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High frequencies of T-cell receptor (TCR) Vβ7+ T-cells are detected among lymphocytes isolated from pancreatic islets of children at the onset of Type 1 diabetes. Peripheral blood mononuclear cells from recently-diagnosed diabetic patients and from patients with diabetes from 2 to 14 years of duration were analyzed to assess whether a preferential expression could also be detected among them. TCR Vβ7 gene expression in patients who converted to diabetes while under our routine clinical monitoring, showed values (> 10%) consistently higher than those measured in age-matched non-diabetic controls (5.1%±1.6) ($p < 0.0005$). Also, among the converters, multiple acute enterovirus infections were detected. These infections appeared to be temporally related with increase in levels of Vβ7 gene transcripts. These data demonstrate that a deviation in the TCR Vβ repertoire is present in circulating T-cells from diabetic patients during disease progression and re-emphasize the importance of Coxsackievirus B infections in the etiology of Type 1 diabetes. If we will be able to recognize the segment of the protein that confers to CVB its Vβ7 gene triggering properties, we will be in the position of generating a vaccine that blocks the diabetogenic process mediated by the virus.

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Appendices (Reprints of published manuscripts):

- **Endocrinology and Metabolism Clinics of North America** 28:841, 1999.
- **Diabetes, Nutrition and Metabolism** 12:3, 1999.
- **Journal of Biological Regulators Homeostatic Agents** 13:14, 1999.
- **Hormone Research** 52:1, 2000.
- **Diabetologia** 43:1484, 2000.
- **Journal of Autoimmunity** 16:3, 2001.
- Book chapter: **Diabetes Mellitus: A Fundamental and Clinical Text**

INTRODUCTION

Type 1 diabetes or Insulin-dependent diabetes mellitus (IDDM) is an autoimmune disease characterized by infiltrating lymphocytes into the islets of Langerhans and destruction of the insulin-producing β-cells (1). Despite many years of intensive studies, the precise cause and mechanism(s) triggering the β-cell-specific attack still remain undetermined. However, twin studies establish that a complex interplay occurs between both genetic and non-genetic factors (i.e., environmental) (2,3). In particular, epidemiological evidence supports a role for infectious agents in Type 1 diabetes development and/or progression (4-10).

Although the possibility of a virus-mediated etiopathogenesis of Type 1 diabetes still remains a central point of discussion, certain viral infections, especially caused by enteroviruses (EV), have long been considered associated with β-cell damage and with the clinical onset of diabetes (4,5,10,11), particularly amongst very young children (12-16). However, the relationship between detection of high titre of antibodies and increased prevalence to Coxsackievirus B (CVB) in recently-diagnosed diabetic patients compared to healthy subjects is inconsistent (6,10,11,15-17). This is especially true amongst certain subgroups, as demonstrated in our population in Pittsburgh (18-20). Recently, it has been shown that children carrying HLA-DQB1 alleles associated with increased Type 1 diabetes risk (i.e., HLA-DQB1* 02 and 03) and with autoimmune markers of subclinical β-cell damage (i.e., autoantibodies) developed T-cell responses against both EV antigens and EV-infected cells (14,21). Moreover, in one report, appearance of islet cell antibody (ICA) was temporally related with occurrence of EV infections (22,23). To date, the direct association of EV infections and Type 1 diabetes has been documented in humans in only a few unusual cases (12,13,24,25). However, in both mice and primates infection with CVB4 has been shown to induce diabetes (26,27).

We have recently shown that lysates of CVB-infected cells are able to specifically stimulate *in vitro* T-cells from healthy donors carrying TCR Vβ3, 7 and 13.1 gene families (28,29). The same TCR gene transcripts were found to be expanded among the T-cells infiltrating the heart of patients suffering from CVB3-associated dilated cardiomyopathy (28). Similarly, the majority of T-cells (~30%) infiltrating the islets of Langherans of diabetic patients who died at the onset of disease, demonstrated a preferential expression of the Vβ7 region of the TCR (30). Immunohistochemical studies performed on both the diseased hearts and pancreata revealed a reactivity against CVB (29), suggesting that this virus might have been responsible for the immune stimulation observed in both diseases (28,30).

Restricted TCR Vβ gene usage and enterovirus infection in Type 1 diabetes

It is generally believed that the destruction of the insulin-producing-β-cells occurs over a prolonged period of time, eventually culminating in the delayed appearance of the disease. The analysis of the TCR repertoire of the T-cells infiltrating the pancreatic islets of Langherans is of importance in this respect since it might represent a direct way to examine T-cells involved in the pathogenesis of Type 1 diabetes. Unfortunately, in humans it is not possible to actually dissect the kinetics of β-cell destruction because specimens from diseased pancreata at different time-points can not be easily obtained.

In this pilot study, we assessed the possibility of studying the TCR Vβ repertoire in the T-cells from peripheral blood of recently-diagnosed diabetic patients. In addition, we evaluated TCR Vβ usage in a longitudinal prospective study of autoimmunity in a group of high-risk first-degree relatives some of whom eventually developed Type 1 diabetes (i.e., converters to diabetes). TCR Vβ usage was also analyzed in an additional group of patients who were suffering from diabetes from 2 to 10 years (i.e., long-standing diabetic patients). Our aim was to determine whether there was a preferential expression of certain TCR Vβ gene families in peripheral blood of these patients at different time-points before, at and after the onset of the disease. Moreover, we assessed if there was any evidence of parallel acute EV infections by testing the sera of these subjects for the presence of EV-RNA and of IgA, IgG and IgM class enterovirus antibodies.

TCR repertoire analysis

The estimated percentage of T-cells bearing different Vβs, extrapolated as a function of the relative abundance of the different TCR gene families, in the peripheral blood of recently-diagnosed Type 1 diabetic patients (31,32) showed a higher abundance of the Vβ7 gene family (mean value 10.5% ± 3.7) compared to the expression pattern observed in the PBMC of age-matched non-diabetic control individuals (5.1% ± 1.6) ($p<0.001$). Fifteen (65%) of the recently-diagnosed diabetic patients had values of Vβ7 above the upper limit of the appropriate control population (8.3%) and this prevalence of skewed Vβ7 was statistically higher ($p<0.001$) compared to the controls. Among the age-matched control population, Vβ4 was the most represented family.

High frequency of circulating Vβ7$^+$ T-cells in recently-diagnosed diabetic patients was also confirmed by FACS analysis. Using a monoclonal antibody anti-human TCR, which only recognizes the Vβ7.1 subfamily, the frequency of pan-TCRαβ$^+$ T-cells carrying the Vβ7.1 chain was always higher (2.9-3.6% of TCRαβ$^+$ T-cells) than the frequency for this Vβ chain detected in normal individuals (1.4-2.3% of TCRαβ$^+$ cells). The difference in absolute values of the detected Vβ7 percentages between the molecular (RT-PCR) and the FACS method could be due to several factors, such as detection of mRNA gene transcripts with one technique and expression of surface protein with the other, specificity of the anti-Vβ antibody limited to the 7.1 subfamily and different denominators each method uses for percentage calculations.

In a time-course analysis of the four converters (33), assessment of TCR Vβ gene expression was performed in blood samples collected from forty months before to seven years after the onset of the disease. The analysis indicated a Vβ7 value consistently above 10% at all time points and in all patients. The specificity of this finding for Type 1 diabetic patients was evident when compared with the range Vβ7 value in PBMC from age-matched control individuals that was instead 5.1%±1.6 (highest 8.3%). All the converters at each time point had values of Vβ7 consistently above the upper limit of the normal control population and this prevalence of skewed Vβ7 was significantly higher ($p<0.0005$) with respect to the controls. The only exception was a sample collected seven years after the onset from converter #3 that showed a value of Vβ7 of 6.3%. The enrichment in Vβ7$^+$ T-cells did not seem to be affected by the anti-CD3 and IL-2 stimulation of the frozen PBMC since TCR analysis performed on freshly processed PBMC from the same patients, showed that levels of Vβ gene expression detected in these two samples

correlate very well.

To rule out the possibility that the skewing in the patients' Vβ repertoire was related only to HLA alleles, we compared the TCR Vβ repertoire pattern of three converters with that of their appropriate HLA-DR and -DQ-matched control subjects (34,35). HLA-matched subjects did not reveal any expansion of the TCR Vβ7 gene family. Moreover, amongst the control population, five individuals carried HLA-haplotypes conferring high Type 1 diabetes risk but did not show increased levels of expression of Vβ7 gene transcripts.

Among the converters, levels of another family, Vβ13.1, also tended to be higher than the mean value calculated amongst the control population. All four patients showed decreased levels of this gene family after the onset of the disease.

Three of the high-risk individuals (all adults) showed values of Vβ7 higher than those detected among the age-matched control population at all time points. In these individuals, Vβ7 values were above the upper limit of the appropriate control population (10.5%) and this prevalence of skewed Vβ7 was significantly higher compared to the controls ($p<0.05$). The fourth individual showed a decreased value of Vβ7 in a blood specimen drawn 8 years after the first sample (from 15.5% to 6.3%). Interestingly, this patient, who was GAD autoantibody positive and ICA-512 autoantibody negative on both occasions, showed decreased ICA values at the second time-point (from 40 to 10 JDF units) which was in contrast to the stability of these titers in the other three individuals. Levels of Vβ13.1 gene family measured in these high-risk individuals tended to be higher than those measured in the control population.

TCR Vβ repertoire was also analyzed in PBMC from patients who were suffering from diabetes from 2 to 14 years. In this analysis, long-standing diabetic patients showed mean levels of Vβ7 ($9.7\% \pm 2.4$) that were statistically different from those detected in the control population ($5.1\% \pm 1.6$) ($p<0.005$). In 79% of these patients (11/14), levels of TCR Vβ7 were above the upper limit of the controls (8.3%) and this prevalence of skewed Vβ7 was significantly higher compared to the control population ($p<0.005$). For seven of these patients, blood samples were also available at the onset of diabetes showing mean levels of Vβ7 of $12.3\% \pm 3.1$ ($p<0.005$ compared to the controls).

EV antibodies and presence of EV-RNA in the sera of the studied individuals

Serological analysis for the presence of IgA and IgG class antibodies against a CVB4-antigen and a synthetic EV-peptide antigen in the recently-diagnosed Type 1 diabetic patients did not show statistical differences in term of both frequency of individuals that were seropositive and in the levels of IgG and IgA class antibodies as compared with the appropriate control population. Acute EV infections, with presence of IgM and/or EV-RNA in the serum, were detected with the same frequency in both recently-diagnosed diabetic patients and controls. This result was actually expected since the majority of kindergarten and school children are equally exposed to such a ubiquitous virus.

Serological analysis (36) performed in the time-course study of the four converters, showed, however, the presence of multiple acute EV-infections, defined by at least two-fold increase in IgA and/or IgG class antibodies to CVB4 and/or by IgM positivity or presence of viremia. In the first converter, three acute infections have been observed approximately at 14, 7

and 2 months before the onset of clinical diabetes, with presence of EV-RNA in the serum in the first infection. The second converter, had one acute infection approximately 5 months before overt diabetes indicated by both IgG and IgM responses. Evidence of an acute infection was also observed in converter #3, though the increase in antibody levels did not quite reach the two-fold increase, as defined in the criteria for considering presence of acute EV-infections in the follow-up study. It is worth noting that, in converter #1, for example, for whom we could determine more time-points before onset, the occurrence of acute infections appears to be temporally related with increase in circulating TCR Vβ7+ T-cells (see Fig. 1). Furthermore, in this converter, detection of EV-RNA coincided with the highest levels of TCR Vβ7 (at -14 before the onset of diabetes). In converter #2 the detection of two acute EV infections (by IgM antibodies) in a period of two years was associated with persistently high expression of Vβ7 almost without oscillation levels. In converter #3 a clear acute EV infection was also associated with Vβ7 increase. Since the specimens tested were collected at 3-6 months intervals and sometimes even longer interval, it was not possible to demonstrate unequivocally how close the TCR repertoire and CVB infection coincides were. Converter #4 did not show evidence of acute infections. However, in this patient the IgG levels remained constantly at very high levels during the entire observation period (37).

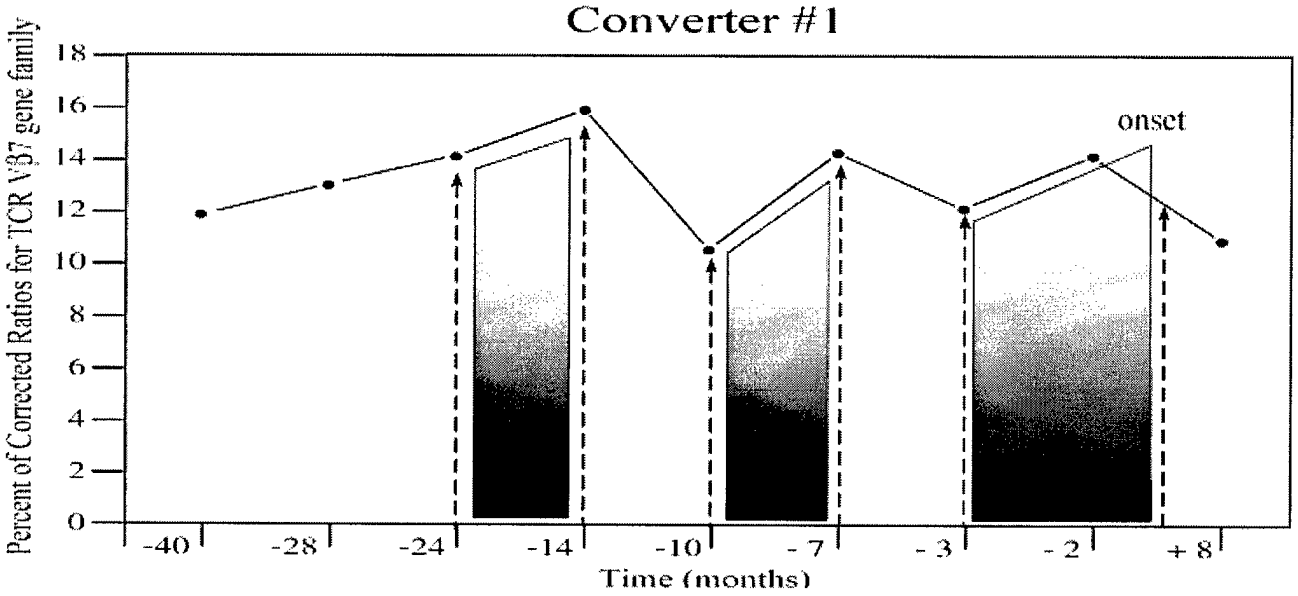


Figure 1. Association of acute enterovirus infections with increase in Vβ7 gene transcript levels in converter #1 for whom a large number of samples prior to diabetes onset was available. Blood specimens collected sequentially during the prediabetic phase were tested for TCR Vβ gene usage and the corresponding sera for the presence of antibodies against several different EV serotypes and EV-RNA. On the Y axis is indicated the percent of TCR Vβ7 gene family measured in PBMC of converter #1 at different time-points during disease progression. On the X axis, the time-points (in months) when collection of blood was actually performed, prior to (-) and consequent to appearance of overt diabetes, are represented. Arrows represent evidence of acute infections and shaded traits demonstrate increase of Vβ7 gene expression occurring at the time of infection (37).

Towards developing an anti-CVB vaccine

In order to develop a vaccine that will be effective against disease producing strains of Coxsackie virus, we have been developing tools that will allow us to localize specific regions of the Coxsackie genome related to the disease process. Having found that the T-cell repertoire of patients with recent onset diabetes (30) or myocarditis (28) is skewed and exhibits characteristics of a superantigen response (29), we have been preparing modified T-cells that will serve as sensitive reporters of this superantigen-like activity.

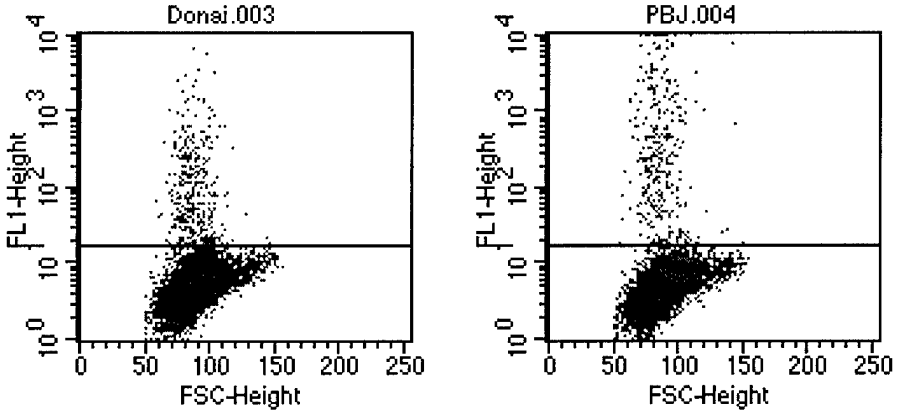
To develop the superantigen-reporter T-cells, we had previously modified Jurkat cells to express β-galactosidase in response to stimulation with PMA and ionomycin or to the SEE superantigen in the presence of B-cells. (The SEE superantigen stimulates Jurkat cells in conjunction with the native Vβ8 containing T-cell receptor of Jurkat cells.) These modified Jurkat cells need to be further modified to also express the T-cell receptors containing Vβ7 or Vβ13.1, which have been associated with the superantigen-like effect that we have observed.

Our early efforts to express Vβ7 T-cell receptors using retrovirus revealed that the LTR driven expression was able to establish neomycin resistance but did not achieve sufficient expression of Vβ7 mRNA relative to the endogenous Vβ8 mRNA. Thus, we began to focus on the promoters involved. Among the stronger promoters, the CMV promoter is susceptible to silencing in T-cells and was not further considered. Instead, initial tests of the SRa promoter, Figure 2, suggested that it had promise, and the SRa promoter was introduced into retroviral vectors as well as vectors for direct transfection of the modified Jurkat cells. The SRa promoter was inserted into the pZeoSV2 vector to create a new vector pZeoSRalpha which was tested by its ability to express eGFP in our LZB#9 Jurkat cells, Figure 3. In parallel, the backbone of the DON-AI retroviral vector was modified to incorporate the SRa promoter after moving the neomycin resistance gene to a position under the control of the LTR to create the retroviral vector pLNSRalpha. While both vectors have produced impressive results when tested using the eGFP sequence, neither has enabled us to express satisfactory amounts of TCR receptors.

As an alternative strategy, we have found that culturing virally transduced cells in the presence of sodium butyrate can enhance the expression of genes whose promoters consist of the viral LTR. We have tested this approach on one of our previous virally transduced cells which only weakly expressed the Vβ7 single chain TCR from the LTR of the MOIN vector. The cells which expressed the highest levels of TCR in the presence of sodium butyrate were sorted by flow cytometry and cloned by limiting dilution. As an example we show the flow cytometric analysis of clone 7.1AM after culture for 3 days with 0.5 mM sodium butyrate, and 2 days after return to normal medium, Figure 4. After longer culture in the absence of sodium butyrate, the TCR expression reverted to the untreated level. Because higher concentrations of sodium butyrate had a deleterious effect on the cells, the growth of the cells in 0.5 mM sodium butyrate was compared to untreated cells. After 3 days the untreated cell number increased about 10 fold while the sodium butyrate treated cells increased by 5 fold. Because more than 40% of the cells at 3 days were expressing easily detectable levels of TCR, it would seem that those cells which are expressing TCR are also dividing albeit with a slightly longer cell cycle time and can be expected to be in good condition. We now grow these cells in 0.5 mM sodium butyrate for 3 days and then culture them in normal medium overnight before an experiment. Several

experiments have confirmed the results of Figure 4.

These sodium butyrate treated cells have also been tested to establish that their activation responses to PMA + ionomycin or to the endogenous Jurkat TCR specific superantigen, SEE are not inhibited, Figure 5. The functionality of the Vβ7 TCR has been tested under similar conditions, using Coxsackie viral lysates in combination with the Raji B-cells, but have not demonstrated significant LacZ activity. However, further testing is underway, using a panel of EBV transformed B-cells which express HLA molecules more similar to those present in the patients we have studied than those of the Raji cells.



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Gate: G1
Gated Events: 20808
Total Events: 25151

Quad	Events	% Gated	Y Mean
UL	0	0.00	***
UR	383	1.84	238.95
LL	0	0.00	***
LR	20425	98.16	4.46

File: PBJ.004
Gate: G1
Gated Events: 20836
Total Events: 24901

Quad	Events	% Gated	Y Mean
UL	0	0.00	***
UR	363	1.74	1144.41
LL	0	0.00	***
LR	20473	98.26	4.30

Figure 2. Expression of eGFP driven by the SRa promoter of pBJ1-Neo.Jurkat LZB#9 cells were transfected, using SuperFect Reagent (Qiagen), with DON-AI and pBJ1-Neo vectors which contained the coding sequence of eGFP. At 48 hours after transfection, the cells were analyzed for green fluorescence by flow cytometry. The DON-AI vector utilizes the CMV promoter which in many cells is a strong promoter but may not be ideal for T-cells. The mean fluorescent intensity of the cells with the SRa promoter is more than four times that of the cells with the CMV promoter.

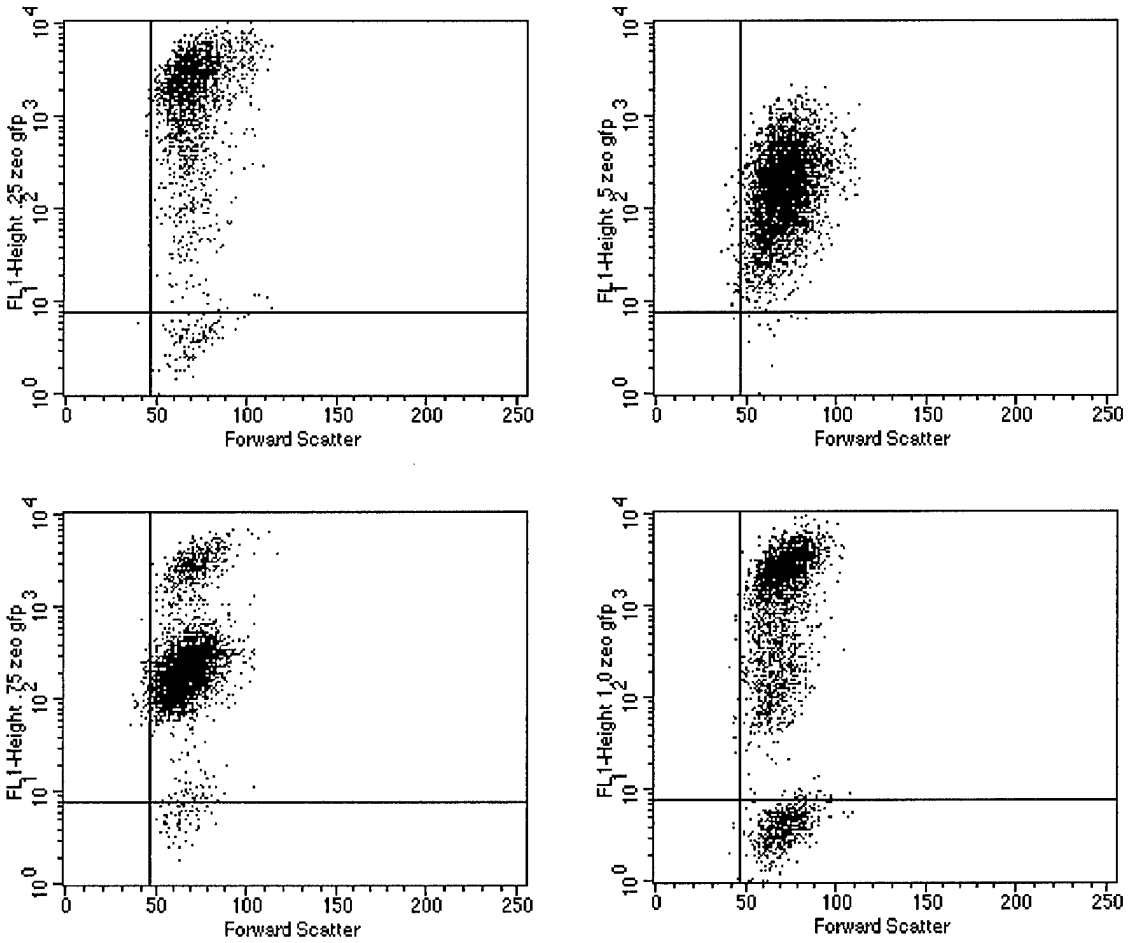

Figure 3. Transfection and selection of Jurkat LZB#9 T-cells with the pZeoR vector encoding eGFP. After transfection using the Superfect reagent (QIAGEN) aliquots of the cells were grown for two weeks in media containing 0.25, 0.50, 0.75 or 1.0 mg/ml Zeocin as indicated on the ordinate of each graph. Dead cells were removed by ficoll gradient centrifugation. The surviving cells were expanded and analyzed by flow cytometry for green fluorescence. All of the Zeocin concentrations used were effective in selecting cells expressing eGFP. Three of the four Zeocin-selected populations contained subpopulations expressing very high cellular concentrations of eGFP.

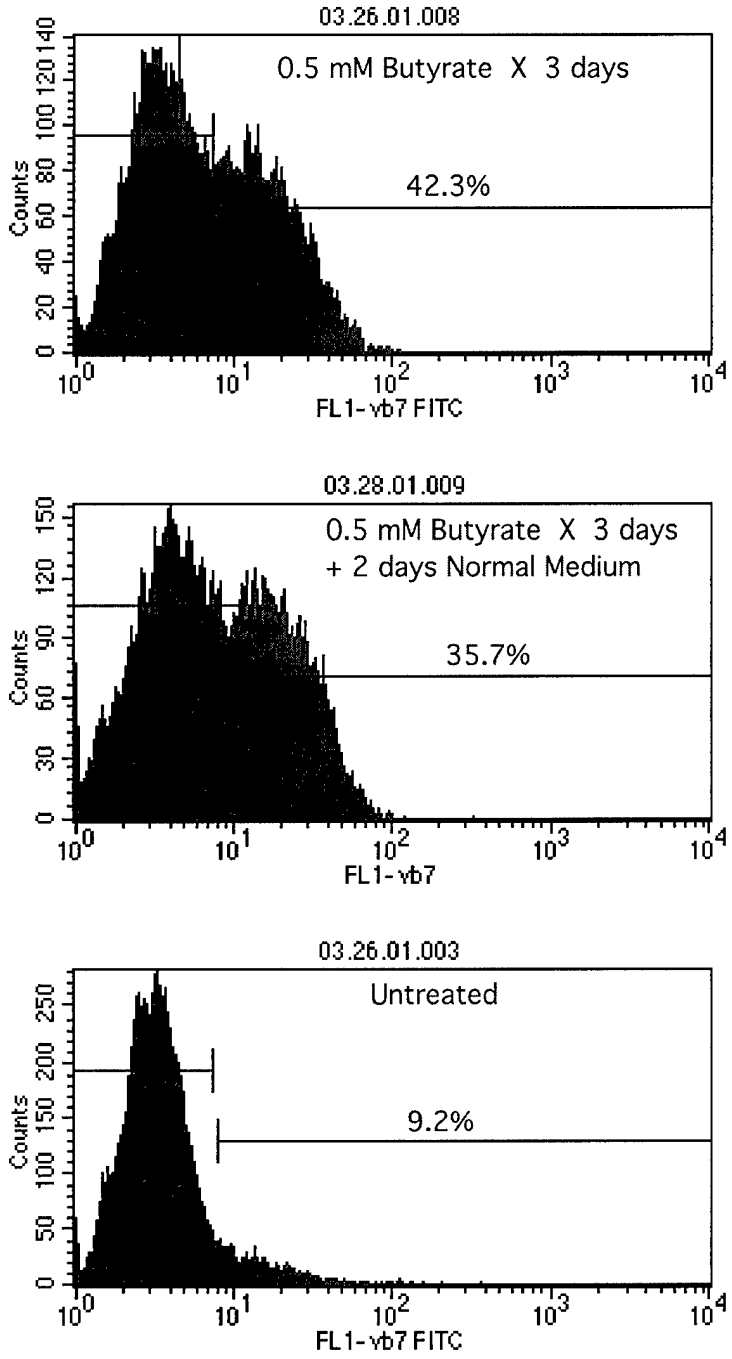

Figure 4. Enhanced expression of Vβ7 TCR following treatment with sodium butyrate. Clone 7.1AM contains a Vβ7.1 single chain TCR which is expressed under the control of the LTR of the MOIN retroviral vector. The cells were grown for 3 days with or without 0.5mM sodium butyrate followed by an additional 2 days in normal medium without sodium butyrate. The cells were stained with FITC labelled anti-Vβ7 antibody, ZOE (Becton Dickinson) and analyzed by flow cytometry. The bars and percentages indicate a conservative estimate of the fraction of cells which are positive relative to the untreated cells .

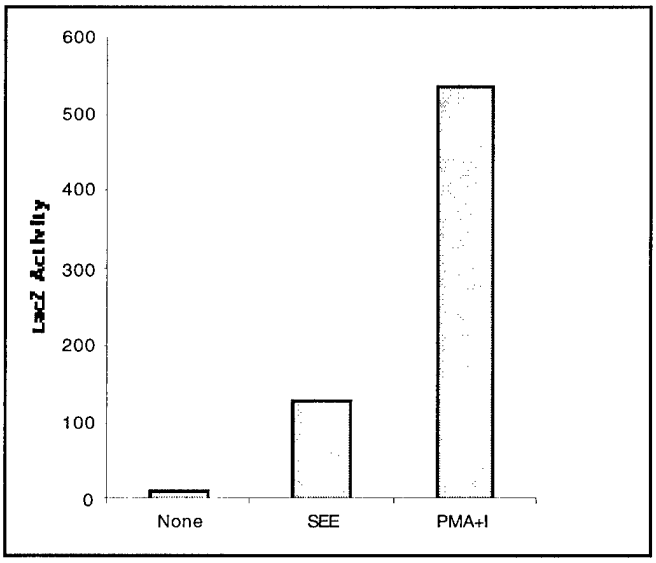

Figure 5. Stimulatory response of sodium butyrate treated cells. Cells from the 7.1AM clone were either co-cultured with Raji B-cells and 20 ng/ml SEE or treated with PMA and ionomycin for 6 hrs. The cells were then collected by centrifugation, lysed, and assayed for β-galactosidase activity using the FluroReporter LacZ Quantitation Kit (Molecular Probes).

KEY RESEARCH ACCOMPLISHMENTS

- Activated T-cells are present on the islets of the pancreas at the moment of the clinical onset of diabetes.
- The repertoire of these T-cells is skewed, showing a predominant percentage of cells carrying the TCR Vβ7 and/or Vβ13.1 chains.
- Coxsackievirus B (CVB) infections can be documented at various times in the clinical history of diabetic patients.
- Lysates from HeLa or Vero cells infected with CVB3 or CVB4 can activate peripheral blood mononuclear cells (PBMC) from non-diabetic individuals *in vitro*.
- The activated T-cells show the same TCR skewing found among the T-cells present in the islet of the pancreas at diabetes onset.
- PBMC collection from patients at the time of the disease onset show a skewing of the same Vβ chain expression as the one found in the islets or among PBMC *in vitro*.
- Blood samples available before the time of the clinical onset in patients who converted to diabetes while under our close monitoring because they were siblings of diabetic probands, showed that this TCR skewing has ups and downs.
- The picks of TCR-specific skewing are time-related with the demonstrated acute infections caused by CVB exposure.
- An *in vitro* system (based on the generation of cell lines presenting at their surface the TCR Vβ7 or the TCR Vβ13.1) to test the triggering properties of different CVB proteins and protein segments has been set up.
- The exhaustive analysis of all CVB proteins will narrow down our search to the one segment responsible for $V\beta7^+$ or $V\beta13.1^+$ T-cell activation. This protein segment could be used for vaccination of genetically-predisposed children.

REPORTABLE OUTCOMES

Peer-Reviewed Articles

- Bertera S, Alexander A, Giannoukakis N, Robbins PD, **Trucco M**: Immunology of Type 1 diabetes: intervention/prevention strategies. **Endocrinology and Metabolism Clinics of North America** 28:841, 1999.
- Friday RP, **Trucco M**, Pietropaolo M: Genetics of type I diabetes mellitus. **Diabetes, Nutrition and Metabolism** 12:3, 1999.
- Luppi P, Alexander A, Bertera S, Noonan K, **Trucco M:** The same HLA-DQ alleles determine either susceptibility or resistance to different Coxsackievirus-mediated autoimmune diseases. **J Biol Regul Homeost Agents** 13:14, 1999.
- Luppi P, **Trucco M**: Immunological models of type 1 diabetes. **Hormone Research** 52:1, 2000.
- Luppi P, Zanone MM, Hyoty H, Rudert WA, Haluszczak C, Alexander AM, Bertera S, Becker D, **Trucco M**: Restricted TCR Vβ gene usage and enterovirus infection in type 1 diabetes: a pilot study. **Diabetologia** 43:1484, 2000.
- Luppi P, Licata A, Haluszczak C, Rudert WA, Trucco G, McGowan FX, Finegold D, Boyle GJ, **Trucco M**: Analysis of TCR Vβ repertoire and cytokine gene expression in patients with idiopathic dilated cardiomyopathy. **J Autoimmunity** 16:3, 2001.

Book Chapter

- Pietropaolo M, **Trucco M**: Major histocompatibility locus and other genes that determine risk of development of insulin-dependent diabetes mellitus. In: LeRoith D, Taylor S, Olefsky JM (Editors), **Diabetes Mellitus: A Fundamental and Clinical Text**, 2nd. Edition, J.B. Lippincott Co., Philadelphia, PA, pp. 399-410, 2000.

CONCLUDING REMARKS

Numerous studies have pointed to the role of infectious agents in the pathogenesis of Type 1 diabetes inducing, accelerating, or enhancing newly formed or already present autoimmune responses. One of the hypotheses is that microbial infections might break "self"-tolerance and trigger activation of autoreactive T-cells (38); activated T-cells, may in turn, destroy the target cells harboring the self-antigen, causing organ damage and loss of function. Thus, the analysis of the T-cell repertoire involved in an organ-specific autoimmune process is very important for the understanding of the pathogenesis of the disease. In a previous report, the analysis of TCR usage in the T-cell population present in the islets of Langherans of two children at the onset of the disease, demonstrated that rearranged Vβ7 mRNA was expressed by the majority of the infiltrating lymphocytes (30).

In the present investigation, we demonstrate the preferential usage of TCR Vβ7 gene family in the peripheral blood of recently-diagnosed Type 1 diabetic patients by both RT-PCR and analysis of protein surface expression by FACS. Such quantitative bias in the Vβ repertoire was also detected years before the clinical onset of the disease in four individuals who had been followed prospectively from months to years prior to the development of Type 1 diabetes. In these patients, another family, Vβ13.1, tended to be higher than in the control population and decreased more promptly than Vβ7 after the onset of the disease. A blood sample available seven years after the onset, showed level of Vβ7 dramatically decreased as well. In long-standing Type 1 diabetic patients, values of Vβ7 were slightly lower as compared to those at the onset of the disease but were still significantly higher than in normal subjects.

These observations appear to be in contrast to previous reports where TCR Vβ repertoire was found to be heterogeneous and there was no consistent patterns of Vβ expression that differentiated diabetic patients from unaffected subjects (39,40). The reason for this is probably due to many factors, including number of individuals analyzed, patient heterogeneity and technical differences. Larger studies are obviously needed to assess whether a bias in the TCR Vβ usage truly characterizes pre-diabetic and diabetic patients.

The skewing of the TCR Vβ repertoire does not seem to be related to the presence of certain HLA alleles, since five of the controls carrying HLA-haplotypes conferring high risk to diabetes, did not show the same TCR repertoire. Moreover, the analysis performed on healthy subjects HLA-DR and –DQ-matched to diabetic patients did not reveal expansion of either Vβ7 or 13.1 gene families.

We recently reported the presence of a T-cell repertoire bias, with overexpression of Vβ7 and Vβ13.1 gene families, in the T-cells infiltrating the myocardium of patients suffering from acute dilated cardiomyopathy, an autoimmune disease generally considered a sequela of a viral myocarditis (28). *In vitro,* exposure of PBMC from healthy donors to lysates of Vero- and HeLa-cells infected with CVB, was capable of specifically activating Vβ7$^+$ and Vβ13.1$^+$ T-cells (28,29). More recently, another group reported on the variable degrees of expansion of Vβ7$^+$ and Vβ13.1$^+$ T-cells among proliferating and recently activated (CD69$^+$) T-cells cultured with CVB-infected cell lysates (41). These data, together with the results generated in the present study, appear to support a role of an environmental factor (i.e., exposure to CVB) for the TCR Vβ bias observed in diabetic patients. Alternatively, the preferential usage of certain Vβ gene families

might be explained by the presence of a T-cell response to an islet-specific (auto)antigen, possibly in cross-reaction with a response to an exogenous (viral) antigen. This study also demonstrates that selective utilization of TCR genes in the periphery may reflect an on-going disease within an affected tissue.

A report of an activated endogenous retrovirus which was claimed to be implicated in the pathogenesis of human Type 1 diabetes (42) was recently disputed (43-45). Although the preferential usage of certain Vβ gene families might be explained by the presence of a T-cell response to an islet-specific (auto)antigen, CVB infections have been linked to Type 1 diabetes since the detection of a CVB4 strain in the pancreas of a diabetic child (24). So far, numerous reports have associated several different EV with pre-diabetic autoimmune episodes and onset of overt diabetes (4,5,10,11-16, 23), although the pathway by which EV may induce diabetes is not well understood.

The serologic analysis performed in our recently-diagnosed Type 1 diabetic patients, showed that the frequency of individuals who were positive for EV-antibodies and RNA was not statistically different than in the control populations. These data were somehow expected because EV are agents of the common cold causing infections in kindergarten and schoolchildren, as also observed in our population in Pittsburgh (18-20). However, in the longitudinal study of the four converters, where serum samples were available for testing every 3-6 months, multiple, sequential acute infections and viremia were detected. In particular, acute infections were observed in samples collected months prior to the clinical onset of diabetes. Other evidence of sequential acute EV infections prior to presentation with Type 1 diabetes has been recently described (4, 46). These data further emphasize the concept that longitudinal prospective studies on sera from diabetic patients taken at short intervals are the method of choice when looking for evidence of acute EV infections.

Although more Type 1 diabetic patients need to be analyzed in prospective studies, it is tempting to speculate that a history of previous environmental insults could have caused a distortion or a deviation of the TCR repertoire, with over-expression of cross-reactive circulating T-cells bearing certain Vβ gene families, an hypothesis recently described for CVB4-induced Type 1 diabetes in mice (47). This could be the reason why we detected a restricted TCR repertoire, with increase in Vβ7 gene transcript levels, in the majority of the recently-diagnosed diabetic patients and in pre-diabetic subjects followed prospectively. Indeed, distortion of the TCR Vβ repertoire, with massive infiltration of T-cells carrying Vβ7 and 13.1 chain transcripts, have been detected in hearts from cases of acute dilated cardiomyopathy patients associated with CVB3 infection (28). Thus, predominance of $V\beta7^+$ T-cells in the peripheral blood from diabetic patients could represent an indirect marker for a previous or present exposure(s) to CVB. The observation of a concomitant increase in the level of this TCR gene transcript at the moment of an acute EV-infection is also supportive of this hypothesis. Since we did not observe high expression of TCR $V\beta7^+$ T-cells in normal individuals who had evidence of EV-antibodies, we can envisage a scenario which in genetically susceptible individuals the persistence of a CVB infection (or multiple exposure to CVB with a diabetogenic potential) may trigger β-cell autoimmunity, with stimulation of resting autoreactive T-cells expressing certain Vβ gene families (i.e., Vβ7 and 13.1) (28,41,47).

Higher levels of TCR Vβ7 and the presence of anti-CVB antibodies in individuals at high

risk for Type 1 diabetes, but so far still without the overt disease, indicate that these individuals were exposed to the same viral infection. However, they were able to circumvent or delay the development of diabetes. In this light, a slowly progressing β-cell destruction can be hypothesized. Indeed, latent autoimmune diabetes developing in adult subjects has been recently recognized as a clearly distinguishable syndrome (48). On the basis of the results obtained on at least one of the high risk individuals in whom the level of Vβ7 gene family decreased seven years after the first assessment, the autoimmune process may have instead subsided. On the other hand, monocyte and T-cell activation, with overproduction of cytokines, is also found in healthy first degree relatives of patients with Type 1 diabetes, suggesting that these individuals have means to counter the potential diabetogenic effects of these immunological abnormalities (49). Although neither the phenotype of the T-cells carrying Vβ7 positive TCRs, nor the cytokine patterns have yet been analyzed in the studied individuals, it is tempting to speculate that in some cases, but not in others, the lymphocyte population may have undergone switching from a predominantly T helper (Th)-1 to a predominantly Th-2 profile (50).

In conclusion, our findings indicate that in peripheral blood from Type 1 diabetic patients there is an expansion of T-cells bearing preferential TCR Vβ gene families. This T-cell expansion is not HLA-related, is evident at the time of clinical onset and up to 40 months prior to the overt diabetes and persists for years. Enteroviral analysis showed the presence of acute infections which were temporally related to increases in preferential Vβ gene families, thus supporting the concept that EV may be etiologically important in the development of Type 1 diabetes.

If we will be able to recognize the segment of the protein that confers to CVB its superantigenic properties we will be in the position of generating a vaccine that will not preserve the children from CVB infections, but will block the triggering of diabetes mediated by the same virus.

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Massimo Trucco, M.D.
Principal Investigator

APPENDICES to
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IMMUNOLOGY OF TYPE 1 DIABETES

Intervention and Prevention Strategies

Suzanne Bertera, BS, Angela Alexander, MS, Nick Giannoukakis, PhD, Paul D. Robbins, PhD, and Massimo Trucco, MD

Insulin-dependent diabetes mellitus (IDDM), or type 1 diabetes, is the outcome of a progressive and selective destruction of the insulin-producing beta cells in the islets of Langerhans in the pancreas. Although evidence of T-cell involvement in the pathogenesis of the disease is incontrovertibly offered by histologic analyses of the pancreata from children at the onset of the disease and confirmed in animal models of the disease such as the nonobese diabetic (NOD) mouse or the BioBreeding rat, the mechanisms that allow or trigger this immunologic invasion of the islets of the pancreas still elude complete understanding. The genetic component of the disease is well-documented, and the linkage with certain alleles at different HLA class II loci has been proven beyond statistical doubt.[20] In some large family studies, other loci were found to be weakly associated with the disease, but these results were not confirmed by additional studies on other family groups.[16, 20] When strict statistical parameters were used to assess the significance of the likelihood ratio between markers and diabetes, even the 5′ region of the insulin gene, the *IDDM2* genetic region, was proven to be "associated" but very weakly "linked" with the disease.[16] Even in the HLA gene complex (i.e., the *IDDM1* genetic region), what is actually genetically transmitted is not the disease itself, but the propensity or the susceptibility to the disease.[78] Despite the finding that the majority of diabetic individuals share the HLA-DQA1*0301, DQB1*0302 or DQA1*0501, DQB1*0201 haplotypes, whereas siblings in the same families seem to be protected from the disease by carrying the DQA1*0102, DQB1*0602 haplotype, in the general population, many individuals with susceptibility haplotypes do not present with any sign of the

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disease. The conclusion of geneticists is that the susceptibility attributable to the genetic component of the disease, even when considering genetic segments with weak statistical associations, constitutes less than 50%.[20] This conclusion is strongly supported by the fact that concordance for the disease in monozygotic twins does not reach 50%.[10] The involvement of a nongenetic component of the disease must be considered. This component can only be offered by the environment.

Many different environmental triggers have been considered during the last 20 years of disease-related studies. They vary from proteins present in cow's milk, frequently used to complement breast-feeding,[40] to viral agents to which children can be frequently exposed before or after their immune system has completely developed.[64]

The genetic HLA linkage and the immunopathologic T-cell presence in the damaged islets, the documented presence of antibodies directed against normal structures of the islet cells in the serum of diabetic patients,[63] the efficient although temporary effect of immunosuppression in delaying the onset of the disease,[7,9] and the evidence that IDDM can be transferred from a diabetic donor to a nondiabetic recipient via bone marrow transplantation[45] strongly indicate the potential autoimmune nature of the pathologic mechanism causing the disease.[8]

Autoimmunity defines a breakdown of self-tolerance whereby immunocompetent cells begin to attack tissues or cells of their own body (i.e., *self*). An important component of the immune mechanism that leads to overt diabetes and that needs to be studied further are self-tissue determinants, the so-called *autoantigens.* Another critical component is the triggering of foreign (i.e., *nonself*) antigens provided by the environment.

Understanding the complex interplay existing between the genetic and the immunologic components of the disease in relationship to autoantigens and foreign antigens is the conditio sine qua non for proposing valuable prevention strategies against diabetes and eventually for implementing reliable and safe intervention approaches.

MODELS OF AUTOIMMUNE DISEASE DEVELOPMENT

In an attempt to present schematically the various perspectives proposed to explain the complex relationship existing among all of the factors involved in IDDM etiopathogenesis, the discussion herein is limited to three models that, more than any others, have gained credence in recent years. All three models attempt to explain how autoreactive T and B lymphocytes may become primed against self-antigens after being somehow exposed to nonself or environmental triggers. The first model, originally proposed by Oldstone,[58] is known as the molecular mimicry hypothesis. The second model, originally formulated by Sprent and collaborators,[76] is based on the induction of the so-called "bystander T-cell proliferation." The third model, which involves superantigens as environmental triggers, was proposed by the authors in 1994 based on unexpected results obtained when studying the T cells infiltrating the pancreata of children who died at the onset of the disease.[18] All of the other explanations proposed to date can be considered variants or combinations of these three hypotheses.

Molecular Mimicry-Activated T-Cell Proliferation

The concept of molecular mimicry was first formally defined by Oldstone as the pathogenetic mechanism for the basis of autoimmune diseases. This

concept can work as a "probe" useful in uncovering the causative agents involved in such diseases.[58, 59] Oldstone's reasoning was based on epidemiologic, clinical, and experimental evidence of an association between infectious agents and autoimmune diseases and on the assumption that epitopes of proteins expressed by these infectious agents can be shared by unrelated molecules encoded by host genes. This postulated antigenic similarity between two completely unrelated molecules can have as a direct consequence the cross-reaction of some T cells physiologically activated against a nonself invader, which start to attack pathologically self-tissues carrying antigenic determinants similar to the nonself antigens. This postulated mechanism can be tested more easily using T lymphocytes and may also be the basis for the production of cross-reacting antibodies by B lymphocytes. Cross-reacting antibodies can be among the autoantibodies frequently found in the serum of diabetic patients.[63]

Experiments supportive of this hypothesis have been performed in humans as well as in mice. Autoimmune diseases other than diabetes have been found to be more suitable for these types of investigations. A classic example is the work by Wucherpfennig and Strominger[82] who used the molecular mimicry hypothesis to explain the etiopathogenesis of multiple sclerosis. In multiple sclerosis, in contrast to diabetes, at least one critical autoantigen present in the central nervous system is known—the myelin basic protein (MBP). MBP is the target recognized by specific T-cell clones found in the blood of patients with multiple sclerosis.

As is suggested for other autoimmune diseases, an environmental component is postulated to be necessary for multiple sclerosis to complement the genetic predisposition associated with the HLA class II alleles. Hence, Wucherpfennig and Strominger first set out to determine the immunodominant segment of MBP that could fit in the binding site of the MHC class II molecule most strongly associated with the disease (i.e., the HLA-DR2) and be recognized by T cells with those receptors most frequently represented in patients with multiple sclerosis. This segment was recognized to encompass amino acids 85 to 99 of MBP. Based on the notion that a degenerate amino acid sequence is to a certain extent allowed without impairment of the most important peptide characteristics, this immunodominant segment was subjected to mutational analysis to define which other amino acids were biochemically permitted at each position. Relatively ubiquitous types of pathogens were then considered as the possible triggers involved in the disease. Several viral peptides were selected out of a protein sequence database and then tested for their ability to activate human MBP (amino acids 85 to 99)–specific T-cell clones established in vitro by the expansion of T cells from the blood of patients with multiple sclerosis. Certain segments from the influenza virus hemagglutinin, among others, were actually able to cross-activate the MBP-specific T-cell clones. The ability to perform in this fashion was recognized to be related more to the conformational shape these peptides could assume than to their linear amino acid sequences. In patients with multiple sclerosis, viral peptide-activated T-cell clones might be able to leave the blood stream, cross the blood-brain barrier, and invade the central nervous system. Here they can recognize MBP on antigen-presenting cells, be expanded, and finally promote the formation of areas of cell degeneration called "plaques," the characteristic lesions of multiple sclerosis (Fig. 1).

A more recent study supporting the molecular mimicry hypothesis between a virus peptide and a normal structure of the mouse cornea has been performed by Cantor and co-workers.[85] In that study, a specific mutation of the herpes simplex virus type 1 (HSV-1) was sufficient to impair the autoimmune mechanism known to be the basis of the development of the herpes stromal keratitis

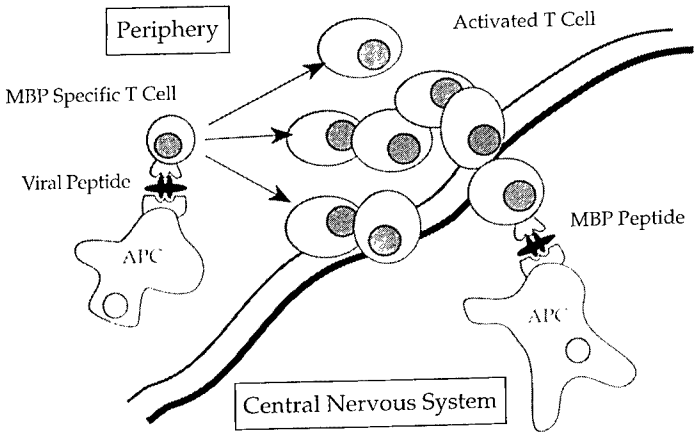

Figure 1. The molecular mimicry hypothesis proposes that in the pathogenesis of multiple sclerosis, T-cell clones with receptors specific for the myelin basic protein (MBP) can recognize in the periphery viral proteins expressing epitopes similar in their tertiary structure to those specific for MBP. Activated T-cell clones can pass the blood-brain barrier, recognize the epitope expressed by the 85-99 AA MBP peptide, and attack structures of the central nervous system. (*Modified from* Wucherpfennig KW, Strominger JL: Molecular mimicry in T-cell mediated autoimmunity: Viral peptides activate human T-cell clones specific for myelin basic protein. Cell 80:695, 1995; with permission.)

of the mouse. A specific peptide from the HSV-1 (KOS) was known to be immunologically relevant in triggering the autoimmune reaction. By artificially inserting a premature stop codon in the KOS wild-type genome at the 5′ of the sequence of the *UL6* gene, which encodes the critical epitope, the mutant virus KOS/UL6^{m} was defective for UL6 expression. Keratogenic T cells from C.AL-20 mice immunized with ultraviolet-irradiated HSV-1 virus adoptively transferred into CB-17 SCID recipients were able to cause a severe type of keratitis after repeated injections of the wild-type virus into the cornea. When the KOS/UL6^{m} was injected instead, the mice did not develop the disease and reacted to KOS/UL6^{m} injections as if they were treated with the completely unrelated type of virus that was used as a negative control. When a second replication-deficient mutant of the KOS virus, KO82, was used for the injections, it behaved as the wild-type virus, promoting keratitis development. KO82 lacks the viral structural protein glycoprotein B but still expresses the *UL6* gene-encoded epitope.

In 1992 Kaufman and collaborators[43] offered perhaps the most relevant suggestion regarding how molecular mimicry may work in determining the autoimmune process that brings about overt IDDM. Their study focused on GAD_{65} as one of the most studied autoantigens against which autoantibodies are frequently detected in the serum of patients with IDDM. GAD_{65} was found to share a six amino acid long segment (PEVKEK) with the coxsackievirus B4 P2-C protein. This segment was then considered the epitope possibly responsible for a molecular mimicry–based immune reaction initiated by a coxsackievirus infection and concluded by GAD-specific T cells attacking endocrine pancreas targets. In support of this hypothesis, it was shown that, in the NOD mouse, immunization with the same peptide induced cross-reactive responses. Directly responsible for these immune responses were MHC-class II–restricted T cells.[74]

The NOD mouse carries the H-2 class II allele that corresponds to the strongest susceptibility HLA-DQ alleles in humans. GAD peptides encompassing the PEVKEK segment, when injected intrathymically, were able to delay the onset of the disease in NOD mice. It was proposed that GAD peptides were able to induce an immunologic tolerance, thus protecting the mouse against the autoimmune attack that generally leads to overt diabetes.[42, 75]

Some technical drawbacks to further research arose. It was difficult, using blood from diabetic patients, to isolate and stimulate T-cell clones able to respond in vitro to both GAD_{65} *and* coxsackievirus B4 P2-C protein.[5] An additional problem was the presence of GAD_{65} not only on the insulin-producing beta cells of the pancreatic islets but also on the alpha and delta cells of the islets and the neuroendocrine cells of the brain.[33]

The lack of recognition of an autoantigen directly associated with the disease onset or of a virus expressly able to cause a diabetes-oriented autoimmune reaction seems to be the main obstacle to the acceptance of the molecular mimicry hypothesis as a convincing explanation of type I diabetes etiopathogenesis. Nevertheless, the evidence of involvement of certain types of viruses and, in particular, of enteroviruses such as the coxsackievirus in the etiopathogenesis of IDDM remains valid, even though the theory of molecular mimicry would not properly explain its specific diabetogenic effect. A different hypothesis that also tries to explain the coxsackievirus involvement in diabetes was recently proposed by the group of Noya Sarvetnick.[38] In their study of the NOD mouse, molecular mimicry evidence after coxsackievirus exposure was compared with evidence supporting a bystander T-cell proliferation effect mediated by the virus.

Bystander T-cell Proliferation Hypothesis

The bystander T-cell proliferation hypothesis[76] is based on the work of two groups of researchers who independently demonstrated that, although viral infections are able to induce vigorous immune responses, the increase in total T-cell numbers is not the result of the expansion of antigen-specific, T-cell clones.[56, 77] In contrast, non-antigen–specific T cells are stimulated by the various cytokines involved in the inflammatory reaction, solely because they are physically present in the area of infection. The bystander (i.e., non-antigen–specific) reaction is not T-cell receptor–dependent and can even be promoted by heterologous viruses as long as they are infectious and able to promote cytokine release. The cytokines most involved in this nonspecific stimulation are the type 1 interferons (IFN) which include IFN-α and IFN-β.[76] Although it is true that antigen-specific T cells are rapidly eliminated from the blood stream, IFN exposure seems to provide protective signals that promote the survival of long-lived resting memory cells.[76]

The bystander T-cell proliferation hypothesis was tested directly against the molecular mimicry hypothesis to explain specifically the immunopathologic *iter* that brings about type 1 diabetes in response to a viral infection.[38] The virus of choice was coxsackievirus B4, for both historical and practical reasons. Coxsackievirus B4 was, in fact, the first virus isolated from patients with IDDM that after being passaged through murine pancreatic beta cells was able to induce diabeteslike disease in mice.[83] Coxsackievirus B4 remains the best example supporting the molecular mimicry hypothesis in IDDM.

Horwitz and collaborators[38] used coxsackievirus B4 to infect B10.H2^{g7} mice carrying the NOD MHC allele, to which presentation of the cross-reactive epitope is restricted, to determine which of the two proposed hypotheses better

explained the mechanism of coxsackievirus B4–induced IDDM. They also used transgenic BDC2.5 mice harboring a diabetogenic T-cell receptor. If the disease mechanism followed the molecular mimicry hypothesis, the B10.H2^{g7} mice carrying the H2 restriction element once exposed to coxsackievirus B4 should have promptly developed autoimmune diabetes. The results showed that, following coxsackievirus B4 infection, the mice generated a strong local immune response but did not develop pancreatic islet autoreactivity. In contrast, coxsackievirus B4 infection in BDC2.5 transgenic mice did generate a rapid diabetic response with all the signs of insulitis and a subsequent destruction of insulin-producing beta cells. It was proposed these results were supportive evidence that IDDM was initiated in these mice by the activation of resting, islet-specific, memory lymphocytes through the bystander mechanism. The immunologic reaction of the BDC2.5 transgenic mice against coxsackievirus B4 was considered an "exaggerated version" of the human preclinical situation in which previously inflicted environmental insults could have caused an expansion of possibly cross-reactive T-cell clones. The viral infection seems to be able to activate indirectly autoreactive T cells that, in turn, can generate initial pancreatic tissue damage. Damaged beta cells release previously ignored self-antigens that can now activate an autoimmune process, rapidly promoting the generation of insulitis and, eventually, overt diabetes.

In their concluding remarks, Horwitz and colleagues offered the explanation that, in humans, enrichment or distortion of the T-cell repertoire, similar to the one artificially created in the T-cell receptor transgenic BCD2.5 mice, could be caused by a history of infections with pathogens expressing superantigens. This superantigen-mediated T-cell activation may render the patient overrepresented for a particular variable segment of the T-cell receptor beta chain. Among the T cells sharing the same T-cell receptor variable region (Vβ), autoreactive T cells may be present that are able to attack specific pancreas structures. This proposed mechanism is based on results published by the authors in 1994,[18] which are perfectly compatible with Sarvetnick's results and conclusions.

Superantigen-Mediated T-Cell Proliferation

Autoreactive T cells can be inappropriately primed to react against self-structures through an encounter with a superantigen. Several pathogenic organisms, including mycoplasma, bacteria, and viruses, can stimulate a vigorous immune response in the infected host through the expression of superantigen proteins.[49] Superantigens seem to have little requirement for processing by antigen-presenting cells, unlike conventional antigens, which are processed and displayed as peptides within the antigen-binding groove of HLA class II molecules. Based on crystallization experiments, superantigens seem to interact as relatively intact molecules with both HLA class II heterodimers and T-cell receptors in an unusual trimolecular configuration that activates many different T cells bearing collectively one (or a few similar) T-cell receptor beta-chain variable region (Vβ)[17, 49] (Fig. 2). Superantigens can activate as much as 30% of the total T-cell pool of an individual depending on the initial frequency of T cells with the relevant Vβ. In contrast, conventional antigens when properly presented can stimulate approximately 1 in 10^5 T cells. This characteristic led to the original definition of "super" antigens.[51] Conversely, T-cell receptor Vα segments seem not to be directly involved in the most common configuration of this activation because the various Vα variable regions are more uniformly represented in the population of superantigen-activated T cells.[49, 51]

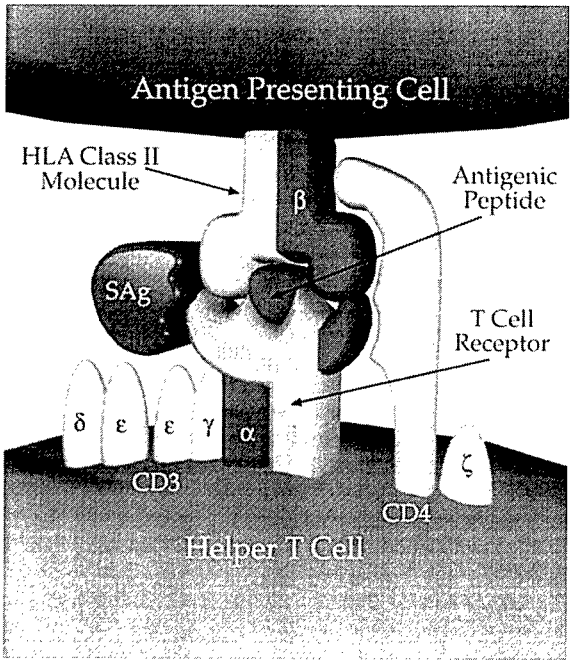

Figure 2. Superantigens (SAg) are proteins, or segments of proteins able to simultaneously bind the major histocompatibility complex ([MHC] in humans, the HLA complex) class II molecule expressed at the surface of an antigen presenting cell, and the T-cell receptor (TCR) molecule on the helper T cell. The contact between the SAg and the variable segment of the β chain of the TCR is sufficient to pass a signal to the CD3 multimolecular family and consequently activate the cell. The HLA molecule-antigenic peptide-TCR complex is normally kept together more firmly by one accessory molecule that, on helper T cells, is CD4. (*Modified from* Conrad B, Trucco M: Superantigens as etiopathogenetic factors in the development of insulin dependent diabetes mellitus. Diab Metab Rev 10:309, 1994; with permission.)

Viral infections could influence development of IDDM through a superantigen effect by recruiting and activating T cells with autoreactive potential. Polyclonal T-cell repertoires with highly restricted T-cell receptor Vβ regions (the hallmark of a superantigen-mediated immune response) have been reported in various autoimmune diseases, including toxic shock syndrome,[15] rheumatoid arthritis,[60] and Kawasaki's disease.[1] Data from the authors' laboratory suggest that the T-cell component of the insulitis present in the pancreas at IDDM onset is characteristic of an immune response against a superantigen, with the reactive cells expressing predominantly Vβ7- and Vβ13.1-positive T-cell receptors.[17, 18] A preferential use of certain Vβ families could be explained by the presence of an oligoclonal T-cell response to a conventional antigen; however, the Vβ skewing together with the presence of a nonskewed Vα repertoire and the polyclonality of the T cells expressing one or the other of the two Vβ families in situ points to an immune response preferentially initiated by a superantigen. In fact, T-cell responses to superantigens are characteristically independent of the variation of the non-Vβ components of the T-cell receptor (Fig. 3).

Because the authors initially did not have blood samples available from

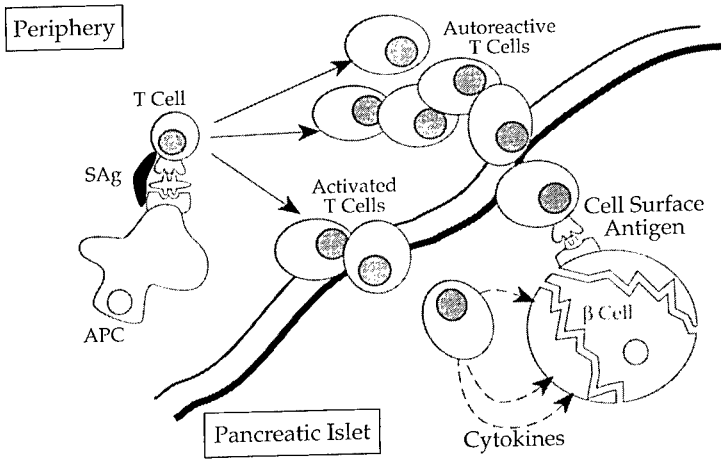

Figure 3. The activation of autoreactive T cells can be triggered by a superantigen (SAg) of viral origin. This immunologic triggering amplifies the damage produced by the virus directly on the pancreas: Autoreactive T cells, sharing the same variable segment of the β chain of their TCR, can directly attack islet-specific targets (e.g., autoantigens), or cause specific β-cell damage, by secreting particular cytokines.

patients who died at the onset of disease, they have only recently been able to demonstrate a skewing of T-cell receptor gene use among peripheral blood mononuclear cells (PBMC) from new onset diabetic patients. High Vβ7 and Vβ13 gene family values were also evident in subjects considered to be at high risk for diabetes who were observed in the authors' diabetes clinic for several months and who subsequently became diabetic.[50] The finding of high levels of Vβ7 and Vβ13.1 gene families present for more than 1 year prior to the onset of IDDM suggests that a viral infection occurred years before the onset of the disease. These results support Sarvetnick's hypothesis[38] and other serologic and epidemiologic data.[36]

All of the superantigen-related diseases described to date have significant HLA associations, perhaps owing to the stronger affinity of certain superantigens for certain HLA alleles.[71] This observation could explain the strong but broad effect of certain HLA-DQ molecules in enhancing or inhibiting T-cell activation and, ultimately, in conferring susceptibility or resistance to IDDM.[52, 78] Moreover, the epidemiology of superantigen-mediated diseases is quite similar to that of IDDM, with marked racial and geographic differences in risk, frequent reports of "outbreaks" with evidence of mini-epidemics, aggregation in families, and strong seasonal patterns. In general, these diseases do not occur in very young children and are frequently associated with moderate viral illnesses that affect the patient months before the onset of the disease.[79]

Understanding the source of the postulated superantigen involved in the development of IDDM became of fundamental importance to support the superantigen-based etiologic hypothesis. Clues came from study of the T-cell infiltrate present in the hearts of three unrelated children who received heart transplants necessitated by the severe consequences of acute viral myocarditis.[48] The T-cell infiltrate was found to be skewed toward use of the same T-cell receptor Vβ gene families as in the diabetic pancreata. Polyclonal Vβ7- and Vβ13.1-positive

T cells were found to be predominant among the cells present in the diseased hearts of these children affected by idiopathic dilated cardiomyopathy, considered a sequela of a coxsackievirus B–induced myocarditis. Furthermore, by challenging PBMC from normal individuals against lysates from Vero cells infected with different strains of coxsackievirus B, it was demonstrated that coxsackievirus B3 provoked in vitro the same T-cell skewing observed in the patients. This finding suggested that coxsackievirus B proteins may exhibit superantigen activity that can lead to the immunopathogenesis characteristic of either idiopathic dilated cardiomyopathy or IDDM.[48] It is possible to explain these findings on the basis of the extremely broad polymorphism involving, in particular, the capsids of different coxsackievirus B. Within each immunologically defined strain of this virus are regions containing large numbers of nucleotide variations frequently resulting in less than 50% homology. The abundance of coxsackievirus B strains that have superantigenic properties has not yet been established. A few strains have been reported to be pancreotropic, whereas several others have been found to be cardiotropic.[84] A pancreotropic strain would be the most likely cause of the initial damage of the pancreas, and the consequent immune response could be enhanced by the superantigenic characteristics of the virus. As a result of the initial beta-cell destruction, a more conventional HLA-restricted, B-cell and T-cell–mediated autoimmune attack may eventually develop against the newly exposed self-antigens. The superantigen-generated abundance of Vβ7-positive cells may increase the probability that some of the self-reactive cells present in situ are sharing the same Vβ. The beta-cell damage expands through an antigen-spreading process[68] that eventually culminates in overt diabetes.

It is not yet known whether the coxsackievirus B encodes a protein segment that is able to act as a superantigen or can instead elicit the expression of a cellular protein, possibly derived from an endogenous retrovirus, that behaves as a superantigen. The hypothesis of an endogenous retrovirus as the source of the superantigen was originally formulated on the basis of the reaction generated by the mouse mammary tumor virus (MMTV).[17] The MMTV is a retrovirus able to encode a superantigen in its long terminal repeat.[14] No convincing evidence of retroviral involvement in human IDDM has been offered.[67]

CD4$^+$ Versus CD8$^+$ Cells in IDDM Etiopathogenesis

T cells able to transfer IDDM in NOD mice have been characterized, suggesting that cloned CD4$^+$ helper T cells alone could transfer the disease in unmanipulated NOD mice.[13] The participation of CD8$^+$ cytotoxic T cells has been proved to accelerate the course of the disease.[81] Transfer of diabetes to NOD-SCID mice occurred with one CD4$^+$ T-cell clone, but a second CD4$^+$ T-cell clone required the cotransfer of CD8$^+$ T cells.[62] Interestingly, both T-cell clones had a cytokine profile consistent with T helper 1 (TH1) cells, used the same Vβ4-positive T-cell receptor gene segment (although they used different Vα region genes), and were directed against undetermined islet cell antigens distinct from insulin, GAD, heat shock proteins, and carboxypeptidase H. These experiments suggest that both helper and cytotoxic T-cell types have a role in diabetes pathogenesis and that TH1 cells may have a different role than TH2 T cells.[41] Early reports attributed a powerful diabetogenic effect to the TH1 cells, which were only limited in their deleterious activity by the insurgency of the TH2 cells. The TH2 cells were then considered the subpopulation of T cells able to limit

the effect of TH1 cells once certain cytokines such as interleukin-4 (IL-4) were secreted in sufficient amounts.

Experiments performed in NOD mice demonstrated that the transgene carrying the gene for IL-4 under the control of the human insulin promoter was able to protect these mice from the development of both insulitis and diabetes.[54] The IL-4 expression, even when limited to the pancreatic beta cells, was also able to induce an efficient functional tolerance that was proven by the inability of these transgenic mice to reject syngeneic pancreatic islets. NOD recipients of IL-4 did not develop diabetes even when they were treated with diabetogenic spleen cells derived from nontreated diabetic NOD donors.

Interleukin-4 has also been shown to have an important protective role against IDDM in humans. In a recent study that still requires confirmation, diabetic siblings were found to have lower frequencies of $CD4^-CD8^-V\alpha24J\alpha Q^+$ T cells when compared with their nondiabetic siblings.[80] $CD4^-CD8^-V\alpha24J\alpha Q^+$ T cells are considered responsible for the early secretory bursts of IL-4 and interferon-γ (IFNγ). In vitro, all of the $V\alpha24J\alpha Q^+$ T-cell clones isolated from diabetic twins secreted only IFNγ, whereas almost all of the clones (76 of 79) isolated from normal twins secreted both IL-4 and IFNγ. Serum IL-4 and IFNγ levels were also found to be drastically different, with IDDM individuals having no detectable IL-4.

PREVENTION AND INTERVENTION STRATEGIES

Currently, the only treatment widely available to alleviate the negative consequences of hyperglycemia is insulin maintenance therapy, which must be continued for the entire lifetime of a diabetic individual. Insulin administration does not completely prevent the life-threatening complications of diabetes that develop over time. Although intensive diabetes management dramatically reduces these complications or at least delays their clinical manifestation,[23] insulin administration cannot be considered a satisfactory therapeutic approach for IDDM.

Prevention

When one considers the fact that type 1 diabetes is the direct consequence of the death of insulin-producing beta cells of the pancreas, it is intuitive that the best approach to spare individuals from the disease would be to prevent beta-cell loss. Methods to act on individuals at risk before the pathologic scenario becomes clinically manifest and then irreversible are being tested in clinical trials in the United States and Europe.

The first problem that must be addressed in any type of preventive intervention consists of recognizing which individuals are actually at risk for the disease. Seventy-five to eighty-five percent of the siblings of diabetic patients in whom disease eventually develops can be recognized by determining whether they carry HLA susceptibility alleles in association with the presence of serum antibodies specific for diabetes-associated autoantigens such as GAD_{65} and IA-2.[46, 63] Although the siblings of a proband (i.e., the first child in a family who receives the diagnosis of diabetes) are the individuals who are most likely genetically and environmentally at risk, the majority of the new cases of type 1 diabetes are actually sporadic, that is, they present in families without any previous history of diabetes. Thus, screening of all children at a certain school age seems to be

too demanding of a task, whereas screening limited to the siblings cannot be completely successful in recognizing all individuals at risk for the disease. The use of the sibling-based approach is complicated even more by the fact that in some of the siblings recognized to be at risk, the disease does not develop. Furthermore, approximately 15% to 25% of the individuals considered genetically at risk will progress to diabetes even though their autoantibody titers are characteristic of individuals protected against the disease (i.e., they are islet cell antibody–negative).[46]

Another problem involves the side effects associated with some of the preventive approaches. The best example is perhaps the strategy undertaken primarily by clinicians in France[7, 9] and Canada[27, 28] in the mid-1980s. Based on the premise that the attack against the beta cells of the pancreas was autoimmune in nature, it was thought that a drug-mediated immunosuppression of the individuals at risk would avoid, or at least delay, the beta-cell destruction and, consequently, the disease onset. In an attempt to implement this type of preventive approach, in addition to the problem of optimal determination of the individual's risk, numerous other critical parameters had to be properly defined. The first parameter was the most appropriate immunosuppressive drug. Several different types of immunosuppressants were available that could be used alone or as part of a cocktail of more than one. Five major classes of immunosuppressive drugs (i.e., steroids, azathioprine, cyclophosphamide, cyclosporine, and methotrexate) could be targeted against different cell types and with different efficiency. Cyclophosphamide acting primarily on B lymphocytes could be the choice in cases in which the pathology seemed to be most associated with high titers of autoantibodies. Nevertheless, this type of therapy seemed more appropriate for diseases such as systemic lupus erythematosus rather than IDDM. The majority of the recognized autoantibodies in the sera of patients with IDDM are generally IgG generated by mature B cells that must be *helped* by T cells. Azathioprine or cyclosporine seemed to be more efficient against T cells. In reality, cyclosporine was proven to have a positive effect on a number of diseases considered autoimmune, including IDDM. Although steroids acted mainly on macrophages, at higher doses they were the optimal complement in a cocktail aimed at the suppression of B- and T-cell activities.[7, 27]

These immunosuppressive approaches were initially considered very promising owing to the dramatic remission of the disease, even in patients with very severe forms. Nevertheless, the initial studies were not carefully controlled trials, and the limitations of this preventive drug-based therapy were soon revealed. Three major drawbacks of these immunosuppressive attempts were recognized by the group of researchers led by Bach in Paris.[7] First, not all patients responded equally well to the same therapeutic protocol. Second, in the majority of the cases, the disease relapsed a few weeks or a few months after termination of the treatment. Third, all of the different agents used for immunosuppressive purposes showed severe side effects that were associated in particular with their prolonged use and high dosage. Nephrotoxicity, the promotion of opportunistic infections, and, of most concern, a possible risk for malignancy were some of the most serious collateral consequences recognized in the immunosuppressed patients. These disadvantages, which are normally acceptable in transplanted patients, were not acceptable from the point of view of a risk-benefit ratio in therapy for or prevention of a disease such as diabetes, in which patients can enjoy a near-normal lifestyle with the appropriate use of insulin injections.

The continuing need for a preventive approach against IDDM that avoids the majority of the negative effects and limitations of immunosuppressive therapy has recently been recognized by the National Institutes of Health. This recognition has promoted financial support of the Diabetes Prevention Trial Type

1 (DPT-1), a nationwide study with the goal of preventing diabetes by insulin administration. The logic on which this trial stands is twofold. The first premise is that reducing the need for physiologic insulin production will prevent beta-cell *overwork*, a suggested but yet not completely proven cause of beta-cell death. The second premise is that if very young recipients can become tolerant to insulin before the autoimmune attack against the cells that produce this protein, the cells will not decrease below a diabetogenic threshold. To meet the requirements of the trial, the individuals to be enrolled must be aged 45 years or younger and must have a brother or sister, child, or parent with type 1 diabetes. Also included are individuals who are aged 20 years or younger and who have a cousin, uncle or aunt, nephew or niece, grandparent, or half-sibling with type 1 diabetes. Potential subjects will be received at one of the numerous DPT-1 trial centers scattered throughout the United States, where they will be tested for the presence of islet cell antibody in serum at no cost. The subjects will also be tested for HLA resistance alleles, such as the HLA-DQB1*0602 allele,[78] and will be excluded from the trial if positive. Individuals with HLA susceptibility alleles and significant titers of islet cell antibody will be enrolled in the trial and randomly assigned to either insulin treatment or a control group (no insulin given). The individuals assigned to the insulin treatment group will be further subdivided into two subgroups based on their degree of risk for developing diabetes over the next 5 years. The first high-risk group will receive injections of low doses of insulin twice a day and will be admitted to a DPT-1 center once a year for 4 days of insulin therapy. The second low-risk group will instead receive oral insulin in the form of a capsule of insulin crystals. The DPT-1 trial is scheduled to last 5 years. The goal is to determine whether there is a benefit of insulin therapy by comparing treated versus untreated groups for the number of individuals who convert to overt diabetes.

The DPT-1 is better organized than the immunosuppressive trials previously completed. Because it is nationwide, it will enroll large enough numbers of individuals in all categories to generate statistically informative answers. A complete compliance to this trial by all of the diabetes centers in the United States is limited by three major considerations, two scientific and the third more ethical in nature. First, the evidence suggesting that the administration of insulin can delay the onset of the disease by "preserving" the still-functional beta cells of an individual at risk is not considered sufficiently convincing by many scientists or clinicians to outweigh the risks involved. Second, the ability to make individuals tolerant to a protein when this protein or peptides derived from it are administered per os has not been proven completely reliable in animal models and even less so in humans. Third, given the acknowledged imprecise selection procedure of individuals at risk, successes in preventing the disease may be confused with cases in which subjects will be spared from IDDM even without any intervention. The unethical price to pay for this intrinsic problem of the trial design is the creation of insulin-dependent patients from potentially absolutely normal individuals. The possibility that 10% of the total number of individuals enrolled in this study could be erroneously considered at risk has been suggested to be too high of a price to pay to demonstrate or negate statistically the utility of procedures not yet scientifically proven as absolutely efficient.

Intervention

The problem of diabetes is even more difficult to approach in individuals in whom the stage of the disease is already set and the progress of its pathologic

development easily foreseen. The goal in these patients should be to reestablish the same finely tuned process that, in normal individuals, can dynamically provide the correct quantity of insulin necessary to metabolize glucose properly. Such a process cannot be faithfully reproduced even with four injections of insulin each day. Transplantation of pancreatic islets seems to be the only real alternative that may help to avoid the dangerous variations of glucose levels in blood that are associated with the major complications of diabetes.

In approximately 46% of 1000 grafts reported, transplant of the entire pancreas alone or with a kidney has been sufficient to render the recipients insulin-independent with normalization of hemoglobin A_{1c} levels.[73] Nevertheless, this type of intervention is not suitable for young patients in whom IDDM develops at puberty. Some of the contraindications are associated with the exocrine secretion of the transplanted pancreas that is generally anastomosed to a segment of the bowel different than the one where the pancreas is physiologically connected. This secretion creates difficulties in definitive postoperative healing and continuous digestive problems, an unacceptable circumstance for young patients who can attain a near-normal lifestyle with proper insulin injections.

The most recent advances in the engineering of non–beta cells as glucose-sensing replacements for insulin production also seem to offer solutions more for adult onset nonautoimmune diabetes than for classic juvenile type 1 diabetes.[57] The microencapsulation of insulin-producing cells or cell lines in synthetic polymers offers an effective remedy only for a limited amount of time. The long-term survival of the encapsulated cells will be hampered by the fibrosis in which the capsules are eventually enclosed. This overlaying fibrotic tissue causes ischemic oxygen deprivation and cytokine-induced damage of the cells contained in these types of polymeric envelopes.[22, 72]

During the past decade, much progress has been made in the field of pancreatic islet transplantation. Despite relative successes in which a transient return to normoglycemia has been achieved when the islets have been transplanted in the liver of the diabetic recipient, much work is needed to realize fully the clinical application of this type of transplantation.[39] Because previous technical problems associated with physical islet replacement no longer limit this application, current efforts should be focused on trying to eliminate the obstacles that remain. The potential reoccurrence of the mechanism that promoted the original autoimmune islet destruction and determination of the mechanism involved in the conventional allorejection of the transplanted islets seem to be the two major problems that must be solved.

Although some optimism is offered by the fact that 33 patients with type 1 diabetes worldwide have become insulin-independent after adult islet allotransplantation,[37] the need for an immunosuppressive regimen strong enough to preserve the transplanted islets from recurrent autoimmunity and allorejection still constitutes a major problem. The source of the islets is, in general, a donor with different histocompatibility antigens than the recipient, and, frequently, more than one donor is necessary to accumulate the number of islets considered optimal for successful treatment. Human pancreatic islets are particularly sensitive to immunosuppressive drugs. In many cases, while the kidney is still functioning, the pancreatic islets injected via the portal vein into the liver stop secreting insulin. Contemporary types of immunosuppressive therapy, such as the use of anti-CD154 monoclonal antibodies, seem to obviate direct damage of the islet.[37] CD154 is the new name of the ligand involved in the CD40-CD40 ligand costimulatory pathway for T-cell activation.[35] The monoclonal antibody 5c8 is anti-CD154–specific and is very effective in prolonging islet allograft

survival in nonhuman primate models of islet allotransplantation[44]; however, by blocking the entire immune system, antibodies such as 5c8 deprive the recipients of their vital defensive help.

The avenue of research that the authors and other investigators have undertaken consists of protecting the transplanted islets from all of the previously mentioned immunologic events acting against their permanent engraftment by using gene therapy approaches to interfere with them.[66] The possibility of transferring potentially therapeutic genes to the islets before transplanting them is very promising because the gene's product will actually be delivered exactly where it is most useful. Secretion of the selected anti-inflammatory factors in situ will not jeopardize the normal protective activities of the immune system everywhere else.

Because pancreatic islets consist of postmitotic cells, efficient vectors such as the murine leukemia virus-based retroviral vectors are unable to integrate stably in islet cells. Of the vectors tested to date, adenoviral vectors seem to be the most efficient in transduction of protective genes into islet cells. Moderate to low multiplicity of infection levels do not result in islet cell dysfunction. First-generation adenoviral vectors have been used to deliver immunosuppressive genes to islets, including cytotoxic T-lymphocyte–associated antigen-4 (CTLA-4), interleukin-10 (IL-10), and transforming growth factor-β (TGF-β1).[3, 4, 69]

The secreted form of CTLA-4, CTLA-4-lg, is a fusion protein that can block the CD28-B7 costimulatory pathway important for T-cell activation and has been shown to prolong, as the inhibitors of other costimulatory pathways (e.g., CD40/CD40L), allograft survival when recombinant protein is administered intravenously at the time of transplantation.[69] In addition, the administration of CTLA-4-lg can induce tolerance to certain allografts or at least allow long-term graft survival. Preliminary reports indicate that expression of CTLA-4-lg locally in allografted islets can double the duration of engraftment. CTLA-4-lg expression following bioballistic gene delivery resulted in a reduction of the infiltration of mononuclear cells into the genetically modified islets.

As mentioned previously, the destruction of beta cells in a variety of murine models is delayed or prevented by T cells producing TH2-type cytokines such as IL-4, IL-10, and TGF-β. The effects of IL-4 have already been described.[54, 80] The properties of IL-10 have also been exploited in the context of a global diabetes-oriented, gene therapy approach.

T-cell–mediated destruction of beta cells in the NOD mouse can be prevented by the transfer of suppressor T-cell clones isolated from the insulitic infiltrate of NOD mice that do not develop diabetes.[53] One of these clones, NY4.2, can also prevent the destruction of syngeneic islet grafts acutely diabetic mice. These clones secrete substantial amounts of IL-10 and TGF-β. In adult NOD mice, diabetes onset is delayed and its incidence significantly decreased when the mice are given recombinant IL-10 (rIL-10).[61] Furthermore, the administration of a noncytolytic IL-10/Fc fusion protein to NOD mice from 5 to 25 weeks of age prevents diabetes.[86] These mice remain disease-free long after the cessation of treatment. In the islets of these mice, the expression of TNF-α and IFN-γ is inhibited. Although transgenic mice have been generated with the IL-10 gene under the control of the insulin promoter, and although the IL-10 is unable to prevent insulitis,[55] the use of a homologue of the cellular IL-10, which is encoded by Epstein-Barr virus, the viral IL-10 (vIL-10), seems to be more promising.[3] Cellular IL-10 has been used to prevent immune rejection of genetically modified tumors,[11] whereas vIL-10 is more efficient in prolonging the survival of allografted organs in rodents.[26, 65] vIL-10 also has the advantage of lacking the growth-promoting effects of cellular IL-10 on some types of cell

targets, which could lead to immunostimulation. Although there are no data on the ability of vIL-10 to modify insulitis and IDDM onset, it is attractive to hypothesize that it may have such effects.

TGF-β1 is an immunosuppressive cytokine that possesses chemoattractant and stimulatory properties. Local expression of TGF-β1 in certain liver and cardiac allografts seems to prolong engraftment.[4] TGF-β1 has also been used alone or in combination with IL-10 to prevent diabetes and to prolong islet allograft survival. Although the initial results are not promising, TGF-β1 may still be considered for diabetes therapy in association with other immunomodulatory genes.

There are good reasons to believe that local gene transfer to islets of several different immunosuppressive cytokines can block the recurrence of autoimmunity as well as the allogeneic immune response and prolong engraftment. Most likely, no one immunosuppressive agent will be sufficient to inhibit the immune response aimed at allograft islets, to block leukocyte infiltrate, and to prevent islet dysfunction and apoptosis. Unfortunately, even low levels of viral gene expression following infection with first-generation adenoviral vectors enhance the immunogenicity of islets following transplantation (Fig. 4). As a result, adenovirus-infected islets are rejected even more quickly than untreated islets. Additional approaches including the use of liposomes and bioballistics have been tried with low transfection efficiencies. Other vectors such as herpes simplex virus,[34] adenoassociated virus,[30] and lentivirus[12] have been tested in the authors' laboratory in an effort to bypass all of the aforementioned limiting aspects of the conventional gene therapy approach. The level of success experienced thus far is sufficient to encourage further studies.

In the authors' experience, the most promising vector seems to be the lentivirus, a nonantigenic vector capable of infecting nondividing cells such as the beta cells of the pancreas. Lentivirus-infected transplanted islet cells are expected to express permanently the therapeutic gene carried by the vector. If the therapeutic gene encodes a secreted protein, it can promote long-term cell survival not only of the beta cells actually infected with the virus but also of the surrounding noninfected beta cells as well. Recent data from the authors' laboratory demonstrate that a lentiviral vector encoding the IL-1 receptor antagonist protein (IRAP) can actually protect mouse and human islets from damage triggered by the proinflammatory cytokine IL-1β.[31, 32]

Interleukin-1 is a proinflammatory polypeptide that is biologically related to TNF-α and is produced in response to infection, toxic injury, trauma, and antigenic challenges. The major source of IL-1 is the macrophage, and its expression can be induced by IFN-γ, although B lymphocytes, endothelial, mesangial, smooth muscle cells, and fibroblasts can produce IL-1 as well.[6, 25] Two different genes encode IL-1, resulting in two polypeptides: IL-1α and IL-1β. The IL-1β is more abundant at the mRNA level and is the major secreted form.[21, 47] Normal human pancreatic beta cells that do not constitutively express Fas become strongly Fas-positive after IL-1β exposure and then become susceptible to Fas-mediated apoptosis.[70] N^G-monomethyl-L-arginine, an inhibitor of nitric oxide synthase, prevents IL-1β–induced functional Fas expression in normal pancreatic beta cells. This selective expression of Fas in beta cells primed by nitric oxide may be responsible for their specific killing because T cells expressing Fas may promote an MHC-unrestricted destruction of the Fas-positive beta cells while sparing neighboring Fas-negative alpha and delta cells[70] (Fig. 5).

Interleukin-1 binds to two cell surface receptors—type I, which transmits its biologic signal, and type II, which is not known to transduce any signal and may act as a decoy molecule to downregulate IL-1–induced inflammation.[29]

Figure 4. Adenovirus-infected islets were transplanted under the kidney capsule of NOD mice. One kidney received NOD islets treated with the adenovirus only. The contralateral kidney was inoculated with islets infected with the adenovirus carrying the IL-1 receptor antagonist protein (IRAP). The antigenic effect of the adenovirus itself is evident by comparing non-treated islets *(A)* with islets infected with the virus only *(B)*.

Illustration continued on opposite page

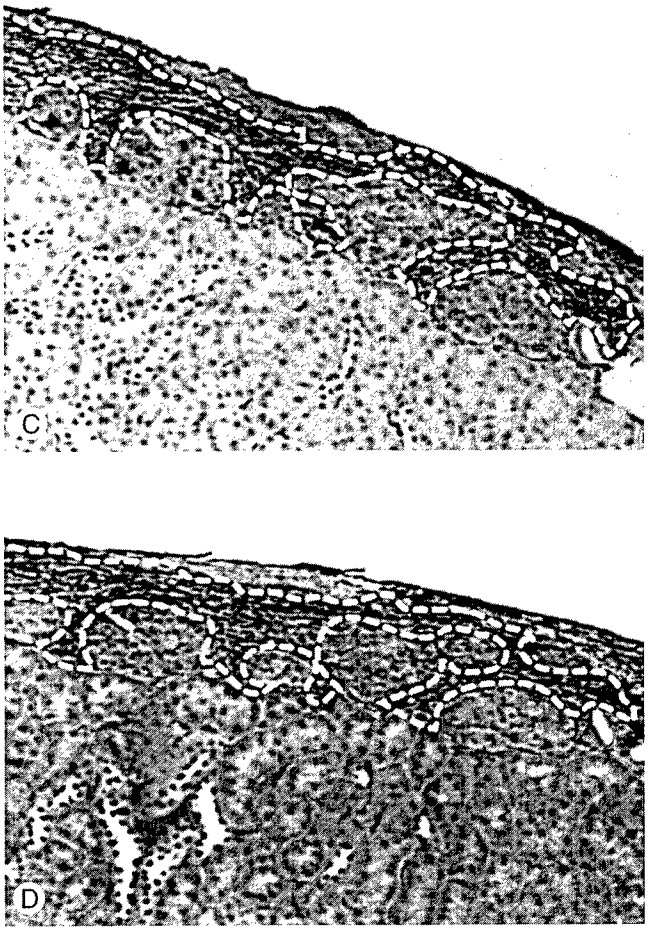

Figure 4 *(Continued).* In *C* and *D,* the effect of IRAP secreted to protect the islets from the recurrent autoimmune attack is revealed by the presence of intact, insulin-producing islets surrounded by a heavy T-cell infiltration *(outline). A* and *C* are labeled with an anti-insulin antibody *(shaded areas)* and contrast dye *(outline).* (Hematoxylin-eosin stain; 320× original magnification.)

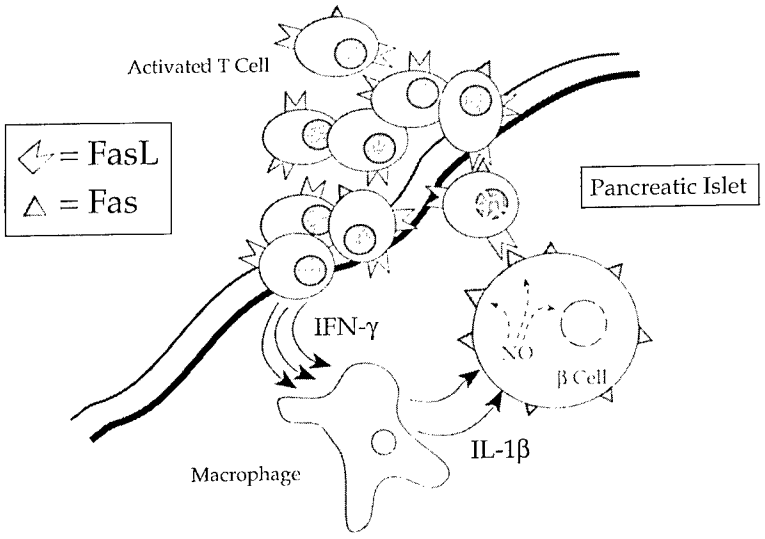

Figure 5. Macrophages, located on the periphery of the islet of Langerhans produce IL-1β in response to IL-2 and IFNγ secreted by activated T-cell clones approaching the islet. IL-1β is able to activate nitric oxide (NO) in the β cells of the islet, thus inducing expression of Fas. The interaction between Fas and FasL will cause apoptosis in pathologic Fas^+ β cells, and in physiologic Fas^+ T cells present in the area of inflammation. Once most of the invading T cells are dead, the apoptosis process has also reduced the number of insulin-secreting β cells to a percentage that cannot efficiently metabolize the blood glucose, thus promoting the onset of diabetes.

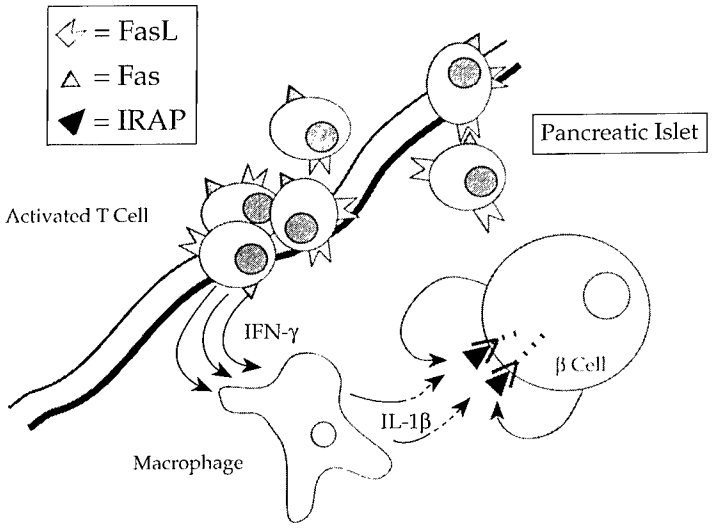

Figure 6. Once transplanted, islet cells infected with viral vectors carrying the IL-1 receptor antagonist protein (IRAP) will be protected against the Fas- and FasL-mediated apoptosis process because the effect of IL-1β on β cells will be impaired.

IRAP is a 17-kd glycoprotein that shares 40% and 39% homology with IL-1β and IL-1α, respectively. IRAP is a competitive inhibitor of IL-1 for the type I receptor and does not possess any signaling capacity.[24] IRAP can completely prevent IL-1–induced inhibition of insulin secretion and can attenuate nitrate formation in isolated murine islets.[19, 87] Moreover, the infusion of recombinant IRAP prevents low-dose, streptozotocin-induced diabetes as well as insulitis in mice. IRAP can also inhibit the apoptosis of beta cells mediated by IL-1β–induced Fas upregulation (Fig. 6).

Because of the differences existing between mouse and human IRAPs, the authors have cloned both genes into viral vectors to be used for infection of mouse and human islets, respectively. The results in vitro[31, 32] and preliminary results in vivo (Fig. 7) seem to encourage further efforts in this direction.

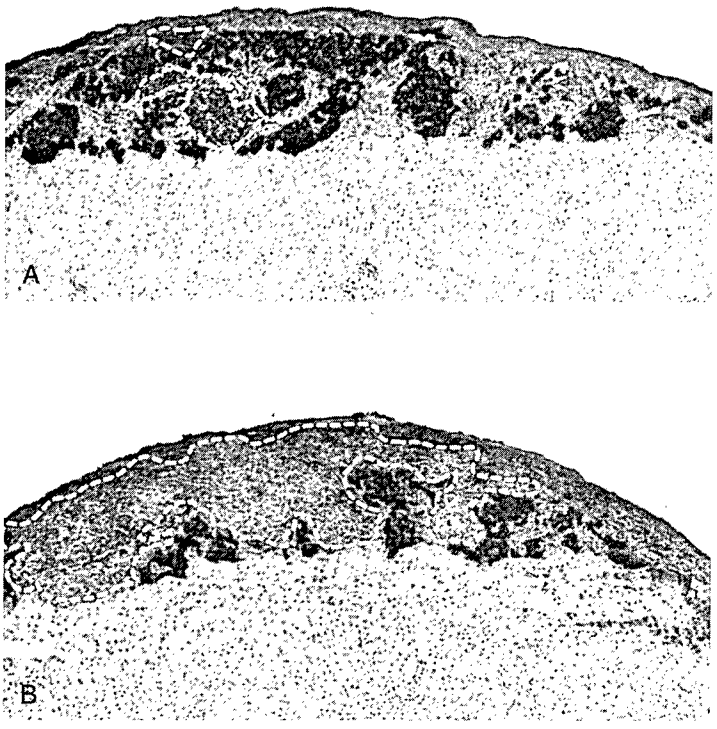

Figure 7. NOD islets treated with a lentiviral vector carrying the IRAP gene *(A)* were transplanted under the kidney capsule of recipient NOD mice. The protection provided by IRAP secretion against invading T cells *(outline)* is more evident when the results of the transplantation of the same number of islets (revealed by anti-insulin antibody, *outline*) treated with the lentivirus carrying the *LacZ* gene in the contralateral kidney are used for comparison *(B)*. (Original magnification 80×.)

CONCLUSION

Based on current knowledge of IDDM initiation and progression mechanisms, new strategies for prevention and intervention can be tested in animal models and eventually implemented in humans.

With the assumption that the molecular mimicry hypothesis can convincingly explain diabetes etiopathogenesis, the route of vaccination with specific, possibly cross-reactive peptides, or with their cDNA should be more systematically pursued. The data obtained in mice using GAD peptides can be used as the blueprint for further studies.[42, 75] The results of the ongoing DPT-1 trial should determine the effects of anti-insulin prophylactic therapy.

If the hypothesis suggesting viruses as the first triggering agents, either as the activators of a bystander reaction or as pancreotropic carriers of superantigens, can be more substantially confirmed, vaccinations against diabetogenic strains of virus can be pursued. This approach will be more applicable in theory than in practice because the number of viruses that could be considered as possible targets is extremely large, and many different strains of each of these viruses exist. A protein segment with superantigenic characteristics would be a more suitable candidate for vaccination purposes because exposure to superantigens at the time when the T-cell repertoire is not yet formed can result in the deletion of the superantigen-reactive T-cell clones.[2]

If the approach aimed at deleting or anergizing "dangerous" autoreactive T-cell clones that, once primed, can eventually be reactivated by viral infections, does not generate the auspicated results, a possible alternative could be an attempt to overpower the autoreactive T cells, forcing the expansion of regulatory T-cell clones by artificially increasing, for example, the amount of TH2 lymphokines in individuals at risk.

As the etiopathogenesis of overt disease is more completely defined and as new powerful technical means are applied to islet cell transplantation, revolutionary solutions to diabetes will be found in the next millennium.

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REVIEW ARTICLE

Genetics of Type 1 diabetes mellitus

R.P. Friday, M. Trucco and M. Pietropaolo

ABSTRACT. **Type 1 diabetes or insulin-dependent diabetes mellitus (IDDM) is the archetypal example of a T cell-mediated autoimmune disease characterised by selective destruction of a single cell type: the insulin-producing β-cells of the pancreatic islets of Langerhans. The pathogenic equation for IDDM presents a complex interrelation of genetic and environmental factors, most of which have yet to be identified. Based on the observed familial aggregation of IDDM, it is certain that there is a decided heritable genetic susceptibility for developing autoimmune diabetes. The well-known association of IDDM with certain human histocompatibility leukocyte antigen (HLA) alleles of the major histocompatibility complex (MHC) was a major step toward understanding the role of inheritance in IDDM. Landmark molecular biological investigations of diabetes HLA susceptibility genes provided great potential for insights into the molecular basis for the autoimmune nature of the disease, beginning a story that continues to unfold. Although the association of certain HLA alleles with IDDM is very strong, this genetic locus is estimated to account for less than 50% of genetic contributions to disease susceptibility. The search for non-HLA susceptibility genes has received great attention in recent years. Albeit genome wide searches are wrought with controversy, such studies have suggested the association of numerous non-MHC loci with Type 1 diabetes that will require careful follow-up investigation. Cell biological and genetic functional analyses will provide clues that are indispensable for further progress. The necessary studies include research on immunological abnormalities that are present many years before the clinical onset of Type 1 diabetes.**

Diab. Nutr. Metab. 12: 3-26, 1999.

INTRODUCTION

Genetic susceptibility to Type 1 diabetes appears to be inherited as a polygenic trait. However, more than 80% of cases of IDDM occur in individuals with no apparent family history of the disease. In the remaining 20%, IDDM aggregates in families. Although it has been suggested that multiple genes play a role in disease susceptibility, there is evidence that two chromosomal regions are associated with and linked to IDDM: the HLA region on chromosome 6p21 (*IDDM1*), and the insulin gene region, (*IDDM2*), on chromosome 11p15. The contribution of these two loci to familial inheritance is approximately 42% for *IDDM1* and 10% for *IDDM2*. As a result of genome wide searches, relationships between many other non-MHC genes and a susceptibility to IDDM have been proposed. These loci along with some potential candidate genes are summarised in Table 1.

Epidemiologic studies support the hypothesized genetic predisposition to developing diabetes with strong evidence for familial clustering of the disease. The overall risk for developing IDDM in North American Caucasian siblings, parents, and offspring of individuals with IDDM ranges from 1% to 15% (1-7) as compared to less than 1% for individuals without affected relatives and 1.2/1000 of the general population (8). It is noteworthy that an increased risk for IDDM seems to be present also in first degree relatives of individuals with classical non-in-

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Key words: Genes, genetics, IDDM, HLA-DQB, genome wide searches.

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Table 1 - *Summary of human IDDM susceptibility loci.*

Chromosome	Locus	Linkage status (according to Lander *et al*) (157)
6p21	*IDDM1*; HLA-DQB	Confirmed
11p15.5	*IDDM2*; *INS* 5' VNTR	Confirmed
15q26	*IDDM3*; *IGF1R*	Suggestive ($p<0.001$, MLS>2.2)
11q13	*IDDM4*; *FGF3*	Confirmed ($p<2.2\times10^{-5}$, MLS>3.6)
6q25	*IDDM5*; *ESR1*	Confirmed ($p<2.2\times10^{-5}$, MLS>3.6)
18q21	*IDDM6*	
2q31	*IDDM7*; IL1, *HOXD8*	Suggestive ($p<0.001$, MLS>2.2)
6q27	*IDDM8*; *IGF2R*	Confirmed ($p<2.2\times10^{-5}$; MLS>3.6)
3q21-q25	*IDDM9*	
10p11.2-q11.2	*IDDM10*	
14q24.3	*IDDM11*	Significant ($p<2.2\times10^{-5}$, MLS>3.6)
2q33	*IDDM12*; CTLA-4	
2q34	*IDDM13*; *IGFBP2*, *IGFPB5*	
6q21	*IDDM15* (distinct from HLA)	
7p	Not assigned; *GCK*; *IGFBP1*, *IGFPB3*	
Xq	Not assigned	
Xp*	Not assigned	Significant ($p=2.7\times10^{-4}$; MLS>3.6)
1q**	Not assigned	Suggestive (MLS=3.31)

The *IDDM* nomenclature is assigned to a locus after linkage has been formally demonstrated, replicated and confirmed in at least three different datasets. Where functional candidate genes are flanked by, or very close to susceptibility markers, they are indicated; MLS=Maximum; LOD=Score; *In MHC HLA-DR3 positive patients (180); **This locus (161) co-localises with loci for SLE (164) and ankylosing spondylitis (165).

sulin-dependent diabetes mellitus (NIDDM) (9-14). Specific HLA haplotypes may account for susceptibility to both IDDM and to a subtype of NIDDM. This latter finding is supported by the occurrence of β-cell humoral autoimmunity in 10-33% of Caucasian adult-onset diabetic patients not treated with insulin (9-12). Therefore, some forms of NIDDM manifest a similar genetic susceptibility and immunologic abnormalities to those characteristic of classical IDDM.

Studies conducted in the United States and Scandinavia have shown that offspring of parents with IDDM have a higher risk for progression to IDDM if the father, rather than the mother, is affected by the disease (7, 15-19). The risk is in fact increased to about 1/40 among offspring of fathers with Type 1 diabetes and 1/66 in offspring of Type 1 diabetic mothers (20). A number of explanations have been proposed for this finding. These include: genomic imprinting of a susceptibility to IDDM, manifesting as an increase in spontaneous abortion by IDDM mothers of foetuses that might develop IDDM (1, 6); maternal environmental factors that determine the foetus' level of tolerance to islet autoantigens (1); or a preferential paternal transmission of HLA diabetogenic alleles (21). The current consensus regarding the pathogenesis of IDDM maintains that both genetic and environmental factors contribute to determine the risk of progression to the clinical stage of disease. This opinion is supported by the observed tendency for IDDM to cluster in families and the suggestive clinical and epidemiological evidence that environmental factors, including exposure to certain viral agents, may play a role in the generation of β-cell autoimmunity (22-27). A discordance rate greater than 50% between monozygotic twins indicates a potential involvement of environmental factors in disease development (28, 29). These observations are further

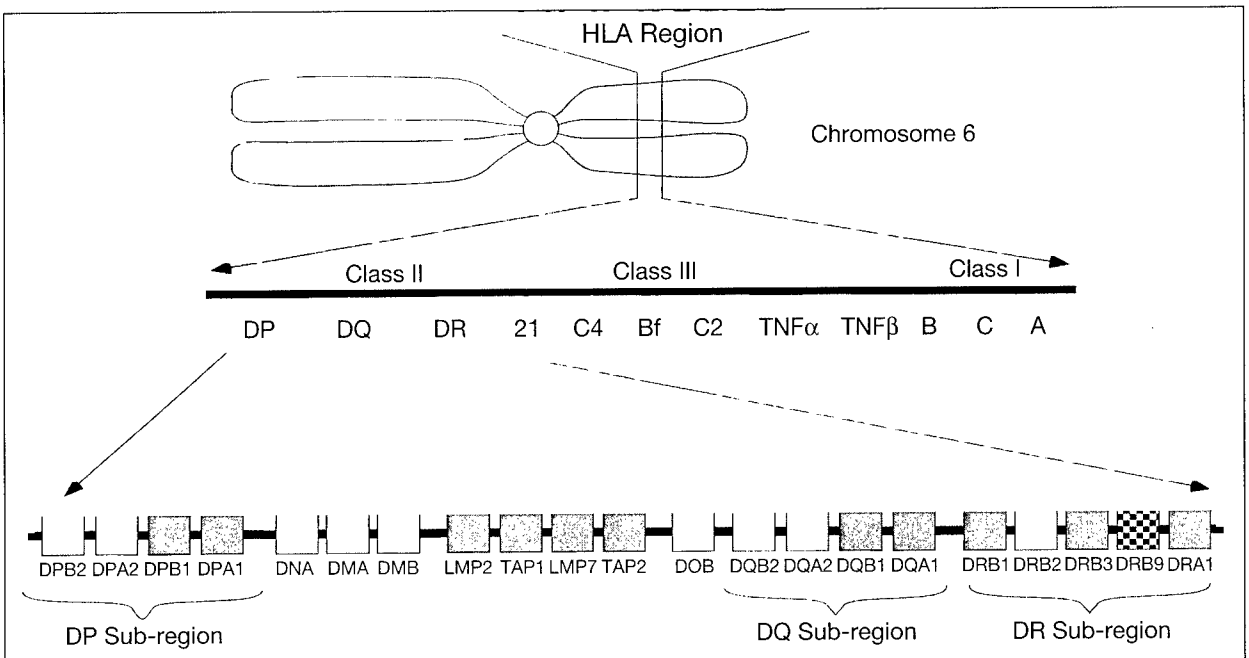

Fig. 1 - *The HLA complex present on human chromosome 6, can be subdivided into 3 regions. The genes present in each region encode class I, class III, and class II molecules, respectively. The class II region, which is closest to the centromere of the chromosome, is conventionally subdivided into 3 additional sub-regions. Among the genes contained in each sub-region, there are some that encode functional molecules (in black), genes that are not functioning,* i.e., *pseudogenes (in white), or that encode proteins not yet characterised (also in white). While DQA1 and DQB1 genes encode for the α and the β chains of a functional HLA-DQ molecule (like DPA1 and DPB1 genes for the chains of a functional HLA-DP molecule), the DRA1 gene encodes for a non-polymorphic α chain able to pair with the products of each of the functional DRB genes. In the example, a DR3 positive haplotype of an individual is shown. DRB1 gene encodes for the DR3 allele, DRB2 is a pseudogene, and DRB3 encodes for the so-called DR52 molecule, which is always found associated with DR3. The DRB9 gene has not been well studied yet but seems to be able to encode for a non-functionally stable protein chain. Transporter associated with antigen processing (TAP) 1 and 2 genes are cytoplasmic transporters of antigenic peptides, while LMP 2 and 7 are genes encoding proteasome subunits. Both these molecules are involved in the antigenic peptide transport from the cytoplasm to the cell surface. Tumor necrosis factor (TNF) α and β genes, located between class I and class III regions are also indicated. The 21-hydroxylase locus encompassing CYP21P and CYP21 genes is simply indicated as "21".*
(Modified from Luppi P., Rossiello M., Faas S., Trucco M., J. Mol. Med. *73: 381-393, 1995).*

supported by the increasing seasonal incidence of IDDM in many Western countries (30, 31).

THE HLA COMPLEX

The short arm of human chromosome 6 (6p21) accommodates a ~3.5 megabase genetic segment containing a group of immune response genes termed the major histocompatibility complex (MHC) (Fig. 1). The principal genes located within the MHC code for human leukocyte antigens, or HLA, two molecular classes of cell surface glycoproteins differing in structure, function, and tissue distribution (Table 2) (Fig. 1).
The class I HLA molecule exists as a heterodimer, consisting of a polymorphic 44 kDa MHC-encoded α or heavy chain in non-covalent association with β_2-microglobulin, a 12 kDa protein encoded by a non-polymorphic gene on chromosome 15. The class I molecule is anchored in the cell membrane only by the heavy chain. This chain contains 338 amino acids and, beginning from the amino terminus, is functionally divided into three regions: an extracellular hydrophilic region (amino acid residues 1 to 281), a transmembrane hydrophobic region (amino acid residues 282 to 306), and an intracytoplasmic hydrophilic region (amino acid residues 307 to 338). The extracellular region is further subdivided into three domains, designated α_1, α_2 and α_3, each of approximately 90 amino acid residues (Fig. 2). The α_1 and α_2 domains comprise the peptide- or antigen-binding region of the molecule.

Table 2 - *Comparison between class I and class II molecules.*

	Class I	Class II
Molecules	HLA-A, B, C	HLA-DR, DQ, DP
Structure	44,000 MW heavy chain 12,000 MW β_2 microglobulin	~34,000 MW α chain ~29,000 MW β chain
Tissue distribution	Virtually on every nucleated cell	Restricted to B cells, macrophages, activated T cells
Function	Bind and present antigenic peptides to CD8+T cells	Bind and present antigenic peptides to CD4+cells

Class II HLA molecules consist of two glycoprotein chains, an α chain of approximately 34 kDa and a β chain of approximately 29 kDa, both encoded within the MHC. As with the class I heavy chain, each class II chain can be divided into three regions (extracellular, transmembrane and intracytoplasmic), but, in contrast to class I molecules, both class II chains are anchored in the cell membrane. Each extracellular region of the class II α and β chains has been further divided into two domains of approximately 90 amino acid residues each, termed α_1, α_2 and β_1, β_2 respectively. The α_1 and β_1 domains together form the peptide-binding region of class II HLA molecules (Fig. 3). Also of note, the class II α_2 and β_2 domains, class I α_3 domain, and β_2-microglobulin all show homology to the constant region domain of immunoglobulins and are therefore classed in the immunoglobulin superfamily.
The genes that encode class I MHC consist of HLA-A,

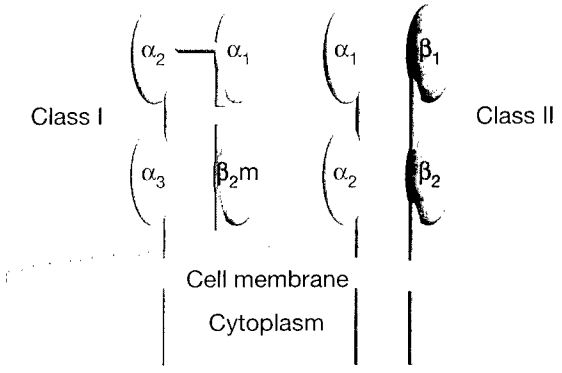

Fig. 2 - *Secondary structure of HLA class I and class II molecules in comparison. Like in the case of immunoglobulins, peptidic sequences that show similarities and are present more than once in the same polypeptidic chain, are called "domains". α_1, α_2 and α_3 constitute the domains of the class I α-chain, while α_1 and α_2 are the domains of the class II α-chain as α_1 and α_2 are the domains characteristic of the class II α-chain. Both class I and class II molecules are composed of non-covalently bound and somewhat different α and β chains. These heterodimers form, at their most external end, a peptide combining site composed of the α_1 and α_2 domains for class I, and α_1 and β_1 domains for class II molecules. The non-polymorphic β_2-microglobulin completes the structure of class I molecules.*
(Modified from Rosner G., Martell J., Trucco M. Bone Marrow Transplantation, *Churchill Livingstone, in press.)*

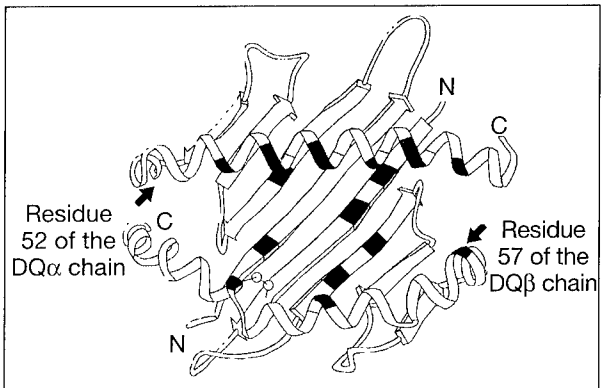

Fig. 3 - *The two outermost domains of HLA class I and class II molecules fold together to form their antigen combining sites in which the processed antigenic peptide can find appropriate lodging. The polymorphic regions of the HLA molecule seen from the top, present on the floor and on the α helices of its groove, are indicated in different nuances of colour from grey to black. The antigenic peptides found in HLA class I molecule grooves are normally 9 amino acid long, while the antigenic peptides most frequently found associated with HLA class II molecules are longer than 9 amino acids and can vary considerably in size. The position of the amino acid 57 of the β chain and that of the α chain in position 52 are indicated.*
(Modified from Trucco M., Diabetes Care *15: 705-715, 1992).*

B, C, whereas class II molecules are encoded by the DR, DQ and DP genes (Fig. 1). Other genes in this cluster include TAP (transporters associated with antigen processing) (32, 33) and LMP (low molecular-weight proteins), both involved in antigen processing (34). A third region of the MHC, denoted class III, codes for several molecules having a variety of functions, namely complement components (C4A, C4B, factor B and C2), tumor necrosis factor (TNF-α and -β) and the 21-hydroxylase genes (CYP21P and CYP21) (Fig. 1).

While class I MHC molecules are expressed in virtually all nucleated cells, class II molecule expression is restricted to B lymphocytes, dendritic cells, macrophages and activated T lymphocytes. Both class I and class II antigens are involved in the presentation of antigen to T cells: cytotoxic T cells ($CD8^+$) mainly recognise antigen in the context of class I, whereas helper/inducer cells ($CD4^+$) usually recognise antigen in the context of the class II molecules.

A number of immunologically mediated diseases, including certain endocrine syndromes, are genetically associated with specific HLA molecules, and several hypotheses have been proposed to explain HLA-disease associations (35, 36). Four of these general hypotheses apply to diseases associated with both class I and class II molecules. The first of these relates to the way in which the antigen-binding cleft of a specific HLA molecule can accept peptides derived from either exogenous (*e.g.*, a viral particle) or endogenous (*e.g.*, an autoantigen) protein antigens. When these T cell stimulatory peptides are processed and presented by antigen presenting cells (APCs), the immune system may respond with the generation of an immune response directed against the antigenic peptide.

The second hypothesis suggests that the actual disease susceptibility genes are not the HLA genes but rather genes encoding α and β chains of the T - cell receptor (TCR). In this scenario, foreign molecules capable of potently stimulating T cells, termed superantigens, can bind to the HLA-peptide-TCR complex, usually in T cells expressing the same Vβ chain segment in their T cell receptor. Because a particular TCR α and β chain combination recognizes a specific antigenic peptide as well as a superantigen in the context of a particular HLA molecule, this may underlie the generation of an immune response directed against a particular foreign or self-peptide. This hypothesis was recently extended when researchers employed X-ray crystallographic data to model HLA and TCR contacts with the staphylococcal enterotoxin E (SEE) superantigen. Their results suggest that the identity of the peptide found in the groove of the HLA molecule may not matter in certain cases of superantigen-driven T cell stimulation (37). In this situation, specificity of superantigen binding and activity would be independent of the HLA-bound peptide and entirely determined by the identity of the HLA molecule and the Vβ gene segment included in the rearranged TCR.

The third hypothesis postulates that a TAP gene product, which normally transports antigenic peptides from the cytoplasm to the endoplasmic reticulum, is defective and therefore predisposes to disease susceptibility. As a result of alterations in antigen processing due to the defective TAP protein, few peptides become available for binding to class I molecules leading to a low surface density of class I-peptide complexes and a high surface density of empty class I molecules. A high density of empty surface class I molecules may bind peptides to which they would otherwise not be exposed intracellularly, for instance viral or bacterial peptides. These newly formed MHC class I-exogenous peptide complexes may account for the induction of an immune response that can be responsible for disease.

The fourth hypothesis, termed molecular mimicry, implies that a foreign antigen such as a viral or bacterial protein shares similarities with a self molecule and because of this similarity the immune response is turned against self target tissues causing an autoimmune response. It is unknown the extent to which these four mechanisms may be acting in the variety of recognised autoimmune disorders, and it should be emphasised that two or more of these or other, as yet unproposed/unexplored immunologic mechanisms may be acting in concert to cause disease.

Some HLA-associated diseases have been linked with polymorphisms of the genes encoding the class II molecule. One typical example is IDDM. Other mechanisms besides those discussed above have been implicated as a result of HLA molecule-peptide interactions, but it should be emphasised that more than one mechanism may be operating concurrently to determine susceptibility to a disease process.

Alleles at multiple loci in close proximity on a single chromosome are usually inherited in combination as a unit. This combination of multiple genes inherited together is termed a haplotype. Because each individual inherits one set of chromosomes from each parent, each individual has two haplotypes for a given physical genetic interval. The HLA

genes found within the MHC on chromosome 6 behave in precisely this manner.

Certain combinations of HLA alleles are found with a frequency greater than expected and as a consequence are not randomly distributed within the general population. This phenomenon is known as linkage disequilibrium, and it is quantified by the difference (△) between the observed and the expected frequencies of a certain allele. One example is given by the HLA-A*0101 and the HLA-B*0801 alleles which are found in Caucasian populations with frequencies of 0.161 and 0.104, respectively. Thus, the expected frequency of the HLA-A*0101, HLA-B*0801 haplotype, that is the probability that these two alleles are found together on the same chromosome, should be 0.161x0.104=0.0167. However, the frequency of this haplotype is instead much higher than this (0.0592), which is almost four times the expected frequency (△=0.0592-0.01670=0.0425).

The observation of linkage disequilibrium and extended haplotype inheritance has given rise to many speculations regarding the reason for its existence. One hypothesis that could explain linkage disequilibrium is that some haplotypes are preferentially protected from genetic recombination and are therefore preserved within the mating population (ancestral haplotypes) (38, 39). The mechanism acting to preserve HLA haplotypes in this manner is not clear, but inhibition of crossing-over during gametic meiosis may explain the cause of gene haplotype associations. Another hypothesis to explain the phenomenon of linkage disequilibrium is that certain haplotypes are preferentially reconstituted by recombination (40). A third, evolution-based "Darwinian" hypothesis holds that certain HLA haplotypes confer a selective advantage and are favoured by natural selection (41).

Regardless of the reason for haplotype preservation, it is clear that the occurrence of HLA haplotypes has wide-ranging implications in terms of inherited disease susceptibility. Linkage disequilibrium is not only observed independently for genes of the class I and class II loci, but also between the MHC regions encoding class I and class II molecules. The common association, or linkage, between class I and class II loci, although not as strong as seen for genes within the two loci independently, may give rise to an extended haplotype.

Although the unit inheritance of certain combinations of HLA-encoding genes adds some level of complexity to MHC gene expression, the genes themselves are codominant and haplotypes follow a simple Mendelian form of transmission in families (Fig. 4). As a consequence of HLA co-dominance, both alleles (one on each chromosome) are expressed from a given HLA locus, while simple Mendelian haplotype inheritance allows us to pre-

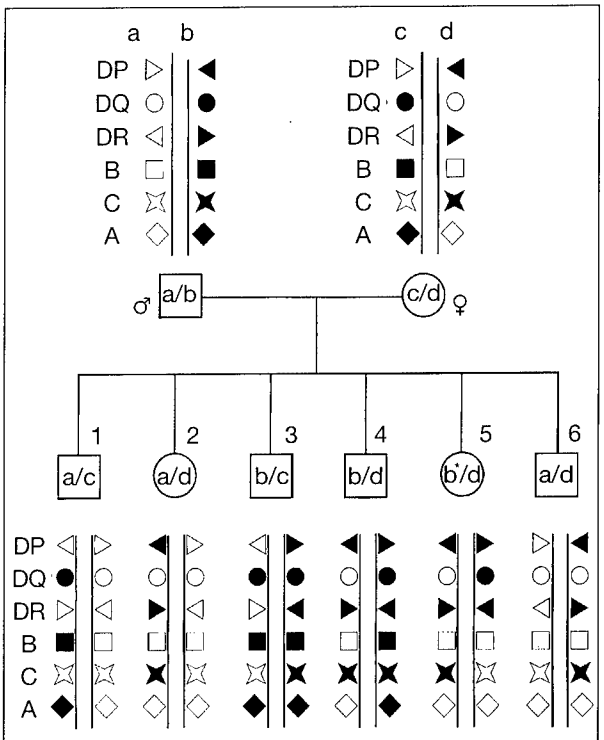

Fig. 4 - *The study of the segregation of HLA alleles through a family is based on the determination of the HLA phenotypes on at least the two parents and a child or on 3 HLA-different members of the same family. Segregation analysis allows the definition of the four haplotypes (normally called a, b, c and d) present in the family, and, consequently the definition of individuals heterozygous or homozygous at certain loci (e.g., sibling 1 is homozygous at C, DR and DP loci, both alleles are white; but heterozygous at A, B and DQ loci, 1 white and 1 black allele), together with the recognition of individuals who share one haplotype only (e.g., siblings 1, 2 and 6 are haploidentical since they share the "a" haplotype), or two haplotypes (e.g., siblings 2 and 6 are HLA identical since they share the "a" and the "d" haplotypes), or none (e.g., siblings 2 and 3 or 1 and 4 are HLA different). Although it is considered a very rare event, it is possible to find individuals, represented here by sibling 5, in which a crossing over between class I and class II gene regions, involving the paternal and maternal haplotypes of the father of this family, cause the "a-b" recombination flagged here with an asterisk: a*.*
(Modified from Rosner G. Martell J. Trucco M. Bone Marrow Transplantation, *Churchill Livingstone, in press.)*

dict the probability of haplotype sharing for offspring of a particular mating. There is a 25% chance that two siblings share the same haplotype and are fully compatible with respect to MHC identity, a 50% chance that they will share one haplotype, and a 25% chance that they share no haplotype and are thus HLA incompatible.

HLA genes and IDDM

Many factors combine to determine the risk of progression to IDDM. These include a family history of diabetes, genotype (*e.g.* HLA haplotype), age, environmental factors, residual insulin secretory capacity, the demonstration of cytoplasmic islet cell antibodies (ICA) on islet tissue sections, and antibodies reacting with characterised islet autoantigens (42, 43). The presence or absence of the others may modify the prognostic significance of any of these risk factors (44). With respect to genotype, polymorphic variants of the class II antigens DQ and DR are recognised as the major contributing genes to HLA-linked susceptibility to autoimmune diabetes (27, 28, 45-47). This notion is supported by transracial studies that have consistently shown the influence of HLA genotypes on the risk of developing Type 1 diabetes (48), the observed conservation of the MHC class II effect in both human and murine autoimmune diabetes (49) and by studies involving MHC transgenic mice (50).

The differences in polymorphic HLA marker frequencies measured between affected and non-affected individuals in case-control studies suggests that a chromosomal region defined by markers on chromosome 6 (location of the MHC) is involved in the disease. In a genetically and clinically heterogeneous diseases such as Type 1 diabetes, approximately 70% of affected individuals carry a specific HLA susceptibility allele (*IDDM1* locus) (51-53). *IDDM1* represents the major IDDM-related susceptibility locus and susceptibility to Type 1 diabetes is mostly conferred by alleles of the DQ region located within the HLA complex. Diabetogenic alleles are not fully penetrant, however, meaning that not every individual who inherits the gene has the disease.

The association between the HLA region and IDDM susceptibility was first documented in case control studies published in the mid-1970s (54, 55). It was observed that the HLA-B8, B15 and B18 alleles showed an increased frequency in patients as compared to a nondiabetic control population. Subsequent serological typing for class II HLA in diabetic individuals revealed a more significant association between HLA-DR and IDDM than that demonstrated for the HLA-B locus (51, 56, 57). In fact, approximately 95% of patients with IDDM in most populations were typed as DR3 and/or DR4, and individuals with heterozygosity for DR3/4 appeared to be most susceptible to progression to IDDM. In contrast, expression of the HLA-DR2 allele was initially considered to be associated with protection from developing Type 1 diabetes.

Although the initial efforts to evaluate HLA associations with IDDM employed serological techniques, advances in molecular biological methods yielding detailed DNA sequence information represent the groundwork from which HLA risk determinants can be refined and prediction of IDDM can be improved. The first of the new methodologies applied to the detailed genetic study of Type 1 diabetes mellitus was restriction fragment length polymorphism (RFLP) analysis (58-60). This technology relies on the differential ability of sequence-specific endonucleases, termed restriction enzymes, to fractionate DNA fragments of a particular size based on DNA base-pair variations within the enzymes' recognition sequences. Detection of differently sized fragments from the DNA of different individuals corresponds to the presence of polymorphisms at the genetic locus under investigation. RFLP analysis allowed researchers to associate serological HLA typings with true inter-individual genetic differences and, for the first time, to evaluate HLA associations with disease at the molecular (DNA) level.

The advent of the polymerase chain reaction (PCR) (61) provided researchers with an even more powerful means of arriving at IDDM susceptibility estimates than the use of serological techniques. PCR amplification of individuals' HLA alleles and direct sequencing of the amplified DNA products has allowed the measurement of single base pair differences previously unrecognised through either serological assessment or RFLP analysis. Studies involving a variety of racial and ethnic groups have revealed that the presence of a specific human DQβ chain variant encoding a neutral amino acid (alanine, valine or serine) other than an aspartic acid residue at codon 57 (non-Asp-57) is strongly associated with IDDM (62-64). In contrast, a negatively charged aspartic acid at position 57 of the DQβ

Table 3 - *Genetic risk estimate for HLA class II in Type 1 diabetes.*

High risk genotypes	Risk in an individual with this genotype
DQB1*0302 (DQ3.2)	1 in 60
DQ3.2/DQ2 (DR3)	1 in 25
DQB1*0302+family history of IDDM	1 in 10
DQ3/DQ2 (DQ3)+family history of IDDM	1 in 4

Adapted from Nepom G.T., *Ann. Rev. Med.* 17-25, 1995.

chain (Asp-57) appears to confer resistance to IDDM progression (45).

Studies aimed at investigating the contribution of the DQα chain to IDDM susceptibility or resistance were initially performed by a group from France. They conducted molecular typing in fifty unrelated IDDM patients and seventy-five randomly selected volunteers using PCR and allele-specific oligonucleotide probes. Their results confirmed the importance of DQβ non-Asp-57 in disease susceptibility, but also implicated a role for the Arg-52 amino acid residue of the DQα chain in the pathogenesis of Type 1 diabetes (65). It should be mentioned that the discovery of these two disease-associated HLA polymorphisms represents a refinement of the IDDM relative risk assessments for the HLA-DR3 and HLA-DR4 disease associations. Because of linkage disequilibrium, the class II HLA extended haplotype inherited with the HLA-DR3 allele includes DQα alleles with the Arg-52 polymorphism and the HLA-DR4 haplotype associates with non-Asp-57 DQβ alleles. The refinement of the DR locus data lies in the fact that the genetically linked DQ associations with IDDM are much stronger than the associations with HLA-DR3 and DR4 (Tables 3, 4).

As mentioned above, HLA-DR3/4 hetereozygotes demonstrate an enhanced susceptibility to Type 1 diabetes as compared to either DR3 or DR4 homozygotes. With our knowledge of the strongly disease associated DQ allele variants, we can begin to translate events at the molecular level into pathogenetic scenarios for the generation of autoimmunity and diabetes progression. Svejgaard *et al* (66, 67) proposed the hypothesis that hybrid HLA molecules are formed by the pairing of an α- and a β-chain, each one deriving from a different copy of the two chromosomes 6 of an individual (Fig. 5). This type of pairing is termed *in trans*, whereas the pairing of chains from the same copy of the chromosome is termed *in cis*. In an individual carrying both the Arg-52 DQα and non-Asp-57 DQβ polymorphisms, the hybrid pairing of these two chains would generate highly "diabetogenic heterodimers", more so than in an individual with either HLA-DQ allele alone. It has been hypothesized that these hybrid molecules would be

Table 4 - *Effect of HLA alleles on Type 1 diabetes susceptibility.*

DQ alleles	Effect	Associated DR
A1*0301, B1*0302	Susceptible	DR4
A1*0501, B1*0201	Susceptible	DR3
A1*0101, B1*0501	Susceptible	DR1
A1*0301, B1*0201	Susceptible (African-Americans)	DR7
A1*0301, B1*0502	Susceptible (Sardinia)	DR2 (DR16)
A1*0301, B1*0303	Susceptible (Japanese)	DR4
A1*0301, B1*0303	Susceptible (Japanese)	DR9
A1*0102, B1*0602	Protective	DR2 (DR15)
A1*0501, B1*0301	Protective	DR5
? B1*0600	Neutral	DR6
A1*0201, B1*0201	Neutral	DR7
A1*0301, B1*0303	Neutral	DR4
A1*0301, B1*0301	Neutral	DR4

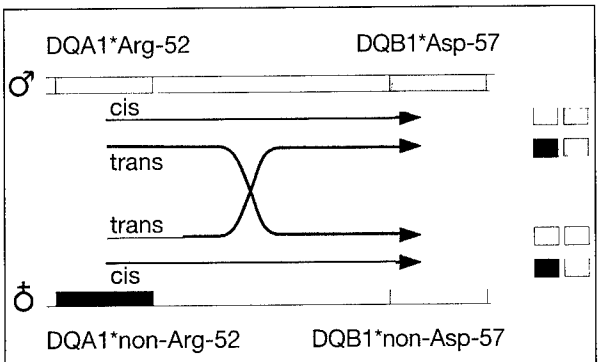

Fig. 5 - *In theory, DQα and DQβ chains generated by the paternal and maternal chromosome 6 can pair* in cis *and* in trans. *Thus, when both parents are segregating IDDM susceptibility alleles at both the DQA1 and DQB1 loci, 4 "diabetogenic" heterodimers can be formed. In this picture, the mother has one susceptibility allele DQB1* non-Asp57 and the father another susceptibility allele DQA1* Arg 52, so that only one diabetogenic heterodimer can be formed* in trans *(two black blocks).*
(Modified from Trucco M., Diabetes Care *15: 705-715, 1992).*

foremost involved in the presentation of the peptide critical for the development of a diabetogenic autoimmune response.

In Caucasians, DQA1*0501/DQB*0201 (Arg-52α) and DQA1*0301/DQB*0302 (non-Asp-57β) heterodimers have the strongest association with diabetes in the context of the high risk HLA-DR3 and DR4 haplotypes, respectively (Table 3). The DRB1 allele is also considered to independently confer susceptibility to Type 1 diabetes (46, 47, 72-78). The frequency of HLA-DR2 is decreased (79, 80) in patients with Type 1 diabetes, and the DQA1*0102/DQB1*0502/DRB1*1601 haplotype accounts for most of the disease susceptibility in DR2-associated cases of Type 1 diabetes (81-86). Therefore, the effect of DR2 haplotypes in diabetes susceptibility is considered to be neutral rather than protective. The effects of various HLA alleles on disease susceptibility are summarised in Table 4.

It has been reported for Type 1 diabetes that HLA susceptibility genes correlate with a young age at disease onset. In particular, IDDM occurring before the age of 15 is highly associated with DQB*0302/DQ2 as compared with IDDM occurring after the age of 15 (87, 88). The risk for IDDM using both genetic and antibody markers has been evaluated in a study of 151 first-degree relatives of probands from the Children's Hospital of Pittsburgh/Allegheny County registries (88). In this prospective study, using a multivariate model, an enhanced relative risk for IDDM was estimated for individuals having 4 HLA-DQ diabetogenic heterodimers as compared to 1 or 2 heterodimers, independently of the islet cell antibody status. Therefore, the greater the potential to generate HLA diabetogenic heterodimers, the higher is the likelihood of developing diabetes during a prospective follow-up in first degree relatives of diabetic probands. This observation was strengthened in several case control studies such as a French study (65) and subsequently by a Spanish study (89). The frequencies of diabetogenic heterodimers (Table 5) were analysed in a population of the Madrid area in 102 individuals with Type 1 diabetes and compared with those of 87 randomly selected non-diabetic control subjects from the general population (Table 5). These findings provide evidence that a quantitative effect promoted by DQα Arg-52 and DQβ non-Asp-57 alleles may account for an enhanced susceptibility to Type 1 diabetes. In other words, the genetic predisposition to IDDM increases as the number of susceptibility alleles in a given individual increases. These data support the molecular hypothesis proposed by Khalil *et al* in which the expression of the heterodimer formed from DQα Arg-52 along with DQβ non-Asp-57 at the cell surface of the MHC increased the diabetogenic effect (65, 90, 91). Dose effect for susceptibility increases with the number of heterodimers that can be formed. Highest risk was for subjects with all 4 possible heterodimers of this type (OR 41, 95% CI: 17-96) (90). Simultaneous expression of 4 different heterodimers of this type explains the very high risk of Caucasian DR3/4 (88).

Because Type 1 diabetes is an autoimmune disease with a long preclinical course (92), the identification of individuals prior to the onset of the disease process provides a real opportunity for predictive testing and for therapeutic intervention. The fact that 4 HLA-DQ diabetic heterodimers are significantly associated with an increased risk for developing IDDM, unlike 0, 1 or 2 HLA-DQ heterodimers, raises the possibility of their use as surrogate predictive markers in individuals who have a significant risk of developing IDDM. An increase in the confidence of our ability to identify seriously at-risk individuals lends support to any future attempts to make therapeutic decisions regarding preventive interventions. At the Children's Hospital of Pittsburgh, a prospec-

Table 5 - *Correlation between quantitative expression of HLA-DQβ Asp-57 and HLA-DQα Arg-52 alleles and risk of development of insulin-dependent diabetes mellitus.*

Genotype	Diabetic patients (*n*=102)		Non-diabetic subjects (*n*=87)		Risk	
DQβ57+DQα52	*n*	Frequency	*n*	Frequency	Relative	Absolute
nD/nD+R/R	66	0.65	6	0.07	30.7	101.3
nD/nD+R/nR	16	0.15	9	0.10	5.2	17.2
nD/nD+nR/nR	2	0.02	10	0.11	0.7	2.3
nD/D+R/R	11	0.11	12	0.14	2.8	9.2
nD/D+R/nR	1	0.01	17	0.20	0.3	1
nD/D+nR/nR	0	0	16	0.18	0.1	0.3
D/D+R/R	3	0.03	6	0.07	1.6	5.3
D/D+R/nR	3	0.03	10	0.12	1	3.3
D/D+nR/nR	0	0	1	0.01	1	3.3
Possible diabetogenic heterodimers						
4	66	0.65	6	0.07	52.4	101.7
2	27	0.26	21	0.24	6.6	12.8
1	1	0.01	17	0.20	0.4	0.8
0	8	0.08	43	0.49	1	1.94

Modified from Gutierrez-Lopez *et al. Diabetologia* 35: 583-588, 1992 (89).

tive family study has been in progress since 1979, and has served as the groundwork for investigating the etiology and prediction of IDDM. To date, we have longitudinally followed over 5300 first degree relatives of IDDM patients, 82 of whom developed the disease. ICA testing was performed in all relatives as an initial screen for islet cell autoimmunity. For the present study, serum samples from 68 prediabetics with at least one year of follow-up before the onset of IDDM (range 1-14.7 years) were analysed. Two hundred forty ICA positive and 199 ICA negative relatives, who did not develop diabetes over time in a prospective follow-up, were evaluated. A survival analysis of the 439 relatives indicated a markedly increased cumulative risk for IDDM in those first degree relatives who can generate 4 heterodimers as compared to relatives who have 0, 1 or 2 diabetogenic heterodimers (Fig. 6). From the original dataset of 439 randomly selected individuals, we then selected 154 family members of IDDM probands based on negativity for antibodies to GAD65 and IA-2, as well as ICA, and performed survival analysis. Remarkably, this analysis showed that in this subset of antibody negative relatives, a condition of 4 heterodimers still confers a cumulative risk of approximately 30% for developing diabetes in a prospective follow-up of 12.5 years. This compares with a risk of 6% for antibody-negative relatives having 0, 1 or 2 diabetogenic heterodimers (Log rank p=0.014) (93). Thus, the fact that 4 HLA-DQ heterodimers can be found in GAD65/IA-2 AA negative individuals who subsequently developed IDDM, substantiates the concept that additional immunological markers may be needed to improve our ability to predict IDDM (94). There is still controversy as to whether DR molecules are also important in IDDM susceptibility (46). Some investigators have argued that DR molecules are not the primary factors which confer IDDM susceptibility (45, 95-97); others believe that the DR locus does play some role in IDDM susceptibility (71, 76, 91,

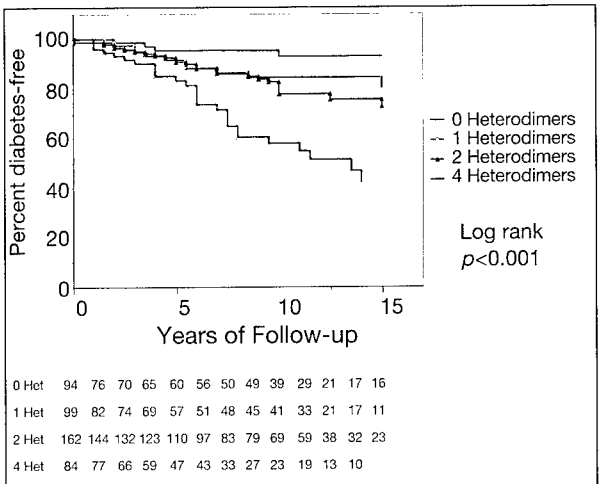

Fig. 6 - *The cumulative risk within 10 years in first degree relatives of Type 1 diabetic patients according to the number of HLA-DQ diabetogenic heterodimers. For the present study, 68 prediabetics (who developed overt diabetes in a prospective follow-up) before the onset of T1DM were analysed and compared with 379 randomly selected relatives who did not develop diabetes over time. First degree relatives of Type 1 diabetic probands were initially evaluated by autoantibody screening at the Children's Hospital of Pittsburgh, as previously reported (88, 181). Four HLA-DQ diabetogenic heterodimers conferred a cumulative risk to develop diabetes of 58% after a follow-up of 14.5 years, compared to a risk of 24%, 20% and 7% for first degree relatives who had 2, 1 or 0 HLA-DQ heterodimers respectively (Log rank p<0.001). These findings provide evidence that a quantitative effect induced by DQβ non-Asp-57 and DQα Arg-52 alleles may account for an enhanced susceptibility to Type 1 diabetes. In other words, the genetic predisposition to Type 1 diabetes increases as the number of susceptibility alleles in a given individual increases.*

98-100). At least three studies have shown an independent effect of the DR molecules in IDDM (96-98, 101-103). In Oriental populations (Chinese and Japanese), susceptibility appears to be conferred by the DRB1*0405 haplotype, whereas DRB1*0403, and DRB1*0406 confer protection in Japanese (101, 104), Sardinians (76) and Spaniards (105). However, the DRB1*0405 haplotype seems to confer strong susceptibility in all ethnic groups, and DRB1*0403 or DRB1*0406 protection (46). The protective effect of DRB1*0403 and DRB1*0406 might overcome the strong susceptibility effect of DQ*0301/*0302. A Belgian group reported that DRB1*0403 protects against IDDM in the high risk DR3/4 heterozygotes (106).

It is commonly accepted that multiple autoantibodies against islet antigens such as glutamic acid decarboxylase (GAD) (107), insulin (108) or IA-2 (109, 110) confer a high risk for developing IDDM in first degree relatives of IDDM patients and, possibly, in the general population (111-115). The combination of HLA susceptibility haplotypes and detectable serum autoantibodies at an age of less than 15 years specifically identifies a very high risk subset of first degree relatives of IDDM patients (78, 116). Because Type 1 diabetes is an autoimmune disease with a long preclinical period (92), the identification of individuals during this preclinical phase of the disease process using both immunological and genetic markers provides a real opportunity for predictive testing. This sets the stage for effective therapeutic intervention during a "window period" of progression to disease onset.

HLA complex and mechanisms of susceptibility or protection from Type 1 diabetes

Amino acid polymorphism at position 57 of the HLA DQB1 chain could influence the interaction between the class II molecule, the peptide antigen and the T cell receptor, consequently controlling the specificity of the immune response to foreign and/or self-antigens (45). However, other residues in the DQβ-chain as well as the DQα-chain appear to be involved in the genetic susceptibility to IDDM.

The importance of class II molecules as players in the pathogenesis of Type 1 diabetes is indicated by studies carried out in a transgenic NOD mouse model. It was demonstrated that the expression of an I-Aβ (equivalent to the human class II DQB allele) transgene carrying Asp-57 instead of Ser-57 prevents these mice from developing diabetes (120, 121). Moreover, expression of Pro-56 instead of the normal His 56 in the I-Aβ chain has the same effect (122). Finally, expression of I-E transgenes (the equivalent of the human HLA-DR allele) appears to confer resistance to the disease (122, 123). Of note, progression to overt diabetes is also prevented when NOD mice are treated with a monoclonal antibody reacting with the murine class II molecule (124). These findings obtained in an animal model of Type 1 diabetes certainly support the role of both HLA-DQ and HLA-DR in human IDDM.

The mechanisms by which the class II genes can influence susceptibility to or protection from IDDM are still the subject of discussion. Brown *et al* (125, 126) have characterised the structure of the crystallized HLA class II molecule. One hypothesis is that

effective antigen binding depends on the conformation of the antigen-binding site on the DQ dimer. The two critical residues, DQα Arg-52 and DQβ Asp-57, are located at opposite ends of the α-helices that form the antigen binding site of the DQ molecule (Fig. 3). It has been postulated that the substitution of an amino acid residue at these positions of the DQ molecule leads to conformational changes of the antigen-binding site and, consequently, to a modification of the affinity of the class II molecule for "diabetogenic" peptide(s) (45). The observation that Asp-57 is involved in hydrogen and salt bonding with both the peptide main chain and the DRα Arg-76 side chain lends support for this hypothesis (126). Theoretically, then, modifications to the DRα Arg-76 residue would also alter the antigen-binding site, but no studies to date have demonstrated such an effect.

Although the actual ratio of *cis*-encoded *vs trans*-encoded DQ heterodimers at the cell surface remains to be determined experimentally, it is possible that moderate differences in chain production translate into large functional differences with respect to antigen presentation and T-cell activation (45). Relatively few class II molecules appear to be required on the surface of an antigen-presenting cell to cross-link the T-cell antigen receptor (TCR) efficiently and initiate a T-cell response (127). On this basis it is easily understandable why the study of *IDDM1* must acknowledge the role of the T-cell, and more specifically the role of particular TCRs in mediating disease. However, the sequence of molecular events that occur during thymocyte maturation, culminating in the expression of a functional TCR, are essentially random. As a result, most recombination events of the gene segments encoding the TCR are nonproductive as a result of out-of-reading-frame joints (41). Fortunately, the number of T-cell precursors undergoing this process is so large that the unselected thymocyte pool still contains 10^{10}-10^{15} distinct antigen specificities from which the peripheral T-cell immune repertoire is shaped.

Once a developing T-cell expresses a functional TCR at the cell surface, it must undergo sequential rounds of positive and negative selection events in the thymus (127, 128). Both of these processes depend upon interactions between the TCR, MHC molecule, and antigenic peptide. Thus, the identity of HLA molecules expressed by an individual control in large part the shaping of the peripheral T-cell repertoire, representing one level at which the HLA-associated genetic susceptibility to IDDM may exert an effect. *Positive selection* of thymocytes occurs as thymic stromal cells bearing MHC molecules (containing self-peptide fragments) engage TCR molecules on the developing thymocytes and direct their continued maturation into functionally mature T-cells. T-cells with "useless" receptors (*i.e.*, those that cannot bind the MHC molecule with sufficient affinity) are not driven to mature and expand, and these cells eventually die. *Negative selection* refers to the poorly understood set of events that specifically eliminates or alternatively "anergises" potentially autoreactive cells, thereby inducing "tolerance" to self (*i.e.*, self-tolerance). During negative selection, factors such as affinity for self-antigen and antigen load likely influence the final outcome of cell death or clonal anergy. Thus, the peripheral T-cell repertoire of each person (including identical twins) is unique (130) and is a consequence of both the random generation of TCRs in the initial unselected thymocyte pool as well as of positive and negative selection events.

Autoimmunity is thought to result from an imbalance between the two functionally opposite processes of *tolerance induction* and *immune responsiveness,* with the scale tipped towards the side of immune responsiveness and reactivity towards self-molecules. In genetically susceptible individuals, certain class II molecules may ineffectively present self-peptides, thereby leading to inadequate negative selection and the preservation of a potentially self-reactive population of T-cells in the periphery. When these self-reactive lymphocytes cannot be held at bay by peripheral tolerance mechanisms, they become free to initiate autoimmune tissue damage. Nepom and Kwok (130) explain the molecular basis of HLA-DQ associations with IDDM exactly on this basis (131). In their disease model, some self-peptides that normally negatively select T-cells paradoxically lead to positive selection and complete T-cell maturation when the MHC molecule is, for example, HLA-DQ3.2.

The HLA-DQ3.2 molecule is encoded by DQA1*0301 and DQB1*0302 genes, which are generally present on the most strongly IDDM-associated haplotype also encompassing HLA-DR4. Due to a characteristic structural motif for peptide binding, the HLA-DQ3.2 can be considered an intrinsically "unstable" MHC class II molecule. If, in a DQ3.2-posi-

tive individual, the T-cells that are negatively selected in the thymus are only those that recognise DQ3.2-peptide complexes in a "stable" high-affinity configuration, the result can easily be the release from the thymus of mature T-cells able to establish a potentially autoimmune repertoire in the periphery. The autoimmune potential of this pool of T-cells results from their ability, in the periphery, to recognise DQ3.2-peptide complexes never encountered in the thymus. These unstable, lower-affinity complexes may have a better chance of forming in the periphery either because of increased "diabetogenic" antigen availability, decreases in competing, high-affinity peptides or other local factors. These differences in antigen presentation between the thymus and the periphery therefore result in the activation of T-cells positively selected for their ability to bind the DQ3.2 molecule only in the context of a high-affinity, tolerising peptide. Figure 7 illustrates these concepts.

Small structural changes, then, may result in large functional changes in the antigen-presenting capabilities of the class II molecules. One might imagine that the cells from a person who is heterozygous for both DQα and DQβ would contain all four chain combinations on their surface. Competition for binding the processed antigen could take place, with effective antigen binding dictated by the conformation of the antigen-binding site on the DQ dimer. Changes at either amino acid DQα-52 or DQβ-57, located at opposite ends of the alpha helices that form the antigen-binding groove (Fig. 3), could alter the configuration of the groove. Changes at both positions, however, would likely inflict a much greater conformational effect on the molecule's antigen-presenting capability. Such conformational differences may be partially responsible for the observed hierarchy in the degree of susceptibility within the group of non-Asp-57 alleles, and for the differences in the degree of protection afforded by each allele within the group of Asp-57 alleles. For example, the protective effect of the Asp-57 allele DQB1*0502 prevails over that of certain susceptible alleles, such as non-Asp-57 DQB1*0501. Conversely, the susceptible allele non-Asp-57 DQB1*0302 dominates over the protective effect of Asp-57 DQB1*0301 .

Positive and negative selection events may also help to explain genetic resistance to IDDM. In many populations, the frequency of the DQB1*0602 allele is rarely found among patients with IDDM (131, 132). This suggests that the HLA molecule encoded by this allele may play a protective role in the disease process. During thymic development, an unidentified diabetogenic peptide can preferentially bind to DQB1*0602 expressed on thymic stromal cells, and, because of the relatively higher affinity and/or avidity of this HLA-peptide interaction with respect to other DQ molecules, it will more efficiently form HLA-

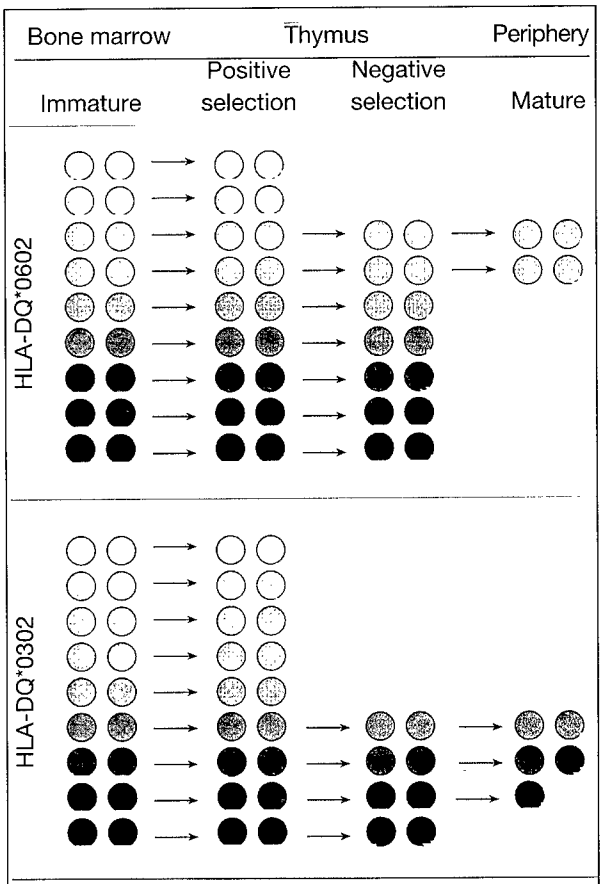

Fig. 7 - *Both positive and negative thymic selections contribute to form the repertoire of mature T-cells in the periphery from the precursors, or immature T-cells, originated in the bone marrow. Individuals carrying HLA-DQ alleles associated with resistance to the disease, like HLA-DQ*0602, will be able to negatively select in the thymus all the T-cells with an high affinity for peptides of the self (●), so that no autoreactive T-cells will be present in their peripheral blood and the chances to develop diabetes will be reduced. Individuals who have instead susceptibility alleles with low affinity for peptides of the self (○) like, for example, HLA-DQ*0302, will negatively select less efficiently autoreactive T-cell clones that will then be present, even if in a relatively small number, among the peripheral T-cells.*
(Modified from Nepom G.T. and Kwok W.T., Reference number: 130).

DQ molecule/antigenic peptide/TCR complexes. This could lead to negative selection and depletion of potentially self-peptide-reactive T cells. Individuals with a typical DQB1*0602 allele can then delete these potentially dangerous T cells during thymic maturation (Fig. 7). The result is protection from developing diabetes. This scenario compares nicely with Nepom and Kwok's susceptibility model in which diabetogenic peptide(s) cannot bind to DQ3.2 molecules in the thymus and, as a consequence, are never "seen" by maturing T-cells (130).

There is some clinical relevance to the observed "protective" effect of the DQB1*0602 allele. At present, carrying the DQB*0602 allele is considered a criterion for exclusion of first degree relatives of diabetic patients from enrolling in clinical trials, such as the U.S.-based Diabetes Prevention Trial 1 (DPT1) (133, 134). This trial has been designed to prevent, by any effective treatment, progression to the clinical onset of IDDM in individuals considered at high risk to develop the disease. However, this does not mean that having the DQB1*0602 allele confers 100% protection from developing IDDM. Individuals with IDDM who are DQB1*0602 positive have been reported, some of whom developed diabetes during prospective monitoring as a first-degree relative of an IDDM patient (135). Thus, although certain HLA alleles may confer strong protection against diabetes, the effect is not absolute. IDDM risk assessment in these individuals will require careful consideration of other factors predisposing to IDDM including a positive family history, autoantibody status, and additional genotypic information regarding other major risk-conferring IDDM genes.

THE INSULIN GENE REGION (*IDDM2*)

Investigation of the insulin gene (*INS*) region on chromosome 11p15 as a premier candidate for genetic association with Type 1 diabetes began in the early 1980s (136-140). Insulin's central role in metabolism and blood glucose homeostasis and its unique distinction as the only known β cell-specific antigen made it a likely frontrunner to account for an inherited susceptibility to diabetes. However, early studies of the human insulin gene and its relation with IDDM, NIDDM and abnormal glucose regulation were inconclusive, presumably due to the relative small sample sizes analysed (136-140). In 1991, Julier *et al* provided evidence of genetic association and linkage for the insulin gene (*IDDM2*) with Type 1 diabetes in a collection of multiplex families from France, USA and North Africa (139). Subsequently, the investigations of Bain *et al* have confirmed the relationship between *IDDM2* and Type 1 diabetes (140). Importantly, Bain *et al*'s finding of significance for the *IDDM2* locus was independent from the influence of HLA alleles (*i.e.*, *IDDM1*) and the parental source of the *IDDM2* susceptibility allele.

Detailed sequence analysis of the insulin gene region identified a polymorphic locus which consists of a variable number of tandem repeats, or VNTR, present within the 5' regulatory region (promoter) adjacent to the coding sequence of the insulin gene. Each repeat element consists of a 14- to 15-bp DNA segment having the consensus nucleotide sequence A(C/T)AGGGGT(G/C)C(T/C/G)(G/A/T)(G/T/A)G(G/C/T) (Fig. 8). The number of repeats within sequenced alleles ranges from 26 to >200, with three classes of alleles identified on the basis of overall size: class I, class II and class III. Class I *INS* VNTR alleles consist of 26-63 repeats, averaging 570 bp in length, and are associated with IDDM susceptibility. Class III alleles consist of 140-200 or more repeats and are considered to be protective from diabetes. In size, class III alleles are the largest variants, averaging over 2.2 kb in length. Finally, class II alleles (1.2 kb average length) are too rare in the populations studied to draw any con-

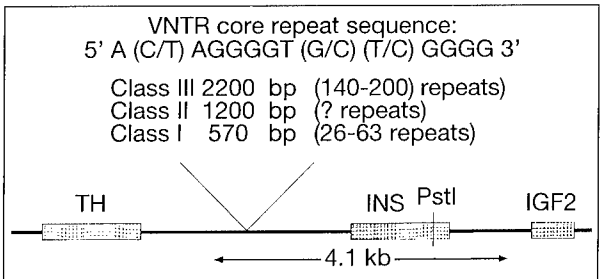

Fig. 8 - *The variable number of tandem repeats (VNTR) in the region 5' of the insulin gene were grouped into three classes. The first with ~40 repeats is called Class I, the one with ~160 repeats is class III, while the number of repeats characteristic of class II genomes has not yet been formally determined. (Modified from Pietropaolo M. Trucco M.: Major histocompatibility locus and other genes that determine the risk of Type I diabetes mellitus. In: LeRoith D., Olefsky J.M., Taylor S.I. (Eds.),* Diabetes mellitus: A fundamental and clinical text. *Lippincot Williams & Wilkins, Philadelphia (in press) (Reference number: 134).*

clusions about their association with IDDM susceptibility (142).

Detailed analyses of the insulin region indicate the presence of a number of additional polymorphisms outside the VNTR region that are in strong linkage disequilibrium with the VNTR itself, which currently appears to be the primary association with IDDM susceptibility. Bennet *et al* reported that a 698-VNTR class I subtype is negatively associated with disease susceptibility, in contrast to other class I alleles which confer susceptibility to disease (142-144).

Biological significance of the VNTR region

A number of studies have suggested that the *INS* VNTR may have a biological role in the genetic regulation of insulin expression (49, 145, 146). The proximity of this polymorphism to the *INS* transcriptional start site (<400 bp upstream) makes this an attractive hypothesis. Furthermore, an association between VNTR polymorphisms and human disease is not unprecedented. It has been suggested that the human *HRAS1* gene, which encodes the H-ras protooncogene and is associated with a genetic susceptibility to certain cancers, is under the allelic effects of a VNTR polymorphism which lies downstream of the gene (147-149). Expansions of nucleotide triplet repeat minisatellites have also been implicated in numerous progressive neurological diseases such as myotonic dystrophy (150). *In vitro* evidence suggests that these genetic elements may exert a regulatory effect by strengthening nucleosome formation, thereby altering local chromatin structure and, consequently, decreasing the efficiency of transcription from nearby genes (150).

Nevertheless, the exact functional role of the *INS* VNTR is still a subject of discussion and debate (151) in view of conflicting reports as to whether class I VNTR-containing promoter constructs induce greater (142, 145) or lesser (146) insulin gene expression *in vitro*. In two early reports (142, 145), it was asserted that class I-associated insulin expression (associated with diabetes susceptibility) was enhanced as compared with class III insulin gene expression, which is notably considered to be associated with protection from Type 1 diabetes. In contrast, Kennedy *et al* (146) have shown that class III VNTR reporter gene constructs had three times higher reporter gene expression than class I VNTR. Nonetheless, the results from Owerbach *et al* (49) do not support those of Kennedy *et al* (146). These controversial results raise the question of whether methodological differences in conducting the experiments may account for the apparent discrepancies. Presently, exactly how the variant insulin gene expression may influence IDDM susceptibility remains to be determined.

THE SEARCH FOR NOVEL NON-MHC SUSCEPTIBILITY GENES

Recent genome-wide searches for IDDM susceptibility genes yielded preliminary evidence for the association of 18 loci with IDDM (152). Because of the large number of markers tested, many of these putative regions suggestive of a genetic linkage with IDDM susceptibility may have occurred by chance alone. It is therefore imperative that these initial observations be confirmed in other datasets as well as in the original dataset using a different set of markers. Saturating the putative locus with many more informative markers is a must to rule in or out the existence of significant, if not true linkage. Further complicating the issue of suggestive linkage from genome-wide scans is the broad range of selection criteria used for inclusion of families in the various datasets. Variations in the age-of-onset of IDDM can make the detection of linkage difficult. Confounding matters even more is the interaction of various disease susceptibility loci that is the hallmark of polygenic diseases like IDDM, in that this interaction (additive, multiplicative or epistatic) adds an additional level of genetic complexity and makes it even more difficult to determine the significance of a LOD (log of the odds) score. Once suggestive linkage to a region has been determined, other powerful association-based tests that take advantage of linkage disequilibrium between a marker and the actual susceptibility alleles can be used to formally implicate a locus and a particular allele with susceptibility (49).

The initial genome scan by Davies *et al* (152) suggested the existence of eighteen potential susceptibility loci (each termed *IDDM*, followed by a number which does not necessarily imply the importance of that locus to susceptibility). The demonstration of significant linkage to some of these loci has been replicated in independent datasets, in some cases implicating a candidate gene. Linkage to other loci originally detected in the scan by Davies *et al* awaits replication. It is important to note that some of the loci which showed suggestive, albeit weak, linkage in the scan by Davies *et al* (152) could

not be confirmed in other populations. This indicates the importance of replication in other datasets, as well as the selection of a dense set of markers to cover as much of the genome as possible. Recent studies have revealed the genetic interaction of certain of these loci and their mode of interaction. For example, *IDDM1* (which is the class II HLA locus) and *IDDM2* (which is the VNTR upstream of the *INS* promoter) interact epistatically (*i.e.* the genes are functionally related, either in a biochemical or a physiological pathway), whereas *IDDM1* and *IDDM4* may act independently (153). In addition, *IDDM4* and *IDDM5* appear to have a greater frequency of shared alleles, identical-by-descent in subgroups of datasets, which have HLA-DR3 in common. The linkage of *IDDM7* is stronger in subgroups lacking HLA-DR3 and finally, *IDDM7* appears to show stronger evidence of linkage in subgroups homozygous for class I VNTR alleles at *IDDM2* (49). All of these results will most likely depend on the nature of the population under study as there are already-established differences among populations in IDDM susceptibility at particular loci. The relative risk of haplotypes at *IDDM2* is dependent upon the presence of HLA-DR4 in a French population (140) but not in American, Belgian, Finnish or British datasets (154).

There is continuous debate, however, as to what criteria should be used when attempting to map non-HLA loci. Historically, in a monogenic disease, the generally-accepted standard for significant linkage is considered to be a LOD score greater than 3 ($p<10^{-4}$). Thomson (155, 156) suggested a statistical level of $p=0.001$ in considering presumable linkage in genome-wide searches, followed by testing of the results in other populations to confirm linkage. Lander and Kruglyak, however, (157) proposed more stringent criteria with a LOD score of 2.2 indicating suggestive linkage ($P<7\times10^{-4}$) and a LOD score of 3.6 ($P<2\times10^{-5}$) to achieve significant linkage (Table 1). Even with a LOD score of 3.6, there is still the possibility of a false positive rate near 5% in genome-wide scans. Moreover, Lander *et al* propose that further replication studies in different populations are necessary in order to verify significant linkage. Very few of the loci indicated in Table 1 will stand the criteria of Lander *et al* for significant linkage. To circumvent this, some investigators propose that HLA exerts a very powerful effect on overall susceptibility and, as a consequence, some loci will be missed although they may play some role in other biochemical or physiological pathways involved in susceptibility. Indeed, one means by which some loci were found has been to stratify based on the number of HLA alleles shared identical-by-descent in sib-pair-based mapping approaches. This method was used to describe *IDDM13* (158). Another approach stratifies based on the presence or absence of autoantibodies (159). To date, there has not been any rigorous statistical analysis on the validity of these stratifications and although they may be justified, the recent failure to find linkage at all described loci except *IDDM1*, *IDDM2*, *IDDM5* and *IDDM8* in more than 500 sib-pairs using stringent criteria (C. Polychronakos, personal communication) may, in part, be explained by the application of such stratifications.

Although much excitement has recently been generated by the results of genome-wide scans, for many polygenic disorders, including Type 1 diabetes, careful and rigorous replication of the original results and gene association studies must be conducted in other populations before any attempts are made by positional cloning or the candidate gene approach to identify potentially elusive sequence variations that could influence genetic susceptibility. The recent publication of two studies with opposing results (160-162) is just one example of controversy which exists in this field.

In sum, genome wide searches are considered only an initial stage in discovering novel susceptibility genes in Type 1 diabetes and in other polygenic diseases. First, linkage must be proven, which is not a small task. Secondly, a large number of families from independent populations are required to confirm linkage and to understand the interactions of putative susceptibility genes.

A PATTERN IS ARISING: CO-LOCALIZATION OF AUTOIMMUNE DISEASE LOCI

Recently, it has been described that the positions of provisional loci found in Type 1 diabetes co-localise or overlap with loci identified in genetic studies of different autoimmune/inflammatory diseases (161, 163). This is consistent with the hypothesis that, like the MHC, some of these provisional loci may involve common susceptibility genes or biochemical pathways that are central to normal immune function. Concannon *et al* (161) identified a novel locus for Type 1 diabetes (MLS=3.31) (Table 1) on human chromo-

some 1q near marker D1S1617. This locus co-localises with loci for systemic lupus erythematosus (SLE) (164) and ankylosing spondylitis (165). In human SLE, genetic variants at this locus are associated with high serum levels of anti-chromatin antibody, and in a mouse model of SLE, similar polymorphisms associate with both anti-chromatin and anti-DNA antibody production (166, 167). This co-localisation of suggestive genetic linkage for three recognised autoimmune diseases indicates that genes at this locus may be involved in a pathway which quantitatively regulates antibody levels, ultimately contributing to the development of the disease phenotype. In summary, particular as-yet-undiscovered genes or pathways may generally contribute to immune dysregulation-a phenomenon detected in many different autoimmune disorders and often observable prior to the onset of overt clinical symptoms (168).

Ten centimorgans is the approximate limit of resolution for a typical first stage genome wide-scan. For example, *IDDM2* found at 11p15.5 (152) is found at the exact position as loci for SLE (169), ankylosing spondylitis (165), asthma (170), and multiple sclerosis (171). All four disease loci have been defined by the same polymorphic marker, D11S922, at 0.323 cM position on human chromosome 11 (172). A candidate gene at 11p15.5 is the insulin gene itself. VNTR polymorphisms in the 5' end of the insulin gene have been associated and linked to IDDM (47). One interpretation of this genetic linkage to insulin as a candidate gene is that there might be an involvement of an imprinted gene (145, 173, 174), which may be under the same transcriptional effects of the VNTR as is the insulin gene. Co-localisation of multiple autoimmune diseases at this location suggests that whatever gene is found to account for the genetic association with *IDDM2* (175) may play a broader role in autoimmune development.

Since this general pattern of locus co-localisation appears not to be found in human non-autoimmune disease (163), it is possible that a pattern of co-localisation of autoimmune disease may be the result of common biological pathways shared among related autoimmune/inflammatory abnormalities in coexisting human autoimmune disorders (163).

CONCLUDING REMARKS

Genome wide scans in Type 1 diabetes have identified over 15 putative loci of statistical significance but only linkage to HLA seems incontestable. It is of interest that familial association of different autoimmune diseases in the same pedigree (163), association of different autoimmune disorders, including IDDM, in the same individual (176, 177), and common clinical parameters of different autoimmune diseases (178, 179) might share a biological basis in a number of autoimmune/inflammatory diseases. Co-localisation and overlapping of candidate loci in autoimmune disease, such as Type 1 diabetes, implies that common biological pathways may be involved in the immunopathogenesis of a subgroup of autoimmune diabetes and other clinically diversified autoimmune disorders.

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The same HLA-DQ alleles determine either susceptibility or resistance to different coxsackievirus-mediated autoimmune diseases

P. LUPPI, A. ALEXANDER, S. BERTERA, K. NOONAN, M. TRUCCO

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ABSTRACT: An important characteristic of autoimmune diseases is their association with major histocompatibility complex class I and class II alleles. In this study, we compared insulin-dependent diabetes mellitus (IDDM) with idiopathic dilated cardiomyopathy (IDC) from a strictly immunologic perspective. Although the target organs are different, being in one case the insulin-producing ß cells of the pancreas and in the other case the myocytes of the heart, many aspects of the tissue-specific immune destruction are common. Similar yet different Coxsackievirus B strains with either pancreotropic or cardiotropic specificity are able to perpetrate the first injury of the respective target tissue. Their shared capacity of inducing a superantigenic reaction further enhances the damage. Once previously secluded autoantigens are then exposed to the immune system, the tissue injury is completed via a more conventional type of immune response. On the basis of the compounded results we obtained, it is possible to propose that the same HLA-DQ molecules which are able to protect the individuals from IDDM (e.g., HLA-DQA1*0102, DQB1*0602) seem to favour the enteroviral attack to the myocardium, while alleles which confer the strongest susceptibility to IDDM (e.g., DQA1*0301, DQB1*0302), seem unable to sustain the immune attack against the heart. (J Biol Regul Homeost Agents 1999; 13: 14-26)

KEY WORDS: *Histocompatibility, Autoimmunity, Insulin-dependent diabetes mellitus, Idiopathic dilated cardiomyopathy, Superantigens*

INTRODUCTION

In 1972, Ceppellini first described the importance of human major histocompatibility complex (HLA) alleles in determining resistance or susceptibility to the development of certain diseases and, in particular, autoimmune diseases (1). These latter disorders are considered to be the outcome of an immune response directed against "self" antigens mistakenly recognized as "foreign". Since his pioneer study, numerous reports have confirmed the existence of an association between certain HLA class I and class II allelic specificities and different diseases. The most classical example of an HLA class I-associated disease is Ankylosing Spondylitis (AS) in which the B27 allele seems to be a "conditio sine qua non" for the development of the typical idiopathic form of the disease (2). In a family study, the presence of the HLA-B27 allele conferred an extremely high relative risk ($\geq$142, $p<10^{-6}$) of developing AS for the carriers, while in a population study, a definite (i.e., based on clinical and radiological parameters) diagnosis of AS was reached in about 20% of B27 positive controls, previously thought to be disease-free (2). Even 25 years after its discovery, the association between HLA-B27 and certain spondyloarthropathies, such as AS, Reiter's syndrome, and reactive arthropathy, continues to attract scientific interest although the molecular mechanism involved in this predisposition still remains to be clarified (3-5).

The HLA class II-associated diseases include a variety of organ-specific and multisystem autoimmune diseases. Among them, rheumatoid arthritis (RA) (6), multiple sclerosis (MS) (7), and insulin-dependent diabetes mellitus (IDDM) (8) have been the most studied. Interestingly, among these three autoimmune disorders, the same HLA class II alleles seem to determine opposite outcomes. On one hand, the HLA-DQA1*0102 and -DQB1*0602 alleles confer the strongest susceptibility to MS (7), but on the other hand, they protect from IDDM development (9,10). In fact, HLA-DQ molecules encompassing amino acids other than Arginine at position 52 of the α chain (α non Arg 52) are coded by DQA1 alleles such as *0102. They are frequently present on the same haplotype with alleles carrying an Aspartic Acid at position 57 of the ß chain (ß Asp 57) that are coded by DQB1 alleles such as *0602. The combination α non Arg 52 and ß Asp 57 confers the strongest protection to IDDM development (11,12). The association between HLA-DQ alleles and IDDM has been confirmed by numerous studies on both animal models and humans, where it has been shown that the genetic predisposition to the disease increases

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TABLE I - EXAMPLES OF THREE AUTOIMMUNE DISEASES AND THEIR HLA ALLELE ASSOCIATIONS

	Rheumatoid Arthritis	Multiple Sclerosis	Type I Diabetes
+	(a) DRB1* 0401 (DR4-Dw4) 0404 (DR4-Dw14) 0405 (DR4-Dw15) (DR1, DRw10, DR6)	(h) DRB1* 1501 (DR2) DQA1* 0102 DQB1* 0602	(h) DRB1* 0401 (DR4) DQA1* 0301 DQB1* 0302 (h) DRB1* 0301 (DR3) DQA1* 0501 DQB1* 0201
AA	DRß 70-74 (neutral → charged)	DRß 86 (Val → Gly)	DQα 52 (Arg → nonArg) DQß 57 (nonAsp → Asp)
–	DRB1* 0402 (DR4-Dw10) 0403 (DR4-Dw13)	(h) DRB1* 0401 DQA1* 0301 DQB1* 0302	(h) DRB1* 1501 (DR2) DQA1* 0102 DQB1* 0602

+ = Positive association; – = Negative association; AA = Amino acid changes in the α or the ß chain which are relevant in allele-disease associations. (a) = Allele; (h) = Haplotype. (6, 7, 8)

with the number of DQ α and ß susceptible alleles able to form "diabetogenic" (i.e., α Arg 52/ß non-Asp 57) heterodimers (13). Similarly, the DRB1*0401, DQA1*0301, DQB1*0302 haplotype, that is one of the two haplotypes that confer the highest susceptibility to IDDM (14,15), is associated with resistance to MS (7). This haplotype contains the allele DRB1*0401 that, like the DRB1*0404 and *0405 alleles, is associated with the highest susceptibility to RA (6) (Tab. I).

The notion that the same HLA class II alleles may play opposite roles in the genesis of different diseases was clear in our mind when we started to study two disorders, IDDM and Idiopathic Dilated Cardiomyopathy (IDC). These diseases are characterized by different anatomical sites of damage (i.e., in one case the pancreatic insulin-producing ß cells; in the other, the myocardium), yet perhaps share a similar etiopathogenesis. In both cases, autoimmunity has been recognized to play a crucial role in disease development (16), while enteroviral infections, mainly mediated by Coxsackievirus B (CVB), seem to trigger the characteristic autoimmune reactions (17).

In 1994, we published compelling evidence supporting the involvement of a superantigen in the etiopathogenesis of IDDM (18,19). Superantigens are protein products of certain micro-organisms (20) known for their ability to engage virtually all T-cells carrying identical or very similar variable segments of their T-cell receptor (TCR) ß chain (Vß) (21,22). Organ damage in both diseases seems to be the result of this powerful immune activation. The search for the source of the "diabetogenic" superantigen thought to be responsible for the immunologic scenario present in the pancreata of children who died at the onset of IDDM (18,19) was first focused on retroviruses, since one of the most studied superantigens is known to be produced by the mouse mammary tumor virus (MMTV) (23). MMTV is an estrogen-sensitive retrovirus responsible, in certain strains of mice, for the development of mammary gland carcinoma. Once this retrovirus has completed its life-cycle in the mammary gland of the mother during lactation, it is transferred to the pups through the milk (24). The presence of the MMTV superantigen in the newborns, i.e., during the initial steps of immunocompetent cell thymic ontogeny, favours the negative selection of T-cells carrying TCR molecules positive for the Vß14 gene family (25,26). The possible presence of endogenous retroviruses that, once activated in the endocrine pancreas, may start to generate superantigen-like molecules was first tested. Exogenous retroviruses were also considered as the agents potentially able to infect the immunocompetent cells of our young patients. In humans, however, the search for a retrovirus as a causative agent of IDDM etiopathogenesis has so far not given any valuable or reliable results (27).

An unexpected clue in the search for the possible source of the IDDM-linked superantigen came from the study of IDC (17). IDC is frequently the complicating outcome of viral myocarditis. Enteroviruses, and especially CVB, are responsible for the initial infection in more than 50% of the cases of myocarditis in North America. Three cases of acute IDC, treated with heart transplantation either at the Children's Hospital of Boston or at our institution, presented the same immunologic characteristics previously observed in the studied IDDM patients (17). The analysis of the TCR repertoire showed that more than half of the T-cells infiltrating the diseased hearts expressed the Vß7 gene family, similar to the pancreata of the IDDM patients (18,19). Other Vß gene families, such as Vß3 and Vß13.1, were also expanded in these tissues. The theoretical conclusion was that CVB could encode the superantigen able to selectively expand a specific T-cell sub-population in the affected tissues from both IDDM and IDC patients (17).

To expand upon our findings, in the study described

hereafter, we used immunohistochemical techniques to investigate the presence of a CVB infection in specimens from the pancreas and the myocardium of our patients. Furthermore, we wanted to assess whether a specific CVB strain was able to selectively stimulate T-cells carrying a restricted set of Vß gene families among the PBMC from healthy or diabetic donors in *in vitro* experiments. Finally, we wanted to determine whether PBMC from donors expressing the same HLA class II alleles characteristically carried by one or the other group of patients, were more reactive to the same strain of CVB than PBMC from donors carrying other alleles.

Materials and methods

Subjects

Pancreas specimens were obtained from #1, a 12 year-old boy, #2, a 13 year-old boy, and #3, a 5 year-old girl. These children shared the same pathologic history: first seen with a chief complaint of vomiting and abdominal pain persisting from previous nights. Physician questioning revealed polyuria and polydipsia that had continued for approximately one month. On the day of admission, the patients were somnolent but responsive, with Kussmaul respirations. Serum glucose levels were in the range of 800 to 1000 mg/dl. Serum acetone was positive. Urinalysis revealed high glucose and ketones, with blood and a trace of proteins. Venous blood gas analysis obtained at admission showed a pH typical of IDDM complicated with diabetic ketoacidosis. Despite fluid rehydration and insulin therapies, the patients soon after became unarousable. Within the next few hours, the patients underwent cardiovascular and respiratory arrest secondary to severe brain swelling with cerebellar tonsillar herniation. The pancreata from the three patients were obtained at autopsy. Pancreatic islet isolation was performed on patient #1 and #2 tissues using a modification of the automated procedure previously described (18). Briefly, the procedure consists of a progressive collagenase digestion of the pancreas retained in a digestion chamber. The islets are then purified on a discontinuous Eurocollins-Ficoll gradient, using a cell separator (COBE, Lakewood, CO). After separation and purification, quantitative and qualitative evaluation of islet number and volume, purity of the preparation, morphologic integrity, and tests of islet viability are routinely performed. The physical condition of the third pancreas was such that islet isolation was not attempted. In this case, mRNA was extracted from total (i.e., exocrinous and endocrinous) pancreas.

PBMC from recently-diagnosed (i.e., within 2 months of the first insulin injection) IDDM patients randomly selected from those seen between January 1996 and June 1997 in the Diabetes Clinic of the Children's Hospital of Pittsburgh, were also tested for their TCR usage.

The IDC patients were #1, a 5 1/2 year-old girl; #2, a 6 month-old boy; and #3, a 14 year-old boy. Here again, the symptomatologic course of the disease was similar in all of them. Healthy until the onset, these children began experiencing several days of fever, vomiting, diarrhea and dehydration, for which they received antipyretics, as well as oral and/or intravenous fluid rehydration. Rapid progression to the signs and symptoms of congestive heart failure led to hospitalization and cardiac evaluation. Findings included radiographic features of cardiomegaly and pulmonary oedema, together with echocardiographic demonstration of globally depressed ventricular function without evidence of structural abnormalities or pericardial effusion. Cardiac catheterization confirmed a poor global ventricular function. Preliminary histological analyses of a few endomyocardial biopsies showed CVB infections, although they were, in general, not completely informative. All patients were diagnosed as having IDC. Due to the rapid and progressive deterioration of cardiopulmonary function (patients #2 and #3) and cardiopulmonary arrest without response to cardiopulmonary resuscitation (patient #1), they were placed on mechanical circulatory assist devices. The patients were listed for heart transplantation after serial echocardiographic examinations documented unremitting severe myocardial dysfunction despite maximal circulatory support. All three patients underwent orthotopic heart transplantation within a few days of decompensation. Intraoperative courses were uncomplicated, and early graft function was good. The patients and the allograft hearts continue to function well approximately 3, 2.5 and 2 years after transplantation, respectively. Anti-rejection therapy with azathioprine, prednisone, and either tacrolimus or cyclosporine, was utilized. The few episodes of clinically silent rejection have responded to methylprednisolone.

Bioptic specimens from the hearts of three additional patients (three boys, 3, 8 and 9 years old) who clinically recovered from an episode of acute IDC without evidence of CVB3 infection, were used as controls.

The pathologist supplied unutilized specimens for these studies. Their use for research was approved by the Institutional Review Boards (IRB) at the Children's Hospital of Boston or at Children's Hospital of Pittsburgh (CHP). This latter IRB also approved the use of both pancreas and heart tissues obtained at the autopsy of an infant who died from renal dysplasia and without histological and histochemical evidence of other abnormalities.

Blood samples from subjects, ranging from 3 to 16 years of age (mean age 10.2 ± 4 7. 9M, 7F), who had been admitted to CHP for orthopaedic intervention were used as "normal" controls, while the blood samples for *in vitro* stimulation studies were obtained from 7 adult blood donors (mean age 36.7 ± 12.3; 2M,

5F). A specific IRB approval covered the use of these samples, as well.

Immunohistochemical procedure

Pancreas and heart fragments obtained, respectively, at the time of the autopsy or at the time of the transplantation, were snap-frozen in isopentane chilled to -150°C, and stored at -80°C. Standard histologic and histochemical techniques were used to prepare 5μm-thick frozen sections. Before staining, the sections were exposed to absolute acetone for 10 mm. Mouse anti-human monoclonal antibodies (mAbs) to Leukocyte Common Antigen (LCA), CD3 (pan-T-cells), CD4 (helper/inducer T-cells), CD8 (cytotoxic/suppressor T-cells), and activated monocytes/macrophages (Ber-MAC3), were obtained from Dako Corporation (Santa Barbara, CA). Mouse anti-human mAbs (Dako) were used to detect insulin and glucagon in the frozen sections of pancreas. The presence of different strains of CVB was determined histologically using mAbs (B1, IgG2a; B3, IgG2a; B4, IgG2b and B6, IgG2a ascites, 1/1000 dilution) from Accurate Chemical & Scientific Corporation (Westbury, NY). Control sections were set up with irrelevant isotype-matched mAbs (IgG1 and IgG2a). Bound mAbs were detected with alkaline phosphatase anti-alkaline phosphatase (APAAP) followed by Fast-Red TR alkaline phosphatase substrate (Dako).

Preparation of RNA and cDNA synthesis

Total cytoplasmic RNA was extracted from pancreas and heart tissues using Trizol® Reagent according to the manufacturer's instructions (Gibco BRL, Life Technologies, Inc., Gaithersburg, MD). Total RNA was prepared from the PBMC of the various subjects using the same protocol. All the RNA samples were maintained in 75% ethanol and stored at -80°C until the cDNA synthesis was performed. For each preparation, 3 μg of total RNA were reverse-transcribed into single-stranded cDNA using the SUPERSCRIPT™ II kit (Gibco BRL Life Technologies).

Molecular typing of HLA class II alleles

Two microliters of the cDNA obtained from either heart or pancreas tissue specimens or PBMC were used for HLA-DR and -DQ molecular typing. In a few individuals, HLA typing was not determined due to the limited quantity of cDNA available. The detection of these HLA class II alleles was performed using a rapid polymerase chain reaction (PCR)-based approach recently developed in our laboratory (28,29). This assay employs sequence-specific priming (SSP) and an exonuclease-released fluorescence (ERF) detection of the amplified product. Briefly, the SSPERF assay is performed by combining in each PCR amplification reaction, the allele-specific primers and a specific double-labelled fluorescent probe, together with control primers and a control double-labelled fluorescent probe. Two different "reporter" dyes are used to distinguish specific and control probes. The fluorescence of the uncleaved probe is "quenched" by a second fluorescent dye bound to the other extreme of the DNA segment. When the probe anneals to its target DNA and is cut by the 5' to 3' exonuclease activity of Taq DNA polymerase, the fluorescent signal is released. Using SSPERF, there is no requirement for post-PCR gel analysis or hybridization; at the end of the reaction, the PCR mixture is directly read in a fluorescence spectrometer.

The various probes are synthesized with one of the three available "reporter" dyes (6-carboxyfluorescein (6-FAM), 4,7,2',4',7',5'-hexachloro-6-carboxyfluorescein (HEX), or tetrachloro-6-carboxy-fluorescein (TET) (all from PE Applied Biosystems, Foster City, CA) at the 5' end. At the 3' end, an amino-modifier C6 dT linker (L) arm nucleotide (Glen Research, Sterling, VA) is added, followed by a 3' phosphate (Phosphalink, PE Applied Biosystems) to prevent extension of the hybridized probe during PCR amplification. Following deprotection, the singly-labelled oligonucleotides are ethanol precipitated, resuspended in 0.25 M $NaHCO_3/Na_2CO_3$ (pH 9.0) and incubated overnight at 37°C with the quencher dye 6-carboxytetramethylrhodamine (TAMRA NHS ester, PE Biosystems). Unreacted dye is removed by passage of the reaction mixture over a PD-10 Sephadex column (Pharmacia Biotech Inc., Piscataway, NJ). FAM-TAMRA, HEX-TAMRA or TET-TAMRA doubly-labelled probes are purified by reverse-phase HPLC using a DeltaPak C18 column (Waters, Bedford, MA) and an acetonitrile gradient (10-80% acetonitrile in 0.1M triethyl ammonium acetate, pH 7.0). Individual fractions are collected, dried down, ethanol precipitated, resuspended in sterile water and evaluated both for DNA concentration and for low (<1) reporter-to-quencher ratios by measuring emission intensities at 518, 538, 556 and 580nm (the approximate maximum emission intensities for FAM, TET, HEX and TAMRA respectively) with the excitation wavelength set at 488nm.

Evaluation of TCR Vα and Vß repertoires

The study of the TCR-Vα and Vß gene segment usage requires individual amplification of at least 29 TCR-Vα and 26 TCR-Vß families and subfamilies (17,18,30). Vα amplification was achieved using a panel of 5'-Vα-specific oligonucleotides (0.5μM) each paired with a 3'-primer-specific for the constant region of the α gene (Cα), tagged with 6-carboxyfluorescein (6-FAM, PE Applied Biosystems). Similarly, Vß amplification employed a panel of 5'-Vß primers and a fluorescent (6-FAM) 3'-Cß oligonucleotide. Vß and Cß oligonucleotide primers were also used at a final concentration of 0.5 μM each in the 50 μl final reaction mixture.

A defined constant region segment of the ß gene

was amplified as an internal positive control in Vα amplification reactions, using a pair of constant region oligonucleotides, 5'-Cβ and 3'-Cβ. This 3'-Cβ primer was labelled with a dye different than 6-FAM, the 4,7,2',7'-Tetrachloro-6-carboxyfluorescein, (6-TET; PE Applied Biosystems). Control primers were used at a final concentration of 0.05 μM. Similarly, as an internal positive control for each Vβ family segment, a pair of constant region oligonucleotides 5'-Cα and 3'-Cα, also tagged with 6-TET, were used at a final concentration of 0.2 μM (17,18,30). Only 28 cycles of PCR were performed to remain in the exponentially increasing part of the reaction (31). PCR conditions were as follows: denaturation at 94°C for 30 seconds, primer annealing at 60°C for 30 seconds and primer extension at 70°C for 45 seconds.

Two microliters of the fluorescently-labelled PCR products corresponding to either the TCR Vα or the Vβ gene families were resolved by electrophoresis on a 16-cm, 5% polyacrylamide non-denaturing gel for 1 hour on an ABI 377 DNA Sequencer and automatically analyzed by GENESCAN software (PE Applied Biosystems). The estimation of the relative abundance of T-cells carrying each Vα or Vβ gene family was estimated by normalization of the fluorescence in each Vα or Vβ band to that in the Cβ or Cα band (the internal positive control) and was expressed as a percentage of the sum of the corrected fluorescence of all the α or β families (i.e., as "corrected" ratios) (31). Two different PCRs were performed for each cDNA sample and the TCR repertoire determined. Average values from the two analyses were then calculated.

Generation of spectratypes

The different lengths of the complementary determining region 3 (CDR3) of the TCR β chain, inclusive of Vβ, (N) Dβ (N), and Jβ segments from the Vβ gene products showing the highest expansion in the samples analyzed were resolved by electrophoresis. Because the CDR3 region of the β chain is the non-germline-encoded hypervariable region thought to carry the specificity for antigen recognition by T-cell (32), an irregular distribution of CDR3 sizes can reveal evidence of an antigen-driven clonal expansion (33). Briefly, 2 μl of the fluorescent PCR product were added to an equivalent amount of formamide/dye loading buffer and heated at 94°C for 2 minutes. Two μl of this mixture were then applied to a pre-warmed 36 cm, 4.25% polyacrylamide native gel and run for 10 hours. Vβ amplification of a polyclonal T-cell population will result in a number of discrete bands. Generally, for each Vβ family, about eight bands at intervals of three nucleotides are seen. The corresponding peaks and their areas (spectratypes) were then displayed and analyzed using GENESCAN analysis.

Infection of Vero and HeLa cells with CVB3

Coxsackievirus titration. The virus was quantitated by the plaque assay method. Ninety-five percent confluent Vero or HeLa cells are obtained by seeding two 12-well plates per each isolate with 1.5 ml of cells at 2.5 x 10^5/ml. Cells become 95% confluent in

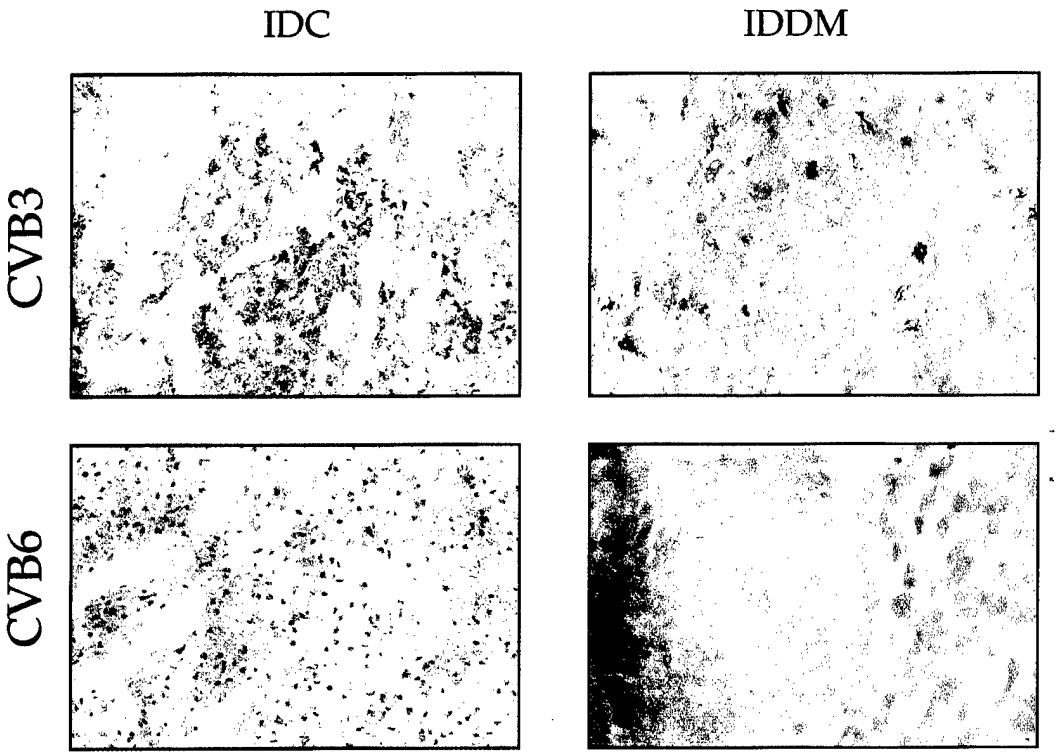

Fig. 1 - Determination in the myocardium of the IDC patients (20x), as well as in the pancreatic islets of IDDM patients (40x) of the CVB3 presence (in red). Immuno-staining with anti-CVB6 mAb was negative in both cases.

1-4 days. The media is aspirated and replaced with 0.1 ml/well of a serial 10-fold dilution (10^{-3} to 10^{-9}) of the viral stock in Minimal Essential Medium (MEM), in triplicates. After viral adsorption at 37°C in 5% CO_2 for 45-60 minutes, 1.5 ml/well of 1% methyl cellulose in MEM is added and the plates incubated for 1-3 days until plaques are visible. The cell layers are stained with crystal violet and the plaques counted.

Preparation of virus stock. Confluent monolayers of Vero or HeLa cell lines in T-175 cm^2 flasks are used 3-4 days post passage. The media is aspirated and replaced with 5 ml of Eagle's MEM supplemented with 10% fetal calf serum and containing $3x10^6$ plaque-forming units of a viral stock to achieve a multiplicity of infection (MOI) of about 0.1. The virus is allowed to absorb at 37° C, 5% CO_2 for 45-60 minutes, and 25 ml of EMEM, 10% fetal calf serum is added. The cultures are incubated for 3-4 days while monitoring cytopathic effect (CPE). At 90-100% CPE, the cells are collected by scraping and then the suspension is centrifuged at 2000 RPM. The supernatant is aliquoted, stored at -70°C, and assayed by plaque titration.

For cell lysate production, the medium and cells are collected as above, but Glycine is added to a final concentration of 100 mM and pH 9.5. The extract is then sonicated and centrifuged as above, aliquoted and stored at -70°C. Finally, the lysate is tested by the plaque assay method to verify that there is no residual CPE.

In vitro *PBMC stimulation with CVB-infected Vero and HeLa cell lysates*

PBMC from healthy adult donors and from recently-diagnosed IDDM patients were isolated from heparinized peripheral venous blood using a Ficoll-Hypaque gradient (1-Step Lymphoprep, Accurate Chemical & Scientific Corporation). For each subject, RNA was extracted from 3×10^6 cells. Also, $1x10^6$ cells/ml were seeded into flat-bottom 24-well culture plates (Corning, New York, NY) and exposed to sonicated cell lysates in complete RPMI-1640 medium supplemented with 10% human AB serum (Normolcera-Plus, Miami, FL).

For one type of experiment, non-infectious lysates from Vero cells exposed to CVB1, CVB3, CVB4 or CVB6, were first tested in dose-response experiments to select the concentration of 2 µl/ml used in the study. Cultures with non-infected Vero cell lysates were used as negative controls, while cultures treated

TABLE II - HLA CLASS II MOLECULAR TYPING OF IDC AND IDDM PATIENTS AND LEVELS OF EXPRESSION OF TCR Vß3, Vß7 AND Vß13.1 IN THE RESPECTIVE TARGET TISSUES

HLA Class II Typing	Vß3*	Vß7	Vß13.1
$CVB3^+$ IDC			
Patient #1 DRB1*07, *13; DQA1*01, *02; DQB1*0201, *0603	21.2	4.4	14.0
Patient #2 DRB1*03, *13; DQA1*01, DQB1*0602, *0603	2.6	55.6	5.0
Patient #3 DRB1*11, *14; DQA1*01, *04; DQB1*0301, *0601	0.8	13.7	9.5
$CVB3^-$ IDC			
Patient #1 DRB1*04, *07; DQA1*02, *03; DQB1*0302, *0303	11.9	6.8	9.7
Patient #2 DRB1*03, *08; DQA1*04; DQB1*0201, *04	5.7	7.2	6.9
Patient #3 DRB1*07, *02; DQA1*01, *02; DQB1*0201, *0501	4.9	8.4	8.9
$CVB3^+$ IDDM			
Patient #1 DRB1*04; DQA1*05, *03; DQB1*0301, *0302	0.7	32.5	6.2
Patient #2 DRB1*01; *03; DQA1*01,*05; DQB1*0201, *0501	3.5	24.5	10.0
Patient #3 DRB1*03, *04; DQA1*03, *05; DQB1*0201, *0302	0.5	45.0	7.0

* The levels of each relevant TCR Vß gene family in the myocardium of IDC patients and in the pancreatic islets of IDDM patients are expressed as median values of percentages of corrected ratios as determined in 3 independent experiments.

with phytohaemagglutinin (PHA) at 5 mg/L were used for positive controls.

In other experiments, non-infectious lysates from HeLa cells exposed to CVB3 were tested in dose-response experiments using different concentrations (2 µl, 5 µl, 10 µl, 20 µl, 40 µl, and 80 µl) at different time points. Cultures with purified human interleukin-2 (hIL-2) (Boehringer Mannheim, Indianapolis, IN) at 5 UI/ml were used for positive controls.

PBMC were cultured at 37°C in 5% CO_2 for 15 days and independently stimulated with each of the different lysates on day 1 and every 3 days thereafter. Purified hIL-2 was added (5 IU/ml) on day 4, 8 and 12. Twelve hours before harvesting, hIL-2 (5 IU/ml) was also added to allow regeneration of potentially modulated TCR (17). At days 4, 9 and 15 of *in vitro* culture, a portion of the cells were harvested, washed, counted and the total RNA extracted. Newly activated (CD71+) cells were isolated from the remaining portion, by immunomagnetic separation, using DynaBeads armed with anti-CD71 antibodies (M-450, Dynal, Inc., Lake Success, NY). RNA extraction was performed directly on cells bound to the beads, and TCR repertoires analyzed.

Results

Immunohistochemical analysis

Heart. The histologic scenario of the cardiac tissue from each of the studied patients was notably heterogeneous with areas of parenchymal damage interspersed with others of apparently normal myocardium. There was a considerable infiltration of LCA^+ white blood cells that were mostly $CD4^+$ and $CD8^+$ T-lymphocytes. $CD4^+$ cells were disseminated within the affected tissues, while $CD8^+$ elements were mostly clustered in multifocal sites of cell damage. Activated monocytes/macrophages were also present in the histological sections, as indicated by their reactivity with Ber-Mac3 mAb (data not shown). Using a mAb specific for the CVB3 serotype, the myocardium of these patients showed a strong reactivity as compared with normal control heart. The immunostaining for CVB1, CVB4 and CVB6 serotypes was negative (Fig. 1).

Pancreas. The histological examination of the frozen preparations of isolated pancreatic islets from diabetic patients revealed a few intact islets with a pronounced LCA^+ white blood cell infiltrate. The infiltrate was abundantly $CD4^+$ and scarcely $CD8^+$. The remaining islets were end-stage islets characterized by the absence of insulin-secreting cells and the presence of elements positive for immunostaining for glucagon (data not shown). Using a mAb specific for the CVB3 and CVB4 serotype, the pancreas of these patients showed a strong reactivity while the immunostaining for CVB1 and CVB6 serotypes was completely negative (Fig. 1).

HLA class II molecular typing of IDDM and IDC patients, normal controls and healthy donors

The HLA class II molecular typing performed on the three IDC patients who were CVB3 positive as determined by immunostaining of the diseased tissues, revealed that all the patients were carrying the DQA1*01 and the DQB1*06 alleles. The same analysis performed on the IDC patients whose diseased hearts were CVB3 negative, revealed that they were not carrying the DQA1*01, DQB1*06 haplotype (Tab. II).

The molecular HLA class II typing of the three IDDM patients who died at the onset of the disease revealed that they were carrying haplotypes conferring the highest susceptibility to the disease. All three patients were heterozygous at both the DQA1 and DQB1 loci and the possibility of forming "diabetogenic" (αArg52-ß non-Asp57) DQ heterodimers (13,14) existed in all the three cases (Tab. II).

The HLA class II molecular typing of randomly selected, normal controls and healthy donors is shown in Tables III and IV, respectively. As expected, their HLA haplotypes were quite different; interestingly, however, normal controls #4, #11, #16 and healthy donors #3 and #7 carried HLA haplotypes associated with high susceptibility for IDDM development. Healthy donors #1, #2, #5 and #6 carried DQ alleles present in all three CVB3-positive IDC patients, i.e., DQA1*01 and DQB1*06.

Analysis of the TCR Vß repertoire of PBMC from normal controls

Semi-quantitative analysis of the TCR Vß repertoire in PBMC from 16 normal controls, showed an equally distributed representation of all the Vß gene families. Amongst these individuals, the Vß4 gene family was the most frequently represented in the T-cells from peripheral blood. Importantly, no enrichment of Vß7 and Vß13.1 in the peripheral blood was seen demonstrating that the skewing found in the patients was neither directly nor solely dependent upon their HLA-DQ phenotype (Tab. III).

Specific TCR Vß skewing of PBMC from healthy donors stimulated with CVB3-infected Vero and HeLa cell lysates

Among the 7 non-diabetic and non-cardiopathic, healthy donors tested against non-infectious lysates of Vero cells infected with different strains of CVB, the four carrying the DQA1*01 and DQB1*06 alleles (i.e., donors #1, 2, 5 and 6) were those who responded more strongly to CVB3 lysates, skewing different combinations of Vß3, Vß7 and Vß13.1 gene families in the activated (i.e., $CD71^+$) PBMC, as compared to the values found in their respective PBMC analyzed before culture. This CVB3 strain seems then able to preferentially activate individuals sharing the DQ

TABLE III - HLA CLASS II PHENOTYPES AND TCR Vß 3, 7 AND 13.1 EXPRESSION VALUES IN THE PBMC OF NORMAL CONTROLS

Normal Controls	HLA DRB1	HLA DQA1	HLA DQB1	Vß3*	Vß7	Vß13.1
1	–	–	–	4.9	3.2	6.1
2	–	–	–	1.4	4.5	7.3
3	02, 11	01	0501, 0602	0.7	4.2	6.2
4	01, 02	01	0501, 0502	6.0	3.9	6.4
5	11, 12	01, 0501	0301, 0501	1.0	8.3	7.9
6	01, 02	01	0501, 0602	9.1	3.5	7.9
7	04	03	0301, 0401/2	6.8	6.9	5.9
8	02, 13	01	0601, 0605	1.2	5.5	6.1
9	–	–	–	4.6	8.2	4.5
10	11, 13	0501	0301	10.6	4.3	5.9
11	03, 04	03, 0501	0201, 0302	5.2	3.7	7.7
12	11 ,13	01, 0501	0603, 0301	11.9	3.9	4.8
13	09, 13	01, 03	0605, 0303	5.9	5.7	7.3
14	02, 09	01, 03	0303, 0601	1.7	6.3	6.8
15	03, 11	0501	0201, 0301	9.1	3.7	6.8
16	03, 12	01, 0501	0201, 0501	5.9	5.8	4.9
Mean				5.4 ± 3.5	5.1 ± 1.6	6.4 ± 1.1

* The levels of each relevant TCR Vß gene family are expressed as percentages of corrected ratios. Mean values ± standard deviation are also indicated

alleles with the IDC patients. One example of selected skewing (i.e., donor #2) is shown in Figure 2, while the values of each relevant Vß gene family in all 7 donors are shown in Table IV. Donors #3 and #7 shared the DQ alleles with the diabetic patients (i.e., the DQ alleles DQA1*0301 and DQB1*0302) (Tab. II). Donor #3 responded to CVB3 infected Vero cell lysates with a three-fold increase of the Vß3 gene family as compared to the value of this family found in her uncultured PBMC. The other two gene families, Vß7 and Vß13.1, did not show any significant expansion. Donor #7 was not reactive to CVB3 infected Vero cell lysates. However, PBMC from diabetic patients carrying IDDM susceptible DQ alleles, tested with the same assay, were actually able to react to the CVB3 infected Vero cell lysate specifically increasing the percentage of Vß7$^+$ T-cells after day 10 of exposure (see below).

CVB3 stimulatory capabilities seem to be somewhat specific for the TCR Vß3, Vß7 and Vß13.1 gene families since the other Vß families did not significantly change in percentage of expression after CVB3 challenge. More importantly, in all donors, cultures with CVB1-, CVB4- and CVB6-infected and uninfected Vero cell lysates failed to significantly increase any of the Vß3, Vß7 and Vß13.1 gene families among newly activated CD71$^+$ T-cells at any time point. The polyclonality of the CD71$^+$ T-cells carrying the most highly expressed Vß gene families after CVB3-infected Vero cell lysate exposure, was demonstrated by the results of GENESCAN spectraype analysis (e.g., Fig. 2).

Since Vero cells are derived from monkeys and HeLa cells from humans, we wanted to assess if we could detect the same Vß gene family expansion with both CVB3-infected cell preparations. To this aim, we repeated previously described experiments, culturing donors' PBMC with different concentrations of lysates obtained from CVB3-infected HeLa cells. As shown in Figure 3, Vß3 and Vß7 gene families were stimulated by almost all the lysate concentrations used on donor #5 at day 11 of culture. The highest values were reached using the highest concentration of CVB3-infected HeLa cell lysates used in this experiment (80 µl). The level of expression of Vß13.1 did not significantly change compared to the value measured in uncultured PBMC.

Analysis of TCR Vß repertoire in PBMC from recently-diagnosed IDDM patients stimulated with CVB3-infected Vero cell lysates

The analysis of the TCR Vß repertoire in PBMC from recently diagnosed IDDM patients showed that, in general, the values of the Vß7 gene family were higher than in the age-matched normal controls even before stimulation. In a recently-diagnosed diabetic patient, the values were 8.8% versus 5.1% ±1.6 of the controls (Fig. 4), when his PBMC were not yet stimulated *in vitro* against CVB-infected cell lysates. Levels of

TABLE IV - SPECIFIC TCR Vß3, 7 AND 13.1 EXPRESSION IN PBMC FROM HEALTHY DONORS EXPOSED TO CVB3-INFECTED VERO CELL LYSATES

Sample from:	Vß3	Vß7	Vß13.1	Vß15
Donor #1: DRB1 *04, *07; DQA1 *01, *03; DQB1 *0302, *0602				
Uncultured PBMC	2.8	9.1	7.2	2.0
$CD71^+$ PBMC	6.9	18.7	10.1	1.5
Donor #2: DRB1 *04, *13; DQA1 *01, *03; DQB1 *0302, *0603				
Uncultured PBMC	0.9	7.7	7.0	1.5
$CD71^+$ PBMC	2.9	12.1	18.4	0.8
Donor #3: DRB1 *03, *04; DQA1 *03, *05; DQB1 *0201, *0302				
Uncultured PBMC	3.5	7.6	7.7	1.6
$CD71^+$ PBMC	12.8	9.8	11.0	1.7
Donor #4: DRB1 *02, *14; DQA1 *01, *05; DQB1 *0301, *0502				
Uncultured PBMC	3.3	8.6	5.6	1.4
$CD71^+$ PBMC	7.1	8.0	13.1	0.6
Donor #5: DRB1 *11, *13; DQA1 *01, *05; DQB1 *0301, *0603				
Uncultured PBMC	3.4	9.4	3.8	1.5
$CD71^+$ PBMC	9.2	10.7	6.0	3.4
Donor #6: DRB1 *02, *07; DQA1 *01, *02; DQB1 *0201, *0602				
Uncultured PBMC	2.2	5.1	6.4	1.3
$CD71^+$ PBMC	5.8	9.3	11.0	0.1
Donor #7: DRB1 *01, *07; DQA1 *01, *02; DQB1 *0201, *0501				
Uncultured PBMC	2.1	5.7	7.1	1.1
$CD71^+$ PBMC	1.9	6.7	6.7	0.5

* Highest values observed during the 15 days of culture are given as examples of TCR Vß3, 7, and 13.1 families of $CD71^+$ PBMC after incubation with CVB3-infected Vero cell lysates, expressed as a percentage of corrected ratios. Uncultured cells were used as base-line controls. Vß15 exemplifies the other unskewed families

Vß13.1 gene family also tended to be higher (9.6%) in the diabetic patient than in the control population (6.4%±1.1). However, when PBMC from this patient were challenged with lysates from CVB3-infected Vero cells, a further increase in the levels of Vß7 gene family was detected among the newly activated (i.e., $CD71^+$) T-cells, after 10 days of culture. This expansion was not detected in PBMC cultured with uninfected or with CVB6-infected Vero cell lysates (Fig. 4).

Discussion

The role played by the alleles at different loci of the HLA complex in many autoimmune diseases should be discussed in the context of the fundamental components constitutive of any immune reaction, since they are most likely involved in the development of these two diseases as well.

The characteristics which define these diseases as "autoimmune" include humoral and cellular immune reactions. The first is represented by the presence of autoantibodies against membrane and cytoplasmic structures of the target tissues in the sera of the patients (34,35). Although for the majority of the autoimmune diseases the critical autoantigens directly involved in triggering them still challenge our specific causative recognition, the most convincing hypothesis supporting an autoreactive type of immune response was offered by Oldstone in his "molecular mimicry" theory of autoimmunity (36). In this theory, he speculated that "foreign" antigens that enter into the body are recognized as such by our immune system that reacts against them using $CD8^+$ T-cells able to efficiently attack and kill the cells hosting the invaders. Other T-cells ($CD4^+$) able to help B-lymphocytes in generating antibody specific for the invader's most antigenic epitopes are also activated. The unusual but possible sharing of some immunogenic characteristics

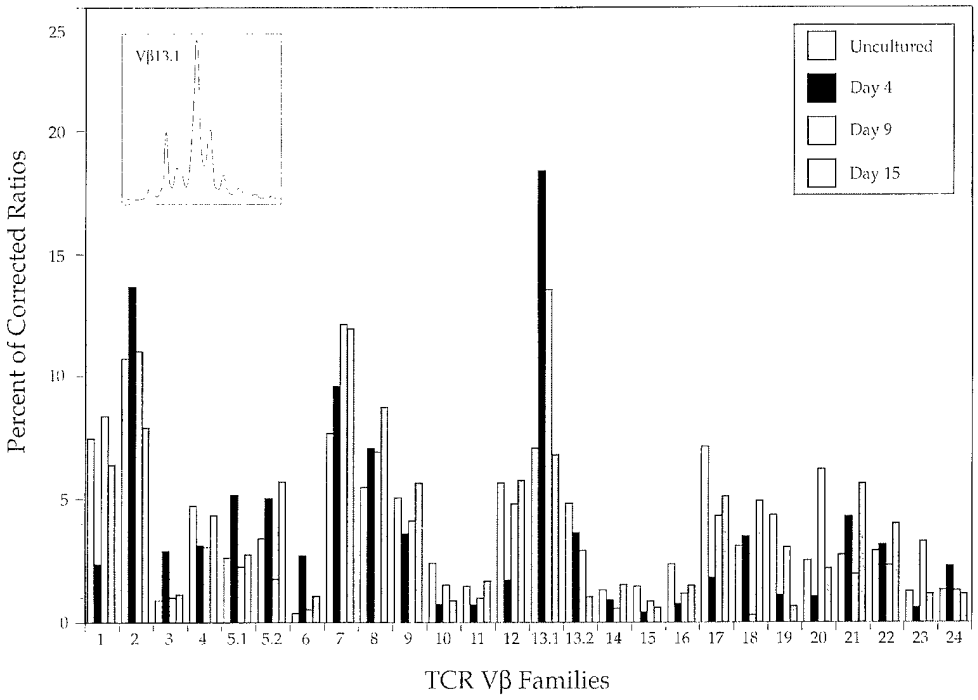

Fig. 2 - TCR Vß repertoire of PBMC from healthy donors cultured with CVB3-infected Vero cell lysates.

Analysis of TCR variable region of the ß chain (Vß) repertoire of donor #2 of Table IV. PBMC, either uncultured or stimulated *in vitro* with cell lysates, were harvested and TCR Vß analysis on CD71⁺ cells performed at 4, 9 and 15 days of culture. Vß13.1 gene family showed the most impressive skewing at day 4 of culture. Spectratype of this family, shown in the upper left corner, indicates the polyclonality of the population of CD71⁺ cells after CVB3 stimulation.

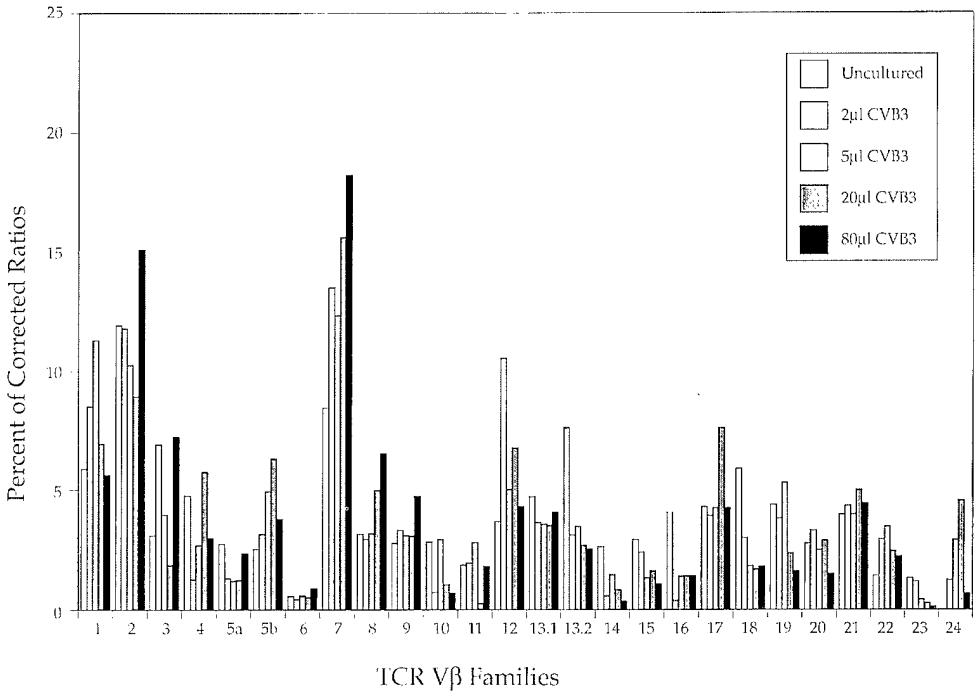

Fig. 3 - TCR Vß repertoire of PBMC from healthy donors cultured with CVB3-infected HeLa cell lysates.

Analysis of TCR variable region of the ß chain (Vß) repertoire of donor #5 of Table IV. Dose-response effect was determined at day 11 of culture. The highest value of Vß7 expression was reached using 80 µl of CVB3-infected HeLa cell lysates.

(i.e., molecular mimicry) between epitopes of the invader with epitopes present in some normal structures of the target organ (i.e., autoantigens), may redirect both T- and B-cell activities so that they mistakenly start to attack "self" structures.

The most limiting aspect of this hypothesis when applied to IDDM or IDC, consists of the fact that there is a high number of normal structures recognized by the various autoantibodies present in the sera of the various patients, but no one seems to be more critical than the other towards disease triggering. Also, the majority, if not all of the best described autoantigens related, for example, to IDDM do not physiologically reside on the membrane of the target cells, but rather in their cytoplasm. Furthermore, these autoantigens do not normally, specifically reside on the target cells of the target tissue only, but are also present in other cells and in other very different tissues. In the case of GAD, one of the most studied autoantigens linked to IDDM etiopathogenesis, it is not only present in the

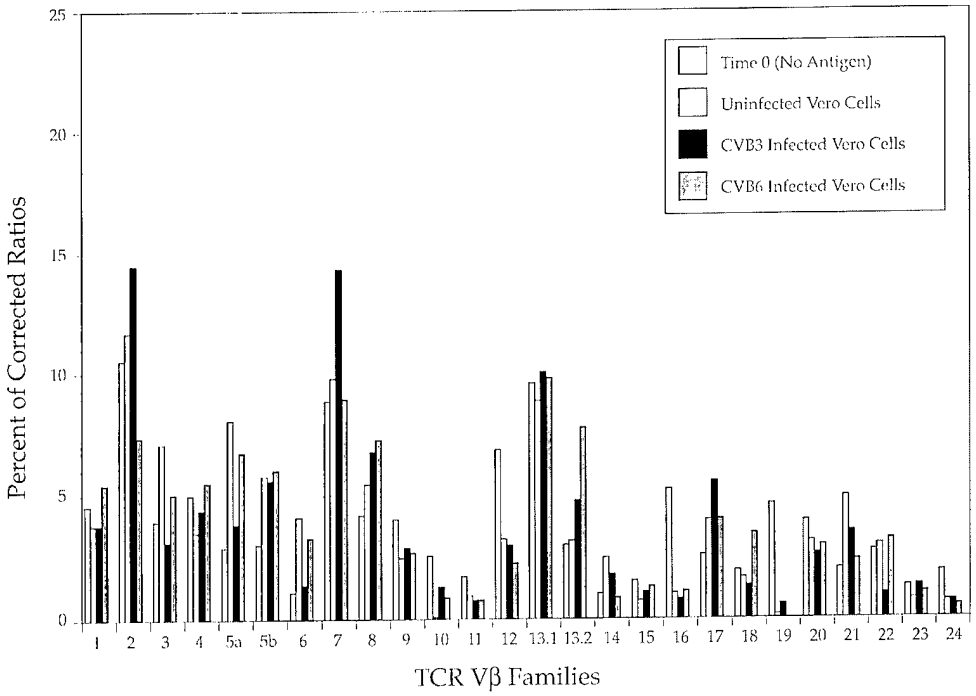

Fig. 4 - TCR Vß repertoire in PBMC from recently-diagnosed diabetic patients cultured with CVB-infected Vero cell lysates.

Analysis of the variable region of the ß chain (Vß) repertoire of a recently-diagnosed diabetic patient at day 10 of culture. A specific expansion of Vß7 gene family was detected only in PBMC cultured in the presence of CVB3-infected Vero cell lysates.

cytoplasm of the ß-cells of the pancreas, but also in other cells of the pancreatic islets and in the cells of the nervous system (37). Finally, T-cells specific for the same autoantigens were rarely found present in the peripheral blood of individuals developing these diseases (38).

Even disregarding this notion, if the restriction normally imposed on the T-cells would explain the importance of certain histocompatibility alleles, either in limiting or in enhancing the susceptibility to the disease, it is still not clear how any specific T-cell can actually be guided to specific targets and how they can specifically kill only certain types of cells if their supposed targets are expressed on other types of cells as well. Furthermore, the particular association of HLA class II alleles to the majority of the autoimmune diseases, seems to suggest that "helper" (i.e., $CD4^+$) rather than "cytotoxic" (i.e., $CD8^+$) T-cells can be activated in the peripheral blood by foreign invaders (39). T-helper-cells of the first type (TH_1) in particular seem to be able to transfer these diseases from one animal to another (40), although $CD8^+$ T-cells are generally considered better target cell killers.

An alternative explanation of the process possibly involved in autoimmune disease pathogenesis may accommodate some of the difficulties we have had in explaining the onset of these diseases just on the basis of classical molecular mimicry and existence of particular autoantigens.

Some foreign invaders have the ability, once encountering the host immune system, to stimulate a very large number of T-cells through a mechanism more permissive than the one normally restricted by the MHC molecules and driven by the TCR. These invaders are able to produce superantigens. Among the numerous T-cells present in the pool activated by a superantigen may exist some normally innocuous, auto-reactive T-cells. These autoreactive T-cells, normally "ignore" the existence of the possible autoantigens, since they never had the chance to be exposed to certain target structures. However, once activated, these T-cells can leave the blood vessel and enter into inflamed tissues. In those tissues in which the autoreactive T-cells finally find their target, a destructive process can then be further enhanced.

A problem still to be solved even using this hypothesis, is why a certain tissue is initially chosen as a target while others are not. This aspect of the process can be explained using the two diseases considered in this study as an example, since we confirmed that CVB could be involved in the etiopathogenesis of both IDC and IDDM. Different CVB strains are known to possess specificities for different targets (41). A cardiotropic strain can cause the first damage of the heart, while a different strain (or a variant within the same strain) with pancreotropic characteristics, may instead cause the initial pancreatic damage that will eventually culminate in overt diabetes. However, some superantigenic properties should be shared by both types of CVB.

Although the healthy donors whose PBMC better reacted to lysates of cells infected with CVB3, carried the same HLA class II alleles as the IDC patients, diabetic patients' PBMC were also stimulated by CVB3-infected cell lysates. Therefore, it is tempting to speculate that another CVB strain exists which is more pancreotropic than the studied CVB3, but perhaps equally able to induce a superantigen-like effect. A superantigen-mediated autoimmune reaction will only amplify the already oriented initial injury,

"opening the doors" to the T-cells' full blunt attack to the target organ.

The presence of mononuclear and dendritic cells in the periductal areas of the islet, once activated by the invading $CD4^+$ T-lymphocytes via their secreted lymphokines (e.g., IL-2 or IFNγ, could be the source of IL-1ß. The autoimmune process leading to IDDM, which brings about the death of the ß cells of the pancreatic islet while sparing islet α and δ cells, finds its specificity in the fact that IL-1ß is capable of inducing Fas expression only on the surface of the ß cells, which are, like the α and δ cells, physiologically Fas-negative (42). IL-1ß-induced Fas antigen expression guides the infiltrating FasL-positive T-cells to cause the apoptosis of the ß cells only. The *in situ* terminal deoxytransferase-catalyzed DNA nick end labeling (TUNEL) test confirmed a specific apoptotic status of only the ß cells in the pancreas of the studied patients (42).

Once the first ß cells are damaged, previously "ignored" self-antigens are recognized in a more conventional fashion by the invading T-cells that share the same TCR Vß gene families. At this point in time, autoantigens will be recognized as conventional antigens in a strictly HLA-restricted mode. $CD8^+$ T-cells will take over after being presented with specific autoantigenic peptides by class I HLA molecules, and will start to progress the damage of the target tissue using, for example, perforin-dependent cytotoxic pathways. This more conventional type of reaction will also be accompanied by the activation of B-lymphocytes that will start to generate the previously described autoantibodies, present in the sera of both IDDM and IDC patients (34,35).

Although superantigens depend on MHC class II molecules for their binding to the TCR-MHC complex, this binding takes place outside the antigen combining site of these molecules and is then not typically MHC allele or, even, species-restricted. A single superantigen can be recognized in the context of multiple class II alleles and isotypes (43). However, different HLA class II alleles display a different capacity to present the same superantigen (44). This characteristic fits nicely with the broad spectrum of resistance or susceptibility-inducing histocompatibility alleles described in association with IDDM and IDC.

Furthermore, the preferential ability of certain HLA class II alleles to bind superantigens seems to be influenced by the presence of certain antigenic peptides in the groove of class II molecules. Numerous crystallographic and binding studies have been performed to assess this hypothesis. It seems clear that different bacterial superantigens (e.g., TSST-1, SEA, SEB) bind to different regions of the class II molecule (43) with a rate of association and dissociation that varies greatly for each superantigen, depending on the presence or the absence of an HLA class II-bound peptide (45).

The influence of non-Vß elements of the TCR and of MHC molecule alleles on superantigen recognition has been suggested to be particularly pronounced for weak TCR/superantigen interactions. Additional contact between TCR non-Vß elements and MHC may influence reactivity by stabilizing or destabilizing the TCR/MHC/superantigen complex (43,45). The superantigen encoded and produced by the different cardiotropic or pancreotropic CVB, may be of this kind.

A very sophisticated, combined effect of $CD4^+$ and $CD8^+$ T-cells, superantigens and autoantigens, HLA class II and class I molecules, seems to be involved in the etiopathogenesis of autoimmune diseases. Our challenge for the future is that of taking advantage of all of the theoretically possible ways of intervention to find the most effective means to avoid the tragic consequences these diseases carry for our patients.

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Review

Hormone Research

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Immunological Models of Type 1 Diabetes

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Key Words

Molecular mimicry · Bystander proliferation · Superantigens · Enteroviruses · Histocompatibility antigens · Tolerance

Abstract

Type 1 diabetes mellitus is a serious health problem that affects several million new people each year. Although it is recognized that type 1 diabetes results from an autoimmune destruction of the insulin-producing β-cells in the pancreatic islets of Langerhans, its etiopathogenesis is still not well understood. A certain genetic phenotype seems to be required, but it is not sufficient per se to trigger diabetes development. Numerous studies have pointed to the role of infectious agents as important environmental factors in breaking 'self'-tolerance and triggering activation of autoreactive T cells. Activated T cells, in turn, destroy target cells harboring the corresponding tissue-specific self-antigen, causing organ damage and loss of function. Several theories have been proposed to explain how environmental factors can initiate and/or perpetuate autoimmunity towards pancreatic β-cells.

Introduction

Type 1 diabetes mellitus results from a selective autoimmune destruction of the insulin-producing β-cells in the pancreatic islets of Langerhans and from associated metabolic abnormalities consequent to impaired glucose homeostasis [1]. The disease is characterized by the appearance of hypoinsulinemia, hyperglycemia and mononuclear cell infiltration of the islets (insulitis). Analysis of pancreatic material from diabetic patients, who unfortunately died at the time of clinical onset, differs from the same analysis performed in animal models where the infiltration consists of a heterogeneous population of cells such as macrophages, dendritic cells, B and CD4+ and CD8+ T lymphocytes. In humans, T lymphocytes predominate in the inflammatory infiltrate [2] and are considered the major disease mediators believed to react with islet cell antigens. Because the specificity of the immune attack is exclusively directed towards β-cells, the role of unique β-cell antigen(s) has been invoked [3], although its actual nature and its involvement in the disease process still needs to be clarified. Uncertainty also exists regarding the pathogenic role played by the autoantibodies which are considered to be the best markers of the ongoing autoimmune process. Autoantibodies specific for a

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number of islet-cell autoantigens, such as insulin, glutamic acid decarboxylase 65 (GAD 65), and IA-2, a cytoplasmic tyrosine phosphatase, are present in the sera of patients with type 1 diabetes for several years before the clinical onset of the disease [4, 5]. However, the direct participation of autoantibodies in the initial immune-mediated attack against the β-cells is probably not relevant. The common belief is that the destruction of the insulin-producing β-cells occurs over a prolonged period of time, eventually culminating in the delayed appearance of the disease [6]. Unfortunately, in humans it is not possible to actually dissect the kinetics of β-cell destruction because specimens from diseased pancreata at different time points cannot be easily obtained. However, the establishment of small animal models, in particular the non-obese diabetic (NOD) mouse strain that has a high frequency of spontaneous diabetes and mimics many of the principal pathological features of the human disease, has greatly facilitated the study of diabetes [7]. Diabetes genesis in these mice has been shown to involve a series of stages in which mononuclear cells first infiltrate the peri-islet regions (peri-insulitis) and slowly progress to an islet invasion. Only when the invaders have destroyed the majority (95%) of the insulin-producing β-cells, overt clinical diabetes eventually develops.

Evidence for the central role played by T cells in β-cell destruction is now extensive. The disease does not appear in NOD mice that are congenitally athymic or that have been neonatally thymectomized, and can be prematurely induced in irradiated NOD mice by the transfer of splenocytes and T lymphocytes from diabetic animals [1, 7]. Both subsets of CD4+ and CD8+ T cells are required for islet invasion and β-cell destruction [8]. However, the relative contribution of each subset of T cells in triggering or perpetuating diabetes is not clear. In particular, it is controversial whether each subset has a unique function. CD8+ T lymphocytes seem to be required to initiate β-cell injury, which in turn could lead to CD4+ T cell priming and subsequent amplification of the response. Nonetheless, several reports have demonstrated that CD4+ T cells by themselves are able to cause rapid onset of the disease when transferred from diseased NOD mice to young healthy NOD animals [7].

Cytokine imbalance also constitutes an important factor in the etiopathogenesis of type 1 diabetes. CD4+ T lymphocytes fall into two major categories: T helper 1 (Th1) cells, which primarily secrete interferon-γ (IFN-γ) and interleukin 2 (IL-2), promote cell-mediated immunity; and Th2 cells, which produce, for example, IL-4 and/or IL-10, downregulate Th1 cell activity and are mainly involved in humoral immunity. Immune responses are regulated by the balance of Th1 and Th2 cytokines. Several reports have associated diabetes with the Th phenotype, leading to the idea that Th1 cells promote disease, while Th2 cells would be protective. The most substantial arguments supporting this concept derived from studies in mice [8]. A diabetogenic T-cell clone from NOD mice which carries a Th1 phenotype [9] can provoke by itself a rapid onset of diabetes in NOD neonates [9]. Th2 cells have been shown to be able to home to the pancreas and to invade the islets of Langerhans. However, they seem to lack the ability to destroy β-cells and trigger diabetes [10]. Modification of the cytokine milieu by the systemic administration of cytokines or monoclonal antibodies to cytokines that favor Th1 or disfavor Th2 cell development generally induce or accelerate diabetes development [11]. IFN-γ gene expression in pancreatic islet-infiltrating mononuclear cells correlate with diabetes onset [12] while transgenic mice carrying a mutation at the IFN-γ receptor showed a drastic effect in limiting both the kinetics and the penetrance of insulitis, thus preventing any signs of diabetes [13]. Interestingly, activated monocytes and T cells able to generate an overproduction of certain Th1-type cytokines have recently been detected in the peripheral blood of recently diagnosed diabetic patients [14].

A critical role for B lymphocytes has also been invoked in the pathogenesis of type 1 diabetes. B-cell-deficient ($Ig\mu^{null}$) [15] or anti-Igμ-treated NOD mice [16] develop neither insulitis nor diabetes. One of the hypotheses to explain B-lymphocyte involvement in diabetes proposes that these cells have more influence on diabetes etiopathogenesis by presenting autoantigens to CD4+ T cells rather than by secreting autoantibodies [7].

Type 1 Diabetes: A Complex Disease

In spite of many years of intensive research, the precise cause and the mechanism(s) triggering the immune-mediated β-cell-specific attack are still undetermined. Furthermore, in the majority of cases diagnosis is made only at the time of acute onset, when ketoacidosis complicates the patients' clinical condition. Insulin therapy is so far the most successful approach to control hyperglycemia and to prevent or delay the occurrence of diabetic complications. Pioneering therapeutic approaches based on the administration of self-antigens (such as insulin or GAD65) to NOD mice and to biobreeding diabetes-prone rats prior to the onset of diabetes, resulted in a delayed onset or in a marked protection from the disease [17, 18].

Although the precise mechanism by which this protection is achieved is not known, it has been proposed that this immunotherapeutic strategy could induce active tolerance by shifting the phenotype of T cells and local cytokine secretion from a Th1 to a Th2 type, so that Th2 cells would protect the target tissue [18]. Similar encouraging results have also been reported in humans and clinical trials of insulin prophylaxis to individuals 'at high risk' for developing diabetes have recently been initiated in several medical centers around the country. This is known as the Diabetes Prevention Trial Type 1, a trial that will last 5 years and is aimed at definitively answering the question whether prophylactic insulin administration is sufficient to block diabetes development in individuals at risk for the disease. Effective methods of type 1 diabetes prevention and treatment would require intervention at an early, preclinical, still asymptomatic stage. Unfortunately, to date there are no 100% reliable tests for the screening and the prediction of individuals at high risk to develop type 1 diabetes in the prodromal period. These individuals are only identified by the presence of disease-associated human histocompatibility complex (HLA)-alleles, serological (i.e. presence of autoantibodies in the serum) and specific metabolic (i.e. intravenous glucose tolerance test) alterations [19]. Approaches to better define individuals at risk for type 1 diabetes have been mainly suffering from the fact that its pathogenesis is multifactorial. As in other autoimmune diseases, e.g. multiple sclerosis and rheumatoid arthritis, many factors contribute to the initiation and/or to the progression of type 1 diabetes. Therefore, a complete understanding of its genesis should not derive from the analysis of one, single, causative factor but rather from the comprehension of the complex interaction amongst different and still mostly unknown events. It is now clear that both host genes and environmental events (i.e. diet and infections) could mediate susceptibility to type 1 diabetes by somehow triggering immune responses toward tissues or organs that should instead be protected by 'self' tolerance. The pattern of inheritance of type 1 diabetes is complex and an increasing number of loci, scattered on many chromosomes throughout the genome, seem to be involved [20]. However, the inheritance of particular major histocompatibility complex (MHC) class II alleles constitutes the strongest genetic risk factor for susceptibility or protection to type 1 diabetes [20]. Like the human disease, diabetes in NOD mice is under polygenic control, with the most important contributor being the MHC complex and the allele H-2^{g7}. MHC molecules are surface glycoproteins located on antigen-presenting cells (APC). Their binding site accepts heterogeneous peptides and present them to T lymphocytes. The I-Aβ^{g7} allele in NOD mice and certain HLA-DQβ alleles encoding for molecules encompassing an amino acid other than aspartic acid (serine, valine, or alanine) at position 57 of the β-chain (β-non-Asp-57) correlate with susceptibility to type 1 diabetes. Conversely, aspartic acid at position 57 of the β-chain (β-Asp-57) is associated with resistance to disease development. Furthermore, it has been shown that the absence of an aspartic acid at position 57 of the DQβ chain is influenced in promoting susceptibility to diabetes by the presence (α-Arg-52) or the absence (α-non-Arg-52) of an arginine residue at position 52 of the DQα chain. Interestingly, position 57 of the DQβ chain and residue 52 of the DQα chain are located at the opposite extremities of the α-helices forming the antigen-binding groove of the MHC molecule on APC. Genetic predisposition to type 1 diabetes increases, in fact, with the number of DQα- and DQβ-susceptible alleles able to form the so-called 'diabetogenic' heterodimers (i.e. DQα Arg-52/DQβ non-Asp 57) [21]. However, since only some of the genetically susceptible individuals develop diabetes, as exemplified by the limited concordance (<50%) for the disease in identical twins, susceptibility at MHC class II seems to be a necessary but not a sufficient predisposing factor. There must then be additional 'environmental' events contributing to the progression toward the overt clinical disease. Indeed, a large body of clinical and epidemiological studies indicates that infections are important in the induction of several autoimmune diseases, type 1 diabetes included.

The Crucial Point in Autoimmunity: Loss of Self-Tolerance

Self-tolerance is a central dogma of immunology that is absolutely required for maintenance of good health. Self-tolerance is based on the peculiar ability of the immune system to discriminate between structures of the 'self', that need to be protected, and 'non-self' structures (i.e. pathogens), that have to be eliminated or sequestered. In autoimmunity, immune responses are pathologically raised against body structures that normally have to be preserved because part of the self. This leads to the idea that in autoimmunity there is a loss of tolerance to self-antigens. In the majority of cases, T lymphocytes constitute the effector elements. These T cells are obviously autoreactive because they destroy targets harboring the corresponding tissue-specific self-antigens. However, according to the principles of the self/non-self discrimina-

tion, autoreactive elements should have been eradicated or rendered irreversibly harmless by the process of negative selection occurring in the thymus during T lymphocyte ontogeny. This mechanism is based on the encounter of T lymphocytes with self-antigens presented in the context of MHC molecules on thymic APCs. Briefly, lymphocytes reacting with high affinity with self-peptides/MHC complex are clonally deleted or functionally inactivated (anergy), while lymphocytes weakly interacting with self-peptides, but strongly with MHC molecules, receive protective signals and are positively selected for export in the periphery. However, studies in animal models and in humans have shown that any healthy immune system contains potentially autoaggressive T cells. In type 1 diabetes patients as well, circulating autoreactive T cells responding to the islet/brain self-antigen GAD have been demonstrated [22]. Several hypotheses have been proposed to explain this phenomenon. One proposes that most T-cell epitopes of a certain autoantigen might not be expressed (cryptic epitopes), or might not be generated in sufficient amount in the thymus to bind to MHC molecules so to be recognized by antigen-specific T cells. In this way, T cells specific for cryptic epitopes escape thymic control. This seems not to be true in the case of myelin basic protein (MBP), one of the most important antigenic targets in multiple sclerosis. MBP is present in the thymus during lymphocyte development and myelin-specific T cells are actually detectable in the blood of laboratory animals and human donors [23]. Therefore, it has been inferred that the mechanism of thymic tolerance though sophisticated is not a perfect one.

In most individuals, circulating autoreactive T cells reside in the immune system throughout the entire life without causing any damage. Potential autoaggressive responses are downregulated and kept in a state of unresponsiveness called 'immunological ignorance' [24]. Since these mechanisms are at work in the periphery, the state of tolerance achieved has been named 'peripheral' (i.e. extrathymic) as opposed to the 'central' one (i.e. thymic). One factor that might have a great impact upon peripheral tolerance is the cytokine milieu in which self-antigens are expressed. For example, the transgenic expression of transforming growth factor β (TGF-β1) in pancreatic islets of NOD mice seems to shift APC preference from B cells to macrophages, thus deviating T-cell responses from a Th1 to a protecting Th2 phenotype preventing diabetes [25]. Peripheral tolerance could also be the outcome of the particular location of certain self-antigens. For example, antigens hidden behind an anatomical barrier (e.g. blood-brain barrier), or confined in small, even if very blood-rich, portion of the body (i.e. islet of Langerhans), or bound to non-professional APC, are somewhat out of sight, so that circulating autoreactive T lymphocytes are prevented to mount an immune attack against them.

The question of how a pathogenic autoimmune response develops has been of interest of immunologists and clinicians for many years. Although many mechanisms have been invoked, it is still not clear how autoimmunity is elicited. From the data available so far in the literature, the triggering point should be identified in a failure of peripheral tolerance. For some reason, circulating autoreactive T cells are no more kept 'under control' and autoimmune responses become evident. Exposure to human viral infections has been recognized as the most important event in inducing, accelerating or enhancing newly formed or already present autoimmune responses. This has been demonstrated in experimental animals and supported by robust evidence in humans [26]. Basically, microbial infections may cross-activate autoreactive T cells. This activation of T cells may occur in the periphery or even locally at the tissue site of the autoimmune damage. In multiple sclerosis and other inflammatory demyelinating brain diseases, activation of encephalitogenic T cells most likely occurs within the peripheral immune system rather than in the brain itself. In fact, only freshly activated T cells are capable of crossing the endothelial blood-brain barrier separating the blood from the brain parenchyma. In other words, activation of circulating autoreactive T lymphocytes occurs in the absence of the nominal self-antigen, which is sequestered within the CNS. In type 1 diabetes as well, numerous hypotheses have been proposed to address the question of how potentially autoaggressive T cells are activated. Most of the literature on this topic focuses on infections caused by enteroviruses, and especially by coxsackievirus B (CVB) [27]. CVB4 is the most commonly detected CVB strain in prediabetic and diabetic patients [28]. A CVB4 strain was isolated from a patient who died at the clinical onset, passaged through murine pancreatic β-cells and found to induce diabetes in mice [29]. Although the pathway by which CVB4 may induce diabetes in humans is not well understood, both the pancreatropic nature of the virus as well as its reported similarity to the islet cell antigen GAD could contribute to its diabetogenicity. Recently, CVB has also been suggested to carry or to induce a superantigen-like activity [30].

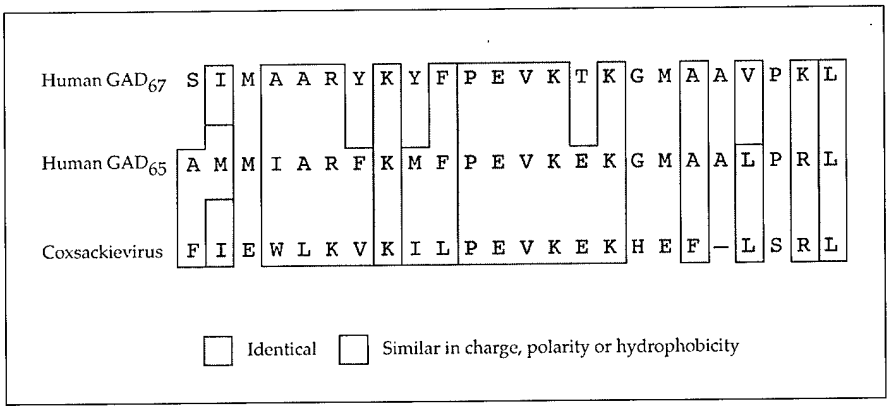

Fig. 1. The amino acid sequence PEVKEK is shared between the P2-C protein of CVB4 and GAD65 autoantigen. This homology is considered to be so far the best example for a molecular mimicry-based explanation of type 1 diabetes etiopathogenesis (modified from Kaufman et al. [33]).

Molecular Mimicry Hypothesis of T Cell Activation

The concept of molecular mimicry as a pathogenic mechanism in T cell-mediated autoimmune diseases has been proposed by Oldstone [26] more than 1 decade ago. Recently, this theory received considerable attention when the activation of autoreactive T cells was reported to be associated with the onset of several diseases [26]. Molecular mimicry postulates that structural similarity (i.e. sharing of linear amino-acid sequences or conformational shapes) occurs between certain pathogen epitopes and self-protein(s) of the host. Autoimmunity may occur when T cell reactivity to an infecting pathogen results in the activation and expansion of T cells cross-reactive against a biologically relevant epitope of an autoantigen. In other words, when the host elicits an immune response against a microbe, a cross-reactive immune response is also generated against a self-protein. The inflammatory response may be self-perpetuating once, being the infectious agent successfully cleared, self-antigens keep to be released as the consequence of tissue destruction. This phenomenon is known as 'determinant spreading' by which the initial immune response directed toward an epitope of a protein antigen spreads to other epitopes of the same protein (intramolecular spreading) and then to different proteins (intermolecular spreading) within the target tissue. One of the best examples of molecular mimicry in autoimmunity is described by Wucherpfennig and Strominger [31] who demonstrated that a significant degree of cross-reactivity existed between CD4+ T-cell-recognized epitopes of viral structural proteins and a certain peptide of MBP. Microbial peptides molecularly mimic myelin when both stereochemically fit into the HLA-DR2 binding groove, the HLA-class II molecule conferring the strongest genetic susceptibility to multiple sclerosis. Extension of this finding to type 1 diabetes suggested that diabetogenic peptides should bear a negative charge at P9 (i.e. in the position corresponding to one of the critical pockets of the HLA antigen binding site) in order to bind preferentially to HLA-DQ susceptibility alleles [32]. In type 1 diabetes pathogenesis as well, there is some evidence that could support the involvement of molecular mimicry. First of all, a sequence similarity has been identified between the P2-C protein of CVB4 (a protein involved in virus replication) and the islet cell 65-kD antigen GAD65 (fig. 1). A stretch of 6 amino acids (PEVKEK) is shared by the two proteins [33]. In the NOD mouse which carries a diabetes-susceptible allele, the immune response against CVB4 activates T cells that cross-react with the GAD65 peptide [34]. Furthermore, in mice cross-reactive immune responses are induced by the same peptide [34]. The shared cross-reactive epitope is a major GAD determinant in 25% newly diagnosed diabetic patients and in 47% of 'high-risk' subjects. In contrast, peripheral blood mononuclear cells (PBMC) from healthy controls did not proliferate as a reaction to both GAD and CVB4 peptides. Recently, Honeyman et al. [35] reported that a dominant epitope present in the protein IA-2 (amino acids 805–820), which is a molecular target of autoimmunity in type 1 diabetes, had 56% identity and 100% similarity for 9 amino acids of VP7, a major immunogenic protein of human rotavirus. Furthermore, the contiguous sequence of VP7 has 75% identity and 92% similarity with a known 12-amino-acid-long T cell epitope of GAD. Also, the immunologically dominant IA-2 peptide showed significant identity and similarity to sequences present in numerous other pathogens, like cytomegalovirus, measles, hepatitis C, herpes, rhino-, hanta- and flaviviruses.

To better address the question of virus-induced autoimmune diseases, transgenic mouse models have been created. One elegant study has used transgenic mice specifically expressing lymphocytic choriomeningitis virus (LCMV) antigens on their pancreatic β-cells [36]. Expression of the transgene does not lead to spontaneous islet cell destruction or infiltration. However, T cells potentially reactive to LCMV antigens are present in the peripheral blood of transgenic mice and when these mice are challenged with exogenous LCMV, they develop diabetes [36]. This suggests that activated LCMV-specific T cells present in the periphery are harmless until they become able to physically encounter the specific antigen also present on pancreatic β-cells. When the mice are infected with LCMV, the immune response specifically generated against the virus not only eliminates the viral infection, but also cross-reacts with the LCMV viral transgene expressed on the β-cells [36]. In addition, these studies demonstrate that a viral gene introduced as early as an animal's egg stage, incorporated in the germline, and eventually expressed in the islet cells does not produce an irreversible state of tolerance. Conceptually, in fact, the viral genes inserted in the germline of transgenic mice become self-antigens and then any antiviral response also becomes an autoimmune response [36]. Based on the results of these experiments, two interrelated but separate events could be envisaged to occur in type 1 diabetes. First, an initial exposure to a virus early in life results in a low level expression of viral genes in the β-cells. This event by itself does not cause disease, especially if the host is hyporesponsive or tolerant to the viral product. Alternatively, the first event could be represented by the expression of a self-protein sharing antigenic determinants with a virus. Later in life, the second event occurs: the infection with the same (or a cross-reactive) agent triggers an immune response designed to clear the new infection which also generates T cells and/or antibodies against the target cells (e.g. β-cells), thus progressing to an autoimmune disease (e.g. diabetes) [36].

Superantigen-Based Model of T Cell Activation

Superantigens are microbial protein products [37] known for their ability to engage virtually all T cells bearing identical or related TCR Vβ chains. Unlike conventional antigens, superantigens bind, in their native conformation, outside of the MHC-binding groove on APCs, tying together the lateral part of this molecule and the Vβ portion of the TCR. The interaction with the Vβ chain is mostly independent of other variable components of the TCR, e.g. Vα, Jα, Dβ and Jβ as well as of the MHC-class II allelic segments carried by the APC (fig. 2). An immune response directed against a superantigen is characterized by the skewing of the TCR Vβ chain representation, manifested by an expansion of T cells expressing particular Vβ families, associated with a random representation of the remaining TCR components, in particular the Vα families. Because the TCR repertoire encompasses a limited number of families with Vβ elements very similar in sequence, any superantigen is able to activate a large fraction of the pool of circulating T cells (5–30%), in contrast to conventional antigens which normally activate $\approx 1 \times 10^5$ T cells. As a result of such a massive T-cell activation, inflammatory Th1-type cytokines are secreted.

Various reports have demonstrated the association of superantigens with the development of several human pathological conditions, autoimmune diseases included. Two typical puberty-onset cases of type 1 diabetes complicated with ketoacidosis were carefully studied for the TCR repertoire of the T cells infiltrating their pancreatic islets [2]. Analysis of the TCR repertoire within these T cells showed an exceptionally high level (>30%) of the Vβ7 gene family, while the TCR Vα usage revealed no selection of any particular gene family. This result was highly suggestive of a superantigen-driven immune response. The same TCR repertoire distortion was reproduced by in vitro cultures of PBMC from normal adult donors with islet-cell membrane preparations of 2 diabetic patients, suggesting that the superantigen was actually present within the pancreatic islet cells. More recently, we had the opportunity to confirm this observation by studying the phenotype of T cells infiltrating the pancreas of another diabetic patient who died at the clinical onset in very similar conditions. Here again, the majority of the pancreas-infiltrating T cells carried the TCR Vβ7 gene family [38]. High frequency of Vβ7+ T cells has also been found in the peripheral blood of newly diagnosed diabetic patients. Furthermore, a longitudinal study on the same cases demonstrated that the Vβ7 expansion was actually already present years before the clinical onset, suggesting that a viral infection, and consequently the exposure to a superantigen, might have occurred at this early stage of the disease process [38]. An activated endogenous retrovirus has been suggested to be implicated in the pathogenesis of human type 1 diabetes [39]. More recently, an envelope protein of this retrovirus has been shown to encode a superantigen that preferentially expands Vβ7+ T cells in type 1 diabetes patients [40]. However, the claimed association between endogenous retroviral transcripts and hu-

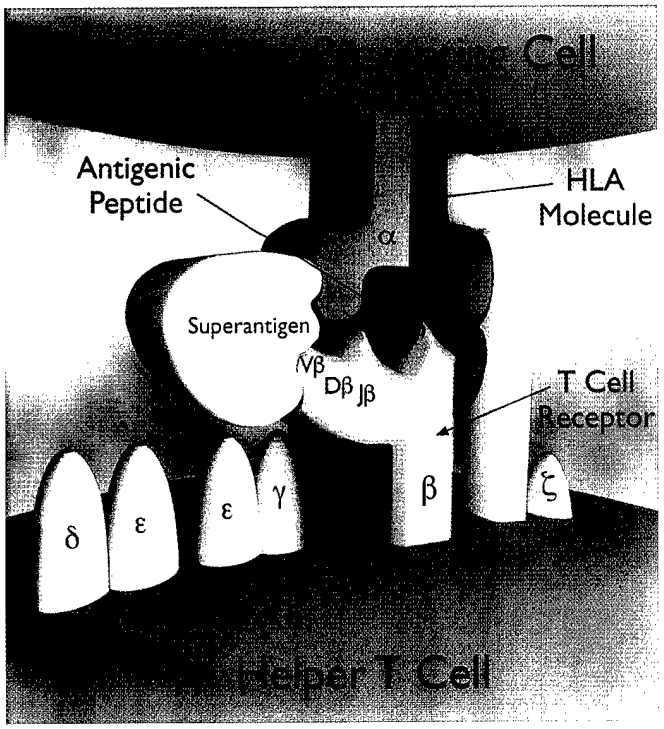

Fig. 2. A schematic representation of the interactions among T cell receptor (TCRαβ), CD3 molecular complex (δ, ε, γ and ζ), HLA class II molecule, antigenic peptide, accessory molecule (CD4) and superantigen during T-cell activation in the presence of an APC.

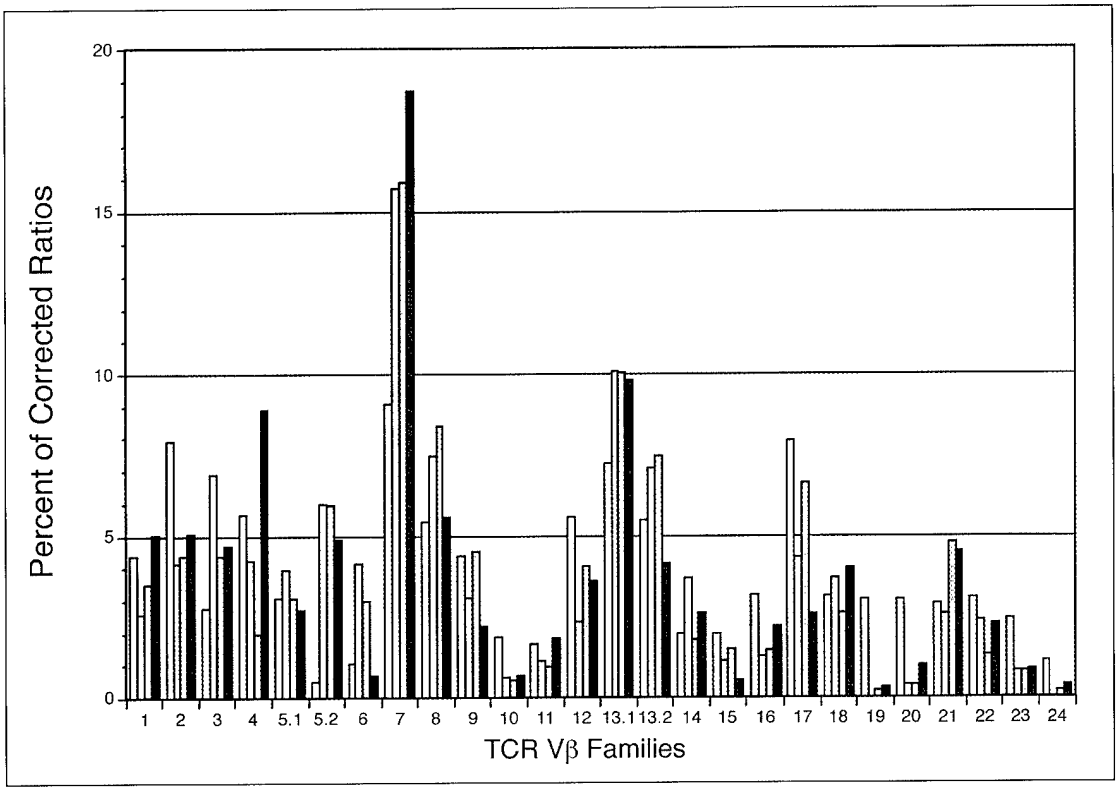

Fig. 3. TCR Vβ repertoire ofPBMC from a healthy donor, cultured with CBV3-infected Vero cell lysates. PBMC, either uncultured (white), or stimulated in vitro with cell lysates, were harvested, and TCR Vβ analysis on CD71+ cells was performed after 4 (yellow), 9 (orange), and 15 (red) days of culture (modified from Luppi et al. [30]).

man type 1 diabetes resulted to be disputable and several reports failed to confirm these exciting observations [41]. Interestingly, in another disease, idiopathic dilated cardiomyopathy, which is considered an end-stage condition of an early, acute myocarditis, we found that the majority of the T cells infiltrating the diseased hearts of patients received at the time of heart transplantation carried the same phenotype detected in the pancreata of diabetic patients [30]. Since CVB is thought to be responsible for more than 50% of myocarditis in North America and has also been incriminated in the pathogenesis of idiopathic dilated cardiomyopathy, we wanted to characterize the immunologic response to this viral infection. The results of our analysis showed that some CVB strains are able to induce an in vitro immune response very similar to that found in vivo in the heart of patients with idiopathic dilated cardiomyopathy and characterized by a skewing of TCR Vβ3, 7 and 13.1 gene families (fig. 3). This immune response also evoked the behavior of a superantigen [30]. Our findings suggested that the autoimmune attack specifically directed against the myocytes was initiated by CVB3 which was also responsible for the further pathologic damage of the patient's heart tissues by means of the triggering of a superantigen-like type of immune response [30]. If we extend these findings to type 1 diabetes, we can hypothesize that a pancreotropic CVB strain (CVB4?) can cause an initial pancreatic damage which is then amplified by the superantigenic features of the virus, associated with cytokine secretion and bystander activation of T cells. Further damage will be the result of exposure of sequestered self-antigens and the activation of potentially autoreactive T cells leading to diabetes.

Bystander (Non-Antigen-Specific) Mechanism of T Cell Activation

T cell proliferation in vivo is presumed to reflect a TCR-mediated polyclonal response directed to various environmental agents. However, viral infections that are generally able to induce vigorous immune responses are more suggestive of a reaction driven by inflammatory cytokines released in the area of infection than driven by the TCR recognition of the foreign antigen [42]. In mice, T cell proliferation in response to viral infections preferentially affects CD8+ T cells with a 'memory-phenotype' ($CD44^{hi}$) [42]. A similar T cell proliferation is obtained after injection of type I interferon (which includes IFN-α and IFN-β) and IFN-γ [42]. Other cytokines have not yet been taken into consideration. This T cell proliferation is not TCR dependent and can even be promoted by other environmental agents as long as they are infectious and able to induce cytokine secretion. IFN-I is also able to potentiate clonal expansion and survival of CD8+ cells responding to specific antigen, suggesting that this cytokine may play a crucial role in the generation and maintenance of long-lived specific memory cells [42]. Basically, local production of IFN-I during a viral infection may act as an adjuvant that increases both the intensity of the response and the survival of early memory T cells. Very recently, a mechanism of local bystander (i.e. non-antigen-specific) activation of potentially autoreactive T cells has been proposed to be involved in the pathogenesis of type 1 diabetes in a model of CVB4-induced diabetes [43]. As mentioned above, CVB4 has been linked to diabetes development both in animals and in humans. The mechanism by which this strain of virus actually triggers the disease is not well understood, and mechanisms of molecular mimicry have been suspected to be at work for a long time. To determine which of the two hypotheses (molecular mimicry or bystander activation of T cells) better explained the mechanism underlying CVB4-induced type 1 diabetes, Horwitz et al. [43] used CVB4 to infect three strains of mice: the NOD, the B10.H2g7 and the BDC2.5 mice. The first two strains of mice carry the MHC allele $H\text{-}2^{g7}$ to which the presentation of the CVB4-GAD65 cross-reactive epitope is restricted. While the NOD mice develop insulitis and spontaneous diabetes by 16–20 weeks of age, the B10.H2g7, which seem to be lacking many other susceptibility factors, do not develop spontaneous diabetes. BDC2.5 mice, instead, have a NOD genetic background and harbor a transgene encoding the rearranged TCR (Vα1; Vβ4) obtained from a diabetic NOD mouse and specific for an islet-granule antigen distinct from GAD65 and not reacting with CVB4. These mice normally show a rampant insulitis at 3 weeks of age but do not develop spontaneous diabetes. The results from the study showed that following CVB4 infection, the NOD and the B10.H2g7 mice generated a strong local immune response (severe pancreatitis) without any signs of islet destruction, islet autoreactivity or diabetes. In contrast, CVB4 infections of the BDC2.5 transgenic mice generated insulitis and rapid islet destruction. Since the transgenic TCR carried by these BDC2.5 mice is not specific for either CVB4 or GAD65, molecular mimicry as the triggering mechanism in type 1 diabetes development was ruled out. In fact, if the molecular mimicry hypothesis was relevant, the animals carrying the diabetogenic MHC allele should have promptly developed disease once infected with CVB4. Viral infections, instead, did not

directly activate virus-specific, islet-cross-reactive T cells. In BDC2.5 mice, diabetes was the outcome of the CVB activation of resting, islet-specific, memory lymphocytes through a bystander reaction driven by local tissue damage and release of sequestered antigen during pancreatic inflammation [43]. An alternative, but not mutually exclusive hypothesis, is that CVB4 could indeed create a proinflammatory 'hot' milieu favoring the differentiation of Th1-type cytokines and the recruitment of accessory cells such as macrophages [44].

The immunologic scenario observed in the CVB4-infected BDC2.5 transgenic mice was considered an 'exaggerated version' of the human preclinical diabetic situation in which genetic predisposition and a history of previous environmental insults could have caused an expansion of cross-reactive T cells leading to peri-insulitis and eventually overt diabetes. Indeed, distortion or deviation of the TCR repertoire with overexpression of certain Vβ families (similarly to the one 'artificially' created in the BDC2.5 transgenic mice) has been detected in the peripheral blood of humans as a consequence, for example, of exposure to certain pathogens with superantigen characteristics [37, 38]. Among these T cells sharing TCR Vβ elements, autoreactive, β-cell-specific elements may be present.

Concluding Remarks

All of the most recent results seem to point to similar mechanisms synergistically acting in destroying the insulin-secreting β cells of the pancreas. Each of the hypotheses presented may explain different aspects of the very complex etiopathogenetic scenario offered by type 1 diabetes. Understanding the complex interplay existing between the genetic and the immunologic components of the disease in relationship to the auto- and foreign antigens, is then the conditio sine qua non for proposing valuable prevention strategies against diabetes, and for eventually implementing reliable and safe intervention approaches.

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Restricted TCR Vβ gene expression and enterovirus infection in Type I diabetes: a pilot study

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Abstract

Aims/hypothesis. High frequencies of T-cell receptor (TCR) $V\beta7^+$ T cells were detected among the lymphocytes isolated from pancreatic islets of children at the onset of Type I (insulin-dependent) diabetes mellitus. We assessed whether a preferential expression of certain TCR $V\beta$ gene families could also be detected among the peripheral blood mononuclear cells from diabetic patients.
Methods. T-cell receptor repertoires were evaluated by using a semi-quantitative RT-PCR-based technique and confirmed by FACS analysis in peripheral blood mononuclear cells from diabetic patients before, at and after onset of the disease. These patients were also tested for exposure to enteroviruses by RT-PCR and by measuring titres of enterovirus-specific antibodies of the IgA, IgG, and IgM classes.
Results. T-cell receptor $V\beta7$ gene family values were higher in recently-diagnosed diabetic patients (10.5 % ± 3.7) than in age-matched non-diabetic control subjects (5.1 % ± 1.6) ($p < 0.001$). In a time-course analysis of people who developed diabetes during clinical monitoring (i. e., converters), T-cell receptor $V\beta7$ gene expression showed values consistently above 10 % ($p < 0.0005$). Long-standing diabetic patients showed lower percentage of $V\beta7$ expression compared to values measured at disease onset. In the longitudinal study of the converters, multiple acute enterovirus infections were also detected. These infections appeared to be temporally related to increased percentage of $V\beta7$ gene transcripts.
Conclusion/interpretation. The deviation in the T-cell receptor $V\beta$ repertoire among circulating T cells from diabetic patients seems to re-emphasize the importance of enterovirus infections in accelerating the progression of Type I diabetes. [Diabetologia (2000) 43: 1484–1497]

Keywords Type I diabetes, etiology, autoimmunity, Coxsackievirus, T cell receptor.

Type I (insulin-dependent) diabetes mellitus is an autoimmune disease characterized by infiltrating lymphocytes in the Langerhans' islets and by destruction of insulin-producing beta cells [1]. Despite many years of intensive studies, the precise cause and mechanisms triggering the beta-cell-specific attack still remain undetermined. The less than 50 % concordance for the disease between monozygotic twins suggests, however, that a complex interplay occurs between genetic and non-genetic factors (i. e., environmental) [2, 3]. In particular, epidemiological evidence supports a role for infectious agents in the development and/or progression of Type I diabetes [4–10].

Although the possibility of a virus-mediated etiopathogenesis of Type I diabetes still remains a central point of discussion, certain viral infections, especially

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Abbreviations: TCR, T-cell receptor; $V\beta$, variable region of the gene encoding the TCR β chain; $C\alpha$, constant region of the gene encoding the TCR α chain; $C\beta$, constant region of the gene encoding the TRC β chain; PBMC, peripheral blood mononuclear cells; RT, reverse-transcriptase; PCR, polymerase chain reaction; EV, enterovirus; CVB, coxsackievirus B; mRNA, messenger RNA; ICA, islet cell antibodies.

those caused by enterovirus (EV), have long been considered associated with beta-cell damage [11–13] and with the clinical onset of diabetes [4–15], particularly amongst very young children [15–19]. Genetically-susceptible subjects with autoimmune markers of beta-cell damage (i.e., autoantibodies) develop T-cell responses against EV antigens and EV-infected cells [12, 20, 21]. The relation between antibodies detected and the increased prevalence of Coxsackievirus B (CVB) infection in recently-diagnosed diabetic patients compared with healthy subjects is, however, often inconsistent [6, 10, 14, 18, 19, 21], as we also found in our previous studies [22–25]. Although, in mice and primates, infection of CVB4 has been shown to induce diabetes [26, 27], the direct association of EV infections and the disease was documented in humans, in only a few anecdotal cases where a CVB4 strain was isolated from the pancreas of a diabetic child [28] and viral RNA sequences were detected in blood samples from children and adults at the onset of Type I diabetes [15, 16, 29].

Several studies proposed Coxsackievirus infection as an environmental trigger for the development of a T-cell autoreactivity by a mechanism of molecular mimicry [30] or by inducing a bystander effect on autoreactive T cells [31]. It has been shown that the timing of a Coxsackievirus infection, rather than a simple documentation of its presence or absence, has important implications in the development of diabetes in NOD mice. In this animal model, CVB4 infection can accelerate the development of Type I diabetes only after a critical amount of autoreactive T cells have accumulated at the periphery of the islets [32]. Based on this finding, we suppose that in humans, like in rodents, the timing of a Coxsackievirus infection is a critical factor in the development of diabetes by activating autoreactive T cells already present in the circulation and possibly in proximity to the islets.

It is generally believed that the destruction of the beta-cells producing insulin occurs over a prolonged period of time, eventually resulting in the delayed onset of the disease [1]. During this period, detection of serum antibodies [33–35] and T-cell sensitization [36] to a vast array of islet-cell antigens signifies the presence of autoimmunity even at early stages in the course of the disease. The analysis of the T-cell receptor (TCR) repertoire of the circulating T cells is important because it might represent a direct means to ascertain the presence of T cells involved in the pathogenesis of Type I diabetes eventually infiltrating the pancreatic islets of Langherans. In humans, it is not possible to actually dissect the kinetics of beta-cell destruction because specimens from diseased pancreata at different time-points can not be easily obtained. In a previous study, TCR $V\beta7$ mRNA was, however, expressed by the majority of the islet infiltrating lymphocytes from two children at the onset of Type I diabetes [37].

We assessed the possibility of showing the skewing of the TCR $V\beta$ repertoire by testing the lymphocytes in the peripheral blood from diabetic patients and of founding whether there is a preferential expression of certain TCR $V\beta$ gene families at different time-points before, at and after the onset of the disease. Accordingly, we evaluated the use of TCR $V\beta$ expression in a group of recently-diagnosed diabetic patients and in a longitudinal prospective study using a group of high-risk first-degree relatives. Some of these relatives eventually developed Type I diabetes (i.e., converters to diabetes) while others did not (i.e., high-risk subjects). The T-cell receptor $V\beta$ expression was also analysed in an additional group of patients who were suffering from diabetes from 2 to 10 years (i.e., long-standing diabetic patients). Moreover, selective $V\beta7^+$ T-cell expansion in response to CVB antigens was ascertained in PBMC of non-diabetic control subjects by flow cytometry analysis. Finally, we assessed if there was any evidence of acute EV infections in the subjects by testing their sera for the presence of EV-RNA and of IgA, IgG and IgM class antibodies against EV.

Materials and methods

Subjects. Twenty-three recently-diagnosed (i.e., within one week of the first insulin injection) children with Type I diabetes (mean age 9.4 years ± 3.8; range 2–16 years; 11 male : 12 female) were consecutively selected from those treated in the Diabetes Center at the Children's Hospital of Pittsburgh (CHP) in July 1996 to July 1998. As part of a prospective study of risk factors of Type I diabetes, we collected peripheral blood mononuclear cells (PBMC) at serial time points, before, at and after the onset of the disease from four high-risk subjects who eventually developed the disease (i.e., converters to diabetes) (mean age at diagnosis 9.2 years ± 2.6; range 7–13 years; 2 male : 2 female) and in four others who have not yet developed the disease (i.e., high-risk subjects) (mean age at the first blood sample taken 21.5 years ± 10.4; range 11–35 years; 2 male : 2 female). In addition, fourteen patients (2 siblings included) with long-standing Type I diabetes (mean: 7.2 years ± 3.4; mean age at diagnosis 6.1 years ± 3.1; range 1–11 years; 6 male : 8 female) were studied. Demographic and clinical data of the subjects studied are summarized in Table 1. These subjects were first-degree relatives of Type I diabetic probands, carried HLA haplotypes conferring susceptibility and had ICA (detected by immunohistochemistry) [33, 34], anti-GAD or anti-ICA512 autoantibodies (detected by radioimmunoassay) or both [35]. Two of the high-risk subjects had borderline, one normal and one low insulin responses to intravenous glucose tolerance but all had normal glycated haemoglobin and plasma glucose concentrations.

The control group consisted of sixteen subjects age-matched and sex-matched to the recently diagnosed diabetic patients (mean age 10.2 years ± 4.7; range 3–16 years; 9 male : 7 female), recruited amongst patients undergoing orthopedic interventions at CHP. An additional group of thirteen healthy adults age-matched and sex-matched to the high-risk subjects (mean age 39 years ± 8.5; range 23–49 years; 5 male : 8 female) was also included. None of the control subjects had a family

Table 1. Demographic and clinical data of subjects studied

Subjects	Number	Time of onset	Mean age at diagnosis (years)	Sex (Male : Female)
Recently-diagnosed	23	Within 1 week	9.4 ± 3.8 (range 2–16)	11:12
Converters	4	From –40 months to + 7 years	9.2 ± 2.6 (range 7–13)	2:2
High-Risks	4	Non-diabetics studied at time points eight years apart	21.5 ± 10.4 (range 11–35)[a]	2:2
Long-Standings	14	Within 1 week and + 2–14 years	6.1 ± 3.1 (range 1–11)	6:8

[a] At the time of the first sample collection

history of diabetes and all were negative for ICA, anti-GAD or anti-ICA512 autoantibodies or both. Specimens from these subjects were also used for the in vitro stimulation studies with CVB-infected HeLa cell lysates.

Molecular HLA typing was done in all subjects for whom the cDNA was available (Table 2) [38, 39]. Informed consent was obtained from each subject participating in this study, approved by the Institutional Review Board of the Children's Hospital of Pittsburgh.

Isolation and culture conditions of peripheral blood mononuclear cells (PBMC). Venous blood was drawn in heparinized tubes and processed for PBMC isolation using a Ficoll-Hypaque gradient (1-Step Lymphoprep, Accurate Chemical & Scientific Corporation, Westbury, N.Y., USA) within a few hours from blood collection. Total RNA was extracted from PBMC obtained from the recently-diagnosed diabetic patients and control subjects, while PBMC from the other subjects were frozen in freezing medium (Gibco BRL Life Technologies, Gaithersburg, Md., USA) and kept in liquid nitrogen until used. To increase the production of functional TCR mRNA, frozen PBMC were stimulated with anti-CD3 (Beckman Coulter, Fullerton, Calif., USA) and recombinant interleukin-2 (rIL-2) (a kind gift from Dr. M.T. Lotze). Frozen PBMC were briefly thawed in RPMI-1640 medium (Gibco BRL) and centrifuged at 1200 rpm for 8 min. Cells were then resuspended in RPMI-1640 medium (Gibco BRL) supplemented with 5% human AB serum (Normolcera-Plus, Miami, Fla., USA) with addition of 5 U/ml of rIL-2 and plated at a concentration of $1 \cdot 10^6$ cells a well into flat-bottom 24-well culture plates (Corning, New York, N.Y., USA). Viability of the cells was checked by Trypan blue exclusion and was estimated to be approximately 80 to 90% in all samples. Peripheral blood mononuclear cells were cultured overnight at 37 °C in 5% CO_2 in an incubator. The next day, anti-CD3-coated plates were prepared using 100 ng/ml anti-CD3 (Beckman Coulter) in each plate and then incubated at 37 °C for at least one hour. After removing unbound antibody, PBMC were transferred to the anti-CD3 treated plates, centrifuged at 500 rpm for 5 min and returned to the incubator for 2 days. On day 3, rIL-2 at a final concentration of 20 U/ml was added to each well and the plates were returned to the incubator for 1 day. On the 4th day of the in vitro culture, cells were harvested, pelletted, counted and total RNA was extracted.

Preparation of RNA and synthesis of cDNA. Total cytoplasmic RNA was extracted from the PBMC from the various subjects using TRIZOL Reagent according to the manufacturer's instructions (Gibco BRL). All RNA samples were maintained in 75% ethanol and stored at –80 °C until cDNA synthesis was carried out. For each preparation, at least 3 µg of total RNA were reverse-transcribed (RT-PCR) into single-stranded cDNA using SUPERSCRIPT II (Gibco BRL).

Evaluation of TCR Vβ repertoires.* The study of the TCR-*Vβ* gene segment expression required individual amplification of at least 26 TCR-*Vβ* families and subfamilies [40, 41] using a panel of 5'-*Vβ* primers together with a 6-carboxyfluorescein (6-FAM, Applied Biosystems, Foster City, Calif., USA) 3'-*Cβ* oligonucleotide. The *Vβ* and *Cβ* oligonucleotide primers were used at a final concentration of 0.5 µmol/l each in the 50 µl final reaction mixture. As an internal positive control for each *Vβ* family segment, a pair of constant region oligonucleotides 5'-*Cα* and 3'-*Cα*, also tagged with 4,7,2',7'-Tetrachloro-6-carboxyfluorescein, (6-TET; Applied Biosystems), were used at a final concentration of 0.2 µmol/l [40, 41]. Twenty-eight cycles of PCR were carried out to remain in the exponentially increasing part of the reaction [42]. Polymerase chain reaction (PCR) conditions were as follows: denaturation at 94 °C for 30 s, primer annealing at 60 °C for 30 s and primer extension at 70 °C for 45 s.

Two microliters of the PCR products, corresponding to the TCR *Vβ* gene families, were resolved by electrophoresis on a 16-cm, 5% polyacrylamide non-denaturing gel for 1 h on an ABI 377 DNA Sequencer (Applied Biosystems). Each gel was then automatically analysed by GeneScan software (Applied Biosystems) which allows for quantitation of fluorescent peak heights and areas corresponding to each band. The relative abundance of T cells carrying each *Vβ* chain transcript was estimated by calculating a ratio of *Vβ* fluorescent and *Cα* fluorescent areas (the internal positive control) for each *Vβ* gene family. The percentage of each *Vβ* gene family expression was computed in relation to the sum of all 26 ratios (i.e., per cent of corrected ratios) according to the following formula:

$$\%\ V\beta_{(i)} = \frac{\text{fluorescence } V\beta_{(i)}/\text{fluorescence } C\alpha_{(i)}}{\sum (\text{fluorescence } V\beta/\text{fluorescence } C\alpha)\ (1...26)} \times 100$$

where "i" specifies an individual *Vβ* family. To ensure reproducibility, two PCRs were independently carried out for the majority of the cDNA samples and the percentage of each *Vβ* gene family expression calculated using the formula shown above. Average values from the two analyses were then calculated.

The presence of a high frequency of TCR $V\beta7^+$ circulating T cells from recently-diagnosed diabetic patients was also checked by flow cytometry analysis. Briefly, 100 µl of whole blood from recently-diagnosed diabetic patients and control subjects was incubated with 20 µl of FITC-conjugated monoclonal antibodies specific for various human *Vβ* chains (Beck-

Table 2. Analysis of HLA-DR and -DQ haplotypes in young and adult control subjects and recently diagnosed diabetic patients

	Sex (M/F)	Age (years)	HLA DRB1	HLA DQA1	HLA DQB1
Young control subjects					
1	M	10	–	–	–
2	F	14	–	–	–
3	M	15	02,11	01	0501, 0602
4	M	14	02, 01	01	0501, 0502
5	M	8	11, 12	01, 05	0301, 0501
6	M	13	01, 02	01	0501, 0602
7	F	16	04	0301	0301, 0601
8	F	13	02, 13	01	0601, 0605
9	F	10	–	–	–
10	F	12	11, 13	05	0301, 0401
11	M	15	03, 04	0301, 05	0201, 0302
12	F	10	11, 13	01, 05	0301, 0603
13	M	3	09, 13	01, 0301	0303, 0605
14	M	3	02, 09	01, 0301	0303, 0601
15	F	4	03, 11	05	0201, 0301
16	M	3	03, 12	01, 05	0201, 0501
Adult control subjects					
17	F	33	02, 14	01, 05	0301, 0502
18	F	30	02, 07	01, 0201	0201, 0602
19	M	27	07	0201	0201
20	M	25	04, 02	01, 0301	0301, 0602
21	M	49	11, 13	01, 05	0301, 0603
22	M	49	01, 07	01, 0301	0301, 0501
23	M	34	04, 07	01, 03	0302, 0602
24	F	40	04, 13	01, 03	0302, 0603
25	F	23	03, 04	03, 04	0201, 0302
26	F	37	03, 04	03, 04	0201, 0302
27	F	42	03, 04	03, 04	0301, 0302
28	F	35	03, 04	03, 04	0201, 0302
29	F	45	03, 04	03, 04	0201, 0302
Recently diagnosed diabetic patients					
1	M	8	03, 04	0301, 05	0201, 0302
2	M	13	03, 04	0301, 05	0201, 0302
3	F	16	04, 08	0301, 04	0302, 0402
4	F	7	03, 04	0301, 05	0201, 0302
5	F	10	04, 08	0301, 0301	0302, 0302
6	F	11	04, 11	0301, 05	0301, 0302
7	M	3	04, 09	0301	0302, 0303
8	M	14	04	0301	0301, 0302
9	F	13	–	–	–
10	M	13	–	–	–
11	M	14	04, 07	0201, 0301	0201
12	M	13	03	05	0201
13	M	2	–	–	–
14	M	8	04, 13	01, 0301	0302, 0602
15	F	11	03, 11	05	0201, 0301
16	M	10	04, 07	0201, 0301	0201, 0302
17	M	7	01, 04	01, 03	0501, 0302
18	F	5	–	0201, 0501	0201, 0301
19	F	10	03, 07	0301, 0501	0201, 0302
20	M	3	—	0102, 0301	0302, 0502
21	M	9	02, 04	01, 0301	0301, 0501
22	F	9	–	–	–
23	F	7	–	–	–

Molecular typing for alleles at HLA-DRB1, DQA1 and DQB1 loci carried out as described previously [38–39]

man Coulter), 20 µl of PE-conjugated monoclonal antibody to pan-TCR $\alpha\beta$ (Coulter, Miami, Fla., USA) and 20 µl of Cy-Chrome-conjugated monoclonal antibody to the leucocyte common antigen (CD45) (Dako Corporation, Carpinteria, Calif., USA) or appropriate negative isotype control. The samples were stained in the dark for 15 min and erythrocytes were then lysed with PharMLyse (Pharmingen International, Becton Dickinson, San Jose, Calif., USA). Samples were then washed twice in FACS media (Hank's balanced salt solution with 0.1% bovine serum albumin and 0.1% sodium azide) and stored in 1% paraformaldheyde (PFA) at 4 °C until run on a Becton Dickinson FACSCalikur Instrument. T-cell receptor $V\beta$ repertoire analysis by flow cytometry was also done on PBMC from normal healthy donors cultured with uninfected

and CVB-infected HeLa cell lysates. Briefly, one milliliter of the cell culture was removed, cells were pelletted, resuspended in phosphate buffered saline (PBS) and aliquoted for flow cytometry staining using the panel of monoclonal antibodies as above. The samples were stained in the dark for 15 min, washed twice in FACS media and then stored in 1% PFA at 4 °C until run on a Becton Dickinson FACScan instrument.

Infection of HeLa cells with Coxsackievirus B (CVB)

Coxsackievirus titres. Coxsackievirus B (CVB) was obtained from the ATCC (catalogue No. VR-30) and quantitated by the plaque assay method. Briefly, 95% confluent HeLa cells were obtained by seeding two 12-well plates for each isolate with 1.5 ml of cells at $2.5 \cdot 10^5$/ml. Cells became 95% confluent in one to four days. The media was aspirated and replaced with 0.1 ml/well of serial tenfold dilutions (10^{-3} to 10^{-9}) of the viral stock in minimal essential medium (MEM) (Gibco BRL), in triplicate. After viral adsorption at 37 °C in 5% CO_2 for 45 to 60 min, 1.5 ml/well of 1% methyl cellulose in MEM was added and the plates incubated for 1–3 days until plaques were visible. The cell layers were stained with crystal violet and the plaques counted.

Preparation of virus stocks and lysates. Confluent monolayers of HeLa cells in T-175 cm^2 flasks were used three to four days post passage. The media was aspirated and replaced with 5 ml of Eagle's minimal essential medium (EMEM) (Gibco BRL) supplemented with 10% fetal calf serum (FCS) containing $3 \cdot 10^6$ plaque forming units of a viral stock to achieve a multiplicity of infection (MOI) of about 0.1. The virus was allowed to absorb at 37 °C, 5% CO_2 for 45 to 60 min and 25 ml of EMEM (Gibco BRL), 10% FCS was added. The cultures were incubated for three to four days while monitoring cytopathic effect (CPE). At 90 to 100% CPE, the cells and the media were collected by scraping and then centrifuged at 2000 rpm. The supernatant was aliquoted, stored at –70 °C and assayed by plaque titration. For cell lysate production, the medium and cells were collected as described above. Glycine was added to a final concentration of 100 mmol/l and pH 9.5. The extract was then sonicated, UV treated, centrifuged as above, aliquoted and stored at –70 °C. Finally, the lysate was tested by the plaque assay method described above to verify that there was no residual CPE.

In vitro PBMC stimulation with CVB-infected HeLa cell lysates. Peripheral blood mononuclear cells ($3 \cdot 10^6$) isolated from thirteen non-diabetic donors were seeded in a volume of 3 ml of RPMI-1640 medium (Gibco BRL) supplemented with 10% human AB serum (Normolcera Plus), into a 0.2 μm ventilation-capped 25 cm^2 tissue polystyrene culture flasks and exposed to lysates of CVB-infected HeLa cells (see above). Cultures with lysates of uninfected HeLa cells were used as negative control cells. Peripheral blood mononuclear cells were cultured at 37 °C in 5% CO_2 for 9 days and independently stimulated with each of the different antigens on day 0 and every 3 days thereafter. Recombinant interleukin-2 (rIL-2) was added (10 U/ml) on day 4. Twelve hours before harvesting, rIL-2 (10 U/ml) was again added to allow regeneration of potentially modulated TCR. At day 5 and 9, 1 ml of the in vitro culture was used for TCR repertoire analysis by flow cytometry as described above.

EV-RNA detection by reverse transcriptase (RT)-PCR and antibody analysis in the serum. Serum samples from the different subjects have been analysed for the presence of EV RNA and EV antibodies. Detection of EV-RNA was done by a RT-PCR method which was carried out from coded samples as described previously [43]. This RT-PCR method amplifies a genome sequence of the highly conserved 5' non-coding region of all EV serotypes and RT-PCR products are identified by a lanthanide chelate labelled EV specific probe in a liquid phase hybridization assay. The detection limit of this assay is 0.015 fg of viral RNA. The IgG and IgA class antibodies to CVB4 antigen and to a synthetic EV peptide were analysed using an enzyme immunoassay (EIA) method described previously [4, 20] and IgM class antibodies using a heavy chain capture EIA using a panel of three EV antigens (CVB3, echovirus 11, and CVA16) [20]. These antibody assays were designed to detect antibodies in several EV serotypes. Virus antigens were also heat-treated at + 56 °C for 15 min to expose antigenic determinants which are cross-reactive between different EV serotypes. The antibody results are expressed as enzyme immunoassay units (EIU) which show the relative antibody activity of the sample compared to positive and negative control sera included in each test and which are therefore comparable between different test runs. All serum samples were tested in duplicate. Samples were considered seropositive if EIU was greater than 15. Diagnosis of acute infection during the follow-up in the four converters was based on the presence of a twofold or greater increase in the concentration of IgA and/or IgG and/or detection of IgM in amounts exceeding cut-off limits defined previously [4] and/or presence of EV-RNA in serum. The IgM and RT-PCR data are shown as categorical variables and are expressed as + /–.

Statistical analysis. The percentage of expression of each $V\beta$ gene family is presented as means ± SD for each study group. For each $V\beta$ gene family, the 100th centile of the appropriate control population was chosen as the upper limit of normal, above which values were considered to be skewed. Differences in the prevalence of skewed $V\beta$ families were assessed using Fischer's exact test. Mean values of $V\beta7$ and $V\beta13.1$ gene families were compared between study groups using the Mann-Whitney U test. A p value of less than 0.05 was considered statistically significant. Concentrations of enteroviral antibodies between the study groups were compared using the Mann-Whitney U test.

Results

TCR repertoire analysis. The estimated percentage of T cells bearing different TCR, extrapolated as a function of the relative abundance of the mRNA from different $V\beta$ gene families, in the peripheral blood of patients recently diagnosed with Type I diabetes is shown in Figure 1. The majority of the patients studied (Table 2) showed a higher abundance of the $V\beta7$ gene family (mean value 10.5% ± 3.7) compared with the expression pattern observed in the PBMC of age-matched non-diabetic control subjects (5.1% ± 1.6) ($p < 0.001$). Fifteen (65%) of the recently-diagnosed diabetic patients had values of $V\beta7$ above the upper limit of the appropriate control group (8.3%) and this prevalence of skewed $V\beta7$ was statistically higher ($p < 0.0005$) compared with the control subjects. In recently-diagnosed diabetic

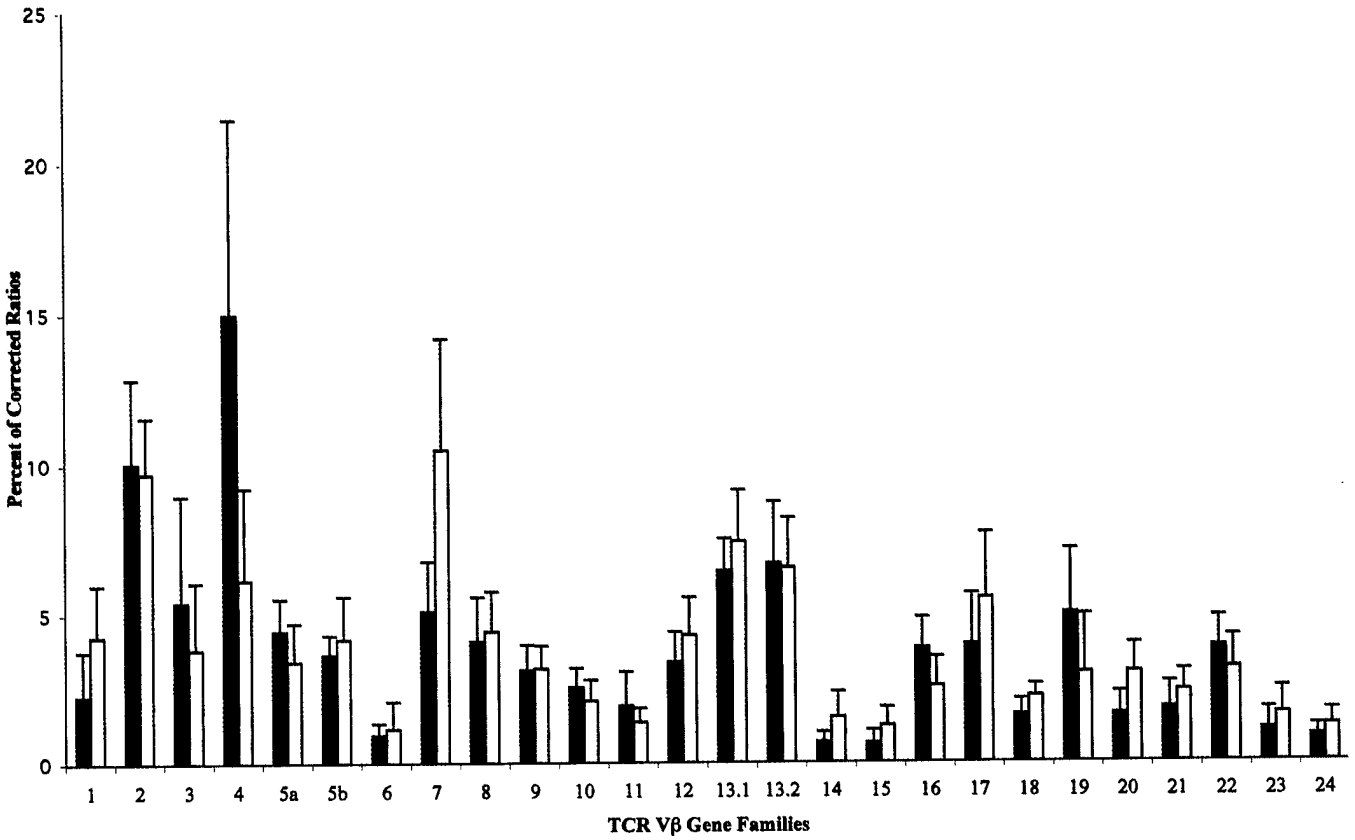

Fig. 1. Analysis of TCR $V\beta$ repertoire in PBMC from recently-diagnosed Type I diabetic patients (□, $n = 23$) and age-matched controls (■, $n = 16$). The value of each TCR Vβ is expressed as a mean percentage (± SD) of corrected ratios. The difference between mean values of $V\beta7^+$ T cells from diabetic patients and normal control subjects is statistically significant ($p < 0.001$)

patients, other $V\beta$ mRNA gene families, like $V\beta$1 and $V\beta$17, tended to be higher than in the control group but failed to reach statistical significance. Among the age-matched control population, the $V\beta4$ family was the most represented although its percentage varied extensively as shown by the large standard deviation (Fig. 1).

A high frequency of circulating V$\beta7^+$ T cells in recently-diagnosed diabetic patients was also confirmed by flow cytometry analysis. Using the only available Vβ7-specific monoclonal antibody that recognizes the Vβ7.1 subfamily, the frequency of pan-TCR$\alpha\beta^+$ T cells carrying the Vβ7.1 chain was higher (2.1–5.8% of TCR$\alpha\beta^+$ T cells) than the frequency for this Vβ chain detected in non-diabetic subjects (1.6–3.2% of TCR$\alpha\beta^+$ cells) (Fig. 2). The difference in raw values of the detected Vβ7 percentages between the molecular (RT-PCR) and the flow cytometry method could depend on several factors, such as detection of mRNA gene transcripts with one technique and expression of surface protein with the other, specificity of the anti-Vβ antibody limited to the 7.1 subfamily and the different denominators each method uses for percentage calculations.

In a time-course analysis of the four converters, TCR $V\beta$ gene expression was assessed in blood samples collected from forty months before the onset of the disease to seven years after. The analysis indicated a $V\beta7$ value consistently above 10% at all time points and in all patients (Table 3). The specificity of this finding for Type I diabetic patients was evident when compared with the range of $V\beta7$ values in PBMC from age-matched control subjects that were 5.1% ± 1.6 (highest 8.3%) (Table 3). All the converters at each time point had values of $V\beta7$ consistently above the upper limit of the normal control group and this prevalence of skewed $V\beta7$ was significantly higher ($p < 0.0005$) compared with control subjects. The only exception was a sample collected seven years after the onset from converter No. 3 that showed a value of $V\beta7$ of 6.3%.

The enrichment in $V\beta7^+$ T cells did not seem to be affected by the anti-CD3 and IL-2 stimulation of the frozen PBMC because TCR analysis on freshly processed PBMC from the same patients showed that the detected percentage of $V\beta$ gene expression in these two samples correlate very well (data not shown), as reported previously [44].

To rule out the possibility that the skewing in the patients' $V\beta$ repertoire was directly related to HLA alleles, we compared the TCR $V\beta$ repertoire pattern

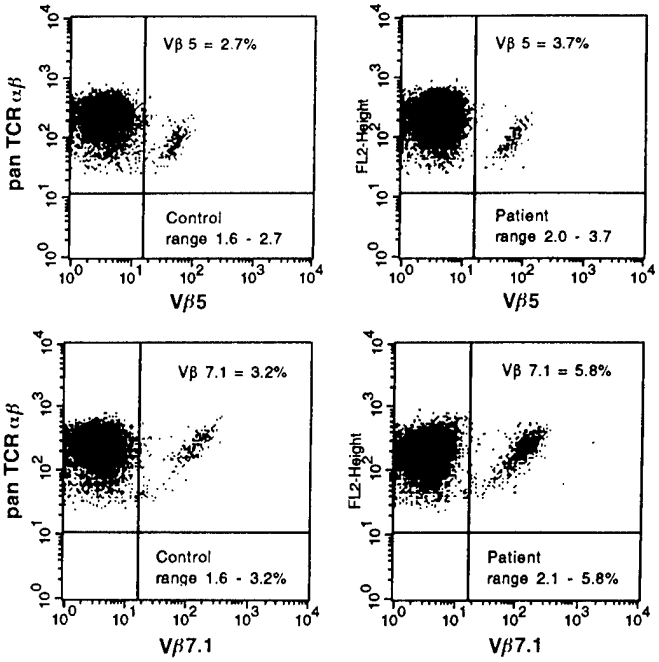

Fig. 2. Flow cytometry analysis of PBMC from one representative, recently-diagnosed diabetic patient (total $n = 23$) and one control subject (total $n = 13$) stained with FITC-conjugated monoclonal antibody against Vβ7.1 chain (clone ZOE) and PE-conjugated monoclonal antibody against pan-TCR$\alpha\beta$, by analysing live gated populations of lymphocytes. The proportion of T cells bearing Vβ7.1 chain is expressed as a percentage of TCR $\alpha\beta^+$ cells within the analysis gates. Shown are the highest value and range of Vβ7.1$^+$ T cells detected in patients and control subjects. Frequency of Vβ5$^+$ T cells is shown as an example of TCR Vβ chains that did not change substantially between diabetic patients and control subjects

Table 3. Time-course analysis of TCR *Vβ7* and *13.1* gene expression in four converters

	Vβ7 %	*Vβ11* %	*Vβ13.1* %
CONVERTER No. 1: HLA-DRB1*03, 04; DQA1*0301, 05; DQB1*0201, 0302			
–40 months	12	1.5	10.5
–28 months	13.3	1.4	8.3
–24 months	14.2	1.6	10
–14 months	16.1	1.3	9.9
–10 months	10.6	1.5	6.9
–7 months	14.4	1.1	8.7
–3 months	12.3	1.4	8.3
–2 months	14.1	1.4	7.5
+ 8 months	11.2	1.8	5.8
CONVERTER No. 2: HLA-DRB1*03, 04; DQ1A*0301, 05; DQB1*0201, 0302			
–40 months	11.4	2.2	9.6
–13 months	10.9	2.8	7.4
–11 months	13.6	2.3	11
–5 months	13.6	1.8	10.4
+ 2 months	13.3	1.7	8.5
+ 24 months	13.6	1.4	5
CONVERTER No. 3: HLA-DRB1*04, 08; DQA1*0301, 04; DQB1*0302, 0402			
–19 months	18.4	1.1	11.7
–Onset	12.8	1.6	10.3
+ 32 months	19.2	0.8	7.4
+ 84 months	6.3	1.2	5.5
CONVERTER No. 4: HLA-DRB1*03, 04; DQA1*0301, 05; DQB1*0201, 0302			
–12 months	16	1.5	9.6
–1 month	16	2	10.7
–Onset	12.2	1.9	9
+ 3 months	10.4	1.4	7
CONTROLS ($n = 16$)	5.1 ± 1.6	1.9 ± 1.2	6.4 ± 1.1

Analysis of TCR *Vβ7* and 13.1 gene families in PBMC from four converters. The relative abundance of T cells carrying each *Vβ* gene transcript is expressed as a percentage of corrected ratios. For each of these subjects, four to nine blood samples were available at several time points, before (–) and after (+) the onset of Type I diabetes. TCR *Vβ11* values are shown as an example of *Vβ* family whose expression did not considerably change throughout the entire period. The mean (± SD) values of TCR *Vβ7*, 11, and 13.1 gene families of normal age-matched control subjects are also shown

of the recently-diagnosed diabetic patients (Table 2) with that of their appropriate HLA-DR-matched and DQ-matched control subjects. The HLA-matched subjects did not reveal any expansion of the TCR *Vβ7* gene family (Fig. 3). Again, values of Vβ7 mRNA expression were significantly higher in the recently-diagnosed diabetic patients than in HLA-matched control subjects ($p = 0.009$). Moreover, amongst the randomly chosen control population, nine subjects carried HLA-haplotypes conferring with a high risk of Type I diabetes (controls No. 4; 11; 16; 19; 25–29) but did not show increased percentage of expression of *Vβ7* gene transcripts.

Among the converters, expression of another gene family, *Vβ13.1*, also tended to be higher than the mean value calculated amongst the control group. All four patients showed decreased expression of this gene family after the onset of the disease (Table 3).

Three of the high-risk subjects (all adults) showed values of *Vβ7* higher than those detected among the age-matched control group at all time points (data not shown). In these subjects, *Vβ7* values were above the upper limit of the appropriate control group (10.5 %) and this prevalence of skewed *Vβ7* was significantly higher compared with the control subjects ($p < 0.05$). The fourth subject showed a decreased value of *Vβ7* in a blood specimen drawn 8 years after the first sample (from 15.5 % to 6.3 %). This patient, who was GAD autoantibody positive and ICA-512 autoantibody negative on both occasions, showed decreased ICA values at the second time-point (from 40 to 10 JDF units) which contrasted with the stability of these titres in the other three subjects. The percentage of the *Vβ13.1* gene family expression measured in these high-risk subjects tended to be higher than those measured in the control group.

The TCR *Vβ* repertoire was also analysed in PBMC from patients who were suffering from diabetes from 2 to 14 years. In this analysis, long-standing diabetic patients showed mean values of *Vβ7*

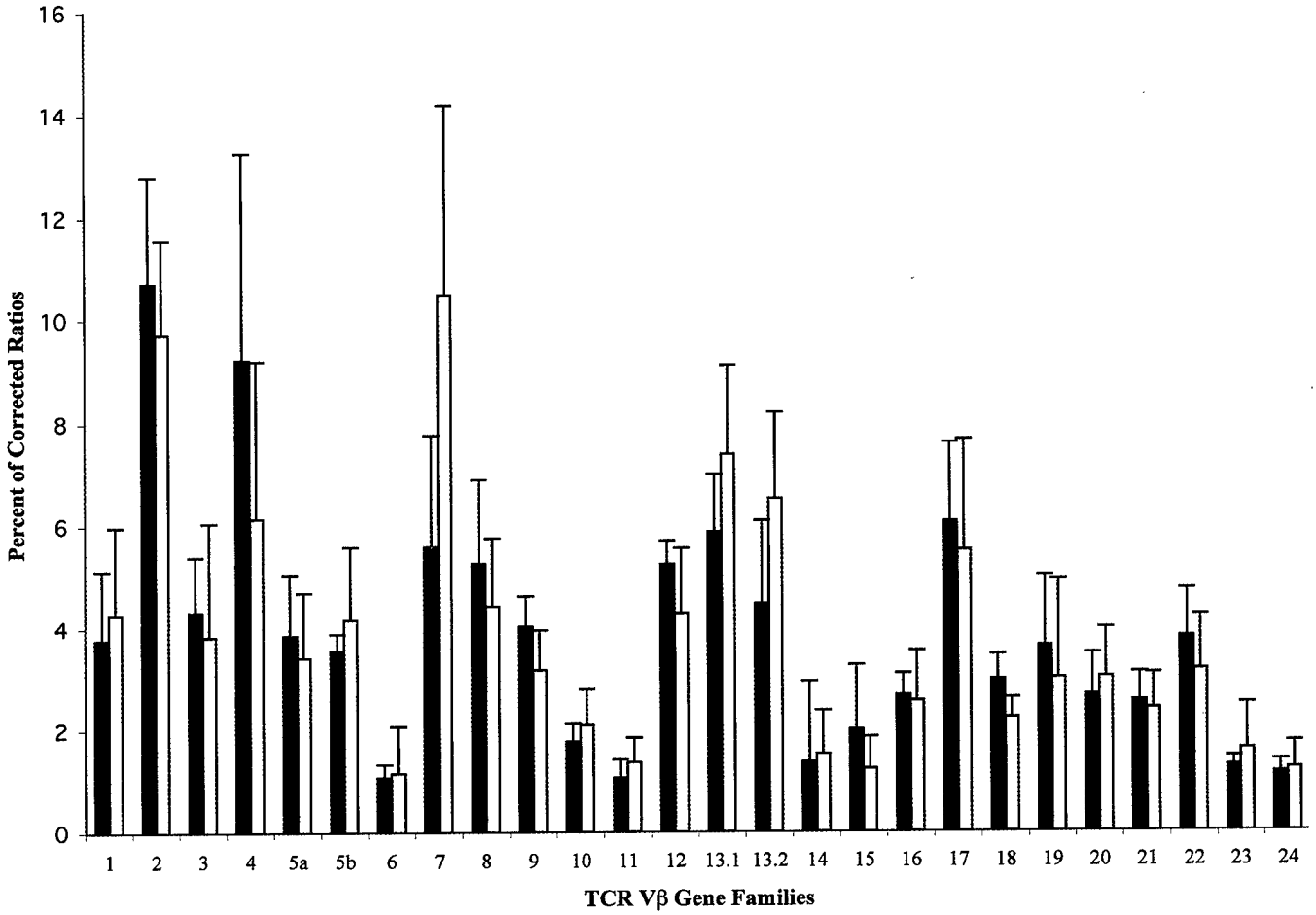

Fig. 3. Analysis of TCR *Vβ* repertoire in PBMC from recently-diagnosed diabetic patients (□, $n = 23$) and their HLA-matched control subjects (■, $n = 6$). The value of each TCR *Vβ* is expressed as a mean percentage (± SD) of corrected ratios. In HLA-matched control subjects, TCR *Vβ* expression by T cells in the peripheral blood was heterogeneous and, in contrast to diabetic patients, no *Vβ7* gene family expansion was observed ($p = 0.009$)

(9.7% ± 2.4) that were statistically different from those detected in the control group (5.1% ± 1.6) ($p < 0.005$) (Fig. 4). In 79% of these patients (11 of 14), the percentage of TCR *Vβ7* was above the upper limit of the control subjects (8.3%) and this prevalence of skewed *Vβ7* was significantly higher than in the control group ($p < 0.005$). For seven of these patients, blood samples were also available at the onset of diabetes showing mean values of *Vβ7* of 12.3% ± 3.1 ($p < 0.005$ compared with the control subjects) (Fig. 4).

In vitro PBMC stimulation with CVB-infected HeLa cell lysates. Flow cytometry analysis of the TCR repertoire of PBMC from 13 normal donors cultured for 9 days with CVB-infected HeLa cell lysates showed variable degrees of expansion of pan-TCR $\alpha\beta^+$ T cells carrying the Vβ 7.1 chain in the majority of the subjects tested (8 of 13) when compared with their PBMC cultured with uninfected-HeLa cell lysates. Whereas expression of this Vβ family increased up to 4 times in the PBMC of different donors, the testing of other Vβ families (e.g., Vβ5) did not show any changes in percentage of positives. The value for the frequency of pan-TCR $\alpha\beta^+$ T cells carrying the Vβ 7.1 and the Vβ5 chains in 3 representative donors is shown in Figure 5. Moreover, we found that PBMC from four donors cultured with CVB-infected HeLa cells showed higher frequency of pan-TCR $\alpha\beta^+$ T cells carrying the Vβ13.1 chain (data not shown). Only one donor's PBMC did not show increase of either Vβ 7.1^+ or Vβ13.1^+ T cells.

EV antibodies and presence of EV-RNA in the sera of the subjects studied. Serological analysis for the presence of IgA and IgG class antibodies against a CVB4-antigen and a synthetic EV-peptide antigen in the patients recently diagnosed with Type I diabetes did not show statistical differences in terms of frequency of subjects that were seropositive and in the concentrations of IgG and IgA class antibodies as compared with the appropriate control group. Acute EV infections, with presence of IgM or EV-RNA, or both, in the serum, were detected with the same frequency in recently-diagnosed diabetic patients and

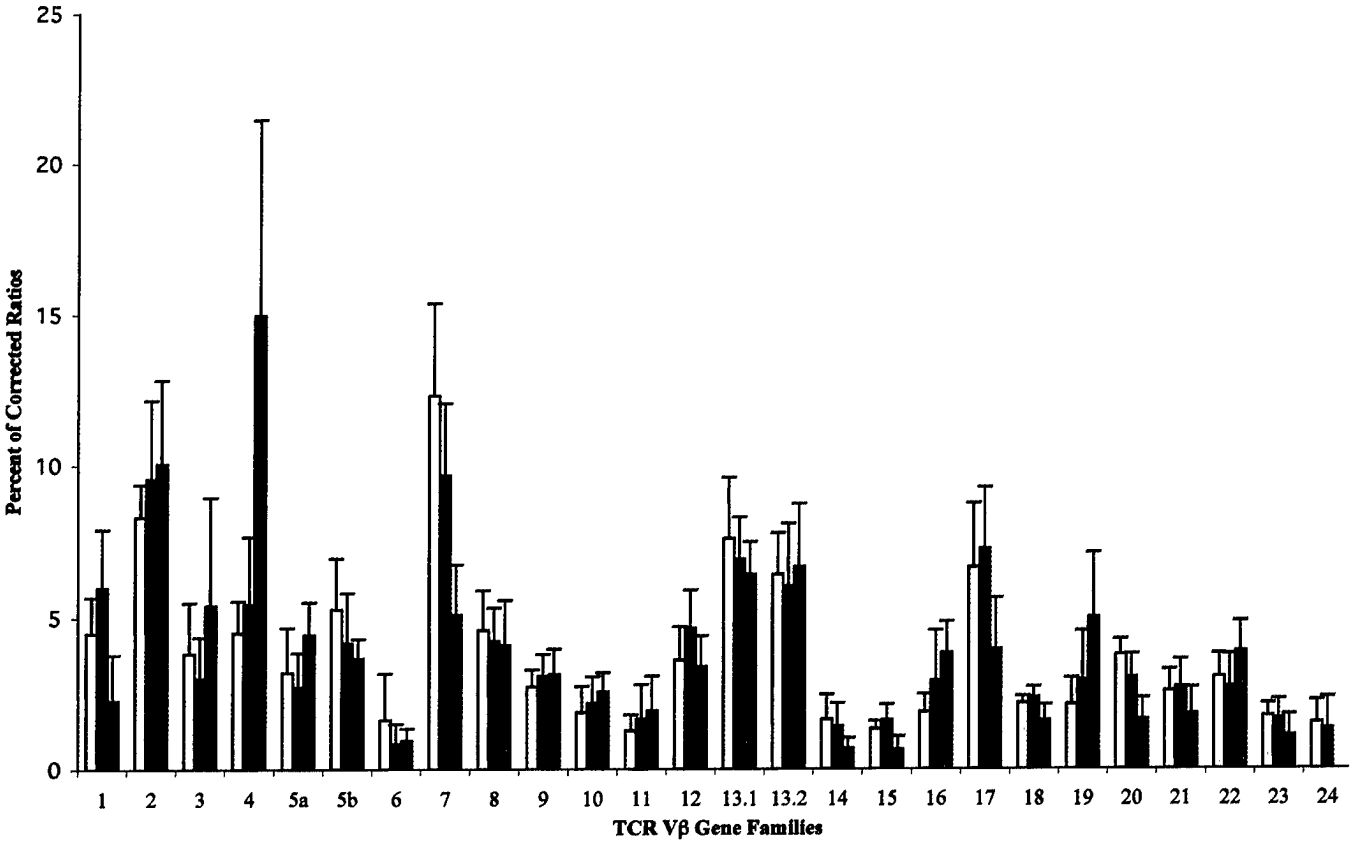

Fig. 4. Analysis of TCR *Vβ* repertoire in PBMC from Type I diabetic patients at onset (□) and after 2 to 14 years (■, after 7.2 years ± 3.4). The value of each TCR *Vβ* is expressed as a mean (± SD) percentage of corrected ratios described in Materials and methods. The mean (± SD) values of TCR *Vβ* gene families from normal control subjects (■, $n = 16$). Long-standing diabetic patients showed mean values of *Vβ7* that were significantly different from those detected in the control subjects ($p < 0.005$). Samples from these patients analysed at the onset of the disease showed significantly higher values of *Vβ7* ($p < 0.005$ to the control subjects)

control subjects (data not shown). This result was expected because the majority of kindergarten and school children are equally exposed to such an ubiquitous virus.

Analysis of the sera of the four converters in the time-course study showed, however, the presence of multiple acute EV-infections, defined by at least a twofold increase in IgA or IgG, or both, class antibodies to CVB4 and/or by IgM positivity or presence of viremia (Table 4). In the first converter, evidence of three acute infections was observed approximately at 14, 7 and 2 months before the onset of clinical diabetes, with the presence of EV-RNA in the serum from the first infection. The second converter had one acute infection approximately 5 months before overt diabetes indicated by both IgG and IgM responses. Evidence of an acute infection was also observed in converter No. 3, though the increase in antibody titres did not quite reach the twofold increase, as indicated in the criteria used to define the presence of acute EV-infections in the follow-up study (Table 4). Of note, converter No. 1, for example, for whom we could analyse more time-points before onset, the occurrence of acute infections is temporally related with an increase in circulating TCR $V\beta7^+$ T cells (Fig. 6). We detected that in this converter EV-RNA coincided with the highest expression of TCR *Vβ7* (at < 14 months before the onset of diabetes) (Table 4, Fig. 6). In converter No. 2 two acute EV infections (detected by IgM antibodies) in a period of two years was associated with persistently high expression of *Vβ7* almost without detectable oscillation. In converter No. 3 occurrence of acute EV infection was also associated with *Vβ7* increase (Tables 3, 4). Because the specimens tested were collected at 3 to 6 months intervals and sometimes at even longer intervals, it was not possible to unequivocally show how close the TCR repertoire and CVB infection coincided. Converter No. 4 did not show evidence of acute infections. In this patient the IgG titre constantly remained, however, very high during the entire observation period (Table 4).

The IgA or IgG, or both, class antibodies were also detected in the sera from high-risk subjects and long-standing diabetic patients showing previous EV infections (data not shown).

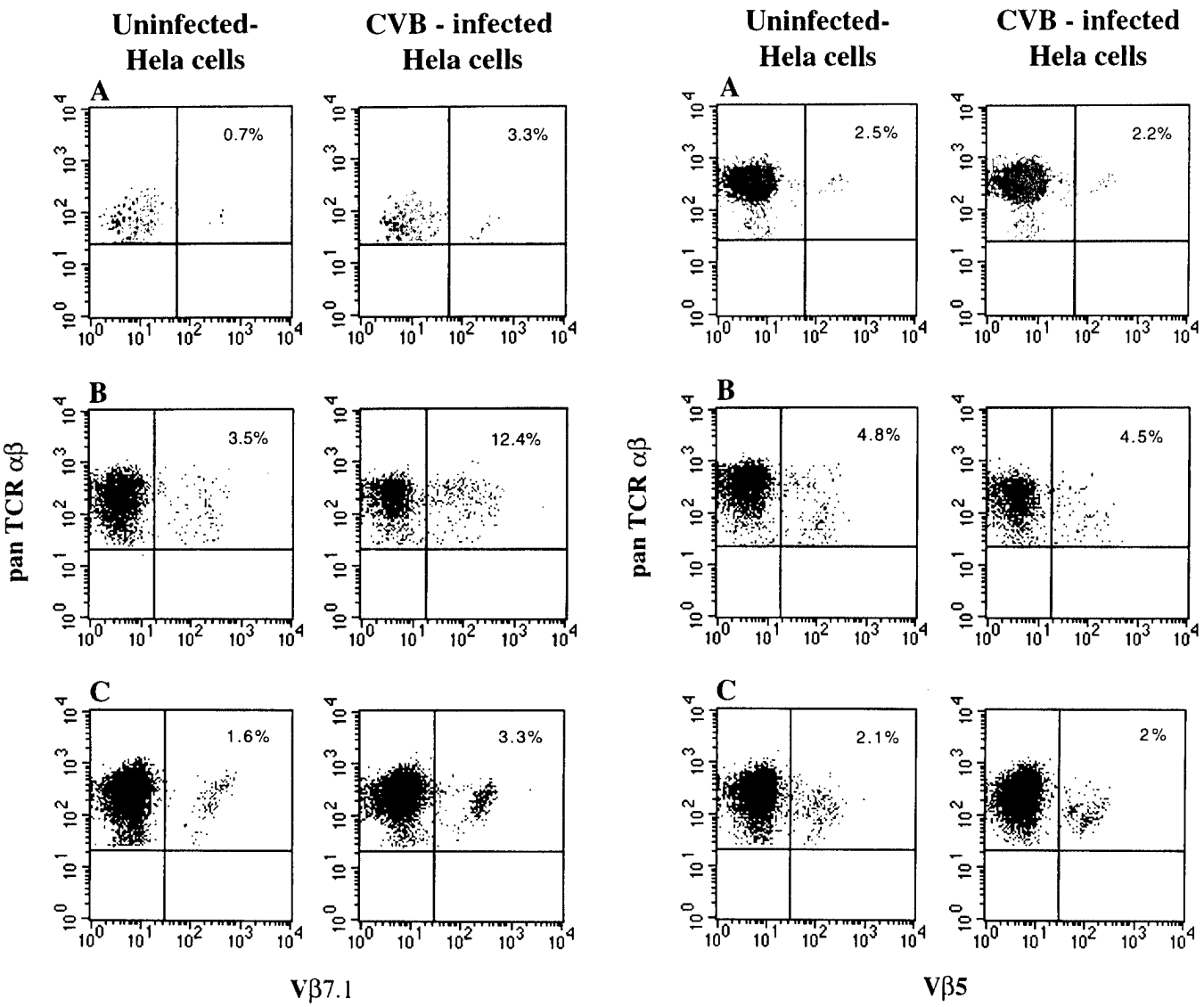

Fig. 5. Flow cytometry analysis of PBMC from three representative normal donors (**A, B, C**) cultured with CVB-uninfected and CVB-infected Hela cell lysates for 9 days. The proportion of T cells bearing Vβ7.1 chain is expressed as a percentage of TCR $\alpha\beta^+$ T cells within the live gated population of lymphocytes. Shown are the values of Vβ7.1$^+$ T cells detected in PBMC cultured with CBV-uninfected and CVB-infected HeLa cell lysates. Frequency of Vβ5$^+$ T cells is also shown as an example of TCR Vβ chain that did not show any change between the two culture conditions

Discussion

Numerous studies have indicated the role of infectious agents in the pathogenesis of Type I diabetes inducing, accelerating, or enhancing newly formed or already present autoimmune responses. One of the hypotheses is that microbial infections might break self-tolerance and trigger activation of autoreactive T cells. Activated T cells, in turn, destroy the target cells harboring the self-antigen, causing organ damage and loss of function [45].

Another hypothesis is based on the evidence that the increase in total T cell numbers associated with viral infections, is caused by the virally-mediated production of cytokines that activate bystander T cells, rather than to a specific antigen (or autoantigen) or the MHC molecule complex recognition [31].

We observed a preferential usage of the TCR *Vβ7* gene family in the peripheral blood of patients recently diagnosed with Type I diabetes by RT-PCR. Analysis of cell surface protein expression by flow cytometry also showed a higher percentage of circulating Vβ7.1 T cells in recently-diagnosed diabetic patients compared with control subjects. Such quantitative bias in the *Vβ* repertoire was also detected years before the clinical onset of the disease in four subjects who had been prospectively followed for months or years before the development of Type I diabetes. A blood sample available seven years after

Table 4. IgA, IgG and IgM class antibody titres against enterovirus (EV) antigens and presence of EV-RNA in converters to Type I diabetes

	EV-IgG (synthetic peptide)	EV-IgA (synthetic peptide)	CVB4-IgG	CVB4-IgA	IgM	RNA
Converter No. 1						
–40 months	12	14	27	34		
–28 months	12	8	26	16		
–24 months	9	5	25	15		
–14 months	6	4	21	12		+
–10 months	7	**3**	24	**15**		
–7 months	7	**41**	20	**45**		
–3 months	3	3	12	14		
–2 months	**3**	3	**14**	12		
+ 8 months	**16**	5	**32**	21		
Converter No. 2						
–40 months	64	33	92	62		
–13 months	40	21	78	39		
–11 months	**47**	22	**87**	48		
–5 months	**105**	28	**313**	79	+ + +	
+ 24 months	94	38	161	88	+	
Converter No. 3						
–19 months	85	4	67	23		
Onset	65	3	50	**16**		
+ 32 months	62	4	67	**28**		
+ 84 months	76	3	81	36		
Converter No. 4						
–12 months	61	8	172	16		
–1 months	96	10	176	17		
Onset	85	11	168	19		
+ 3 months	103	10	162	17		

The value for each IgA and IgG class antibody is expressed in enzyme immunoassay units (EIU). IgM and RT-PCR data are shown as categorical variables and are expressed as + /–. In bold, is shown the presence of acute EV infections in sequential samples shown as a twofold or greater increase in antibody value. Converter No. 3 had a marginal increase in CVB4-IgA which was highly suggestive for acute enterovirus infection

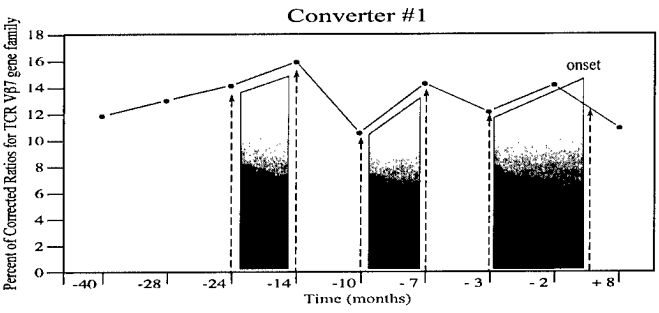

Fig. 6. Association of acute enterovirus infections with increase in *Vβ7* gene transcript in converter No. 1 for whom a large number of samples before onset of diabetes was available. Blood specimens collected sequentially during the prediabetic phase were tested for TCR *Vβ* gene expression and the corresponding sera for the presence of antibodies against several different EV serotypes and EV-RNA. The Y axis indicates the percent of TCR *Vβ7* gene family. The X axis indicates the time-points (in months) when blood was collected before (–) and consequent to appearance of overt diabetes. Arrows represent evidence of acute infections and shaded traits show increase of *Vβ7* gene expression occurring at the time of infection (see Table 4)

the onset of the disease subsequently showed that the expression of *Vβ7* decreased dramatically. In these patients, another family, *Vβ13.1*, tended to be higher at the onset of the disease than in the control group and decreased more rapidly than *Vβ7* after the onset of the disease. We concluded that the activation of autoreactive T-cell clones is mediated by a mechanism which is not very specific (because more than one Vβ chain is involved, i.e., Vβ7 and Vβ13.1) yet certainly not totally aspecific (the two chains involved are always Vβ7 or Vβ13.1 or both). A possible immunologic mechanism involving a superantigen was suggested previously [37, 40] but other slightly different explanations can also be considered [31, 32]. Apart from the true mechanism involved, it seems evident that in the majority of the cases studied, the expression of the *Vβ7* gene was skewed more pronouncedly than *Vβ13.1*, so that quantitative variations of *Vβ7* were more easily detectable than those of *Vβ13.1*. This difference in quantity also explains why variations of *Vβ13.1* in the converters (for whom we test the TCR repertoire at different time-points) could actually be detected, whereas in new-onset patients (for whom we frequently can only test one sample) the increase in

$V\beta$13.1 gene expression, although present, did not reach statistical significance. Thus, because the skewing of $V\beta$13.1 was, in all cases studied, quantitatively lower than the skewing of $V\beta$7, the $V\beta$13.1 increase became more rapidly undetectable over time than the rise in $V\beta$7. Because the evaluation of the various $V\beta$ families is a relative ascertainment of TCR β chain gene usage expressed as a percentage of the total, smaller increases (e.g., $V\beta$13) are more difficult to define than larger increases (e.g., $V\beta$7). In long-standing Type I diabetic patients, values of $V\beta$7 were slightly lower as compared with those at the onset of the disease but were still statistically higher than in normal subjects. The TCR repertoire analysis on our young control subjects showed an expansion of the $V\beta$4 gene family. This gene family showed the highest variability among different subjects, possibly representing a population of T cells easily activated in young children, whose activation, however, quickly disappears over time.

The observation of a preferential $V\beta$7 gene expression in peripheral blood of diabetic patients is in contrast to previous reports where the TCR $V\beta$ repertoire was found to be heterogeneous and where there was no consistent pattern of $V\beta$ expression that differentiated diabetic patients from subjects that were not affected [46, 47]. The reason for this probably depends on many factors, including the number of subjects analysed, patient heterogeneity and technical differences. Larger studies are needed to assess whether a bias in the TCR $V\beta$ expression truly characterizes pre-diabetic and diabetic patients.

The skewing of the TCR $V\beta$ repertoire is not only related to the presence of certain HLA alleles because nine of the control subjects carrying HLA-haplotypes conferring high risk to diabetes did not show the same TCR repertoire. Moreover, the analysis done on healthy subjects HLA-DR-matched and DQ-matched to the diabetic patients did not show expansion of either $V\beta$7 or *13.1* gene families.

We previously reported the presence of a T-cell repertoire bias with overexpression of $V\beta$7 gene family among the T cells infiltrating the islets of Langerhans of patients suffering from Type I diabetes [37]. Our results indicate that a preferential usage of the same $V\beta$ gene families can also be detected amongst the circulating T cells of diabetic patients. This suggests that the selective use of TCR genes in the periphery reflect an on-going infiltration within the affected tissue possibly mediated by T cells sharing the same autoreactive TCRs.

The CVB infections have been linked to Type I diabetes once a CVB4 strain was isolated from the pancreas of a diabetic child [28]. So far, reports have associated several different EV with pre-diabetic autoimmune episodes and with the onset of overt diabetes [4, 5, 10, 12–19], although the pathway by which EV could actually induce diabetes in humans is not understood very well.

The analysis of the sera from our recently-diagnosed Type I diabetic patients, showed that the numbers of subjects who were positive for EV-antibodies and EV-RNA was not statistically different than in the control groups. We anticipated these data because EV are agents of the common cold causing infections in kindergarten and school children, as reported previously [23–25]. In the longitudinal study of the four converters, however, where serum samples were available for testing every 3–6 months, multiple, sequential acute infections and viremia were detected. In particular, acute infections were observed in samples collected months before the clinical onset of diabetes. Other evidence of sequential acute EV infections before onset of Type I diabetes was described recently [4, 48]. These data further emphasize the concept that longitudinal prospective studies on sera from diabetic patients taken at short intervals are necessary when looking for evidence of acute EV infections. Our results confirmed that the timing of EV infections play a critical part in the development of diabetes, as shown in the NOD mouse [32] and, more recently, in humans [13].

Although more Type I diabetic patients need to be analysed in prospective studies, we speculate that a history of environmental factors (i.e., Coxsackievirus infections) cause an activation of autoreactive T cells bearing certain $V\beta$ gene families already present in pre-diabetic subjects. In vitro, exposure of PBMC from certain healthy donors to lysates of HeLa-cells infected with CVB, was capable of selectively activating $V\beta7^+$ and $V\beta13.1^+$ T cells. These results agree with previously published data showing that variable degrees of expansion of $V\beta7^+$ and $V\beta13.1^+$ are found among proliferating and recently activated ($CD69^+$ and/or $CD71^+$) T cells from both healthy control subjects and diabetic patients cultured with CVB-infected cell lysates [40, 49, 50]. The presence of increased percentage of TCR $V\beta$7 gene expression at the moment of acute EV-infections was observed in at least two of the diabetic patients. Results obtained in the mouse also support this general hypothesis [31].

In short, we presented data showing an expansion of T cells bearing preferential TCR $V\beta$ gene families in the peripheral blood of Type I diabetic patients. This T-cell expansion was not solely HLA-related, it was already somewhat higher than in healthy control subjects months before overt diabetes, it was evident at the time of clinical onset and persisted for years. Also, enteroviral analysis on the sera of these subjects showed signs of acute infections months before overt diabetes. These results, with the evidence that CVB can induce preferential TCR $V\beta$ activation, support the hypothesis that EV is aetiologically important in the development of Type I diabetes by acceler-

ating, if not initiating the process of autoimmune beta-cell destruction.

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Analysis of TCR Vβ Repertoire and Cytokine Gene Expression in Patients with Idiopathic Dilated Cardiomyopathy

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Although the etiopathogenesis of idiopathic dilated cardiomyopathy (IDC) is still unclear, it is widely accepted that a complex interplay between viral infections and immune mechanisms is the basis of disease genesis. Previously, we showed that heart-infiltrating T cells of patients suffering from acute, fulminant Coxsackie virus $B3^+$-IDC shared a preferential usage of three variable gene segments of the T cell receptor β chain-(TCR-Vβ) encoding families Vβ3, 7 and 13.1. This indicated the possible presence of a superantigen-driven immune response. Here, we further investigated the IDC immunological scenario by analysing different phenotypes of heart-infiltrating cells: TCR repertoires, cytokine expression and presence of enterovirus-specific antigens. IDC patients who underwent heart transplantation at different times after the onset of heart failure were studied. A cardiac infiltrate of $CD4^+$ and $CD8^+$ T cells was present together with activated macrophages. Furthermore, the same Vβ gene families, previously found to be skewed in hearts from fulminant cases of $CVB3^+$-IDC, together with two additional Vβ gene families, Vβ1 and 5B, were increased. IL-1β, IL-2, IL-6 and IFN-γ were expressed in the myocardium while others, like IL-4 were not. In conclusion, an orchestrated complex of immune mechanisms seems to be the basis of IDC etiopathogenesis.

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Key words: T cell receptor, cytokine, coxsackie virus B, cardiomyopathy, myocarditis

Introduction

Idiopathic dilated cardiomyopathy (IDC) is characterized by the dilatation and impaired contractility of the myocardium eventually leading to terminal heart failure. IDC represents the primary indication for heart transplantation in both adults and children beyond the neonatal period [1]. Many cases of IDC are of unknown origin, but (at least in adolescents and young adults) IDC is often considered to be the possible sequelae of a prolonged or recurrent myocarditis [2]. Although little more is known, its pathogenesis is generally believed to be multifactorial, involving genetic predisposition, immune mechanisms and viral infections.

Group B Coxsackie viruses (CVBs) are the agents most commonly involved in the etiology of acute myocarditis in humans. The presence of CVB-specific neutralizing IgM and IgG antibodies has frequently been observed in the sera of patients suffering from acute and chronic myocarditis [3, 4]. However, despite indirect evidence of persistent CVB infections, attempts to detect CVB genome in the affected tissue, by both molecular techniques and immunohistochemistry, have produced results that varied considerably between different reports [2, 5–7]. These reported discrepancies could reflect the fact that after the first injury, the majority of the viral particles are cleared from the cardiac tissue and, depending on the stage of the disease and the viral load, the persistence of viral

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genome is not easily detected by standard techniques. Recently, it has been proposed that cell-mediated immune mechanisms associated with the secretion of certain cytokines are the basis of the pathogenesis of chronic myocardial inflammation progressing to IDC [8]. Most studies acknowledge the important immunopathological role of heart infiltrating $CD4^+$ and $CD8^+$ T-lymphocytes in the pathogenesis of IDC [9–11]. Furthermore, the expression of immunoregulatory cytokines like interferon-γ (IFN-γ) and interleukin-2 (IL-2) and of the pro-inflammatory cytokines interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumour necrosis factor (TNF)-α, have been proposed to play important roles in heart muscle disease [12].

Experimental evidence suggests that there are two phases of myocardial damage associated with viral infection: (1) an early, often fulminant myocarditis, in which the cardiotropic virus causes, directly or indirectly, the myocardial injury; and (2) a later, more prolonged 'chronic active myocarditis', that appears to be the result of autoimmune phenomena specifically directed against the myocytes. Immune-mediated mechanisms are also believed to be crucial in the development of fulminant myocardial failure that may be observed in some patients with acute myocarditis.

Recently, we showed that a restricted T cell receptor (TCR) Vβ repertoire is present amongst the T cells infiltrating the hearts of patients suffering from acute, fulminant CVB3-mediated myocarditis that rapidly progressed to IDC. This remarkable abundance of TCR Vβ3, Vβ7 and Vβ13.1 mRNA gene transcripts was not only found in the myocardium but also in the mediastinal lymph nodes and the thymus of our patients. The TCR Vα repertoire analysis showed, instead, a normal pattern of expression. The characterization of a limited number of TCR Vβ families amongst the cells directly involved in these early and acute heart diseases suggested that the infiltrating T cells might have been specifically reactive against a CVB-superantigen [11].

In this new study, we investigated further the immunological mechanisms involved in the pathogenesis of IDC by analysing the phenotypes of infiltrating cells and the expression of TCR genes in six additional patients clinically diagnosed as suffering from IDC who underwent cardiac transplantation at different times after the onset of heart failure. In particular, we were interested to see if in the myocardium from these patients it was still possible to detect expanded T cell subpopulations that were using the same restricted set of TCR Vβ gene segments as we detected in hearts of acute, fulminant IDC-patients. We also examined the presence of enterovirus in the hearts of these patients by both molecular and immunohistochemistry techniques. In view of the importance of cytokines in heart disease, we also quantified mRNA gene transcripts for several cytokines in the myocardium and peripheral blood of these same IDC patients. Finally, all the patients were typed for their human leukocyte antigens (HLA) class II alleles.

Materials and Methods

Patients/tissue samples

We studied the explanted hearts of six patients diagnosed as suffering from IDC and admitted to the Children's Hospital of Boston (CHB) and the Children's Hospital of Pittsburgh (CHP) for cardiac transplantation between December 1995 and July 1998. These comprised of: a) the hearts from three patients (patients 1–3) previously described as suffering from an acute-onset CVB3-IDC [11] and of the hearts from three new patients (patients 4, 6, 7) who underwent cardiac transplantation within 2 weeks–4 years from the initial onset of symptoms. For the majority of the patients, both right (RV) and left ventricle (LV) specimens were available together with peripheral blood. We also studied endomyocardial biopsies performed on an additional patient (patient 5) who clinically recovered from an acute IDC after treatment with intravenous γ-globulin and corticosteroids. All the patients showed radiographic features characteristic of marked cardiomegaly and echocardiographic evidence of globally depressed ventricular function with left ventricular dilatation and impaired systolic contraction (left ventricular ejection fraction of less than 50%). Another two patients (patients 8 and 9) with the clinical diagnosis of 'suspected myocarditis' were also included in this study. Endomyocardial biopsies and peripheral blood samples were obtained from these patients two weeks after the onset of symptoms. The Institutional Review Board (IRB) at the CHB or at the CHP approved the use of the heart specimens for research. This latter IRB also approved the use of blood samples from 18 healthy children ranging from 3 to 16 years of age who had no family history of heart diseases. These samples were used as normal controls for TCR Vβ repertoire and cytokine gene expression analyses. All the patients were HLA class II molecularly typed [13, 14]. Demographic and clinical data of IDC and myocarditis patients are described in Table 1. In some cases, cytokine mRNA assessment and TCR Vβ repertoire were incomplete due to the limited availability of cDNA.

Immunohistochemistry

Specimens of cardiac tissue obtained from IDC patients at the time of transplantation were snap-frozen in isopentane chilled to −150°C, and stored at −80°C. Immunohistochemistry was performed on cryostat sections (0.5-μm) using the immunoalkaline phosphatase anti-alkaline phosphatase (APAAP) technique according to Mason [15]. Briefly, heart sections were incubated with the following mouse monoclonal antibodies (mAbs) specific for CD4 and CD8 cells, and activated monocytes/macrophages (Ber-MAC3) (DAKO Corporation, Santa Barbara, CA, USA). The presence of different strains of CVB was determined histologically using mAbs (B-Blend, IgG1; B3, IgG2a;

Table 1. *Demographic and clinical data of the patients*

Patient	Age*/ sex	Clinical diagnosis	Time from onset to biopsy/ transplantation	Medical history	HLA-DRB1	HLA-DQA1	HLA-DQB1
1§	$5\frac{1}{2}$ yrs/F	Fulminant IDC	6 days	Fever and recurrent vomiting	07, 13	01, 0201	0201, 0603
2§	6 months/M	Acute IDC	2 weeks	Viral syndrome	13, 15	01	0602, 0603
3§	15 yrs/M	Acute IDC	1 month	Flu-like illness	11, 14	01, 05	0502, 0601
4	8 yrs/M	Severe IDC	3 month	Flu-like syndrome, vomiting	04, 07	0201, 0301	0302, 0303
5	12 yrs/M	Clinically recovered from acute IDC	1 month	Fever, malaise, cough	03, 08	04	0201, 04
6	7 yrs/M	Severe IDC	4 years		04, 13/14	01, 03	0302, 0605
7	3 months/F	Familial IDC	2 weeks	Family history of IDC	04, 08	03	06
8	14 yrs/F	Myocarditis	2 weeks			01	0501, 0603
9	16 yrs/F	Myocarditis	2 weeks			0201	0201, 0301

*Age at time of biopsy/transplantation; §data for patients 1–3 previously described [11].
IDC=Idiopathic dilated cardiomyopathy; F=female; M=male; HLA=human leukocytes antigens-DRB1, DQA 1 and DQB1.

B6, IgG2a ascites, 1/1,000 dilution) (Accurate Chemical & Scientific Corporation, Westbury, NY, USA). Control sections were treated with irrelevant isotype-matched mAbs (IgG1 and IgG2a). Sections were then incubated with rabbit anti-mouse immunoglobulin (DAKO). The APAAP immunocomplex (DAKO) was applied and then detected using Fast-Red TR as substrate (DAKO).

Peripheral blood leukocytes (PBL) isolation

Peripheral blood was collected at the time of the surgical procedure. Leukocytes were isolated using Ficoll, washed extensively in HBSS, counted and used for RNA extraction.

Detection of picornavirus genome

For the detection of CVB genome, reverse transcriptase (RT)–PCR using a 5′ sense primer (5′-CAC GGA CAC CCA AAG TA-3′) and 3′-antisense primer (5′-ACC TTT GTA CGC CTG TT-3′) was performed [16]. The amplified DNA was tested for the presence of enterovirus sequence with nested primers Coxprim 3 and Coxprim 4 which amplify a 298-bp segment located in the 5′-non-coding region of all sequenced picornaviruses with exception of Hepatitis A [16]. Twenty microlitres of the PCR products were separated on an agarose gel and visualized with ethidium bromide.

RNA extraction and RT–PCR

Specimens from explanted hearts and endomyocardial biopsies were frozen immediately in dry ice. Total RNA was extracted from the myocardium and PBL using TRIZOL® Reagent according to the manufacturer's instructions (Gibco BRL, Life Tech., Inc., Gaithersburg, MD, USA). All the RNA samples were kept in 75% ethanol and stored at −80°C until RT–PCR was performed. For each preparation, at least 3 μg of total RNA were reverse-transcribed into single-stranded cDNA using SUPERSCRIPT™ II (Gibco BRL) and used for TCR Vβ repertoire and cytokine mRNA gene transcript analyses.

Evaluation of TCR Vβ repertoires

The study of the TCR Vβ gene segment usage requires individual amplification of at least 26 TCR Vβ families and subfamilies, as previously described [17, 18]. Two microlitres of the PCR mixtures containing the Vβ gene family amplified cDNA were resolved by electrophoresis on an ABI 377 DNA Sequencer and automatically analysed by GENESCAN 2.1 software (Applied Biosystems Inc.) [18]. For the controls, levels of each Vβ gene family were expressed as the mean±standard deviation (SD) for each study group. For each patient, a value for any TCR Vβ gene family which exceed 2 SD above the mean of the controls was considered to be a significant deviation of the TCR repertoire.

Competitive RT–PCR for IL-4 and IFN-γ mRNA expression

cDNA prepared from myocardial specimens from the majority of patients was used for IL-4 and IFN-γ-specific amplification. Primer pairs for IL-4 specific amplification were as follows: (5′) ACC ACG GAC ACA AGT GCG ATA (sense) and (3′) 5′GTT GGC TTC CTT CAC AGG ACA G (3′) (anti-sense). These primers amplify a 333-bp segment located in position 128–460 of the mRNA sequence. Constant amounts of cDNA samples were amplified in the presence of serial dilutions of a IL-4 specific competitor cDNA

(PCR MIMIC; Clontech Lab., Inc., Palo Alto, CA, USA) following the manufacturer's instructions. The size of this competitor product was 504 bp. Primers pairs for IFN-γ-specific amplification were as follows: (5′) TGG GTT CTC TTG GCT GTT ACT (3′) (sense) and (5′) CTT CCC TGT TTT AGC TGC TG (3′) (anti-sense). These primers amplify a 409-bp segment located in position 173–582 of the mRNA sequence. IFN-γ specific competitor cDNA (PCR MIMIC; Clontech Lab.) was used in a competitive PCR amplification as described above for IL-4 specific amplification. The size of the IFN-γ competitor PCR product was 557-bp. Cytokine amplification was performed with a PE Applied Biosystem DNA Thermal Cycler with 35 cycles of the following sequence: denaturation at 95°C for 20 s, primer annealing at 63°C for 30 s, primer extension at 72°C for 30 s. PCR products were separated by electrophoresis on 1.6% agarose gels and visualized using ethidium bromide or GelStar® nucleic acid gel stain (FMC BioProducts, Rockland, ME, USA) and photographed (55 film; Polaroid, Cambridge, MA, USA). Densitometric analysis of the negative images was performed using a Molecular Dynamics Densitometer. After subtraction of the background values, the density ratio of the competitor band to the target mRNA was determined and the amount of cytokine mRNA was calculated by interpolation between the known amounts of competitor which produced densities nearest to the target densities. Equal aliquots of cDNA were analysed for IFN-γ and IL-4 and the final result was expressed as the number of molecules of specific mRNA per μg of total RNA.

Quantitation of IL-1β, IL-2 and IL-6 mRNA gene expression

Total RNA extracted from the myocardial specimens or the PBL was used for assessment of IL-1β, IL-2 and IL-6 mRNA gene expression using a sensitive and specific colorimetric microplate assay (Quantikine® mRNA, R&D SYSTEMS, Minneapolis, MN, USA). Briefly, 50 μl of cytokine-specific probe was added to each well together with appropriately diluted RNA sample. For each experiment, 10–20 μg of total RNA from each myocardium specimen and 3–6 μg of total RNA from PBL of each patient were used and run in duplicate. A calibration curve was also determined in each assay. The plates were incubated, washed and developed according to the manufacturer's instructions. The optical density was read at both 490 and 650 nm. The duplicate results for each sample were averaged and corrected for the average optical density of the zero calibration wells. Final results were expressed as the number of molecules of specific mRNA per μg of total RNA.

Results

Immunohistochemical analysis

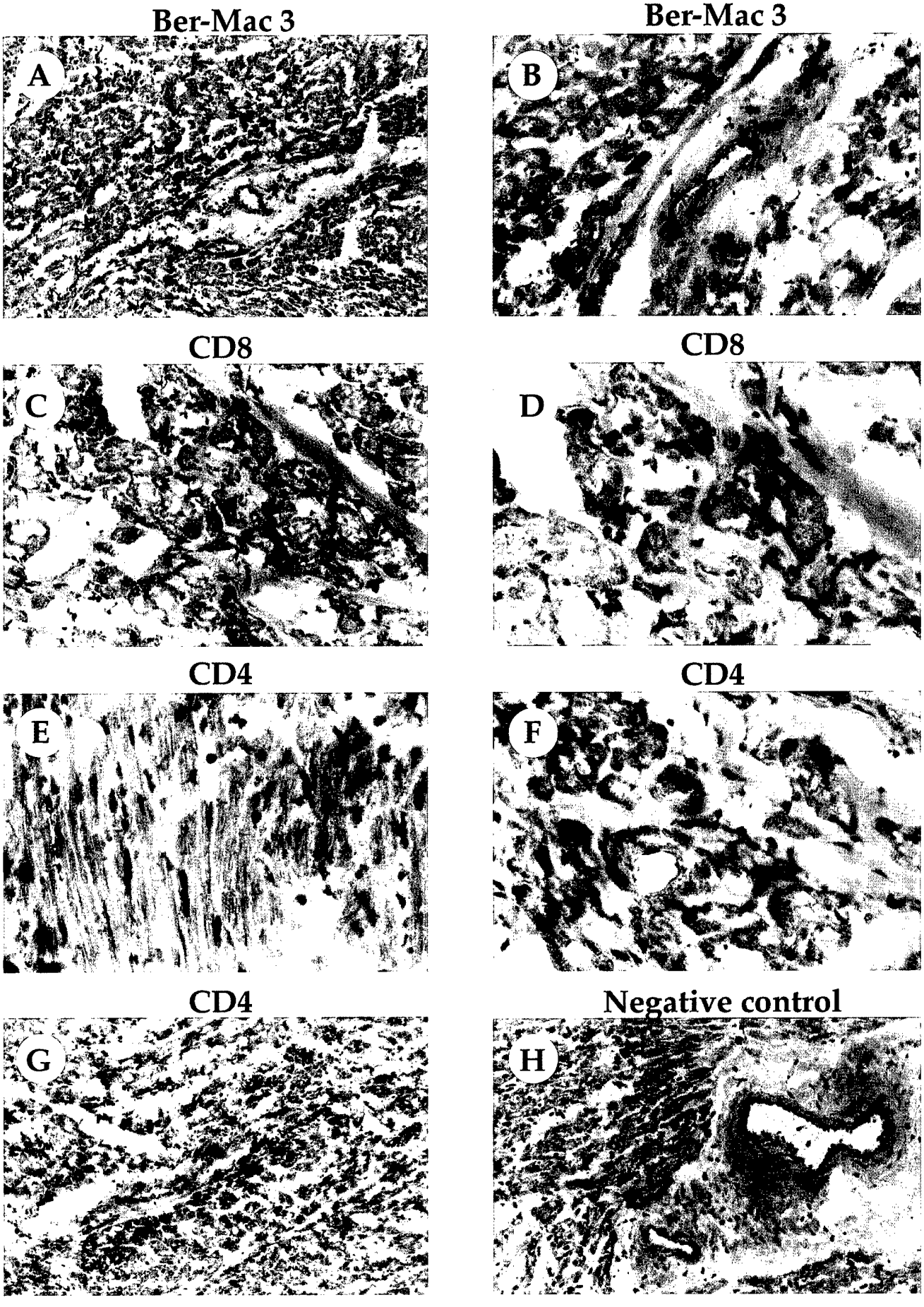
Expression of Ber-MAC3, CD4, and CD8 in hearts of IDC patients

By using the Ber-MAC3 mAb specific for stimulated monocytes and resident tissue macrophages [19], we detected a massive infiltrate of cells in all the specimens tested (Figure 1). Immunostaining against CD4 and CD8 showed the presence of an abundant infiltrate of cells between the myocytes, in the perivascular tissue and adjacent to blood vessels. While no signs of fibrosis were found in the majority of the sections analysed, areas of extensive damage and necrosis were frequently present. However, in patient 7, focal areas of scar tissue were detected interspersed within areas of apparent normal cardiac tissue. In this patient, CD4- and CD8-specific immunostaining appeared weaker and leukocytes looked smaller in size, with a redued cytoplasm. Since only biopsy material was available from patients 5, 8, and 9 it was not possible to perform the immunohistochemical analysis for these cases.

Detection of CVB antigens in specimens of IDC patients

Using CVB-blend mAb specific for all serotypes of CVB (B1–B6) we found that amongst the IDC patients tested, the myocardium of patients 1, 2, 3 and 4 showed strong reactivity while patients 6 and 7 were negative. The same hearts were positive for CVB3 serotype as demonstrated by staining with the specific mAb (Figure 2). The staining was localized in the same areas found positive for Ber-MAC3, CD4, and CD8. Within the cardiac tissue, stronger positivity was detected in areas of extensive necrosis. In these areas, the infiltrating CVB^+-cells appeared between the myofibres, which looked irregular in shape, and within the connective tissue or around blood vessels (Figure 2). The staining appeared granular, both in the cytoplasm and in the surrounding space. In patient 1, where mediastinal lymph node tissues were available, a selective positivity for CVB3 was also detected. CVB6 immunostaining was negative in all samples.

Figure 1. Immunostaining of heart from patient 4 for Ber-MAC3, CD4 and CD8. A. Massive infiltration of Ber-$MAC3^+$ elements are showed (red) between the myofibres and in the perivascular tissue. Myofibres appear irregular in shape (10×). B. Ber-MAC3 elements cross the wall of a blood vessel (20×). C. Infiltrating $CD8^+$ lymphocytes are showed (red) in an area of prominent necrosis and connective tissue between myocytes (20×). D. Infiltrating $CD8^+$ elements at a higher magnification (40×). E. $CD4^+$ cells between myofibres (40×). F. and G. Remarkable infiltration of $CD4^+$ cells (red) in the affected myocardium (40×; 10×). H. IgG1 isotype negative control (20×).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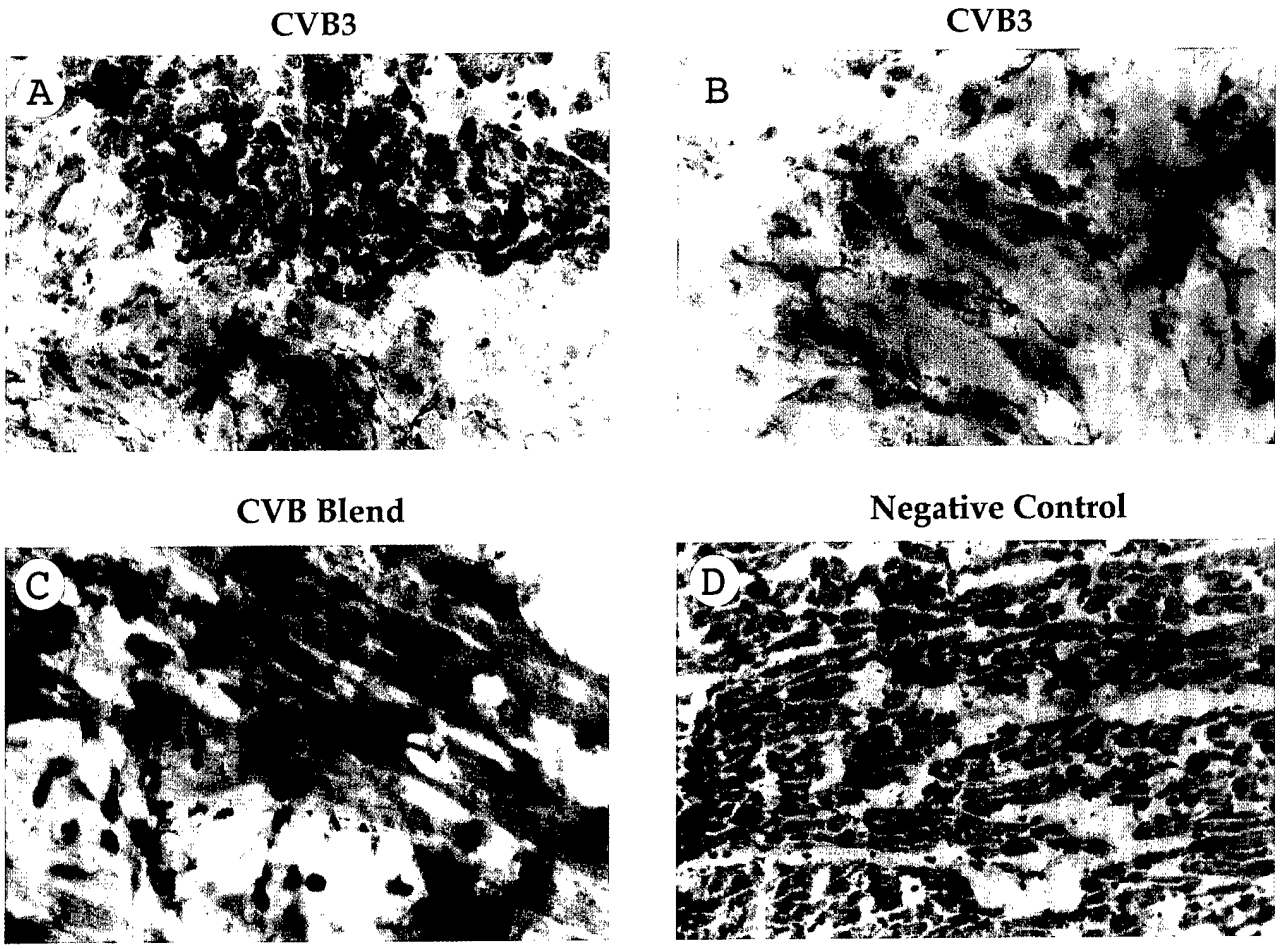

Figure 2. Coxsackie virus B immunostaining of heart from patient 4. A, B. CVB3 serotype immunostaining revealed by APAAP in red. Positive staining was within the necrotic areas of the myocardium; $CVB3^+$-elements between irregular myofibres are also shown (20×; 40×). C. CVB Blend (B1–B6 serotypes) positive elements between myofibres (40×). D. IgG2a isotype negative control (20×).

Detection of picornavirus by RT–PCR

Amongst heart specimens, we were able to amplify the CVB genome in patient 1 only [11].

TCR repertoire analysis of infiltrating T cells in the hearts of IDC patients

The estimated percentages of T cells bearing different Vβs, extrapolated as a function of the relative abundance of the different TCR gene families, in the myocardium of IDC patients are shown in Figure 3 and Table 2. In the majority of the patients there was a different combination of expansion of Vβ3, 7 and 13.1 gene families as compared to the expression of these families in PBL from normal, healthy controls. In patients 5, 6 and 7, we also detected higher abundance of the TCR Vβ1 gene family as compared with the expression of this family in the PBL of healthy age-matched children (Figure 3 and Table 2). In these patients, TCR Vβ1 expansion was greater than 2 SD above the value measured in PBL from normal controls. Furthermore, high frequency of $V\beta5B^+$ T cells, was detected in hearts of patients 4–6. These patients showed TCR Vβ5B expansion greater than 2 SD above the value measured in PBL from age-matched, controls. TCR Vβ analysis on patient 7, who was suffering from a familial IDC, showed a remarkable expansion of T cells carrying the Vβ17 gene family (Figure 3 and Table 2). In the endomyocardial biopsies of the two myocarditis patients (patients 8 and 9), the TCR Vβ repertoire was not interpretable, possibly because of paucity of the T cell infiltrates in the tissue available from these patients. TCR repertoire analysis of the patients' PBL was only possible in patient 4 where high frequencies of $V\beta7^+$ (11.2%) and $V\beta13.1^+$ (15.9%) T cells were detected as compared with the frequency measured in peripheal blood from controls (5.5%±2.1) and 6.3%±1.1, respectively) (data not shown).

Expression of cytokine gene transcripts in hearts of IDC patients

IL-4 and IFN-γ mRNA expression

For each patient from whom the cDNA was available, IL-4 and IFN-γ mRNA expression was determined by

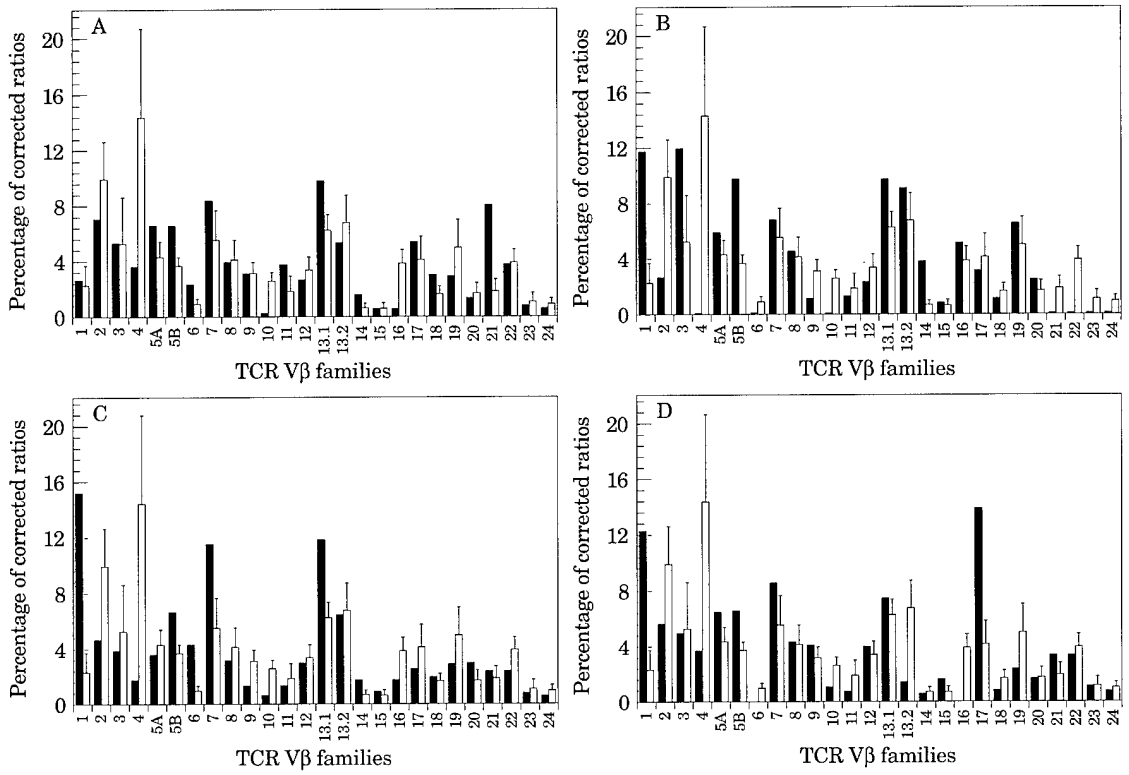

Figure 3. T cell receptor (TCR) Vβ repertoires of T cells infiltrating the heart of IDC patients. Analysis of TCR variable region of the β chain (Vβ) repertoire of T cells infiltrating the heart (■) of IDC patients (A) 4, (B) 5, (C) 6 and (D) 7 and the PBL (□) of normal controls (n=18).

Table 2. *T cell receptor (TCR) Vβ expression in IDC heart specimens*

Patients	Vβ1	Vβ5B	Vβ3	Vβ7	Vβ13.1	Vβ17
1*	2.1	4.4	21.2	4.4	14	4
2*	3.1	2.3	7.4	55.6	6.1	3.4
3*	4.5	3.6	0.8	13.7	9.5	4.3
4	2.7	7.3	5.7	8.4	10.7	6.3
5	11.7	9.8	11.9	6.8	9.7	3.1
6	17.5	7.5	4	13.2	13.2	3.3
7	12.3	6.6	4.9	8.6	7.4	13.8
PBL controls (n=18)	2.3±1.4	3.7±0.6	5.2±3.3	5.5±2.1	6.3±1.1	4.1±1.7

The value of each TCR variable segment of the β-chain (Vβ) is expressed as a percentage of corrected ratios. Shown are the highest value measured in the heart of IDC patients.
*Data (Patients 1–3) for TCR Vβ3, 7 and 13.1 previous reported [11]. Mean values (±SD) for PBL from normal controls.

using a PCR MIMIC system based on a competitive PCR approach. An example of these analyses is shown in Figure 4. Relative abundance of IFN-γ mRNA molecules was measured in all the hearts of IDC patients compared to the number of mRNA molecules detected for IL-4 (Table 3). Relative abundance of IFN-γ mRNA molecules was also detected in mediastinal lymph node of patient 1. The highest relative abundance of IFN-γ mRNA was measured in patient 4 (100,000 mRNA molecules/μg of RNA used in the cDNA synthesis).

IL-1β, IL-2 and IL-6 mRNA expression

For each specimen where the RNA was available, the expression of IL-1β, IL-2 and IL-6 gene transcripts in the hearts of IDC patients was quantified. The same RNA, extracted from the heart of each patient, was tested for IL-1β, IL-2 and IL-6 mRNA expression. The cytokine which showed the highest level of expression in the hearts of IDC patients tested in this study was IL-1β (ranging from $1{,}200\times10^3$ to $6{,}400\times10^3$ mRNA molecules/μg RNA) (Table 4). Interestingly,

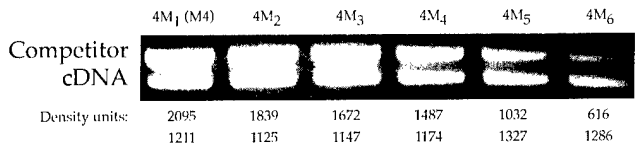

Figure 4. Competitive PCR analysis for IFN-γ. An example of competitive PCR is shown in which the quantitation of IFN-γ mRNA in lymph node was performed on patient 1 specimen. Briefly, cDNA prepared from specimens from IDC patients was amplified in the presence of two-fold serial dilutions of a specific competitor cDNA for IFN-γ, as described in Material and Methods. In the first lane the amount of competitor was 15,000 molecules. In all cases, the upper band is due to amplification of the competitor cDNA and the lower band is due to amplification of the target cDNA. The quantification of the amount of the target cDNA in the samples is based on determination of which two-fold serial dilutions gives target and competitor bands of equal intensity. The particular two-fold competitive dilution is shown above each pair of bands and the densitometric measurement is shown beneath the bands. In this example, the dilution where the target and the MIMICS bands are of equal intensity is between $4M_4$ and $4M_5$ dilutions and corresponded to 14,000 mRNA molecule/μg.

Table 3. *IL-4 and IFN-γ mRNA gene transcripts in hearts of IDC patients*

Patient	Source	IL-4 mRNA molecules	IFNγ mRNA molecules
1	Heart	28	733
	Lymphonode	50	14,400
2	LV	<1	<1
	RV	<1	<1
3	LV	3.5	280
	RV	<1	<1
4	LV	10,500	100,000
	RV	930	<1
5	Biopsy	n.d.	n.d.
6	LV	<1	<1
	RV	<1	12,750
7	Heart	<1	3
8	Biopsy	n.d.	n.d.
9	Biopsy	n.d.	n.d.

Equal aliquots of cDNA were analysed for IL-4 and IFN-γ and the final result is expressed as the number of molecules of specific mRNA per μg of total RNA.
LV=Left ventricle; RV=right ventricle; n.d.=not determined.

the highest number of mRNA molecules for IL-β was measured in the right ventricle of patient 4 where strong immunostaining for the CVB3 serotype was also detected. Furthermore, IL-1β was the cytokine most highly expressed in the lymph node of patient 1. The cytokine which showed the lowest levels of expression was IL-2. In all the specimens, we detected less than the minimum amount of IL-2 mRNA molecules detected by our assay (200 molecules of mRNA/μg of RNA). Levels of IL-1β, IL-2 and IL-6 mRNA molecules did not correlate with duration of symptoms. Interestingly, the lowest level of IL-6 mRNA molecules was measured in the heart of patient 7 who was diagnosed as suffering from a familial IDC and had neither evidence of viral-like illness before the onset nor was positive for CVB as ascertained immunologically.

Expression of cytokine gene transcripts in PBL of IDC and myocarditis patients

IL-1β was the cytokine that showed the greater expression in the hearts of IDC patients. We wanted to investigate mRNA levels for this cytokine in PBL from the same IDC and myocarditis patients. For each individual where RNA was available, the number of mRNA molecules for this cytokine was calculated and is shown in Table 5. Among the patients, the highest values of mRNA molecules for IL-1β were measured in one patient (patient 2) with acute, fulminant IDC and in the two myocarditis patients (patients 8 and 9). Only one patient (patient 3) with acute, fulminant IDC showed levels of IL-1β mRNA molecules similar to the values measured in PBL from normal controls (Table 5). Low levels of IL-1β mRNA molecules were measured in patient 6 who was suffering from a severe IDC and was transplanted after 4 years from the onset and in patient 7 who was diagnosed as suffering from a familial IDC.

HLA class II molecular typing

HLA class II molecular typing was performed on cDNA from cardiac tissue of IDC patients. This analysis revealed that the majority of the patients carried the DQA* 01 (55%) and DQB* 06-alleles (66%) (Table 1).

Discussion

The pathogenesis of IDC is admittedly complex. Presumed infectious and/or autoimmune myocarditis is believed to produce almost all of the IDC cases seen in the United States children beyond infancy [2]. Enteroviruses, and especially the CVB group, have been associated with the etiology of myocarditis and its progression to IDC [3, 4, 11]. Although a post-viral pathogenesis for IDC is now widely accepted, the actual role of viral infections in IDC has not been well defined. In part, this is due to the inability to detect the presence of virus in all cases of IDC strongly suspected to have resulted from an infectious myocarditis. Several investigators have reported the persistence of live enterovirus or enteroviral CVB3-RNA in endomyocardial biopsies of both myocarditis and IDC patients [2, 5–7]. However, it is still unclear whether IDC is the result of a direct cytopathic effect of viruses on the myocytes or merely a consequence of infection-induced immune responses against myocardial cells.

Table 4. *Cytokines mRNA gene transcripts in the heart of IDC patients*

Patient	Source	IL-1 mRNA 10^3 molecules	IL-2 mRNA 10^3 molecules	IL-6 mRNA 10^3 molecules
1	Heart	2600	<0.2	1.1
	Lymph node	4700	<0.2	2.5
2	LV	4900	<0.2	0.8
	RV	1200	<0.2	
3	LV	4100	<0.2	1.4
	RV	2900	<0.2	3.3
4	LV	5000	<0.2	1.9
	RV	6400	<0.2	1.9
5	Biopsy	n.d.	n.d.	n.d.
6	LV	5300	<0.2	<1
	RV	5100	<0.2	<1
7	Heart	3800	<0.2	0.7
8	Biopsy	n.d.	n.d.	n.d.
9	Biopsy	n.d.	n.d.	n.d.

For each cytokine, final result is expressed as the number of molecules of specific mRNA per μg of total RNA.
LV=Left ventricle; RV=right ventricle; n.d.=not determined.

Table 5. *IL-1β mRNA gene expression in peripheral blood*

Patient's PBL	Clinical diagnosis	IL-1β mRNA 10^3 molecules
1	Fulminant IDC	n.d.
2	Acute IDC	17,500
3	Acute IDC	800
4	Severe IDC	n.d.
5	Clinically recovered from acute IDC	n.d.
6	Severe IDC	600
7	Familial IDC	1200
8	Myocarditis	18,300
9	Myocarditis	10,600
PBL from controls (n=4)		3200±3600

Final result is expressed as the number of molecules of specific mRNA per μg of total RNA.
PBL=Peripheral blood leukocytes; n.d.=not determined.

Cell-mediated and humoral immune responses are considered crucial in development of a sustained myocardial damage leading to IDC [8]. Recently, we found that the majority of T cells infiltrating the myocardium of patients suffering from acute, fulminant CVB3$^+$-IDC carry a restricted set of rearranged TCR genes with an expansion of Vβ3, 7 and 13.1 gene families. The polyclonality of the T cell populations, together with the presence of a non-restricted Vα repertoire was suggestive of an immune response initiated by a CVB3-superantigen [11]. Superantigens are protein products from micro-organisms well known for their ability to bind major histocompatibility (MHC)-class II proteins and stimulate proliferation of T cells bearing specific Vβ sequences as part of their α/β TCR [20].

Here, we wanted to further investigate the immunological scenario of the infiltrating T cells in IDC patients who underwent cardiac transplantation at different times after the onset of symptoms. The immunohistochemical analysis of the myocardial inflammatory infiltrate showed the presence of CD4$^+$ (helper) and CD8$^+$ (cytotoxic) T-lymphocytes in addition to activated monocytes and resident tissue macrophages. Strong and selective immunostaining for the CVB3 serotype was observed in the myocardium of patients 1, 2, 3 and 4. The first three patients were suffering from acute, fulminant IDC while patient 4 had severe IDC and was transplanted 3 months after the onset of disease. However, presence of enterovirus RNA was detected by RT–PCR only in patient 1. This is in agreement with other reports where detection of the enteroviral genome has generally been unsuccessful [3, 7]. Again, immunohistochemical analysis showed CVB3 specific-antigens and it may be the method to choose when looking for evidence of enterovirus infections in hearts of IDC patients.

Analysis of TCR Vβ repertoire of the tissue infiltrate showed expansion of Vβ3, 7 and 13.1 gene families in the majority of the samples tested. The highest values for these Vβ gene families were measured in heart from the first three patients who were suffering from acute, fulminant IDC. In the patients with less acute forms of the disease (patients 4–7), Vβ1 and 5B gene transcripts were also found to be skewed. The finding of expansion of three Vβ gene families, 3, 7 and 13.1, in the heart specimens from our patients is very suggestive of an immune response initiated by a CVB. In fact, skewing of these TCR Vβ3, 7 and 13.1 gene families has been previously detected in stimulated T cells from normal, adult donors cultured with lysates of Vero [11] and HeLa cells [21] infected with CVB3. Interesting, in these patients the medical history records flu-like illness, with fever, vomiting, malaise and cough before the onset of cardiac failure. CVB could produce or induce the expression of a superantigen that triggered the preferential usage of

certain Vβ families and could have been responsible for the first, viral-induced direct or indirect organ damage as it has been previously suggested for cases of acute, fulminant IDC [11]. Although the patient survives this initial phase, the myocyte injury may result in the release of intracellular constituents which cause further cytokine release (beyond that produced by the viral infection) thereby increasing the infiltration of inflammatory cells, such as macrophages, natural killer cells and T-lymphocytes with new antigen specificities. The increased frequency of T cells carrying TCR Vβ1 and 5B gene families in the hearts of some IDC patients (patients 4–7) may thus be the result of an ensuing autoimmune phenomenon, where infiltrating T cells recognize specific autoantigens released by the damaged myocytes, a phenomenon described as antigen determinant spreading [22]. There may also be a supporting role for autoantibodies to cardiac constituents like myosin, adenine nucleotide transporter, and/or branched chain ketoacid dehydrogenase [8, 23]. Persistent expansion of infiltrating T cells bearing Vβ3, 7 and 13.1 gene transcripts might be the result of a persistent or recurrent CVB-infection. The hypothesis could also be supported by the data on peripheral blood of patient 4 where high frequencies of circulating $V\beta7^+$ and 13.1^+ T cells were detected. In fact, selective utilization of TCR genes in the periphery may reflect an on-going disease within an affected tissue. The only subject who did not show increase of Vβ3, 7 and 13.1 gene family was patient 7 who had IDC of a different aetiology, being diagnosed with a familial dilated cardiomyopathy.

Activated T cells can elaborate a number of different cytokines in response to antigens. The T-helper 1 (Th1) subset, for example, releases inflammatory cytokines such as IL-2, IL-6, IFN-γ and TNF-α, all of which has been shown to be involved in myocarditis and IDC pathogenesis [24–26]. Th2 cells, by contrast, produce cytokines such as IL-4, IL-5 and IL-10. These mediators regulate the migration and tissue infiltration of leukocytes, modulate the outcome of cardiotropic virus infection and enhance its autoimmune sequela, and induce depressant effects on myocardial contractility [24–26]. In addition, a recent report demonstrated that TNF-α, IL-1β and IL-6 are secreted by human monocytes and fibroblast in response to CVB3 infection [27]. This demonstrates that immune response to infection is an important factor in IDC pathogenesis.

In this study, we measured mRNA gene expression for several inflammatory cytokines in the hearts of IDC patients. In the tested specimen, the cytokine detected with the highest abundance was IL-1β, followed by IL-6. Interestingly, the highest level of expression of mRNA molecules for both IL-1β and IFN-γ was observed in the right ventricle of patient 4 that appeared more strongly positive for CVB3. Although we did not examine the physical localization of cytokines, it can be envisaged that these mediators may be synthesized and released locally in the myocardium by infiltrating lymphocytes and macrophages. Furthermore, one report showed both IL-1β mRNA and protein product in endothelial cells of small vessels, the interstitial regions between the myocytes and in the myocytes themselves [28]. While the numbers of individuals tested was limited, it is certainly worthwhile to note that IL-1β mRNA gene expression in the PBL from two myocarditis patients and from one IDC showed very high number of mRNA molecules compared to both the expression in PBL from normal controls and from another two IDC patients. These results are also in agreement with another study showing elevated levels of IL-1β in plasma of patients with acute myocarditis but not in plasma of IDC patients [25]. Lack of detection of plasma IL-1β in IDC patients could be due to compartmentalization of the cytokine within areas of the myocardium itself, as suggested by Francis *et al.* [28].

In agreement with our previous report [11] we have found that the majority of our IDC patients carried the DQA1* 01 and DQB1* 06-alleles. Although this finding is suggesting a possible genetic predisposition to the disease, many more patients should be examined to determine the frequency of this phenomenon.

In conclusion, our data demonstrate that autoimmune mechanisms are important factors in the natural history of IDC. CVB-induced myocardial injury showed TCR Vβ3, 7 and 13.1 gene families expansion in heart infiltrating T cells. This may be a prerequisite for the induction of cardiac autoimmunity. As tissue damage accrues, recognition of additional cardiac antigen may occur which cause recruitment of T cells with additional antigen specificities (Vβ1, 5B and 17). When there is no evidence of CVB-infection the Vβ repertoire is not skewed towards any specific gene families.

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CHAPTER 38

Major Histocompatibility Locus and Other Genes That Determine the Risk of Development of Type 1 Diabetes Mellitus

Massimo Pietropaolo and Massimo Trucco

tudies aimed at identifying susceptibility genes for type 1 diaetes mellitus (DM) have initially focused on those encoding ıe highly polymorphic human leukocyte antigen (HLA) moleıles. To date, the influence of HLA on type 1 DM remains nquestioned, although a number of other candidate genes ave also been evaluated for their potential role in disease susptibility. Also, a genome-wide search for genes affecting type DM susceptibility has been undertaken by several groups sing microsatellite markers to construct high-resolution ıman genetic linkage maps (1,2). The results of these studies ıve confirmed the strong linkage between type 1 DM and the LA region, now called the *IDDM1* susceptibility region, and vealed weaker associations between the disease and 18 other romosomal regions that may contain susceptibility loci (Table -1). While the majority of these non-HLA genes were found eakly associated with the disease in some large family studies, eir association was not confirmed by other studies on different family groups (3). Even the region 5′ of the insulin gene, the so-called *IDDM2* susceptibility region, appeared not to be linked with the disease when more stringent statistical parameters were used (3). Because of the large number of markers tested, the identification of linkage with many putative regions of type 1 DM susceptibility may have occurred by chance alone. Replication of these observations in the initial as well as in other data sets will be necessary to confirm significant linkage. Furthermore, it would be useful to saturate any putative locus of association with many more informative markers. To complicate the confirmation of linkage is the wide range of selection criteria used for inclusion of families in the various genome scans. Also, variations in the age of onset can make the detection of linkage difficult. The interaction of the various disease susceptibility loci, the hallmark of polygenic diseases like type 1 DM, may confuse matters even more. This interaction (additive, multiplicative, or epistatic) adds an additional level of difficulty in determining the significance of a logarithm of odds (LOD) score.

For example, *IDDM1* and *IDDM2* interact epistatically; the genes present in these two susceptibility regions seem to be functionally related, either in a biochemical or a physiologic manner, whereas *IDDM1* and, for example, *IDDM4*, i.e., the region defined by the fibroblast growth factor-3 (FGF-3) gene, may act independently (4). In addition, *IDDM4* and *IDDM5*, i.e., the region containing the estrogen receptor-1 (ESR-1) gene, appear to have a greater frequency of shared alleles, identical by descent in subgroups of data sets that share the HLA-DR3 allele. On the contrary, the linkage of *IDDM7*, i.e., the region containing the interleukin-1 (IL-1) gene, is stronger in subgroups homozygous for class I variable number of tandemly repeated (VNTR) alleles at *IDDM2*, but lacking HLA-DR3 (5). All of these results will most likely depend on the nature of the

TABLE 38-1. Human type 1 diabetes mellitus (DM) susceptibility regions

Chromosome	Locus[a]	Association[b]
6p21	*IDDM1; HLA*	Confirmed
11p15.5	*IDDM2; INS* 5′ VNTR	Confirmed
15q26	*IDDM3; IGF1R*	Suggestive ($p<.001$, MLS>2.2)
11q13	*IDDM4; FGF3*	Confirmed ($p<2.2\times10^{-5}$, MLS>3.6)
6q25	*IDDM5; ESR*	Confirmed ($p<2.2\times10^{-5}$, MLS>3.6)
18q21	*IDDM6*	
2q31	*IDDM7; IL-1, HOXD8*	Suggestive ($p<.001$, MLS>2.2)
6q27	*IDDM8; IGF2R*	Confirmed ($p<2.2\times10^{-5}$, MLS>3.6)
3q21–q25	*IDDM9*	
10p11.2-q11.2	*IDDM10*	
14q24.3–q31	*IDDM11*	Significant ($p<2.2\times10^{-5}$, MLS>3.6)
2q33	*IDDM12; CTLA-4*	
2q34	*IDDM13; IGFBP2, IGFBP5*	
6p21	*IDDM15* (distinct from HLA)	
7p	Not assigned; *GCK, IGFBP1, IGFBP3*	
Xq	Not assigned	

[a]The type 1 DM nomenclature is assigned to a locus after linkage has been formally demonstrated, replicated and confirmed in at least thee different data sets. Where functional candidate genes are flanked by, or very close to, susceptibility markers, they are indicated (7).

[b]See ref. 10.

MLS, maximum LOD score; HLA, human leukocyte antigen; VNTR, variable number of tandem repeats; ESR, estrogen receptor.

population under study as there are already-established differences among populations in type 1 DM susceptibility at particular loci; the relative risk of alleles at *IDDM2* is dependent on the presence of HLA-DR4 in a French population (6) but not in American, Belgian, Finnish, or British data sets (7). Once the suggestive linkage to a region has been determined, other powerful association-based tests, which take advantage of linkage disequilibrium between new markers and other susceptibility alleles, can be used to formally implicate that locus and/or a particular allele with susceptibility (5).

There is continuous debate, however, as to what criteria should be used when attempting to map non-HLA loci. Historically, in a classic genetic disease, the generally accepted standard for "significant" linkage is considered to be a LOD score greater than 3 ($p < 10^{-4}$). Glenys Thomson (8,9) suggested a statistical level of $p = .001$ for "presumable" linkage in genome-wide searches, to be confirmed by the results in other populations. Lander and Kruglyak (10) proposed instead more stringent criteria for suggestive linkage with a LOD score threshold of 2.2 ($p < 7 \times 10^{-4}$) and a LOD score of at least 3.6 ($p < 2 \times 10^{-5}$) to achieve significant linkage. Even with a LOD score of 3.6, there is still a possibility of a false-positive rate of 5% in genome-wide scans. Lander and Kruglyak also proposed that further replication studies in different populations are necessary to verify significant linkage. Thus, very few of the loci indicated in Table 38-1 will stand the criteria of Lander and Kruglyak for significant linkage. Therefore, genome-wide searches should be considered only an initial stage in discovering potential susceptibility genes in type 1 DM other than the ones included in the *IDDM1* region.

THE MAJOR HISTOCOMPATIBILITY COMPLEX (*IDDM1*)

In humans, the major histocompatibility complex (MHC) comprises a cluster of genes (11) that encode class I (HLA-A, -B, -C) and class II (HLA-DR, -DQ, -DP) molecules; genes in the class III region of the MHC encode such unrelated products as tumor necrosis factor, complement, and 21-hydroxylase (Fig. 38-1) (11). Both MHC class I and class II protein products consist of separately encoded α- and β-chains. The class I α-chain is polymorphic and noncovalently associated with its monomorphic β-chain, the β_2-microglobulin encoded on chromosome 15 (12). In contrast, class II molecules contain highly variable domains in both α-and β-chains, and are encoded by polymorphic genes within the HLA complex. Class I proteins are expressed on virtually all nucleated cells, whereas class II molecules are found on selected cell types such as B lymphocytes, macrophages, dendritic cells, and activated T cells. Both class I and class II proteins function similarly to present antigen to T cells; cytotoxic T cells primarily recognize antigen in the context of class I, whereas helper/inducer T cells usually recognize antigen associated with class II molecules (13).

Early studies suggested that type 1 DM patients more likely expressed HLA-B8 and -B15 alleles than nondiabetic controls (14,15). More significant associations between HLA-DR and type 1 DM became apparent once these HLA class II molecules were identified with HLA-DR3 and -DR4 alleles found to be strongly linked with type 1 DM susceptibility (16,17). (The original association with the HLA-B locus is now thought to be a function of linkage disequilibrium between DR3/4 and B8/15

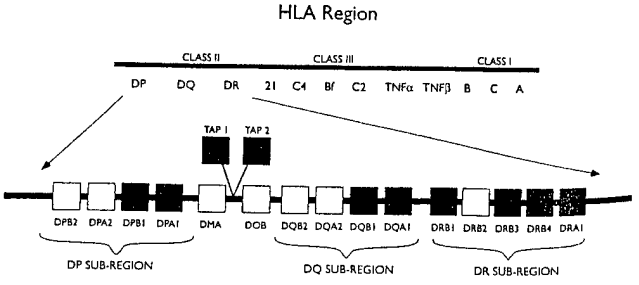


Figure 38-1. Schematic representation of the human leukocyte antigen (HLA) complex on chromosome 6. The genes that encode a protein product are indicated in black; the genes encoding non-functional products, or products that have not been characterized, are indicated in white.

locus antigens.) Subsequent studies revealed that approximately 95% of patients with type 1 DM were heterozygous for DR3/4 or expressed at least one of these alleles. Heterozygous individuals appeared to be more susceptible to type 1 DM development than DR4/4 or DR3/3 homozygotes. Expression of the HLA-DR2 allele, by contrast, was highly associated with resistance to type 1 DM development (17,18).

The application of newly developed molecular biology techniques to study the genetic basis of disease has permitted a more detailed analysis of the association between HLA genes and type 1 DM susceptibility. Restriction fragment length polymorphism (RFLP) analysis was the first such method to be used (19). During RFLP analysis, a set of restriction enzymes cleave the genomic deoxyribonucleic acid (DNA) of interest. The resulting fragments are separated by size with gel electrophoresis and are immobilized on a nitrocellulose filter. Particular sequences contained within the fragments are recognized by specific DNA probes, which reveal a "fingerprint" characteristic of each person's DNA. RFLP analysis of DNA from type 1 DM patients and nondiabetic controls revealed an even stronger association between the HLA-DQ locus and disease susceptibility than had been described previously for HLA-DR (20,21). The importance of HLA-DQ in type 1 DM susceptibility was underscored by DNA sequencing analysis of the MHC (H-2) genes of the nonobese diabetic (NOD) mouse strain (22). In addition to their susceptibility to diabetes development, these mice also have an unusual MHC expression pattern that permits an experimental dissection of the potential role of different class II gene products in type 1 DM development. Due to a mutation within the class II I-E locus (the murine homologue of the human HLA-DR), mice of this strain express only class II I-A molecules (equivalent to the human HLA-DQ). The nucleotide sequences of the I-A α- and β-chain genes from NOD mice were compared with those from nonobese normal (NON) control mice. Nucleotide differences between the two strains were found only at codons 56 and 57 of the I-Aβ gene; these changes permitted a serine residue substitution at position 57 in NOD mice, whereas aspartic acid was found at the same position in NON mice (22). Subsequent analysis of the DNA sequences of human HLA-DQ genes confirmed that position 57 of the DQβ-chain was strongly associated with type 1 DM incidence; a negatively charged aspartic acid at position 57 (Asp-57) correlated with "resistance" to type 1 DM, whereas alleles in which a neutral amino acid such as alanine, valine, or serine was present (non–Asp-57) correlated with increased susceptibility to the disease (23,24).

Deoxyribonucleic acid sequencing, although precise, is a costly and laborious technique unsuited for large population studies. The development of the polymerase chain reaction (PCR) enabled researchers to perform the first large-scale characterization of the HLA-DQ alleles of individuals whose families have multiple members with type 1 DM (25). Twenty-seven multiplex families were examined with the use of PCR and allele-specific oligonucleotide probes able to distinguish a single base difference. Among the families examined, approximately 96% of the diabetic haplotypes contained non–Asp-57 sequences, compared with 19% of nondiabetic haplotypes ($p < .0001$). In probands homozygous for non–Asp-57, the relative risk of developing diabetes was calculated as 107, an order of magnitude higher than the risk predicted by the best serologic markers, DR3 and DR4 (26).

The importance of Asp-57 in conferring resistance to disease was emphasized as additional studies were performed on diabetic and nondiabetic individuals in populations with differing incidence of type 1 DM (27,28). Molecular typing of the alleles at the HLA-DQ loci revealed the genotypic frequency of non–Asp-57 alleles among these groups; this calculation was then compared with data collected through epidemiologic type 1 DM registries. The non–Asp-57 marker was highly associated with type 1 DM incidence in all populations studied (as measured by relative risk). These studies also demonstrated a positive correlation between the frequency of the disease and the frequency of the marker in almost every population examined (27–32). The Japanese were the only population to exhibit a higher than expected frequency of Asp-57 in type 1 DM patients relative to nondiabetic controls (33,34). These and other studies suggested that susceptibility or resistance to type 1 DM is a polygenic phenomenon, with HLA-DQ being the most sensitive genetic marker available for determining risk (35,36).

The contribution of the HLA-DQα chain to type 1 DM susceptibility or resistance was also examined in a study by Khalil et al. (37), who used PCR and specific oligonucleotide probes to molecularly type 50 unrelated type 1 DM patients and 75 randomly selected healthy controls. These results implicated a role for the Arg-52 residue of the HLA-DQα chain in disease susceptibility and confirmed the importance of HLA-DQβ non–Asp-57. Furthermore, this study as well as others suggested that susceptibility to disease was increased in patients bearing DQ alleles that could form multiple "diabetogenic" dimers through the association of DQ chains encoded on the same chromosome (in *cis*) or on separate chromosomes (in *trans*) (38–40). The pairing of *cis*-encoded and *trans*-encoded α- and β-chains permits highly polymorphic class II molecules to achieve even greater diversity (41,42) (shown schematically in Fig. 38-2). In the Khalil study, subjects were grouped on the basis of their genetic potential to form four possible heterodimeric HLA-DQ molecules through either *cis* or *trans* pairing (37). Susceptible dimers (αS-βS) were defined as DQβ non–Asp-57-DQα Arg-52, and protective dimers (αP-βP) were defined as DQβ Asp-57-DQα non–Arg-52. The frequency of subjects with type 1 DM correlated closely with the expected frequency of the formation of the αS-βS heterodimer. Thus, subjects affected with type 1 DM could form only αS-βS heterodimers, whereas the disease was not observed in subjects who could generate only αS-βP and αP-βS or αP-βP DQ molecules (37). The formation of hybrid DQ molecules through the association of α- and β-chains encoded in *trans* was also implicated in the development of disease in an analysis of a multiplex family (38). The incidence of type 1 DM (which occurred in three of six offspring of the nondiabetic parents) could be traced only to the diabetogenic DQ heterodimer formed in these children through the pairing of the father's α-chain (Arg-52) with the mother's β-chain (non–Asp-57) (43).

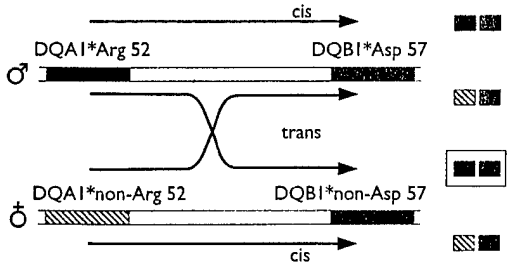


Figure 38-2. Associations of DQα- and DQβ-chains encoded either in cis or in trans can result in the formation of different DQ heterodimers, some of which may be "diabetogenic" (e.g., Arg-52α, non-Asp-57β). In this example, only one diabetogenic heterodimer (indicated by the double black boxes) can be formed by the association in trans of the α-chain encoded by the paternal (#) haplotype (Arg-52) with the β-chain encoded by the maternal (*) haplotype (non-Asp-57). (Adapted from Trucco M. To be or not to be Asp 57, that is the question. *Diabetes Care* 1992;15:705.)

The ability to form hybrid diabetogenic class II molecules may also help explain the apparent discrepancies observed between HLA haplotypes, the presence of islet cell antibodies (ICAs) in the patients' sera [a classic "prediabetes" marker used to follow relatives of type 1 DM probands (44)], and type 1 DM incidence. In a study of 151 first-degree relatives of probands from the Pittsburgh type 1 DM registry, 74 were ICA positive (irrespective of their HLA haplotype), but only 23% of these ICA-positive individuals went on to develop type 1 DM within the 4.9-year duration of the study (39). A comparison of ICA positivity, the potential to generate diabetogenic HLA dimers, and the incidence of disease in these subjects indicated that the greater the possible number of diabetogenic heterodimers, the higher the incidence of disease. Relatives who were ICA positive and αS-βS homozygotes were estimated to be 229.3 times more likely to develop type 1 DM than those who were ICA negative and could not form diabetogenic heterodimers (39). Similarly, a study in Spain examined the annual incidence of type 1 DM relative to the expression of non–Asp-57 and Arg-52 alleles (40) (Table 38-2). Subjects who could not generate any diabetogenic heterodimers experienced an incidence of disease roughly similar to that of subjects who could generate only one (1.9 versus 0.8/100,000/year). The annual incidence increased among those who could generate two (12.8/100,000/year) or four diabetogenic heterodimers (101.7/100,000/year). This is an extraordinarily high value when compared with the incidence calculated in the same area by conventional epidemiologic methods (11.8/100,000/year) that do not fractionate the population based on HLA phenotypes (45). Taken together, these studies suggest that the genetic predisposition to type 1 DM increases, in a dose-dependent fashion, as the number of susceptible alleles in a given individual increases. Thus, individuals whose haplotypes comprise two non–Asp-57 alleles and two Arg-52 alleles would appear to have the greatest probability of generating diabetogenic molecules and developing the disease (39,40,43).

In 1987, Bjorkman et al. (46) succeeded in deciphering the precise structure of the crystallized HLA-A2 molecule, a triumph that shed considerable light on the molecular implications of amino acid changes at various positions in these class I molecules. Their analysis revealed that the antigenic peptide, processed by the antigen-presenting cell (APC) from which the A2 molecules were purified, was still intact within the molecular pocket (the so-called Bjorkman's groove) that functions as the antigen-binding site (Fig. 38-3). It appeared that changes in the amino acids deep within the groove might alter the structural pockets that accommodate particular peptide side chains. Such changes would thus inhibit or enhance peptide binding, and in turn, recognition by a given T cell. Conversely, changes in amino acids on the exposed surface of the α-helices that actually form the groove might interfere with the molecular interactions between the HLA molecule/antigenic peptide complex and the T-cell receptor. The characterization of the crystal structure of the DR1 class II molecule permitted closer scrutiny of the potential implications of amino acid changes at certain positions (47–49). A schematic representation of the class II molecule's antigen-binding groove is shown in Fig. 38-4. Residue 57 of the DR1β-chain faces the inside of the groove at one end of the peptide binding site; an amino acid change at this position could easily translate into a critical conformational modification of the antigen-binding site. Asp-57 is involved in hydrogen and salt bonding with both the peptide main chain and the DRα Arg-76 side chain; thus, changes in the DRα Arg-76 residue would also be expected to alter the antigen-binding site (47,48). DQ molecules have yet to be crystallized and their structure analyzed, but on the basis of crystallographic evidence obtained in its mouse counterpart, the I-A^K molecule (50), it is likely that these critical residues will be positioned similarly in these class II molecules.

Small structural changes, then, may result in large functional changes in the antigen-presenting capabilities of the class II molecules. One might imagine that the cells from a person who is heterozygous for both DQα and DQβ would contain all four chain combinations on their surface. Competition for binding the processed antigen could take place, with effective antigen binding dictated by the conformation of the antigen-binding site on the DQ dimer. Changes at either amino acid DQα-52 or DQβ-57, located at opposite ends of the α-helices that form the

TABLE 38-2. The influence of HLA-DQα and HLA-DQβ genotypes and the possible formation of diabetogenic heterodimers or the risk of development of type 1 diabetes mellitus[a]

Genotype			Diabetic patients ($n=102$)		Nondiabetic subjects ($n=87$)		Risk	
DQβ57	+	DQα52	*n*	Freq.	*n*	Freq.	Relative	Absolute
■ ■	+	■ ■	66	0.65	6	0.07	30.7	101.3
■ ■	+	■ ◪	16	0.15	9	0.10	5.2	17.2
■ ■	+	◪ ◪	2	0.02	10	0.11	0.7	2.3
■ ◭	+	■ ■	11	0.11	12	0.14	2.8	9.2
■ ◭	+	■ ◪	1	0.01	17	0.20	0.3	1.0
■ ◭	+	◪ ◪	0	0.00	16	0.18	0.1	0.3
◭ ◭	+	■ ■	3	0.03	6	0.07	1.6	5.3
◭ ◭	+	■ ◪	3	0.03	10	0.12	1.0	3.3
◭ ◭	+	◪ ◪	0	0.00	1	0.01	1.0	3.3
Possible diabetogenic heterodimers								
	4		66	0.65	6	0.07	52.4	101.7
	2		27	0.26	21	0.24	6.6	12.8
	1		1	0.01	17	0.20	0.4	0.8
	0		8	0.08	43	0.49	1.0	1.94

[a]The genotype of subjects at positions DQβ57 and DQα52 are indicated (see Fig. 38-2) as follows: susceptibility loci DQβ non-Asp-57 (black boxes), or DQα Arg-52 (black boxes); protective loci DQβ Asp-57 (striped triangles) and DQα non-Arg-52 (striped boxes).

Absolute = absolute risk×100,000 per year; Freq. = frequency of the genotype among the population studied; *n* = number of persons (subgroup).

Adapted from Gutierrez-Lopez MD, Bertera S, Chantres MT, et al. Susceptibility to type I diabetes in Spanish patients correlates quantitatively with expression of HLA-DQα Arg 52 and HLA-DQβ non-Asp 57 alleles. *Diabetologia* 1992;35:583.

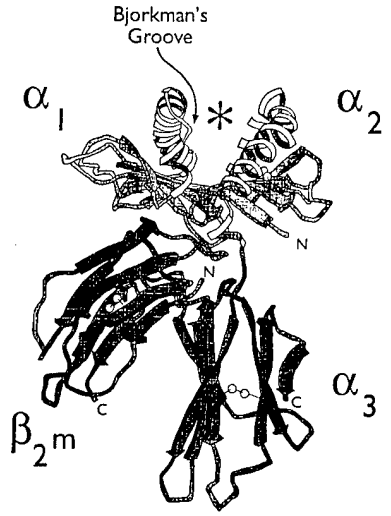
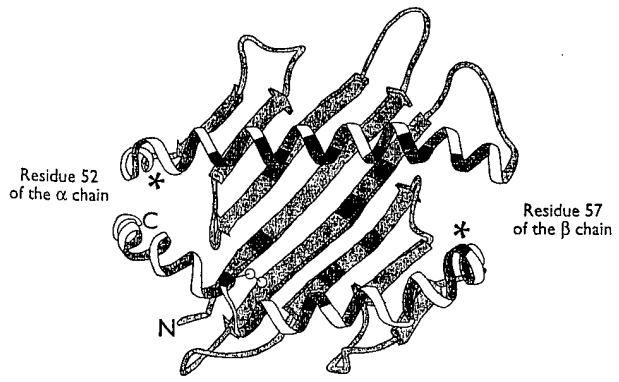


Figure 38-3. Schematic representation of the crystallized HLA-A2 class I molecule. The peptide antigen present within the antigen-binding site (Bjorkman's groove) is indicated with an asterisk. (Adapted with permission from Bjorkman P, Saper M, Samraoni W, et al. Structure of the human class I histocompatibility antigen, HLA-A2. *Nature* 1987;329:512.)

antigen-binding groove (Fig. 38-4), could alter the configuration of the groove. Changes at both positions, however, would likely inflict a much greater conformational effect on the molecule's antigen-presentation capability. Such conformational differences may be partially responsible for the observed hierarchy in the degree of susceptibility within the group of non–Asp-57 alleles, and for the differences in the degree of protection afforded by each allele within the group of Asp-57 alleles. For example, the protective effect of the Asp-57 allele DQβ1*0502 prevails over that of certain susceptible alleles, such as non–Asp-57 DQβ1*0501. Conversely, the susceptible allele non–Asp-57 DQβ1*0302 dominates over the protective effect of Asp-57 DQβ1*0301 (51).

Competition for antigen binding would also be influenced by the relative abundance of each form of heterodimer on the cell surface, which in turn is likely influenced by two factors:

1. Certain DQα– and β-chains appear to be under structural constraints that limit the formation of dimers between them. For example, the β-chain of the DQβ1*0501 allele does not couple efficiently with α-chains of the DQA1*0301 or DQA1*0501 alleles (52). Thus, persons who are heterozygous for these alleles would not be expected to readily form significant numbers of hybrid molecules from the *trans*-encoded genes.
2. Studies of the promoter regions of these genes suggest that the levels of transcription of the DQα– and β-chain genes may differ among allelic variants (53,54). These studies imply that a chain encoded by one gene may be synthesized in larger amounts than a chain encoded by the other allele, thereby increasing the probability of its participating in dimerization.



gure 38-4. The antigen binding site of an HLA class II DR1 molecule, own from the top. (Adapted with permission from Stern LJ, Brown JH, rdetzky TS, et al. Crystal structure of the human class II MHC protein LA-DR1 complexed with an influenza peptide. *Nature* 1994;368:215.)

Although the actual ratio of *cis*-encoded versus *trans*-encoded DQ heterodimers at the cell surface remains to be determined experimentally, it is possible that moderate differences in chain production translate into large functional differences with respect to antigen presentation and T-cell activation (43). Relatively few class II molecules appear to be required on the surface of an APC to cross-link the T-cell antigen receptor (TCR) efficiently and initiate a T-cell response (55). On this basis it is easily understandable why the study of *IDDM1* must acknowledge the role of the T cell, and more specifically the role of particular TCRs in mediating disease.

The TCR on a given peripheral T cell is composed of separately encoded α- and β-chains that are disulfide linked; these dimers must form a molecular complex with the multichain CD3 molecule to be functionally expressed at the cell surface. T cells undergo a maturation process that occurs primarily in the thymus during fetal life. During this process, precursor stem cells initially from the fetal liver and then from bone marrow enter the thymic *anlage*, where they are induced to rearrange their germline TCRα and β genes (56). The sequential steps involved in TCR gene rearrangement are outlined in Fig. 38-5, and result in the junction of a variable (V), diversity (D), joining (J), and constant (C) region gene segment. TCR gene rearrangements are essentially random, and most are nonproductive as a result of out-of-frame joints; however, these rearrangements are requisite for the expression of generally a single functional αβ TCR at the cell surface, and their essentially random nature ensures an extremely large (10^{10}–10^{15}) repertoire of distinct antigen specificities present in the unselected thymocyte pool.

Once a T cell expresses a functional TCR at the cell surface, it is subject to either positive or negative selection events in the thymus (57,58). Both positive selection and negative selection depend on interactions between the TCR, MHC molecule, and antigenic peptide. *Positive selection* occurs as thymic stromal cells bearing MHC molecules (containing self-peptide fragments) engage TCR molecules on the developing thymocytes and direct their continued maturation into functionally mature T cells. T cells with "useless" receptors (i.e., those that cannot bind with sufficient affinity the MHC molecule) are not driven to mature and expand, and these cells eventually die. *Negative selection* refers to the poorly understood set of events that specifically eliminates or alternatively "anergizes" potentially autoreactive cells, thereby inducing "tolerance" to self. During negative selection, factors such as affinity for self antigen and antigen load likely influence the final outcome of cell death or clonal anergy. Thus, the peripheral T-cell repertoire of each person (including identical twins) is unique (59), and is a consequence of both the random generation of TCRs in the initial unselected thymocyte pool as well as of positive and negative selection events.

The two functionally opposite processes, *tolerance induction* and *immune responsiveness*, each depend on the presence of class I and class II molecules with appropriate structures (dictated by the genes encoding them) that are able to present critical anti-

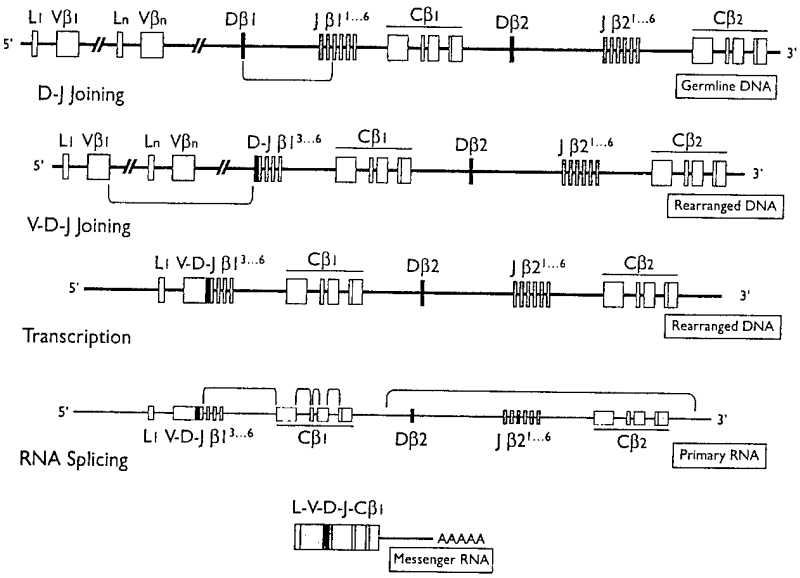


Figure 38-5. The human TCR β-chain locus on chromosome 7 encompasses 75 different variable segments, each with its own leader sequence (Vβ1–Vβn and L1–Ln). These are associated with two gene clusters, one composed of one diversity segment (Dβ1), six joining segments ($J\beta1^{1...6}$), and one constant segment (Cβ1) encoding the β1 chain, and the other with the $D\beta2/J\beta2^{1...6}/C\beta2$ segment encoding the β2 chain. Two somatic recombination events take place to generate the version of the rearranged DNA that is eventually transcribed. The D-J joining event occurs first, followed by the V-D-J event. The rearranged DNA is then transcribed into the primary RNA transcript. This transcript is converted into messenger RNA (mRNA) through an RNA splicing event. The TCR β-chain protein will be made after translation, processing, and glycosylation. The leader sequence is removed once the TCR β-chain is in place on the cell membrane.

genic peptides. In genetically susceptible individuals, certain class II molecules may ineffectively present self peptides, thereby leading to inadequate negative selection of T-cell populations that could later become activated to manifest an autoimmune response. Nepom and Kwok (60) explain the molecular basis of HLA-DQ associations with type 1 DM exactly on this basis. Paradoxically, some self peptides that normally negatively select T cells are likely to lead to positive selection when the MHC molecule is, for example, the HLA-DQ3.2.

The HLA-DQ3.2 molecule is encoded by DQA1*0301 and DQβ1*0302 genes that are generally present on the most strongly type 1 DM-associated haplotype also encompassing HLA-DR4. Due to a characteristic structural motif for peptide binding, the HLA-DQ3.2 can be considered an intrinsically "unstable" MHC class II molecule. If in a DQ3.2-positive individual, the T cells that are negatively selected in the thymus are only those that recognize DQ3.2-peptide complexes in a "stable" high-affinity configuration, the result can easily be the release from the thymus of mature T cells able to establish a potentially autoimmune repertoire in the periphery. Figure 38-6 illustrates these concepts.

Positive and negative selection events can also explain genetic resistance to type 1 DM. In many populations, the frequency of the DQβ1*0602 allele is rarely found among patients with type 1 DM (30,61,62). This suggests that this allele may play a protective role in the disease process. During thymic development, an unidentified diabetogenic peptide can preferentially bind to the DQβ1*0602 molecule, and because of the relatively higher affinity and/or avidity it has with this than with other DQ molecules, it will form HLA-DQ molecule/antigenic peptide/TCR complexes more efficiently than other molecules. This could lead to negative selection and depletion of potentially self-peptide–reactive T cells. Individuals with a typical DQβ*0602 allele can then delete these potentially dangerous T cells during thymic maturation and therefore are protected from developing diabetes (Fig. 38-6).

In some cases then, mature T cells that have left the thymus are able to invade the pancreatic islets and cause first insulitis and then type 1 DM. In NOD mice, $CD4^+$ helper T (TH) cells, cloned from those present in the infiltrated islets, could transfer by themselves the disease in unmanipulated recipient NOD mice (63). However, the participation of $CD8^+$ cytotoxic cells (TC) has been shown to be able to accelerate the course of the

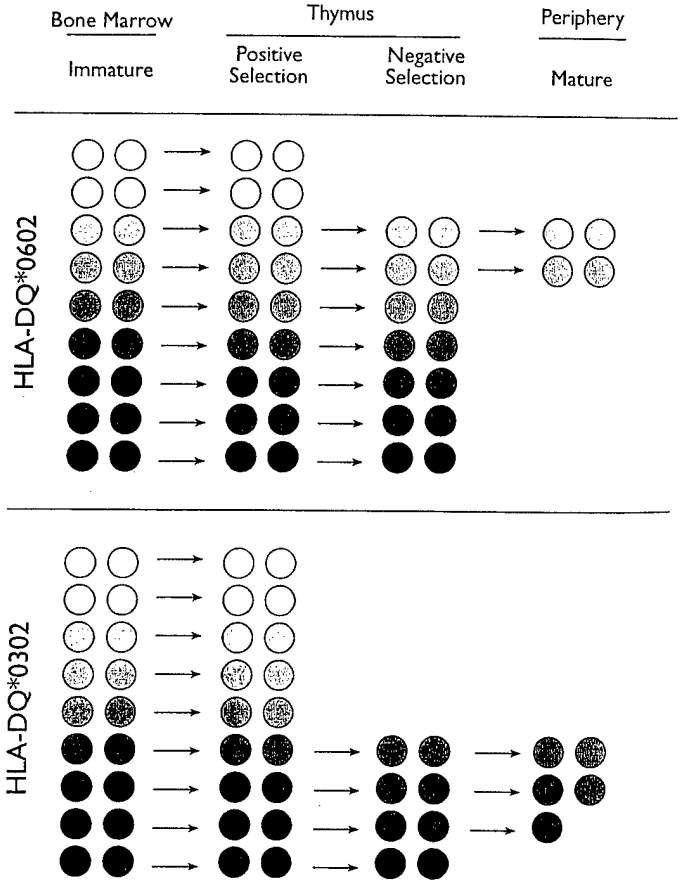


Figure 38-6. Both positive and negative thymic selections contribute to form the repertoire of mature T cells in the periphery from the precursors, or immature T cells, originated in the bone marrow. Individuals carrying HLA-DQ alleles associated with resistance to the disease, such as HLA-DQ*0602, will be able to negatively select in the thymus all the T cells with an high affinity for peptides of the self (●), so that no autoreactive T cells will be present in their peripheral blood and the chances to develop diabetes will be reduced. Individuals who have instead susceptibility alleles with low affinity for peptides of the self (○), such as, for example, HLA-DQ*0302, will negatively select less efficiently autoreactive T-cell clones that will then be present, even if in a relatively small number, among the peripheral T cells. (Modified from Nepom GT, Kwok WT. Molecular basis for HLA-DQ associations with IDDM. *Diabetes* 1998;47:1177.)

disease (64). Transfer of diabetes to NOD–severe combined immunodeficiency disease (SCID) mice occurred with one $CD4^+$ T-cell clone, but a second $CD4^+$ T-cell clone required the cotransfer of $CD8^+$ T cells (65). Interestingly, both T-cell clones had a cytokine profile consistent with TH1 cells, used the same $V\beta4^+$ TCR gene segment (although they used different Vα region genes), and were directed against undetermined islet cell antigens distinct from insulin, glutamic acid decarboxylase (GAD), heat shock proteins, and carboxypeptidase H. These experiments suggest that both TH and TC cell types play a role in diabetes pathogenesis, but also attracted a lot of attention to the role played by TH1 versus TH2 T cells (66). The first reports attributed a very powerful diabetogenic power to the TH1 cells that were only limited in their deleterious activity by the insurgency of the TH2 cells. TH2 cells were then considered the subpopulation of T cells able to limit the effect of TH1 cells once certain cytokines such as IL-4 were secreted in sufficient amounts.

Experiments performed in the NOD mice demonstrated that the transgene carrying the IL-4 gene under the control of the human insulin promoter was able to protect these mice from the development of both insulitis and diabetes (67). The importance of IL-4 was then confirmed in humans when a series of individuals considered at risk for type 1 DM were directly compared with diabetic patients (68). Although these data still need to be confirmed, they showed that diabetic siblings had lower frequencies of CD4–CD8–Vα24′OαQ$^+$ T cells than their nondiabetic siblings. CD4–CD8–Vα24′OαQ$^+$ T cells are considered the cells responsible for the early secretory bursts of IL-4 and IFNα. Also, all the Vα24′OαQ$^+$ T-cell clones isolated from diabetic twins secreted only interferon-α (IFN-α) upon *in vitro* stimulation, while almost all the clones isolated from the at-risk nonprogressors and from the normal twins secreted both IL-4 and IFN-α. Furthermore, type 1 DM individuals did not have detectable levels of IL-4 in their sera.

In type 1 DM patients, the combination of HLA association and T-lymphocyte involvement, together with the documented presence in the serum of the diabetic patients of antibodies directed against normal structures of the islet cells (i.e., autoantibodies) (69), the efficient, although temporary, effect of immunosuppression in delaying the onset of the disease (70), and the evidence that type 1 DM can be transferred from a diabetic donor to a nondiabetic recipient via bone marrow transplantation (71), strongly indicate the potential "autoimmune" nature of the pathologic mechanism able to cause the disease. Autoimmunity defines a breakdown of tolerance, whereby immunocompetent cells begin to attack tissues or cells of their own body (i.e., "self").

In 1987, Oldstone (72) defined "molecular mimicry" as the pathogenetic mechanism at the basis of autoimmune diseases. His reasoning was based on epidemiologic, clinical, and experimental evidence of an association of infectious agents with autoimmune diseases and on the assumption that similar structures (i.e., epitopes) can be shared by molecules encoded by dissimilar genes, as, for example, a virus and a normal host self-determinant (73). This postulated antigenic similarity between two completely unrelated molecules can have as a direct consequence the cross-reaction of some T cells that, although physiologically activated against a foreign (i.e., nonself) invader, start to pathologically attack normal (i.e., self) tissues carrying antigenic determinants similar to the nonself antigens. Perhaps the most relevant argument in support of molecular mimicry as the mechanism responsible for determining the autoimmune process that leads to overt type 1 DM is the one reported by Kaufman and collaborators (74) in 1992. In this study, GAD_{65} was chosen as one of the most studied autoantigens against which autoantibodies are frequently detected in the serum of type 1 DM patients. GAD_{65} was found to share a 6 amino acid long segment (PEVKEK) with the Coxsackie virus B4 P2-C protein. This segment was then considered the epitope possibly responsible for a molecular mimicry–based immune reaction, initiated by a Coxsackie virus infection and concluded by GAD-specific T cells attacking endocrine pancreas targets. Also, intrathymically injected GAD peptides encompassing the PEVKEK segment were able to delay the onset of the disease in NOD mice. The explanation proposed was that GAD peptides were able to induce an immunologically relevant tolerance, and able to protect the mouse against the autoimmune attack that generally culminates in overt diabetes (75,76).

The initial enthusiasm generated by the latter findings, however, was questioned on the basis that GAD_{65} is present not only on the insulin-producing β-cells of the pancreas, but also on the α- and δ-cells of the pancreatic islets in addition to neuroendocrine cells of the brain (77). Also, the search for T-cell clones able to react with the peptide did not give the expected results since it was difficult, using blood from diabetic patients, to isolate and stimulate T-cell clones able to respond *in vitro* to both GAD_{65} *and* Coxsackie virus B4 P2-C protein (78).

What seems to primarily hamper the demonstration of a molecular mimicry involvement in type 1 DM is the lack of recognition of an autoantigen causatively involved with the disease, or a virus directly able to trigger a DM-oriented autoimmune mechanism. In the case of enteroviruses like Coxsackie virus as well, molecular mimicry seems not to be the best hypothesis to explain the mechanisms involved in the etiopathogenesis of type 1 DM. A more convincing hypothesis that seems to explain the Coxsackie virus involvement in diabetes better than molecular mimicry was recently proposed by the group of Nora Sarvetnick (79). In this study conducted on the NOD mouse model, molecular mimicry evidence after Coxsackie virus exposure was compared with evidence supporting instead a bystander T-cell proliferation effect mediated by the virus (80).

To determine which of the two proposed hypotheses better explains the mechanism underlying Coxsackie virus B4 (CVB4)-induced type 1 DM, Horwitz and collaborators (79) used CVB4 to infect $BI0.H2^{g7}$ mice carrying the NOD MHC allele to which presentation of the cross-reactive epitope is restricted. They also used BDC2.5 mice harboring a diabetogenic T-cell receptor as a transgene. The results showed that following CVB4 infection, the $BIO.H2^{g7}$ mice generated a strong local immune response without any signs of pancreatic islet autoreactivity. On the basis of the molecular mimicry hypothesis, these mice, carrying the diabetogenic MHC restriction element, should instead promptly develop diabetes once exposed to CVB4. On the contrary, CVB4 infection in BDC2.5 mice generated a very quick diabetic response with all the signs of insulitis and a marked loss of insulin-producing β-cells. These results were interpreted as evidence that in these mice, type 1 DM was the result of the activation of resting, islet-specific, memory lymphocytes through the bystander mechanism.

The bystander T-cell proliferation hypothesis is based on the evidence that although viral infections are in general able to induce vigorous immune responses, the increase in total T-cell numbers is not due to the expansion of antigen-specific T-cell clones (81,82). Actually, the number of antigen-specific T cells remains consistently low, while non–antigen-specific T cells seem to be stimulated solely because they are physically present in the area of infection. The bystander (i.e., non–antigen specific) reaction is then not TCR dependent and can even be promoted by heterologous viruses as long as they are infectious and able to promote cytokine release. Among the cytokines most involved in this nonspecific stimulation are the type 1 interferons (IFN-1), which include IFN-α and IFN-β (80).

Although antigen-specific T cells are rapidly eliminated from the bloodstream, long-lived resting memory cells remain after receiving protective signals from the IFN-1 exposure.

The immunologic reaction of the BDC2.5 transgenic mice against CVB4 was considered an "exaggerated version" of the human preclinical situation in which previously inflicted, environmental insults could have caused an expansion of possibly cross-reactive T cell clones (79). The viral infection seems to be able to indirectly activate autoreactive T cells that will then generate an initial pancreatic tissue damage. The first damaged β-cells, in turn, will release previously "ignored" self antigens, which, once properly presented, can activate an autoimmune process able to rapidly proceed to the generation of insulitis and eventually overt diabetes. In their concluding remarks, the authors of this study offer the explanation that in humans, enrichment or distortion of the T-cell repertoire, similar to the one artificially created in the TCR transgenic BCD2.5 mice, could be due to a history of infections with pathogens expressing superantigens. This superantigen-mediated T-cell activation may render the patient overrepresented for a particular variable segment of the TCR β-chain. Among the T cells sharing this TCR Vβ segment, autoreactive T cells able to attack specific pancreas structures can also be present.

To date, a number of pathogenic organisms, including mycoplasma, bacteria, and viruses, have been shown to stimulate a vigorous immune response in the infected host through the expression of superantigen proteins (83). Unlike conventional foreign antigens, which are processed by APCs and displayed as peptides within the antigen binding groove of HLA class II molecules, superantigens appear to have little requirement for processing. Based on crystallization experiments, they are presumed to interact as relatively intact molecules with both HLA class II heterodimers and TCRs in an unusual trimolecular configuration that activates many different T cells bearing collectively one (or a few similar) TCR β-chain variable region (Vβ) (84) (Fig. 38-7). Thus, in contrast to conventional antigens that, when properly presented, can stimulate only ~1 in 10^5 T cells, superantigens can activate up to 30% of the total T-cell pool, depending on the initial frequency of T cells with the relevant Vβ. Vα segments seem not to be directly involved in the most common configuration of this activation, and the various Vα variable regions are more uniformly represented in the population of superantigen-activated T cells (84). Because superantigens do not activate T cells in a classic MHC-restricted fashion, the positive and negative selection events that normally occur in the thymus may not effectively constrain a population of T cells with potential reactivity to these antigens. Thus, T cells that can respond normally to a given foreign antigen with no ill-effects (e.g., no self reactivity) may also be activated via their TCR Vβ chain by a superantigen. Similarly, potentially autoreactive T cells that remain in the periphery in an anergized state may become superantigen-reactivated, via their Vβ chain, to carry out their effector functions. Polyclonal T-cell repertoires with highly restricted TCR Vβ regions (the hallmark of a superantigen-mediated immune response) have been reported in various autoimmune diseases, including toxic shock syndrome (85), rheumatoid arthritis (86), and Kawasaki's disease (87).

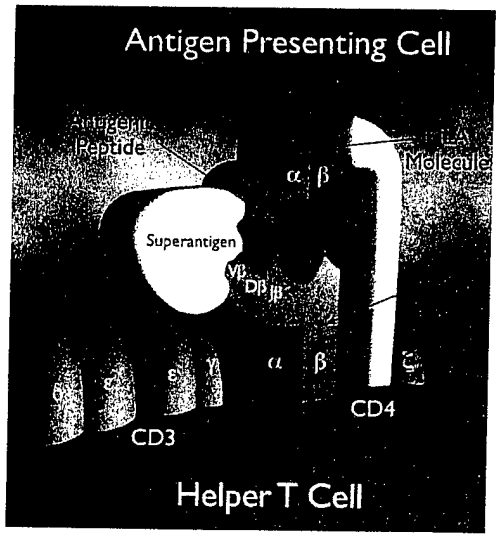


Figure 38-7. A schematic representation of the interactions among the T-cell receptor (TCRαβ), CD3 components (δ, ε, τ, and *z*), HLa class II molecule, peptide, accessory molecule (CD4) and superantigen during T-cell activation by an antigen-presenting cell. (Adapted with permission from Trucco M, LaPorte R. Exposure to superantigens as an immunogenetic explanation of type I diabetes mini-epidemics. *J Pediatr Endocrinol & Metabol* 1995;8:3–10.)

Data from our laboratory suggest that the T-cell component of the insulitis present in the pancreas at the type 1 DM onset is characteristic of an immune response against a superantigen, with the reactive cells expressing predominantly Vβ7- and Vβ13.1-positive T-cell receptors (88). This Vβ skewing together with the presence of a nonskewed Vα repertoire and the polyclonality of the T cells expressing one or the other of the two Vβ families *in situ* points to an immune response preferentially initiated by a superantigen rather than by a conventional antigen. A skewing of TCR gene usage was also found among peripheral blood mononuclear cells (PBMCs) from new-onset DM patients (89). High Vβ7 and Vβ13 gene family values were also evident in subjects considered to be at high risk for developing DM, who had been followed in our diabetes clinic for several months, and subsequently became diabetic. The finding of a high level of Vβ7 and Vβ13.1 gene families already present more than 1 year prior to the onset of type 1 DM suggests that a viral infection might actually have occurred years before the onset of the disease that, because of repetitive exposures to or persistence of the virus, stays sufficiently active to eventually cause the disease onset. This is in accordance with Sarvetnick's group's (79) hypothesis and with published serologic and epidemiologic studies (90).

Among the superantigen-related diseases described to date, all have significant HLA associations, perhaps due to the stronger affinity of certain superantigens to certain HLA alleles (91). This observation could explain the strong but broad effect of certain HLA-DQ molecules in enhancing or inhibiting T-cell activation, and ultimately in conferring susceptibility or resistance to type 1 DM (23,26,28,43). Moreover, the epidemiology of superantigen-mediated diseases is quite similar to the one of type 1 DM, with marked racial and geographic differences in risk, frequent reports of "outbreaks" with evidence of miniepidemics, aggregation in families, and strong seasonal patterns. In general, these diseases do not occur in very young children and are frequently associated with moderate viral illnesses that affect the patient months before the onset of the disease (92).

Very recently, studies on patients with myocarditis shed some light on the possible source of the postulated superantigen (93). The T-cell infiltrate in the hearts of three children who underwent transplantation because of the severe consequences (i.e., idiopathic dilated cardiomyopathy, IDC) caused by acute viral myocarditis was found to be skewed toward usage of the same Vβ gene families as in the diabetic pancreata. Polyclonal $V\beta7^+$ and $V\beta13.1^+$ T cells were found to be predominant among the cells present in the diseased hearts of these IDC patients. Furthermore, by challenging PBMCs from normal individuals

against lysates from Vero cells infected with different strains of CVB, we were also able to show that Coxsackie virus B3 (CVB3) was able to provoke *in vitro* the same T-cell skewing we observed *in vivo* (93). This suggested that some CVB protein may exhibit superantigen activity that can lead to the immunopathogenesis characteristic either of IDC or of type 1 DM. The extremely broad polymorphism of the different strains of CVB involving, in particular, their capsids may possibly explain our findings. A formal demonstration of the superantigenic characteristics of CVB, as well as of the abundance of CVB strains that may have superantigenic properties, still awaits to be provided; however, few CVB strains have been reported to be pancreotropic, while several others have been found to be cardiotropic (94).

The first damage of the pancreas should be caused by a pancreotropic strain, while the consequent immune response could be enhanced by the superantigenic characteristics of the virus. As a result of the initial β-cell destruction, a more conventional HLA-restricted, B- and T-cell–mediated autoimmune attack may eventually develop against the newly exposed self antigens. The superantigen-generated abundance of $V\beta7^+$ cells may increase the probability that some of the self-reactive cells present *in situ* are also sharing the same Vβ. The β-cell damage expands through an antigen-spreading process that will eventually culminate in overt diabetes (95). Alternatively, the insulin-producing β-cells may be bystanders rather than direct targets of the immune response. The pancreatic β-cells could possibly be destroyed because of their exquisite sensitivity to certain cytokines produced during T-cell activation (96,97). IL-1 is a proinflammatory polypeptide that is biologically related to tumor necrosis factor-α (TNF-α) and is produced as a result of infection, toxic injury, trauma, and antigenic challenges (98). The major source of IL-1 is the macrophage and its expression can be induced by IFN-α, although B lymphocytes, endothelial cells, mesangeal cells, smooth muscle cells, and fibroblasts can produce it as well (99). Two different genes encode IL-1 resulting in two polypeptides: IL-1α and IL-1β (100). IL-1β is more abundant at the messenger ribonucleic acid (mRNA) level and is the major secreted form (101). Normal human pancreatic β-cells that do not constitutively express Fas become strongly Fas-positive after IL-1β exposure, and then susceptible to Fas-mediated apoptosis (102). N^G-monomethyl-L-arginine, an inhibitor of nitric oxide (NO) synthase, prevents IL-1β–induced functional Fas expression in normal pancreatic β-cells. This selective expression of Fas in β-cells primed by NO may be responsible for their specific killing since T cells expressing FasL may promote an MHC-unrestricted destruction of the Fas-positive β-cells, while sparing neighboring Fas-negative α- and δ-cells (Fig. 38-8). It is perhaps not by chance, then, that the IL-1 gene maps in the *IDDM7* susceptibility region (1).

It is not yet known whether the CVB itself encodes a protein segment able to act as a superantigen, or if it is instead able to elicit the expression of a cellular protein, possibly derived from an endogenous retrovirus, that behaves as a superantigen (103). However, no convincing evidence of retroviral involvement in human type 1 DM has been offered so far (104).

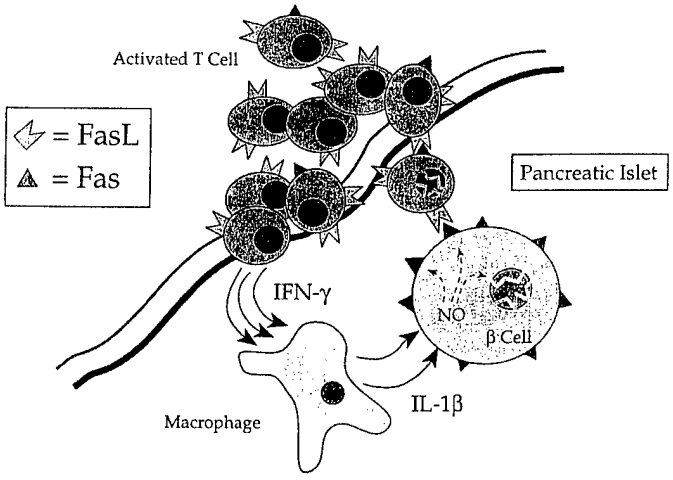


Figure 38-8. Macrophages, present in a small number in the periphery of the pancreatic islet, are activated by T cells approaching the islet. Activated T cells secrete interferon-γ (IFN-γ) and interleukin-2 (IL-2). Activated macrophages in turn start to secrete IL-1β that is able to hyperregulate the production of nitric oxide (NO) in the insulin-secreting β-cells, promoting Fas expression. Activated T cells express Fas ligand (FasL) molecules at their surface, thus causing apoptosis in the pathologically Fas-positive β-cells. Since T cells physiologically express Fas on their surface as well, they will also undergo apoptosis, physiologically concluding the cycle of the immune reaction against a specific foreign antigen.

THE INSULIN GENE REGION (*IDDM2*)

The discovery more than a decade ago of a polymorphism upstream of the insulin gene promoter on chromosome 11p15.5 consisting of a VNTR DNA sequence, led to observations suggesting an association of this region with both type 1 DM and type 2 DM (105–107). Until recently, further scrutiny of the initial association between type 1 DM and this locus was suspended because of subsequent data suggesting a relatively weak influence of this locus on type 1 DM susceptibility (108). In 1991, however, Julier et al. (6) reported a significant association with specific polymorphisms at this locus and linkage with type 1 DM susceptibility in DR4-positive individuals. This association was further refined to polymorphisms in and around the insulin gene, and in later studies it was shown that susceptibility was not limited to any specific HLA-DR haplotype (109). Fine mapping of the susceptibility locus has recently shown it to be the VNTR itself upstream of the insulin gene promoter (110). The different alleles at the VNTR can be subdivided into three general categories based on the number of tandem repeats of the consensus sequence: A(C/T)AGGGGT(G/C)C(T/C/G)(G/A/T)(G/T/A)G(G/C/T) (5). Class I alleles consist of 26 to 63 repeats, on average 570 base pair (bp) in length, and are associated with type 1 DM susceptibility. Class III alleles consist of 140 to 200 repeats and are considered protective. In size, they are the largest class, on average > 2.2 kilobase (kb) (Fig. 38-9). Class II alleles (1.2 kb size, on average) were too rare in the populations studied to draw any conclusion about their association with type 1 DM susceptibility (110).

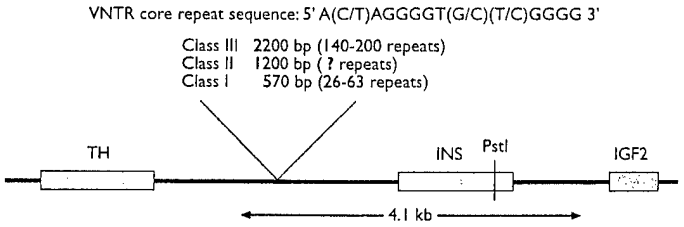


Figure 38-9. The variable number of tandem repeats (VNTRs) in the region 5′ of the insulin gene were grouped into three classes. Class I encompasses ~40 repeats, Class III ~160 repeats, whereas the number of repeats for Class II sequences has not yet been defined.

In vivo, in the fetal pancreas, transcription of insulin from chromosomes with class I VNTR alleles is higher than from chromosomes with class III alleles. In contrast, in the fetal thymus the class III alleles are associated with increased insulin gene expression and mRNA levels (111,112). *In vitro*, the results are not too clear. In one study, reporter gene activity was greater in constructs with class I VNTR than in those with class III. The opposite was shown in two other studies, one assaying the effects of the VNTR on reporter gene activity and the other quantitating insulin transcripts from VNTR-*INS* mini-gene constructs (5,113,114). The discrepancy between the different *in vitro* data was originally attributed to the choice of the class I allele used. This explanation is not completely convincing now in light of the findings of McGinnis and Spielman (115), who showed that in fact there was no special attribute to this particular class I allele in that it was equally transmitted as the nonprotective class I allele from class I "protective"/"nonprotective" heterozygotes to diabetics. In the study of Catignani-Kennedy et al. (113), this class I allele, which was initially believed to be protective (110), was used in the class I VNTR construct. The functional effects of the VNTR appear to be then more complex than initially thought. Other observations suggest that it may be under parent-of-origin effects and could act as a long-distance enhancer of the nearby insulin-like growth factor II gene in a tissue-specific manner (116).

Type 1 DM is a disease that is preferentially transmitted from affected fathers to affected children. The effect is two- to fourfold in favor of paternal transmission. In such cases of parent-of-origin–dependent transmission distortion, the underlying modification of disease transmission can be due to genomic imprinting acting at one or more functionally linked loci. In its broadest sense, genomic imprinting describes the behavior of a gene based on the sex of the transmitting parent. At the molecular level, this behavior often translates into monoallelic gene transcription (116,117).

In conclusion, a number of studies suggest that the VNTR region has a biologic role in the gene expression of insulin (5). Nevertheless, the exact role of VNTR is still a subject of discussion in view of conflicting reports as to whether the class I VNTRs induce greater (110) or lesser (118) insulin gene expression. In two early reports, it was stated that class I–associated insulin expression was actually associated with type 1 DM susceptibility and enhanced as compared with class III insulin gene expression (6,109). Class III expression was notably considered to be associated with protection from type 1 DM. Conversely, Kennedy et al. (118) have shown that class III VNTR reporter gene constructs had three times higher reporter gene expression than class I VNTR. Nonetheless, findings from Owerbach and Gabbay (5) do not support those of Kennedy et al. These controversial results raise the possibility that either methodologic differences in conducting the experiments (e.g., method of DNA transfection) or different cell lines used for DNA transfection, or different reporter gene constructs, etc., may account for some of these discrepancies. Presently, the mechanisms how the variant insulin gene expression may influence type 1 DM susceptibility remains to be conclusively determined.

IMMUNOGLOBULIN HEAVY CHAIN GENES (*IDDM11*)

The genes encoding the immunoglobulin (Ig) heavy chains are located on the long arm of chromosome 14. Serologically defined allotypes of the IgGγ heavy chains (called Gm allotypes) have been described and examined for their association with a number of diseases, including type 1 DM (119). Although these Gm antigens are strictly defined as IgG heavy-chain epitopes, the genetic region that is the object of disease association studies (by linkage analysis) also includes loci that encode the other Ig heavy chains (IgA, IgD, IgE, and IgM). Thus far, numerous association studies have offered little evidence that Gm-region genes directly influence type 1 DM susceptibility (120). However, a number of studies suggest that these genes, or unrelated loci in strong linkage disequilibrium with them, may indirectly affect susceptibility to type 1 DM. For example, diabetics heterozygous for DR3 and DR4 have a significantly higher frequency of non-G2m(23) allotypes than do diabetics with other HLA genotypes (120). The absence of the G2m(23) allotype also appears to be associated with certain TCRβ alleles in patients with type 1 DM compared with their unaffected siblings (119). The precise interactions, however, between the Gm region and these associated alleles must be further characterized, and their overall effect on type 1 DM susceptibility remains to be determined.

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CHAPTER 39

Association Between Immune-Mediated (Type 1) Diabetes Mellitus and Other Autoimmune Diseases

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Diabetes mellitus (DM) represents a collection of genetically determined disorders affecting the metabolism of carbohydrates, proteins, and fats associated with a relative or absolute insufficiency of insulin secretion and various degrees of insulin resistance. It is characterized by fasting hyperglycemia with long-term atherosclerotic and microangiopathic vascular diseases and neuropathy. The differences between various forms of DM (juvenile-onset type, maturity-onset type) are expressed in terms of their etiologies and pathogeneses (genetic, environmental, and immunologic), natural histories, and responses to treatment. Whereas type 1 DM is that due to an absolute, primary deficiency of pancreatic insulin, its most common cause is immune-mediated pancreatic β-cell destruction (immune-mediated diabetes, or IMD).

The advent of systematic and quantitative studies of the pancreas made it possible for Gepts (1) to describe a specific loss of insulin-producing β-cells in association with the clinical onset of type 1 DM. This work was of major significance because it was later determined that diabetic patients who needed insulin treatment more often acquired autoimmune thyroid disease. Conversely, patients with various autoimmune diseases, including Graves' disease, Hashimoto's thyroiditis, pernicious anemia, or Addison's disease, showed an increased prevalence of type 1 DM or IMD (2).

HISTORICAL BACKGROUND

Since the late 1970s, researchers have been accumulating a large body of evidence indicating that IMD, as well as other endocrine diseases associated with it, have autoimmune pathogeneses (3–5).

Since the inception of modern immunology, immune responses directed against self-structures have been considered potentially harmful. It was believed initially that autoimmune responses are restricted to disease states and do not occur under normal conditions. This idea soon needed modification because autoimmune phenomena were subsequently found greatly to outnumber disease-associated states in their occurrence. In normal people, the destruction of autoreactive T cells occurs in the thymus for all self-antigens (autoantigens) that can be expressed in this gland, as restricted by human leukocyte antigen (HLA) molecules (*central tolerance*). Many self-antigens,