Characterization of the T-Cell Response to Coxsackievirus B4

Evidence That Effector Memory Cells Predominate in Patients With Type 1 Diabetes

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Most of the evidence linking enterovirus (EV) infection with the development and/or acceleration of type 1 diabetes is indirect. Few studies have examined T-cell responses to these viruses, and therefore the nature of the viral targets and the immune cells involved in antiviral responses remain unclear. In the present study, we examined the characteristics of the T-cell response to the EV Coxsackievirus B4 (CVB4) in patients with type 1 diabetes and healthy control subjects. We find that CVB4-specific T-cells preferentially target the envelope proteins VP1, VP2, and VP3, and that the response to these and other CVB4 proteins differs markedly in type 1 diabetic patients compared with nondiabetic control subjects. The frequency of T-cell proliferative responses against VP2 was significantly reduced in type 1 diabetic patients compared with control subjects, especially in patients tested near to diagnosis ($P < 0.001$). In contrast, median levels of γ-interferon (IFN-γ) production by T-cells in response to the CVB4 antigens tested were generally high in new-onset type 1 diabetic patients, who produced significantly higher levels in response to VP3 compared with healthy subjects ($P < 0.05$) and patients with long-standing disease ($P < 0.05$). New-onset type 1 diabetic patients also had higher levels in response to P2C compared with healthy subjects ($P < 0.005$) and to VP2 compared with patients with long-standing disease ($P < 0.05$). These results suggest that the quality of the immune response to CVB4 antigens differs significantly between type 1 diabetic patients and control subjects, with a predominance of primed effector (IFN-γ–producing) memory cells near to disease diagnosis. The data are consistent with the notion that the diagnosis of type 1 diabetes is associated with recent or persistent exposure to EV antigens. *Diabetes* 51:1745–1753, 2002

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CVB4, Coxsackievirus B4; EV, enterovirus; IFN-γ, γ-interferon; IL, interleukin; mAb, monoclonal antibody; MBP, maltose binding protein; PBMC, peripheral blood mononuclear cells; T_{CM}, central memory T-cell; T_{EM}, effector memory T-cell; TT, tetanus toxoid.

Epidemiological studies (1–4) and research on identical twins (5) indicate that autoimmune diseases such as type 1 diabetes have a strong environmental component to their pathogenesis. There is a considerable body of evidence suggesting the involvement of several groups of viruses, but particularly those of the *Enterovirus* genus, in the development and/or acceleration of type 1 diabetes. These studies include the isolation of Coxsackievirus B4 (CVB4) from the pancreas of a child at diagnosis of type 1 diabetes (6), several epidemiological and prospective studies showing that some cases of type 1 diabetes are strongly associated with enterovirus (EV) infections (1–4), case-control studies that show an increased prevalence and levels of IgM antibodies to CVB in newly diagnosed patients (7–9), several case studies in which diabetes develops after an EV infection (10–13), and a higher frequency of EV RNA in the serum of type 1 diabetic patients at diagnosis (14–17).

Several mechanisms have been proposed to explain this putative link with type 1 diabetes pathogenesis, including "molecular mimicry" (18,19), bystander activation (20), and superantigen effects (21,22). Dissection of these models requires an understanding of the nature of the T-cell response to candidate viruses, but few studies have focused on T-cell/virus interactions. Those studies that have been performed have tended to explore a single theme, namely molecular mimicry and the relevance of the amino acid sequence similarity (the so-called PEVKEK region) shared between the P2C nonstructural protein of CVB4 and the autoantigen GAD65 (23–27). There is the potential for such studies to ignore other mechanisms by which viruses could induce islet autoimmunity. Moreover, in relation to molecular mimicry, it is well established that similarities between regions of antigens leading to T-cell cross-reactivity are the consequence of shared structural features rather than simply a reflection of sequence identity (28). Such structural similarities are difficult to identify by database searches, requiring instead the prior characterization of T-cell reactivity to the candidate environmental and self-antigen.

In a recent study, we showed that upregulation of the CD69 activation marker on CD4 T-cells expressing particular variable T-cell receptor β-chains after culture with crude CVB4 viral lysates was more prevalent in type 1

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TABLE 1
Characteristics of the study groups

	Control subjects	Long-term type 1 diabetic subjects	Newly diagnosed type 1 diabetic subject
n	20	19	21
M/F (*n*)	13/7	13/6	12/9
Age (years)	31.2 ± 8.5	25.1 ± 8.8	29.9 ± 4
Time since diagnosis (years)	N/A	5 (7 months–30 years)	0.24 (2 weeks–5 months)
HLA DR 04/X*	35	42.1	33.3
HLA DR 03/X	30	21	19.1
HLA DR 04/03	5	26.3	23.8
HLA DR X/X	30	10.5	23.8

*HLA DRB1 04/X includes 04/04 homozygotes; HLA DRB1 03/X includes 03/03 homozygotes. Data are means ± SD, median (range), and %, unless otherwise indicated.

diabetic patients than control subjects (22). We have therefore taken a more systematic approach to examine T-cell reactivity to CVB4 antigens in type 1 diabetes by developing protocols for the generation of viral coat and nonstructural proteins from E2, the diabetogenic strain of CVB4 (29). Here, we report the analysis of T-cell proliferation and cytokine production to these antigens in patients with type 1 diabetes and healthy nondiabetic control subjects. Our results suggest that the viral coat proteins are major targets of the antiviral T-cell responses to CVB4. In addition, we show that the phenotype of responder T-cells differs between patients and control subjects, with a predominance of primed effector memory cells associated with type 1 diabetes.

RESEARCH DESIGN AND METHODS

Subjects. A total of 60 subjects were studied. There were 21 type 1 diabetic patients, who were recruited as consecutive newly diagnosed cases during the period between March 2000 and July 2001. An additional 19 long-standing type 1 diabetic patients were recruited during the same period from the King's College Hospital Diabetes Service (courtesy of Professor S.A. Amiel). Also, 20 healthy donors were recruited from laboratory and hospital staff during the same period. Control subjects were matched for age and sex, had a distribution of HLA genotypes similar to that of the type 1 diabetic patients, and were recruited during seasons similar to those of the patients to avoid possible confounding effects of virus exposure. Characteristics of the subjects studied are shown in Table 1. Blood was drawn with the consent of all subjects, and appropriate permission was obtained from the institute's ethical review board. HLA genotyping was performed by PCR–single-strand conformation polymorphism (SSCP) analysis.

Antigens. The following antigens were used in the T-cell assays: tetanus toxoid (TT; Pasteur Merieux MSD, Berkshire, U.K.); uninfected, CVB4-infected (JVB strain), or influenza A–infected cell lysates (Institute Virion, Zürich, Switzerland); and individual recombinant CVB4 antigens (E2 diabetogenic strain) (30) comprising the VP1, VP2, VP3, and VP4 structural proteins and the P2C nonstructural protein. cDNAs encoding these CVB4 proteins were cloned, expressed, and purified as previously described (29). Briefly, the sequences of the individual genes were amplified from cDNA encoding the entire CVB4 E2 genome (kind gift of Prof. J.W. Yoon, University of Calgary, Canada), using *Pfu* polymerase (Invitrogen, Groningen, the Netherlands), and cloned into the pMAL-c2 vector (New England Biolabs, Hertford, U.K.) to produce recombinant fusion proteins with the maltose binding protein (MBP) at the NH_2 terminus of the viral protein. After transformation of JM109 cells and induction of protein synthesis by adding isopropyl β-D-thiogalactopyranoside, cells were harvested and the fusion protein purified using an amylose resin column (New England Biolabs). After purification, protein was dialysed overnight against PBS, pH 7.5, filter-sterilized, and stored at −20°C until use. As a control in the assays, the MBP protein alone was also purified in the same way as the MBP viral fusion proteins.

Proliferation assays. Peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll-Hypaque density gradient centrifugation of heparinized blood, washed twice in Hank's buffered saline, and resuspended at 1×10^6 cells/ml in RPMI 1640 medium (Life Technologies, Paisley, U.K.) supplemented with 10% AB+ human serum (PAA Laboratories, Linz, Austria), 50 IU/ml penicillin, and 50 μg/ml streptomycin (Life Technologies). PBMCs (10^5 cells/well) were cultured in triplicate in U-bottomed 96-well plates with medium alone, 100 ng/ml TT, 5 μl/ml (~0.2 μg protein lysate/ml final concentration) of either uninfected or virus-infected Vero cell lysates, or purified viral antigens or MBP at 10 μg/ml. These concentrations were shown to give optimal proliferation in preliminary and previous studies (29). After 6 days at 37°C, 0.5 μCi/well [^{3}H]thymidine was added, and the cultures were harvested after a further 18 h of incubation onto glass fiber filters. Proliferation was measured as [^{3}H]thymidine incorporation determined by direct β-plate counting in a Matrix 9600 counter (Packard Instruments, Pangbourne, U.K.). Results are expressed as a stimulation index (SI), which calculated as the mean counts with antigen divided by the mean counts with relevant control preparation (i.e., uninfected Vero cell lysate or MBP protein). For TT responses, the SI was calculated using the following formula: mean counts with antigen/mean counts with medium alone. An SI >3 is considered positive by convention. Mean counts (means ± SE) in healthy donors and newly diagnosed and long-standing type 1 diabetic patients, respectively, were 535 ± 95, 711 ± 45, and 560 ± 131 cpm in the presence of medium alone; 802 ± 178, 620 ± 134, and 736 ± 212 cpm with uninfected influenza lysate; 792 ± 186, 973 ± 248, and 632 ± 174 cpm with uninfected CVB4 lysate; and 738 ± 123, 986 ± 120, and 765 ± 172 cpm with MBP.

Cytokine production. IFN-γ and interleukin (IL)-4 were measured by enzyme-linked immunosorbent assay as previously described (29) in PBMC culture supernatants harvested after 7 days in culture with the antigens. In preliminary studies, no major differences were seen when collecting the supernatants on days 3, 4, or 7. Briefly, 96-well plates were coated with capture monoclonal antibodies (mAbs; BD Pharmingen, Oxford, U.K.). Plates were washed and blocked with 0.1% BSA. After washing, standards (78–20,000 pg/ml for IFN-γ or 31–8,000 pg/ml for IL-4, both from BD Pharmingen) and samples were added to the wells and incubated at 4°C overnight. A second biotin-labeled mAb (BD Pharmingen) specific for each cytokine was added after washing the plates four times, and after a 90-min incubation at room temperature and a further wash, streptavidin-alkaline phosphatase (Sigma Chemical, Poole, U.K.) was added to the wells. For developing, P-nitrophenyl phosphate (Sigma) was added to the wells and color development was analyzed at 405 nm in a Microplate Reader (Molecular Devices, Wokingham, U.K.). Typically, no IFN-γ was seen in the presence of control antigens or medium alone; when detected, these values were subtracted from those in the presence of antigen.

Statistical analysis. Comparisons of the frequency of the different subject groups responding (by either proliferation or cytokine production) to the different antigens was made using Fisher's exact test. The levels of cytokines made by the different subject groups were compared using the Mann-Whitney *U* test. Analyses were performed using the Prism statistical package.

RESULTS

Production and purification of the CVB4 E2 proteins. The cDNA sequences that encode the four structural (VP1–4) and the nonstructural protein P2C of the diabetogenic E2 strain of CVB4 were subcloned into the pMAL-c2 vector. This strategy allows the production of each protein as a soluble recombinant fusion protein with the MBP and purification by affinity chromatography on an amylose column as previously described (29). The five

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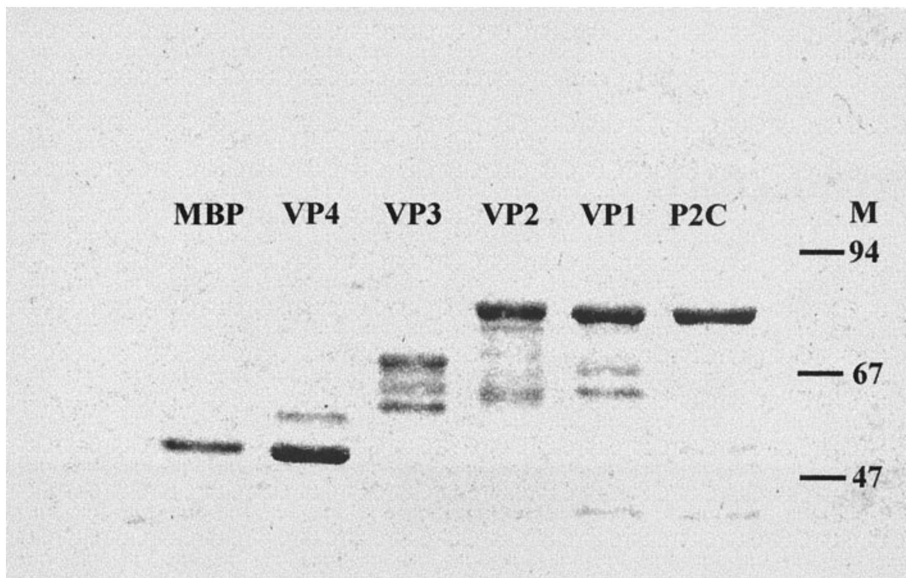

FIG. 1. PAGE analysis and Coomassie staining showing the five viral CVB4 fusion proteins (VP4 to P2C lanes) and the control antigen MBP (MBP lane). M, molecular weight markers (in kDa).

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viral recombinant proteins were more than 95% pure, as assessed by PAGE electrophoresis and Coomassie staining (Fig. 1).

Purified MBP protein alone was used as a control antigen for the proliferation assays as previously published (29). In addition, we used the same expression system to generate an MBP-VP2 fusion protein from the reference JVB strain of CVB4 (30), with a similar level of purity (data not shown).

Anti-CVB4 T-cell response: proliferation. A similar prevalence and median level (SI) of T-cell proliferation to the recall antigens TT and influenza A lysate was seen in all of the study groups (Fig. 2*A* and *B*).

Significant proliferation (SI >3.0) against CVB4-infected lysate was observed in 7 of 20 (35%) healthy donors, 3 of 21 (14.3%) newly diagnosed type 1 diabetic patients, and 4 of 19 (21%) long-standing type 1 diabetic patients. These prevalences were not statistically different, but newly diagnosed type 1 diabetic patients tended to have a lower frequency of significant proliferation compared with control subjects ($P = 0.11$).

To characterize the major T-cell targets in CVB4, we examined T-cell proliferation to the four coat proteins and the nonstructural protein P2C. For the five individual CVB4 antigens, the frequency of positive proliferative responses varied from moderate to low prevalence. The most prevalent response was seen to VP2 (17 of 60 subjects, 28.3%), followed by VP1 (10 of 60, 16.7%), VP3 (7 of 60, 11.7%), P2C (6 of 60, 10%), and VP4 (3 of 60, 5%) (Fig. 2*E*–*H*). There was a marked difference in the response to the dominant protein of those we examined, VP2, between patients and control subjects. Of the 20 control subjects, 11 (55%) tested showed positive T-cell proliferation (SI >3.0) against VP2 compared with 2 of 21 (9.5%) newly diagnosed type 1 diabetic patients ($P < 0.0001$) and 4 of 19 (21.1%) long-standing type 1 diabetic patients ($P = 0.007$). For the other coat proteins, there was no significant difference in the frequency of positive T-cell proliferation responses between the study groups.

Considering the responses to different CVB4 proteins together, a significant proliferative response against one or more of the five viral antigens was seen more frequently in healthy subjects (13 of 20, 65%) than in newly diagnosed type 1 diabetic patients (7 of 21, 33.3%; $P = 0.008$), whereas patients with long-standing type 1 diabetes had a prevalence of responses (42.1%) similar to control subjects.

To examine whether this difference in T-cell response against VP2 could reflect exposure to different virus strains, in a limited number of subjects ($n = 19$; 3 healthy control subjects and 7 long-standing and 9 newly diagnosed type 1 diabetic subjects), we were also able to measure T-cell proliferation against VP2 from the prototypic JBV strain (30). A good correlation was found between T-cell proliferative responses against the E2 and JBV VP2 antigens ($r^2 = 0.31$, $P = 0.013$) (Fig. 3). This suggests that the minor amino acid differences between the proteins of different CVB4 strains do not influence T-cell reactivity, although this preliminary finding will need to be confirmed in larger studies.

Anti-CVB4 T-cell response: cytokine responses. To further examine the T-cell response to CVB4 proteins, we measured production of the cytokines IFN-γ and IL-4 in the supernatant of antigen-stimulated PBMCs. Responses were considered positive when cytokines were detectable above the limit of sensitivity of the assay (78 pg/ml for IFN-γ and 31 pg/ml for IL-4). In general, the response against all of the viral antigens tested, including the influenza A lysate, was dominated by IFN-γ, whereas IL-4 production was rarely observed, suggesting a T helper 1–type response (Fig. 4*A* and *B*). In support of this, we detected IL-4 among the newly diagnosed type 1 diabetic patients less frequently when compared with healthy control subjects or long-term type 1 diabetic patients, particularly in response to the VP3 and VP4 antigens (for VP4, $P = 0.004$ for newly diagnosed patients compared with healthy control subjects) (Fig. 4*B*).

Median levels of IFN-γ production after stimulation with the control recall antigens TT and influenza A were similar in the study groups. However, we noted that IFN-γ production in newly diagnosed type 1 diabetic patients and, to a lesser extent, in long-standing type 1 diabetic patients tended to be higher in the presence of the CVB4 antigens compared with control subjects (Fig. 5).

Most notably, the median level of IFN-γ production to

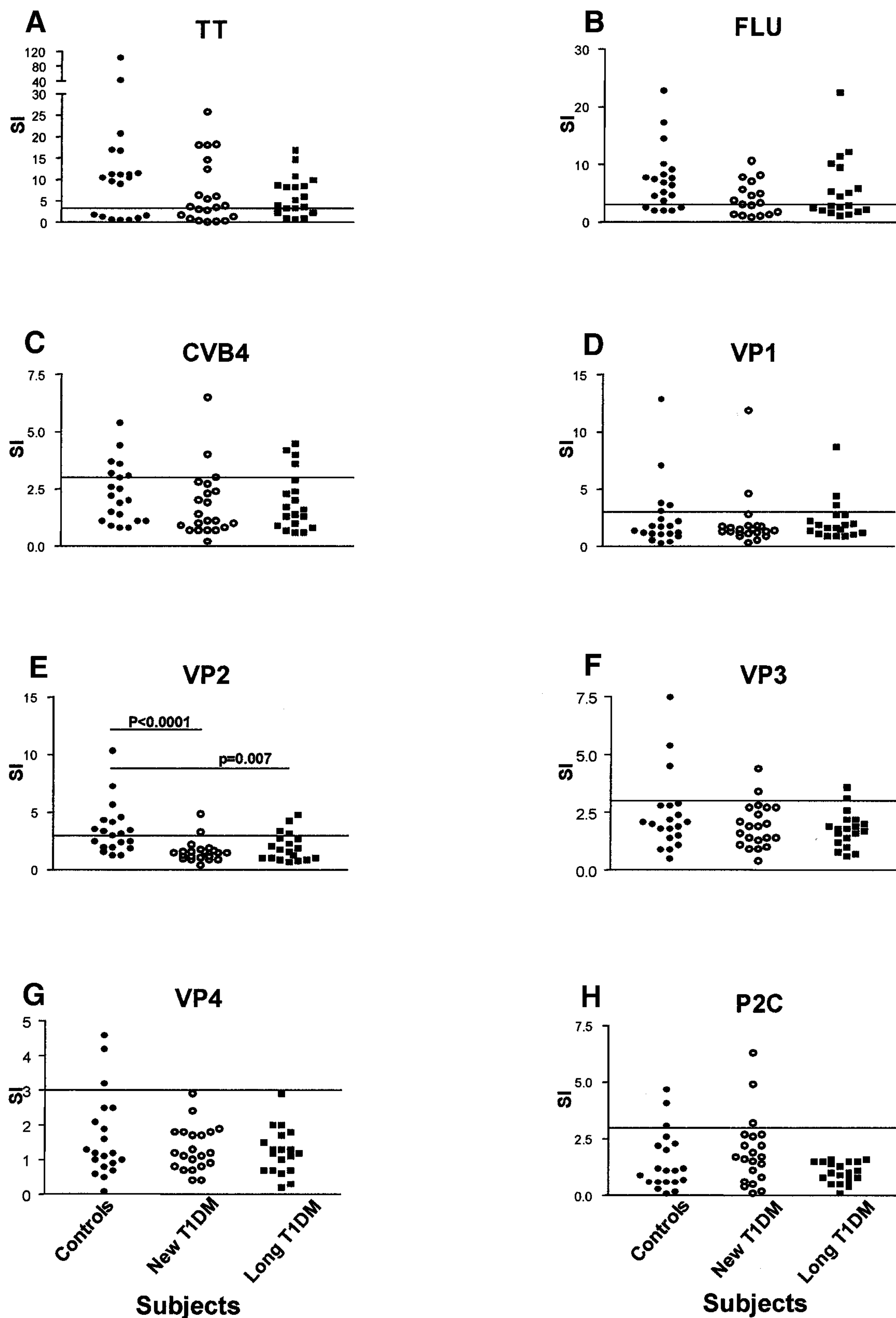

FIG. 2. Proliferation responses against the control and viral antigens in the different study groups. Controls, healthy nondiabetic donors; FLU, influenza A virus; Long T1DM, long-standing type 1 diabetic patients; New T1DM, newly diagnosed type 1 diabetic patients. *P* values are indicated in the corresponding figure when <0.05. Horizontal line represents the conventional cutoff of positivity for proliferation assays (SI = 3.0).

VP3 antigen was highest in the new-onset type 1 diabetic patients ($P = 0.012$ vs. control subjects and $P = 0.008$ vs. long-standing patients) (Fig. 5*F*). In addition, the median level of IFN-γ production to VP2 was highest in the new-onset type 1 diabetic patients ($P = 0.027$ vs. long-standing patients) (Fig. 5*E*), and similar results were seen

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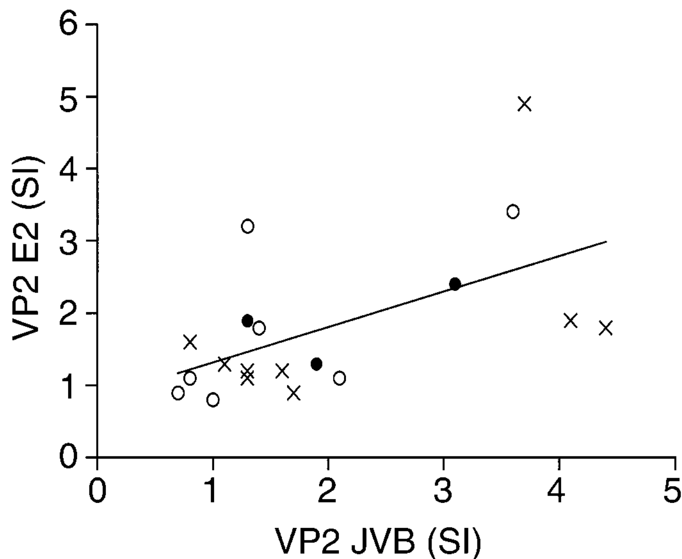

FIG. 3. Correlation between T-cell proliferation responses against the VP2 coat proteins from the E2 (*y*-axis) and JVB (*x*-axis) strains of CVB4 in 19 individuals. Values are expressed as stimulation indexes (SI). A good correlation is seen between T-cell proliferative responses to E2 and JBV VP2 antigens (r^2 = 0.31; P = 0.013). ●, healthy control subjects; ×, newly diagnosed type 1 diabetic patients; ○, long-standing type 1 diabetic patients.

with IFN-γ production to P2C (P = 0.003 vs. healthy control subjects) (Fig. 5*H*).

In general, the pattern of T-cell responsiveness to CVB4 antigens in patients with type 1 diabetes, especially those that were newly diagnosed, indicated low levels of T-cell proliferation, but raised levels of IFN-γ production. This pattern is most clearly seen when proliferation and IFN-γ values for individual antigens in individual subjects are plotted against each other (Fig. 6). This analysis for the viral proteins that elicit the greatest IFN-γ production, namely VP2 (Fig. 6*A*), VP3 (Fig. 6*B*), and P2C (Fig. 6*C*), are shown. This analysis shows that those subjects that produce higher amounts of IFN-γ show low proliferative responses, and vice versa, independently of the antigen tested.

HLA genotype and CVB4 T-cell response. Because HLA genotype is potentially important in determining immune responsiveness to CVB4 antigens and is unequivocally linked to type 1 diabetes susceptibility, we determined the HLA DRB1 genotypes of all subjects tested (Table 1). The frequencies of subjects carrying the HLA DR4/X (X being non-DR3), the HLA DR3/X (X being non-DR4), or the DRX/X (X being haplotypes other than DR4 or DR3) genotypes were similar among all subject groups (Table 1).

Correlations between HLA genotype and the presence of a detectable T-cell response (proliferation or cytokine production), were only found among healthy control subjects. Of the 11 control subjects proliferating against VP2, 7 were HLA-DR4/X, compared with 1 of 9 nonresponders having HLA-DR4/X (P = 0.028). Similarly, all 3 control subjects proliferating against VP3 and all 3 of those proliferating against VP4 were HLA-DR4/X, compared with 5 of 17 nonresponders (P = 0.049). Only 3 of 15 control subjects producing IFN-γ in response to VP2 were HLA-DR3/X, compared with 4 of 5 nonresponders (P = 0.031).

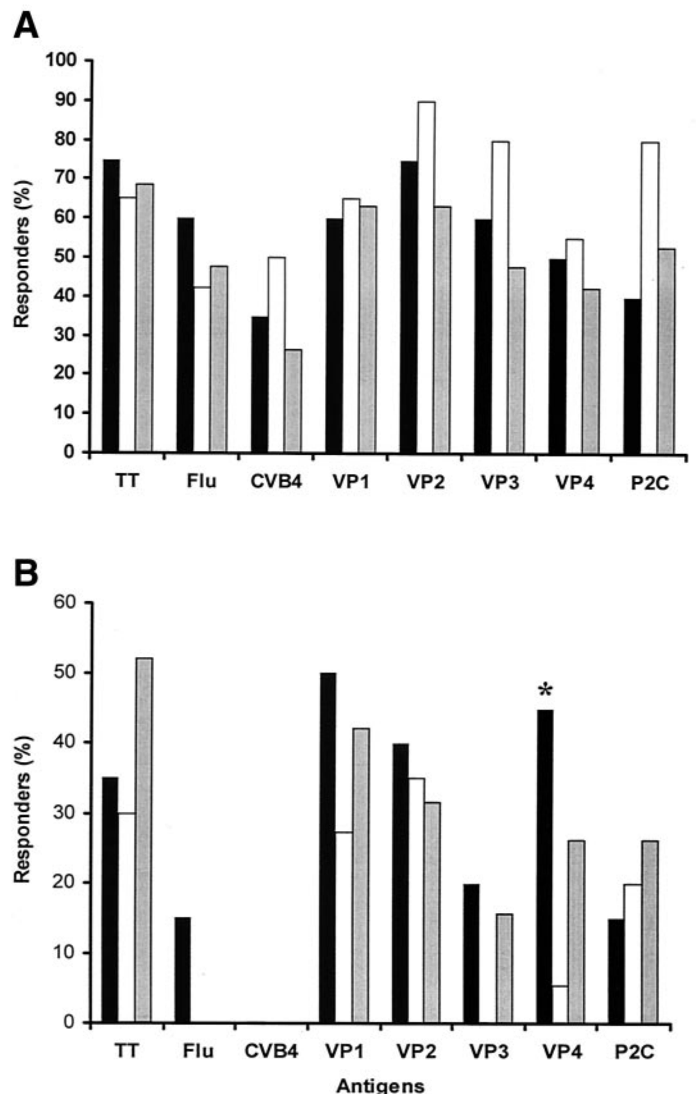

FIG. 4. Frequency of IFN-γ (*A*) and IL-4 (*B*) producers against the different antigens tested. ■, healthy donors; □, newly diagnosed type 1 diabetic patients; ▨, long-standing type 1 diabetic patients. The *x*-axis shows the antigens tested, and the *y*-axis shows the percentage of subjects producing the cytokine. *P = 0.004 for IL-4 production by newly diagnosed versus healthy control subjects. Flu, influenza A virus.

DISCUSSION

In the present study, we have focused on examining the T-cell response to CVB4, a candidate viral trigger and/or disease accelerant in type 1 diabetes. Using a novel approach to obtain soluble, purified viral components (29), we have shown that both structural (VP1-VP4) and non-structural (P2C) proteins can be targeted. In addition, we demonstrate that the quality of the T-cell response to CVB4 differs in key respects between healthy individuals and type 1 diabetic patients near to diagnosis. These data add weight to the view that EVs may play a role in type 1 diabetes pathogenesis.

In the present study, we demonstrate that type 1 diabetic patients show a different quality of T-cell response to CVB4 when compared with healthy nondiabetic control subjects. This was seen most clearly in the lower frequency of T-cell proliferation to CVB4 antigens in patients, with responses to VP2 being significantly reduced, contrasted with the markedly enhanced production of the

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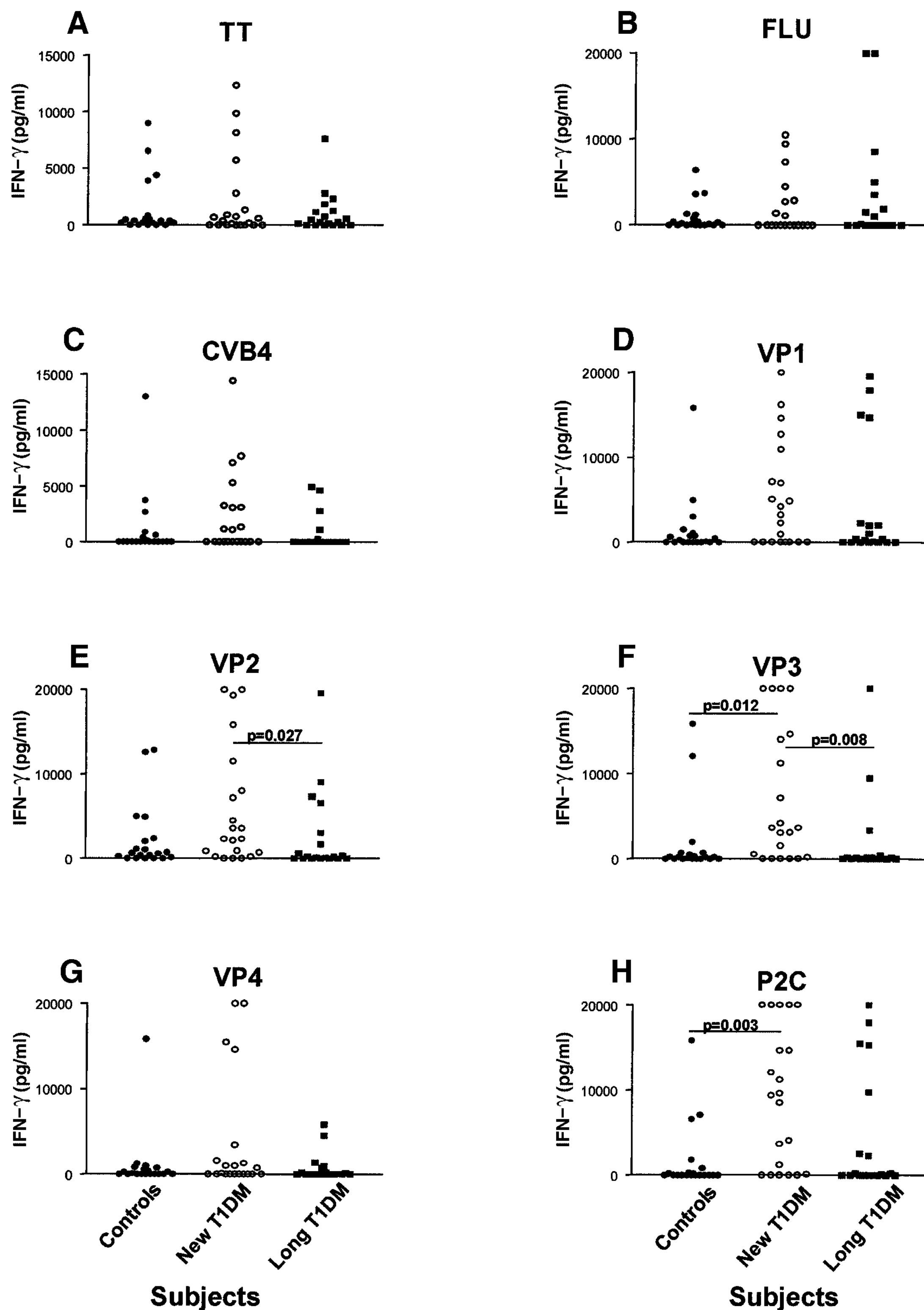

FIG. 5. IFN-γ production (pg/ml) against the different control and viral antigens in the different subject groups. Controls: healthy nondiabetic donors; Long T1DM: long-standing type 1 diabetic patients; New T1DM: newly diagnosed type 1 diabetic patients. *P* values are indicated in the corresponding figure when <0.05.

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inflammatory cytokine IFN-γ in response to most CVB4 antigens tested, and especially VP3. These differences were most pronounced near to diagnosis, and a trend remained in patients with long-standing disease, suggesting that the bias toward IFN-γ production and away from proliferation in response to CVB4 is established in the pre- or peridiabetic period. The low proliferative response against CVB4 antigens at disease onset is in agreement with recent studies showing reduced T-cell proliferation against EV-infected cell lysates or selected EV peptides at

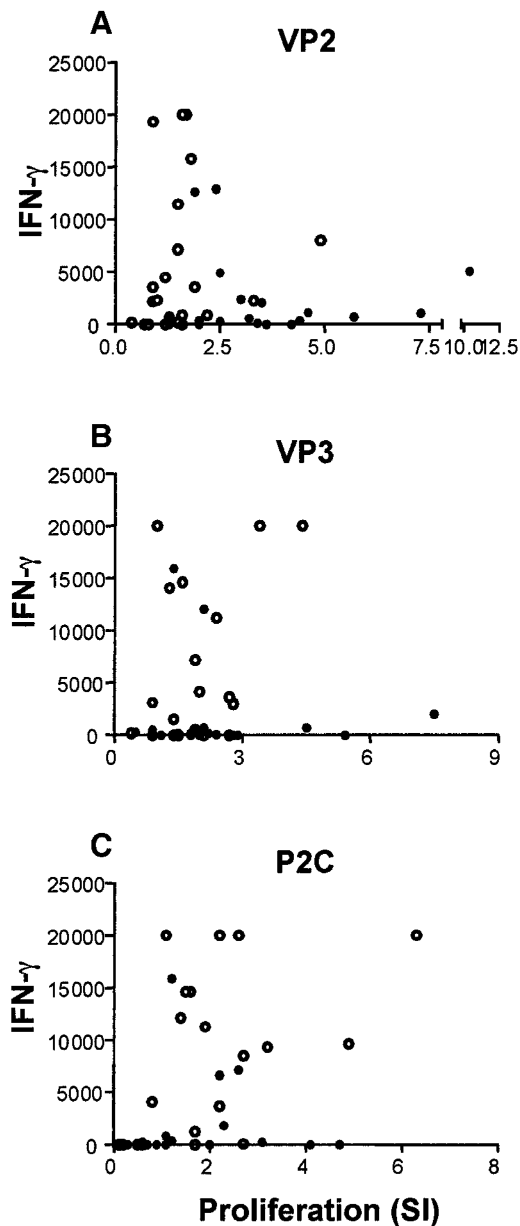

FIG. 6. Scatter plot of proliferation versus IFN-γ production among the healthy donors and newly diagnosed type 1 diabetic subjects against the CVB4 VP2 (*A*), VP3 (*B*), and P2C (*C*) antigens. ●, healthy donors; ○, newly diagnosed type 1 diabetic patients. Responses to these CVB4 antigens are seen as IFN-γ production or proliferation, but rarely both.

the time of diagnosis (25,31). These studies were interpreted as indicating that CVB4-reactive T-cells are absent from the circulation because of redistribution into the inflamed pancreas (31). Our results indicate that this is not the case. On the contrary, there is an abundance of circulating primed CVB4-specific responder T-cells in patients with type 1 diabetes. The ability to distinguish between proliferating and cytokine-producing T-cells is of importance, therefore, in investigating T-cell responses to candidate viruses involved in the pathogenesis of type 1 diabetes.

The relative lack of proliferation, but enhanced IFN-γ production by anti-CVB4 memory T-cells, in type 1 diabetic patients is attributable to a different balance of phenotypically distinct populations of memory cells, when compared with control subjects. Two broadly defined phenotypes of memory T-cells have been characterized extensively in the recent studies of Lanzavecchia, Sallusto, and colleagues (32,33). Primed (memory) T-cells with the capacity to proliferate have been termed "central memory," or T_{CM} cells. These cells lack immediate effector function and predominantly produce IL-2, the major T-cell growth factor to support proliferation, as well as express CCR7, a chemokine receptor that directs homing to lymph nodes. In contrast, the primed memory cell subset that produces the proinflammatory cytokine IFN-γ during an immune response has been termed the "effector memory" subset, or T_{EM}. T_{EM} cells do not express CCR7 and are therefore present in the circulation and available to make antigen-specific responses at sites of infection or tissue inflammation.

Recent studies have focused on defining the relationship between T_{CM} and T_{EM} cell subsets specific for the same antigen (33). T_{CM} cells in lymph nodes have a lower activation threshold and faster kinetics compared with naive T-cells. After activation, T_{CM} cells proliferate and acquire effector function, in effect becoming T_{EM} cells. It appears that the major stimuli promoting differentiation to IFN-γ–secreting T_{EM} cells are antigen presentation by dendritic cells plus the dendritic cell–derived cytokine IL-12. It is apparent that prolonged antigen-specific stimulation through the T-cell receptor promotes T_{EM} development (34). In combination with our own findings, these data suggest that the explanation for the relative predominance of CVB4-specific T_{EM} cells at diagnosis of diabetes lies in either a recent or persistent exposure to CVB4 infection.

Among the coat proteins, we found that responses are typically made against VP2>VP3>VP1, with VP4 rarely recognized by T-cells. VP1–3 antigens demonstrate the most amino acid sequence variability across the *Enterovirus* genus, whereas VP4 is highly conserved (35). Similar findings have been reported in relation to the immune response to the influenza A virus, in which the two main targets of the T-cell response, the hemagglutinin and neuraminidase glycoproteins, are also the most variable structural proteins of the virus (36). VP1–3 proteins delimit a narrow depression, called the "canyon," which has been demonstrated to be the receptor binding site of the Coxsackie-adenovirus receptor (CAR) used for viral entry (37), and CD4 T helper responses against these proteins may be important in generating protective levels of antibodies that inhibit infectivity. Our results also confirm previous observations (29,31) showing that the nonstructural P2C protein is targeted by T-cells during an anti-CVB4 immune response.

Interest in analyzing the immune response to EVs stems from the need to examine the numerous proposed mechanisms through which these micro-organisms could initiate (e.g., by molecular mimicry) or accelerate (e.g., by bystander activation) progression to type 1 diabetes. The results from our work highlight some of the problems previously encountered in trying to identify T-cell targets in the CVB4 virus (23–26,38–45). First, the predominant use of proliferation to assess T-cell reactivity will fail to detect most circulating cells with the capacity to respond, as we have highlighted in the present study. In other

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reports, either whole virus–infected cell lysates (45) or poliovirus instead of Coxsackievirus have been used as a source of antigen (38–42), despite the fact that these viruses have major differences at the protein level (35). Studies have also been carried out using animal models without genetic susceptibility to autoimmune diabetes (38–40), in which the T-cell epitopes identified are of doubtful relevance to human type 1 diabetes.

Much previous work has focused on immune responses against the region of sequence similarity between GAD65 and P2C, the so-called PEVKEK region (23–26,43,44). At present, there are no definitive data to support a role for this region in initiating islet autoimmunity through molecular mimicry, but our results indicate that enhanced T_{EM}-cell reactivity to P2C is significantly associated with diabetes diagnosis. However, it is possible that other CVB4 antigens may also harbor cross-reactive epitopes. T-cell cross-reactivity is dependent on shared structural features of peptide epitopes. These may not require amino acid sequence identity and are therefore difficult to identify by database searches (46). Such structural homologies require characterization of the T-cell response to the candidate environmental and self-antigen in order to be elucidated, and our approach therefore represents an important step in this direction.

In summary, the present work highlights the importance of a comprehensive analysis of T-cell reactivity in relation to potentially diabetogenic viruses. In addition, we reveal a diabetes-related phenotype of CVB4-reactive T-cells, which adds weight to the notion that EVs play a role in the development of the disease.

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