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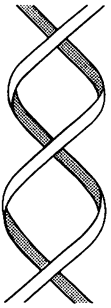

AIMS AND SCOPE

The journal publishes original research findings (and reviews solicited by the Editor) on the subject of the identification of markers associated with the disease processes whether or not they are an integral part of the pathological lesion. The disease markers may be a genetic host factor predisposing to the disease or the occurrence of cell-surface markers, enzymes or other components, either in altered forms, abnormal concentrations or with abnormal tissue distribution. This journal is designed to provide a forum for publications dealing with original observations in this developing field on any aspect of the general topic including:

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- ☐ Population studies of new and existing markers, designed to elucidate information on their normal distribution as well as that in disease states
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- ☐ Family studies of markers in disease
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DISEASE MARKERS, VOL. 9, 273–280 (1991)

THE LEVEL AND THE PERSISTENCE OF ISLET CELL ANTIBODIES IN HEALTHY SCHOOLCHILDREN ARE ASSOCIATED WITH POLYMORPHIC RESIDUES OF THE HLA-DQβ CHAIN

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SUMMARY

Cytoplasmic islet cell antibodies (ICA) were determined in a group of non-diabetic Caucasian schoolchildren ($n=4208$). The prevalence rate for ICA positivity was 1·05 per cent (95 per cent confidence interval: 0·8–1·4 per cent). Analysis of HLA risk factors revealed that HLA-DRB1*03 ($p<0·01$), HLA-DRB1*04 ($p<0·01$) and HLA haplotypes with non-charged amino acids (non-Asp) at codon 57 of the HLA-DQβ ($p<0·01$) chain were significantly increased when compared to controls. High levels of islet cell antibodies, i.e. Juvenile Diabetes Federation units (JDF units) equal to or greater than 30 JDF units were found to be associated with amino acids other than aspartic acid at codon 57 of the DQβ chain molecule. Also the persistence of circulating ICA was found to be associated with non-Asp homozygosity of the proband ($p<0·03$).

KEY WORDS HLA Insulin-dependent diabetes mellitus Islet cell antibodies

INTRODUCTION

Cytoplasmic islet cell antibodies (ICA) are a major serological hallmark of insulin-dependent diabetes mellitus (IDDM) and prediabetic insulitis. It has been shown in family studies that the persistence of ICA over a period of several months, the presence of high titer ICA, their complement-fixing ability, and the detection of radiobinding insulin autoantibodies are independently correlated with progression to IDDM in non-diabetic first-degree relatives (Tarn *et al.*, 1988; Srikanta *et al.*, 1985; Ziegler *et al.*, 1990; Riley *et al.*, 1990). Immunoprecipitating antibodies to the 64 000 Mr (64 KD) islet cell protein, now recognized as glutamate decarboxylase, have been detected in the prediabetic period in first-degree relatives of IDDM patients who later developed IDDM (Atkinson *et al.*, 1990). However, the laborious methods of detection have so far not permitted large-scale testing for 64 KD antibodies, so that ICA are still applied for the primary screening for IDDM risk.

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Genes of the major histocompatibility complex (MHC) have been shown to constitute the major part of the immunogenetic background in IDDM patients. More than 90 per cent of Caucasian Type 1 diabetic individuals type as HLA-DRB1*03 and/or HLA-DRB1*04 (Trucco *et al.*, 1989). Particular alleles at the HLA-DQB1 locus, which are characterized by the presence or absence of aspartic acid at amino acid at position 57 of the DQβ chain, are even more intimately associated to disease susceptibility (Todd *et al.*, 1987; Morel *et al.*, 1988). For this reason, molecular typing of the HLA-DQB1 alleles now provides a valuable genetic risk marker for IDDM in Caucasians (Dorman *et al.*, 1991). In addition, the Barts-Windsor family study has shown that the cumulative risk of a non-diabetic relative is dependent on haplotype-sharing of IDDM-associated HLA risk markers with the diabetic individual (Tarn *et al.*, 1988).

Since most of our present knowledge on the natural course of prediabetic insulitis is derived from studies of non-diabetic relatives of IDDM patients, a critical gap exists in our knowledge of the epidemiology of ICA and their corresponding risk factors in the general population (Boehm *et al.*, 1991). Over 90 per cent of newly diagnosed IDDM patients lack a family history of the disease, so that an epidemiologic approach is required to obtain a clear picture of the risk factors. Only six population based studies from Japan, Spain, the Netherlands, Italy, Finland, and the USA have in part addressed this issue so far. All studies have provided limited or no data on the correlation of ICA with risk factors that had already been established for first-degree relatives (reviewed in Lipton *et al.*, 1989).

To address the above questions, we have studied a large group of schoolchildren in a homogenous German population, which allowed us to establish prevalence rates for ICA positivity. Also, HLA risk factors were measured to analyze their association with the presence and the persistence of ICA as well as the ICA levels at a given point of time.

MATERIALS AND METHODS

In a cross-sectional study between June 1988 and June 1989, 4208 schoolchildren from a homogenous population in the southern part of Germany were screened for the presence of ICA in the serum. The total population of schoolchildren comprised 8031 individuals (3730 males and 4301 females). The median age was 13·9 years; the effective range 6 to 21 years. The study was performed according to the principles of the Declaration of Helsinki.

Islet cell antibodies (ICA) were determined by indirect immunofluorescence testing on unfixed cryostat sections of human pancreas of a donor with blood group 0. The high quality of ICA testing was evaluated by the Juvenile Diabetes Federation ICA-Workshop (Second JDW ICA Proficiency Program; LAB ID No. 116). Results were transferred to Juvenile Diabetes Federation units (JDF units) using the standard curve of the ICA-Workshop. The lower limit of detection in the assay was 5 JDF units throughout the study.

HLA-DRB1 phenotypes were determined by the two-color fluorescence technique according to van Rood and coworkers (1976). HLA-DQB1 genotypes were analyzed as described (Morel *et al.*, 1988; Saiki 1987) using *in vitro* amplified

genomic DNA and a panel of sequence specific oligonucleotide probes (SSOs). The recent nomenclature for factors of the HLA system was used (Bodmer *et al.*, 1991).

Confidence intervals for ICA prevalence were calculated using the Poisson distribution. The HLA data were analyzed by the chi-square test; 2 by 2 contingency tables were performed, using the Yates correction to estimate the statistical significance (Svejgaard *et al.*, 1980).

RESULTS

Forty-four out of 4208 individuals (25 males and 19 females) were ICA positive (ICA+). An overall prevalence of 1·05 per cent (95 per cent confidence interval: 0·8–1·4 per cent) of ICA positivity was observed.

Thirty-six out of 44 ICA+ individuals, 808 local non-diabetic control subjects, and 185 local IDDM patients with onset of disease before age 22 were HLA-DRB1 phenotyped using serological methods. In addition 36 ICA+ schoolchildren, 104 IDDM patients, and 123 controls were DNA typed. The HLA-DRB1*03 ($p<0·01$), and the HLA-DRB1*04 ($p<0·01$) phenotypes were found to be increased in ICA+ schoolchildren compared to controls. No significant difference in the prevalence of HLA-DRB1 types (the following alleles were tested: HLA-DRB1*01, *02, *03, *04, *05, *06, *07, and *08), could be observed between IDDM patients and the ICA+ children. The HLA-DQB1 DNA analysis showed that 61·1 per cent of the ICA+ schoolchildren were non-Asp homozygous at codon 57 of the DQβ chain or non-Asp/'blank'. Of the ICA positive schoolchildren 91·6 per cent had at least one non-Asp HLA haplotype. In contrast, a non-Asp haplotype was found in 71·5 per cent of the local controls ($p<0·01$ when compared to ICA+). All IDDM patients revealed at least one non-Asp HLA-haplotype and 84·6 per cent were non-Asp homozygous. This difference was significant when compared to ICA+ subjects ($p<0·002$) as well as the local controls ($p<0·01$). The HLA-DNA data are summarized in Table 1.

Three groups were formed in order to analyze the association of ICA levels and HLA risk factors. The groups consisted of either HLA-DRB1*03+, or HLA-DRB1*04+ ('high-risk' HLA factors), or HLA-DRB1*02 and/or HLA-DRB1*07 positive schoolchildren ('low-risk' HLA factors). The rationale behind this strategy was to split the ICA+ healthy individuals into subgroups with well-known HLA risk factors, i.e. HLA-DRB1*03 and HLA-DRB1*04, or protective HLA factors, i.e. HLA-DRB1*02 and HLA-DRB1*07 (Figure 1). The mean ICA level measured in JDF units was $5·8 \pm 6·1$ in DRB1*04+ schoolchildren, and $5·9 \pm 5·1$ in DRB1*03 positives. In the group of DRB1*02 and/or DRB1*07 positive probands, the mean level was found to be $6·6 \pm 4·5$ JDF units. No significant difference was found.

In summary, no association between the ICA level and any HLA-DRB1 phenotype could be observed. In ICA+, Asp homozygous individuals, the mean level was found to be $3·8 \pm 3·7$ JDF units. Asp/non-Asp heterozygotes revealed a mean titer of $5·8 \pm 4·8$ JDF units. In contrast, non-Asp homozygotes showed significantly elevated mean ICA levels of $10·3 \pm 5·3$ JDF units. In contrast non-Asp homozygotes showed significantly elevated mean ICA levels of $10·3 \pm 5·3$ JDF units ($p<0·003$) when compared to the latter groups (Figure 1). A high level of islet cell antibodies of

Table 1. Relation of charged and non-charged amino acids at codon 57 of the HLA-DQβ chain and ICA positivity

Haplotype at codon 57	Controls ($n=12$)	ICA+ ($n=36$) No (% with haplotype)	IDDM ($n=104$)
Asp/Asp	35 (28·4)	3 (8·3)*	0 (0)
Asp/non-Asp	47 (38·2)	11 (30·5)**	16 (15·4)
Non-Asp/non-Asp	41 (33·4)	22 (61·1)***	88 (84·6)
Any haplotype including one non-Asp allele	88 (71·5)	33 (91·6)****	100 (100)

Asp: HLA-haplotype with aspartic acid at codon 57 of the DQβ chain. Asp+ haplotypes correspond to the following HLA-DQB1 alleles: HLA-DQB1*0301, -0303, -0401, -0402, -0503, -0601, and 0602. Non-Asp: HLA haplotype with a non-charged amino acid at codon 57. Non-asp+ haplotypes correspond to the following DQB1 alleles: HLA-DQB1*0201, -0302, -0501, -0502, -0504, -0603, -0604, and -0605 (see Marsh and Bodmer, 1991).
The statistical analysis was done within the local group of controls, IDDM patients, and ICA+ schoolchildren. *ICA+ versus controls: $p<0·01$; ICA+ versus IDDM patients: not significant (NS). **ICA+ versus controls: NS; ICA+ versus diabetics: NS. ***ICA+ versus controls: $p<0·01$; ICA+ versus IDDM: $p<0·02$. ****ICA+ versus controls: $p<0·01$; ICA+ versus IDDM: NS.

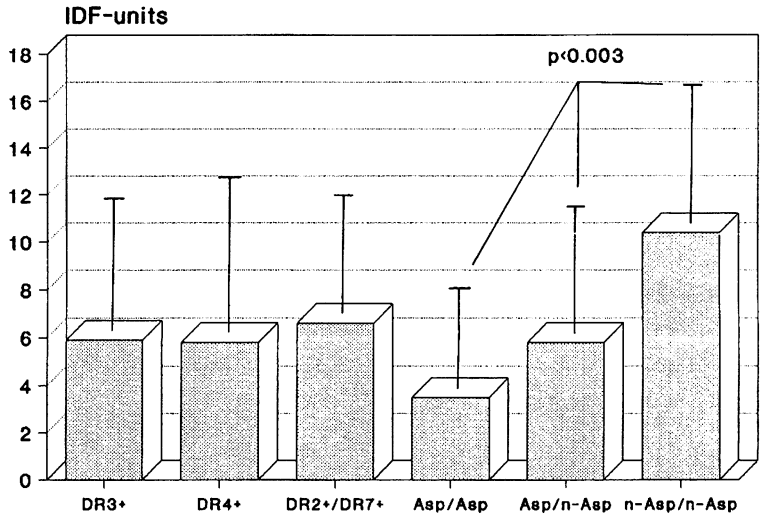

Figure 1. Subgroups of the ICA+ schoolchildren are shown. ICA levels are given as Juvenile Diabetes Federation units (±SEM). A significant difference between non-Asp homozygous ICA+ schoolchildren and Asp-homozygous or non-Asp/Asp-heterozygous individuals was observed ($p<0·003$). No association with HLA-DRB1 phenotypes can be seen

≥30 JDF units was found in 13 individuals. Three probands had at least one Asp positive HLA-haplotype, whereas 10 individuals were non-Asp homozygous.

Schoolchildren, who were tested as ICA+ during the initial survey, were retested after 6 months. Their first blood sample was retested together with the second sample using the same source of pancreas. ICA were designated as persistent, when

Table 2. Persistence of islet cell antibody positivity in relation to codon 57 of the HLA-DQβ chain

Haplotype at codon 57	ICA negative	ICA positive	Total
Asp/Asp	2	1	3
Asp/non-Asp	6	3	9
Non-Asp/non-Asp	5	13*	18
Total-	13	17	30

*Statistically significant, when non-Asp homozygotes are compared to non-Asp/non-Asp heterozygous and Asp homozygous individuals ($p < 0{\cdot}03$).

still positive in a second sample drawn from the proband. Thirty out of 44 initially ICA + individuals were tested 6 months after the first ICA analysis. Thirteen (43 per cent) schoolchildren were found to be negative, 17 (57 per cent) children positive. Seventy-six per cent of these healthy individuals that were persistently ICA + genotyped as non-Asp homozygous. Three individuals were heterozygous Asp/non-Asp at coden 57 of the DQβ chain, and one individual was found to be Asp homozygous (Table 2). No association with HLA-DR phenotypes could be observed.

DISCUSSION

A number of studies have examined the prevalence rates of islet cell antibodies in different ethnic groups (for review see Lipton and LaPorte, 1989). Prevalence rates were found to range from 0·24 per cent in Dutch children (Bruining *et al.*, 1988); 0·35 per cent in a Spanish population (Bergua *et al.*, 1987) and 4·1 per cent in a Finnish population (Karjalainen, 1990). In our survey, which is the first population study where cytoplasmic islet cell antibodies were expressed in JDF units using the protocol of the Juvenile Diabetes Federation ICA-Workshop, the prevalence of ICA positives was found to be 1·05 per cent with a confidence interval of 0·8 to 1·4 per cent.

While there is much information on factors indicating high risk for the future development of IDDM in first-degree relatives of IDDM patients, only limited data are available to indicate the possible risk factors in the general population. Since more than 90 per cent of IDDM patients lack a family history of IDDM, population based data are required.

So far virtually no information has been provided to calculate the immunogenetic risk factors associated with ICA positivity in a random population. The Barts-Windsor family study showed the high ICA levels as well as complement-fixing ICA indicated an increased risk for the future development of IDDM in non-diabetic first-degree relatives (Bonifacio *et al.*, 1990). An impact of ICA levels on the cumulative risk for IDDM has also been shown by Riley and colleagues (1990) in Gainsville and in the family study from Boston, which employed the protein A assay which is known to detect ICA of 80 JDF units and above (Ziegler *et al.*, 1990). As is

evident in the above mentioned family studies, our survey also showed that ICA levels were associated with IDDM specific immunogenetic high-risk markers.

Our survey shows that immunogenetic risk markers in health ICA+ school-children resemble the immunogenetic background of IDDM patients from the same geographic area. We have also confirmed the data from Todd and coworkers (1987), who emphasized a strong association of polymorphic residues of the HLA-DQβ chain with IDDM in Caucasians. Our immunogenetic data suggest that the genetic background of the ICA+ children is not completely congruent with the high-risk markers found in IDDM patients. The design of the study has excluded ICA+ non-Asp homozygous subjects that were already diabetic. In this way, the healthy ICA+ population may be slightly enriched at the initial survey with those ICA+ individuals who will not become diabetic.

In the Barts-Windsor family study, the cumulative risk for IDDM in the non-diabetic relatives was associated with haplotype sharing of diabetes associated haplotypes, i.e. HLA-DRB1*03 and HLA-DRB1*04 positivity (Tarn *et al.*, 1988). Our study is the first to analyze the association of polymorphisms at position 57 of the HLA-DQβ chain and ICA positivity. In our population survey, high levels of ICA, i.e. greater than or equal to 17 and/or 30 JDF units, respectively, were found more often in non-Asp positives. Individuals with an Asp+ HLA haplotype had lower levels of ICA. At the population level we were also able to demonstrate that retesting for ICA after several months was required to demonstrate ICA persistency which was closely correlated with the immunogenetic IDDM marker non-Asp at codon 57 of the DQβ chain.

We show here for the first time that the molecular basis for the presence of cytoplasmic islet cell antibodies in a random population may be HLA haplotypes with non-charged amino acids at position 57 of the HLA-DQβ chain. The observed HLA risk factors, which were also prevalent in the local group of IDDM patients, were not only associated with ICA positivity but also with the persistence of circulating islet cell antibodies.

The genetic control of the antibody response was shown to depend on so-called immune response genes (Ir-genes); (Levine *et al.*, 1963; McDevitt *et al.*, 1965). A complex of Ir-genes and antigen directs the T-cell reaction (Wraith *et al.*, 1989). In organ specific autoimmune diseases, autoantibody production is suggested to be dependent on specific T-cell help and more directly on the antigen binding of immunogenic peptides by the HLA molecules (Buus *et al.*, 1987; Zwollo *et al.*, 1991). The herein described HLA risk markers may be associated with factors that provide help for the generation of autoantibodies or may be associated with the lack of β-cell specific suppressor phenomena. We favour the hypothesis that the observed HLA risk factors may be involved in the regulation of the immune response against islet cell antigens. T-cell cloning, the further characterization of the, to date, poorly characterized islet cell antigens, will help to clarify this question (Pankewycz *et al.*, 1991).

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