III. MATERIALS AND METHODS

A. Subjects

Because the distribution of HLA allele is different in various ethnic groups, therefore, only one ethnic group, native northern Thai, was selected. The subjects were selected from leprosy patients and normal persons who were born in northern Thailand. Burmese, Chinese and hill tribal groups were excluded. The leprosy group included 143 unrelated patients who came from different families. All were diagnosed and treated at the McKean Rehabilitation Institute, Chiang Mai. The majority of this patients group came from 6 provinces in northern Thailand: Chiang Mai, Chiang Rai, Lampang, Lampoon, Nan and Pitsanulok. The clinical types of leprosy were determined by experienced leprologists in the Institute according to Ridley & Jopling's classification (Ridley and Jopling, 1966). The normal controls consisted of 120 healthy adult blood donors and volunteers who came from different families and had no known family history of leprosy. The majority of the control group was blood donors at the Blood Bank Unit of Maharaj Nakorn Chiang Mai Hospital.

B. Preparation of genomic DNA

Genomic DNA was prepared from peripheral blood leukocytes by the method of Miller et al. (1988) as follows: Peripheral venous blood (10 ml) was collected, mixed well with 50 µl of 0.5 M EDTA and centrifuged at 700 x g for 10 min in order to separate blood cells from plasma. After, the plasma was removed without touching the buffy coat, red cells were lysed by adding red cell lysis solution (10 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 10mM NaCl) to a final volume of 50 ml. Lysed red cell and debris were then removed by slow centrifigation (1000 rpm for 10 min) such that only the white cells, but not red cell ghosts, formed pellet at the bottom of the tubes. The pellet was resuspended in 3 ml of nucleic lysis buffer (10 mM Tris-HCl, pH 8.0, 400 mM NaCl, 2 mM EDTA), then 50 µl of 10% SDS and 50 µl of 10 mg/ml proteinase K were added. The solution was incubated at 55°C overnight and the remaining proteins were precipitated by adding 1.25 ml of saturated NaCl and standing at 4°C for 20 min.

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The solution was centrifuged at 800 x g for 25 min resulting in the formation of visible protein pellet. The fluid component which contained DNA was next transferred to a new tube. The DNA was recovered by precipitating with 2 volume of absolute ethanol. After washing the pellet twice with 70% ethanol and letting dry at room temperature, DNA was resuspended in 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA and stored at 4°C.

The concentration of genomic DNA was determined by measuring the UV light absorbance at 260 nm. DNA concentration was adjusted to 100 μg/ml and checked by running 0.5 μg of the DNA on 0.7% agarose gel and staining with 0.5 mg/ml of ethidium bromide against known amount of phage lambda DNA.

C. Amplification of HLA-DQA1 and HLA-DQB1 loci by PCR

1. HLA-DQA1 and HLA-DQB1 PCR primers

The oligonucleotide primers used in the PCR reaction were recommended by the Eleventh International Histocompatibility Workshop for use as primer pairs in the amplification of HLA-DQA1 and HLA-DQB1 genes. They were designed for amplification of the polymorphic second exons of HLA-DQA1 and HLA-DQB1 loci which encoded 76 (11th-87th) amino acids and 71 (13th-84th) amino acids of the alpha1 and beta 1 chains, respectively. The positions of the locus-specific primer pairs that correspond to the sequence of HLA-DQA1 and HLA-DQB1 alleles were shown in Figures 2 and 3 and the nucleotide sequences of these primers were shown in Table 2.

2 Amplification of HLA-DQA1 and HLA-DQB1 loci by PCR

The genomic DNA was amplified in 50 μl volume of the mixture containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM of each of the four dNTP, 1.5-2.0 mM $MgCl_2$ (1.5 mM for HLA-DQA1 amplification and 2.0 mM $MgCl_2$ for HLA-DQB1 amplification), 25 pmol of each primer, 1 unit of Taq DNA polymerase (Perkin Elmer Cetus, California, U.S.A.). After mixing the solution, 2 drops of mineral oil were added prevent evaporation. The amplification of HLA-DQA1 locus was allowed to proceed for 30 cycles by using the following parameters: denaturation, 94°C for 30 sec, annealing 56°C for 1 min, extention 72°C for 1.5 min and the last extention step was at 72°C for 8.5 min. The amplification of HLA-DQB1 locus was allowed to

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Table 2. Properties of HLA-DQA1 and HLA-DQB1 PCR primers.

HLA loci	Sequence (5' to 3')	position*	Tm (C)	Length of products (bp)
DQA1	ATG GTG TAA ACT TGT ACC AGT	32-52	58	229
	TTG GTA GCA GCG GTA GAG TTG	240-260	64	
DQB1	CAT GTG CTA CTT CAC CAA CGG	39-60	64	214
	CTG GTA GTT GTG TCT GCA CAC	232-252	64	

* : position indicates 5'-end of the primer maps the site of the second exon of the HLA-DQA1 and HLA-DQB1 loci.

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proceed for 30 cycles according to the following parameter: denaturation, 96°C for 30 sec, annealing, 57°C for 1 min, extention, 72°C for 2 min, and the last extention step was at 72°C for 8.5 min.

3. Agarose gel electrophoresis

In order to check that all amplified DNA are of the correct size and sufficient quantity for the hybridization, 3 µl of the PCR products were analyzed by electrophoresis through a 1.5% agarose gel in Tris/acetate/EDTA (TAE) buffer at 100 volts for 30 min. HaeIII-digested Phi X 174 DNA was used as the size marker. Following the staining with ethidium bromide (0.5 mg/ml) and destaining with water, the DNA fragments of 229 and 214 base pairs from the HLA-DQA1 and HLA-DQB1 amplifications, respectively, were clearly visible.

D. Dot blot hybridization

1. Dot spotting of amplified DNA on nylon membrane

Three microliters of amplified DNA (approximately 50 ng) were dotted on several nylon membranes according to the spotting order shown in Figure 4. The filters were denatured in 0.4 N NaOH for 5 min and neutralized in 10X SSPE buffer (1.5 M NaCl, 0.1 M NaH_2PO_4 (pH 7.4), 10 mM EDTA) for 10 min. Then the amplified products were fixed onto the nylon membrane by illuminating both sides with 254 nm UV light to 0.51 Joule/cm^2. After drying the membranes at room temperature, they were kept at 4°C.

2. Sequence specific oligonucleotide (SSO) probes

Eleven HLA-DQA1 SSO probes and 17 HLA-DQB1 SSO probes were recommended by the Eleventh Histocompatibility Workshop. Nine HLA-DQA1 and 17 HLA-DQB1 alleles, respectively, can be identified with these probes. The specificities and corresponding alleles of these HLA-DQA1 and -DQB1 SSO probes are shown in Tables 3 and 4.

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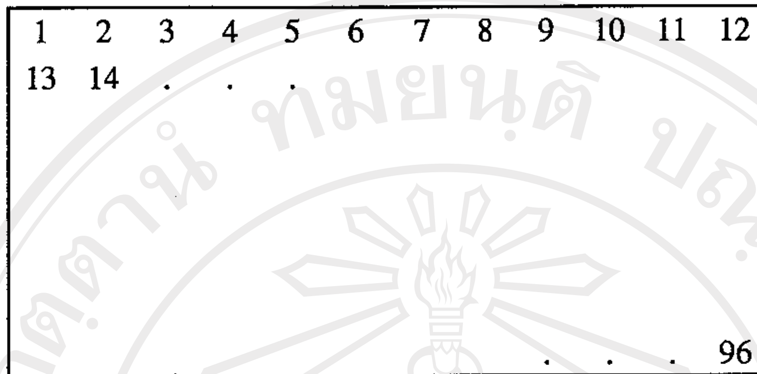

Figure 4. Dot spotting order of PCR products on nylon membrane.

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Table 5. Sequence and specificities of HLA-DQA1 SSO probes

DQA1 SSO	SEQUENCE (5'->3')	ALLELE SPECIFICITY
2502	TGGCCAGTTCACCCATGA	0103+0201+0601
3401	GAGATGAGGAGTTCTACG	0101
3402	GAGACGAGCAGTTCTACG	0102+0103+0501
3403	GAGATGAGCAGTTCTACG	0401+0601
4102	ACCTGGAGAAGAAGGAGA	0103
5501	TCAGCAAATTTGGAGGTT	0101+0102+0103
5502	TCCACAGACTTAGATTTG	0201
5503	TCCGCAGATTTAGAAGAT	03+03012+0302
5504	TCAGACAATTTAGATTTG	0401+0501+0601
6903	ATCGCTGTCCTAAAACAT	0501+03012
6902	ATCGTCTGTGCTAAAACAT	0201+03+0302

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Table 6. Sequence and specificities of HLA-DQB1 SSO probes

DQB1 SSO	SEQUENCE (5'->3')	ALLELE SPECIFICITY
2301	GACCGAGCTCGTGCGGGG	0401
2302	AACGGGACCGAGCGCGTG	03031+0402
2601	CGGGGTGTGACCAGACAC	0501+0502+05031+05032
2603	CGTCTTGTGACCAGATAC	0602+0302+03031+0302
2604	CGTCTTGTAACCAGACAC	0603+0604
2606	CGTCTTGTAACCAGATAC	0605
3702	AGGAGGACGTGCGCTTCG	0601
4501	GACGTGGAGGTGTACCGG	0301+0304
4901	GGTGTACCGGGCAGTGAC	0501
5701	GCGGCCTGTTGCCGAGTA	0501+0604+0605
5702	GCGGCCTAGCGCCGAGTA	0502+0504
5703	GGCGGCCTGACGCCGAGT	05031+0601
5704	GCGGCCTGATGCCGAGTA	05032+0602+0603
5705	GGCTGCCTGCCGCCGAGT	0201
5706	GGCCGCCTGACGCCGAGT	0301+03031+03032
5707	GGCCGCCTGCCGCCGAGT	0302+0304
0504	TCCTGGAGGAGGACCGGG	0504+0401+0402

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3. Preparation of digoxygenin-11-ddUTP-labeled SSO probes

The SSO probes were enzymetically labeled at their 3' end with terminal deoxynucleotidyl transferase by incorporation of a single digoxygenin-conjugated dideoxyuridine triphosphate (DIG-ddUTP) by using DIG-oligonucleotide 3' end labeling kit (Boehringer Mannheim Biochemica). Twenty-five pmol (5 pmol/μl) of each SSO probe were 3' end-labeled at 37°C for 2 hours with 25 units of terminal deoxynucleotidyl transferase and 1 pmol of DIG-11-ddUTP in a final volume of 20 μl. The reaction mixture contained 5 μl of 5X reaction buffer (1M potassium cacodylate, 125 mM Tris-HCl (pH 6.6), 1.25 mg/ml bovine serum albumin and 0.1 mmol $CoCl_2$). The DIG-labeled SSO probes were kept at -20°C and used in the hybridization directly without removing of unincorporated DIG-ddUTP.

4. Hybridization with SSO probes

The dot-blotted membranes were prehybridized with 0.1 ml/cm^2 of hybridization buffer (6X SSPE, 5X Denhardt's solution, 0.1% sarcosine sodium, 0.02% SDS) at 42°C for 1 hour in a sealed plastic bag in order to block non-specific binding. The DIG-ddUTP-labeled probe was added directly to the hybridization buffer in a final concentration of 0.5 pmol/ml. After hybridization at 42°C for 2 hours with shaking every 30 min, the membranes were washed twice in 2X SSPE buffer (0.3 M NaCl, 0.02 M NaH_2PO_4, 2 mM EDTA), 0.1% SDS at room temperature for 5 min, then washed twice in tetramethylammonium chloride (TMAC) solution (50 mM Tris-HCl (pH 8.0), 3.0 M TMAC, 2 mM EDTA, 0.1% SDS) at room temperature for 10 min, washed again in TMAC solution at 57°C for 15 min and then washed twice in 2X SSPE buffer at room temperature for 10 min.

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E. Chemiluminescent detection of DIG-ddUTP

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The DIG-labeled SSO probe that was hybridized to the target DNA was detected by using the DIG-chemiluminescent detection kit (Boehringer Mannheim Biochemica). The probe was detected by adding alkaline phosphatase-conjugated anti-DIG antibody and chemiluminescent substrate, AMPPD. Dephosphorylation of AMPPD catalyzed by alkaline phosphatase results in light emission which can be documented on X-ray film.

The hybridized membrane was washed in buffer 1 (100 mM maleic acid, 150 mM NaCl, pH 7.5) at RT for 10 min. In order to block non-specific binding, the membrane was soaked in buffer 2 (1%(W/V) blocking reagent provided in the kit in buffer 1) at RT for 30 min. Then 1:10,000 dilution of alkaline phosphatase-conjugated anti-DIG antibody (75 mU phosphatase activity/ml) in buffer 2 was placed over the membranes at RT for 30 min. Unbound antibody was removed by washing twice with washing buffer (0.3% Tween 20 in buffer 1) at RT for 15 min. Next, the membrane was equilibrated in buffer 3 (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) at RT for 5 min, then 1:100 dilution of AMPPD (100 mg/ml) in buffer 3 was spreaded over membrane at RT for 5 min. After dripping off excess liquid, the damp membrane was sealed in a PhotoGene development folder (BRL) and then incubated at 37°C for 15 min in order to allow efficient enzyme reaction and fastest results. Then the membrane was exposed to an X-ray film (Agfa) at RT for 2 hours to overnight according to the hybridizing activity of each SSO probe.

F. Dehybridization

In order to reuse the hybridized membrane, bound SSO probe was removed by dehybridization in the following steps. First, the membrane was washed in distilled water for 5 min, then the membrane was incubated with the dehybridization solution (0.2 N NaOH, 0.1% SDS) at 42°C for 30 min with shaking, then the membrane was neutralized in 2X SSC (pH 7.5) for 5 min, then it was ready to be used again.

G. Assignment of HLA alleles

The dot spot reactivities were graded as positive or negative by judging relative intensity in the following scoring system:

0	means	not done
1	means	negative (definite)
2	means	negative (probable)
4	means	indefinite
6	means	positive (probable)
8	means	positive (definite)
10	means	positive (definite, more than double intensity)

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After grading the dot spot signal, the assignment of allelic forms of the HLA-DQA1 and HLA-DQB1 alleles was made on the basis of combined positive signal from 10 HLA-DQA1 or 17 HLA-DQB1 SSO probes according to Tables 5 and 6. The frequency of each allele in the leprosy and normal control groups was then determined. In the next step, the statistical test will be employed in order to determine the level of statistical significance of the association of each allele to the clinical forms of leprosy.

H. Statistical analysis

1. Relative risk

In order to determine the association between each particular antigen or allele form of the HLA system and disease, the relative risk (R.R.) is usually calculated. The relative risk defines how many times more frequent the disease is detected in individuals carrying the antigen/allele as compared to individuals lacking the antigen/allele (Svejguard et al., 1975). The formula of calculation of the relative risk is:

$$\text{R.R.} = \theta = (\text{Allele+/Allele-})\text{disease} \div (\text{Allele+/Allele-})\text{control}.$$

If the relative risk is greater than 1, an association between the disease and the antigen is indicated; the greater the relative risk value, the stronger association.

This associated determination can be measured by using allele frequency and antigen frequency. The allele frequency was obtained by direct counting the actual number of the allele carried in each individual, presuming that all individuals were diploid with regards to the two loci tested, whereas the antigen frequency was obtained by counting of number of individuals who carried the allele. Thus, a homozygote would contribute two units to the allele frequency, but yielded only one unit to the antigen frequency.

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Table 7. Hybridization specificity of HLA-DQA1 SSO probes

DQA1 SSO	HLA - DQA1 allele								
	*0101	*0102	*0103	*0201	*03	*03012	*0401	*0501	*0601
2502	-	-	+	+	-	-	-	-	+
3401	+	-	-	-	-	-	-	-	-
3402	-	+	+	-	-	-	-	+	-
3403	-	-	-	-	-	-	+	-	+
4102	-	-	+	-	-	-	-	-	-
5501	+	+	+	-	-	-	-	-	-
5502	-	-	-	+	-	-	-	-	-
5503	-	-	-	-	+	+	-	-	-
5504	-	-	-	-	-	-	+	+	+
6903	-	-	-	-	-	+	-	+	-
6902	-	-	-	+	+	-	-	-	-

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Table 8. Hybridization specificity of HLA-DQB1 SSO probes.

DQB1 SSO	HLA-DQB1 allele																	
	*0501	*0502	*5031	*05032	*0504	*0601	*0602	*0603	*0604	*0605	*0201	*0301	*0302	*03031	*03032	*0304	*0401	*0402
2301	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
2302	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+
2601	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2603	-	-	-	-	-	-	+	-	-	-	-	-	+	+	+	-	-	-
2604	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-
2606	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
3702	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
4501	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-
4901	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5701	+	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-
5702	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
5703	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
5704	-	-	-	+	-	-	+	+	-	-	-	-	-	-	-	-	-	-
5705	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
5706	-	-	-	-	-	-	-	-	-	-	-	+	C	+	+	C	-	-
5707	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-
0504	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	+

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C = cross reactivity

2. Etiologic fraction (EF)

An alternative method for determining association, the etiologic fraction (EF) or population attributable risk (PAR) (MacMahon and Pugh, 1970), determines how strong of association compared between one allele and the disease (Miettinen, 1974). PAR or EF is defined as the proportion of case attributable to the exposure, out of the total number of cases developed in the total population. The formula for calculation of PAR or EF is:

$$PAR = EF = \delta = (FAD-FAP)/(1-FAP)$$

Here FAD stands for the frequency of antigen/allele among the individuals with disease, and FAP stands for the frequency of antigen/allele in the general population. If δ is equal to 0, there is no association between the disease and the antigen/allele. If there is a positive association, δ takes a value between 0 and 1. When δ is 1, the association between the disease and the antigen/allele is complete, which means that all individuals with the disease carry the antigen/allele.

3. Preventive fraction (PF)

An analogous function to the etiologic fraction is used when an association is negative. This measure, called the preventive fraction (PF) (Miettinen, 1974) represents how large a reduction in the number of cases has the exposure caused by its presence in the population. The formula for calculation of the preventive fraction is:

$$PF = FAP-FAD/1-FAP$$

If there is a negative association, PF takes a value between 0 and 1. When PF is 1, the association between the disease and the antigen/allele is negative completely, which means that all individuals with the disease do not carry the antigen/allele.

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4. Significance testing

Comparison between the various alleles in patients with leprosy and controls was made by using chi square test for two by two tables in order to answer first, whether there is a significant difference between the frequency of antigen or allelic forms in patients and controls, second, how strong is the association between antigen/ allele and the disease. For testing the statistical significance of the HLA and disease, the Woolf's method (Woolf, 1955) and Haldane's method (Haldane, 1955) were used. The formula for the Woolf 's method is:

$$\chi^2 = wy^2$$

$y^2 = \ln x$
$x = ad/bc$
$w = 1/v$
$v = 1/a+1/b+1/c+1/d$

a means numbers of patients with the antigen (allele),
b means numbers of patients without the antigen (allele),
c means numbers of controls with the antigen (allele),
d means numbers of controls without the antigen (allele).

When samples are small (≤ 5), the formula given by Haldane can be used:

$x = (2a+1)(2d+1)/(2b+1)(2c+1)$,
$v = (1/a+1) + (1/b+1) + (1/c+1) + (1/d+1)$.

After the chi square value was calculated, the probability value (p value) was obtained from the chi square table. The p value was corrected by multiplying by the number of alleles tested at each locus. If p value is less than 0.05, the difference is significant.

5. Hardy-Weinberg analysis

In order to investigate whether the distribution of the HLA-DQA1 and HLA-DQB1 genotypes in northern Thai population was in equilibrium, the Hardy-Weinberg

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analysis was performed. The Hardy-Weinberg equilibrium means that the distribution of alleles of a given locus fits the binomial distribution according to Hardy-Weinberg law and that the gene frequencies of all given alleles remain constant from generation to generation. The conditions that allows such equilibrium in the population are that there is random mating, no migration, no selection, no new mutation and no genetic drift (Thompson, 1986). It is assumed that large and constant normal population without these confounding factors is in Hardy-Weinberg equilibrium. If the normal control group is in Hardy-Weinberg equilibrium, it means that our normal control samples are good representative samples.

The binomial distribution of the locus that contains two alleles according to Hardy-Weinberg law is:

$$(p + q)^2 = 1$$
$$p^2 + 2pg + q^2 = 1$$

where p and q are allele frequencies of the first allele and 2nd allele, respectively.

p^2 is the allele frequency of a combination of the fisrt allele and first allele (homozygous of the first allele).

2pq is the allele frequency of a combination of the first allele and the second allele (heterozygous of the first allele and second allele).

q^2 is the allele frequency of a combination of the second allele and second allele (homozygous of the second allele).

If the locus contains multiple alleles, the Hardy-Weinberg law is applied as:

$$(p + q + r + ...)^2 = 1$$
$$p^2 + q^2 + r^2 + ... + 2pq + 2pr + 2qr + ... = 1$$

p, q, r, ... are allele frequencies of 1st, 2nd, 3rd, ... alleles, respectively.

The expected genotype frequencies as this law can calculate is as follows:

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homozygous genotype frequency = (allele frequency)^2,

heterozygous genotype frequency = 2 x 1st allele frequency x 2nd allele frequency.

The comparison between the distribution of expected genotype frequency distribution according to the Hardy-Weinberg law and the observed genotype frequency was made using chi-square test. The formula of chi-square test is:

$$\chi^2 = \Sigma (O-E)^2 / E$$

where O = Observed frequency of the combination of each genotype,
E = Expected frequency of the combination of each genotype,
the degree of freedom = number of genotype of the given loci - 1.

If the p value is more than 0.05, the difference is not significant. This means that the distribution of observed frequencies fits the distribution of expected frequencies according to the Hardy-Weinberg law and that the distribution of alleles of this locus is in equilibrium.

6. Linkage disequilibrium analysis

Linkage disequilibrium is the tendency of specific combination of two alleles or more linked loci to occur together on the same chromosome more frequently than would be expected by chance (Benjamin and Lewin, 1993). Since the family study was not performed, the chromosomal phase (actual linkage) of each individual was unknown. However, strong linkage of the HLA-DQA1 and -DQB1 alleles may be detected without performing actual family study. This was done by comparing, for a particular pair of HLA-DQA1 allele and HLA-DQB1 allele, the actual haplotype frequency (determined by direct counting) with the expected haplotype frequency (determined by mutiplying the allele frequencies of the two alleles). In the counting of the actual haplotype frequency, a double homozygote (such as an individual with HLA-DQA1*0101/*0101-HLA-DQB1*0501/*0501) was counted as four units of linked haplotype. For a double heterozygote, it was assumed that any of the four possible haplotypic combination occurred at an equal frequency. Thus, there were four possible

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haplotypes in a double heterozygote, each was counted as one unit of linked haplotype. Significant linkage disequilibrium was identified by employing the chi-square test or Fisher's exact test.

Linkage disequilibrium is calculated by determining the difference (Δ) between the observed frequency of a particular combination of allele at two loci and the frequency expected for random association according to the formula (Paul, 1991):

$$\Delta (\%) = (Fobs - pq) \times 100$$

p = frequency of allele "a" at loci 1.
q = frequency of allele "b" at loci 2.

The significance of such difference is then tested by employing the chi-square test. The formula of the chi-square is (Vogel and Motulsky, 1986):

$$\chi^2 = (ad-bc)^2N / (a+b)(c+d)(a+c)(b+d)$$

where, a = allele frequency of individuals who carried both of allele a and allele b,
b = allele frequency of individuals who carreid allele b but not allele a,
c = allele frequency of individuals who carried allele a but not allele b,
d = allele frequency of individuals who not carried both of allele a and allele b,
N = total number of individuals in the population (a + b + c + d),
and the degree of freedom = 1.

If the p value is less than 0.05, the allele a and allele b are in linkage disequilibrium.

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