Virology **293,** 217–224 (2002)
doi:10.1006/viro.2001.1259, available online at http://www.idealibrary.com on IDEAL®

w metadata, citation and similar papers at core.ac.uk
brought to you by CO
provided by Elsevier - Publisher Conne

T Cell Epitopes in Coxsackievirus B4 Structural Proteins Concentrate in Regions Conserved between Enteroviruses

Jane Marttila,*,†,[1] Heikki Hyöty,*,‡ Pekka Vilja,‡ Taina Härkönen,§ Annu Alho,¶ Merja Roivainen,§ Timo Hyypiä,¶ and Jorma Ilonen*,†

**JDRF Center for Prevention of Type 1 Diabetes in Finland; †Department of Virology, University of Turku, Turku, Finland; ‡Department of Virology, University of Tampere Medical School and Tampere University Hospital, Tampere, Finland; §National Public Health Institute, Helsinki, Finland; and ¶Department of Virology, Haartman Institute, University of Helsinki, Helsinki, Finland*

Received June 27, 2001; returned to author for revision August 10, 2001; accepted October 9, 2001

The present study aimed to characterize systematically the target epitopes of T cell responses in CBV4 structural proteins. These were studied by synthesizing 86 overlapping 20-aa-long peptides covering the known sequence of CBV4 structural proteins and analyzing the proliferation responses of 18 CBV4-specific T cell lines against these peptides. Recognized peptides differed depending on the HLA-DR genotype of the T cell donor. They were concentrated to the VP4 and VP2 regions as six of seven common peptide epitopes located in this region, whereas there was only one in the VP3 region and none in the VP1 region. Peptides from conserved areas were recognized more often (on average, 15% of them stimulated each T cell line) than those derived from variable areas (3%) ($P < 0.0001$, Fisher's exact test). Some conserved peptides inducing T cell responsiveness in most subjects were identified, a knowledge which can be useful in the development of new synthetic vaccines. © 2002 Elsevier Science (USA)

Key Words: coxsackievirus B4 (CBV4); enterovirus; T cell epitope; HLA; motif; cell-mediated immunity; T cell cross-reactivity.

INTRODUCTION

Enteroviruses are small, nonenveloped RNA viruses belonging to the family of Picornaviridae. The enterovirus genome is a single, positive-strand RNA molecule of about 7500 nt. The P1 region codes for the four structural proteins, which form the virus capsid: the major capsid proteins VP1–3 are on the virion surface, while VP4 is internal. VP4 and VP2 are synthesized as a precursor protein VP0, which undergoes a maturation cleavage late in the capsid assembly. The P2 and P3 regions encode seven nonstructural proteins 2A, 2B, 2C, 3A, 3B, 3C, and 3D, which are responsible for functions needed in replication (Rueckert, 1996). Comparison of enterovirus genomes in the capsid-coding region has indicated the presence of four genetic clusters among human enteroviruses. Cluster A contains 11 coxsackievirus A serotypes (CAVs) and enterovirus 71. Cluster B contains all coxsackievirus B serotypes (CBVs), CAV9 serotype, and echoviruses; cluster C contains polioviruses and 11 CAV serotypes, and cluster D contains enteroviruses 68 and 70 (Hyypiä *et al.,* 1997; Oberste *et al.,* 1999; Pöyry *et al.,* 1996). Highest similarity between enterovirus strains is seen within the clusters and for example within cluster B the serotypes share at least 96% of amino acid (aa) in their VP4 protein. Most variable is the VP1 protein (at least 78% similarity) and VP2 and VP3, sharing at least 85 and 83% of aa, respectively. The nonstructural proteins show considerably less variation than structural proteins (Pöyry *et al.,* 1996).

Enteroviruses are common human pathogens. Most infections are subclinical or accompanied by mild common cold-type symptoms, but several distinct complications occur, such as meningitis, myocarditis, and sepsis-like infections in newborns (Melnick, 1996). Increasing evidence also implicates enteroviruses among environmental triggers in the pathogenesis of type 1 diabetes (Hyöty *et al.,* 1998). Immunity against enterovirus infections is created by the presence of neutralizing antibodies, which by definition are serotype specific. Localization of neutralizing antigenic sites of polioviruses has been well characterized by using antigenic variants and monoclonal antibodies. The major neutralizing antigenic sites are located in the VP1 protein and, to a lesser extent, in the VP2 and VP3 polypeptides (Hogle *et al.,* 1985; Mateu, 1995; Minor *et al.,* 1986). The significance of cell-mediated immunity in enterovirus infections has not been extensively analyzed but it may be involved in the development of disease-associated complications (Beck *et al.,* 1990; Henke *et al.,* 1995). Cell-mediated immunity directed against enteroviruses is largely cross-reactive

[1] To whom correspondence and reprint requests should be addressed at Department of Virology, University of Turku, Kiinamyllynkatu 13, FIN-20520 Turku, Finland. Fax: +358 2 251 3303. E-mail: jane.marttila@utu.fi.

0042-6822/02 $35.00
© 2002 Elsevier Science (USA)
All rights reserved.

as enterovirus antigens have been recognized by T cells established from donor without neutralizing antibodies to the tested enterovirus serotype (Beck and Tracy, 1990; Cello *et al.*, 1996) and T cell lines specific to different enteroviruses have also been recognizing widely other enterovirus antigens (Graham *et al.*, 1993).

In this study we systematically mapped T cell epitopes from CBV4 structural proteins. This was made by analyzing the responsiveness of 18 CBV4-specific T cell lines against a panel of eighty-six 20-aa-long synthetic peptides covering the sequence of CBV4 structural proteins. We demonstrated that the exact peptide epitopes recognized by T cells were defined by the HLA genotype of the donor and were able show that these epitopes were concentrated to the conserved areas of the structural proteins.

RESULTS

CBV4-specific T cell response to synthetic peptides and *in vitro* produced proteins

Eighteen coxsackievirus B4 (CBV4)-specific T cell lines were established from 18 donors and their responses to 86 synthetic peptides were analyzed. These 20-aa-long peptides overlapped with each other by 10 aa and covered the whole sequence of the CBV4 structural proteins VP1, VP2, VP3, and VP4. In the first phase the epitopes were mapped by using 17 pools of five to six peptides and dissecting the responses by including single peptides of stimulating pools in the second test (Fig. 1). Recognized peptides differed markedly between subjects and depended on the HLA genotype of the donor (Table 1). T cell lines established from each donor recognized up to 15 different peptides from CBV4 structural proteins. Nine T cell lines of 15 were stimulated by CBV3-VP1 protein and six of them also recognized synthetic peptide(s) and/or peptide pool(s) from VP1 region. The other six T cell lines, which did not respond to CBV3-VP1 protein, did not respond to any of the synthetic peptides covering the CBV4 VP1 sequence ($P = 0.028$, Fisher's exact test). All 17 tested T cell lines had response to CBV4-VP0 protein and also recognized peptides covering the sequence of VP0 (including VP4 and VP2 proteins).

Phenotype of the CBV4-specific T cell lines

The CBV4-specific T cell lines contained mixed phenotype of $CD4^+$ and $CD8^+$ T cells. An average of 42% of all $CD3^+$ T cells contained the CD4 molecule and 27% contained the CD8 molecule when the phenotype of the CBV4-specific T cell lines was analyzed by flow cytometry (Facscan, Becton–Dickinson) using FITC- or phycoerythrin-conjugated mAb (Coulter) to the CD4 and CD8 T cell surface markers. After depletion of CD8-positive T cells, one randomly selected CBV4-specific T cell line was further cultured and the pattern of recognized peptides was analyzed. This new T cell line contained only 1% $CD8^+$ T cells and recognized all the same peptides as the original one (containing 10% $CD8^+$ T cells) (data not shown).

Effects of HLA genotypes of the donor: Common T cell epitopes and HLA-DR motifs

Variability in HLA molecule defines which type of peptides are bound and presented to T cells. Some rules of amino acids in key or anchor positions have been formulated and patterns of amino acids demanded are called motifs (Rammensee *et al.*, 1995). Peptide 27 (LRTNNSATIVMPYINSVPMD) from VP2 protein stimulated T cells most often, as it was recognized by altogether nine T cell lines (Table 1). It mainly stimulated T cell lines established from donors carrying HLA-DR1 and/or -DR2 molecules but also two T cell lines established from donors with HLA-DR4 or HLA-DR9 recognized it. The overlapping area between peptides 26 and 27 contained sequence **L**RT**N**N**S**ATI and the middle of peptide 27 contained sequence **A**TIV**M**PYI**N**, which both exactly fit the DR1 motif (actual motif is shown in bold) (Rammensee *et al.*, 1995). Most DR1-positive lines stimulated by peptide 27 also recognized peptide 26. Peptide 28 was recognized by five T cell lines and three of them were established from donors with HLA-DR2 molecule and also recognized peptide 27. No complete DR2 motif was found either in peptide 27 or 28 but in the overlapping area there is motif MPY**I**NS**V** with some DR2 anchor positions (Rammensee *et al.*, 1995).

Both peptides 3 and 9 were recognized by seven T cell lines and peptide 4 was recognized by six T cell lines. Peptide 3 (GNSIIHYTNINYYKDAASNS) was recognized with T cell lines established from donors with HLA-DR1 and/or -DR2 and/or -DR3 molecule. It contains HLA-DR2 motif **I**IH**Y**TN**I** and partially HLA-DR3 motif **I**IHYT**N**IN**Y** but none of the HLA-DR1 motif (Rammensee *et al.*, 1995). Peptide 4 (NYYKDAASNSANRQDFTQDP) was recognized by T cell lines established from donors sharing HLA-DR1 and/or -DR2 molecule or HLA-DR9 molecule. It contains HLA-DR1 motif **Y**KD**AA**S**N**SA but none of HLA-DR2 motif (Rammensee *et al.*, 1995). Peptide 9 (RVRSITLGNSTITTQECANV) did not contain any clear HLA-DR1 or HLA-DR2 motifs but it is likely that DR1 epitope was located in the overlapping region with peptide 8 because T cell lines established from donors sharing HLA-DR1 molecule also had response with peptide 8. In the same manner T cell lines established from donors with HLA-DR2 molecule also had a sometimes response to peptide 10 and so the HLA-DR2 epitope probably located in the overlapping region with peptide 10.

Peptide 38 (RNLMEIAEVDSVVPINNLKA) was recognized by five T cell lines, four of them established from

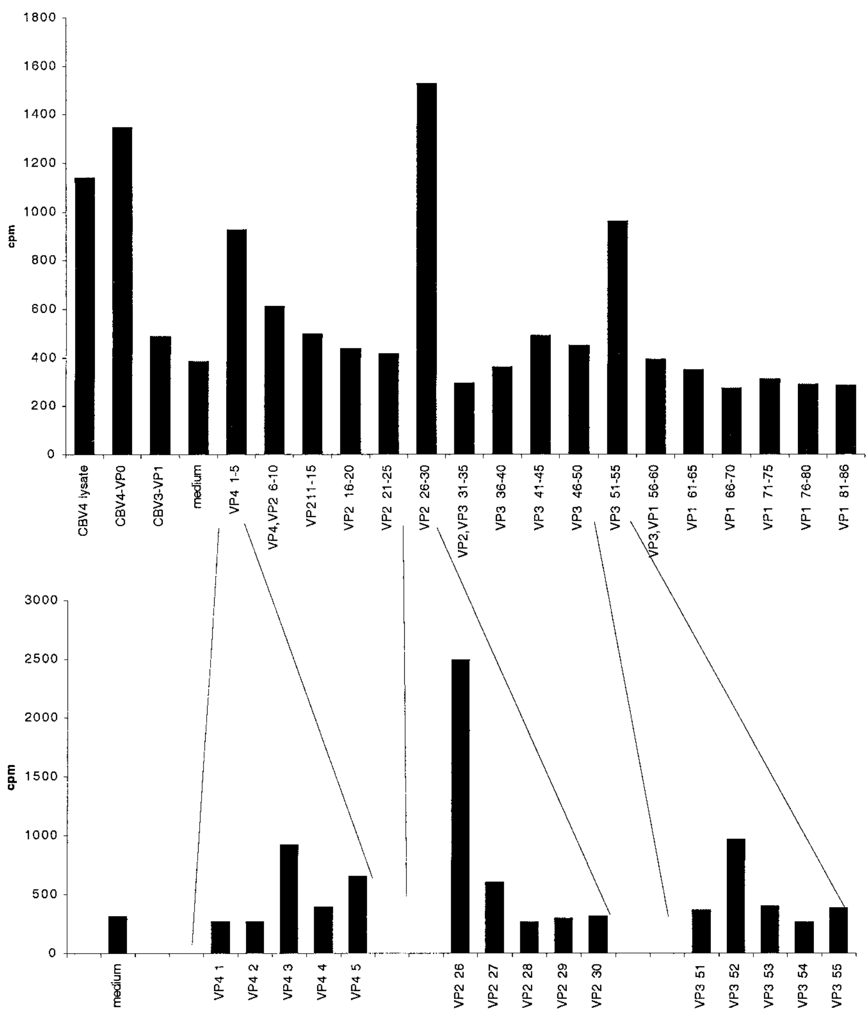

FIG. 1. The epitopes detected by coxsackievirus B4 (CBV4 lysate)-specific T cell lines were mapped by using 17 pools of five to six peptides in the first phase (upper figures) and dissecting the responses by including single peptides of stimulating pools in the second test (lower figures). Medium, culture medium without the antigen; CBV4-VP0, GST protein including CBV4 VP4 and VP2 proteins; CBV3-VP1, GST protein including CBV3 VP1 protein.

TABLE 1

Positive Peptide Responses (SI > 2.0) of 18 CBV4-Specific Human T Cell Lines to 20-aa-long Peptides with 10 aa Overlaps Covering CBV4 Structural Proteins

Peptide	Protein	Amino acids	Sequence	HLA-DR types of the donors of the responding T-cell lines
1	VP4	1–20	MGAQVSTQKTGAHETSLSAS	DR2,4
3	VP4	21–40	GNSIIHYTNINYYKDAASNS	DR1; DR1,8; DR2; DR1,3; DR2,3; DR1,2; DR2,7
4	VP4	31–50	NYYKDAASNSANRQDFTQDP	DR1; DR1,8; DR2; DR2,7; DR1,2; DR9
5	VP4	41–60	ANRQDFTQDPSKFTEPVKDV	DR2,4; DR1,3; DR1,6
6	VP4	51–70	SKFTEPVKDVMIKSLPALNS	DR2,4
7	VP2	61–80	MIKSLPALNSPTVEECGYSD	DR2,4
8	VP2	71–90	PTVEECGYSDRVRSITLGNS	DR1,3; DR1
9	VP2	81–100	RVRSITLGNSTITTQECANV	DR1; DR1,3; DR2; DR2,3; DR2,4; DR2,5; DR1,2
10	VP2	91–110	TITTQECANVVVGYGVWPDY	DR2,4; DR2
12	VP2	111–130	LSDEEATAEDQPTQPDVATC	DR9
13	VP2	121–140	QPTQPDVATCRFYTLNSVKW	DR1,3; DR1,3; DR1
14	VP2	131–150	RFYTLNSVKWEMQSAGWWWK	DR1; DR1,3; DR1,3
16	VP2	151–170	FPDALSEMGLFGQNMQYHYL	DR1
17	VP2	161–180	FGQNMQYHYLGRSGYTIHVQ	DR1
18	VP2	171–190	GRSGYTIHVQCNASKFHQGC	DR1
19	VP2	181–200	CNASKFHQGCLLVVCVPEAE	DR1; DR1,3
22	VP2	211–230	AYGDLCGGETAKSFEQNAAT	DR1,2
23	VP2	221–240	AKSFEQNAATGKTAVQTAVC	DR1,3
24	VP2	231–250	GKTAVQTAVCNAGMGVGVGN	DR1,3; DR2
26	VP2	251–270	LTIYPHQWINLRTNNSATIV	DR1; DR6,7; DR1,3; DR1,3; DR1,6
27	VP2	261–280	LRTNNSATIVMPYINSVPMD	DR1; DR2,7; DR2; DR1,3; DR1,6; DR2,4; DR2,7; DR9; DR4
28	VP2	271–290	MPYINSVPMDNMFRHNNFTL	DR2,7; DR2,4; DR2,7; DR9; DR4
29	VP2	281–300	NMFRHNNFTLMIIPFAPLDY	DR2,7; DR6,7; DR4
33	VP3	321–340	YNGLRLAGHQGLPTMLTPGS	DR2,7
36	VP3	351–370	SPSAMPQFDVTPEMNIPGQV	DR2,7; DR2,7; DR9
37	VP3	361–380	TPEMNIPGQVRNLMEIAEVD	DR4
38	VP3	371–390	RNLMEIAEVDSVVPINNLKA	DR2,7; DR2,4; DR2,7; DR9; DR4
39	VP3	381–400	SVVPINNLKANLMTMEAYRV	DR2,4; DR4
40	VP3	391–410	NLMTMEAYRVQVRSTDEMGG	DR2,4
41	VP3	401–420	QVRSTDEMGGQIFGFPLQPG	DR2,7
42	VP3	411–430	QIFGFPLQPGASSVLQRTLL	DR2,7
43	VP3	421–440	ASSVLQRTLLGEILNYYTHW	DR2,7
44	VP3	431–450	GEILNYYTHWSGSLKLTFVF	DR2,4; DR2,7; DR1,2; DR4
45	VP3	441–460	SGSLKLTFVFCGSAMATGKF	DR1; DR4
52	VP3	511–530	DDKYTASGFISCWYQTNVIV	DR1; DR1,8; DR1,3
55	VP3	541–560	MCFVSACNDFSVRMLRDTQF	DR2,4; DR1,2
68	VP1	671–690	LRRKMEMFTYIRCDMELTFV	DR4
73	VP1	721–740	VPTSVNDYVWQTSTNPSIFW	DR1,4
74	VP1	731–750	QTSTNPSIFWTEGNAPPRMS	DR1,4
75	VP1	741–760	TEGNAPPRMSIPFMSIGNAY	DR1,6; DR2,3; DR2,4; DR1,4
76	VP1	751–770	IPFMSIGNAYTMFYDGWSNF	DR1,4
78	VP1	771–790	SRDGIYGYNSLNNMGTIYAR	DR1,4; DR4
79	VP1	781–800	LNNMGTIYARHVNDSSPGGL	DR4
80	VP1	791–810	HVNDSSPGGLTSTIRIYFKP	DR4
84	VP1	831–850	SVNFDVEAVTAERASLITTG	DR9

DR2- and/or DR4-positive donors. It contains two HLA-DR2 motifs, **L**MEIAE**V** and **V** V PIN**NL**, and one HLA-DR4 motif **V**VPI**N**N**NL**K**A** (Rammensee *et al.,* 1995). T cells established from donors with HLA-DR4 also recognized the overlapping peptide 39 sharing the same HLA-DR4 motif area. The recognized HLA-DR2 epitope located probably in the middle of the peptide (**L**MEIAE**V**) because T cells established from HLA-DR2-positive donors did not recognize either peptide 37 or 39.

T cell lines established by different enterovirus antigens

T cell lines originally raised against one enterovirus antigen also widely recognized other enterovirus antigens. Three CBV4-specific T cell lines established from different donors recognized almost all other tested enterovirus lysate antigens (CBV3, EV11, CAV16, and PV1) (Table 2). When specific T cell lines were also estab-

TABLE 2

Three CBV4-Specific T Cell Lines Established from Different Subjects Recognized Widely also Other Enterovirus Antigens

CBV4-specific T cell lines	Antigen (SI)				
	CBV4	CBV3	EV11	CAV16	PV1
Subject 1	5.5	3.6	6.4	1.5	2.3
Subject 2	2.1	3.8	2.5	3.8	3.3
Subject 3	3.7	1.6	2.7	2.1	1.6

Note. The responses are expressed in stimulation index, as defined under Materials and Methods. Neutralizing antibodies of the subjects: (1) CBV4−, CBV3−, E11+; (2) CBV4+, CBV3+, EV11+; (3) CBV4−, CBV3−, EV11+. Neutralizing antibodies against CAV16 or PV1 were not analyzed, but all subjects had a valid polio vaccination history.

lished using CBV4-, CBV3-, EV11-, CAV16, and PV1 antigens, most lines from three donors had positive responses to CBV4 lysate antigen. Almost the same CBV4 peptides were also recognized without any respect which enterovirus serotype was used to initiate the line (Table 3).

T cell epitopes in various structural proteins and variability between different enteroviruses

Degree of the variability was estimated by comparing the sequences of other CBV serotypes to the sequence of CBV4. VP4 is very conserved between CBV serotypes, as from the 69 aa only four to seven are different (average of 8% variability). Instead, the variability is high in VP1 protein as 80–88 of 284 aa are different when compared to CBV4 (average of 29% variability). In VP2 (261 aa) and VP3 (238 aa) the number of different aa were 41–54 (average of 18% variability) and 37–52 aa (average of 19% variability), respectively (Table 4). Eighteen CBV4-specific T cell lines recognized one of the six VP4 peptides 19 times, one of the 26 VP2 peptides 50 times, one of the 24 VP3 peptides 27 times, and one of the 29 VP1 peptides 13 times (Table 4). Percentage of recognized peptide was calculated with the formula [(number of recognized peptide)/(number of T cell line × number of peptide)] × 100%. Thus on average 18% of VP4 peptides, 11% of the VP2 peptides, 6% of VP3 peptides but only 2% of VP1 peptides were recognized when positive responses with all the 18 CBV4 specific T cell lines were analyzed (Table 4).

TABLE 4

Number of T Cell Responses to Synthetic Peptides Derived from Various CBV4 Structural Proteins

CBV4 peptides (number of the peptides in each protein)	Number of peptides recognized by 18 T cell lines	Percentage of recognized peptides (%)	Degree of variability between CBVs (%)
VP4 peptides ($n = 6$)	19	18	8
VP2 peptides ($n = 26$)	50	11	18
VP3 peptides ($n = 24$)	27	6	19
VP1 peptides ($n = 29$)	13	2	29

Note. Percentage of recognized peptides was calculated with the formula [(number of the recognized peptide)/(number of T cell lines × number of peptide)] × 100%. Degree of the variability in various structural proteins was calculated when sequences of other CBVs were compared to sequence of CBV4.

T cell recognition and variability in the peptides between enteroviruses

The variability in various peptides was compared using sequences of CBV3, CBV4, EV11, CAV16, and PV1 viruses and two groups of peptides were selected. The first group contained peptides 1, 3, 4, 5, 6, 7, 8, 9, 10, 13, 16, 17, 18, 19, 26, 27, 34, 44, 45, 46, 48, 49, and 74, which were highly conserved (over 76% identity, mean 81% identity). Another group contained peptides 20, 21, 22, 23, 24, 39, 40, 41, 56, 57, 58, 64, 65, 66, 69, 70, 77, 78, 83, 84,

TABLE 3

Five T-Cell Lines Were Established from Three Subjects Using CBV4−, CBV3−, EV11−, CAV16−, and PV1 Antigens

Target antigen of the T cell line	Antigen					
	Subject 1		Subject 2		Subject 3	
	CBV4 (SI)	CBV4 peptides with positive response	CBV4 (SI)	CBV4 peptides with positive response	CBV4 (SI)	CBV4 peptides with positive response
CBV4	5.5	7, 9, 13	2.1	26, 27	3.7	8, 9, 13, 19, 26, 27
CBV3	2.5	6, 7, 9, 10, 11, 12, 14, 56	1.2	—	6.0	8, 9, 13, 14, 16, 19, 27
EV11	4.7	9	1.6	26, 27	8.1	3, 4, 8, 9, 13, 14, 16, 17, 19, 26
CAV16	1.4	—	3.0	26, 27	2.6	—
PV1	1.0	—	1.4	–	6.1	—

Note. Responses to CBV4 antigen are expressed in stimulation index. CBV4 peptides with positive response (SI > 2) were shown.

TABLE 5

Percentage of Recognized Peptides When All CBV4-Specific T Cell Lines Were Included (all) or When T Cell Lines from Donors with (CBV4-ab+) or without (CBV4-ab−) CBV4-Specific Neutralizing Antibodies Against CBV4 Were Included

Donors	Highly conserved peptides ($n = 23$)	Less conserved peptides ($n = 21$)	CBV4-specific peptides ($n = 8$)
All ($n = 18$)	15%[a]	3%[a]	3%
CBV4-ab+ ($n = 10$)	13%[b]	2%	4%
CBV4-ab− ($n = 6$)	14%[b]	5%	2%

Note. For peptides included, see Results.
[a] $P < 0.0001$.
[b] $P =$ ns.

and 85, which were less conserved (less than 49% identity, mean 32% identity). Percentage of recognized peptides was 15% in the group of conserved peptides but only 3% in the group of peptides with high variability ($P <$ 0.0001, Fisher's exact test) (Table 5).

Neutralizing antibodies in the donors

Neutralizing antibodies to CBV1, CBV2, CBV3, CBV4, CBV5, CBV6, EV6, EV9, EV11, and CAV9 were tested from sera of 19 donors. They all had neutralizing antibodies to at least one of the tested enterovirus serotypes. Two donors had antibodies to nine examined serotypes and one donor had antibodies against only one serotype, but the average seropositivity was five. Eleven donors had neutralizing antibodies to CBV4. CBV4-specific T cell lines have been established from 16 tested donors and 10 of them had neutralizing antibodies against CBV4. The percentage of CBV4 peptides recognized by 10 T cell lines, which were established from CBV4-seropositive donors (VP4 17%, VP2 10%, VP3 4%, and VP1 2%, respectively), was comparable to that of peptides recognized by all T cell lines (Table 4).

Eight peptides (21, 22, 23, 40, 65, 66, 84, and 85) were defined specific to CBV4 because they had less than 10 common amino acids with other CB viruses and CAV16, PV1, and EV11. Ten CBV4-specific T cell lines established from donors with neutralizing antibodies to CBV4 responded three times to any of CBV4-specific peptides (4%), while six CBV4-specific T cell lines established from donors without CBV4-specific neutralizing antibodies responded once to these peptides (2%). This difference was not significant by Fisher's exact test (Table 5). T cell lines, established from donors with or without CBV4-specific neutralizing antibodies, did not show any differences in responses to either less conserved or highly conserved peptides (Table 5).

DISCUSSION

This is the first study in which the localization of epitopes recognized by human T cells in enterovirus structural proteins has been systematically studied. We generated a panel of CBV4-specific T cell lines from healthy donors representing various HLA genotypes and tested their responses to 20-aa-long synthetic peptides covering the whole sequence of CBV4 structural proteins. The use of CBV4-specific T cell lines ensures that the detected epitopes are naturally processed epitopes of CBV4 proteins. Direct stimulation of peripheral blood lymphocytes by synthetic peptides may produce nonspecific reactivity due to the described wide cross-reactivity of T cell recognition (Wucherpfennig and Strominger, 1995).

T cell epitopes of all enterovirus structural proteins have not been previously characterized using this type of study design. Enteroviral T cell epitopes cross-reactive between various serotypes have been earlier reported by using direct stimulation of peripheral blood lymphocytes with fifteen 9- to 19-aa-long synthetic peptides covering conserved regions in structural proteins (Cello *et al.,* 1996). T cell epitopes were localized mainly in the capsid proteins VP2 and VP3 and to a lesser extent in VP1 but none in the VP4 protein (Cello *et al.,* 1996). Lack of the responses in the VP4 protein in this study is not surprising because it is possible that their two short VP4 peptides (12- to 13-aa-long) did not include any minimal epitopes. Also few poliovirus epitopes have been described near neutralizing antibody epitopes in VP1 of PV1 and PV3 (Graham *et al.,* 1993; Simons *et al.,* 1993).

In the present study the found epitopes were concentrated to the regions which are conserved among different enteroviruses, indicating that high cross-reactivity between enteroviruses might be expected. Only few epitopes could be mapped to a variable region. Six of the seven dominant peptide epitopes 3, 4, 9, 26, 27, and 28 were found in the VP4 and VP2 regions, whereas there was only one dominant peptide epitope in VP3 region (peptide 38) and none in the VP1 region. All of the 18 tested CBV4-specific T cell lines recognized peptides covering the sequence of CBV4-VP2 and/or -VP4 proteins, whereas only six T cell lines of 15 recognized some of the CBV4-VP1 peptides. All six CBV4-specific T cell lines with positive response to CBV4-VP1 peptide(s) also had positive response to CBV3-VP1 protein even if the identity of the VP1 proteins between CBV4 and CBV3 was only 69%. In concordance with that, the sequence of these VP1 epitopes was also relatively conserved as compared with rest of VP1 protein. As expected, all lines responding to CBV4 VP2 and/or VP4 peptides were also recognizing the *in vitro* produced CBV4-VP0 protein containing these two polypeptide chains.

After depletion of CD8-positive T cells CBV4-specific T-cell line recognized all the same peptides as the orig-

inal one. So in all likelihood the T cell epitopes found in our study were mainly mediated by CD4-positive T cells. The function of CD8-positive T cells, which were involved in our T cell lines, remains unknown. In our recent work we found that the T cell epitopes on the CBV4 2C protein were widely distributed, but HLA-DR identical subjects recognized highly similar epitopes and we suggested that HLA-DR genotype of the donors mainly defines the pattern of the recognized peptide epitopes (Marttila *et al.,* 2001). The previous studies were instead made without HLA genotype information of the donors (Cello *et al.,* 1996; Graham *et al.,* 1993). In this study, we also found that the pattern of recognized peptides was defined by the HLA II genotype of the donor and that the established HLA-DR motifs (Rammensee *et al.,* 1995) could to a certain extent explain the HLA-specific recognition.

We also found that the pattern of recognized peptides was similar regardless of the CBV4-specific neutralizing antibodies of the T cell donor and that T cells from donors with CBV4-specific neutralizing antibodies did not significantly recognize CBV4 specific peptides more often. It is obvious that the targets of the T cell immunity against enteroviruses are very different from the structures recognized by humoral immunity. Neutralizing antibodies are serotype specific by definition and the main target of the antibodies is variable VP1 protein, whereas T cell immunity is highly cross-reactive between different enterovirus serotypes and the main targets are the conserved regions mainly in the VP4 and VP2 proteins.

We thus detected an inverse correlation between the degree of variability in the proteins between enteroviruses and the number of T cell epitopes which were recognized within them. Epitopes shared by several serotypes are recognized in repeated infections and responsiveness may thus be enhanced, or alternatively, the probability of an encounter with these specific epitopes is higher. The results of previous studies emphasizing the significance of the highly conserved nonstructural 2C protein of CBV4 as a target of T cell responsiveness are also in line with the present observations (Marttila *et al.,* 2001; Varela-Calvino *et al.,* 2000). On average as much as 17% of 20-aa-long synthetic peptides covering this protein were stimulating a panel of T cell lines (Marttila *et al.,* 2001). This figure is very similar to the percentage of 18% found for VP4 peptides in this study.

The mapping of antigenic epitopes is of apparent importance for the development of subcomponent or synthetic epitope-based enterovirus vaccines in the future. Although neutralizing antibodies are needed for protection against enterovirus infections, their production is dependent on T cell help. Several conserved regions with high cross-reactivity between different enterovirus serotypes mainly in the VP4 and VP2 proteins are available and might be connected to structures able to induce neutralizing antibodies. Large variation of the recognized T cell epitopes between individuals with different HLA-DR genotypes should be taken into account and peptides recognized by all specificities selected. The question whether some T cell responses may be harmful because of the molecular mimicry between viral and tissue structures and may participate in the pathogenesis of autoimmune diseases must also be solved before safe vaccines can be designed.

MATERIALS AND METHODS

Enterovirus lysate antigens

Coxsackievirus B4 (JVB), coxsackievirus B3 (Nancy), echovirus 11 (Gregory), coxsackievirus A16 (G-10), and poliovirus 1 (Sabin) were grown in ML-2 LLC cells (ATCC). Infected cell cultures were harvested when cells detached freely from the flask surface. The infected cells and supernatant fluids were frozen and quickly thawed three times. The suspensions were clarified by centrifugation (8000 rpm, 5 min) and the supernatants were used as antigens. The antigens were inactivated with beta-propiolactone. Their protein concentrations were measured using the Pierce BCA protein assay reagent (Pierce, Rockford, IL).

CBV-GST proteins

The fusion proteins were prepared as described elsewhere (Härkönen *et al.,* 1997). Full-length CBV3 or CBV4 cDNA was used as a template to amplify the genes by PCR. The VP1 gene of CBV3 and VP0 gene of CBV4 were cloned into pGEX vector (Pharmacia, Uppsala, Sweden) and expressed as GST fusion proteins (GST-VP0 or GST-VP1) in *Escherichia coli.* GST-VP0 and GST-VP1 were purified using a glutathione–sepharose column (GST Gene Fusion System, Pharmacia Biotech, Uppsala, Sweden).

Peptides

Eighty-six 20-amino-acid-long synthetic peptides with 10 aa overlaps covered the whole CBV4 structural proteins sequences of the JBV strain (Jenkins *et al.,* 1987). The peptides were synthesized by F-moc [*N*-(9-fluorenyl)-methoxycarbonyl] chemistry on a simultaneous multiple peptide synthesizer (SMPS 350, Zinsser Analytic, Frankfurt a/M, Germany). Purity of the peptides was analyzed by reversed-phase HPLC (Hewlett–Packard 1100 series, Waldbronn, Germany).

T cell lines

Eighteen laboratory staff members donated blood for the study. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood by Ficoll–Paque (Uppsala, Sweden) gradient centrifugation. PBMC (2×10^6/ml) were incubated with the CBV4 antigen (10 μg/ml) in RPMI 1640 medium supplemented with 10% human AB serum, gentamycin sulphate (10 μg/ml),

HEPES buffer solution 1 M (20 μl/ml), and 3% glutamine (10 μl/ml). After 7 days, fresh medium supplemented with 0.5 ng/ml recombinant human IL-2 (R&D Systems Inc., Minneapolis, MN) was added every second or third day. After a total of 14 days from the beginning, the T cells were restimulated with both CBV4 antigen and irradiated (30 Gy) autologous antigen presenting cells (2×10^6 PBMC/ml). T cell lines were restimulated at 7-day intervals; IL-2 was added 2 days after antigen stimulation and at 2- to 3-day intervals thereafter. All T cell lines had more than two cycles of stimulation with CBV4 virus antigen, irradiated autologous APC, and IL-2. The T cell lines did not respond to control cell lysate or to GST protein. An average of 93% of cells were CD3 positive and 42% of them were CD4 positive and 27% CD8 positive when the phenotype of 10 T cell lines was analyzed by flow cytometry (Facscan, Becton–Dickinson, Mountain View, CA) using FITC- or phycoerythrin-conjugated mAb (Coulter, Hialeah, FL) for the CD3, CD4, and CD8 T cell surface markers, respectively.

Lymphocyte proliferation assay

Triplicate cultures of $5–10 \times 10^3$ T cells per well were incubated with 2×10^4 antigen-presenting cells (PBMC) and antigen in 200 μl volumes in 96-well round-bottomed microtiter plates for 2 days. Tritiated thymidine (2 μCi/ml) was added 18 h before harvesting the cultures. Incorporated radioactivity was measured by 1450 Microbeta Plus liquid scintillation counter (Wallac, Turku, Finland). Stimulation index (SI) was calculated by dividing the median of stimulated triplicate culture wells by the median of the unstimulated wells.

HLA typing

HLA typing of blood donors was done using panels of commercial antisera (Biotest, Dreieich, Germany) in standard microlymphocytotoxicity test. B cells for HLA class II typing were enriched using immunomagnetic beads (Dynal, Oslo, Norway).

Neutralizing antibodies

Serotype-specific antibodies against coxsackievirus B serotypes 1 to 6, echovirus serotypes 6, 9, and 11, and coxsackievirus A serotype 9 were analyzed using standard plaque neutralization assay. Titers below 4 were considered negative.

ACKNOWLEDGMENTS

We thank Anne Suominen and Terttu Laurén for technical assistance. This work was supported by the Sigrid Juselius Foundation, the Academy of Finland, and Juvenile Diabetes Foundation International.

REFERENCES

Beck, M. A., Chapman, N. M., McManus, B. M., Mullican, J. C., and Tracy, S. (1990). Secondary enterovirus infection in the murine model of myocarditis. Pathologic and immunologic aspects. *Am. J. Pathol.* **136,** 669–681.

Beck, M. A., and Tracy, S. M. (1990). Evidence for a group-specific enteroviral antigen(s) recognized by human T cells. *J. Clin. Microbiol.* **28,** 1822–1827.

Cello, J., Strannegard, O., and Svennerholm, B. (1996). A study of the cellular immune response to enteroviruses in humans: identification of cross-reactive T cell epitopes on the structural proteins of enteroviruses. *J. Gen. Virol.* **77,** 2097–2108.

Graham, S., Wang, E. C., Jenkins, O., and Borysiewicz, L. K. (1993). Analysis of the human T-cell response to picornaviruses: Identification of T-cell epitopes close to B-cell epitopes in poliovirus. *J. Virol.* **67,** 1627–1637.

Härkönen, T., Hovi, T., and Roivainen, M. (1997). Expression of Coxsackievirus B4 proteins VP0 and 2C in Escherichia coli and generation of virus protein recognizing antisera. *J. Virol. Methods* **69,** 147–158.

Henke, A., Huber, S., Stelzner, A., and Whitton, J. L. (1995). The role of $CD8^+$ T lymphocytes in coxsackievirus B3-induced myocarditis. *J. Virol.* **69,** 6720–6728.

Hogle, J. M., Chow, M., and Filman, D. J. (1985). Three-dimensional structure of poliovirus at 2.9 A resolution. *Science* **229,** 1358–1365.

Hyöty, H., Hiltunen, M., and Lönnrot, M. (1998). Enterovirus infections and insulin dependent diabetes mellitus—Evidence for causality. *Clin. Diagn. Virol.* **9,** 77–84.

Hyypiä, T., Hovi, T., Knowles, N. J., and Stanway, G. (1997). Classification of enteroviruses based on molecular and biological properties. *J. Gen. Virol.* **78,** 1–11.

Jenkins, O., Booth, J. D., Minor, P. D., and Almond, J. W. (1987). The complete nucleotide sequence of coxsackievirus B4 and its comparison to other members of the Picornaviridae. *J. Gen. Virol.* **68,** 1835–1848.

Marttila, J., Juhela, S., Vaarala, O., Hyöty, H., Roivainen, M., Hinkkanen, A., Vilja, P., Simell, O., and Ilonen, J. (2001). Responses of coxsackievirus B4-specific T-cell lines to 2C protein—characterization of epitopes with special reference to the GAD65 homology region. *Virology* **284,** 131–141.

Mateu, M. G. (1995). Antibody recognition of picornaviruses and escape from neutralization: A structural view. *Virus Res.* **38,** 1–24.

Melnick, J. L. (1996). Enteroviruses: Polioviruses, coxsackieviruses, echoviruses, and newer enteroviruses. *In* "Fields Virology" (B. M. Fields, D. M. Knipe, P. M. Howley, *et al.,* Eds.), 3rd ed. Lippincott-Raven, Philadelphia, PA.

Minor, P. D., Ferguson, M., Evans, D. M., Almond, J. W., and Icenogle, J. P. (1986). Antigenic structure of polioviruses of serotypes 1, 2 and 3. *J. Gen. Virol.* **67,** 1283–1291.

Oberste, M. S., Maher, K., Kilpatrick, D. R., and Pallansch, M. A. (1999). Molecular evolution of the human enteroviruses: Correlation of serotype with VP1 sequence and application to picornavirus classification. *J. Virol.* **73,** 1941–1948.

Pöyry, T., Kinnunen, L., Hyypiä, T., Brown, B., Horsnell, C., Hovi, T., and Stanway, G. (1996). Genetic and phylogenetic clustering of enteroviruses. *J. Gen. Virol.* **77,** 1699–1717.

Rammensee, H. G., Friede, T., and Stevanoviic, S. (1995). MHC ligands and peptide motifs: First listing. *Immunogenetics* **41,** 178–228.

Rueckert, R. R. (1996). Picornaviridae: The Viruses and Their Replication. *In* "Fields Virology" (B. M. Fields, D. M. Knipe, P. M. Howley, *et al.,* Eds.), 3rd ed. Lippincott-Raven, Philadelphia, PA.

Simons, J., Kutubuddin, M., and Chow, M. (1993). Characterization of poliovirus-specific T lymphocytes in the peripheral blood of Sabin-vaccinated humans. *J. Virol.* **67,** 1262–1268.

Varela-Calvino, R., Sgarbi, G., Arif, S., and Peakman, M. (2000). T-cell reactivity to the P2C nonstructural protein of a diabetogenic strain of coxsackievirus B4. *Virology* **274,** 56–64.

Wucherpfennig, K. W., and Strominger, J. L. (1995). Molecular mimicry in T cell-mediated autoimmunity: Viral peptides activate human T cell clones specific for myelin basic protein. *Cell* **80,** 695–705.