Reaction Sequencing Kit (ABI/Perkin Elmer, Foster City, California) for 25 cycles of 96C° for 10 seconds, 50C° for 5 seconds, and 60C° for 4 minutes. The samples were then ramped to 4C°. The products were ethanol washed to remove unincorporated labeled nucleotides. The samples were added to 35 uL of 95% alcohol, vortexed, and incubated on ice for 10 minutes. At 4C°, the samples were centrifuged at maximum speed for 15 minutes. After the supernatant was removed, the pellet was vacuum dried and then resuspended in 25 uL of Template Suppression Reagent (ABI/Perkin Elmer, Foster City, California). These samples were vortexed, spun, heated to 95C°, vortexed and spun again before they were sequenced using the ABI PRISM™ 310 Genetic Analyzer (ABI/Perkin Elmer, Foster City, California).

Electrophoresis of TAP1 and TAP2

Products were electrophoresed using a Mini-PROTEAN® II Cell electrophoresis chamber (Bio-Rad, Richmond, California) and a Model 200/2.0 Power Supply (Bio-Rad, Richmond, California). A 6% polyacrylamide gel with the dimensions 80 mm x 73 mm x 0.75 mm was used [1.74% (w/v) acrylamide (Bio-Rad, Richmond, California), 0.06% N,N'-methylene bisacrylamide (Bio-Rad, Richmond, California)], TAE [40 mM Tris(hydroxymethyl)aminomethane (Sigma Chemical Company, St. Louis, Missouri), 0.114% glacial acetic acid (EM Industries Incorporated, Gibbstown, New Jersey), 1 mM EDTA (Sigma Chemical Company, St. Louis, Missouri) pH 8.0], 0.001% N,N,N',N'-Tetramethylethylenediamine (TEMED) (Boehringer Mannheim Corporation, Indianapolis, Indiana), and 0.08% ammonium persulfate (J. T. Baker Incorporated, Phillipsburg, New Jersey).

The 10 µL PCR sample was mixed with 1µL of 6X gel-loading buffer [0.25% bromophenol blue (Sigma Chemical Company, St. Louis, Missouri), 0.25% xylene cyanol (Sigma Chemical Company, St. Louis, Missouri), and 40% (w/v) sucrose (Mallinckrodt Chemical Incorporated, Paris, Kentucky) in ddHOH]. On the gel 5 µL of this mixture was loaded along with one lane of a molecular weight standard, which includes DNA fragments of 1000, 700, 500, 400, 300, 200, 100, and 50 base pairs (Boehringer Mannheim Corporation, Indianapolis, Indiana). A current of 200 volts

was applied until the bromophenol blue dye front migrated approximately 2/3 the length of the gel (15-20 minutes).

Electrophoresis of LMP2

The 20 µL PCR sample was mixed with 5µL of 6X gel-loading buffer [0.25% bromophenol blue (Sigma Chemical Company, St. Louis, Missouri), 0.25% xylene cyanol (Sigma Chemical Company, St. Louis, Missouri), and 40% (w/v) sucrose (Mallinckrodt Chemical Incorporated, Paris, Kentucky) in ddHOH]. The 6% acrylamide gel with dimensions of 0.5mm x 16cm x 20cm was loaded with 10 uL of sample [1.74% (w/v) acrylamide (Bio-Rad, Richmond, California), 0.06% N,N'-methylene bisacrylamide (Bio-Rad, Richmond, California)], TBE [89 mM Tris(hydroxymethyl)aminomethane (Sigma Chemical Company, St. Louis, Missouri), 89 mM boric acid (J.T. Baker Incorporated, Phillipsburg, New Jersey), 2 mM EDTA (Sigma Chemical Company, St. Louis, Missouri) pH 8.0], 0.001% N,N,N',N'-Tetramethylethylenediamine (TEMED) (Boehringer Mannheim Corporation, Indianapolis, Indiana), and 0.0008% ammonium persulfate (J. T. Baker Incorporated, Phillipsburg, New Jersey)). One lane of a molecular weight standard, which includes DNA fragments of 1000, 700, 500, 400, 300, 200, 100, and 50 base pairs (Boehringer Mannheim Corporation, Indianapolis, Indiana) was also loaded onto the gel. The samples were electrophoresed using a PROTEAN® IIxi Cell electrophoresis chamber (Bio-Rad, Richmond,

California) and a Model 1000/500 Power Supply (Bio-Rad, Richmond, California). The gel was cooled with water and electrophoresed at 100-125 volts until the bromophenol blue and xylene cyanol dye fronts separated the gel into thirds (approximately 4 hours).

Detection and Analysis

The gels were placed into approximately 100-500 mL of ddHOH with one drop of ethidium bromide (10 mg/mL) (Calbiochem-Behring Corporation, La Jolla, California) and incubated with gentle rotation at room temperature for 10 minutes. DNA bands were visualized using the Chromato-Vue transilluminator model 75-36 (UVP Incorporated, San Gabriel, California) at the wavelength of 254 nm. The gels were photographed with type 667 black and white Polaroid® film (Polaroid Corporation, Cambridge, Massachusetts) using a Fotodyne FCR-10 camera (Fotodyne Incorporated, Hartland, Wisconsin). Film was exposed for one second at *f*=8 and developed for one minute.

Statistical Analysis

Statistical evaluations used the chi-square method or Fisher's Exact test where appropriate using a 95% confidence interval (p=0.05). Graph Pad Prism (Graph Pad Software Incorporated) was the computer program used to do the chi-square analysis. Comparisons were made between group 1

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(diabetic DR3, DR4) and group 4 (normal DR3, DR4); group 2 (diabetic with one DR3 or DR4) and group 5 (normal with one DR3 or DR4); and group 3 (diabetic no DR3 or DR4) and group 6 (normal no DR3 or DR4) (refer to Figure 1) for each polymorphic site. A dominant mode of inheritance that considers the frequency of each allele along with a recessive mode of inheritance which considers the frequency of a homozygous type compared to the other outcomes was evaluated.

RESULTS

Samples for this study included 43 random Puerto Rican IDDM samples and 31 random Puerto Rican normal controls. Also included were 21 samples from four complete first generation Puerto Rican families. Each family included one member with IDDM. Sample populations were divided into three subgroups for a total of six groups. The subgroups were based on the DR allele and include DR3/DR4, DR3 or 4/DRX, and DRX/DRX. The X denotes any allele but DR3 or DR4, however some samples could be homozygous DR3 or homozygous DR4. The subgrouping was done to separate the traditional DR3/DR4 IDDM risk haplotype and to further separate samples with a risk allele (DR3 or DR4).

In typing for the TAP and LMP polymorphisms within this population, positive controls were run with every ARMS PCR reaction to verify reaction conditions were sufficient for amplification. As controls were unknown for the LMP2 loci, HTCL were sequenced to obtain appropriate homozygous and heterozygous controls used in further typing analysis. Sequence analysis results of the three HTCL used as controls for LMP2 are shown in Figure 5.

Polymorphisms for the TAP and LMP genes at six sites were determined by an ARMS PCR typing method. After amplification with allele specific primers, the PCR products were electrophoresed on acrylamide gels and the DNA was stained with ethidium bromide. The banding patterns

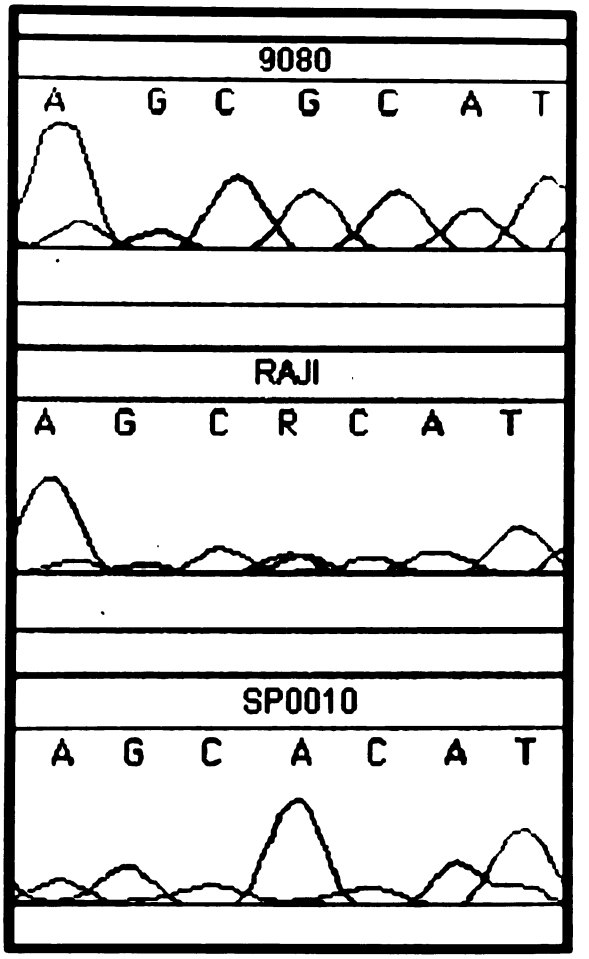


Figure 5. Electorpherogram
Sequence data for the cell lines used as controls in the LMP2 ARMS typing. Cell line 9080 is homozygous for guanosine at codon 60 (AG CGC AT). SP0010 is homozygous for adenosine at codon 60 (AG CAC AT). RAJI is heterozygous at codon 60 (AG CRC AT). R= adenosine or guanosine (purine).

observed were compared to a molecular weight sizing standard and assigned a genotype. See Figure 6-11 for a representation of each of the TAP and LMP polymorphic banding patterns observed on an ethidium bromide stained gel.

For TAP1 Amino Acid Position 333, Figure 6, the control band appears at 533bp, the val band appears at 351bp and the ile band appears at 241bp. The blank was negative. For TAP1 Amino Acid Position 637, Figure 7, the control band appears at 429bp, the asp band appears at 307bp and the gly band appears at 180bp. The blank was negative. For TAP2 Amino Acid Position 379, Figure 8, the control band appears at 427bp, the val band appears at 328bp and the ile band appears at 158bp. The blank was negative. For TAP2 Amino Acid Position 565, Figure 9, the control band appears at 400bp, the ala band appears at 298bp and the thr band appears at 161bp. The blank was negative. For TAP2 Amino Acid Position 665, Figure 10, the control band appears at 408bp, the ala band appears at 326bp and the thr band appears at 141bp. The blank was negative. For LMP2 Amino Acid Position 60, Figure 11, the control band appears at 252bp, the Arg band appears at 231bp and the His band appears at 60bp. The blank was negative.

Samples were assigned a type based on the size of the PCR fragment. They were assigned either one of two possible homozygous types or a heterozygous type. Samples with only

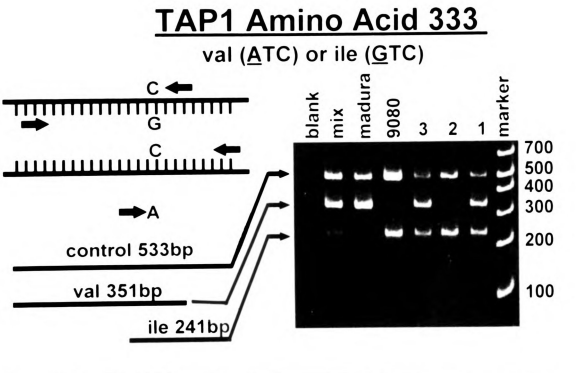


Figure 6. TAP1 Amino Acid 333 ARMS Diagram
A representation of the ARMS PCR for the TAP1/333 system. Control band appears at 533bp. Valine band is 351bp and the isoleucine band is 241bp.

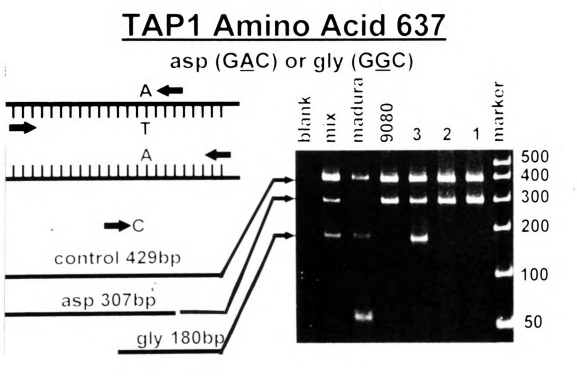


Figure 7. TAP1 Amino Acid 637 ARMS Diagram
A representation of the ARMS PCR for the TAP1/637 system. Control band appears at 429bp. Aspartic acid band is 307bp and the glycine band is 180bp.

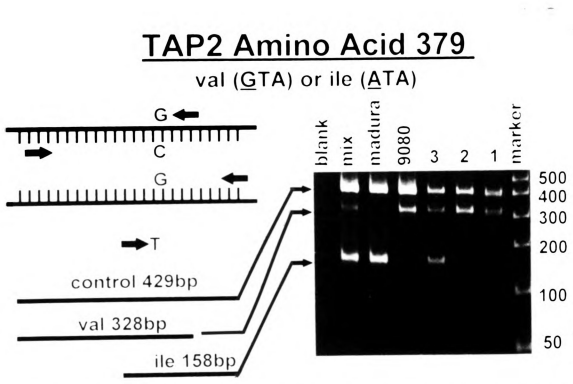


Figure 8. TAP2 Amino Acid 379 ARMS Diagram
A representation of the ARMS PCR for the TAP2/379 system. Control band appears at 427bp. Valine band is 328bp and the isoleucine band is 158bp.

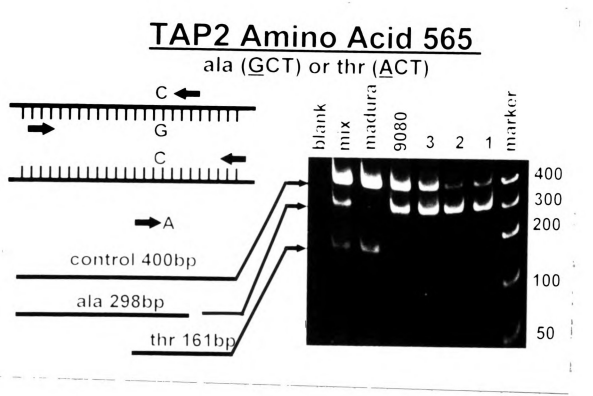


Figure 9. TAP2 Amino Acid 565 ARMS Diagram
A representation of the ARMS PCR for the TAP2/565 system. Control band appears at 400bp. Alanine band is 298bp and the threonine band is 161bp.

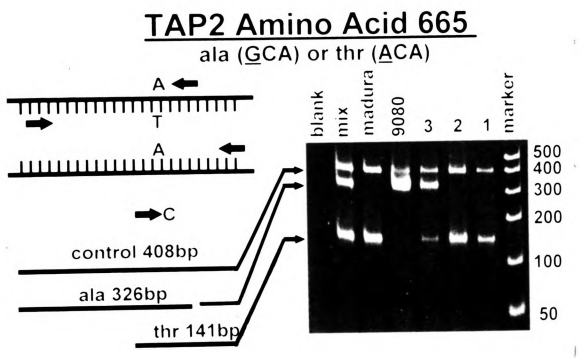


Figure 10. TAP2 Amino Acid 665 ARMS Diagram
A representation of the ARMS PCR for the TAP2/665 system. Control band appears at 408bp. Alanine band is 326bp and the threonine band is 141bp.

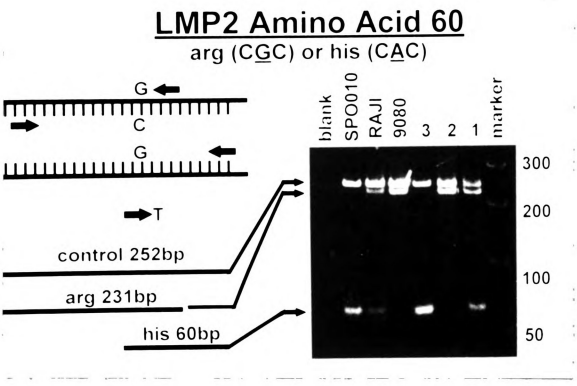


Figure 11. LMP2 Amino Acid 60 ARMS Diagram
A representation of the ARMS PCR for the LMP2/60 system. Control band appears at 252bp. Arginine band is 231bp and the histidine band is 60bp.

Table 5. Typing Results
Listed are the combined typing results for TAP1 position 333 and 637, TAP2 position 379, 565, and 665, and LMP2 position 60 according to their specific catagories.

	Diabetic DR3 DR4 (n=11)	Diabetic DR3 or 4 DRX (n=28)	Diabetic DRX DRX (n=4)	Normal DR3 DR4 (n=2)	Normal DR3 or 4 DRX (n=6)	Normal DRX DRX (n=23)
TAP1 POS 333	val,x =2 val,ile=5 ile,x =4	val,x=3 val,ile=7 ile,x=18	val,x=0 val,ile=1 ile,x=3	val,x=0 val,ile=1 ile,x=1	val,x=1 val,ile=1 ile,x=4	val,x=1 val,ile=6 ile,x=16
TAP1 POS 637	asp,x=4 asp,gly=5 gly,x=2	asp,x=18 asp,gly=7 gly,x=3	asp,x=3 asp,gly=1 gly,x=0	asp,x=1 asp,gly=1 gly,x=0	asp,x=4 asp,gly=2 gly,x=0	asp,x=16 asp,gly=6 gly,x=1
TAP2 POS 379	val,x=11 val,ile=0 ile,x=0	val,x=23 val,ile=5 ile,x=0	val,x=3 val,ile=1 ile,x=0	val,x=2 val,ile=0 ile,x=0	val,x=6 val,ile=0 ile,x=0	val,x=17 val,ile=5 ile,x=1
TAP2 POS 565	ala,x=11 ala,thr=0 thr,x=0	ala,x=25 ala,thr=3 thr,x=0	ala,x=3 ala,thr=1 thr,x=0	ala,x=2 ala,thr=0 thr,x=0	ala,x=6 ala,thr=0 thr,x=0	ala,x=22 ala,thr=1 thr,x=0
TAP2 POS 665	ala,x=0 ala,thr=3 thr,x=8	ala,x=0 ala,thr=7 thr,x=21	ala,x=0 ala,thr=3 thr,x=1	ala,x=0 ala,thr=0 thr,x=2	ala,x=0 ala,thr=2 thr,x=4	ala,x=2 ala,thr=11 thr,x=10
LMP2 POS 60	arg,x=8 arg,his=3 his,x=0	arg,x=14 arg,his=12 his,x=2	arg,x=2 arg,his=2 his,x=0	arg,x=1 arg,his=1 his,x=0	arg,x=5 arg,his=1 his,x=0	arg,x=13 arg,his=8 his,x=2

an allele specific band are assumed to be homozygous at that loci. Refer to Table 5 for data collected from these typings. Figure 12 illustrates the family haplotype analysis. Two samples out of 1140 PCR reactions showed aberrant results. Additional typing of these samples was performed and they were assigned a type based on the most prevalent type of that sample.

Statistical analysis of the data was done using the Graph Pad Prism statistical program. Results of the chi-square analysis for a dominant affect is shown in Table 6 while analysis for a recessive affect is shown in Table 7. Results for dominant affect compared the frequencies of each amino acid in the population. Recessive affects were tested by comparing the frequency of one homozygous type to other possible outcomes (including homozygosity for the other allele). Then the frequency of the other homozygous allele was compared to the other possible outcomes.

Table 6. Dominant Inheritance Results
Chi-square analysis for a dominant mode of inheritance (typically found in IDDM protective alleles). Chi-square as well as Fisher's Exact test was performed with a 95% confidence interval (p=0.05). There were no significant outcomes.

Chi-Square analysis for Dominant Testing (p=0.05)					
Group	chi-square,df	p-value	Outcome	Fisher's Exact p-value	Outcome
TAP1/333 DR3/4	0.36, 1	0.55	Not Significant	1.00	Not Significant
TAP1/333 DR3 or 4/X	0.01751, 1	0.8947	Not Significant	1.00	Not Significant
TAP1/333 DRX/X	0.1174, 1	0.7319	Not Significant	0.8640	Not Significant
TAP1/637 DR3/4	0.3619, 1	0.5474	Not Significant	1.00	Not Significant
TAP1/637 DR3 or 4/X	0.2464, 1	0.6196	Not Significant	1.00	Not Significant
TAP1/637 DRX/X	0.1174, 1	0.7319	Not Significant	1.00	Not Significant
TAP2/379 DR3/4	N/A	N/A	Not Significant	N/A	Not Significant
TAP2/379 DR3 or 4/X	1.156, 1	0.2822	Not Significant	0.5772	Not Significant
TAP2/379 DRX/X	0.03987, 1	0.8417	Not Significant	1.00	Not Significant
TAP2/565 DR3/4	N/A	N/A	Not Significant	N/A	Not Significant
TAP2/565 DR3 or 4/X	0.6725, 1	0.4122	Not Significant	1.00	Not Significant
TAP2/565 DRX/X	2.037, 1	0.1535	Not Significant	0.2767	Not Significant
TAP2/665 DR3/4	0.6166, 1	0.4323	Not Significant	1.00	Not Significant
TAP2/665 DR3 or 4/X	0.1494, 1	0.6991	Not Significant	0.6541	Not Significant
TAP2/665 DRX/X	0.07337, 1	0.7865	Not Significant	1.00	Not Significant
LMP2/60 DR3/4	0.3357, 1	0.5623	Not Significant	0.5107	Not Significant
LMP2/60 DR3 or 4/X	2.159, 1	0.1418	Not Significant	0.2692	Not Significant
LMP2/60 DRX/X	0.004193, 1	0.9484	Not Significant	1.00	Not Significant

Table 7. Recessive Inheritance Results
Chi-square analysis for a recessive mode of inheritance (typically found in IDDM susceptibility alleles). Chi-square as well as Fisher's Exact test was performed with a 95% confidence interval (p=0.05). There were no significant outcomes.

Chi-Square analysis for Recessive Testing (p=0.05)					
Group	chi-square, df	p-value	Outcome	Fisher's Exact p-value	Outcome
TAP1/333-val DR3/4	0.4298, 1	0.5121	Not Significant	1.00	Not Significant
TAP1/333-val DR3 or 4/X	0.1687, 1	0.6813	Not Significant	0.5585	Not Significant
TAP1/333-val DRX/X	0.1806, 1	0.6709	Not Significant	1.00	Not Significant
TAP1/333-ile DR3/4	0.1330, 1	0.7154	Not Significant	1.00	Not Significant
TAP1/333-ile DR3 or 4/X	0.01227, 1	0.9118	Not Significant	1.00	Not Significant
TAP1/333-ile DRX/X	0.04827, 1	0.08261	Not Significant	1.00	Not Significant
TAP1/637-asp DR3/4	0.6300, 1	0.4274	Not Significant	1.00	Not Significant
TAP1/637-asp DR3 or 4/X	0.01227, 1	0.9118	Not Significant	1.00	Not Significant
TAP1/637-asp DRX/X	0.04827, 1	0.8261	Not Significant	1.00	Not Significant
TAP1/637-gly DR3/4	0.2182, 1	0.6404	Not Significant	1.00	Not Significant
TAP1/637-gly DR3 or 4/X	0.7051, 1	0.4011	Not Significant	1.00	Not Significant
TAP1/637-gly DRX/X	0.1806, 1	0.6709	Not Significant	1.00	Not Significant
TAP2/379-ala DR3/4	N/A	N/A	Not Significant	N/A	Not Significant
TAP2/379-ala DR3 or 4/X	1.256, 1	0.2624	Not Significant	0.5585	Not Significant
TAP2/379-ala DRX/X	0.002096, 1	0.9635	Not Significant	1.00	Not Significant
TAP2/379-thr DR3/4	N/A	N/A	Not Significant	N/A	Not Significant
TAP2/379-thr DR3 or 4/X	N/A	N/A	Not Significant	N/A	Not Significant
TAP2/379-thr DRX/X	0.1728, 1	0.6776	Not Significant	1.00	Not Significant

Table 7 cont.

Group	chi-square,df	p-value	Outcome	Fisher's Exact p-value	Outcome
TAP2/565-ala DR3/4	N/A	N/A	Not Significant	N/A	Not Significant
TAP2/565-ala DR3 or 4/X	0.7051, 1	0.4011	Not Significant	1.00	Not Significant
TAP2/565-ala DRX/X	2.119, 1	0.1455	Not Significant	0.2792	Not Significant
TAP2/565-thr DR3/4	N/A	N/A	Not Significant	N/A	Not Significant
TAP2/565-thr DR3 or 4/X	N/A	N/A	Not Significant	N/A	Not Significant
TAP2/565-thr DRX/X	N/A	N/A	Not Significant	N/A	Not Significant
TAP2/665-ala DR3/4	N/A	N/A	Not Significant	N/A	Not Significant
TAP2/665-ala DR3 or 4/X	N/A	N/A	Not Significant	N/A	Not Significant
TAP2/665-ala DRX/X	0.3590, 1	00.5491	Not Significant	1.00	Not Significant
TAP2/665-thr DR3/4	0.7091, 1	0.3997	Not Significant	1.00	Not Significant
TAP2/665-thr DR3 or 4/X	0.1763, 1	0.6746	Not Significant	0.6445	Not Significant
TAP2/665-thr DRX/X	0.4819, 1	0.4876	Not Significant	0.6239	Not Significant
LMP2/60-arg DR3/4	0.4104, 1	0.5218	Not Significant	1.00	Not Significant
LMP2/60-arg DR3 or 4/X	2.227, 1	0.1356	Not Significant	0.1960	Not Significant
LMP2/60-arg DRX/X	0.05870, 1	0.8086	Not Significant	1.00	Not Significant
LMP2/60-his DR3/4	N/A	N/A	Not Significant	N/A	Not Significant
LMP2/60-his DR3 or 4/X	0.4554, 1	0.4998	Not Significant	1.00	Not Significant
LMP2/60-his DRX/X	0.3757, 1	0.5399	Not Significant	1.00	Not Significant

Family 1

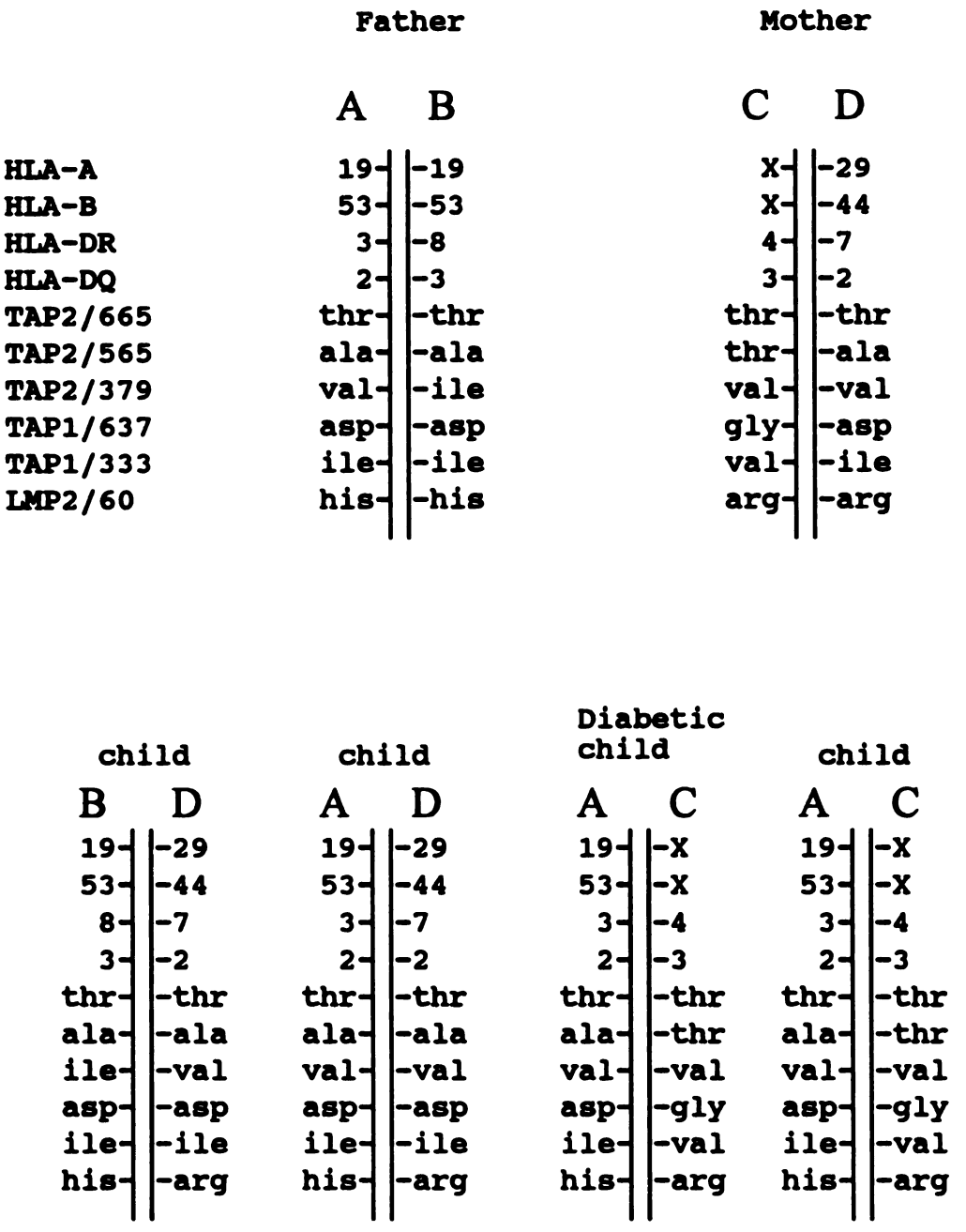


Figure 12. Extended HLA Haplotypes from Family Studies
HLA haplotypes derived from family studies include the class I antigens (HLA-A and HLA-B), class II antigens (HLA-DR and HLA-DQ), and TAP1, TAP2, and LMP2 polymorphisms. The large A and B designates paternal haplotypes, while the large C and C designates maternal haplotypes. An undetermined allele is denoted by X.

Family 2

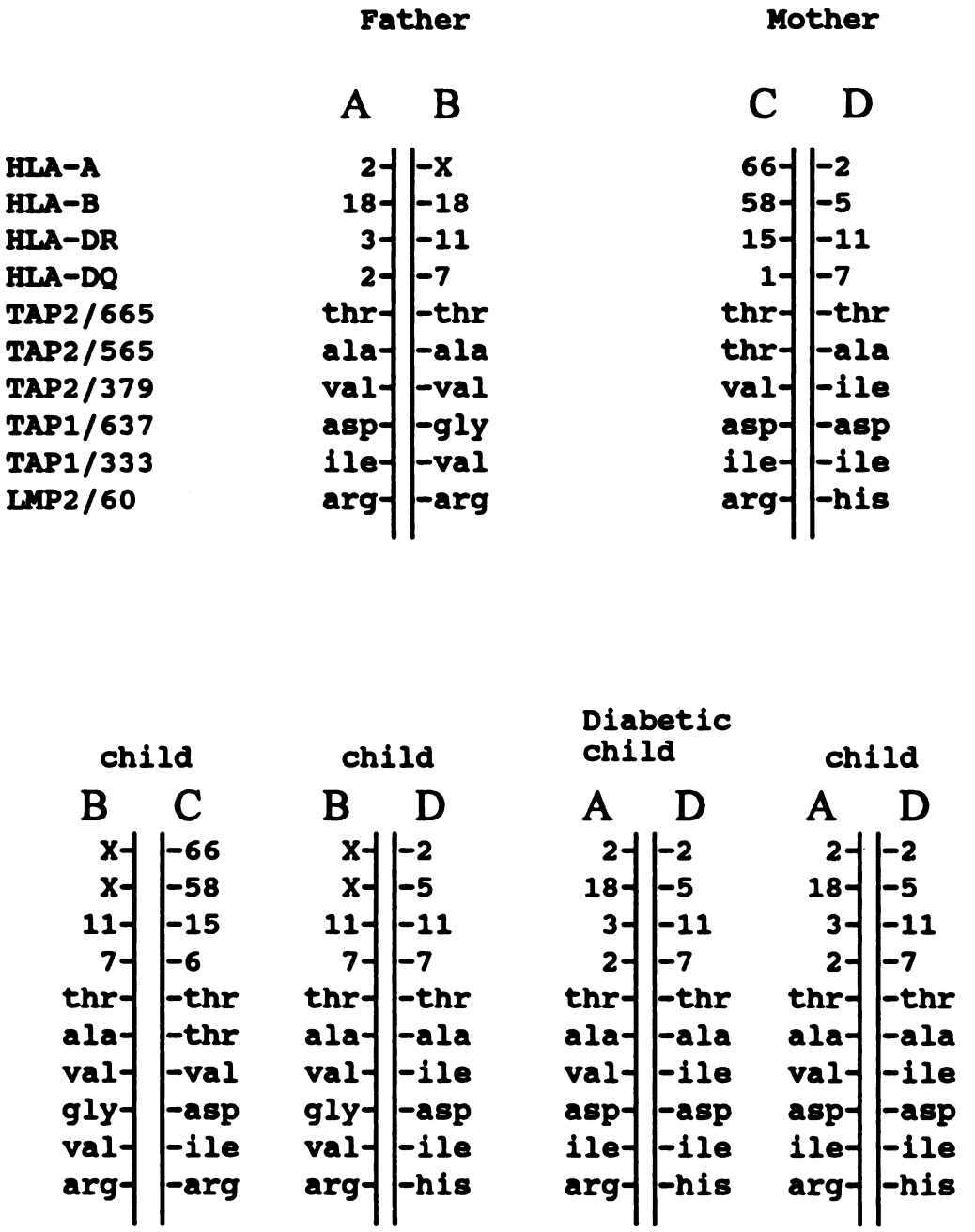
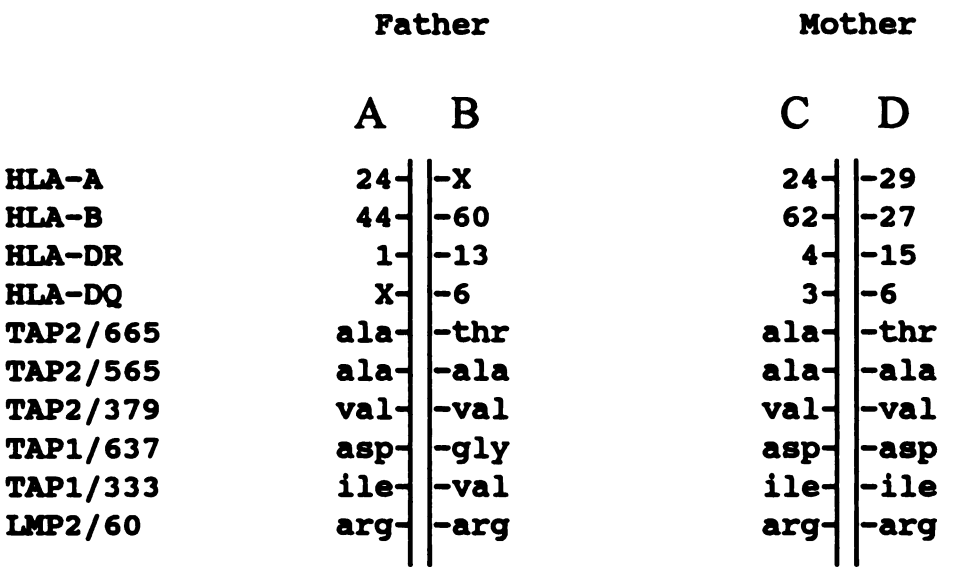


Figure 12 cont.

Family 3



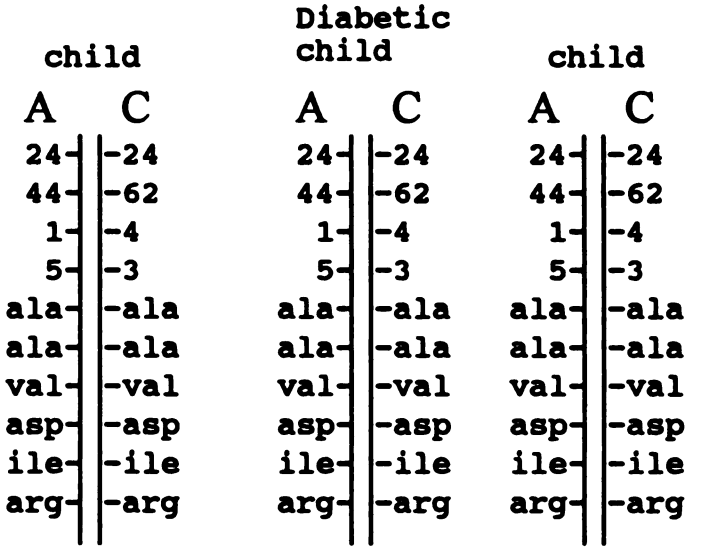
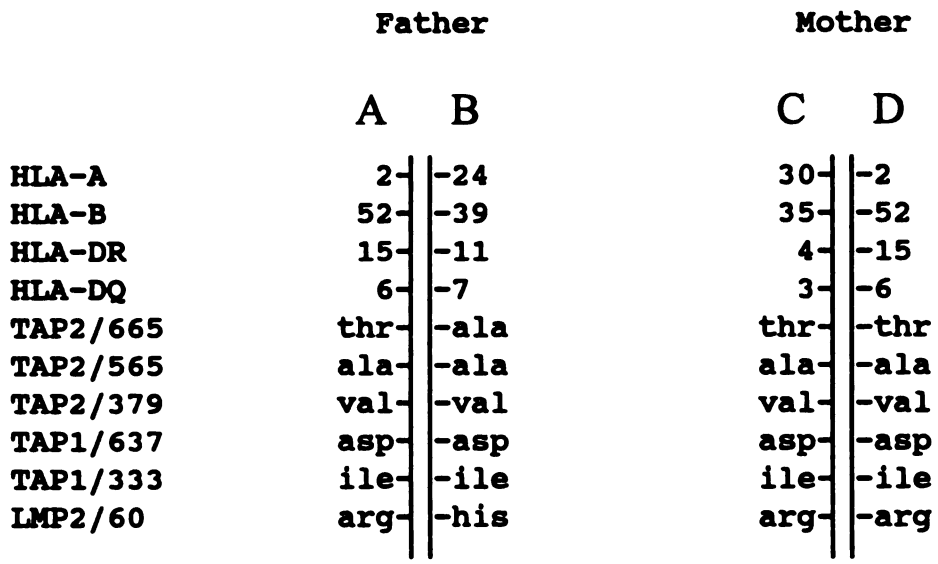


Figure 12. cont.

Family 4



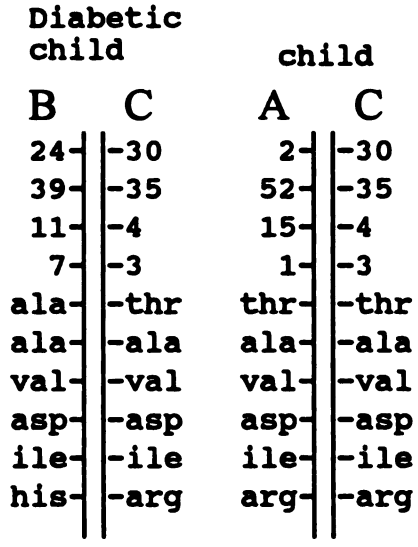


Figure 12. cont.

DISCUSSION

Development of LMP2 ARMS PCR

ARMS PCR has been a typing method used to detect known mutations in a selected nucleotide sequence. This method used a set of four primers. Two primers flank the mutation site and amplify a control fragment. The other two primers were specific for one of the two known polymorphisms. One allele specific primer was positioned in the sense direction, the other allele specific primer, in the antisense direction. The allele specific primer in the sense direction was paired with the antisense control primer and amplified a fragment of determined length if that allele was present. The other allele specific primer in the antisense direction was paired with the sense control primer and amplified a fragment of determined length if that allele was present (See figure 13).

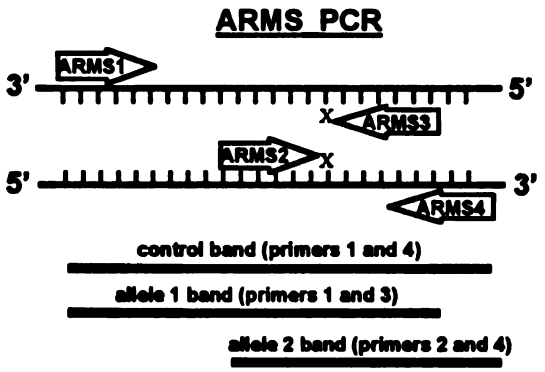


Figure 13. ARMS PCR
An illustration of the ARMS PCR method. Two out of four primers serve as controls. The other two primers are allele specific and amplify, with their corresponding control primer, a fragment of a specified length.

Primers for ARMS PCR amplification of TAP alleles came from published primer sequences. LMP2 allele specific primers were developed specifically for this research project. This enabled the LMP2 polymorphism to also be typed by an ARMS based method. The LMP2 allele specific primers, LMP2Arg and LMP2His, have a 3' terminal primer/template G/T mismatch for the Arg allele and a T/G mismatch for the His allele. It has been shown that *Taq* polymerase lacks a 3' to 5' exonuclease activity (Newton). The lack of a 3' to 5' repair mechanism is essential to have a refractory PCR amplification system. To increase the specificity of the refractory PCR reaction, the third nucleotide from the 3' end of the allele specific primer was purposefully mismatched (Newton). The mismatches most refractory to amplification were purine/purine or pyrimidine/pyrimidine pairings (Newton). In development of the LMP2 allele specific primers, a deliberate mismatch incorporated into the third nucleotide in from the 3' end of the sequence is primer/template T/C in the Arg specific primer and A/A in the His primer to enhance specificity.

Inhibition of PCR

The ARMS primer sets were initially screened against HTCL that had been previously typed at the TAP1 and TAP2 loci (Powis, 1993 and Carrington) and sequenced at the LMP2 loci to verify the accuracy of the typing results. While

attempting to run initial typings with the Puerto Rican samples, non-amplification was noted in several samples as no allele or control PCR products appeared.

The frozen whole blood samples obtained from Puerto Rico were at least five years old. Many of these samples have gone through repeated freeze and thaw cycles. These conditions lead to cell lysis and degradation of DNA. The hemoglobin released from red blood cells is known to be a PCR inhibitor (Wiedbrauk). Because of the less than ideal sample quality, an inhibitor to the PCR process within the non-amplified samples was suspected when non-amplification occurred.

There were several ways to reverse the affects of PCR inhibitors. A detergent, solvent, or protein may be added or the inhibitor can be diluted out of the sample. Tween-20 (Polyoxyethylene sorbitan monolaurate) is a detergent that reverses inhibitory effects of 0.01% SDS (Sodium dodecyl sulfate) on *Taq* polymerase (Varadaraj). Glycerol is an organic solvent that destabilizes double stranded DNA and improves strand separation. Glycerol is thought to either eliminate the formation of secondary structures or affect the thermal activity of *Taq* polymerase (Lu). Another method to minimize inhibition of PCR is to add bovine serum albumin (BSA). BSA has been shown to suppress the actions of inhibitory factors in DNA samples from mummies dated to 1912 (Lin). Diluting out the inhibitor is another means of

eliminating PCR inhibition. PCR inhibitory effects are concentration dependent (Wiedbrauk).

To test this hypothesis, Puerto Rican genomic DNA that previously did not amplify, was subject to an additional ARMS PCR with either 2% Tween-20, 2% glycerol, 2% BSA, or DNA dilution with water (1:2, 1:4, and 1:8). Optimal results were obtained with a 1:8 dilution as demonstrated in Figure 14. Therefore, DNA isolated from whole blood samples at Michigan State University were subsequently diluted 1:8 and used for typing.

Data Analysis

After typing each sample in duplicate, results were analyzed using a chi-square analysis with Fisher's Exact test to correct for small sample numbers. Susceptibility to IDDM is inherited in a recessive mode while genes conferring protection to IDDM are inherited in a dominant mode (She). Statistical analysis of this data revealed no association with any allele that was typed for and IDDM. Analysis considered dominant (presence of an allele) and recessive (homozygosity of an allele) patterns of inheritance.

Haplotype analysis

Within the four families, there were no haplotypes that predominately associated with IDDM. Even between unrelated diabetics with the same DR antigens, haplotypes including

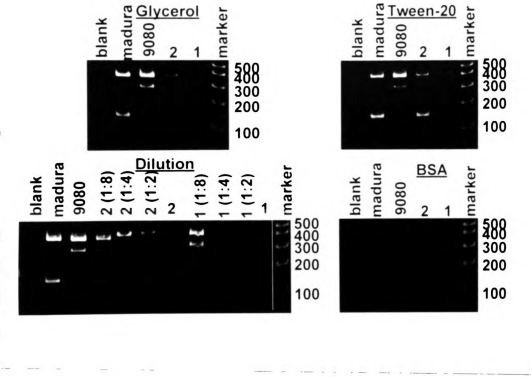


Figure 14. Inhibition of PCR
Above are several gels with different methods of overcoming PCR inhibition. 2% glycerol, 2% BSA, 2% Tween-20, 1:2, and 1:4 dilutions were not as effective as a 1:8 dilution of genomic DNA. This gave a final genomic DNA concentration of 0.0125ug/ul which reduced the amount of DNA in the PCR reaction by 1/8 as compared to the undiluted sample.

the A, B, DR, DQ, TAP and LMP alleles, were not common. Within a family there were diabetic and non-diabetic siblings that shared the same haplotypes. These non-diabetic siblings should be noted and watched for IDDM development.

Conclusions

No statistical significance was evident in this Puerto Rican population for the TAP and LMP polymorphisms tested for. There was no TAP or LMP allele associated with IDDM in a dominant or recessive manner. Other studies have found TAP2B to be predisposing to IDDM, however, this was later found to be secondary to linkage disequilibrium to DR4 (Ronningen, 1993). Samples for this study were collected in order to minimize the DR4 and TAP2B linkage. If a TAP or LMP allele was a predisposing or protective factor, then data in all subgroups (DR3/DR4, DR3 or 4/DRX, and DRX/DRX) should have been significant.

Because there is no association with TAP or LMP genes in this Puerto Rican population and other studies have shown DP not to be significant, a conclusion based on this and other data is that the IDDM susceptibility gene is telomeric to the TAP and LMP gene region and possibly maps between TAP2 and the DQ and DR region. The possibility still exists that susceptibility to IDDM is based upon an interaction of DR and DQ and possibly other genes within the MHC region as

the relative risk is additive when more loci are included (Huang).

SUMMARY

Puerto Ricans diabetics and controls were typed for polymorphisms within the TAP and LMP genes. This population was chosen to supplement, in a supportive or refutive manner, the Caucasian population data. The Puerto Rican population with its Hispanic, African, and Native American mixture provided an ideal comparison to facilitate racial haplotype minimization. However, there were no significant findings between IDDM and the TAP and LMP polymorphism typed for in the Puerto Ricans. Sixteen extended HLA haplotypes were described in this population. With further haplotype and disease association information, these haplotypes may be used as a probability predictor for the occurrence of IDDM. If an individual has a haplotype that has been previously associated with IDDM, then that person would be at a higher risk for developing IDDM.

RECOMMENDATIONS

Because of the limited number of samples included in this study, it would be beneficial to repeat the analysis on a larger sampling of this population. Other newly discovered polymorphic loci within the TAP and LMP genes could also be included along with LMP7 polymorphisms.

Other studies could focus on the region between TAP2 and DQ looking for a yet unknown gene that contributes to IDDM susceptibility or resistance. Promoter region polymorphisms would also be of benefit to study within this region. As promoters regulate transcription and gene expression, a polymorphic or mutant promoter may lead to a less efficient or altered method of gene expression that would be associated with antigen processing or presentation and ultimately lead to disease.

Large scale familial haplotype analysis would be beneficial to better determine the extended haplotypes associated with DR3 and/or DR4. From this information, a prediction of the occurrence of the disease can be made.

Because genetic factors are not the only influence on the development of IDDM, studies could focus on the peptide presented by the MHC molecule during the onset of the disease. Characterization of this peptide could provide useful information on the target of the immunological response. This knowledge would help in understanding what the autoimmune reaction was actually against and the

mechanisms behind it. This information could lead to effective treatment or prevention of the disease.

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