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## Biomarkers of susceptibility to type 1 diabetes with special reference to the Indian population

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**Type 1 diabetes (T1D) is a polygenic autoimmune disease. Susceptibility to T1D is strongly linked to a major genetic locus that is the major histocompatibility complex (MHC) and several other minor loci including insulin, CTLA4 that contribute to diabetes risk in an epistatic way. MHC harbours genes whose primary function is to govern immune responsiveness. Being the most polymorphic genomic region known in humans, MHC serves as a very exciting minigenome model for studying susceptibility to T1D. We have observed enormous diversity in HLA class I and class II genes in the north Indian population and identified several ‘novel alleles’ and ‘unique haplotypes’. For example, multiple DR3+ve autoimmunity favouring haplotypes have been identified, some of which are unique to the Asian north Indian T1D patients. Our molecular studies have revealed that (i) the classical Caucasian autoimmunity favouring AH8.1 (HLA-A1 B8 DR3) is rare in the Indian population and has been replaced by a variant AH8.1v that differs from the Caucasian AH8.1 at several gene loci, (ii) AH8.2 (HLA-A26 B8 DR3) is the most common DR3 positive haplotype in this population and resembles the Indian AH8.1v rather than Caucasian AH8.1, and (iii) there are additional HLA-DR3 haplotypes HLA-A24 B8 DR3 (AH8.3), A3 B8 DR3 (AH8.4) and A31 B8 DR 3 (AH 8.5) that occur in the Indian population. The studies have led to a hypothesis that AH8.1 and AH8.1v might have co-evolved from a common ancestor but preferential divergence of AH8.2 over AH8.1 leading to survival advantage might have been driven by vigorous pathogenic challenges encountered by the Indian population. These studies have important implications in our understanding of disease pathogenesis, identification of high risk individuals, disease diagnosis, disease management and immunological therapeutic approaches.**

**Key words** Autoimmunity - CTLA4 - haplotypes - HLA - insulin - MHC - type 1 diabetes

Type 1 diabetes (T1D) is a chronic autoimmune disease, characterized by irreversible autoimmune destruction of the insulin secreting  $\beta$ -cells of the islets in the pancreas. The disease occurs worldwide, usually develops at a younger age, although it may develop at any age. The symptoms appear when the  $\beta$ -cell mass gets reduced by approximately 90 per cent leading to severe insulin deficiency and hyperglycaemia. The latter is due to hepatic overproduction of glucose by glycogenolysis and gluconeogenesis and decreased cellular uptake of glucose from the circulation. In the absence of insulin, there is also an increase in fat breakdown and fatty acid oxidation, resulting in the excessive production of ketones. If not treated, these metabolic disturbances lead progressively to central nervous system depression, coma and death. Therefore, T1D requires life long treatment with exogenous insulin for survival.

Over the last two decades, the incidence of diabetes has increased in an epidemic-like fashion worldwide. T1D is the second most common chronic disease during childhood and the most common form of diabetes in children (1.7 of 1000 children) around the globe. It is estimated to increase from 4.4 million in 2000 to approximately 5.4 million in 2010 worldwide<sup>1</sup>. The rate of incidence of the disease is consistently increasing in many countries; the highest being in northern Europe, particularly in Finland (37.4/ 100,000 per year) and Sweden (34.6/ 100,000 per year)<sup>2</sup>. The lowest incidence has been reported from the Zunyi province of China (0.1/ 100,000 per year)<sup>3</sup>. In the rest of Europe, the incidence varies between 7.0 and 19.0 cases / 100,000 per year, except in Sardinia (36.0/ 100,000 per year), where the incidence is the highest in the world after Finland. Although the definitive figures are not available, the incidence of T1D in southern region of India has been reported to be 10.5 cases / 100,000 per year<sup>4</sup>.

Based upon the aetiology, diabetes can be divided into four main groups: (i) Type 1 diabetes (previously called as insulin-dependent diabetes mellitus) involves absolute insulin deficiency due to an

autoimmune destruction of the insulin producing  $\beta$ -cells in the islets of Langerhans, (ii) Type 2 diabetes (previously called as non-insulin-dependent diabetes mellitus) is characterized by relative insulin deficiency due to decreased effect of insulin in the target tissue *e.g.* muscle and adipose tissue (insulin resistance) or due to a secretory defect of insulin with or without insulin resistance, (iii) Gestational diabetes is characterized by carbohydrate intolerance resulting in hyperglycaemia of variable severity with onset or first recognition during pregnancy, and (iv) MMDM (Malnutrition modulated diabetes mellitus).

### Immunological aspects

T1D is an autoimmune disease in which the pancreatic islets show abundant infiltration by mononuclear cells that include dendritic cells, macrophages and T cells. The islet-specific autoimmune process is characterized by the appearance of circulating islet autoantibodies.

### Humoral immunity in T1D

Although beta cell damage is a T cell mediated process, antibody responses have been of great significance in allowing accurate diagnosis and prediction of 'at risk' individuals to develop T1D. Circulating cytoplasmic islet cell autoantibodies (ICAs), insulin autoantibodies (IAAs) and antibodies to a 65 kD antigen (glutamic acid decarboxylase or GAD) are present at diagnosis in approximately 70- 80 per cent of patients<sup>5,6</sup>, whereas GAD65 and insulinoma-associated protein-2 (IA-2) antibodies were found to be present in approximately 20- 26 per cent of cases of type 1 diabetes from north India<sup>7,8</sup>. Generally, the appearance of autoantibodies precedes the clinical onset of symptoms by several years. These antibodies may be directed towards multiple  $\beta$ -cell targets and are a useful tool to predict the risk of developing type 1 diabetes<sup>9</sup>. Interestingly, some people fail to develop disease even when autoantibodies are present, suggesting an important role of host determined factors.

**Proinsulin and insulin autoantibodies (IAA):** The first known  $\beta$ -cell protein to which an autoimmune response has been documented in T1D patients is insulin<sup>10</sup>. Using an improved assay system, nearly 70 per cent of the newly diagnosed patients have been reported to have circulating antibodies to insulin<sup>5</sup>. Further, antibodies reactive to proinsulin, but not to insulin, have been found among a group of newly diagnosed T1D patients before the commencement of insulin treatment<sup>11</sup>.

**Glutamic acid decarboxylase autoantibodies (GAD):** Newly diagnosed patients have an autoantigen GAD, an islet cell protein of 65kDa<sup>12</sup>. GAD exists in two forms, GAD65 (65kDa; 585 amino acids) and GAD67 (67kDa; 593 amino acids). Only the former is expressed in the  $\beta$ -cells of human islets. GAD is the rate limiting enzyme required in the conversion of glutamic acid to  $\gamma$ -aminobutyric acid (GABA). GAD is expressed in the peripheral nervous systems, pancreatic islets, epithelial cells of the fallopian tube, and spermatozoa. Thus, unlike insulin, it is not a  $\beta$ -cell specific protein. Up to 70 per cent of the new-onset T1D patients have antibodies to GAD65, compared to 4 per cent of healthy controls<sup>6</sup>.

**Anti-ICA69 (Islet cell autoantigen of 69 kDa) antibodies:** Expression studies have shown that ICA69 mRNA is detected in islets and brain with low levels detectable in heart, thyroid and kidney. Sera from subjects at risk for T1D development have shown positivity for ICA69 antibodies in 43 per cent of ICA+ first-degree relatives who later developed T1D. Subsequent studies have shown that 30 per cent of recently diagnosed T1D patients had detectable anti-ICA69 antibodies<sup>13</sup>.

**Insulinoma-associated protein 2 autoantibodies (IA-2):** Sera from diabetic patients immunoprecipitate a 64-kDa protein which, on mild trypsin treatment, leads to the production of fragments of 40 and 37kDa. The former represents amino acids 653-979 of insulinoma-associated protein 2 (IA-2), and is a member of the tyrosine phosphatase family<sup>14</sup>. In addition to  $\beta$ -cells, IA-2 is enriched in the secretory

granules of neuroendocrine cells. The full-length protein has a luminal domain (amino acid 1-576), a single transmembrane domain (577-601) and a cytoplasmic tail (602-979) containing a tyrosine phosphatase motif. Initial studies with the full-length IA-2 protein have indicated that 54-66 per cent of diabetic patients have circulating antibodies to IA-2<sup>14,15</sup>.

**Additional antibody targets:** Continued studies of serum samples from recently diagnosed T1D patients or those with longer disease duration have led to the identification of additional antibody targets. For example antibodies against autoantigens like heat shock protein (HSP)-60<sup>16</sup>, HSP-70<sup>17</sup>, HSP-90<sup>18</sup>, carboxypeptidase (CPE)<sup>19</sup>, *etc.*, have all been demonstrated in these patients.

### Cellular immunity in T1D

While extremely useful for prediction, antibodies do not directly contribute to  $\beta$ -cell damage, as it is now well established that T1D is a T cell mediated disease, requiring contributions from both helper T cells as well as  $\beta$  cell specific cytotoxic T cells<sup>20</sup>. The presence of autoantibodies against different peptides of pancreatic islet  $\beta$ -cells suggests that major histocompatibility complex (MHC) class II-restricted CD4+ T cells identify the antigenic targets of the pathogenic  $\beta$ -cell reactive cells. There is considerable overlap among the antigens, and even their specific epitopes, targeted by these cells. Soluble HLA-class II tetramers containing a peptide corresponding to an immunodominant epitope from human GAD65 have been used to analyze peripheral blood T cells of newly diagnosed type 1 diabetic patients and at-risk subjects<sup>21</sup>. These studies have led to the identification of human GAD65 peptides recognized by CD4+ T cells. Similar results have been obtained by using a combination of chromatography and mass spectrometry of GAD65 peptides bound by HLA-DR4 molecules<sup>22</sup>. Using a murine insulin dependent diabetes model of NOD mouse, Kaufman and colleagues<sup>23</sup> reported spontaneous loss of T cell

tolerance to GAD protein and lack of disease development in these animals. This suggests that GAD65 may be one of the early CD4+ T cell targeted autoantigens. Additionally, other protein antigens including IA2<sup>24</sup>, HSP60<sup>25</sup>, ICA69<sup>26</sup>, *etc.*, have been found to elicit CD4+ T cell responses in recently onset T1D patients.

Autoreactive cytotoxic T cells recognize peptide epitopes displayed on the islet  $\beta$ -cell surface in the context of HLA class I molecules. These 8-10 amino acid epitopes are considered to be derived primarily from islet  $\beta$ -cell proteins. The islet  $\beta$ -cells, which express class I (but not class II) MHC molecules, can be directly recognized and killed by cytotoxic CD8+ T cells specific for  $\beta$ -cell peptides. Various studies have shown that CD8+ T cells are able to recognize the HLA class I associated peptide epitopes on insulin and proinsulin<sup>27,28</sup>, islet amyloid polypeptide (IAPP)<sup>29</sup>, glutamic acid decarboxylase-65 (GAD65)<sup>30</sup>, islet specific glucose-6-phosphatase catalytic subunit-related protein (IGRP)<sup>31</sup> and may be other proteins.

### Genetic predisposition to type 1 diabetes

The rate of beta cell destruction in T1D varies from patient to patient, but tends to be more aggressive in infants and young children. Hence, the disease usually presents during childhood or adolescence, although it may develop much later in life. The variation in age at onset could be indicative of disease heterogeneity, with different mechanisms leading to pancreatic destruction in childhood onset versus adult onset diabetes. This clearly reflects the involvement of different genetic and environmental susceptibility determinants.

The genetic contribution to susceptibility to T1D has been demonstrated with twin studies where the concordance rates differ significantly between monozygotic (approximately 50%) and dizygotic twins (6%)<sup>32</sup>. When the first twin of a twin-pair develops type 1 diabetes after age 25, the risk of the

second monozygotic twin developing type 1 diabetes is less than 5 per cent with long-term follow up<sup>33</sup>. However, the risk of second twin to develop T1D increases to 60 per cent in case the first one develops disease prior to age 6. In case of monozygotic twins, expression of anti-islet autoantibodies directly correlates with progression to diabetes. Essentially, all such monozygotic twins who express anti-islet autoantibodies (to GAD, IA-2/ ICA-12, insulin, measured by radioimmunoassays) also progress to diabetes over time<sup>34</sup>. In contrast, dizygotic twin pairs with one patient with T1D, have a low risk of expressing anti-islet autoantibodies, a risk similar to that of siblings. Similarly, the life time risk of developing diabetes is about 10-12 times higher among first degree relatives of diabetic individuals (5-6%) compared to the healthy white population (0.4%)<sup>35</sup>.

### T1D: a polygenic disease

It has been suggested that type 1 diabetes is a polygenic disease. Susceptibility to T1D is linked to a major genetic locus MHC and several other minor loci contribute to diabetes risk in an epistatic way. It has been postulated that environmental factors such as certain viral infections and possibly, nutritional and/or chemical agents, when superimposed on genetic factors, may lead to the activation of disease.

Both association studies and linkage analysis using various analytical methods have been used to identify multiple susceptibility loci, and these are conventionally abbreviated as IDDM and a number, *e.g.*, IDDM1, IDDM2, *etc.*, with the number usually reflecting the order in which such loci were reported. Using the candidate gene approach, association studies dating about two decades ago provided evidence for the first two susceptibility loci, the HLA region (IDDM1) and the insulin gene (INS) locus (IDDM2)<sup>36</sup>. A comprehensive list of the currently known susceptibility loci (IDDM1 to IDDM18) is shown in Table I with LOD scores from the combined genome screen of 767 families analyzed by Cox and co-workers (HBDI and BDA repository)<sup>33</sup>.

### Major histocompatibility complex and IDDM1

Although more than 18 IDDM genetic loci have been implicated, about 50 per cent of the genetic risk of developing diabetes is conferred by the MHC region which is referred as IDDM1<sup>37</sup>. Thus HLA region is the largest genetic contributor to T1D susceptibility as suggested by several studies involving variable ethnic groups.

Human MHC gene cluster spans a region of about 4000 kb length ( $4 \times 10^6$  nucleotides) on the short arm of chromosome 6 in the distal portion of the 6p 21.3 band. Studies on the structural organization of MHC molecules have revealed a total of 224 genes in this region of which 128 are assumed to be functional genes and 96 are pseudogenes. More than 40 per cent of the genes have one or more assigned immune functions<sup>38</sup>. These genes are arranged in three distinct sets of molecules, each comprising of a cluster of immune response genes (Fig. 1). The most centromeric

segment is the class II region that spans around 1100 kb and contains the HLA-DP, DQ and DR loci, which are found as pairs, encoding the  $\alpha$  and  $\beta$  chains. These chains encode the heterodimeric class II protein molecules expressed at the cell surface of antigen presenting cells (macrophages, dendritic cells, Kupffer cells, Langerhans cells, B cells, activated T cells). The class I region on the other hand, lies at the telomeric end and contains the classical HLA-A, B and C and related loci, spread over a region of approximately 2 Mb. HLA class I molecules are expressed ubiquitously on almost all nucleated cells.

The HLA genes that are involved in immune regulation are located mainly in the class I and class II regions, which are structurally and functionally different. Class III genes (central genes) are placed between class I and class II regions. The MHC class III region consists of genes involved in the complement system, and others including tumour necrosis factor (TNF) and HSP.

**Table I.** Known susceptibility loci for type 1 diabetes and their genetic contribution expressed as Log (base 10) Od (LOD) score

Locus	Chromosome	Candidate genes	Markers	LOD
<i>IDDM1</i>	6p21.3	<i>HLA-DR/DQ</i>	-	65.8
<i>IDDM2</i>	11p15.5	<i>INSULIN VNTR</i>	-	4.28
	16q22-24		D16S3098	3.93
<i>IDDM3</i>	15q26		D15S107	<1.56
<i>IDDM4</i>	11q13.3	<i>MDU1, ZFM1, RT6, ICE, LRP5, FADD, CD3</i>	FGF3, D11S1917	<1.56
<i>IDDM5</i>	6q25	<i>SUMO4, MnSOD</i>	ESR, a046Xa9	1.96
<i>IDDM6</i>	18q12-q21	<i>JK (Kidd), ZNF236</i>	D18S487, D18S64	<1.56
<i>IDDM7</i>	2q31-33	<i>NEUROD</i>	D2S152, D251391	2.62
<i>IDDM8</i>	6q25-27		D6S281, D6S264, D6S446	1.81
<i>IDDM9</i>	3q21-25		D3S1303, D10S193	<1.56
<i>IDDM10</i>	10p11-q11		D10S565	2.8
<i>IDDM11</i>	14q24.3-q31	<i>ENSA, EL-IL</i>	D14S67	<1.56
<i>IDDM12</i>	2q33	<i>CTLA-4</i>	(AT)n 3' UTR, A/G Exon 1	2.62
<i>IDDM13</i>	2q34	<i>IGFBP2, IGFBP5, NEUROD, HOXD8</i>	D2S137, D2S164, D2S1471	2.62
<i>IDDM15</i>	6q21		D6S283, D6S434, D6S1580	<1.56
<i>IDDM17</i>	10q25		D10S1750, D10S1773	1.56
<i>IDDM18</i>	5q31.1-33.1	<i>IL12B</i>	IL12B	<1.56

The IDDM14 and IDDM16 denominations have not been assigned to any locus

Source: Ref. 33

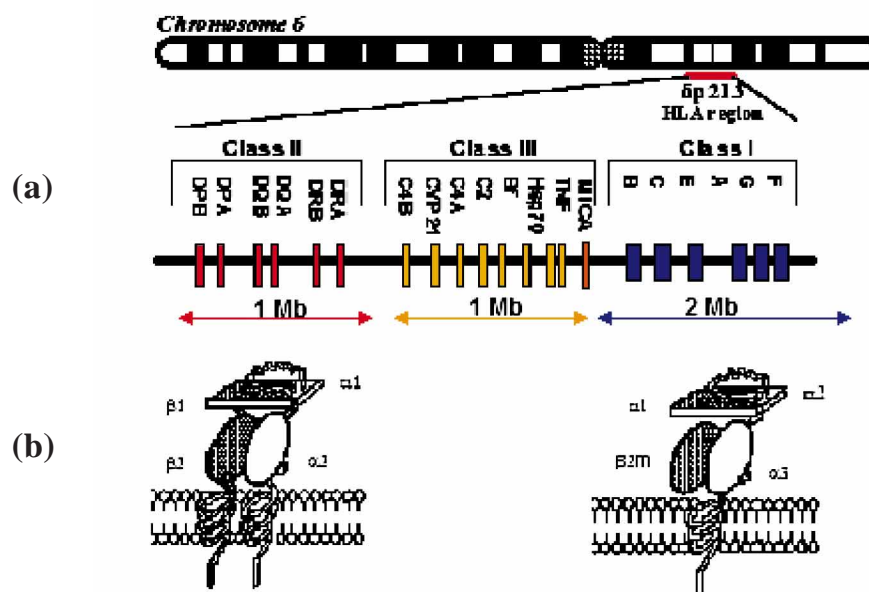
## HLA and type 1 diabetes

Worldwide, type 1 diabetes has been reported to be strongly associated with HLA-DR3 or DR4 or both alleles, whereas HLA-DR15 has been found to be protective in several populations<sup>39-43</sup>. In addition, DQ2 (DQB1\*0201-DQA1\*0501) and DQ8 (DQB1\*0302-DQA1\*0301) which occur in strong linkage disequilibrium with DR3 and DR4 respectively are strongly associated with the disease in several Caucasian populations specially when present in DR3/DR4 heterozygous condition<sup>44</sup>. Therefore, the synergistic effect of DR3/DR4 heterozygosity is usually considered at par with that of DR3 homozygosity in most populations. The HLA-DR3-DQ2 and DR4-DQ8 are referred to as 'high risk class II haplotypes' in this disease. The HLA - DQB1-DQA1-DRB1 haplotypes associated with susceptibility or resistance to T1D in various populations are shown in Table II.

Some studies have reported on the modulatory effect of DPB1-alleles on the DQ-DR-associated risk of T1D. For example the DPB1\*0301 is positively

associated with Mexican- American T1D patients carrying high-risk DQ-DR genotypes, and this association is not due to a linkage disequilibrium between DP and DQ alleles<sup>45</sup>. Similarly studies among Caucasian multiplex families from the US have shown DPB1\*0301 to be associated with T1D patients not having the DQ2-DR3/DQ8-DR4 genotypes<sup>46</sup>. Another study on T1D families from Sardinia and UK has shown that DPB1\*0402 conferred some protection<sup>47</sup>. Among Caucasians, the high risk associated with DPB1\*0202 has been found on the predisposing DR3-B18 ancestral haplotype (AH18.2)<sup>48</sup>. Thus suggested susceptibility of the DP locus in T1D is conferred by DPB1\*0301 and DPB1\*0202 alleles, and protection by DPB1\*0402.

Further linkage studies on extended MHC haplotypes have confirmed the importance of DR3 extended haplotypes, most importantly the ancestral haplotype AH8.1 (HLA-A1-B8-DR3-DQ2). The AH8.1 is of interest not only because of its high frequency in most Caucasian populations, but also because it is associated with markers of immunological hyper-reactivity and multiple



**Fig. 1.** Human major histocompatibility complex (MHC): (a) Chromosomal location and gene map showing multiple genes within MHC region on the short arm of chromosome 6 (6p21.3). (b) Pictorial view of HLA class I and class II molecular structures showing peptide binding cleft formed between  $\alpha 1$  and  $\alpha 2$  domains in case of class I, and  $\alpha 1$  and  $\beta 1$  domains in case of class II molecules.

autoimmune diseases including T1D and fast progression to AIDS.

### MHC studies in the Indian population

Molecular studies on the north Indian T1D patients have revealed a positive association of HLA-DR3 but not of DR4 with the disease<sup>39</sup>. Preliminary investigations have revealed that HLA-DR4 associated effect might be more relevant among the south Indian T1D patients. An analysis of the HLA-A-B-DR haplotypes revealed the presence of multiple DR3+ve haplotypes in the north Indian population and patients with autoimmune disorders *e.g.*, Celiac

disease<sup>49</sup> and type 1 diabetes<sup>41</sup>. In the latter disease, another DR3+ve haplotype, namely HLA-A2-B50-DR3, designated as ancestral haplotype AH50.2 is also positively associated. It is interesting to note that the common Caucasian extended haplotype, namely ancestral haplotype AH 8.1 is rarely observed among the north Indians. Instead, it has been replaced by a unique haplotype AH 8.2 (HLA-A26-B8-DR3-DQ2). The underlying autoimmune basis of DR3+ve haplotypes led us to characterize other genes in the MHC region so as to define extended ancestral haplotypes (AH) both at the population level as well as in patients with T1D.

**Table II.** Association of HLA class II haplotypes with T1D in various populations

DQB1-DQA1-DRB1 haplotype	Association with T1D	Populations
*0201-*0501-*0301	Susceptible	Asian Indians and most populations
*0302-*0301/02-*0401/02/04/05	Susceptible	Asian Indians, Caucasians, Orientals
*0302-*0301/02-*0403/06	Dominant protection	Asian Indians, Caucasians
*0401-*0301/02-*0405	Susceptible	Japanese
*0201-*0301-*0405	Susceptible	Greek
*0301-*0501-*1201/02	Susceptible	Taiwanese
*0602-*0102-*1501	Protection	Asian Indians, Caucasians, Orientals
*0303-*0301/02-*0901	Susceptible	Chinese, Koreans, Japanese
*0302-*0301-*0802	Protection	Japanese
*0601-*0103-*0803	Susceptible	Taiwanese
*0201-*0201-*0701	Susceptible	Blacks
*0601-*0102/03-*1502	Protection	Japanese, Taiwanese

Source: Ref...39

**Table III.** Disease associated multiple DR3+ve haplotypes in Asian Indians as compared to their distribution in caucasians. AH8.1v, AH8.2, and AH50.2 are 'unique haplotypes' in Asian Indians, not reported in any other population so far. AH8.3 AH8.4 and AH8.5 are additional T1D associated haplotypes encountered in Asian Indians

Haplotypes*	DRB1	DRB3	Bf	TNF-A -308	HLA-B	HLA-C	HLA-A	Distribution
AH8.1	0301	0101	S	2	0801	0701	0101	Caucasians <sup>40,41,49</sup>
AH8.1v	0301	0202	F	1	0801	0702	01	Asian Indians
AH8.2	0301	0202	F	1	0801	0702	26	Asian Indians <sup>40</sup>
AH8.3	0301	0202	F	1	0801	0702	24	Asian Indians
AH8.4	0301	0202	F	1	0801	0702	03	Asian Indians
AH8.5	0301	0202	F	1	0801	0702	31	Asian Indians
AH50.2	0301	0202	S	1	5001	0602	02	Asian Indians

\*Ancestral haplotype (AH) refers to the extended HLA haplotypes in which alleles at multiple loci occur in strong linkage disequilibrium with each other and preferentially occur in different ethnic groups. Numbers in parenthesis denote the reference cited in the text (unpublished data)

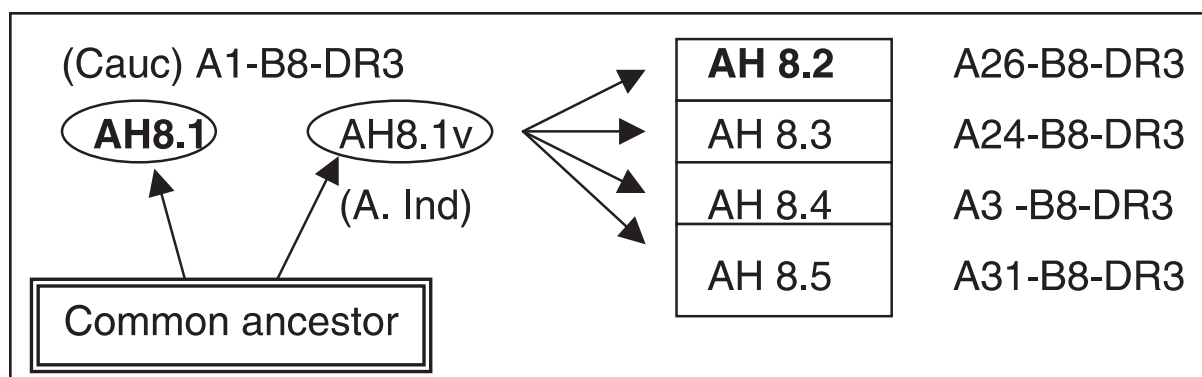
S-slow; F-fast; Bf-factor B; TNF- tumour necrosis factor. Other haplotypes are from our unpublished data

Fine molecular analysis employing multiple microsatellite markers and SNPs interspersed in the HLA class I, class II and class III regions revealed that the Indian and European diabetic conserved haplotypes share HLA-B\*0801, DRB1\*0301 and DQB1\*02 but differ at several loci including the C locus, DRB3 and all the central MHC loci tested<sup>40</sup> suggesting coincidental association of B8 and DR3 on two otherwise unrelated haplotypes (Table III). The exact distribution of AH8.2 and its association with T1D in major population groups of India has not been fully elucidated. Similarly the mechanism of generation and preference of AH8.2 over AH8.1 in the Indian population is not clear. It is possible that due to a survival advantage of B8-DR3 haplotypes in north Indians as imposed by vigorous immune response to various pathogens may have led to independent evolution of AH8.2 and other related haplotypes.

Detailed molecular analysis carried out by us has revealed that AH8.1 haplotype carrying HLA-A\*01 that is characteristic of the European population although occurs in the Indian population, albeit with much reduced frequency, shows remarkable differences at several loci. The Indian AH8.1 is a variant (AH8.1v) that differs at HLA-C, TNF-308, DRB3 and may be other yet unknown loci (Kaur *et al* unpublished observations). In this respect, it resembles more like AH8.2. Among Indian T1D

patients, additional disease conferring DR3+ve haplotypes namely HLA-A24-B8-DR3, HLA-A3-B8-DR3 and HLA-A31-B8-DR3 may be implicated. In analogy to AH8.1, AH8.2, *etc.*, we propose to designate these haplotypes as AH8.3, AH8.4 and AH8.5 (Fig. 2). Their role in the development of T1D in patients who otherwise lack AH8.2 needs to be further evaluated. These haplotypes differ from the more common Caucasian AH8.1 at multiple loci except HLA-B and –DR B1. It is possible that the AH8.1 and its variant form (AH8.1v) have evolved independently from a common ancestor. Under environmental influences, the latter has acquired preferential occurrence among the Asian Indians, while the other disease associated DR3+ve haplotypes might have evolved from it. Interestingly all of these haplotypes are disease associated with the highest risk conferred by AH8.2 both in Celiac disease as well as type 1 diabetes. Further studies are required to elucidate the evolutionary advantage of multiple DR3+ve haplotypes in the Indian population.

Caucasian diabetes patients lacking HLA-DR3 usually show a strong association with HLA-DR4 and its two common molecular subtypes - DRB1\*0401 and \*0404<sup>39</sup>. Our studies indicate that while DR4 association in T1D is lacking among north Indian subjects<sup>39,41</sup>, patients from the Chennai region show positive association with DR4 family of alleles<sup>41</sup>.



**Fig. 2.** A hypothetical model for the origin and evolution of multiple autoimmunity favouring DR3+ve haplotypes. The haplotypes in bold are the most commonly occurring B8-DR3+ve haplotypes: AH8.1 in Caucasians (Cauc) and AH8.2 in Asian Indians (A. Ind). AH8.1v represents a variant form of AH8.1 that occurs only amongst Asian Indians.

However, the exact molecular subtypes involved are not clear. A case control study in both these population groups involving a reasonable number of patients and ethnically matched controls can help in understanding the role of HLA-DR4 in Indian diabetic subjects.

### **Functional evidence for the role of HLA-DR and -DQ molecules in pathogenesis of type 1 diabetes**

A direct role for HLA-DR and HLA-DQ molecules in the pathogenesis of type 1 diabetes has been demonstrated in studies using transgenic mice. The expression of human DR3 (DRB1\*03) and/ or DQ8 (DQA1\*0301-DQB1\*0302) in the B10 mice was shown to induce a loss of immune tolerance to GAD, a potential  $\alpha$  cell antigen<sup>9,50</sup>. No immune reactivity to GAD was seen in transgenic mice which expressed DR3 or DQ8 in combination with DQ6.2 (DQA1\*0102-DQB1\*0602)<sup>50</sup> proving the dominant protective role for DQ6.2. In another study involving C57BL/6 HLA transgenic mice, the expression of DQ8 or DR4 (DRB1\*0401) alone was sufficient to induce spontaneous diabetes, but only in those mice which expressed T cell co-stimulatory molecule B7.1, on their pancreatic  $\beta$  cells<sup>51</sup>.

### **Molecular structure of HLA-DR/DQ molecules explains T1D susceptibility**

Primary function of HLA-class II molecules is presentation of antigen (foreign and self) to the CD4+ T helper cells leading to their proliferation and activation and subsequent cytotoxic T lymphocyte (CTL) induced response at the pathologic sites. X-ray crystallography and computer based three dimensional modeling approaches have suggested that HLA molecules associated with susceptibility to type 1 diabetes share chemical and geometric properties in their peptide binding cleft. These characteristics are strikingly different from those of the protective HLA molecules, which show similarity to each other<sup>52,53</sup>. These structural differences between predisposing and protective molecules may

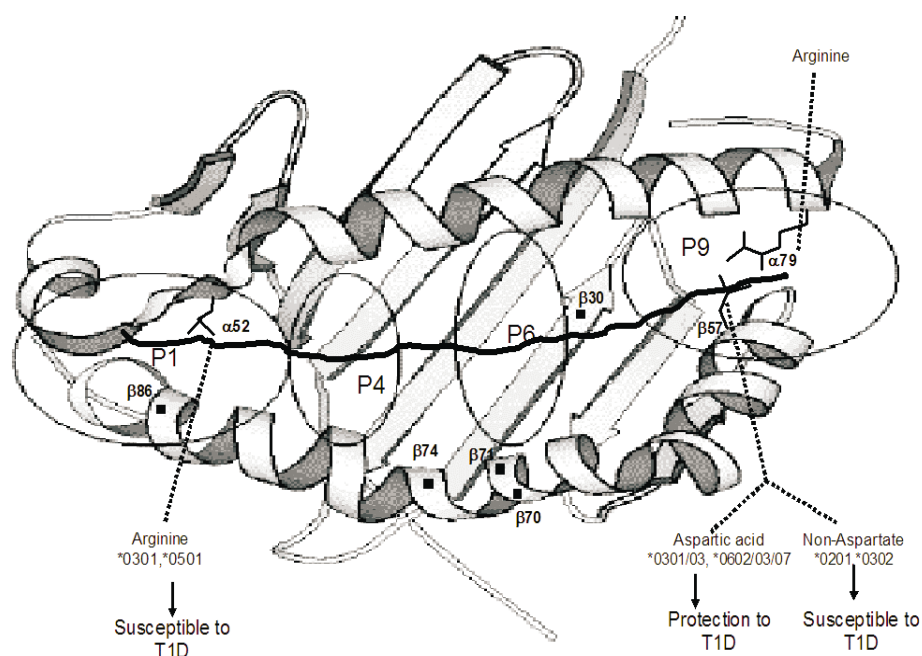
result in their functional differences in (i) peptide selectivity and binding affinity, (ii) TCR interaction, and (iii) molecular stability on the surface of the antigen presenting cells.

The antigen binding clefts of the DR and DQ molecules are quite similar in architecture and size with the carboxyl and amino terminal ends being open than closed. The groove can therefore accommodate longer peptides of approximately 12-25 amino acids although the conserved residues distributed through the central part of the groove over the binding sites are nonamers. These residues form hydrogen bonds with the amino and carboxyl groups along the backbone of the peptide<sup>54</sup>. Amino acid side chains of the peptide also slot into a series of deep cavities within the binding cleft, termed “pockets” (Fig. 3). These pockets are highly polymorphic and their structure provides a basis for the “peptide binding motif” of the molecule that results in the preferential binding of particular amino acid residues at crucial anchor points along the peptide. Structurally distinct HLA molecules favour different peptide binding motifs<sup>55,56</sup> dictated by the shape and size of the pockets and may therefore interact differently with a given antigenic peptide. The key determinants of the binding motif are pockets 1, 4 and 9 (designated as P1, P4 and P9 respectively).

The ‘P1 Pocket’ is much deeper than others, particularly so in the ‘predisposing HLA molecules’ compared to that of the ‘protective molecule’. For example, P1 in the predisposing DR4 molecule encoded by DRB1\*0401 or 0405 contains a glycine residue at position 86 of the  $\beta$  chain ( $\beta$ 86), which favours binding to large aromatic side chains in the peptide. In contrast, the valine residue in the protective DR4 molecule encoded by DRB1\*0403 favours small or medium sized hydrophobic residues in the peptide for binding<sup>52</sup>. Similarly, it has been seen that P1 pockets of the predisposing DQ2 and DQ8 molecules are much deeper than that of the protective DQ6.2 molecule<sup>52</sup>.

The 'P4 pocket' plays an important role in peptide binding selectivity in the DR molecule. For example, the alanine residue at  $\beta 74$  in the susceptible DR4 (DRB1\*0401) molecule results in greater affinity for acidic residues. In contrast, glutamate at the same position in the protective DR4 molecule encoded by DRB1\*0403 creates an incompatible pocket which does not bind to the acidic anchor residues<sup>57</sup>. On the contrary, the P4 pockets in DQ2, DQ8 and DQ6.2 molecules are similar suggesting no importance in determining susceptibility to diabetes<sup>52,53</sup>. Presence of valine at  $\beta 86$  and of glutamate at  $\beta 74$  in DRB1\*0403 might explain the dominant protection conferred by this allele in Asian Indian T1D subjects. Incidentally, this allele comprises the bulk of the DR4 family of alleles in the north Indian population<sup>58</sup>. Whether this could be correlated to the observed lack of DR4 association and the relative decreased incidence of the disease in this population compared to the Caucasians is not fully clear. Further studies comprising different ethnic groups in India will be useful in this regards.

The 'P9 Pocket' plays an important role in peptide binding in autoimmune diseases, particularly type 1 diabetes (Fig. 3). In general, the protective HLA class II molecules (DQ6.2) carry an aspartate residue at position 57 in the  $\beta$  peptide chain (Asp $\beta 57$ ), whereas those that predispose to the disease carry an uncharged amino acid residue at this position (non-Asp $\beta 57$ , carrying generally valine, alanine and serine), although there are some exceptions to this rule<sup>59</sup>. Residue  $\beta 57$  is located within P9 and plays an important role in determining the structure of this pocket. In HLA molecules which carry aspartate at position 57 in the  $\beta$  peptide chain, a salt bridge is formed between this negatively charged residue and a conserved positively charged residue (arginine) at position  $\alpha 76$  (in DR molecules) or  $\alpha 79$  (in DQ molecules)<sup>53,60</sup>. This alters the shape of P9 relative to that seen in non-Asp $\beta 57$  molecules and hence alters the binding preference for particular anchor residue in a peptide. The non-Asp $\beta 57$  molecules preferentially bind to peptides with an acidic (negatively charged) residue at the P9 anchor point, because this residue can form a stabilizing salt bridge



**Fig. 3.** The peptide binding cleft of an HLA-DQ molecule showing the binding pockets P1, P4, P6 and P9. Important amino acid residues implicated in the peptide binding are shown. A hypothetical peptide is shown as a solid horizontal line. Presence of aspartic acid at  $\beta 57$  position confers protection due mainly to formation of a salt bridge with arginine residue at  $\alpha 79$  position. Non-aspartate at  $\beta 57$  prevents the formation of salt bridge and hence confers predisposition to T1D. Source: Modified from Ref.64.

with the unopposed Arg  $\alpha$ 76 or Arg  $\alpha$ 79 residues<sup>53,55,61</sup>. However DQ2 molecule, which prefers large hydrophobic residues in P9, does not support this explanation. This could be attributed to the neighbouring residues, which produce a larger P9 pocket than found in other non-Asp $\beta$ 57 molecules like DQ8<sup>55,62</sup>. This shows the importance of the morphology of the entire pocket rather than the effect of a single residue. Nevertheless, the amino acid residue at  $\beta$ 57 influences the peptide binding affinity and selectivity of DR and DQ molecules<sup>53,61,63</sup>. Further, it has been seen that HLA-DQB1 alleles that encode a nonaspartate amino acid (Ser, Ala or Val) at position 57 in combination with HLA- DQA1 alleles with arginine at position 52, especially the DQA1\*0301-DQB1\*0201 heterodimer whether placed in trans (on DR3/DR4 haplotypes in most populations or DR3/DR9 haplotype in Chinese) or cis (DR7 haplotype in Blacks) configuration exhibit stronger positive association with T1D than DQB1 or DQA1 alone. This highlights the synergistic effect of DR3/DR4 heterozygosity in this disease. A list of the DQB1 alleles with or without Asp57 and DQA1 allele encoding Arg52 is given in Table IV.

In summary, structural differences between the predisposing and protective HLA molecules may result in differences in their ability to bind diabetogenic peptides thereby dictating susceptibility or protection to diabetes.

### Molecular stability of MHC

A more stable HLA molecule on the cell membrane may lead to an extended half life of HLA-peptide

complex which might influence disease risk by altering the strength of the interaction with pathogenically relevant T cells and ultimately affecting their activation status. Generally, it has been seen that the protective HLA-DQ molecules are more stable than the predisposing HLA-DQ molecule. It has been shown that the Asp $\beta$ 57 residue is crucial for the stability of the protective DQ6.2 molecule<sup>64,65</sup>. On the other hand, soluble DQ heterodimers encoded by DQA1\*0201-DQB1\*0302 and DQA1\*0201-DQB1\*0303 have similar stability, despite differing only at residue  $\beta$ 57 (Ala versus Asp, respectively)<sup>63</sup>. This suggests that other residues may also play an important role in defining the stability of an HLA class II molecule.

Possible mechanisms by which HLA molecules influence the development of type 1 diabetes are not clear. Differential ability of the protective and predisposing HLA molecules to present diabetogenic peptides, their interaction with the T-cell receptor (TCR) of autoreactive T cells and ensuing damage to the islet  $\beta$ -cells are all important. Further, the stability of the HLA-peptide-TCR complex depends on the stability of surface expression of HLA molecules. In case of predisposing HLA molecules, the less stable complex with self antigen in thymus may result in inefficient removal and escape of autoreactive T cells and release into the periphery. In contrast, a protective HLA molecule may form a stable complex leading to their efficient deletion. A protective HLA molecule therefore is not able to bind well to diabetogenic peptides in the periphery failing to activate an autoimmune T cell response, whereas a predisposing HLA molecule may be.

**Table IV.** Susceptibility/resistance conferring DQB1 and DQA1 alleles based on the presence of Asp<sup>57</sup> or Arg<sup>52</sup> respectively

Asp <sup>57</sup> or Arg <sup>52</sup>	Encoding alleles	Association
DQ $\beta$ -Asp <sup>57</sup>	*0301, *0303, *0402,*0602,*0603,*0607	Protection
DQ $\beta$ -non-Asp <sup>57</sup>	*0201, *0302, *0501, *0604	Susceptibility
DQ $\alpha$ -Arg <sup>52</sup>	*0301, *0501	Susceptibility
DQ $\alpha$ -non-Arg <sup>52</sup>	*0102, *0103	Protection

DQ $\beta$ -non Asp<sup>57</sup> alleles include those carrying valine, serine and alanine at this position. Asp<sup>57</sup> (aspartate at 57<sup>th</sup> position); Arg<sup>52</sup> (arginine at 52<sup>nd</sup> position)

The outcome of an immune response depends on differences between the proinflammatory and regulatory phenotype of T cells. The protective and predisposing HLA molecules may interact differently with autoreactive T cells via their TCR, affecting the phenotype (proinflammatory vs regulatory) or their activation status (proliferative vs anergised). Although several possible mechanisms by which an HLA molecule can influence the development of type 1 diabetes have been proposed, but these still remain illusive.

### Other MHC genes around IDDM1

Some of the MHC class III (central region) genes may also be involved in conferring risk to T1D. This region encodes molecules with a variety of functions, including complement components (C4A, C4B, factor B and C2), tumour necrosis factor (TNF), heat shock protein Hsp70 and 21-hydroxylase (CYP21). Of these, complement and TNF proteins play an important role in immune surveillance pathways. Our ongoing genetic studies on factor B have indicated a positive correlation of Bf\*S allele with type 1 diabetes in north Indians (unpublished observations).

In general, the central region genes are associated secondarily to the disease associated DR and DQ alleles through strong linkage disequilibrium. These include for example, the transporter associated with antigen processing (*TAP*) genes, the large multifactorial protease (*LMP*), tumour necrosis factor (*TNF*) and B cell associated transcript (*BAT*) genes. *BAT2* gene lies within the class III region of the MHC. The frequency of BAT2.9 allele has been reported to be significantly increased in T1D particularly patients with younger age of disease onset. On the contrary, the frequency of BAT2.12 allele has been found to be significantly decreased in these patients as compared with the control subjects. This suggests that the BAT2 microsatellite polymorphism is associated with age-at-onset of T1D and possibly with the inflammatory process of pancreatic  $\beta$ -cell destruction during the development of T1D. However, this association is not independent

of TNF $\alpha$ , as the BAT2.9 allele is found to be strongly associated with TNF $\alpha$ 9 in the young-onset T1D patients<sup>66</sup>.

Several studies have shown an association of TNF- $\alpha$  allele 2<sup>67</sup> with T1D which could actually be due to a strong linkage disequilibrium of HLA-DR3 with TNF- $\alpha$  alleles. Mapping studies using microsatellite markers have identified non class II regions that may contain additional susceptibility genes. Significant associations have been reported with the D6S273 microsatellite (located centromeric of the TNF locus) and the D6S2223 microsatellite (located telomeric of HLA-F)<sup>68,69</sup>.

### MIC-A and type 1 diabetes

A distinct family of MHC genes designated MHC class I chain-related genes (*MIC*) has been identified within the class III region<sup>70</sup>. The *MIC* family consists of three pseudogenes *MIC-C*, *MIC-D* and *MIC-E* and two functional genes namely *MIC-A* and *MIC-B*<sup>71</sup>. The latter are located telomeric to the TNF $\alpha$  genes between B cell associated transcript (*BAT-1*) and *HLA-B* genes.

The *MIC-A* and *MIC-B* genes contain long open-reading frames encoding MHC class I molecules with three extracellular domains ( $\alpha$ 1,  $\alpha$ 2, and  $\alpha$ 3), a transmembrane segment and a cytoplasmic tail, each encoded by a separate exon. Sequence analysis of the *MIC-A* gene has revealed a trinucleotide repeat (GCT) microsatellite polymorphism within the transmembrane region<sup>72</sup> (Fig. 4). So far, five alleles of exon 5 of the *MIC-A* gene, each consisting of 4, 5, 6 and 9 repetitions of GCT and five repetitions of GCT with an additional nucleotide insertion (GGCT), have been identified<sup>72</sup>. These alleles have accordingly been named A4, A5, A6, A9 and A5.1, and their sizes correspond to 179, 182, 185, 194 and 183 bp respectively. *MIC-A* and *MIC-B* are stress-inducible surface molecules that are not associated with  $\beta$ 2 microglobulin and peptides. They are expressed in the intestinal epithelium and in epithelial tumours and are recognized by a subset of  $\gamma\delta$  T cells which are mostly found in epithelial sites.

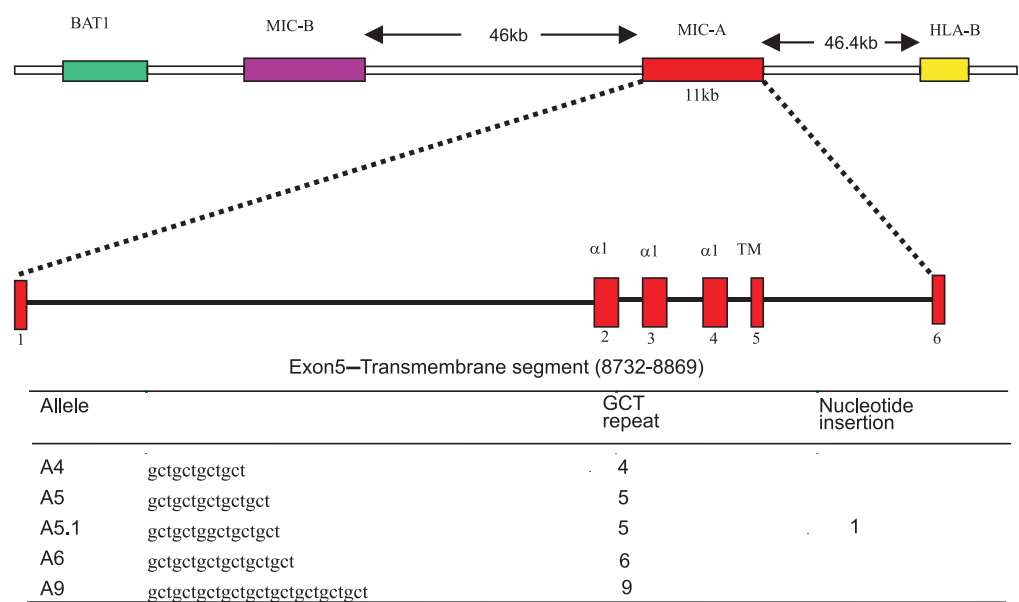
Many studies have shown an association of MIC-A alleles with T1D in HBDI (Human Biological Data Interchange) families from the USA, Korea, Latvia, China and Japan. In these families transmission of MIC-A allele 5 and 5.1 from parent to the offspring was found to be significantly more frequent than expected suggesting a positive association of MIC-A5 and A5.1 with T1D<sup>72,73</sup>. Among Asian patients, MIC-A6 and A9 were found to be negatively associated with the disease.

IDDM2: insulin- VNTR and type 1 diabetes

At least two candidate genes in T1D, namely insulin-VNTR and *CTLA-4* contribute a combined second most inheritable risk of about 15 per cent after MHC. Of these, the former is involved in the regulation of insulin expression while the latter is important in the regulation of T cell function. Several studies have shown a strong association of the variable number of tandem repeat (VNTR) polymorphism in the insulin gene with type 1 diabetes<sup>36,43,73-75</sup>. These tandem repeats are located 596 bp upstream of the translational start site of the

insulin gene on chromosome 11p15.5<sup>76</sup> (Fig. 5). Based on the number of tandem repeats of 14-15 bp sequences, these VNTR alleles are grouped into three classes: class I alleles (20-63 repeats), class II alleles (64-139 repeats) and class III alleles (140-210 repeats). The short class I alleles are generally predisposing, especially when in homozygous state, they confer more than two fold relative risk for type 1 diabetes whereas class III alleles are associated with dominant protection<sup>76</sup>. Nevertheless there are some exceptions to this pattern of both class I and class III allelic association<sup>75</sup>. Class I alleles are found in nearly 80 per cent of Caucasian chromosomes, class III alleles occur less common (nearly 20%) whereas class II alleles are most infrequent<sup>78</sup>.

The exact mechanism by which the insulin VNTR alleles influence the risk of type 1 diabetes is not well understood. These polymorphisms regulate the expression of two downstream genes: the insulin and the insulin-like growth factor 2 (IGF2). These two growth factors play important roles in disease pathogenesis as insulin and its precursors are potential target autoantigens for  $\beta$ -cell destruction.



**Fig. 4.** Gene organization of *MIC-A* on chromosome 6p 21.3 with *HLA-B* oriented towards the telomeric side and *MIC-B* towards centromeric side. The trinucleotide GCT repeats are located within exon 5 that codes for transmembrane portion of *MIC-A*. Depending on the number of repeats the *MIC-A* alleles are named as A4, A5, A5.1 (with one extra insertion of G nucleotide), A6, A9.

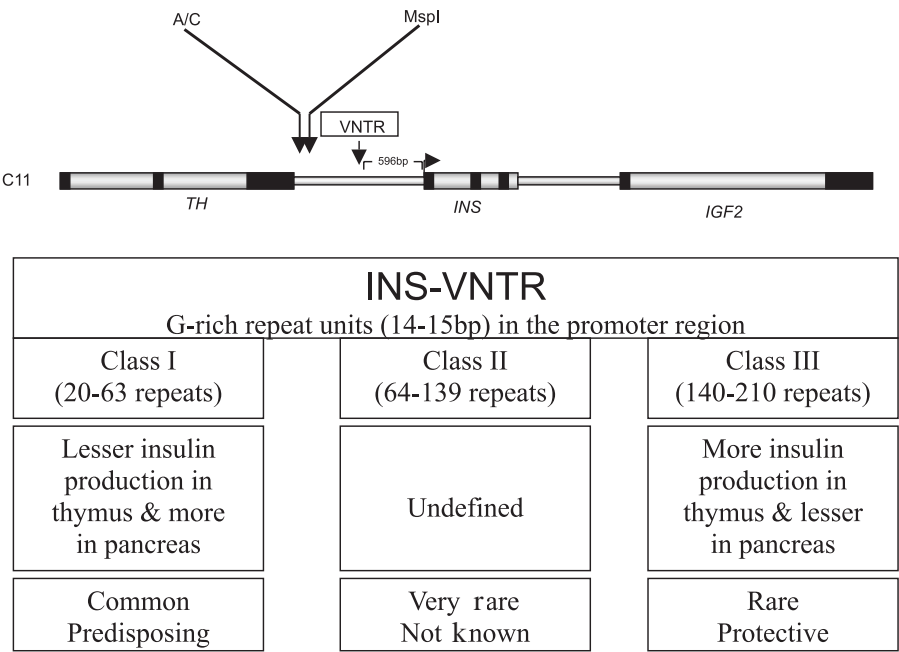
Studies have shown a transcription of the insulin gene and other  $\beta$ -cell autoantigens, albeit at low levels in the thymus of mice<sup>79</sup> as well as in humans<sup>80,81</sup>. The insulin mRNA expression levels have been found to correlate with allelic variations at the VNTR locus<sup>81</sup>. Whereas the increased transcription of the insulin gene in thymus is associated with protective class III alleles, the low levels of transcription are found in the case of predisposing class I alleles. This situation is much the reverse in pancreas, *i.e.*, higher insulin mRNA expression is associated with the class I alleles<sup>80</sup>. This suggests that negative selection of autoreactive thymocytes is dose dependent and raised concentrations of pre-proinsulin in the thymus may promote an efficient deletion of autoreactive T cells for insulin and its precursors. This leads to central immune tolerance to an important autoantigen in the pathogenesis of diabetes. This mechanism may explain the closer positive association of homozygous class I alleles having lower intrathymic insulin expression and higher expression in the pancreas thus increasing the risk to diabetes and dominant protective effect of class III alleles because

of comparatively higher expression in thymus and lower expression in pancreas.

The insulin growth factor 2 gene product (*IGF 2*) also contributes towards IDDM2 associated susceptibility to diabetes because of its critical role in T cell development and thymic negative selection. It has some homology to proinsulin<sup>82</sup>, therefore, it may also act as a selecting peptide for insulin reactive T cells. The contributory role of *IGF2* gene appears to be independent of the insulin VNTR alleles as it has been shown that VNTR class I and class III alleles are associated with similar level of *IGF2* expression in thymus and pancreas<sup>82,83</sup>. However, increased expression of *IGF2* in the placenta is associated with class I alleles suggesting that it may influence intrauterine growth and birth size, which are risk factors for type 1 diabetes<sup>84,85</sup>.

**IDDM 12: CTLA-4 gene and type 1 diabetes**

Cytotoxic T lymphocyte associated antigen-4 (CTLA-4) along with *CD28* and *ICOS* genes are



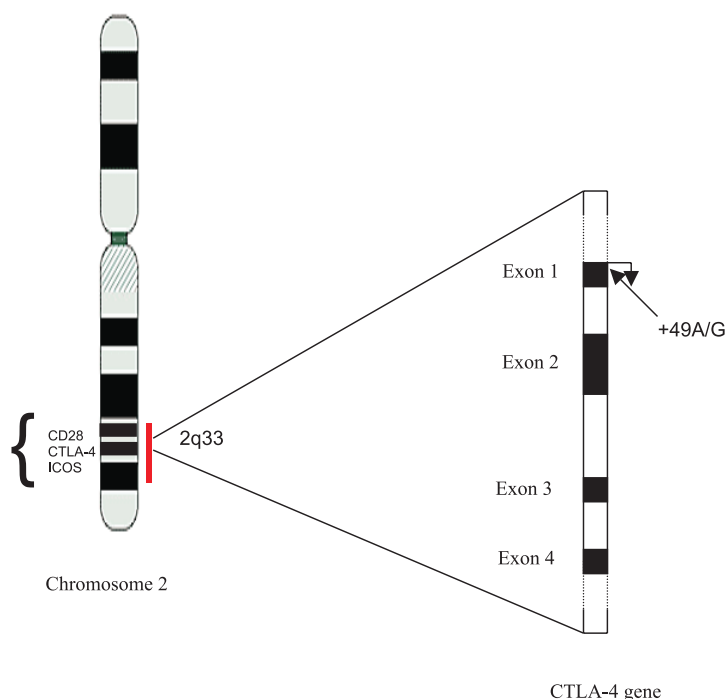
**Fig. 5.** Insulin gene map showing the type 1 diabetes associated VNTRs located 596 bp upstream of the transcription site in the promoter region of the gene. The lower half of the figure depicts a correlation between different classes of INS VNTR alleles and insulin production thereby leading to susceptibility or resistance to T1D. The dark shaded boxes depict exons while light shaded boxes are intronic regions. *Source:* Modified from Ref.77.

located on chromosome 2 (2q31-35) in humans<sup>86-90</sup> (Fig. 6). Within this 23 centimorgan (CM) interval, the 2q33 region (referred as IDDM12) is associated with autoimmune diseases. Susceptibility to T1D has been reported to be associated strongly with a single nucleotide polymorphism (SNP) in the 5' end of CTLA-4 rather than polymorphism at the 3' UTR<sup>89</sup>.

CTLA-4 plays an important role in the regulation of T cell function by downregulating antigen-activated immune responses. Given the function of CTLA-4 as a negative regulatory molecule of the immune system in general and that of HLA class II in the presentation of a specific antigen to initiate an immune response to the antigen, it is reasonable to speculate that a susceptible allele at CTLA-4 leads to autoimmunity, while the specific HLA haplotypes target the autoimmune attack to pancreatic islets through recruitment of specific CTLs and may be the natural killer (NK) cells. Two isoforms are known

for CTLA-4: (i) the full-length transmembrane glycoprotein expressed transiently on the surface of activated CD4 and CD8 T cells, also detected on B cells, is the main isoform found in adult thymocytes, and (ii) the soluble form. The latter is generated by alternative splicing of transmembrane domain and is mainly expressed by inactivated T cells<sup>91</sup>. The full length CTLA-4 receptor binds to the ligand B7 expressed on the surface of antigen presenting cells (APCs) having an antagonistic role of its counter receptor CD28. Its expression is upregulated only 2-3 days following T cell activation and is merely a fraction of the expression level of constitutively expressed co-stimulator, CD28. Thus CTLA-4 is a low abundance but high avidity receptor on the surface of T cells.

*CTLA-4* gene consists of four exons, the first encodes a V-like domain of 116 amino acids. An A to G substitution at nucleotide 49 in exon 1 results



**Fig. 6.** Gene map of *CTLA-4* on chromosome 2q33 region. Various polymorphisms in *CTLA-4* gene in the 5'UTR, 3'UTR regulatory regions and exon 1 are shown in the map and have been studied extensively in T1D. The dark shaded boxes depict exons while light shaded boxes are intronic regions.

in an amino acid substitution (Thr/Ala) in the leader peptide. In addition to this, several CTLA-4 polymorphisms have been identified in humans, which have been used in genetic studies. These include polymorphisms in the 5' flanking promoter region, an (AT)<sub>n</sub> repeat in the 3' UTR, and a recently described A6230G SNP (initially described as CT60 by Ueda *et al*<sup>89</sup>) outside the predicted CTLA-4 polyadenylation site<sup>92</sup>. A49G polymorphism affects the cell surface expression of CTLA-4 molecule. The G49 allele of the signal peptide has been consistently over-transmitted from heterozygous parents to the affected offspring as observed by transmission disequilibrium test (TDT) in various ethnic groups<sup>93</sup>. Preliminary studies conducted by us in T1D patients from North India have confirmed the similar association of G49 allele with the disease (unpublished observation). In particular, patients with young age at onset of disease are found to carry significantly increased frequency of GG genotypes. This association is better supported by a study showing increased proliferation of T cells in individuals homozygous for the predisposing G49 allele at the signal peptide coding SNP<sup>94,95</sup>. These investigators also showed that this allele negatively affects the downregulation of T cell activation in response to IL-2<sup>95</sup>. A recent review has addressed the molecular basis underlying these findings and postulated that a threonine to alanine (Thr 17 Ala) amino acid substitution in a highly conserved position in the signal peptide of *CTLA-4* may alter early events in its post-translational process<sup>96</sup>. Alternatively it could be a marker in linkage disequilibrium with the causative polymorphism.

### Other T1D susceptibility genes

Although unconfirmed by linkage analysis *IDDM6*, *IDDM7*, *IDDM10*, and *IDDM13* also show evidence for an association with type 1 diabetes in affected family based and case control studies<sup>90,97-100</sup>. Although, fine mapping of the intervals has not yet determined the identity of the putative diabetes susceptibility genes, certain

intervals contain important candidate genes. For example, *IDDM4* maps close to the galanin (*GALN*) and FAS-associated death domain (*FADD*) genes. *GALN* is a neuropeptide that may influence insulin secretion from the pancreas<sup>101</sup>. *FADD* is a cytoplasmic protein involved in the regulation of T cell apoptosis, which may be an important mechanism in type 1 diabetes<sup>101</sup>. In a study involving T1D affected sib pairs, neither of these genes could be significantly associated or in linkage with the disease<sup>101</sup>.

*IDDM7* maps close to the *NeuroD/BETA2* gene on chromosome 2q32. *NeuroD/BETA2*, is an insulin gene transcription factor that plays an important role in the development of the pancreatic islet  $\beta$ -cells. Some studies have shown the association of heterozygosity for the Ala45Thr variant of this gene with type 1 diabetes<sup>101,103</sup> although others have failed to confirm these findings<sup>104</sup>. The effect of *NeuroD/BETA2*, may, however be independent of *IDDM7*<sup>103</sup>.

*IDDM13* is located on human chromosome 2 (2q34) close to the *NRAMP1* gene, which is a host resistance gene. *NRAMP1* is a divalent cation transporter that plays a key role in macrophage activation. It affects the function of macrophages in a pleiotropic manner, which includes the expression of chemokines, IL-1 $\beta$ , TNF- $\alpha$  inducible nitric oxide synthetase and MHC class II molecules<sup>105</sup>. In the promoter region of *NRAMP1*, six alleles have been reported. An association of this polymorphism to autoimmune and infectious diseases has been reported<sup>105,106</sup>. For example, the allele 3 is associated with rheumatoid arthritis and also shows increased transmission to diabetic siblings with a first or second degree relative with rheumatoid arthritis. *NRAMP1* does not show an association in those families which only have sibs affected with type 1 diabetes<sup>107</sup>.

### Type 1 diabetes and ICAM-1 gene polymorphism

The intercellular adhesion molecule-1 (ICAM-1) is a well-characterized surface glycoprotein, known to be expressed on macrophages, thymic

epithelial cells, vascular endothelial cells and activated lymphocytes. It is involved in various leukocyte functions including antigen presentation and extravasation into lymphoid tissues and inflamed non-lymphoid tissues<sup>108,109</sup>. In addition to these, expression of ICAM-1 has been shown on pancreatic islet  $\beta$ -cells by electron microscopy<sup>110</sup> suggesting that it may be involved in the killer lymphocyte-mediated cytolysis of pancreatic islet  $\beta$ -cells. Therefore, this may be implicated in the pathogenesis of autoimmune diseases including type 1 diabetes. Two single nucleotide polymorphisms have been described in exon 4 and exon 6, which change the amino acids G241R (glycine  $\rightarrow$  arginine) and E469K (glutamic acid  $\rightarrow$  lysine) respectively<sup>111</sup>. Recently, Masataka *et al*<sup>112</sup> have shown an association between *ICAM-1* gene polymorphism and age of onset of type 1 diabetes.

#### ***PTPN22* gene polymorphism and type 1 diabetes**

The *PTPN22* (protein tyrosine phosphatase N22) gene is located on chromosome 1p13 and encodes a lymphoid-specific phosphatase known as Lyp. It performs the function of dephosphorylation of Lck, Fyn and ZAP-10 proteins which are known to be important in T cell signaling. Lyp binds to C-terminal Src kinase tyrosine kinase (Csk) resulting in the downregulation of activated T cells, because Csk is an important suppressor of kinases that mediate T cell activation. In addition, Lyp has been shown to play a negative regulatory role in T cell signaling by binding to the adaptor molecule Grb2 (growth factor receptor-bound protein 2)<sup>113</sup>.

Recently, a positive association of a single nucleotide polymorphism C1858T in *PTPN22* gene with T1D has been reported in a non-Hispanic white population from North America, and an Italian population<sup>114,115</sup>. The 1858T is a rare allele with a substitution from arginine (R) to tryptophan (W) at position 620, which results in disruption of the proline-rich binding motif PxxPxR, which is important for Lyp binding to both Csk and Grb2<sup>113,114</sup>.

Besides type 1 diabetes, the 1858T allele has also been reported to be positively associated with other autoimmune diseases including rheumatoid arthritis and systemic lupus erythematosus<sup>116,117</sup>.

#### ***FOXP3* gene polymorphism and type 1 diabetes**

*FOXP3/scurfin* gene located on human chromosome Xp11.23 encodes a protein that is a member of the forkhead/ wing-helix family of transcriptional regulators, and is specifically expressed in naturally occurring CD25+CD4+ regulatory T cells<sup>118,119</sup>. In a retroviral gene transfer experiment, the transfer of *FOXP3* has been shown to convert naïve T cells towards a regulatory T cell phenotype similar to the naturally occurring regulatory T cells<sup>119</sup>. These findings show that *FOXP3* works as a master regulatory gene for the development of regulatory T cells. Since an impaired T cell activity can lead to an autoimmune disease<sup>120,121</sup>, the requirement of regulatory T cells as important components in the homeostasis of the immune system cannot be ignored. A mutation in the *FOXP3* gene results in a rare recessive monogenetic disorder called IPEX (immune dysregulation, polyendocrinopathy including type 1 diabetes, enteropathy and X-linked syndrome)<sup>122</sup>. An autoimmune disease is supposed to develop as a result of misbalance between pro-inflammatory and anti-inflammatory cytokines indicating that the dysregulation of *FOXP3/scurfin* gene expression may lead to the development of autoimmune diabetes. Recently, Bassuny *et al*<sup>123</sup> have reported an association of a functional microsatellite polymorphism (GT)<sub>n</sub> in *FOXP3* gene with susceptibility to type 1 diabetes in the Japanese population. However similar studies conducted in other populations failed to confirm any such association<sup>124,125</sup>.

Type 1 diabetes is thus a complex, polygenic autoimmune disease with multifactorial inheritance. To date HLA genes are the strongest risk contributors, which include HLA-DQ and DR alleles/

haplotypes, although their influence may be modified by other genes within the MHC or outside of it. Although several genes have been implicated (*IDDM1* to *IDDM18*), functional studies are necessary to understand their exact influence on the overall disease susceptibility and/ or protection. In depth, information is also desirable on the autoantigenic peptides of critical importance, their binding affinity on the particular MHC and the type and specificity of T cells involved in the cascade events of immunological injury of the pancreatic  $\beta$ -islets. Further, the influence of environmental factors in influencing susceptibility to type 1 diabetes is not clearly defined. After HLA genes, the insulin *VNTRs* and *CTLA-4* genes together provide the second most evident genetic contribution. Clearly, the effect of non-HLA genes on type 1 diabetes susceptibility is modest, as compared with the extremely strong effect of HLA-linked genes. Nevertheless, identification of these genes will provide important information on biological pathways in the development of type 1 diabetes, leading to the definition of effective methods for prediction, prevention and intervention in the disease. Such a knowledge will also help in instituting personalized molecular medicine approaches that may have individual variation depending upon the genetic makeup.

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