V. DISCUSSION

Polymerase chain reaction and sequence-specific oligonucleotide (PCR-SSO) hybridization is a rapid method for the detection of previously known nucleotide sequence variations. In this study PCR-SSO hybridization was employed to define HLA-DQA1 and HLA-DQB1 alleles in order to determine the association between HLA-DQA1 and HLA-DQB1 allelic forms and leprosy in northern Thailand. In order to assign the HLA-DQ alleles, the second polymorphic exons of the HLA-DQA1 and -DQB1 genes are amplified by PCR and the nucleotide sequence variations of these HLA-DQ alleles detected by hybridization with SSO probes. The antigen/allele frequencies of the leprosy patients or various subtypes of leprosy are compared with those of normal control group. This analysis allows us to identify exactly which one of the HLA-DQ alleles is associated with leprosy or subtypes of leprosy in northern Thai population.

Classically, HLA phenotyping is performed by using serologic methods (WHO, 1991). However, these techniques are limited by the availability of polyclonal monospecific antisera, monoclonal antibodies and homozygous typing cells. Lately, the molecular cloning and sequencing of HLA genes reveals nucleotide sequence variations that are responsible for the various structural heterogeneity of the HLA antigens (Paul, 1993). DNA sequencing is the most correct method to identify variants of the HLA class II loci but, because of the technical difficulty, it is not practical for large-scale population studies (Paul, 1993). The RFLP, PCR-RFLP (Salazar et al., 1992) and PCR-SSO help to identify known sequence variations without the need for sequencing. The PCR-RFLP is a new technique that incorporates digestion of the PCR amplified product with restriction endonucleases and the resolution of the cleaved fragments by gel electrophoresis (Hui and Bidwell, 1993). Currently, this method is capable of distinguishing only 14 HLA-DQB1 allelic forms and can not discriminate DQB1*0602 and DQB1*0603 alleles. On the other hand, the PCR-SSO technique is relatively simple, rapid and readily applicable to large scale population study. It also provides the ability to define a great number of alleles and can discriminate down to one base pair difference.

Up to now, there are a total of 13 known HLA-DQA1 alleles (Marsh and Bodmer, 1993). The PCR-SSO hybridization method as recommended in 11th

International Histocompatibility Workshop is capable of identifying nine allelic forms (Reference protocol, 1991). It can not distinguish: a) the DQA1*03011 allele from *0302 allele, and b) the DQA1*05011 allele from *05012 and *05013 allele due to the identical nucleotide sequence in the second exon region that is flanked by the HLA-DQA1 primer pair employed. However, the nucleotide differences between all of these three pairs (DQA1*03011-*0302, DQA1*05011-*05012, and DQA1*05011-*05013) do not lead to variation at the level of amino acid sequence of the HLA-DQα chain (Marsh, 1991). In addition, this PCR-SSO method can not distinguish HLA-DQA1*0104 from *0101; this is due to a point mutation in the first exon of this gene (Marsh and Bodmer, 1993). The amino acid interchange (Asp → Gly) at the second amino acid position of the first extracellular domain, however, is located outside of the peptide binding groove of the HLA class II molecule (Brown et al, submitted).

In this study we employ ten HLA-DQA1 SSO probes which are sufficient to distinguish nine HLA-DQA1 alleles. We find that, with an exception of four samples, all HLA-DQA1 alleles and HLA-DQA1 genotypes can be identified by these ten probes. In all four cases (N136, N140, N203, L114), there were positive reactions with the DQA1 SSO 3402, 5503, 5504 and 6903 probes. Such pattern of hybridization is compatible with two genotypes: HLA-DQA1*03/*05 and DQA1*03012/*05 genotypes. The problem in these cases is that the positive signal of SSO 6903 is due to the hybridization with the DQA1*05 allele; thus, the differentiation between HLA-DQA1*03 and -DQA1*03012 alleles, which is decided by the presence or absence of positive hybridization with SSO 6903 probe, can be definitely made. To solve this problem, an additional probe, DQA1 SSO 6902, is employed to discriminate between these two possiblities. This is because the DQA1 SSO 6902 probe hybridizes only to DQA1*03, but not DQA1*05 and DQA1*03012. When all four samples are found positive with the DQA1 SSO 6902 probe, they are definitely assigned as HLA-DQA1*03/05. So, HLA-DQA1 typing in northern Thai population may require upto eleven probes, e.g. 10 probes that are included in the BSHI HLA-class II PCR-SSO kit plus DQA1 SSO 6902.

The other two samples with non-typable HLA-DQA1 alleles are N59 and L153. The N59 DNA sample does not hybridize with any probes, so it HLA-DQA1 genotype cannot be assigned. Three possibilities that may its explain the problem are: 1) the nucleotide sequence of the PCR primer binding region(s) has been

mutated such that the primer(s) cannot anneal under the conditions used, 2) there is Taq DNA polymerase inhibitor in the DNA solution, 3) other components of the DNA solution are not optimal for the PCR. In the case of L153, this DNA sample hybridizes with the DQA1 SSO 2502, 3401, 3402, 5501 and 5502 probes and, because the unexpected hybridization with the DQA1 SSO 5502 probe, cannot be assigned the HLA-DQA1 genotype. So, this sample was rediluted from the original DNA stock, re-amplified, and re-hybridized with all five positive probes. The second hybridization result was the same as the first one. In order to further investigate whether the unusual hybridization of L153 was due to a new HLA-DQA1 allele or to DNA contamination, the PCR products were ligated to pBluescript II SK+/- vector and transformed into competent *Escherichia coli*. Transformed colonies were screened on agar plates containing ampicillin and X-gal and positive colonies were grown in five ml of LB broth overnight. The recombinant plasmids were cut with HindIII to identify clones with inserted DNA. When three positive clones were sequenced by the dideoxy chain termination method (Sanger et al., 1977), we found that, the original DNA sample contains three allelic forms: HLA-DQA1*0101, *0102, and *0201. This result indicates that L153 DNA is most likely to be contaminated with other DNA sample.

Of all possible 19 HLA-DQB1 alleles, our use of 17 HLA-DQB1 SSO probes as included in the BSHI HLA-class II PCR-SSO kit allows the determination of 18 alleles. An unidentifiable allele, HLA-DQB1*0606, is due to a point mutation at the codon position 74. The detection of this allele requires a new SSO probe. From the use of 17 probes, every alleles and genotypes of HLA-DQB1 allele of all samples can be assigned, except for three samples: N205, L90, L13. Samples N205 and L90 hybridize with the HLA-DQB1 SSO 2601, 5702, 4901, 5701 and 2603 probes whereas L13 hybridizes with HLA-DQB1 SSO 2601, 5702 and 2603. The hybridization with SSO 2603 probe indicates that there are unusual DQB1*0501 or *0502 alleles in the three samples. The hybridization with the DQB1 SSO 2603 probe is unlikely to be due to contamination because of the lack of other positive hybridizations, such as with DQB1 SSO 5705, 5706, and 5707 probes. It is quite possible that in this population there exists a DQB1*0502 allele that is mutated such that the DQB1 SSO 2603 probe can hybridize with it. Such unusual allele can be further characterized by subcloning and sequencing. It is a potential candidate for a new HLA-DQB1 allele. The new mutation may be located at the 75th to 90th codon

positions (Jonsson et al, 1987). Another possibility to explan this problem is: the HLA-DQB1*0502 allele may have mutation in some base pairs that cause the cross reaction of hybridization. In the case of L90, may contain such mutated sequence in only one chromosome, because DQB1 SSO 2601 still hybridize with another usual DQB1* 0502 allele.

Most HLA-DQA1 and HLA-DQB1 alleles that have been found earlier in other populations are also found in this panel of northern Thais but, the frequencies of many alleles are different. As expected, the gene frequencies of HLA-DQA1 and HLA-DQB1 alleles in this panel of northern Thais are quite similar to other oriental populations but are different from Caucasian populations (Djoulah et al., 1994, Cerna et al., 1992, Ronninggen et al.,1991; Doherty et al., 1992; Lango et al., 1993; Yanagawa, 1993). We find the HLA-DQA1*0102, DQA1*0101 and DQA1*03 alleles at high frequency (28.8%, 25.5% and 19.2%, respectively). This is similar to the northern Chinese population (HLA-DQA1*0301 and DQA1*0102 at 28.6% and 14.8%, respectively; Gao et al., 1991), and Shanghai Chinese population (HLA-DQA1*0301, -DQA1*0102 and DQA1*0103 at 28.7%, 16.3% and 16.3%, respectively; Wang et al., 1993), and Filipino population (DQB1*0502 and DQB1*0301 at 28.5% and 18.5%; Bugawan, et al., 1994). On the contrary, the alleles DQA1*0501 and DQA1*0201 are dominant in the Caucasoid populations (Djoulah et al., 1994, Cerna et al., 1992, Ronninggen et al., 1991; Doherty et al., 1992; Lango et al., 1993; Yanagawa, 1993). In British Caucasoid, the alleles DQA1*03 and DQA1*0501 are the most frequent (23.7% and 22%, respectively; Doherty, et. al., 1992). The alleles DQA*0501, DQA*0201 and DQA*0301 are found at the frequencies of 38.3%, 14.9% and 14.9%, respectively. These allele frequencies are also found in Western Algerian (Djoulah et al., 1994). In Norwegian Caucasian, the alleles DQA1*0102 and DQA1*0501 were detected at the high frequencies of 42% and 40%, respectively (Ronningen et al., 1990). Clearly, the dominant HLA-DQA1 alleles of northern Thais, especially the DQA1*0102 allele, are quite similar to those of other oriental populations. Also as observed in the oriental groups, the allele DQA1*0501 is detected at low frequency in northern Thais, while it is the dominant allele in most Caucasoid populations.

With regards to the distribution of HLA-DQB1 alleles, we find the DQB1*0502 allele to be the most frequent (35.4%). The other common alleles,

DQB1*0301 and DQB1*03032, are found at only 15.8% and 12.1%, respectively. Similar to those of northern Thais, the alleles DQB1*0502 and DQB1*0301 are found very frequently (28.1% and 18.5%, respectively) in the Filipino population (Teodorica, 1994). On the other hand, the two most frequent HLA-DQB1 alleles in the Chinese populations, such as northern Chinese and Shanghai Chinese, turn out to be DQB1*0303 and DQB1*0301 (Gao et al., 1991; Wang et al., 1993). In the British Caucasoid, the alleles DQB1*0201, DQB1*0302 and DQB1*0301 are found most frequently (23.2%, 16.4% and 16.1%, respectively; Doherty et al., 1992). In the Norwegian, DQB1*0201 and DQB1*0602 are found at 44% and 33%, respectively (Ronningen et al., 1990). In Africa, such as Algeria, DQB1*0301 and DQB1*0201 are also the predominant HLA-DQB1 alleles (35.1% and 24.5%, respectively; Djoulah et al., 1994). Different from the caucasian populations, the Gypsies from Hangary inherit the alleles DQB1*0503 and DQB1*0301 more frequently than other alleles (Cerna et al., 1992). Thus, the allele HLA-DQB1*0502 appears to be a predominant allele in northern Thais as well as in the Filipino population. It is very rare in various Caucasian populations (Ronninggen et al.,1991; Doherty et al., 1992; Lango et al., 1993; Yanagawa, 1993). The two other common alleles in northern Thais, DQB1*0301 and DQB1*03032, are also found readily in northern Chinese and Shanghai Chinese. In contrast, the allele DQB1*0201 is found frequently in various Caucasian groups, but only rarely in the oriental populations.

In the present study, there are upto eight alleles that are absent from the normal northern Thais: HLA*DQA1*03012, DQA1*0401, DQB1*05032, DQB1*0504, DQB1*0604, DQB1*0605, DQB1*03031, and DQB1*0402. Some of these alleles were also absent from northern Chinese (e.g., DQB1*0604 and DQB1*0504), Shanghai Chinese (e.g., DQB1*0402), and Filipino (e.g., DQB1*0603) (Wang et al., 1993; Teodorica, 1994).

Hardy-Weinberg equilibrium analysis of the HLA-DQA1 and HLA-DQB1 genotype distribution of the northern Thai population shows that both of the HLA-DQA1 and HLA-DQB1 loci are in equilibrium. This means that the genotype frequency distribution fits the binomial expansion-prediction and these genotype frequencies remain constant from generations to generations (Thompson, et al., 1986). When the leprosy and subtypes of leprosy are analyzed in the same manner, they are also in Hardy-Weinberg equilibrium.

The association between the HLA-DQA1 and -DQB1 loci in normal northern Thais is determined by linkage disequilibrium analysis. This parameter defines the tendency of specific combinations of two alleles at two or more linked loci to occur together on the same chromosome more frequently than would be expected by chance (Benjamin and Lewin, 1993). The linkage disequilibrium may be important in the study of disease susceptible genes because the true susceptible genes give stronger association with disease than other linked-genes (Vogel and Motulsky, 1986). From the analysis of normal northern Thais, there are 11 HLA-DQA1-DQB1 haplotypes with significant linkage disequilibrium. They are identical to those in northern Chinese and Shanghai Chinese population (Vogelsky, 1986). As expected, some of the known HLA-DQA1-DQB1 haplotypes with linkage disequilibrium of the other races are not found in northern Thais. Examples of the latter cases include: the DQA1*03-DQB1*03032 haplotype in British Caucasoid (Doherty, 1992), the DQA1*03-DQB1*03032, DQA1*0102-DQB1*0502, DQA1*0501-DQB1*0201 and DQA1*0601-DQB1*0301 haplotypes in Algeria (Djoulah, 1994).

The main objective of this study is to determine and compare the distribution of HLA-DQA1 and HLA-DQB1 alleles in different types of leprosy patients with normal northern Thais. None of the seven HLA-DQA1 alleles is found at significantly increased or decreased frequencies in leprosy patients or various subtypes of leprosy patients when compared with normal controls. Eventhough the allele DQA1*0601 is found initially to be decreased in TT patients, the difference is not significant after correcting the p value with the number of alleles tested for. Comparisons of the HLA-DQA1 allele frequency between the same groups of subjects also give the same results as in the case of the HLA-DQA1 antigen frequency. Similarly, none of the HLA-DQB1 alleles is significantly increased or decreased in leprosy or types of leprosy when campared with the normal controls. The result of our study is in concordance with the study of HLA-DQ allele typing in leprosy patients by RFLP technique (Kanjanahaluethai et al., 1994). They find no significant association of any particular HLA-DQA1 band with leprosy. However, they observe that one of the two HLA-DQA2 RFLP alleles is significantly reduced in leprosy patients (Kanjanahaluethai et al., 1994). The HLA-DQA2 gene is in close linkage with the HLA-DQA1 gene, but whether it is

expressible is still unknown (Auffray et al., 1984). Our PCR-SSO method cannot identify the HLA-DQA2 alleles (Tiercy et al., 1993).

Our result is different from the study of Schauf et al (1985) who compared the HLA class II antigens between 32 tuberculoid leprosy patients and 35 lepromatous leprosy patients with 32 normal controls in northern Thai population. They found that the HLA-DR2 and HLA-DQw1 antigens were associated with tuberculoid leprosy patients (relative risk of 21.4 and 18.7, respectively). When we reclassify the sixteen HLA-DQB1 alleles into three corresponding HLA-DQ antigens (HLA-DQw1, -DQw2 and -DQw3) and compared the distribution of deduced HLA-DQ antigens between the two types of leprosy and controls, we still detect no association. There are two possible explanations for the difference of these two studies.

1). The sample sizes of the study of Schauf et al seem too small, causing sampling error.

2). The patients or control groups of the two studies may be different. This is suggested by the discrepancy of the antigen frequencies of HLA-DQ antigens. They find the following frequencies for the HLA-DQw1, -w2, and -w3 antigens: 65.6%, 0%, and 77.7%, respectively, in the normal controls while we find 55.2%, 8.6%, and 32.6% of the same antigens. This situation may reflect the differences in the criteria of selection of the normal and/or patient groups. It is well known that northern Thailand is composed of many ethnic groups. We have deliberately selected only one ethnic group, northern Thai ethnic group, and excluded Chinese, Burmese and hill tribal ethnic groups from both the normal controls and leprosy patient groups. We do not know that Schauf et al had been aware of the population heterogeneity and had exercised care in the sample selection.

Up to now, there is only one other study on leprosy-HLA class II association performed at the DNA level (Rani et al., 1993). This was done in north India by comparing 41 LL patients, 25 BL patients, 28 TT patients with 47 normal controls. They found that: 1) the allele DQB1*0601 was significantly more frequent in leprosy patients (60% vs 17%, R.R. = 7.4), 2) the allele HLA-DQA1*0102 was significantly more frequent in leprosy patients (40.9% vs 19.1%, R.R. = 2.9), 3) the allele HLA-DQA1*0103 was significantly more frequent only in LL patients (27.6% vs 51%, R.R. = 2.8), and 4) the allele HLA-DRB*15 was most strongly associated with leprosy, multibacillary leprosy, and TT patients

(75.3% vs 21.3%, R.R. = 11.3 for leprosy; 81.5% vs 21.3%, R.R. = 16.3% for multibacillary leprosy; 60.7% vs 21.3%, R.R. = 5.7% for TT patients) (Rani et al., 1993). Some possible explanations for the difference between this study and our study are:

1). A leprosy-associated allele, such as HLA-DQA1*0103, appears in the northern Thai population at a very low frequency (4.2%), while it is found in northern India at a relatively much higher frequency (27.6%). If such rare allele really contributes to the pathogenesis of leprosy, a statistically significant association will be more difficult to be detected in northern Thailand due to the relative paucity of cases.

2). The association of HLA-DQA1*0102, DQA1*0103 and DQB1*0601 with leprosy in northern India may be due secondarily to the linkage disequilibrium of these HLA-DQ alleles with HLA-DRB1*15. Rani et al. (1993) showed that when the allele DRB1*15 is excluded, the significant associations of leprosy with the alleles DQA1*0102, DQA1*0103 and DQB1*0601 are no longer observed. On the contrary, when the alleles DQA1*0102 or DQA1*0103 or DQB1*0601 are excluded, the significant association with HLA-DRB*15 is still seen. It is possible that if a HLA-DRB allele is actually associated with leprosy in northern Thailand, such HLA-DRB allele may not be in strong linkage disequilibrium with any HLA-DQ allele to allow detection of association of HLA-DQ allele with leprosy.

3). The northern Thai and northern India populations are different in certain genetic background. The background may exert different levels of influence primarily on the functioning of HLA molecule and secondarily to the pathogenesis of leprosy in different populations. Thus, HLA-DQ association with leprosy may be observed only in certain ethnic groups.

Association between HLA and various autoimmune diseases are consistent and widely accepted and the association is primarily with HLA-DQ alleles (Altmann et al., 1991). But association between HLA and many infectious diseases are inconsistent in different ethnic groups (Hill, 1992). Hill (1992) described the possible explanation for the inconsistent association between infectious diseases and HLA as the following:

1). The associated HLA alleles in the early studies of the infectious disease and HLA was observed without correction of the p value by multiplying with the number of compared alleles. These caused false-positive association.

2). Case and control groups are not in matching groups.

3). The small sample size of most infectious disease studies cause unconvincing results. Because the infectious patients samples are difficult to collect in large group while the autoimmune disease' patients usually accumulate in specialist clinic.

4). The variation of the infectious agents may cause different HLA association.

An example of interesting association between HLA and infectious disease is malaria in over 2000 western African population (Hill, 1991). HLA class I antigen, HLA-Bw53 and HLA class II haplotype, DRB1*1302-DQB1*0501 are associated with reduced susceptibility to severe malaria. It is interesting that both of these HLA types are much more frequent in western African. It is suggested that natural selection by malaria had contributed to be increased of those HLA types frequencies in African population. In the case of the other infectious diseases, association between HLA and disease may be found in the large population through natural selection by the infectious pathogen.

Although our results suggest that the differences in HLA-DQA1 and HLA-DQB1 genotypes of northern Thai individuals do not influence the development of various clinical outcomes of leprosy, further studies are needed to examine whether other linked genes, such as HLA-DR and -DP genes, are associated with the outcomes of *Mycobacterium leprae* infection in this population.