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Analysis of the Human T-Cell Response to Picornaviruses: Identification of T-Cell Epitopes Close to B-Cell Epitopes in Poliovirus

SHEK GRAHAM,[1]†* EDDIE C. Y. WANG,[1] OWEN JENKINS,[2] AND LESZEK K. BORYSIEWICZ[1]

Department of Medicine, University of Wales College of Medicine, Heath Park, Cardiff CF4 4XN,[1] and Smithkline Beecham Pharmaceuticals, Biosciences Research Center, Epsom, Surrey KT18 5XQ,[2] United Kingdom

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Little is known about the nature and specificity of T-cell-mediated responses to picornaviruses in humans. In this study, the nature of the T-cell response to seven picornaviruses, including polioviruses, coxsackieviruses B3 and B4, human rhinovirus 14, and encephalomyocarditis virus, was determined. Twenty-nine individuals responded to poliovirus type 3, coxsackievirus B3, and encephalomyocarditis virus by proliferation of T cells, and from such cultures, 130 virus-specific T-cell lines were established. T-cell lines generated in response to encephalomyocarditis virus were exclusively strain specific. However, the majority of T-cell lines established in response to viruses, other than encephalomyocarditis virus, were cross-reactive to each other. Their cross-reactivity was confirmed in 2 of the 30 picornavirus-specific clonally derived T-cell lines from two subjects, but the majority of these lines were serotype specific. T-cell epitopes adjacent to each of the B-cell antigenic sites in VP1 of poliovirus type 3 were identified. The response to the region adjacent to B-cell antigenic site 1 (residues 97 to 114) was dominant between individuals. The localization of this major CD4 T-cell epitope may permit the construction of chimeric viruses utilizing the natural picornavirus T-cell response to augment production of antibody specific for inserted sequences.

Picornaviruses are widely prevalent, small nonenveloped RNA viruses with a single-stranded positive-sense genome (7.5 to 8.8 kb) (42). Members of the genus infect insects and mammals and are subdivided into enteroviruses (polioviruses, coxsackieviruses A and B, and echoviruses), aphthoviruses, cardioviruses, and rhinoviruses. These produce a range of diseases such as paralytic poliomyelitis, aseptic meningitis (coxsackievirus and echoviruses), pericarditis and myocarditis (coxsackievirus B3), and the common cold (human rhinovirus) (32). In addition, enteroviruses have been invoked as an etiological agent in a number of chronic disorders, e.g., diabetes and myocarditis, in which the pathogenesis may be mediated by induction of autoimmunity (5).

Host protection after infection requires an effective neutralizing antibody response (12, 33, 41). However, there is little information about the functional role of picornavirus-specific T cells. The nature and specificity of this T-cell response remain uncharacterized. $CD4^+$ T cells are essential for the generation of effective neutralizing antibodies, as shown in experimental studies with foot-and-mouth disease virus (9, 10). Cytotoxic $CD8^+$ T cells specific for picornaviruses have been described. Hepatitis A infection is associated with a strong major histocompatibility complex class I-restricted response which is found in liver-infiltrating lymphocytes during clinically evident hepatitis (47). $CD8^+$ coxsackievirus-specific cytotoxic T lymphocytes have been identified in mice infected with coxsackievirus B3 (22). In the latter example, other cytotoxic T lymphocytes which lyse normal and infected myocardial cells via recognition of virus-induced host determinants are generated (21). This provides a possible mechanism whereby viral infection may be associated with organ-specific tissue injury; yet conventional evidence for the presence of virus in the affected organ is difficult to demonstrate.

We have sought to determine the nature and specificity of the normal T-cell response to picornaviruses in humans. As has been shown in a number of studies (23, 40, 44, 46), there is a large overlap in the primary sequences of the picornaviral polyproteins. T cells recognize such primary sequences present in peptide fragments of virus-encoded products associated with major histocompatibility complex class I (HLA-A, -B, and -C determinants [$CD8^+$ cells]) or major histocompatibility complex class II (HLA-DR, -DP, and -DQ [$CD4^+$ cells]) in humans. In this study, we show that the picornavirus-induced $CD4^+$ T-cell response can be both cross-reactive between different picornaviruses and serotype specific at a clonal level. These results, together with a comparison of picornavirus polyprotein primary structure, may be used to predict the possible fine specificity of picornavirus-specific $CD4^+$ T-cell epitopes. We used this approach with overlapping synthetic peptides to define T-cell epitopes in VP1 of poliovirus type 3 (PV3), which are close to B-cell antigenic site 1 (36, 39).

MATERIALS AND METHODS

Cell lines. HeLa cells, a clone of HeLa, HeLa (Ohio) (H.Ohio), RD cells (human embryonal rhabdomyosarcoma cells), and Hep2c cells (European Collection of Animal Cell Cultures, Porton Down, United Kingdom); F2002 cells (whole embryonic human fibroblasts), BGM cells, and Vero cells (green monkey kidney fibroblasts) (Flow Laboratories, Irvine, Scotland); and L-A2 cells (mouse fibroblast carcinoma L cells transfected with the human class I HLA-A2

* Corresponding author.
† Present address: Department of Medical Parasitology, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, United Kingdom.

gene; N. Holmes, Cambridge, United Kingdom) were grown in 150-cm^2 flasks in minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS), 1% nonessential amino acids, 2 mM L-glutamine, 100 μg of streptomycin per liter, and 1×10^5 IU of penicillin per liter (MEM-10) and passaged every 3 to 4 days.

MLA144 cells (gibbon lymphosarcoma lymphoblasts which constitutively secrete interleukin 2 [IL-2]), OKT 3, OKT 4, and OKT 8 (mouse hybridoma cells; American Type Culture Collection) cells were grown in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 100 μg of streptomycin per liter, and 1×10^5 IU of penicillin per liter (RPMI-10), at 2×10^5 cells per ml, and the supernatant was harvested every 3 to 4 days, clarified at 800 g, and stored at −20°C. The cell lines were shown by DNA staining to be mycoplasma free (7).

Virus growth. Poliovirus Sabin types 1, 2, and 3 (PV1, PV2, and PV3, respectively), coxsackievirus types B3 (CVB3) and B4 (CVB4), human rhinovirus 14 (HRV14), and encephalomyocarditis virus (EMCV) seed stocks (O. Jenkins, Reading, United Kingdom, and A. Nash, Cambridge, United Kingdom) were grown in serum-free MEM in H.Ohio cells. Both cell-associated virus and thrice freeze-thawed infected cells and cell supernatant virus were harvested. These were used as secondary seed stocks from which all subsequent virus supplies were grown. All virus stocks were grown at 37°C, apart from HRV14, which was grown at 34°C. Infectious virus was estimated by a modification of the Karber method (17) with a 50% tissue culture infective dose ($TCID_{50}$) assay with a Biomek 1000 automated laboratory workstation (Beckman Instruments, Palo Alto, Calif.) (1). Cells were assayed on H.Ohio cells in 96-well flat-bottomed plates by using 8 replicate wells at each dilution of virus. Sucrose-gradient-purified poliovirus (K. Katrak, NIBSC, Hertfordshire, United Kingdom) prepared by the method of Minor (35) was also used. Virus stocks were shown to be mycoplasma free by DNA staining and coculturing and were stored at −70°C until use. Virus preparations for use as inactivated antigens were prepared by being heated at 60°C for 45 min, after which no infectious virus was detectable with a $TCID_{50}$ assay.

Virus stocks were also checked for purity against possible laboratory cross-contamination by a modified neutralization assay utilizing monospecific anti-PV1, -PV2, -PV3, -CVB3, and -CVB4 polyclonal sera and polyspecific anti-poliovirus and anti-coxsackievirus antisera (PHLS, Colindale, United Kingdom). One-hundred $TCID_{50}$ of virus was added to each well of a 96-well plate containing H.Ohio cells, at various dilutions of antiserum, by using replicates of 32 wells per serum dilution (35).

Peptide synthesis. Peptides covering the entire sequence of VP1 of P3/Leon $12a_1b$ (46) were synthesized by T-boc chemistry as described by Houghten (19). Methylbenzhydrylamine resin–HCl (100 to 200 mesh, Peninsula Labs., Europe Ltd.) was sealed inside a polypropylene bag (72-μm-pore size) and soaked overnight in dichloromethane. Amino acids were sequentially added to the amino terminus of the elongating synthetic peptide. After synthesis was complete, peptides were deprotected and removed from the resin by hydrogen-fluoride cleavage (20). Twenty-five peptides, the majority of which were 18-mers, were synthesized, each overlapping by six residues. In addition, nine 15-mer peptides, overlapping by 12 residues, were synthesized encompassing amino acids 82 to 117 of VP1 in PV3 (Fig. 1). Peptides were >70% pure, as assessed by high-performance liquid chromatography, and were used without further purification.

A

301*	1[a]	G I E D L I S E V A Q G A L T L S L	18[b]
302	13	A L T L S L P K Q Q D S L P D T K A	30
303	25	L P D T K A S G P A H S K E V P A L	42
304	37	K E V P A L T A V E T G A T N P L A	54
305	49	A T N P L A P S D T V Q T R H V V Q	66
306	61	T R H V V Q R R S R S E S T I E S F	78
307	73	S T I E S F F A R G A C V A I I E V	90
308	85	V A I I E V D N E Q P T T R A Q K L	102
309	97	T R A Q K L F A M W R I T Y K D T V	114
310	109	T Y K D T V Q L R R K L E F F T Y S	126
311	121	E F F T Y S R F D M E F T F V V T A	138
312	133	T F V V T A N F T N A N N G H A L N	150
313	145	N G H A L N Q V Y Q I M Y I P P G A	162
314	157	Y I P P G A P T P K S W D D Y T W Q	174
315	169	D D Y T W Q T S S N P S I F Y T Y G	186
316	181	I F Y T Y G A A P A R I S V P Y V G	198
317	193	S V P Y V G L A N A Y S H F Y D G F	210
318	205	H F Y D G F A K V P L K T D A N D Q	222
319	217	T D A N D Q I G D S L Y S A M T V D	234
320	229	S A M T V D D F G V L A V R V V N D	246
321	241	V R V V N D H N P T K V T S K V R I	258
322	253	T S K V R I Y M K P K H V R V W	268
323	263	K H V R V W C P R P P R A V P Y Y G	280
324	275	A V P Y Y G P G V D Y R N N L D P L S	293
325	288	N L D P L S E K G L T T Y	300

B

350	82	G A C V A I I E V D N E Q P T	96
351	85	V A I I E V D N E Q P T T R A	99
352	88	I E V D N E Q P T T R A Q K L	102
353	91	D N E Q P T T R A Q K L F A M	105
354	94	Q P T T R A Q K L F A M W R I	108
355	97	T R A Q K L F A M W R I T Y K	111
356	100	Q K L F A M W R I T Y K D T V	114
357	103	F A M W R I T Y K D T V Q L R	117
358	106	W R I T Y K D T V Q L R R K L	120

FIG. 1. Synthetic peptides of PV3 VP1. The peptide number is indicated first (*) and the first and last amino residue numbers for each peptide are indicated by "a" and "b," respectively. (A) Twenty-five mainly 18-mer peptides overlapping by six amino acid residues were synthesized to encompass the entire VP1 sequence of P3/Leon $12a_1b$ (45). (B) Peptides 350 to 358 were synthesized, each of which was a 15-mer overlapping by 12 residues, encompassing the sequence covered by peptides 308 and 309.

Proliferation of PBMCs. Blood samples from 29 normal subjects of both sexes, 20 to 45 years of age, were anticoagulated with preservative-free heparin (CP Pharmaceuticals, Wrexham, United Kingdom) at 50 IU/10 ml of blood.

Peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation on Lymphoprep (Nycomed Pharma, Oslo, Norway) and washed three times in phosphate-buffered saline prior to use. PBMCs were resuspended in RPMI-AB, with 10% human AB serum to replace FCS, at 2×10^6 cells per ml. Aliquots of 100 μl of cells were plated out into 96-well flat-bottomed microtiter plates.

Infectious supernatant picornavirus, cell-associated picornavirus preparations (freeze-thawed and whole infected H.Ohio cells), and purified poliovirus virions were compared with equivalent heat-inactivated picornavirus preparations in a series of proliferation assays. Although the results were often comparable, heat-inactivated supernatant virus yielded the most consistent results, giving the lowest background proliferation in response to H.Ohio cells. As a control for H.Ohio antigens present in the virus preparations, mock-infected H.Ohio cells were sonicated for 15 s to lyse the cells and release H.Ohio antigens into the supernatant, which was then used as the background control antigen. This preparation was shown to give proliferative results similar to those obtained with EMCV or CVB3 by using PV3-specific clonally derived T-cell lines.

Several different concentrations ($TCID_{50}$ equivalent, 5×10^6 to 5×10^8) of heat-inactivated picornavirus antigens, from the supernatant of virus-infected cells, and control antigens were added in 100-μl aliquots in triplicate or quadruplicate. The plates were incubated for 5 days and pulsed with 1 μCi of [^{3}H]methyl-thymidine (5 mCi/mmol) (Amersham International, Aylesbury, United Kingdom) per well for 14 to 16 h before radiolabelled cells were harvested onto fiberglass Titertek filters with a Skatron Titertek cell harvester. Radioactive thymidine incorporation was measured on a Packard 1500 beta counter (Canberra Packard, Groningen, The Netherlands).

Results are expressed as stimulation indices (S.I.s), where S.I. = (cpm measured in response to virus preparation)/(cpm to H.Ohio sonicate alone), where differences between subjects' responses to a number of different picornaviruses were to be compared in different experiments. However, in most studies, the equation Δ cpm = test result (cpm) − H.Ohio sonicate alone (cpm) was used to give more accurate quantitative results, because results were to be compared within each experiment.

A similar assay was used to test PBMCs from eight subjects against each of the 25 synthetic PV3 VP1 peptides across a concentration range of 1 to 100 μg/ml.

In vitro culture and stimulation of PBMCs. PBMCs were isolated from 10 normal volunteers and resuspended at 2×10^6 cells per ml in RPMI-AB, as described above. Aliquots of 1.5 ml were plated out into a 24-well plate with optimal concentrations of heat-inactivated picornavirus antigen, as determined by prior PBMC proliferation (5×10^7 to 5×10^8 $TCID_{50}$/ml). Eight of these subjects were used in studies to examine the cross-reactivities of seven picornaviruses against each other by raising a panel of picornavirus-specific T-cell lines and testing each one against each of the picornaviruses in the panel. PBMCs from six of these subjects and from another two subjects were used to generate peptide-specific T-cell lines by culturing the cells with a peptide pool made of the synthetic PV3 VP1 peptides (10 μg/ml) and incubating them for 5 days. At this stage, all wells from cell lines exhibiting good growth were divided in two, and all wells were given 5 IU of recombinant IL-2 (rIL-2; Boehringer Mannheim, Lewes, United Kingdom) per ml.

Cultures were maintained by regularly dividing wells, adding fresh medium and rIL-2 every 3 to 4 days. RPMI supplemented with 10% Myoclone FCS serum (RPMI-M; GIBCO) was used after cells had been in culture for 5 days. Myoclone serum is especially selected for its low levels of endotoxins and its ability to specifically support the growth of hybridomas. These qualities appear to give better batch reproducibility and lower background proliferation than standard FCS batches. In addition, every 7 to 10 days, the cultures were given 1×10^6 to 2×10^6 fresh autologous irradiated (2.5 krad) PBMCs per well, as feeder cells, and antigen.

Aliquots of the in vitro-stimulated PBMCs were examined by proliferation assay after 11 days. The cells were incubated with 2×10^5 autologous irradiated PBMCs per well, which had been previously pulsed with picornavirus antigens overnight, in 96-well flat-bottomed plates (27). [^{3}H]thymidine (1 μCi) was added to each well after 48 h, and the cells were harvested after a 14- to 16-h incubation.

$CD4^+$ and $CD8^+$ depletion of PBMCs prior to proliferation assay. Anti-$CD4^+$- and anti-$CD8^+$-coated magnetic beads (Dynal, Merseyside, United Kingdom) were used to deplete fresh PBMCs from two subjects of either $CD4^+$ or $CD8^+$ T cells (49). Briefly, PBMCs were depleted of the appropriate T-cell subset by allowing the adherence of either $CD4^+$ or $CD8^+$ T cells to the antibody-coated beads and removing the magnetic beads and the attached T-cell subset from the cell suspension. The $CD4^+$- or $CD8^+$-depleted cells were cultured at 2×10^5 cells per well in a 6-day proliferation assay with either PV3 or peptide 309. The cells were pulsed with [^{3}H]thymidine, as above, and harvested.

Growth of picornavirus-specific T-cell clones. After 7 to 11 days in culture, viable PBMCs (an aliquot from two subjects) were separated on Lymphoprep and cloned by limiting dilution in Terasaki plates at 0, 1, 2, 5, 10, 25, 50, 100, 250, and 500 cells per well onto 2×10^4 irradiated autologous PBMCs (26) or in 96-well microtiter plates on 10^5 irradiated autologous PBMCs. All cloning procedures were carried out in RPMI-M with 20 IU of rIL-2 per ml and 20% MLA144 supernatant in the Terasaki plates or 5 IU of rIL-2 per ml in the 96-well plates, with optimal concentrations of heat-inactivated virus-containing supernatant. The 96-well plates were refed with fresh RPMI-M and rIL-2 every 3 to 4 days.

All wells with growing cells, as determined by phase-contrast microscopy, were moved into 24-well plates with 2×10^6 irradiated autologous PBMCs per well in RPMI-M supplemented with 5 IU of rIL-2 per ml and 20% MLA144 supernatant (T-cell culture medium). Only those wells that came from plates well within the clonal distribution of outgrowth, estimated by extrapolation from the limiting dilution analysis of growth (29), were continued. These clonally derived lines were maintained in T-cell culture medium and given 10^6 irradiated allogeneic PBMCs per well as feeder cells every 7 to 10 days. The allogeneic PBMCs used were surface oxidized with galactose oxidase and neuraminidase to generate Schiff bases (14) and to aid antigen-free stimulation of the clonally derived T-cell lines.

Phenotypic analysis. The phenotypes of fresh PBMCs, T-cell lines, and clonally derived T-cell lines were examined by staining 10^5 cells for 30 min with either anti-CD4, anti-CD8, or anti-CD3 mouse anti-human monoclonal antibodies (see above) and then incubating them with goat anti-mouse fluorescein isothiocyanate-conjugated immunoglobulin G (Dako, Buckinghamshire, United Kingdom) for 25 min. The cells were then examined by an Epics Profile flow cytometer (Coulter Electronics, Luton, United Kingdom).

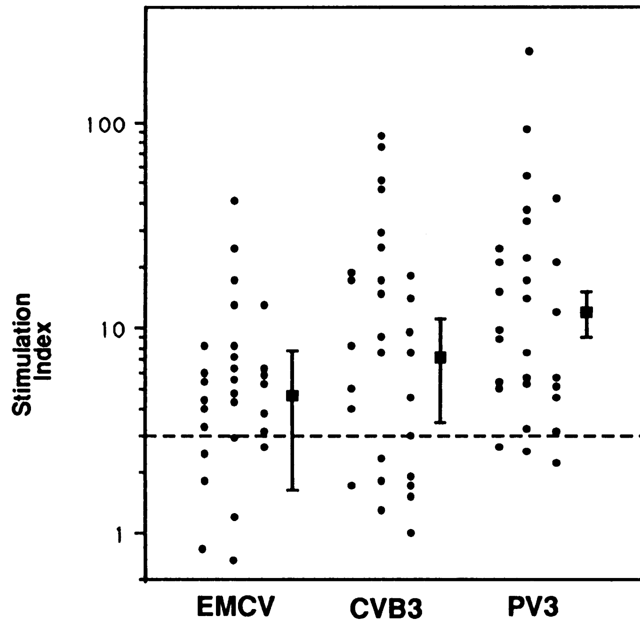

FIG. 2. The proliferative response of PBMCs from 29 subjects assayed against PV3, CVB3, and EMCV, as described in Materials and Methods. Virus was used across a concentration range of 5×10^7 to 5×10^8 $TCID_{50}$/ml. The S.I. of each subject's response was plotted on a logarithmic scale for each virus (●) with H.Ohio sonicate as the background control (variation between subjects was 100 to 500 cpm). The mean and SD of the proliferative response of the population for each virus (■) are marked and were calculated from the logarithm of each S.I. in order to normalize the curve. S.I.s of >3.0 were considered significant (48).

TABLE 1. Proliferation of PBMCs in response to picornaviruses

Test virus	Proliferation of PBMCs from subject[a]				
	JB	JS	RS	EW	TB
PV1	88 ± 2	11 ± <1	27 ± 1	9 ± <1	3 ± <1
PV2	100 ± 4	14 ± <1	48 ± 2	27 ± 1	2 ± <1
PV3	118 ± 3	12 ± <1	14 ± <1	31 ± 1	2 ± <1
CVB3	76 ± 2	18 ± <1	52 ± 2	12 ± <1	1 ± <1
CVB4	86 ± 3	11 ± <1	41 ± 2	2 ± <1	2 ± <1
HRV14	107 ± 3	8 ± <1	52 ± 1	30 ± 1	3 ± <1
EMCV	41 ± 1	1 ± <1	12 ± <1	2 ± <1	2 ± <1
PPD[b]	282 ± 10	239 ± 8	86 ± 2	127 ± 5	152 ± 8

[a] Proliferation of PBMCs is expressed as the S.I. ± standard error, with background responses to H.Ohio sonicate varying between 100 and 500 cpm.
[b] PPD, purified protein derivative of tuberculin, used at 100 to 200 IU/ml, is a T- and B-cell mitogen.

RESULTS

Virus yields. To obtain optimum virus yields from a single cell line, minimizing variables such as the antigenic background, peak virus yields of both freeze-thawed virus-infected cells and supernatant from CVB3-infected cultures in seven different cell lines were examined. H.Ohio cells consistently gave the highest yields of infectious virus (5×10^7 to 5×10^8 $TCID_{50}$/ml). Similar experiments with the other picornaviruses showed that virus growth in this cell line gave the best overall yields of virus for the majority of the seven picornaviruses tested (data not shown).

Further experiments also showed that supernatant from virus-infected cells contained virus of sufficiently high titer (5×10^7 to 3×10^9 $TCID_{50}$) to use in proliferation and cloning experiments. The background proliferation in response to this antigen was much less than that to cell-associated virus (data not shown), and these supernatants were used in all subsequent experiments. In all cases, virus was neutralized by its serotype-specific serum, but was not neutralized by serum against another picornavirus.

Proliferation of PBMCs in response to picornavirus antigens. PBMCs from 29 normal volunteers were cocultured in vitro with cell supernatant virus, and a wide range of responses to picornaviruses among the normal individuals tested were observed (Fig. 2). In each case, multiple dilutions of each antigenic preparation were used. Results are expressed as the maximal response across the range of antigen dilutions rather than a comparison with a single protein or $TCID_{50}$ equivalent value because the antigenicity of an individual preparation may vary. Peak responses were observed at a concentration equivalent to 5×10^7 to 5×10^8 $TCID_{50}$/ml. There were minor differences between subjects in the concentration of each antigen required to elicit a maximal response.

However, there was variability between subjects in the maximum responses (Table 1). PBMCs from subjects JB and RS proliferated in response to each of the viruses tested. Subject JB had recently received an inactivated poliovirus vaccination. Most subjects studied showed a significant response to each of the picornaviruses, suggesting that coxsackieviruses and HRV14 may be commonly encountered by a normal population.

Of the 29 subjects tested, 21 showed proliferation in response to EMCV (Fig. 2), a picornavirus not commonly associated with human infection (50). In most subjects, there was no significant difference between the magnitude of T-cell proliferation in response to viruses such as EMCV, CVB3, and PV3, even though all but one of the subjects had received routine poliovirus immunization in childhood. However, 26 of 29 subjects responded to PV3 (S.I., >3), whereas 8 of 29 subjects did not make a significant response to EMCV and CVB3.

The response to purified poliovirus was similar to that with both supernatant and cell-associated virus preparations, suggesting that the T-cell response is predominantly to the structural virion capsid proteins. In addition, heat-inactivation of the antigens resulted in a greater proliferative response than that to the native antigen (Table 2). Several subjects were tested between 3 and 8 times, with some subjects tested over 20 times, and the results were reproducible. $CD4^+$ and $CD8^+$ depletion of PBMCs showed that the response was due to the $CD4^+$ T-cell population (see Table 5).

Proliferation of cultured PBMCs in response to picornavirus antigens. Two possible explanations for the results observed (Fig. 2 and Table 1) were that there may be a significant cross-reactive T-cell response between structurally similar viruses or that there is a significant amount of environmental exposure to agents such as EMCV. To examine this, PBMCs were cultured in vitro in the presence of each of the picornaviruses. The T-cell lines generated were then tested to determine whether or not a cross-reactive response against the other picornaviruses could be elicited. Cell lines were also tested against heat-inactivated Epstein-Barr virus and vaccinia virus antigens. In addition, six cell lines from human cytomegalovirus-seropositive subjects were also tested against human cytomegalovirus. No proliferation in response to any of the non-picornavirus antigens was ob-

TABLE 2. Proliferation of PBMCs in response to heat-inactivated sucrose-gradient-purified and supernatant picornavirus antigens

Antigen[a]	Proliferation of PBMCs from subject[b]			
	RS	JS	EW	JB
PV1				
SGP	65 ± 1.8	8 ± 0.2	22 ± 0.8	71 ± 1.4
SUP	73 ± 1.5	12 ± 0.2	26 ± 0.3	77 ± 1.3
SGP HI	87 ± 1.6	10 ± 0.3	25 ± 0.4	76 ± 1.6
SUP HI	81 ± 0.7	14 ± 1.2	31 ± 0.9	79 ± 2.1
PV3				
SGP	85 ± 1.2	9 ± 0.6	5 ± 0.4	63 ± 1.6
SUP	101 ± 2.1	15 ± 0.5	20 ± 1.0	82 ± 1.1
SGP HI	94 ± 3.3	12 ± 0.5	13 ± 0.6	75 ± 2.7
SUP HI	124 ± 3.8	15 ± 1.2	24 ± 1.4	104 ± 3.7

[a] Abbreviations: SGP, sucrose-gradient-purified virus; SUP, supernatant viral antigens from infected H.Ohio cells; HI, heat-inactivated at 60°C for 45 min.

[b] Results are expressed as peak S.I.s ± SE, calculated with H.Ohio sonicate as background control for supernatant virus (300 to 850 cpm) and medium as background control for purified virus (200 to 500 cpm). The peak response to viral antigens required 5×10^7 to 5×10^8 $TCID_{50}$/ml.

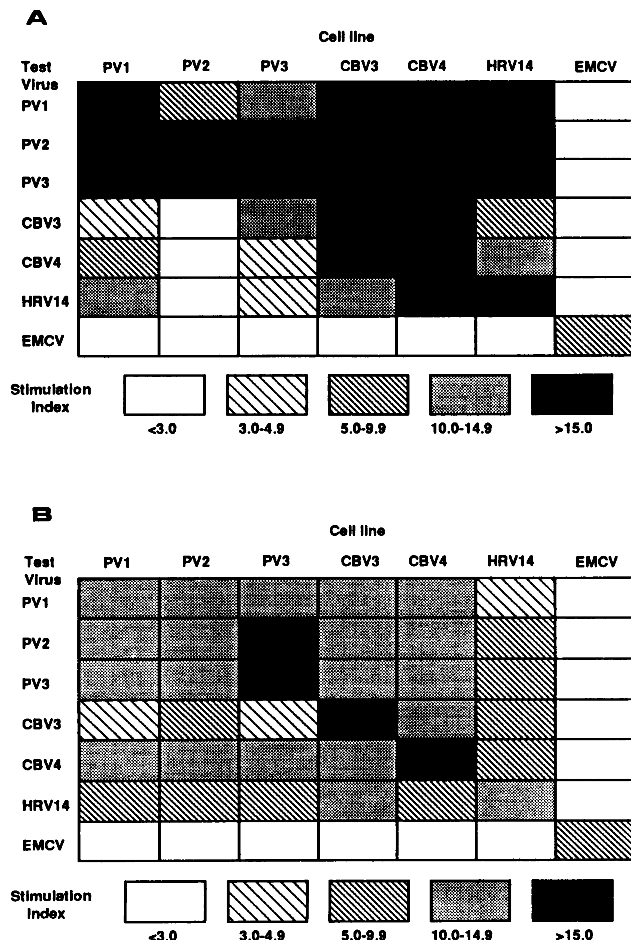

FIG. 3. Cross-reactivities of picornavirus-specific T-cell lines. PBMCs from subjects JB (A) and LB (B) were aliquoted into 24-well plates at 2×10^6 cells per ml in RPMI-AB and stimulated with one of seven picornaviruses. After 11 to 14 days, each T-cell line was assayed for proliferation in response to a panel of picornaviruses, including the original stimulating virus. The results are expressed as the maximal S.I. (SD, <10%). Proliferation in response to the H.Ohio sonicate background control varied between 230 and 1,050 cpm.

TABLE 3. Proliferation of picornavirus-specific clonally derived T-cell lines derived from PBMCs of subject LB

Clonal line[a]	Proliferation in response to test virus[b]				
	PV1	PV2	CVB3	CVB4	EMCV
CVB3-D3	1.9	2.0	42	2.8	1.5
CVB3-D4	1.1	1.0	35	0.9	0.9
CVB4-B7	1.8	2.2	2.1	25	1.3
CVB3-C2	0.9	1.2	110	85	0.8

[a] CVB3-D3, -D4, and -B7 are picornavirus serotype-specific clonally derived T-cell lines. CVB3-C2 is a clonally derived T-cell line cross-reacting between coxsackievirus B serotypes.

[b] Results are expressed as S.I.s with the response to H.Ohio sonicate as the background control (<180 cpm) for each line; standard deviation [SD], <10%).

served. Each of the eight subjects tested had a different proliferative profile in response to the picornaviruses tested and the results from two subjects are detailed (Fig. 3).

In all subjects studied (eight of eight), there was no cross-reactive response with EMCV, suggesting that the proliferation in response to EMCV in fresh PBMCs was in response to this virus alone. All subjects exhibited some broad cross-reactivity between the three polioviruses. Good proliferative responses to all seven picornaviruses were observed in subject JB (Fig. 3A). The background level of proliferation in response to H.Ohio sonicate was particularly

TABLE 4. Proliferation in response to PV3 and CVB3 of picornavirus-specific clonally derived T-cell lines derived from PBMCs of subject JS

Clonally derived line[a]	Proliferation in response to test virus[b]	
	PV3	CVB3
PV3-1	212	1.1
PV3-2	35	0.9
PV3-3	5.1	2.1
PV3-4	43	1.4
PV3-6	98	1.7
PV3-13	36	2.1
PV3-18	144	1.7
PV3-19	14	1.4
PV3-20	77	1.5
PV3-23	24	2.3
PV3-27	52	2.8
PV3-28	69	1.8
PV3-31	13	2.1
PV3-44	124	1.3
PV3-45	22	1.6
PV3-46	115	1.4
PV3-47	73	0.9
PV3-53	12	2.1
PV3-60	14	1.8
PV3-61	22	1.5
PV3-62	15	1.3
PV3-65	35	2.9
CVB3-159	2.7	65
CVB3-151[c]	47	66
T-PV3-10	222	1.4
T-PV3-11	30	2.0

[a] Clonally derived T-cell lines were obtained by limiting dilution in round-bottomed microtiter plates, except for those derived from cloning by hanging drop in Terasaki plates (T-PV3-10 and -11).

[b] Results are expressed as S.I.s with H.Ohio sonicate as background control (proliferation in response to H.Ohio sonicate, <200 cpm; SD, <10%).

[c] Cross-reactive clonally derived T-cell line.

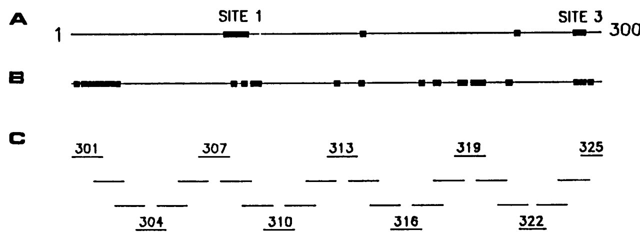

FIG. 4. (A) VP1 capsid protein of P3/Leon $12a_1b$ (45). The blocked-in areas are residues known to contribute to the immunodominant B-cell antigenic site 1 of PV3. The residues that contribute to site 3 are also marked (36, 37, 39). (B) Residues which vary between all three poliovirus serotypes (blocked-in areas), as assessed by visual analysis of the amino acid sequence (46). (C) Sequence alignment of the initial 25 synthetic VP1 peptides. Peptides were synthesized by using T-boc chemistry (19).

high in this subject, but the general pattern of cross-reactive proliferation in all virus-specific T-cell lines, except that generated against EMCV, is typical of the majority of the subjects tested. CVB4 and the three poliovirus serotypes cross-reacted very strongly in all of the cell lines tested, with the exception of EMCV.

Subject LB (Fig. 3B) showed good proliferative responses to PV1, and lesser responses to PV2 and PV3. The cell line generated in response to PV2 was poliovirus specific and failed to cross-react to a significant level (S.I., <3) with any of the other picornaviruses (48). The CVB4 T-cell line cross-reacted very strongly with PV2 and PV3, but the poliovirus cell lines did not cross-react as strongly with CVB4.

The ratio of $CD4^+$ to $CD8^+$ T cells did not change appreciably during short-term bulk culture in the presence of inactivated virus, implying that there was no preferential outgrowth of a particular T-cell phenotype under the culture conditions used.

Proliferation of clonally derived T-cell lines in response to picornaviruses. The results suggested that specific stimulation with EMCV may occur in vivo but that considerable cross-reactivity was present. In order to ascertain whether the cross-reactive responses seen with T-cell lines could be demonstrated at the clonal level, a number of picornavirus-specific clonally derived T-cell lines were grown in both inverted Terasaki plates (26) and 96-well round-bottomed plates. Of the 30 picornavirus-specific clonally derived T-cell lines obtained from the two subjects, all were $CD4^+$. The majority of clonally derived T-cell lines obtained were PV3 specific. Two cross-reactive clonally derived T-cell lines were also obtained, both of which were generated in response to CVB3 but in different subjects. The clonally derived T-cell line CVB3-C2 responded to both CVB3 and CVB4 by proliferation, and line CVB3-151 responded to both CVB3 and PV3 (Table 3 and Table 4). Although the probability that each of these cell lines was clonal was >99.9% (29), it has not been possible to reclone these clonally derived T-cell lines, and in the absence of absolute proof as to the clonality of these lines, it remains possible that two or more $CD4^+$ T-cell populations were present.

Proliferation of PBMCs in response to synthetic peptides of PV3 VP1. The serotype-specific nature of some of the PV3-specific clonally derived T-cell lines suggested that the epitope or epitopes recognized by such lines may lie in VP1, the most hypervariable of the picornavirus capsid proteins. PBMCs from eight subjects were stimulated with each of 25 synthetic peptides synthesized from VP1 of PV3. The position of the VP1 residues involved in B-cell antigenic sites 1 and 3, the amino acid variability of this protein, and the spatial relationship of each of the 25 synthetic peptides to VP1 of P3/Leon $12a_1b$ are shown in Fig. 4. The responses of PBMCs from two subjects to these peptides are shown in Fig. 5. Subjects LB and EW both responded to a restricted region—peptide 309, encompassing residues 97 to 114 of VP1.

Six of eight subjects tested made a strong response to peptide 308 and/or peptide 309. The observed response of PBMCs is mostly mediated by $CD4^+$, because depletion of PBMCs of $CD4^+$ T cells abrogated the normal proliferative response of fresh PBMCs to either PV3 or peptide 309, $CD4^+$ depletion of PBMCs resulting in <2% of the proliferation of $CD8^+$-depleted T cells (Table 5).

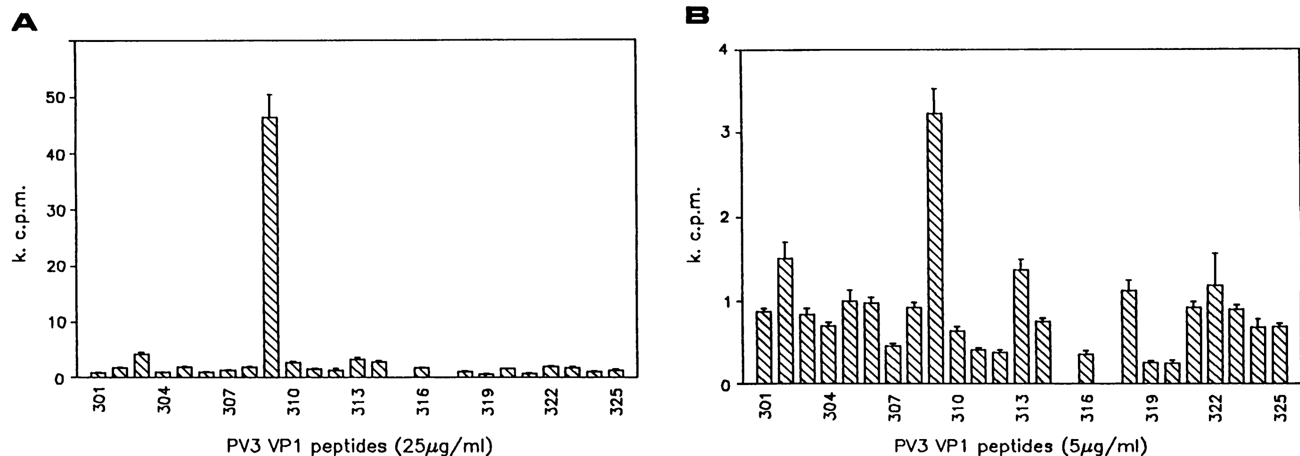

FIG. 5. Proliferation of PBMCs from subjects LB (A) and EW (B) in response to synthetic peptides of the VP1 protein of PV3. PBMCs were resuspended in RPMI-AB and tested at 2×10^5 cells per well in triplicate. The cells were incubated for 6 days with each peptide at a range of concentrations, as described in Materials and Methods. The Δ cpm of each response is plotted together with its SD (response to the medium control was <300 cpm). Peptides 315 and 317 were not available for testing.

TABLE 5. Proliferation of PBMCs in response to PV3 and peptide 309 on depletion of $CD4^+$ and $CD8^+$ T-cell subsets

Subject (antigen)	Proliferative response of[a]:		
	Whole PBMCs	$CD4^+$-depleted PBMCs[b]	$CD8^+$-depleted PBMCs[c]
EW (PV3)	18	0.3	23
EW (peptide 309)	24	0.3	27
SG (PV3)	9.6	1.8	16
SG (peptide 309)	11	2.1	17

[a] Results are expressed as the S.I.s of proliferation stimulated by either PV3 with H.Ohio cell sonicate as the background control (range, 300 to 700 cpm) or peptide 309 with medium alone as the peptide control (range, 200 to 400 cpm).
[b] Depletion of $CD4^+$ subset from PBMCs was 98% for EW and 85% for SG.
[c] Depletion of $CD8^+$ subset was 95% for EW and 80% for SG.

Proliferation of PV3-specific and VP1 peptide-specific T-cell lines in response to synthetic peptides of PV3 VP1. Most of the response of fresh PBMCs to the VP1 peptides was to the hypervariable regions of the molecule (Fig. 4B and Table 6). We wished to determine whether this specificity was maintained in PV3-stimulated T-cell lines and whether the peptide could induce virus-reactive T cells. Thus, both PV3-stimulated and peptide-stimulated T-cell lines were assayed for their responses to peptide. Twelve of 12 T-cell lines from eight subjects responded to several peptides, sometimes, but not always, mirroring the response seen on stimulation of fresh PBMCs from the same subjects (Fig. 6). The five peptide-generated T-cell lines also responded to PV3 and cross-reacted with several of the other picornaviruses tested but not with EMCV (Table 7).

Further delineation of PV3 epitopes in subjects LB, EW, and MA. An additional nine peptides covering the sequences of peptides 308 and 309 were synthesized (Fig. 1B). The amino acid sequence of this region and the relative positions of these nine synthetic peptides are shown in Fig. 7. The dose-response curves of PBMCs from subjects LB, EW, and MA in response to some of these peptides are shown in Fig. 8. LB responded strongly to both peptides 355 and 356, with the response decreasing in the order of peptides 309 > 356 > 355, proliferation being strongly dose-dependent. There was little or no response to the other peptides tested. EW responded in a different manner to these peptides (Fig. 8B), only responding at concentrations of peptide exceeding 25 μg/ml. Subject MA responded particularly well to peptide 354 (Fig. 8C) and to peptide 353 at higher concentrations.

DISCUSSION

This study demonstrates a picornavirus-specific T-cell response in normal, healthy subjects to a number of different strains. Picornavirus-specific T-cell lines cross-react, although the response to EMCV was specific. Exclusively serotype-specific and cross-reactive responses have been shown at the clonal level. In addition, the use of synthetic overlapping peptides has identified T-cell epitopes, which in each instance overlap or are adjacent to the linear portion of B-cell antigenic sites in VP1 (36, 37, 39).

Virus culture conditions were initially optimized so that high-titer virus was obtained from the supernatant of infected cell cultures derived from a single cell line, thus giving reproducible proliferative results, with low levels of background activity on stimulation of PBMCs. The individual polyclonal T-cell responses of 29 subjects against up to seven human picornaviruses (PV1, PV2, PV3, CVB3, CVB4, HRV14, and EMCV) varied. Interestingly, 21 of 29 subjects responded specifically to EMCV. Although, the T-cell response to these viruses was reproducible, responses

TABLE 6. Summary of PBMC responses to PV3 VP1-derived synthetic peptides

Peptide[a]	Response to PV3 VP1 peptide by PBMCs from subject[b]							
	JW	MA	JS	EW	LB	SG	JB	TB
301	−	−	−	−	−	−	−	−
302	−	−	−	+	−	−	−	−
303	−	−	−	−	−	−	−	−
304	+	−	−	−	−	−	+	+
305	−	−	−	−	−	+	−	−
306	−	−	−	−	−	+	−	−
307	−	−	−	−	−	−	−	−
308	−	+	−	−	−	−	+	−
309	+	+	−	+	+	+	−	−
310	−	−	−	−	−	−	−	−
311	−	−	−	−	−	−	−	−
312	−	−	−	−	−	−	−	−
313	−	−	+	+	+	−	+	−
314	−	−	−	−	+	+	−	+
316	−	−	−	−	−	−	−	−
318	−	−	−	−	−	−	−	−
319	−	−	−	−	−	−	−	−
320	−	−	−	−	−	−	−	−
321	−	−	−	−	−	−	+	−
322	−	−	−	+	−	+	+	+
323	−	−	−	−	−	+	−	+
324	−	−	−	−	−	−	−	−
325	−	−	−	−	−	−	−	−
Mean ± SE	2,728 ± 1,898	1,332 ± 1,479	1,670 ± 1,130	890 ± 595	3,655 ± 9,148	2,433 ± 2,045	2,403 ± 2,031	1,231 ± 717

[a] The response to peptides 315 and 317 was not tested.
[b] +, response to peptide >2SE above the mean for all peptides tested; −, response to peptide <2SE above the mean for all peptides tested.

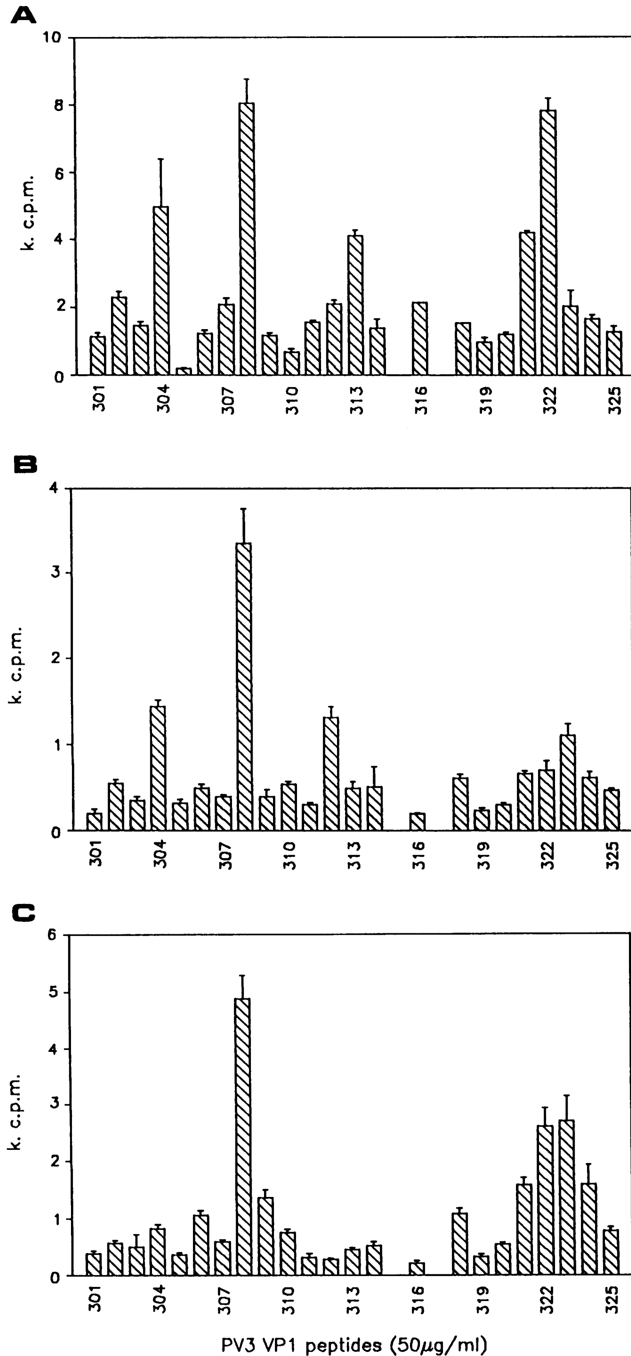

FIG. 6. Proliferation of T cells from subject JB in response to synthetic peptides of the VP1 protein of PV3. PBMCs (A) were incubated with peptide, as described in the legend to Fig. 5. A PV3-stimulated T-cell line (B) and a VP1 peptide-stimulated T-cell line generated from a pool of all of the VP1 peptides (C) were incubated for 3 days with peptide. The Δ cpm of each response is plotted together with its SD (response to medium control was <300 cpm). Peptides 315 and 317 were not available for testing.

TABLE 7. Proliferative response to picornaviruses of T-cell lines generated in response to a pool of 25 PV3 VP1-derived peptides

Virus	Proliferative response of T-cell lines from subject[a]				
	JB	JW	SG	EW	JS
PV1	17	4.3	7.3	11	2.3
PV2	21	4.7	9.2	14	2.5
PV3	28	7.2	13	12	5.3
CVB3	15	3.8	4.6	7.5	1.9
CVB4	17	5.6	6.8	9.0	2.6
HRV14	12	6.1	8.7	11	1.4
EMCV	1.3	0.9	2.1	2.3	0.7

[a] Results are expressed as S.I.s with H.Ohio sonicate as background control for each virus (SD, <10%; control values, 300 to 1,100 cpm).

to these viruses in an individual changed with infection in vivo and after poliovirus vaccination (16a).

To address the question of T-cell cross-reactivity between these viruses with extensively conserved primary polyprotein structures (40), 130 virus-specific T-cell lines were raised against the seven viruses studied. In each instance, the proliferative response to each of the viruses in the panel was then estimated. Six of seven picornaviruses generally gave rise to cross-reactive T-cell lines, as observed in studies of experimental murine (3, 25, 28), and bovine (9) infections and in previous studies with human T cells (4). In each case, EMCV-specific lines were the exception, because they did not cross-react against any of the other six picornaviruses. EMCV is the most distant of the picornaviruses studied, at the level of primary structure (40), which may explain the lack of cross-reactivity. The apparent specificity of this response suggests that human infection with EMCV, of which isolated instances are documented (11, 43, 50), may be more prevalent. Alternatively, a closely related, hitherto unidentified cardiovirus or aphthovirus could have induced cross-reactive T-cell responses which would not have been detected by the restricted panel of picornaviruses used in this study.

Although cross-reactivity in polyclonal T-cell populations was observed, we wished to establish whether cross-reactivity occurred at the level of the single T-cell epitope. Picornavirus-specific clonally derived T-cell lines were generated by a number of techniques, and both exclusively serotype-specific and broadly cross-reactive clonally derived lines which cross-reacted between serotypes and even different picornavirus strains were found. The conditions used to initiate and expand these clonally derived T-cell lines may have favored a high number of serotype-specific lines. We are investigating whether further stimulation of virus-specific T-cell lines with other picornaviruses before cloning may generate more cross-reactive clones.

The location of epitopes recognized by $CD4^+$ T cells was

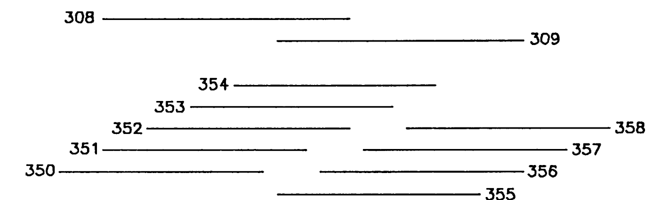

FIG. 7. Amino acid sequence of VP1 region encompassing peptides 308 and 309, and positions of peptides 350 to 358 in relation to the original synthetic peptides 308 and 309 of PV3 VP1. The peptides were synthesized as described in Materials and Methods.

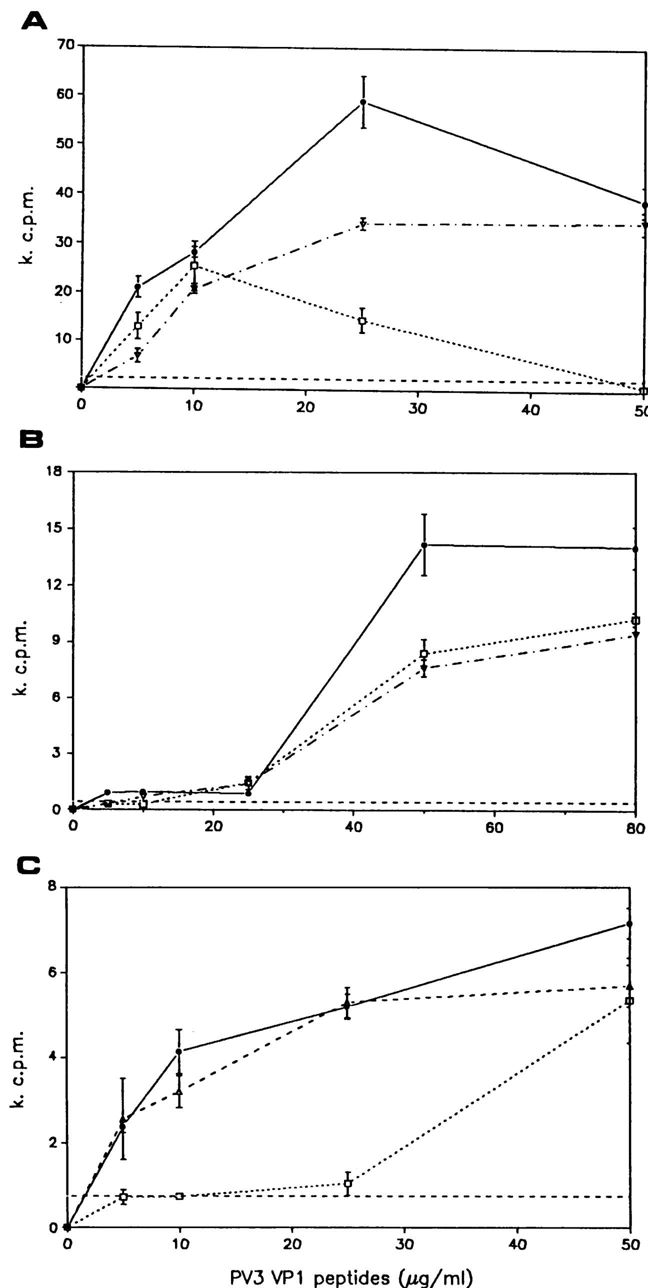

FIG. 8. Dose-response curves of proliferation of PBMCs from three subjects in response to VP1 peptides of PV3. Results are expressed as Δ cpm ± SE, with medium alone as the background control (control response was <500 cpm). (A and B) Responses of subjects LB and EW, respectively, to synthetic peptides 309 (●), 355 (□), and 356 (▽). The mean response, calculated over all concentrations tested, to peptides 354 and 357 is plotted as a peptide control (– – –). (C) Response of subject MA to peptides 308 (●), 354 (▽), and 353 (□). The mean response to peptides 352 and 356 is plotted as a control (– – –).

then considered. $CD4^+$ cells recognize purified PV1 and PV3, as well as heat-inactivated or live-virus-containing supernatants, which additionally contain nonstructural proteins released from infected cells. This suggested that the predominant response may be directed against the structural capsid proteins. Furthermore, the majority of clonally derived T-cell lines were group specific. By sequence analysis, VP1 was identified as the most variable of the four capsid proteins (46), and peptides encompassing the entire sequence were synthesized. Using fresh PBMCs, one or more T-cell epitopes in VP1 of PV3 was identified in all eight subjects. In each instance, the identified epitope was within or adjacent to the B-cell sites identified by neutralizing monoclonal antibodies (36, 39). The predominant site in six of eight individuals was located in peptides 308 and 309 within or next to B-cell antigenic site 1. We attempted to further localize this epitope in three subjects by using a subset of shorter overlapping peptides. This suggests that two subjects (LB and EW) recognized a similar determinant (encompassed by peptides 355, 356, and 309: probable sequence [residues 97 to 114], TRAQKLFAMWRITY KDTV). The third subject (MA) recognized a different epitope within the same region (encompassed by peptides 308, 309, 353, and 354: probable sequence [residues 91 to 108], DNEQPTTRAQKLFAMWRI). Further mapping of these responses is in progress, making use of peptides with single amino acid deletions and substitutions.

Interestingly, a similar site in PV1 has been shown to be a T-cell epitope in mice (28). The identification of these T-cell epitopes, either overlapping or adjacent to the B-cell antigenic site 1 in PV3 in humans, suggests that the murine PV1 T-cell epitope may be recognized by human T cells. The requirement for spatial proximity of T- and B-cell epitopes has been suggested in a number of antibody responses. A naturally occurring T-cell epitope, overlapping a B-cell antigenic site, was previously shown in mice, with the hemagglutinin of influenza A virus (2, 16). Other recent studies have identified adjacent human B- and T-cell epitopes in Murray Valley encephalitis virus (31) and herpes simplex virus (18). This suggests that efficient presentation of virus antigens to elicit a functional, neutralizing antibody requires an adjacent T-cell epitope. It may be that such adjacent epitopes are also required for efficient production of antibodies specific for nonviral antigens, as in *Streptococcus mutans* infection (30).

The identification of such T-cell epitopes could allow the development of these viruses as vectors for simple subunit or single epitope vaccines. Alternatively, peptides which artificially place T-cell and B-cell epitopes adjacent to one another could be synthesized. Such peptides have already been used to generate efficient specific antibody responses (8, 15, 34). However, poliovirus could be modified to express both the T-cell epitope identified and relevant B-cell epitopes inserted into antigenic site 1 of PV3. Poliovirus chimeras have been constructed to exchange the residues comprising the linear portion of B-cell antigenic site 1 (6, 13, 24, 38). However, the T-cell epitope we have identified is disrupted in these chimeras, and this may account for the relative inefficiency of these synthetic viruses in inducing an antibody response (38). Thus, further identification of T-cell epitopes will be fundamental to the determination of the correct site for insertion of B-cell epitopes in chimeric viruses in order to generate effective B-cell immunity.

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