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T Cell Activation by Coxsackievirus B4 Antigens in Type 1 Diabetes Mellitus: Evidence for Selective TCR Vβ Usage Without Superantigenic Activity[1]

Ruben Varela-Calvino,* Gianluca Sgarbi,* Lucy R. Wedderburn,† Colin M. Dayan,‡ Jenny Tremble,* and Mark Peakman[2]*

Numerous clinical and epidemiological studies link enteroviruses such as the Coxsackie virus group with the autoimmune disease type 1 diabetes mellitus (DM). In addition, there are reports that patients with type 1 DM are characterized by skewing of TCR Vβ chain selection among peripheral blood and intraislet T lymphocytes. To examine these issues, we analyzed TCR Vβ chain-specific up-regulation of the early T cell activation marker, CD69, on CD4 T cells after incubation with Coxsackievirus B4 (CVB4) Ags. CD4 T cells bearing the Vβ chains 2, 7, and 8 were the most frequently activated by CVB4. Up-regulation of CD69 by different TCR families was significantly more frequent in new onset type 1 DM patients ($p = 0.04$), 100% of whom ($n = 8$) showed activation of CD4 T cells bearing Vβ8, compared with 50% of control subjects ($n = 8$; $p = 0.04$). T cell proliferation after incubation with CVB4 Ags required live, nonfixed APCs, suggesting that the selective expansion of CD4 T cells with particular Vβ chains resulted from conventional antigen processing and presentation rather than superantigen activity. Heteroduplex analysis of TCR Vβ chain usage after CVB4 stimulation indicated a relatively polyclonal, rather than oligo- or monoclonal response to viral Ags. These results provide evidence that new-onset patients with type 1 DM and healthy controls are primed against CVB4, and that CD4 T cell responses to the virus have a selective TCR Vβ chain usage which is driven by viral Ags rather than a superantigen. ***The Journal of Immunology,*** **2001, 167: 3513–3520.**

Type 1 diabetes mellitus (DM)[3] is an autoimmune disease characterized by lymphocytic infiltration of the islets of Langerhans and destruction of the insulin-producing β cells. Studies showing geographical variability in disease prevalence, as well as increasing incidence of type 1 DM despite a stable gene pool, suggest that environmental factors play a major role in the development of this autoimmune disease (1, 2). In particular, numerous studies have suggested an association between enterovirus infections and the development of type 1 DM. This association is based on virus isolation from the pancreas of newly diagnosed patients (3), several epidemiological and prospective studies showing that some cases of type 1 DM are strongly associated with enterovirus infections (4–6), and case-control studies that show an increased prevalence and levels of IgM Abs to Coxsackievirus B (CVB) in newly diagnosed patients (7). Initially, Gamble and Cumming (8) reported an association with CVB, but more recently there has been evidence for the involvement of a wider range of enteroviruses, including Coxsackievirus A and echoviruses (9–12).

Several mechanisms have been proposed to explain this link between virus infections and type 1 DM, among them the so-called "molecular mimicry" theory (13, 14), as well as bystander activation of autoreactive T cells by virus-specific T cells during an ongoing infection (15). More recently, it has been suggested that viral protein(s) with superantigenic activity (16) may be active in the development of type 1 DM. In studies on two children with recent onset type 1 DM, a marked overrepresentation of mRNA encoding the TCR Vβ7 chain among islet infiltrating lymphocytes was observed (16). In a more recent study of PBLs (17), TCR Vβ7 mRNA was found to predominate in type 1 DM patients at diagnosis compared with healthy individuals and patients with long-term diabetes. To date, there has been no adequate explanation for this TCR Vβ skewing (18–24). However, a potential clue to the etiology of the TCR Vβ skewing in type 1 DM patients may be found in a study on T cells infiltrating the hearts of children with proven CVB3-associated acute myocarditis (25). The study showed skewing of TCR Vβ mRNA toward expression of Vβ transcripts (7, 3, and 13.1) similar to those seen in islets from diabetic children (16), thus providing a link between Coxsackieviruses and TCR Vβ skewing.

In some of the above studies, spectratype analysis indicated that expansion of selected TCR Vβ chain transcripts was poly rather than oligo- or monoclonal. This argued against an Ag-driven T cell expansion and in favor of the possibility that during CVB infection, protein(s) drives TCR Vβ skewing through a superantigen effect. In the present study, we investigated this possibility directly by examining TCR Vβ-specific up-regulation of the early activation marker, CD69, on CD4 and CD8 T cells, following exposure to CVB4 viral proteins. Our results show that under these conditions CVB4 proteins activate mainly CD4 T cells compared with

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[3] Abbreviations used in this paper: DM, diabetes mellitus; CVB/CVB4, Coxsackievirus B/Coxsackievirus B4; TSST-1, toxic shock syndrome toxin-1; TT, tetanus toxoid; SEB, staphylococcal enterotoxin B; SI, stimulation index.

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CD8 T cells. We show that the activation does indeed target selected T cells according to Vβ chain expression, including Vβ chains previously shown to be preferentially represented in islets of Langerhans at diagnosis of type 1 DM. However, we provide evidence that this TCR Vβ activation requires viral Ag processing and is therefore unlikely to be the result of superantigen activity.

Materials and Methods

Subjects

Sixteen subjects were studied. There were eight (six males, two females) healthy nondiabetic donors without family history of the disease and eight (five males, three females) newly diagnosed type 1 DM patients (all within 2 wk of diagnosis). Mean age (±SD) for the healthy donors was 34.4 ± 6.8 years and for the diabetic patients 30.4 ± 5.7. Blood was drawn with the consent of all subjects and appropriate permission from the Institute's Ethical Review Board. The matched healthy controls and type 1 DM patients were tested during the same time period to control for seasonal variations in exposure to CVB4. HLA genotyping was performed by PCR-single-strand conformation polymorphism.

CD69 up-regulation studies

PBMCs were obtained by Ficoll-Hypaque centrifugation of heparinized blood, washed twice in HBSS, 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). Suspended PBMCs were distributed in a 24-well tissue culture plate (1 ml/well) supplemented with medium alone, toxic shock syndrome toxin-1 (TSST-1; 100 ng/ml; Toxin Technology, Sarasota, FL), or either control uninfected or CVB4-infected Vero cell lysate (JVB strain; 5 μl/ml; ≈0.2 μg of protein lysate/ml; Institute Virion, Zurich, Switzerland) and incubated for 5 h at 37°C. Cell lysates were prepared as previously described (26). In control experiments, influenza-infected and control cell lysates (Institute Virion) were used as a source of Ags.

After incubation, cells were collected and washed (300 × *g* for 10 min at 4°C) twice with PBS containing 5% FCS (PAA Laboratories). After washing, cells were resuspended in 1 ml of PBS-FCS, aliquoted, and stained for 30 min at 4°C with CD4-APC (clone SK3), CD8-PerCP (clone SK1; both from BD Biosciences, Erembodegem, Belgium), CD69-PE (clone CH/4; Serotec, Oxford, U.K.), and 1 of 16 FITC-labeled Vβ Abs: anti-human Vβ1 (clone BL37.2), 2 (clone MPB2D5), 3.1 (clone 8F10), 5.1 (clone LC4), 6.7 (clone OT145), 7 (clone ZOE), 8 (56C5.2), 11 (clone C21), 12 (clone VER2.32.1), 13.1 (clone IMMU222), 14 (clone CASS1.1.3), 16 (clone TAMAYA1.2), 17 (clone C1), 20 (clone ELL1.4), 21.3 (clone IG125), and 22 (clone IMMU546; all from Serotec). Cells were also stained with the corresponding fluorochrome and isotype control Abs (either from BD Biosciences or Serotec), and nonspecific staining subtracted was from the appropriate populations. After staining, cells were washed twice with PBS-FCS and resuspended in PBS for analysis using a FACSCalibur flow cytometer (BD Biosciences). Ten thousand CD4-positive events and 5000 CD8-positive events were collected. Data were analyzed using the CellQuest program (BD Biosciences). The panel of 16 Vβ-specific mAbs we used represents the major TCR Vβ families and stains ~60–70% of TCR-positive cells.

Detection of CD69 up-regulation on specific $V\beta^+$ T cells

To demonstrate that T cells bearing a specific Vβ chain showed CD69 up-regulation after exposure to CBV Ags, we used two separate analyses. This approach was first validated in preliminary studies using TSST-1 as the stimulus, as discussed in *Results*. Only those Vβ families that showed significant CD69 up-regulation as judged by both analyses were considered positive. The two separate analyses are illustrated in Fig. 1 and are based on a direct comparison between PBMCs incubated with CVB-infected and uninfected lysates.

In the first analysis, our approach was similar to that described previously in the demonstration of superantigenic activity associated with EBV (27). Activation of $V\beta^+$ cells was considered positive if the proportion of a specific Vβ subset expressing CD69 was higher after stimulation with the CVB-infected lysate compared with the control uninfected lysate. The proportion of a specific Vβ subset expressing CD69 is given by the formula quadrant 2/quadrant 2 + 4 (Fig. 1).

In the second analysis, we examined the contribution of each Vβ family to the total number of cells activated (i.e., total $CD69^+$) by CVB. The total population of cells activated to express CD69 by a given stimulus is represented by quadrant 1 + quadrant 2. For each patient and control, a mean value for quadrant 1 + quadrant 2 was obtained from the 16 separate Vβ analyses. This value was used as the denominator, and values in quadrant 2 for each Vβ analysis were used as the numerator. This is given by the formula quadrant 2/$(\text{quadrant 1 + 2})_{\text{MEAN}}$. Activation of a specific Vβ family was considered to contribute to total CD69 up-regulation when quadrant 2/$(\text{quadrant 1 + 2})_{\text{MEAN}}$ equated to greater than zero.

Only those Vβ families in which there was evidence of CD69 up-regulation by CVB4 proteins (first analysis) and evidence that this up-regulation contributed to total CD69 up-regulation by CVB4 (second analysis) were considered positive. The rationale for using a combination of two separate analyses is that it takes into account those Vβ families that are poorly represented numerically, in which small changes in the numbers of activated cells appear as large proportional changes, although overall these cells represent a very small percentage of cells activated by the virus. Reproducibility of this analytical approach was assessed by preliminary experiments conducted on samples 3–4 wk apart obtained from healthy control subjects. A representative example is subject C7, who showed positive activation of TCR Vβ families 1, 2, 5, 6, 11, 16, and 17 on first analysis, with the remaining families negative. An identical pattern of positivity and negativity was seen in the second sample.

Proliferation assays

PBMCs (10^5 cells/well) were cultured in triplicate in U-bottom 96-well plates with medium alone, 100 ng/ml tetanus toxoid (TT; Pasteur Merieux MSD, Berkshire, U.K.), 10 ng/ml staphylococcal enterotoxin B (SEB) (Toxin Technology) and 5 μl/ml (≈0.2 μg of protein lysate/ml final concentration) of either uninfected or CVB4-infected Vero cell lysate. These concentrations were shown to give optimal proliferation in preliminary and previous studies (26). 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 beta plate counting in a Matrix 9600 counter (Packard Instrument, Pangbourne, U.K.). Results are expressed as a stimulation index (SI: mean counts with Ag/mean counts with relevant control preparation (i.e., uninfected Vero cell lysate). For SEB and TT responses, the SI was calculated using the formula: mean counts with Ag/mean counts with medium alone. An SI above 3 is considered positive by convention. Mean counts in the presence of uninfected lysate were 684 cpm (range, 206-2417) and in the presence of medium alone 489 cpm (range, 61–1532).

To study the possibility that a superantigen is encoded in the CVB4 virus genome, a responder population ($CD3^+HLA\text{-}DR^-$ cells) and an APC population ($CD19^+$) were isolated from total PBMCs by positive selection using microbeads as suggested by the manufacturer (Miltenyi Biotec, Surrey, U.K.). Briefly, cells were resuspended at 10^7/80 μl in PBS containing 0.5% BSA and 2 mM EDTA. Twenty microliters of the corresponding magnetic beads (CD3 or CD19) was added to the cells and incubated at 6–12°C for 15 min. Cells were isolated using a type MS^+/RS^+ column, prewashed with PBS/BSA/EDTA, and placed in a magnetic MACS separator. The cell suspension was applied to the column and washed three times with 500 μl buffer. The column was then removed from the magnet and retained cells were eluted in 1 ml of PBS/BSA/EDTA. The $CD3^+$ population was depleted of $HLA\text{-}DR^+$ cells using HLA-DR Dynabeads (Dynal, Wirral, U.K.) according to the manufacturer's instructions, and the CD3/HLA-DR- population was washed with HBSS and resuspended at 10^6 cells/ml in RPMI 1640 medium (RPMI 1640/10% AB^+ serum). The $CD19^+$ cells were resuspended at 4×10^6 cells/ml in RPMI 1640 medium and divided into two tubes. One tube was left unmanipulated (APCs nonfixed) and to the second the same volume of 2% paraformaldehyde in PBS was added to the cells, incubated for 10 min at room temperature, washed twice, and resuspended in RPMI 1640 medium at 4×10^6 cells/ml. The purities of the isolated populations were assessed by flow cytometry and for the responder population ($CD3^+HLA\text{-}DR^-$ cells) was 95% or greater, with only 0.35–2% of $CD19^+$ cells (B cells), and for the APC population ($CD19^+$) purity was 87–99%.

To set up the proliferation assays, responder cells (10^5 cells/well) and APCs (4×10^4 cells/well), either nonfixed or fixed with paraformaldehyde, were cultured in triplicate in U-bottom 96-well plates with the corresponding Ag (SEB, TT, CVB4-infected lysate, or no Ag) in a total volume of 200 μl. Total unmanipulated PBMCs were also incubated with the same Ags. After 5 days in culture, the cells were labeled with [^{3}H]thymidine and harvested as described above. Results are expressed as a SI (mean counts with Ag/mean counts with medium alone).

Heteroduplex analysis of TCR usage

A detailed description of this analysis can be seen in the study by Maini et al. (28). In the context of the current study, this technique is able to provide a direct visualization of clonal expansion of T cells according to particular

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Vβ usage. Briefly, PBMCs ($4-6 \times 10^6$) were harvested after 6 days in culture with either uninfected or CVB4-infected cell lysates. Total RNA was extracted using the SV Total RNA Isolation System kit (Promega, Southampton, U.K.) and cDNA was synthesized using the RETROscript (Ambion, Oxon, U.K.).

Twenty-six different PCRs, for both cells incubated with uninfected and CVB4-infected lysates, were performed using a consensus internal Cβ primer (5′-CACCCACGAGCTCAGCTCCACGTGGTC-3′) and 26 TCRBV primers as described before (29) in a final volume of 50 μl. Hot-start PCR was conducted with initial denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 1 min, with a final extension period at 72°C for 10 min. PCR products were visualized on a 1% agarose gel. DNA carriers consisting of cDNAs encoding BV1–24 cloned from T cell clones or lines as described previously and provided by G. Casorati (Unitá d'Immunochimica, DBIT, Istituto Scientifico, Milan, Italy) (29) were amplified using the same TCRBV primers and conditions as those for the sample cDNAs, but using a different Cβ primer, external Cβ primer (5′-TGCTGACCCCACTGTCGACCTCCT TCCCATT-3′) which is 30-bp 3′ to the internal Cβ. A 20-μl aliquot of each sample PCR product was mixed with 400 ng of the appropriate BV-matched carrier product, denatured at 95°C for 5 min, and allowed to reanneal at 50°C for 1 h. The mixtures were loaded on a 12% nondenaturing polyacrylamide gel and resolved at 10 mA for 16 h at 4°C. For initial visualization, heteroduplex gels were stained with ethidium bromide for 30 min and then blotted onto nylon Hybond N^+ membranes in 20× SSC. The DNA was denatured and fixed to the membrane by a 20-min incubation in 0.4 M NaOH. The external Cβ primer was biotin-labeled using the BrightStar Psoralen-Biotin kit (Ambion). Membranes were prehybridized for at least 2 h at 42°C with prehybridization buffer (6× SSC containing 5× Denhardt's, 0.1% SDS, and 100 μg/ml sonicated salmon sperm DNA; Sigma, Poole, U.K.) and hybridized for 16 h at 42°C with hybridization buffer (6× SSC, 0.1% SDS) containing 10 ng/ml labeled probe. Membranes were washed in 5× SSC/0.1% SDS for 20 min at room temperature and then twice in 1× SSC/0.1% SDS for 15 min each at 42°C. To detect the biotinylated probe, we used a chemiluminescent detection kit (BrightStar BioDetect; Ambion) following the manufacturer's instructions. Filters were exposed to x-ray films at room temperature for 2–16 h.

Statistical analysis

Comparisons of the frequency of activation of different Vβ chains was made using Fisher's exact test.

Results

Detection of TCR Vβ skewing by expression of the early activation marker CD69

Up-regulation of the early activation marker CD69 has been used previously to identify superantigen activity in EBV (27). Using a similar approach, we incubated PBMCs with TSST-1, a superantigen known to favor TCR Vβ 2 expansion, for 5 h and, after harvesting, cells were stained for CD4, CD69, and different Vβ chains. According to the two criteria described (see *Materials and Methods* and Fig. 1), T cells carrying Vβ2 chains were activated by TSST-1 as well as some Vβ 3^+ cells (data not shown). These data confirm that this experimental approach is able to identify superantigenic activity.

Activation of T cells with CVB4 Ags elicits CD69 expression on cells bearing selected TCR Vβ chains

A typical example of the results obtained in our analyses on PBMCs from patients with type 1 DM and control subjects, using CVB4 lysate as the antigenic stimulus, is shown in Fig. 1. As stated above, two criteria were used to determine whether CVB4 activates specific Vβ populations: 1) an increase in the proportion of cells of a specific Vβ population expressing CD69 in the presence of CVB4 Ags and 2) an increase in specific $V\beta^+CD69^+$ cells as a proportion of the total number of cells induced to express CD69 by CVB4 Ags.

In the case depicted in Fig. 1 for CD4 T cells from a type 1 DM patient (D1, Table I), several Vβ families show an increase in their percentage of CD69 expression after incubation with CVB4 Ags compared with uninfected lysates. Increases vary from 29.8% for

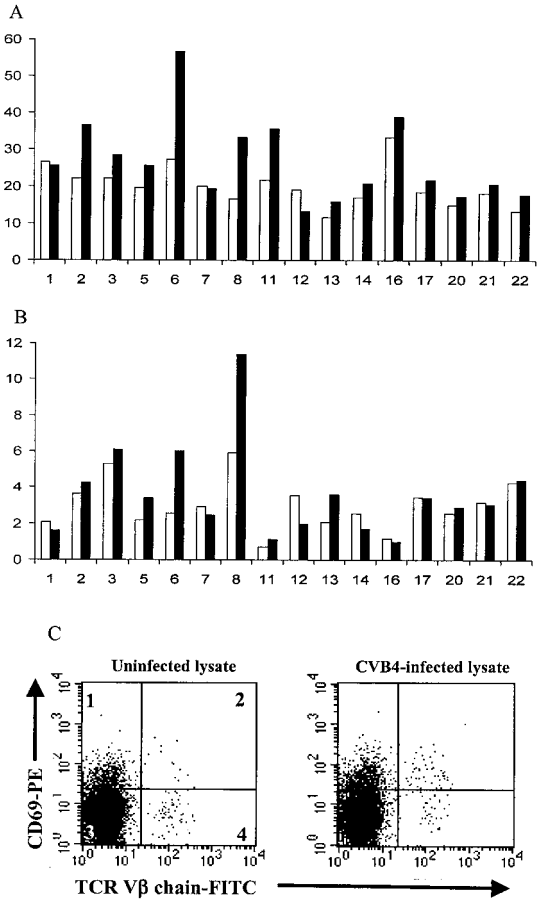

FIGURE 1. Representative analysis of CD69 up-regulation by CD4 T cells stimulated with CVB4 Ags in a patient with new-onset type 1 DM. PBMCs were stimulated with CVB4 or control Ags and Vβ-specific CD69 up-regulation was analyzed using the two criteria described in *Materials and Methods*. *A*, Number of cells expressing a specific Vβ chain that up-regulates the early activation marker CD69 in response CVB4 Ags compared with control lysates. Results expressed as a percentage of the total number of cells expressing that Vβ chain (i.e., quadrant 2/quadrants 2 + 4 in *C*). *B*, Contribution of specific $V\beta^+CD69-+$ activated cells to the total number of T cells up-regulating CD69. Results are expressed as a percentage of the total activated population (i.e., quadrant 2/quadrants 1 + 2 in *C*). *C*, Flow cytometric plot of a single Vβ chain analyzed for CD69 up-regulation after incubation with either uninfected or CVB4-infected lysates. □, Control, uninfected lysates; ■, CVB4-infected lysates.

Vβ6 to 2.4% for Vβ20, (Fig. 1*A*). Some Vβ families (1, 7, and 12) show no increase compared with the uninfected lysates.

After incubation with CVB4 lysates, there is an increase from 11.0 to 14.1% in the size of the population of $CD4^+$ cells coexpressing CD69 (mean of quadrants 1 + 2 for all 16 Vβ families analyzed). In the case shown, the major component of this increase is from the Vβ8 and Vβ6 families (Fig. 1*B*), although other Vβ cells also contribute. Several other Vβ families also contribute to the increase in CD69 expression after incubation with CVB4, the exceptions being TCR Vβ 1, 7, 12, 14, 16, 17, and 21 (Fig. 1*B*). This analysis indicates that in this patient, CD4 T cells having TCR Vβ chains 2, 3, 5, 6, 8, 11, 13, 20, and 22 were activated by the CVB4-infected lysate and also contribute to the total virus-activated CD69 population. In this individual, therefore, these Vβ chains were considered positive.

We went on to analyze eight patients with new-onset type 1 DM and eight healthy nondiabetic control subjects in the same way, applying the two-step criteria to identify Vβ families in which

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Table I. *Vβ-specific CD69 up-regulation on CD4 T cells in response to incubation with CVB4 Ags*[a]

Subject	HLA-DR Genotype	Vβ Chain															
		1	2	3	5	6	7	8	11	12	13	14	16	17	20	21	22
C1	03,15			X[b]	X	X	X	X	X		X	X		X	X		
C2	04,15			X			X		X	X	X	X				X	X
C3	03,15							X			X						X
C4	04,15		X		X			X	ND	X			ND	X		X	
C5	03,07	X					X	X	X	X	X		X	X	X		
C6	01,13					X	X		X	X	X		X		X		
C7	04,04	X	X		X	X			X				X	X			
C8	03,03	X	X		X					ND					X	X	X
% positive		37.5	37.5	25	50	37.5	50	50	71.4	57.1	62.5	25	42.9	50	50	37.5	37.5
D1	03,04		X	X	X	X		X	X		X				X		X
D2	01,01			X	X		X	X		X			X		X		
D3	01,08		X				X	X	ND	X			ND	X	X	X	
D4	03,04	X	X			X	X	X		X				X	X		X
D5	03,03		X					X	X			X	X	X		X	X
D6	03,04	X		X	X	X	X	X	ND	X						X	
D7	04,07		X	X	X	X	X	X	X	X	X		X	X		X	
D8	03,13	X	X	X			X	X	X			X	X			X	X
% positive		37.5	75	62.5	50	50	75	100*	66.7	62.5	25	25	57.1	50	50	62.5	50
Total positive (%)		37.5	56.3	43.8	50	43.8	62.5	75	69.2	60	43.8	25	50	50	50	50	43.8

[a] C1–8, Control subjects; D1–8, patients with type 1 DM.
[b] X, positive response to CVB4; ND, not done.
*, $p < 0.05$ compared with healthy controls.

CD69 was up-regulated by CVB4. In the CD4 T cell compartment, considering all the subjects tested together (16 in total), we observed that certain Vβ families showed CD69 up-regulation more frequently. CD69 up-regulation on TCR Vβ8 cells was seen in 75% (12 of 16 subjects) and Vβ2, 7, 11, and 12 in 56.3% (9 of 16), 62.5% (10 of 16), 69.2% (9 of 13), and 60% (9 of 15) subjects, respectively (Table I). T cells expressing remaining Vβ chains were activated in 50% of cases or less.

However, when we compared the two groups of subjects, we observed differences in the Vβ activation patterns of type 1 DM patients compared with healthy control subjects. Among type 1 DM patients, 100% (8 of 8) subjects showed clear up-regulation of CD69 CD4 T cells bearing TCR Vβ8 chains compared with 50% (4 of 8) of healthy subjects ($p = 0.04$; Table I). CD4 T cells with other TCR Vβ chains were more frequently activated in patients than control subjects, most notably in the cell populations expressing Vβ2 (75% in type 1 DM patients vs 37.5% in control subjects), Vβ3 (62.5 vs 25%), Vβ7 (75 vs 50%), and Vβ21 (62.5 vs 37.5%), although these differences in frequency failed to reach statistical significance (Table I). In contrast, Vβ13-expressing cells were more frequently activated in control subjects (62.5 vs 25% in type 1 DM patients), although this difference did not reach statistical significance. Overall, coculture of CVB4 Ags with PBMCs from type 1 DM patients induced Vβ chain-specific CD69 up-regulation more frequently (70 positive of 125 tests, 56%) than PBMCs from control subjects (56 of 125 tests, 45%; $p = 0.04$). Although the number of cases studied is small, there did not appear to be any relationship between HLA-DR genotype and Vβ-specific CD69 up-regulation (Table I).

In the CD8 compartment, CD69 up-regulation was lower in magnitude and frequency than for CD4 T lymphocytes (Table II). Considering all the subjects, only CD8 T cells bearing TCR Vβ1 chains were activated in >50% of all test subjects. There were no significant differences between type 1 DM patients and control subjects in the frequency of CD8 Vβ chain-specific activation.

To examine the viral specificity of the Vβ TCR-related activation we observed, we also analyzed CD69 up-regulation after coculture with influenza viral cell lysates, compared with the appropriate control cell lysates, when sufficient cells were available. The pattern of Vβ-specific CD69 up-regulation on CD4 T cells by influenza viral cell lysates was consistently different than that seen with CVB4 lysates, as shown for subject C7 in Fig. 2.

Selective activation of TCR Vβ-bearing T cells requires processing of CVB4 Ags

In the light of evidence that CVB4 Ags induce CD69 expression on selected populations of T cells according to Vβ chain expression, our next step was to examine whether this could be due to a superantigen effect. Superantigens bind directly to MHC class II molecules, outside the conventional peptide binding groove, and stimulate T cells regardless of Ag specificity via engagement with the TCR Vβ chain. Their interaction with MHC class II molecules and subsequent T cell activation does not require conventional Ag processing and presentation by APCs. We designed an assay to distinguish between T cell activation driven by superantigen and that driven by conventionally processed Ag. In this assay, APCs and responder T cells (HLA-DR depleted) were purified and the APCs either fixed with paraformaldehyde or left unmanipulated. Responder T cells were stimulated to proliferate in the presence of the superantigen SEB irrespective of whether fixed or nonfixed APCs were used (SIs, 54.6 and 47.1, respectively; Fig. 3*A*). In contrast, as expected, the conventional recall Ag TT, which requires processing by APCs for T cell recognition, only induced T cell proliferation in the presence of live, unfixed APCs (Fig. 3*B*). Under identical conditions, the ability of CVB4 Ag lysates to induce T cell proliferation was examined. In the case of CVB4, the results were similar to those for the recall Ag TT, in that proliferation only occurred in the presence of live, unfixed APCs (Fig. 3*C*). None of the cases tested showed significant proliferation to CVB4 Ags in the presence of fixed APCs (SI < 2.0). These data were acquired in three separate experiments on three healthy individuals (cases C6, C7, and C8 in Table I) and argue against the possibility that the CVB4 lysates contain Ags capable of inducing T cell proliferation or Vβ skewing through a superantigen effect.

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Table II. *Vβ-specific CD69 up-regulation on CD8 T cells in response to incubation with CVB4 Ags*

Subject	HLA-DR Genotype	Vβ Chain 1	2	3	5	6	7	8	11	12	13	14	16	17	20	21	22
C1	03,15		X[b]	X	X	X				X		X		X	X	X	X
C2	04,15	X					X		X	X	X	X	X		X	X	X
C3	03,15		X	X				X	X		X			X			X
C4	04,15								ND				ND				
C5	03,07			X				X				X			X		
C6	01,13		X					X		X			X	X			
C7	04,04	X	X	X		X	X		X	X	X			X	X	X	X
C8	03,03	X	X										X		X		
% positive		37.5	62.5	50	12.5	25	25	37.5	42.8	50	37.5	37.5	42.8	50	62.5	37.5	50
D1	03,04	X				X		X						X			
D2	01,01			X		X				X		X		X			
D3	01,08	X		X		X			ND				ND				X
D4	03,04	X			X	X			X					X		X	X
D5	03,03	X				X						X	X				
D6	03,04						X	X		X		X		X			X
D7	04,07	X					X	X		X		X	X			X	
D8	03,13	X	X	X	X	X			X			X			X		
% positive		75	12.5	37.5	25	75	25	37.5	28.6	37.5	0	62.5	28.6	50	12.5	25	37.5
Total positive (%)		56.3	31.3	37.5	18.8	50	18.8	37.5	21.4	37.5	12.5	43.8	28.6	50	25	25	37.5

[a] C1–8, Control subjects; D1–8, patients with type 1 DM.
[b] X, Positive response to CVB4; ND, not done.

CVB4 induces clonal expansions in Vβ families that show CD69 up-regulation

Expression of CD69 is a marker of early T cell activation, typically seen within 6 h of stimulation, and as such it is frequently used for analysis of Ag-specific T cell activation. However, its expression gives limited information on the functional fate of the activated cell. To examine whether patterns of TCR Vβ-specific T cell proliferation taking place over several days mirror those seen using CD69 analysis, we used heteroduplex analysis of TCR Vβ expression. This approach is able to identify clonal and oligoclonal T cell expansion within a specific Vβ family after antigenic stimulation (28–30).

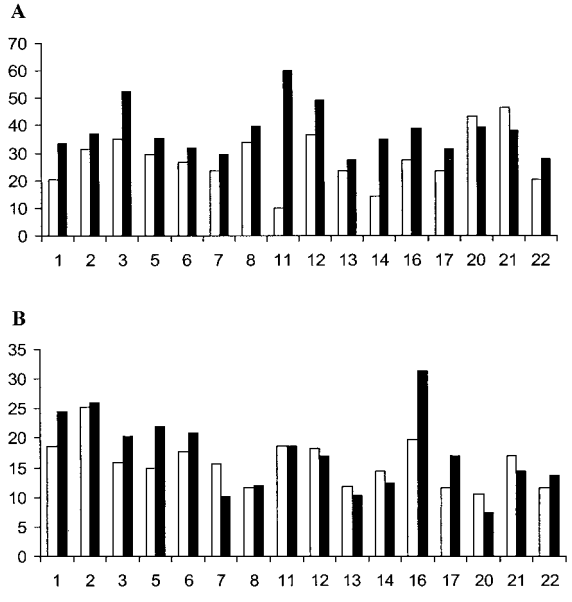

FIGURE 2. Comparison of CD69 up-regulation on CD4 T cells induced with CVB4 Ags and influenza Ags. PBMCs from a control donor were stimulated with influenza (*A*) or CVB4 Ags (*B*) and Vβ-specific CD69 up-regulation was analyzed. Data shown are the number of cells expressing a specific Vβ chain that up-regulates the early activation marker CD69 in response to viral Ags compared with control lysates (i.e., quadrant 2/quadrants 2 + 4 in Fig. 1*C*). A different pattern of Vβ-specific CD69 up-regulation is seen for the two different viral Ags. □, Control, uninfected lysates; ■, virus-infected lysates.

After 6 days in culture with either uninfected or CVB4-infected lysates, cDNA prepared from PBMCs was analyzed using this technique. One limitation of an RT-PCR based TCR detection technique is that for $CD4^+$ cells, in vitro culture is often required to expand clones to a size that is readily detectable. As we and others have previously described (26, 31), T cell proliferative responses to CVB4 are typically CD4 T cell dominated and only detectable at low levels in healthy individuals and type 1 DM patients. Proliferation assays performed on PBMCs from subjects in the present study showed a similar pattern of low reactivity. For this reason, sufficient cDNA for heteroduplex analysis of TCR Vβ usage was available on only a limited number of subjects.

Healthy donor 7 shows CD69 up-regulation of CD4 T cells bearing Vβ chains 1, 2, 5, and 6 (Table I), but not Vβ7 or 8 cells. As shown in Fig. 4*A*, numerous CVB4-specific CD4 T cell clones are generated in this subject after 6 days of in vitro stimulation with CVB4. After CVB4 Ag stimulation, the highest frequency of novel clones is seen among T cells bearing TCR Vβ1, 5.1, 5.2, and 6, mirroring those Vβ families up-regulating CD69 in this individual. Similarly, Vβ families not induced to express CD69 (Vβ chains 7 and 8) are associated with fewer novel CVB4-specific clones.

Fig. 4*B* shows similar results for two patients with type 1 DM. Subject D7 shows CD69 up-regulation among CD4 T cells with TCR Vβ5 and 8 chains (Table I). Heteroduplex analysis showed that, although there are fewer novel clonal bands after virus stimulation compared with the healthy donor, there is a correlation between CD69 up-regulation and the appearance of CVB4-specific clonal bands associated with the Vβ8 and 5 families (Fig. 4*B*). Fainter bands could also be seen sporadically in TCR Vβ chains that do not show CD69 up-regulation, probably due to a lower level of activation of some Vβ-bearing T cells by CVB4 proteins or bystander activation (Fig. 4*B*). A similar picture emerges from

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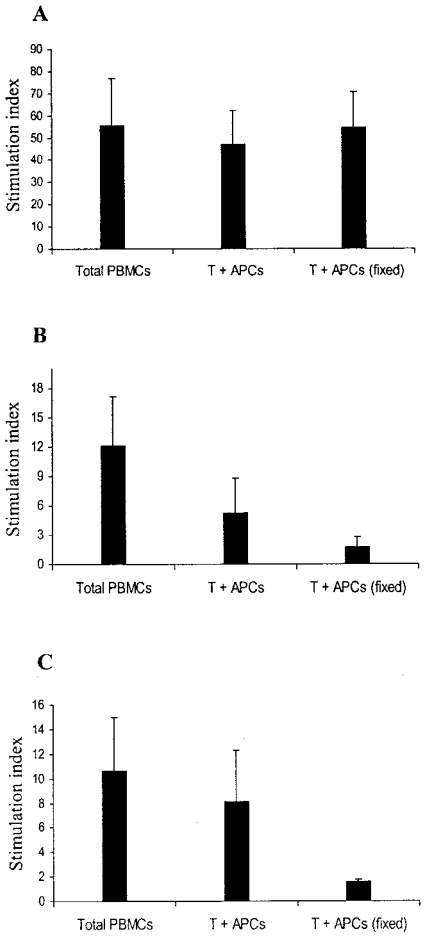

FIGURE 3. Effect of APC fixation on T cell activation by superantigen, recall Ag, and CVB4. Unfractionated total PBMCs, $CD3^+$ T cells cultured with unmanipulated $CD19^+$ B cells (T plus APCs) or $CD3^+$ T cells cultured with paraformaldehyde-fixed $CD19^+$ B cells (T plus APCs (fixed)) were incubated with SEB superantigen (*A*), TT recall Ag (*B*), and CVB4 cell lysates (*C*) and cell proliferation was measured by incorporation of $[^3H]$thymidine after 6 days. Proliferation results are expressed as SI as indicated in *Materials and Methods*. The results indicate that superantigens such as SEB do not require processing by live APCs to stimulate T cell proliferation, whereas recall Ag such as TT do. CVB4 Ags behave as TT, requiring processing by live APCs to achieve T cell activation. Bars represent the means of SIs from three separate experiments on three individuals (cases C6, C7, and C8, Table I). Error bars, +1 SD.

analysis of the second type 1 DM patient (D4). In this subject, CD69 up-regulation is detected in CD4 T cells with TCR Vβ chains 1 and 8 (Table I). Again, strong CVB4-specific T cell clonal bands are detected in association with the same TCR Vβ chains that up-regulate CD69 (Fig. 4*B*).

Analysis of the number of Ag-specific novel TCR clonal bands across the repertoire suggested that overall the response to CVB4 Ags involved multiple T cell clones. The pattern is typical of an oligoclonal, Ag-driven response and is quite distinct from heteroduplex analysis of TCR Vβ expression by T cells stimulated with a superantigen such as SEB or a mitogen such as PHA. Under these conditions of polyclonal stimulation, a smear is seen, representing many hundreds or thousands of distinct clones (32).

The correlation we observed between the analysis of Vβ-specific CD69 up-regulation and TCR Vβ expansion by heteroduplex analysis suggests that clones bearing selected TCR Vβ chains and showing early evidence of activation by CVB4 Ags proceed to expansion in vitro.

Discussion

In the present study, we have addressed the questions of whether there is preferential TCR Vβ chain usage by T cells specific for CVB4 and whether the CVB4 genome encodes a protein with superantigen activity. Our results indicate that the host response to CVB4 typically involves recruitment of CD4 T cells bearing numerous different Vβ chains. In particular, CVB4-activated T cells used Vβ chains 2, 7, and 8 in the majority of subjects. Analysis of CD69 up-regulation and use of the heteroduplex technique to analyze TCR Vβ usage associated with clonal CD4 T cell proliferation indicate that CVB4 induces both early activation and proliferation of clones bearing the same Vβ chain. The most important finding in this study, however, is that using a proliferation assay designed to assess the requirement for Ag processing in T cell activation, we demonstrate that CVB4 Ags only induce T cell proliferation after conventional Ag processing and presentation by APCs, rendering it unlikely that the CVB4 genome encodes a protein with superantigenic activity.

Our work was prompted by consideration of three separate studies published in recent years. First, in the study of Conrad et al. (16), an expansion of T cells bearing Vβ7 transcripts was noted in the islets of Langerhans of two children who died at diagnosis of type 1 DM. Subsequently, the same group reported modest but significant expansions of similar Vβ7 cells in the peripheral blood of patients with type 1 DM and those at risk of developing the disease (17). A possible link between these cells and Coxsackievirus was made in a report describing overrepresentation of selected Vβ transcripts, including Vβ7, in association with CVB3 in the diseased hearts of children with proven Coxsackie-mediated myocarditis (25). Our study was designed to test the hypothesis that CVB4 viral Ags induce Vβ-specific T cell activation and to examine whether this is achieved through a superantigenic effect.

After the initial report of Vβ skewing in the pancreas of type 1 DM patients at diagnosis (16), the effect of a superantigen was invoked as a possible explanation (33). Having demonstrated selective TCR Vβ activation in the current study, we examined whether such effects could be due to a CVB4-encoded superantigen. However, after treatment of $CD19^+$ B lymphocytes with paraformaldehyde, which abrogates Ag uptake and processing, these APCs could not sustain CVB4-induced T cell proliferation. These experiments argue against the existence of a superantigen effect operating on those Vβ chains we were able to study. However, there remains the possibility that during natural infection with CVB4, proteolytic processing of the viral polyprotein in infected cells generates polypeptide fragments with superantigenic effects. A similar dependence on proteolytic processing to display superantigenic activity has been demonstrated for the prototypic viral superantigen, the mouse mammary tumor virus superantigen (34). In this study, it was demonstrated that proteolytic processing of the viral superantigen 7 by furin was essential for the protein to display superantigenic activity. However, given the general ubiquity of intracellular expression of furin and other proteases, we consider it unlikely that lack of protease activity has masked a CVB4 superantigen. Our interpretation that presentation of CVB4 Ags by conventional means to T cells may induce preferential use of particular TCR Vβ chains is supported by previous reports, such as the preferential usage of T cells bearing TCR Vβ17 in recognition of the influenza A virus matrix peptide 57–68 (35).

Our results show that patients with type 1 DM exhibit a high frequency of Vβ-associated CD69 up-regulation after incubation with CVB4 Ags. In addition, we noted that some Vβ chains were more frequently up-regulated in new onset type 1 DM patients than control subjects. However, the number of cases we have studied to

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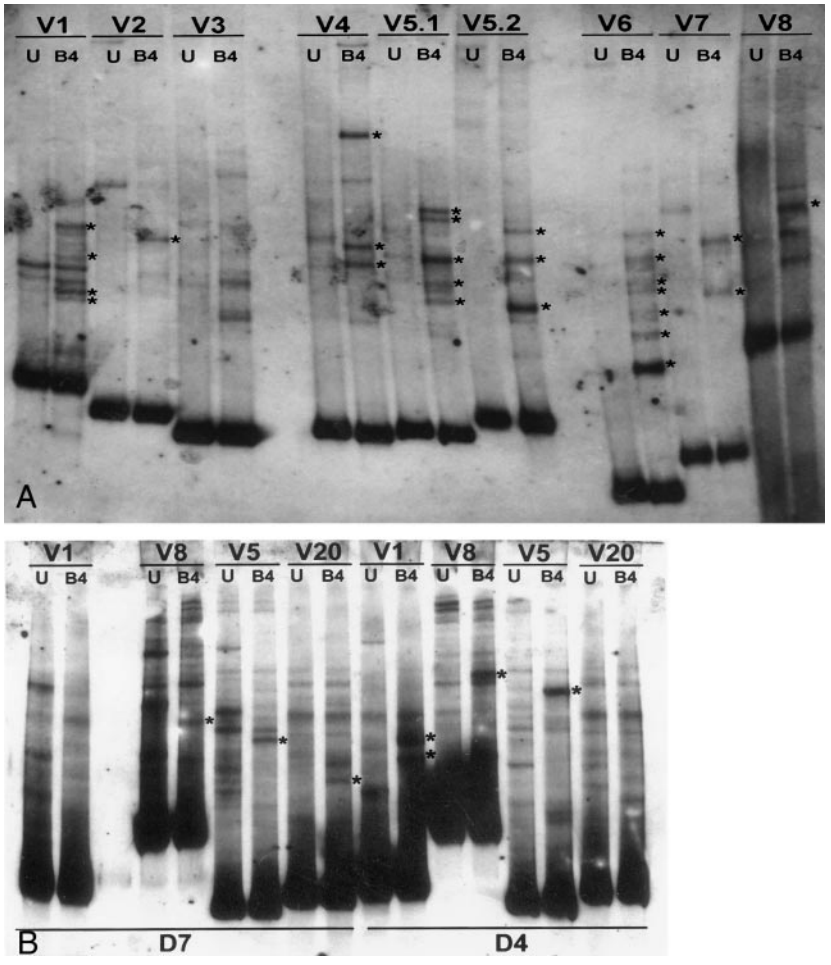

FIGURE 4. Heteroduplex analysis of TCR Vβ expression by T cell clones activated by CVB4 Ags. *A*, Healthy control subject C7. For each TCR Vβ shown, analyses represent cells stimulated with CVB4 lysates (B4) or control uninfected lysates (U). Bands generated from CVB4-specific T cell clones have no corresponding band generated by the control preparation and are labeled (*). Healthy donor C7 shows CVB4-induced CD69 up-regulation by CD4 T cells bearing Vβ chains 1, 2, 5, and 6 (Table I), but not Vβ7 or 8 cells. As shown, a higher frequency of clones is seen among T cells bearing TCR Vβ1, 5.1, 5.2, and 6, mirroring those Vβ families up-regulating CD69 in this individual. Similarly, Vβ families not induced to express CD69 (Vβ chains 7 and 8) are associated with proliferation of fewer novel CVB4-specific clones. *B*, Type 1 DM patients D7 and D4. Subject D7 shows CD69 up-regulation among CD4 T cells with TCR Vβ5 and 8 chains (Table I). Heteroduplex analysis shows the appearance of CVB4-specific clonal bands associated with the Vβ8 and 5 families. Similarly, patient D4, who showed CD69 up-regulation in association with TCR Vβ chains 1 and 8 (Table I), shows CVB4-specific T cell clonal bands in association with the same TCR Vβ chains.

date is small, and this precludes us from demonstrating with confidence whether patients with type 1 DM and healthy controls differ in the frequency and selectivity of Vβ-associated CD69 up-regulation. We found that among CD4 T cells, those having the TCR Vβ2, 7, and 8 chains showed the highest frequency of CD69 up-regulation by CVB4. In the case of Vβ8, all newly diagnosed type 1 DM patients show activation of CD4 T cells bearing this TCR Vβ, and this frequency is significantly higher than in healthy control subjects. Although these analyses were performed on small numbers of subjects and require confirmation in larger cohorts, two preliminary conclusions can be drawn. First, our data suggest that the CD4 T cell response to CVB4 is dominated by the Vβ8 family of T cells. Second, the higher frequency of CVB4-induced Vβ8 CD4 T cell activation seen in patients with type 1 DM could reflect a higher frequency of primed T cells in the peripheral blood at the time of diagnosis, consistent with a recent exposure to the virus. There is a large body of work examining serological responses to enteroviruses in association with type 1 DM, and numerous studies are consistent with there being a higher prevalence of enterovirus infections near to diagnosis. Epidemiological studies and case reports also support a role for enteroviruses in the disease pathogenesis (4–10). However, very few studies have examined T cell responses to the virus.

Preferential activation of CD4 T cells bearing selected TCR Vβ chains is of potential importance in the light of postmortem studies in which TCR Vβ usage has been analyzed in the pancreas of patients with new-onset type 1 DM. In particular, Hanninen et al. (36) showed that T cells having TCR Vβ8 chains were the most highly represented Vβ family (8–14 cells per islet) in the islet infiltrate of a pancreas obtained at diagnosis. In a similar study by Somoza et al. (37), only three distinct Vβ chains were clearly detectable by RT-PCR in the intrapancreatic lymphocyte preparation, of which one was Vβ8. These studies supporting a role for Vβ8-expressing T cells in intraislet pathogenic events in type 1 DM are balanced by postmortem studies in which Vβ7 T cells were overrepresented (16), and analysis of pancreatic biopsy specimens in Japanese type 1 DM patients in which no dominant Vβ usage could be seen (38). These differences could be due to disease heterogeneity or differences in methodology. The tentative hypothesis that the CVB4-induced Vβ8 expansion we observe reflects intrapancreatic events such as infection with pancreas-tropic enteroviruses will therefore require further experimental confirmation.

Other studies have examined Vβ skewing of T cells in the peripheral blood, although the approaches used are not strictly comparable to the current work. TCR Vβ7 chain expansion was found in a pilot study of type 1 DM patients and first-degree relatives at risk of the disease, and a temporal relationship between this observation and enterovirus infection was suggested by the data (17). Our results show that CD4 T cells with Vβ7 chains are among the three Vβ T cell families most frequently activated by CVB4 in vitro.

In conclusion, our data show that CVB4 induces a skewed activation of CD4 T cells, but only following processing and presentation of viral Ags by APCs. This suggests that CVB4 is not capable of interacting with T cells via superantigen effects. Additional studies will be required to extend our preliminary finding that Vβ selection by CVB4 may be more frequent in patients with type 1 DM. The fact that some of the Vβ chains utilized by the

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virus have been detected in infiltrated islets of Langerhans supports the proposal that enteroviruses such as CVB4 may have a role in type 1 DM pathogenesis.

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