Elicitation of T-cell responses by structural and non-structural proteins of coxsackievirus B4

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Coxsackievirus B4 (CV-B4) belongs to the genus Enterovirus within the family Picornaviridae. To investigate target proteins recognized by T-cells in human enterovirus B infections, virus-encoded structural [VP0 (VP4 and VP2), VP1, VP3] and non-structural (2A, 2B, 2C, 3C and 3D) proteins were expressed and purified in Escherichia coli. Peripheral blood of 19 healthy adult donors was used to create enterovirus-specific T-cell lines by repeated stimulation with CV-B4 cell lysate antigen. T-cell lines responded in individual patterns, and responses to all purified proteins were observed. The most often recognized enteroviral protein was VP0, which is the fusion between the most conserved structural proteins, VP4 and VP2. T-cell responses to VP0 were detected in 15 of the 19 (79 %) donor lines. Non-structural 2C protein was recognized in 11 of the 19 (58 %) lines, and 11 of the 19 (58 %) lines also had a response to 3D protein. Furthermore, responses to other non-structural proteins (2A, 2B and 3C) were also detected. T-cell responses did not correlate clearly to the individual HLA-DR-DQ phenotype or the history of past coxsackie B virus infections of the donors.

INTRODUCTION

Coxsackievirus B4 (CV-B4) is a human enterovirus, which are common pathogens. Currently, more than 100 human-infecting enterovirus types have been identified. Taxonomically, they are assigned to the genus Enterovirus, which is the most diverse genus of the family Picornaviridae (Adams et al., 2013). Enteroviruses are non-enveloped viruses with a ssRNA genome of positive polarity (Tuthill et al., 2010). The viral capsids are composed of 60 units of four structural proteins (VP1–VP4). VP1–3 proteins are located on the virus surface, whilst VP4 protein is in the interior. In infected cells, the viral genome is translated as a single, large polyprotein, which is cleaved proteolytically to individual viral proteins by the virus-encoded proteases 2A and 3C (Davies et al., 1991). All seven non-structural proteins (2A, 2B, 2C, 3A, 3B, 3C and 3D) possess different functions and are produced sequentially during co- and post-translational events (Tuthill et al., 2010).

Enteroviruses cause a range of diseases from subclinical infections to more severe disorders. These include poliomyelitis, myocarditis, conjunctivitis, and hand, foot and mouth disease (Grist et al., 1978; Tuthill et al., 2010; Whitton et al., 2005). Enteroviruses are also the most common cause of aseptic meningitis with a viral aetiology (Rotbart, 1995). Although common agents of acute infections, enteroviruses, and particularly coxsackievirus B types, may also be involved in the pathogenesis of type 1 diabetes (Craig et al., 2013). It has been suggested that enteroviruses either directly infect pancreatic islet β-cells leading to cell destruction (Colli et al., 2011; Ylipaasto et al., 2004) or trigger a T-cell immune response, which evokes pro-inflammatory mediators and the activation of the immune cells, which attack and destroy β-cells in pancreatic islets (Eizirik et al., 2009; Hämäläinen et al., 2014).

CV-B4 is one of the most-used model enteroviruses for T-cell activation studies (Yoon et al., 1979). The cellular immune responses to the non-structural 2C protein have largely been studied due to homology with the diabetes-associated auto-antigen glutamic acid decarboxylase 65 (GAD65), which has led to the concept of molecular mimicry between enteroviruses and human proteins as an explanation for the evolution of type 1 diabetes in childhood (Ellis et al., 2005; Varela-Calvino et al., 2000, 2004; Vreugdenhil et al., 1998). However, T-cell epitopes in the structural viral proteins (VPs) of CV-B4 have also been described. Conserved VP4 and VP2 proteins induce more responsiveness than the highly variable VP1, which is the most heterologous viral protein and is currently used for the typing of enteroviruses (Marttila et al., 2002; Oberste et al., 2003). Specific epitopes recognized by CD4+ T-cells have recently been identified in the 3D [viral RNA-dependent RNA polymerase (RdRp)] protein of human enterovirus 71 (Dang et al., 2014), whilst a previous study on coxsackievirus
B3 (CV-B3) recognized CD8+ T-cell epitopes in 3C and 3D proteins (Weinzierl et al., 2008) as well.

In the present study, the structural and non-structural proteins of CV-B4 were produced in Escherichia coli, and purified proteins were used to elicit T-cell responses in CV-B4-induced enterovirus-specific T-cell lines from healthy individuals in order to characterize the immunogenicity of the viral proteins. It was demonstrated that all structural and non-structural CV-B4 proteins elicited T-cell responses, but that these responses varied among individuals. In addition, and because of the importance of HLA class II molecules in the presentation of the protein antigens to the CD4+ T-cells, HLA-DR-DQ genotypes of all test subjects were also determined, but no clear correlation between either the T-cell responses to CV-B4 proteins and specific HLA class II haplotypes or the patient infection history was detected.

RESULTS

Establishment of specific T-cell lines

In order to test the potency of individual CV-B4 proteins to stimulate T-cell responses, specific T-cell lines were established from the peripheral blood of healthy adult donors by using CV-B4 lysate antigen as a trigger. Lysate virus antigen was generated by infecting LLC-MK2 cells (ATCC) with CV-B4 at an m.o.i. of 2, and was collected at the high peak of virus replication (based on the early-stage cytopathic effect). By using four to five cycles of stimulation with a mixture of CV-B4 lysate antigen, irradiated autologous antigen-presenting cells and IL-2, T-cell lines lost their responsiveness towards tuberculin purified protein derivative (PPD) and tetanus toxoid (TT) (which as a rule stimulate peripheral blood lymphocytes). Instead, they acquired and maintained a specific proliferative response to CV-B4 lysate antigen. Fig. 1 demonstrates the development of the antigen specificity in two representative T-cell lines (subject nos 4 and 10) that were generated and used in the study. PBMCs collected from both subjects responded to CV-B4-infected cell lysate, purified CV-B4 virus, PPD and TT when comparing the c.p.m. with the background cultures with medium alone. During the restimulations over a 5-week period the PPD- and TT-specific responses disappeared, and cells responding to CV-B4 became prevalent within the cell population. The response was higher against purified CV-B4 than against CV-B4 lysate antigen, indicating that structural proteins may stimulate T-cells more effectively than other viral proteins (see below). However, this may also have been due to the dose–response or the presence of inhibitory factors in the cell lysate. In further assays, PBMCs from the other donors were stimulated in a similar manner.

Responses of CV-B4-induced enterovirus-specific T-cell lines to viral proteins

All CV-B4 proteins except 3A and 3B were expressed and purified in E. coli and used in cell stimulation studies (Table 1, Fig. 2). Enterovirus-specific T-cell lines were established from 19 healthy adult subjects and their proliferation responses to structural and non-structural proteins of CV-B4 as well as to TT and PPD control
antigens were studied (Table 2). The HLA-DR-DQ genotypes of the study subjects were also determined and correlated with the responses from the stimulation studies. T-cell responses to the CV-B4 proteins (VP0, VP1, VP3, 2A, 2B, 2C, 3C and 3D) were measured by using two concentrations, 0.1 and 1.0 \( \mu g \) ml \(^{-1}\). A strong correlation between responses was obtained with these two concentrations, with Spearman’s rank correlation coefficient (\( r_s \)) varying from 0.531 to 0.939 and \( P \) values from values less than 0.0001 to 0.0192. As a rule, stronger responses were obtained with the higher protein concentration, and these values are presented in Tables 2 and 3. All except one of the T-cell lines also responded to the purified virus, which is in accordance with the high frequency of responses to the structural proteins of CV-B4. Most T-cell lines (12 of 19) were responsive to two or all three of the structural proteins. In contrast, subject nos 5 and 18 had strong responses to one or two non-structural proteins but no responses to the structural proteins. All of the studied CV-B4 proteins were recognized by at least one of the generated T-cell lines, even though the number of donors was limited. In 15 of the 19 T-cell lines, a proliferative response to VP0 was detected, but 11 T-cell lines also showed a response to non-structural proteins 2C and 3D (Table 2). This indicated that T-cell responses are not specific to capsid protein(s) but occur commonly in response to any expressed CV-B4 protein.

**Correlation of HLA class II haplotype and patient infection history with CV-B4 protein-induced T-cell responses**

The HLA class II haplotypes of the study subjects were determined, and the strength of T-cell responses to CV-B4 proteins was compared between the study subjects with or without specific HLA-DR-DQ haplotypes using the Mann–Whitney \( U \) test. Haplotypes found in at least two study subjects \((n=9)\) were tested for responses to eight different viral proteins in a total of 72 assays. Subjects positive for HLA-(DR15)-DQB1*06 : 02 had higher responses to the 2A \((P=0.014)\) and 2B \((P=0.044)\) proteins compared with those without this haplotype, and the HLA-(DR3)-DQA1*05-DQB1*02 haplotype was associated with a higher response to the 3D protein \((P=0.016)\), but these differences did not remain significant when multiplied by the number of comparisons made. Serum antibody levels to CV-B1–6 were also determined by a microneutralization test (Table 2). Samples from 16 of the 19 study subjects contained neutralizing antibodies to at least one of the studied CV-B strains. No correlations were found between the number of CV-B serotypes recognized by individual sera and the responsiveness to any of the tested viral proteins when tested by Spearman’s rank correlation (\( r_s \)) varied from \(-0.146\) to 0.164 and \( P \) values from 0.873 to 0.503.

**Variation of T-cell responses against viral antigen**

In order to study the internal variation of stimulation with the same CV-B4 protein antigen, several T-cell lines were generated using cells from the same subject. Four and three independent T-cell lines were generated using cells from subject nos 1 and 11, respectively (Fig. 3). The T-cell lines from subject no 1 (Fig. 3a) had repeated responses in all
Table 2. Responses of CV-B4-induced enterovirus-specific T-cell lines established from the donors against medium, TT, PPD, CV-B4 and CV-B4 proteins

Results are shown as stimulation index (SI) value against the background (medium) values (c.p.m.).

<table>
<thead>
<tr>
<th>Subject</th>
<th>HLA-DR-DQ haplotype 1</th>
<th>HLA-DR-DQ haplotype 2</th>
<th>Medium (c.p.m.)</th>
<th>TT (SI)</th>
<th>PPD (SI)</th>
<th>CBV4 (SI)</th>
<th>pCBV4 (SI)</th>
<th>VP0 (SI)</th>
<th>VP1 (SI)</th>
<th>VP3 (SI)</th>
<th>2A (SI)</th>
<th>2B (SI)</th>
<th>2C (SI)</th>
<th>3C (SI)</th>
<th>3D (SI)</th>
<th>CV-B antibodies</th>
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<td>1.6</td>
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generated T-cell lines to proteins VP0, VP1 and 3D. T-cell responses above control values to 2C and 2B were seen in three generated T-cell lines. In the cell lines derived from subject no. 11 (Fig. 3b), strong responses to VP0, 2B and 2C were seen in the first T-cell line but not in the others. The variation may be due to the frequency of specific T-cells in the cell population that was used to generate the T-cell lines.

**Correlations between T-cell responses to various CV-B4 proteins**

The established T-cell lines had stronger responses to the purified CV-B4 particles (pCV-B4) than to CV-B4 lysate antigen (CV-B4) (Fig. 1). This may have been due to higher amounts of cellular components in the lysate antigen including inhibitory substances. T-cell responses to specific proteins showed distinct patterns (Table 3). Responses to non-structural proteins 2A and 2B were most strongly correlated \( r_s = 0.906, P < 0.0001 \), and correlations were also detected between the responses to both of these and the responses to 3C, which, in contrast, correlated with responses to 2C. The responses to the non-structural proteins thus tended to correlate with each other except for the responses to the 3D protein, which did not correlate significantly with any other protein responses. The responses to 2A and 2B also displayed a significant correlation with the responses to the structural proteins VP0 and VP3 of purified CV-B4. Furthermore, the responses to structural protein VP0, composed of VP2 and VP4 proteins, and VP3 correlated with each other, but the responses to VP1 did not correlate with any other proteins tested.

**DISCUSSION**

The concept of protective immunity in enterovirus infections has largely been based on the development of neutralizing virus-type-specific antibodies that are directed

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**Table 3. Correlations between the strength of the T-cell responses to various CV-B4 proteins**

Only \( P \) values showing statistically significant correlations are shown (* \( P < 0.05 \); ** \( P < 0.01 \); *** \( P < 0.001 \)). CV-B4 was used at 10 μg ml\(^{-1}\) and viral proteins at 1 μg ml\(^{-1}\).

<table>
<thead>
<tr>
<th>Protein</th>
<th>CV-B4</th>
<th>pCV-B4</th>
<th>VP0</th>
<th>VP1</th>
<th>VP3</th>
<th>2A</th>
<th>2B</th>
<th>2C</th>
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<th>3D</th>
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**Fig. 3.** Responses to control antigens and enterovirus proteins in T-cell lines established independently from two subjects at different time points. The results are shown for four (a; subject no. 1) and three (b; subject no. 11) different lines as stimulation indices (SI) against the background (medium) values (c.p.m.).
against viral capsid structures and that possibly prevent the binding of the virus via VP1 protein to the cell-surface receptors (Jun et al., 1997; Tauriainen et al., 2011). Most of the studies dealing with the cell-mediated immunity have therefore focused on the viral structural proteins, even though it is widely known that the surface of viral proteins in the interior of the capsid and non-structural viral proteins are potentially immunogenic at some stage of acute infection. It has been described that both CD4+ and CD8+ cells respond to the non-structural proteins during influenza and hepatitis C infections (Ferrari et al., 1994; Jameson et al., 1998; Kembel et al., 2008). In the case of CV-B4, epitopes in the structural and 2C proteins have previously been mapped using CD4+ T-cell lines (Marttila et al., 2001, 2002), whilst the HLA class I-restricted CD8+ epitopes have been mapped in the 3C and 3D proteins of CV-B3 (Weinzierl et al., 2008). The latter study identified four CD8+ epitopes, which were widely shared by different human type B enteroviruses.

The approach of the current study was based on the production and purification of CV-B4 proteins in bacterial cells and their use in T-cell proliferation assays. Whilst these proteins were estimated to reach a purity of 80–95% (Fig. 2 and data not shown), it can be argued that they may still contain bacterial contaminants (e.g. endotoxin, heat-shock proteins), which may affect cell proliferation and interfere with detection of the antigen-specific T-cell responses (Roep et al., 1999). However, in light of our findings, we believe that possible contaminants were not interfering with the interpretation of the results, as the enterovirus-specific T-cell lines generated from the same subject during the study responded differentially to different viral proteins, and because we obtained T-cell responses to all viral proteins used in the study but in different combinations. Thus, there was no clear pattern that could have arisen from contaminants in the viral protein preparations processed in a similar manner in a heterologous protein production system.

By using CV-B4-infected cell lysate antigen to generate specific T-cell lines from the peripheral blood samples of healthy adult volunteers, we ensured that all CV-B4 proteins expressed during viral infection in the cells were present to stimulate T-cells (Table 2). We found that enterovirus-specific T-cells targeted not only the capsid proteins VP1 and VP0 but also the non-structural proteins 2C and 3D, which are essential in virus replication. As 3D was recognized as often as 2C by T-cells, it may therefore possess novel dominant immunogenic epitope(s) as well as the 2C protein, which has been described previously. In addition, all the non-structural proteins were also immunogenic, indicating that most if not all of the viral proteins are capable of inducing T-cell-mediated immune responses. To demonstrate the consistency of the T-cell responses, we also generated several individual T-cell lines from two study subjects (Fig. 2). The fact that their responses to the tested proteins were not always repeatable may have been due to the low precursor frequency of specific T-cells and the associated variability in the T-cell repertoire selected against the relatively small cell population, which was used to generate the T-cell lines. This is to some extent supported by the observation that the pattern of responsiveness was best repeated in the T-cell lines with high proliferation responses to the viral lysate antigen used in the induction (data not shown). As enterovirus infections are very common, it is also possible (although unlikely) that the infection history of the study subjects had changed during the study period and affected the pattern of responsiveness.

The determined class II HLA-DR and HLA-DQ genotypes of the donors did not show any clear associations with the T-cell responses to CV-B4 proteins, as the responsiveness to any of the proteins was not limited to the subjects possessing a unique haplotype. This suggests that each viral protein possesses several epitopes, which are recognized in the context of various HLA alleles. Because of the small number of subjects in the panel, it has to be noted that one would expect only very strong associations to be detected. We did not detect any significant association between the infection history and the responsiveness to the CV-B4 proteins. The three subjects without any CV-B group antibodies also had strong responses to several individual proteins, demonstrating a large cross-reactivity of these proteins within the enterovirus group. This is also in accordance with a recent study showing cross-reactivity against coxsackievirus A16 and poliovirus in VP2-dominated CD4+ T-cell responses against enterovirus 71 and poliovirus (Tan et al., 2013). However, two of these subjects did not respond to VP1 protein and one had only a low response, which emphasizes the fact that there is high structural variation in VP1 among picornavirus types.

Strong correlations were detected, in particular in responses between non-structural proteins 2A and 2B, which also correlated with responses to 2C and 3C as well as to structural proteins VP0 and VP3. All these proteins possess relatively small sequence variation among the virus types in the species Human enterovirus B, but the number of CV-B infections experienced as detected by neutralization tests did not correlate with the strength of the responsiveness to these proteins. The independence of the responses to 3D and lack of correlation with the other responses was conspicuous, as was the lack of correlations of the responsiveness to the VP1 protein, which can be explained by it being the most variable enteroviral protein. Its variability is most probably caused by its location on the outer surface of the virus particle by which it is exposed to the immunological pressure of the host, and also by possession of epitopes recognized by neutralizing antibodies. The present results relate to our previous results with synthetic peptides where fewer T-cell epitopes were identified in VP1 than in other capsid proteins (Marttila et al., 2002). Many factors including similarities between viral proteins, HLA restrictions of the dominant peptides and protein expression levels within cell during infections can contribute to the correlations or lack of them.
Although there are several open questions regarding the origin of the T-cell responses, our data suggest that target epitopes of cellular immunity exist in all studied enterovirus proteins.

Overall, these data demonstrate the wide recognition of various enteroviral proteins by infection-induced T-cells and also that there is wide individual variation in the targets that give rise to these responses, which does not seem to have a clear association with HLA-DR/DQ specificity or CV-B infection history as detected by neutralizing antibodies.

METHODS

**Plasmid constructs.** Sequences coding for VP0 (nt 950–1732, aa 70–330), VP1 (nt 2447–3298, aa 569–853), VP3 (nt 1733–2446, aa 331–568), 2A (nt 3299–3739, aa 853–999), 2B (nt 3740–4036, aa 1000–1098), 3C (nt 5357–5905, aa 1538–1721), 3D (nt 5906–7292, aa 1722–2183) were amplified by PCR using the reference JVB strain CV-B4 (GenBank accession no. X05690; Jenkins et al., 1987) cDNA as the template. Overlapping primers were designed to amplify non-linear peptide regions between the adjacent proteins that reside in the polyprotein but not in the individual proteins. This was done to ensure that possible immunogenicity and responses to these regions within the polyprotein were detected. PCR fragments of the expected sizes were excised from the agarose gels, eluted with a QIAquick Gel Extraction kit (Qiagen) and subcloned in frame with pet15b vector (Merck Chemicals) to produce N-terminally His-tagged fusion proteins. CV-B4 2C protein was produced as a glutathione S-transferase fusion in T7 Express cells (New England Biolabs) as described previously (Härkönen et al., 1997). Expression of the proteins 3A and 3B as a 3AB fusion protein was not successful. The primer pairs are listed in Table 1.

**Expression and purification of recombinant CV-B4 proteins.** The recombinant plasmids and control pet-15b vector were transformed into E. coli T7-expressing cells (New England Biolabs). The transformed bacteria were grown to an OD of 0.6 in Luria-Bertani medium supplemented with ampicillin (100 μg ml⁻¹), and protein expression was induced with 1 mM IPTG. After induction for 3 h at 37 °C, the cells were collected and stored at −20 °C until purification. The bacterial pellets were resuspended in BugBuster reagent (Novagen) and incubated at room temperature for 20 min or until cell lysis was evident. The lysate was centrifuged (10 000 g, 15 min) using an Eppendorf 5430 (Eppendorf AG) and supernatant was removed. The pellet was washed three times in TBS buffer [20 mM Tris/HCl (pH 8), 150 mM NaCl], supplemented with 1% Triton X-100 and protease inhibitors (Complete MINI; Roche) and once in TBS alone. The sample was centrifuged (10 000 g, 5 min) between washes. The pellet was suspended in 500 μl TBS supplemented with 8 M urea and 10 mM β-mercaptoethanol. The urea was removed by stepwise dialysis against 2 M urea in TBS and TBS, respectively. Proteins were quantified using the Bradford method (Pierce).

**SDS-PAGE and Western blotting.** Proteins were separated by SDS-PAGE under reducing conditions (Laemmli, 1970) and stained with Coomassie blue. For Western blot analysis, the separated proteins were transferred to nitrocellulose membrane (Hybond-C; Amersham). After blocking with 4% (w/v) non-fat milk in PBS, the blots were probed by incubation with His-specific antiserum (H-129; Sigma), and bound antibodies were visualized with HRP-conjugated anti-rabbit antibody (Santa Cruz), followed by peroxidase substrate.

**Generation of T-cell lines.** PBMCs were isolated by Ficoll density centrifugation and incubated at a cell density of 2 × 10⁶–3 × 10⁶ ml⁻¹. Isolated cells were incubated with CV-B4 virus (strain JVB) antigen lysate (10 μg ml⁻¹) in RPMI 1640 supplemented with 10% human serum, 10 μg gentamicin sulphate ml⁻¹, 10 mM HEPES buffer and 3% glucose (10 μl ml⁻¹). Preparation of the lysate antigen has been described previously (Marttila et al., 2002). The lysate virus was inactivated by incubation at 56 °C for 30 min. After 7 days, the cell medium was replaced with fresh medium supplemented with 0.5 ng recombinant human IL-2 (R&D Systems) ml⁻¹, and the medium replacement was repeated every third day. After 14 days of incubation, the T-cells were restimulated both with the CV-B4 virus antigen and with γ-irradiated (30 Gy) autologous antigen-presentation cells (PBMCs; 2 × 10⁶–3 × 10⁶ cells ml⁻¹). Continuous T-cell lines were restimulated at 7-day intervals and further expanded with IL-2 2 days after antigen stimulation and at 2/3-day intervals thereafter. All T-cell lines had at least four cycles of stimulation with the CV-B4 virus antigen, γ-irradiated autologous antigen-presenting cells and IL-2. The cells were tested for antigen specificity in a lymphocyte proliferation assay.

**Lymphocyte proliferation assay.** The antigen specificity of the T-cell lines was tested by incubating 10⁵ T-cells with 2 × 10⁵–3 × 10⁵ γ-irradiated autologous PBMCs in 200 μl culture medium in 96-well tissue culture plates (Nunclon 96-well Microtest plate). The cells were incubated in triplicate with the medium alone or with TT, PPD, structural proteins VP0, VP1, VP3 and non-structural proteins 2A, 2B, 2C, 3D at 0.1 and 1 μg ml⁻¹. After 1 day of incubation, 2 μCi [³H]thymidine ml⁻¹ (Amersham) (1 Ci = 3.7 × 10¹² Bq) was added in 25 μl medium and incubation was continued for 16–18 h. Cultures were harvested onto glass fibre filters and [³H]thymidine incorporation was measured using a Microbeta Plus Liquid Scintillation Counter (Perkin-Elmer). The results of the lymphocyte proliferation were expressed as stimulation index (SI) values, defined as c.p.m. in the presence of the antigen divided by c.p.m. in medium alone. An SI value of ≥2 is considered positive by convention.

**PBMC proliferation assay.** At the time point when the T-cell lines were initiated, the T-cell responses to control antigens TT, PPD, CV-B4 and purified CV-B4 were measured by using a 6-day PBMC proliferation assay. PBMCs (10⁵ cells) were incubated with antigen in 200 μl culture medium in 96-well tissue culture plates (Nunclon 96-well Microtest plate). After 5 days of incubation, 2 μCi [³H]thymidine ml⁻¹ was added in 25 μl medium, and incubation was continued for 16–18 h. Cultures were harvested onto glass fibre filters and [³H]thymidine incorporation measured using a Microbeta Plus Liquid Scintillation Counter.

**Determination of antibody levels in human sera by micro-neutralization.** The serum antibody levels to coxsackievirus B1–6 (prototype strains; ATCC) were determined by a microneutralization test. Threefold dilutions starting from 1:10 were prepared in M199 medium containing 1% FCS. Diluted antisera were incubated with 100 p.f.u. CV-B1–6 for 60 min at 37 °C and added to confluent monolayers of 50 000 LLC-MK2 cells on 96-well microtitre plates (Nunclon 96-well Microtest plate). The plates were incubated for 6 days, and the virus growth was observed daily starting from day 3 until day 6 before staining with crystal violet. Viral titres were determined as the highest dilution of the serum samples capable of neutralizing the virus.

**HLA genotyping.** Typing for HLA-DR-DQ haplotypes was carried out using a lanthanide-labelled oligonucleotide hybridization method as described previously (Hermann et al., 2003).

**Statistical analysis.** Spearman correlations were used to explore the associations between the T-cell responses. Dichotomized responses...
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