V. DISCUSSION

The selective amplification of DNA and hybridization with oligonucleotide defined alleles on the basis of their variable sequences. Many new alleles have now been discriminated with greater refinement than the older techniques (Bidwell et al., 1990, Navarrete et al., 1993). In this study the sequence specific oligonucleotide (SSO) probes were selected from the 11th International Histocompatibility Workshop reference protocol to identify 8, 17 and 48 of the HLA-DQA1, HLA-DQB1 and HLA-DRB1 alleles, respectively (Kimura et al., 1991). Many additional alleles, 5 of HLA-DQA1, 9 of HLA-DQB1 and 87 of HLA-DRB1 were recently reported (Marsh and Bodmer, 1995), but some were not typed in this study such as the alleles HLA-DQA1*05012, *05013, HLA-DQB1*06012, *06052, HLA-DRB1*11012 were not typed because the nucleic acid substitution does not result in amino acid substitution. Some alleles are not typed because there are no specific SSO probes.

Ten selected DQA SSO probes from the workshop protocol and 2 designed probes typed 13 alleles of HLA-DQA1 locus except 4 alleles: the HLA-DQA1*0104, *05012, *05013 and *0503. The nucleic acid sequence in the PCR amplified region of the alleles HLA-DQA1*0101 and *0104 is similar but they have one base difference outside this region (at nucleic acid position 5) (Marsh and Bodmer, 1995). Thus, the HLA-DQA1*0101 allele may be reported as the HLA-DQA1*0101/0104 allele. Similar to the case of the allele HLA-DQA1*0503 which resemble the HLA-DQA1*0501 allele except the nucleic acid position 479 which stand outside the PCR amplified region (Marsh and Bodmer, 1995). Therefore, the HLA-DQA1*0501 allele may be reported as the HLA-DQA1*0501/0503 allele.

Twenty-five alleles of HLA-DQB1 were published, the HLA-DQB1*03031 was deleted (Marsh and Bodmer, 1995). There were 9 new alleles; HLA-DQB1*06012, *06052, *0606, *0607, *0608, *0609, *0202, *0304 and *0305. The 17 selected DQB SSO can type the HLA-DQB1*0304 and *0305 but cannot type some new alleles of the established HLA-DQB1*06 (i.e. HLA-DQB1*0606, *0607, *0608 and *0609 alleles) and HLA-DQB1*0202 allele. These alleles have similar hybridization pattern for the designed probes and have to be distinguished from each other by other specific probes. Because of an extremely low frequency in the population, or in some case, none of the subjects show the particular pattern. We decide not to attempt to type these alleles.

There are 33 of 87 new alleles of HLA-DRB1 locus that cannot be typed by the 24 chosen DRB SSO probes. If using the additional of 8 DRB SSO probes; 2809, 3709, 3712, 5706, 7006, 7008, 7009 and DRB DR8, the 21 of 33 new alleles can be typed. However, there are 12 remaining alleles cannot be typed. The new alleles mostly distributed into 2 main subgroup as follows.

1) The HLA-DR2 group has six new alleles; HLA-DRB1*1504, *1505, *1603, *1604, *1605 and *1606 (Marsh and Bodmer, 1995). The hybridization pattern of all new HLA-DRB1*16 alleles is similar to that of the allele HLA-DRB1*1601. However, the HLA-DRB1*1601 pattern was not found in our patient and control subjects. On the other hand, the HLA-DRB1*1503, *1504 and *1505 alleles cannot be differentiated from the allele HLA-DRB1*1501 with the probes that we used. Although, DRB SSO 2813 and DRB SSO 7011 probes can be used to discriminate all new HLA-DRB1*15 alleles. In case of HLA-DRB1*1501 homozygote, the set of probes that we used would give hybridization pattern which can be interpreted either as a HLA-DRB1*1501 homozygote or a HLA-DRB1*1503 or *1504 or *1505 heterozygote. Thus, the HLA-DRB1*1501 homozygote should be more correctly assigned as HLA-DRB1*1501/1503/1504/1505 heterozygote. Actually, no subject in this patient group was a candidate for a HLA-DRB1*1501 homozygote.

2) The HLA-DR52 associated DRB1 group. This group has 66 new alleles (Marsh and Bodmer, 1995). Many of them can be typed by the DRB SSO probes that we used. There are 2 new alleles, HLA-DRB1*1112 and *1410 that can not be distinguished. The hybridization pattern of the allele HLA-DRB1*1112 is identical with that of HLA-DRB1*1101. The nucleic acid position 110 of the HLA-DRB1*1112 differs from that of the HLA-DRB1*1101 allele. This problem can be solved by designing the new probe cover this region. Another new allele, HLA-DRB1*1410, is similar to HLA-DRB1*1401, but it hybridizes with the probe DRB SSO 1004 in stead of DRB SSO 1003. It is grouped as the HLA-DRB1*04 allele.

Early studies of the association of HLA genes with Graves' disease had been conducted mostly in Caucasian populations (Farid et al., 1979, Dahlbreg et al., 1981, Stenszky et al., 1985). These studies almost invariably revealed strong association of the HLA-DR3 allele with the development of Graves' disease. Association of Graves' disease with the HLA-DQA*0501 allele in the Caucasian population has also been detected (Yanagawa et al, 1993, 1994; Badenhoop et al, 1995), but has not been confirmed in a more recent study (Cuddihy and Bahn, 1996). Recently, a few studies were done on

several Oriental populations but no association with the HLA-DR3 allele was detected. Many of these studies employed a small number of subjects, especially that of the male subgroup. Such low number of subjects may be inadequate for analysis in the light of an increasing number of HLA alleles currently detectable by molecular genetic techniques. In our study of 108 female and 70 male Graves' disease patients who resided in the upper northern provinces of Thailand, we observed a positive association of the HLA-DQB1*03032 allele with Graves' disease only in the subgroup of early-onset patients (the age of onset of disease below 31 years) but not in all patients combined. Similar to other studies done in Oriental groups, we also do not detect the association of HLA-DR3 allele with Graves' disease.

Similar HLA association with early onset of Graves' disease have been reported in other Oriental populations although these did not involve the same allele that we detected. For example, previous studies of Chinese and Japanese patients with Graves' disease revealed a significantly increased frequency of the HLA-Bw46 allele with early age of onset of disease (Yeo et al., 1989; Onuma et al., 1994). In the same way, Asians Indian patients carrying the phenotypes HLA-A10 and HLA-B8 were more prone to develop Graves' disease at a younger age (Tandon et al., 1990). One explanation for this observation is the possibility that Graves' disease may be divided as early-onset subgroup and late-onset subgroup with regards to HLA association, especially in the Oriental populations. A precedence for this is in the case of diabetes mellitus in which the early-onset and insulin-dependent subgroup is associated with HLA-DQw3.2 (HLA-DQA1*0301-DQB1*0302) whereas the late-onset and non-insulin dependent subgroup is not associated with the HLA allele (Todd et al., 1987; Auwera et al., 1995). However, this association has been observed in both Caucasian and Oriental populations, especially the Japanese (Thorsby and Rønningen, 1992). Thus, disparity in term of HLA association in the case of diabetes mellitus may actually reflect the fact that IDDM and NIDDM are two distinct diseases with different underlying pathogenetic mechnisms. The first explanation is unlikely because Graves' disease is most likely representing only a continuum of one pathological entity; no subdivision of these patients with regards to age has been observed and/or proposed. The more likely explanation for the association of HLA-DQB1*03032 allele with Graves' disease only in the subgroup of early-onset patients is due to non-random sampling of the patient group resulting in selection bias. Another possible reason is the role of HLA gene increases the susceptibility of disease. This susceptibility gene may

accelerate the disease expression. Thus, the younger patients were significant associated with a particular HLA allele.

As mentioned earlier, the observation found in most studies involving Caucasian populations that there is a strong association between the HLA-DR3 allele with Graves' disease cannot be reproduced in similar studies of the Oriental groups. The lack of association of the allele HLA-DR3 with Graves' disease in the Oriental populations correlates with low prevalence of this allele in most Oriental populations (~5% vs ~14%) (Cerna et al., 1993, Huang et al., 1995, Rønningen et al., 1990, Gao et al., 1991, Wang et al., 1993, Doherty et al., 1992). In this situation, it is possible that, in contrast to Caucasians, the major susceptibility gene for Graves' disease in Oriental population lies in/nearby the HLA-DQ locus rather than the HLA-DR locus. In at least two studies, the association appears to involve only the HLA-DQB locus. For examples, Graves' disease has been shown to associate with the HLA-DQB1*0303 allele in Hong Kong Chinese (R.R. = 3.2; n = 90) (Cavan et al., 1994). In another study, HLA association with Graves' disease involves serologic determinant governed by the HLA-DQB allele, such as the HLA-DQw4 allele in the Japanese (R.R. = 2.8 ; n = 88) (Inoue et al., 1992). On the other hand, a study of Singaporean Chinese revealed no positive association of any HLA class II alleles (HLA-DQA1, -DQB, -DRB1,-DRB3, -DRB4, -DRB5 and -DPB1 loci were considered) with Graves' disease (n = 33; HLA-DR3 = 11.4% antigen frequency in normal control) (Chan et al., 1993). Similarly, our results agreed with the results of Chan et al. (1993) although we studied only the HLA-DQA1, -DQB and -DRB1 loci (n = 174; HLA-DR3 = 10.3% antigen frequency in normal control). The discrepancy is unlikely to be due to a low number of cases studied.

The differentiation of HLA alleles based on primary sequences is very fine. These alleles differ by at least one nucleic acid position. However, the nucleotide substitution in each position that classifies all HLA alleles dose not always change the amino acid sequence or the fine structure of HLA molecules. Thus, the association (or lack of association) between a particular HLA allele and Graves' disease may not correlate with the functional molecule that actually influences disease susceptibility. Known example of specific amino difference of the HLA molecules which is associated with disease susceptibility was first described by Todd et al. (1987) who noticed that all class II haplotypes which were not positively associated, but were neutral or negatively associated with IDDM possessed in common an aspartic acid residue at the position 57 of HLA-DQβ chain. Similarly, Badenhoop et al. (1995) found that the HLA-DQA1 alleles with an

arginine at position 52 and the HLA-DQB1 alleles with a non-aspartic acid residue at the position 57 were significantly associated with IDDM, Graves' disease and Addison's disease. This structural difference prompted several investigators to study functional correlates. According to Charron (1990) the negatively charged aspartic acid residue at the position 57 of HLA-DQβ1 chain was thought to form salt bridge with arginine residue at the position 79 of HLA-DQα1 chain as in the HLA-DR molecule (Brown et al., 1993). Indeed, Kwok found that the polymorphism at the position 57 of HLA-DQB1 molecule could either enhance or abrogate peptide binding, depending on the peptide involved (Kwok et al., 1995). The comparison between HLA-DQA1*0301/DQB1*0302 and HLA-DQA1*0301/DQB1*0302m57 which are identical except at codon 57 altered the affinity of the λR12-24 or AYK peptides. Commonly, the λR12-24 peptide preferentially bind HLA-DQB1*0302. The binding activity of HLA-DQB1*0302m57 was less 4-fold of the HLA-DQB1*0302. Similar to the interaction of the AYK peptide which dose not commonly bind the HLA-DQB1*0302 molecule, the mutagenesis at position 57 of HLA-DQB1*0302 increased the binding activity more than 2-fold. So, the substitution of the amino acid residue which change the structure or property of the binding groove is now known to change its binding affinity to antigenic peptides (Brown et al., 1988).

When we divided the HLA class II alleles based on the presence of polymorphic amino acids within the peptide binding groove, we found that the alleles with non-Asp DQ β57 residue was not associated with Graves' disease or the absence of Graves' disease. This is different from previous finding in Graves' disease in the Caucasian populations (Badenhoop et al., 1995). The most interesting finding in this study is the association of alleles with either Tyr^{25} and Leu^{69} in the HLA-DQA molecule with Graves' disease. The amino acid position 25 in other alleles of the HLA-DQA molecule (or the corresponding position 22 of HLA-DRA and position 24 of some H2-A allele) is phenylalanine (Brown et al., 1988). The only structural difference between tyrosine and phenylalanine is the presence of an additional hydroxy group in the benzene ring of tyrosine. The functional difference caused by the presence of a hydroxyl group at this amino acid position is not yet clear. The problem is compounded by the lack of the three-dimensional structure of HLA-DQ molecule. However, the conservation of the three-dimensional structure of the MHC class I and II molecules (Brown et al., 1993; Ghosh et al., 1995) allows direct comparison of the HLA-DR and DQ molecules. Based on the crystal structure of HLA-DR1 molecule, the residue 22 of the HLA-DR alpha chain is located on the sidewall of the binding cavity as part of the outermost strand of the two beta-pleated sheet strands which

help form the base and sidewall of peptide binding groove (Brown et al., 1993). In the HLA-DR3 molecules, Phe^{22} represents one of the CLIP-contacting residues in pocket 3 (Ghosh et al, 1995). In both HLA-DR1 and -DR-3 molecules, pocket 1 is a deep cavity located at one end of the binding groove; it is lined with highly conserved amino acids, many of which are quite hydrophobic (Ghosh et al, 1995), and exhibits preferences for the hydrophobic side chain of bound peptide site (Stern et al, 1994; O'Sullivan et al, 1991; Hammer et al, 1992). The pocket 3 is located close to pocket 1 but is smaller, more shallow and less hydrophobic (Ghosh et al., 1995). Based on these structural properties of Phe^{22} in the HLA-DR molecules, the substitution of Phe^{25} with Tyr^{25} at the corresponding position in the HLA-DQ molecule may result in two changes. First, the larger size of the side chain may restrict the choice of peptides that can fit into the groove of the HLA-DQA1*0501/0301/0101/0102 alleles as compared with other alleles with Phe^{25}, such as HLA-DQA1*0201/0401/0601/0103 molecules. Second, the hydroxyl group at the end of the Tyr^{25} residue may decrease the hydrophobicity of the pocket 3 and, by this way, alter the binding affinity of these pockets with the appropriate side chain of bound peptide. Quite similar substitution such as the substitution of Phe^{26} by His^{26} in the H2-A alpha chain in the non-responder $H2\text{-}A^d$ allele of mice has been shown to be responsible for the lack of binding of the HEL 52-61 peptide to $H2\text{-}A^d$ molecule (Brown et al., 1988).

An additional residue that is associated with Graves' disease in this study, Leu^{69} of HLA-DQA molecule, is located on the alpha helical portion of the alpha chain inside the peptide binding groove. This DQA residue corresponds to an aspartate residue at the position 66 of HLA-DRA molecule and valine/glycine/glutamic acid at the position 74 of the H2-A molecules (Brown et al., 1988). In the three dimensional structure of HLA-DR1-HA 306-318 and HLA-DR3-CLIP 87-101 molecules the position Asp^{66} lies on an α helical strand which forms a shallow pocket 6. This pocket locates between the first inner α helix strand of the α-chain and the last inner β-pleated sheet strand of the β-chain close to the α helical strand of the α-chain (Stern et al., 1994; Ghosh et al., 1995). This pocket is formed between the α helical region and β-pleated sheet near the pocket 3. Approximately 41 % of the surface area of this pocket is formed by conserved residues including asparagine and valine (Ghosh et al., 1995). They accommodate the side chains of Thr^{313} of HA 306-318 and Pro^{96} of CLIP87-101 peptides (Stern et al., 1994; Ghosh et al., 1995). Smaller residues are generally preferred at this position in other peptides (Hammer et al., 1993). The substitution of Asp^{66} of HLA-DRA molecule by Leu may alter the size

of pocket and its overall charge and hydrophobicity and may affect the binding of certain antigenic peptide.

If the association of HLA-DQA1 alleles containing either Tyr^{25} and Leu^{69} in the HLA-DQA chain with Graves' disease is also found in other populations, this association may truly represent yet another HLA-linked susceptibility factor for the development of Graves' disease. Comparison of the HLA-DQA1 alleles containing either Tyr^{25} and Leu^{69} in the HLA-DQA chain in Graves' disease patients and control in a Caucasian population (Yanagawa et al., 1994) and a Singaporean Chinese population (Chan et al., 1993) indicated that our observed association is not consistent in at least two other studies. Data from both of the studies revealed that the frequencies of Graves' disease patients and control subjects who bear Tyr^{25} in the HLA-DQA1 molecules were not significantly different (Yanagawa et al., 1994; Chan et al., 1993). Again, the association between HLA-DQA1 alleles containing either Tyr^{25} and Leu^{69} in the HLA-DQA chain with Graves' disease falls into the same category as HLA-DR3 (Farid et al., 1979; Dahlberg et al., 1981; Baldini et al., 1995) and non-Asp57-bearing HLA-DQB1 alleles in Caucasian populations (Badenhoop et al., 1995) and HLA-B46 in Oriental populations (Yeo et al., 1989; Dong et al., 1992; Onuma et al., 1994) because the association is not found in all populations studied.

One possible explanation for the inconsistency of HLA-disease association with regards to population is the difference in HLA allele frequency in various populations, especially when small sample size was used for the study of this uncommon disease. It is clear that Graves' disease is mediated by autoantibodies specific for the thyroid-stimulating hormone receptor (TSHR). Approximately 90 % of Graves' patients has thyroid-stimulating autoantibodies (TSAb) and 67% of this group react with recombinant human TSHR extracellular domain (Soliman et al., 1995). The activation occurred by mimicking the thyroid stimulating hormone (TSH) action elicit hyperthyroidism in Graves' disease (Song et al., 1996). Thus, the pathogenesis is not directly T cell-based (Ludgate and Vassart, 1995), eventhough the plasma cells undergo clonal expansion only in response to cytokine stimulation and CD4 T cell are the source of these (Abbas et al., 1994). The selection of pathogenic HLA molecules and autoreactive T cells may be based on the variety of TSHR peptides as has been shown that T-cells in patients with Graves' disease were directed to a variety of epitopes of TSHR including peptides in regions 158-176, 217-263 and 329-362 (Soliman et al., 1995). Therefore, it is conceivable that many different HLA molecules such as HLA-DR3 and HLA-B46, which showed strong

association with Graves' disease in different races, may actually be all involved in the pathogenesis of this disease. However, the chance of detecting the statistically significant association of a particular allele of HLA molecules would depend on the prevalence of that allele in each population. As mentioned above, the lack of association of the allele HLA-DR3 with Graves' disease in the Oriental populations correlates with low prevalence of this allele in most Oriental groups. In contrast, the allele HLA-B46 is much more frequently found in Oriental populations than in Caucasian populations (21% vs 0.2 %) (Imanishi et al., 1992). Thus, eventhough both alleles play some role in disease susceptibilty, a high prevalence of HLA-DR3 may allow the detection of statistically important association with Graves' disease only in the more prevalent Caucasian population, but not the Oriental group, and vice versa.

Another possible explanation may reflect the presence of non-HLA susceptibility factors. In an effort to explain the lack of a common HLA susceptibility marker with Graves' disease when comparing patients from different races, Caven et al. (1994) suggested that the difference was due to the influence of non-HLA factors. Moreover, their study also provided strong evidence for sex difference in the effect of HLA marker on disease susceptibilty. The HLA-B46, -DR9 and -DQB1*0303 were associated with Graves' disease only in male group but not with female patients. Moreover, the contribution of HLA-associated susceptibility to Graves' disease was documented in a subgroup of male thyrotoxic patients who manifested periodic paralysis (Caven et al., 1994). The cytokines are other factors involving the pathogenesis of Graves' disease. The non-specific IL-2 and IFN-γ may involve the aberrant expression of HLA-DR on thyroid cell which progress as the antigen presenting cell (DeGroot, 1996). Other cytokines such as IL-1α, IL-6, IL-8 and possibly TNF-α which increase in Graves' thyroid follicular cell (TFC) could be important in the early phase of autoimmune thyroid disease by enhancing the number and activation of intrathyroidal lymphocytes (Weetman, 1994). These cytokines also enhance the aberrant expression of adhesion molecules on the relative thyroid cell (Nagashima et al., 1994).

Additional non-HLA-linked molecule that may serve as an underlying basis for the development of Graves' disease is CTLA-4. The CTLA-4 molecule is expressed on activated T cells and represents another ligand for B7, which is necessary to provide the costimulatory signal during T cell activation (Linsley et al., 1992). The CTLA-4 gene is quite heterogeneous with regards to AT-rich repeats at the 3'-untranslated portion. The 106-base allele (containing 17 repeats) was associated with susceptibility to Graves'

disease (27 % vs 13.5 % in normal population; RR = 2.82) (Yanagawa et al., 1995). This polymorphism is thought to be important in the determination of mRNA stability (Shaw and Kamen, 1986). In addition to its presence in the CTLA-4 gene, Yanagawa proposed that the mRNA of allele 106 is less stable than other alleles (Yanagawa et al., 1995). The adhesion molecules; ICAM-1, LFA-1, VLA-4, ELAM-1, also play potentially important role in the pathogenesis of Graves' disease (Weetman et al., 1994). Nakashima et al (1994) reported the strong expression of ICAM-1 on capillary or post capillary endothelium cells, and LFA-1 on the mononuclear cells around the post capillary endothelium cells in thyroid tissue in Graves' disease patients, but none or weak expression in normal thyroid tissue. The VLA-4 was expressed on the infiltrating mononuclear cells, especially in the mantle zone of lymphoid follicle like area and germinal center. The VCAM-1 was expressed on follicular dendritic-like cells in the germinal centers. These molecules were not found in normal thyroid glands. The LFA-1/ICAM-1 and ELAM-1 may be responsible for the migration of mononuclear cells into the thyroid gland of patients with Graves' disease. The VLA-4/VCAM-1 play a critical role in the cellular interaction that led to the formation of B-memory cells and the excess production of antibodies (Koopman and Pals, 1992). The expression of these accessory molecules was thought to represent the secondary response which was likely to exacerbate autoimmune injury (Weetman et al., 1994).

In contrast to the positive association of certain alleles with Graves' disease as mentioned above, there was a significant negative association between the alleles HLA-DQA1*0601 and -DRB1*1202 with Graves' disease (RR = 0.21, pc = 0.00007; RR = 0.41, pc = 0.005). The preventive fraction conferred by the alleles HLA-DQA1*0601 and HLA-DRB1*1202 were 9 % and 11 %, respectively. This indicates that if the HLA-DQA1*0601 and HLA-DRB1*1202 alleles are involved in the prevention of Graves' disease, then the HLA-DQA1*0601 and HLA-DRB1*1202 alleles had reduced 9 % and 11 %, respectively, of cases that would have developed in the population in the absence of these two alleles. In a few studies, the protective effect of HLA alleles with Graves' disease have been reported; these include the alleles HLA-DRB1*1501 (RR = 0.28, p = 0.025) and HLA-DQB1*0301 (RR = 0.38, p = 0.017) in Singaporean Chinese patients (Chan et al., 1993) and the allele HLA-DQB1*0501 (RR = 0.12, $p < 0.05$) of Japanese patients (Tamai et al., 1994). Thus, similar to the difference in the HLA class II alleles that are associated with the occurence of Graves' disease, the HLA class II alleles that are associated with reduction of Graves' disease are also varied in various populations. One

possible explanation is the lack of immunogenicity of bound peptide or lack of peptide binding in certain HLA alleles. The study of binding capacity of HLA class II molecules to various human β_2M (self) and murine β_2M (non-self) peptide homologs demonstrated that the HLA-DR5 and -DR2 molecules exhibited weak binding capacity with 3 and 6, respectively, of self peptides. The human β_2M1-16 peptide could bind to the HLA-DR5 and -DR2 molecules but had not immunogenicity activity. The remaining peptides had less immunogenicity except that of HLA-DR2 to β_2M55-70 peptide (Sette et al., 1992). The HLA-DRB1*1202 and *1501 alleles correspond to the HLA-DR5 and -DR2 serological specificities, respectively. Conceivably, these alleles may protect the development of Graves' disease at the level of peptide binding.

Although the actual mechanism of which certain HLA class II alleles are involved in the protection against Graves' disease remains unknown, basic research in small animals has shown some interesting findings. In an animal model of experimental autoimmune disease, collagen-induced arthritis (CIA) in rodents which shares many pathological aspects with human rheumatoid arthritis (RA), the gene controlling the CIA susceptibility has been narrowed down to the MHC class II H-2A molecule, especially those of the q, w3 and w17 haplotypes (Holmdahl et al., 1989). In the q, w3 and w17 haplotypes, the H-2E molecule is not functional because of mutations in the Ea and Eb genes (Begovich et al., 1990). The transgenic mice for Eb^d gene showed the dramatic decrease both in the incidence and severity of CIA (Gonzalez-gay et al., 1994). In human, RA is linked to the HLA-DRB1 locus, which is analogous to mouse H-2Eb. The study of protective effect of HLA-DRB1 molecules to RA and murine H-2Eb molecules to CIA demonstrated that certain sequences of the hypervariable region 3 (HV3) of HLA-DRB1 and H-2Eb molecules were involved in strong RA and CIA protection (Zanelli et al., 1995). This third diversity region is located in the margin of the antigen binding cleft formed by the α-helical portion of the HLA-DRβ1 chain that includes the polymorphic residues numbers: 57-86 (Winchester, 1994). At the positions 67, 70, 71 and 74 of the HLA-DRB1/H-2Eb molecules, there were six polymorphic amino acid patterns that showed strong RA and CIA protection. These polymorphic patterns were: FDRA/L, LQA/KA, IDRQ and IDEA of the HLA-DRβ1 and FQRA and IDAS of murine H-2Eb molecules. Interestingly, the allele HLA-DRB1*1202 that was associated with protection to Graves' disease contains the pattern FDRA at the positions 67, 70, 71 and 74. Thus, the allele HLA-DRB1*1202 falls into one of thesix protective patterns of DRβ1 molecule. This pattern is also found in HV3 of HLA-DRB1*1501 molecule which was associated with protective phenotype in

Singaporean Graves' patients. The exact structural reason for this protection is not yet clear. From the crystallographic analysis of HLA-DR1 molecule (Brown et al., 1993), residues 67, 70 and 71 are located near the pronounced kink in the β-chain helical region. This region forms the highest point of the structure and also might be in the position to interact with T cell receptor, especially the position 71. In one study, the residue 70 forms hydrogen bond with the side chain of a peptide antigen, PKYVKQNTLKLAT, in the HLA-DR1 molecule (Stern et al., 1994). This shared epitope structure appears to be a contact site determining the specificity of the interaction between the side chain of the bound peptide and the HLA molecule.

In the northern Thai population the HLA-DQA1*0601 allele is found in strong linkage disequilibrium with the HLA-DRB1*1202 allele. Indeed, ten HLA-DQA1*0601 positive patients were all positive with HLA-DRB1*1202 allele. In our patient population, an uncommon haplotype: HLA-DRB1*1202-DQA1*0601-DQB1*0301 was significantly decreased in Graves' disease patients (RR = 0.24, *pc* = 0.002). Collectively, these haplotypes, however, was present in only 2.9% of all the patients. In contrast, the HLA-DRB1*1202-DQA1*0601-DQB1*0301 haplotype is a common extended haplotype in central Thais and Chiang Mai population with haplotype frequencies of approximately 13 % and 11.1 %, respectively (Sujirachato et al., 1994; HAF Stephen, et al., personal communication). This result from our study provides another piece of information on a possible effect of genetic factor on reduced risk of Graves' disease development. However, no evidence of the association of this haplotype with Graves' disease has been found in other populations and the protective mechanism, if this is true, is not yet known.

When relapse and exacerbation were considered together, relapse/exacerbation in Graves' disease patients were associated with the HLA-DQB1*03032 allele (RR = 2.33, *pc* = 0.02) and the HLA-DRB1*09012 allele (RR = 2.20, *pc* = 0.04). However, when further subdivided, only the exacerbation group was associated with HLA-DQB1*03032 allele (RR = 2.83, *pc* = 0.04), and the HLA-DRB1*09012 allele (RR = 3.67, *pc* = 0.05). The etiologic fraction confered by the allele HLA-DQB1*03032 and -DRB1*09012 were 17.9 % and 20.9 %, respectively. This means that if the HLA-DQB1*03032 and HLA-DRB1*09012 were two of the causes of the disease, 17.9 % and 20.9 % of the cases were developed among the HLA-DQB1*03032 and -DRB1*09012 positive individuals. The common HLA-DRB1*09012-DQA1*03-DQB1*03032 haplotype was found 20.6 % of Graves' patients and 12.4 % of normal controls (HAF Stephen et al., personal communication). The difference was not significant statistically. The HLA-DQB1*0303

allele was also positively associated with Graves' disease in Hong Kong Chinese (Cavan et al., 1994). In contrast, the association of HLA-DR3 with Graves' disease in Caucasian is well established as a predictive parameter for relapse (Baldini et al., 1995; Ratanachaiyavong et al., 1990). This discrepancy may be caused by the difference in the distribution of HLA alleles in each population as discussed previously.

Patients with periodic paralysis mostly inherited the HLA-DQA1*03, HLA-DQB1*0502, HLA-DRB1*1401 and *09012 alleles. We did not find the specific HLA allele which is associated with thyrotoxic periodic paralysis in our patients. Thyrotoxic periodic paralysis is a rare syndrome with greater frequency in Oriental, especially Chinese and Japanese patients (Ober, 1992). It was seen only with male Graves' patients (Tamai et al., 1986). When these authors investigated the HLA-antigen in 35 male Japanese patients with periodic paralysis in comparison with 165 normal males, the HLA-A2, HLA-Cw3 and HLA-DRw8 alleles were significantly associated with Graves' disease with periodic paralysis ($p < 0.001$) (Tamai et al., 1986). The HLA-DRw8 antigen occurred in 63 % of the patients with paralysis while 29% of patients without periodic paralysis presented this antigen (Tamai et al., 1986). No specific HLA association with thyrotoxic periodic paralysis in 31 male Hong Kong Chinese Graves' patients except the HLA-B46-DR9-DQB1*0303 haplotype which was weakly associated (Cavan et al., 1994). Yeo reported the HLA-A2 and HLA-Bw22 were positive associated with thyrotoxic periodic paralysis in Singaporean Chinese (Yeo et al.,1978).

The distribution of the HLA-DR alleles in male patients differs from those of the female group. In male patients, the four most commonly found alleles are: HLA-DRB1*09012, *1602, *1401 and *1501 in a decreasing order. In the female patients, however, the alleles *1501 and *1502 were the third and fourth most common alleles instead. By considering the distribution of HLA alleles in normal population, the HLA-DRB1*0901, *1501, *1201, *07 and *1401 were highly predominant in other Asian populations such as Chinese, Japanese and Korean (Geng et al., 1995). Similar to the frequencies of HLA-DR9 (DRB1*09) allele among male from mainland China and male Singaporean Chinese Graves' patients that presented more frequent than those of female patients (Yeo et al., 1989, Cavan et al., 1994).

The sex distribution of Graves' disease shows a decided preponderance of females. Females are mostly found this disease than males in ratio 7:1 to 10:1 (Feliciano, 1992). This suggests that sex hormones or other factors associated with the inheritance of two X chromosome are likely enhance the development of Graves' disease in female. However,

of interest, the stronger HLA associations with Graves' disease were usually found in male patients (Yanagawa et al., 1994; Caven et al., 1994; Yeo et al., 1989). This simply means that in order to manifest the disease phenotype in a situation where it is not favored to occur, a greater number of susceptibility genes need to be present. This may imply that in males patients the HLA contribution to genetics is greater (Winchester et al., 1994).