T-Cell Responses to Predicted Amphipathic Peptides of Varicella-Zoster Virus Glycoproteins II and IV

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Four peptides from the predicted amino acid sequences of varicella-zoster virus (VZV) glycoproteins II and IV selected for potential amphipathicity and a terminal lysine residue were synthesized. The peptides elicited weak proliferative responses by T cells with the CD4⁺ UCHL1⁺ CD45R⁻ phenotype from the blood of VZV-immune individuals. The frequency of responder cells in individuals with specific response to peptides was 1:80,000 or fewer blood mononuclear cells, and the number of peptides responded to did not correlate with the proliferative response to VZV antigen. Of 40 peptide-specific T-cell clones obtained by limiting dilution, 10 were restimulated by extracted VZV antigen in the presence of autologous antigen-presenting cells. A total of 50% of these clones lysed HLA class II-positive lymphoblasts which had been preincubated with the appropriate peptide, and 2 of 15 cytotoxic clones lysed lymphoblast targets superinfected with VZV. The data indicated that T cells with specificity for putative VZV peptides may readily be cultured from the subset of blood mononuclear cells which bears the phenotype associated with memory.

Varicella-zoster virus (VZV) is medically important as the cause of shingles, which, in the elderly, is commonly followed by postherpetic neuralgia (5). Previous observations link severe or lethal primary VZV infections with immunosuppression (11) and an increased frequency of virus reactivation from latency with increasing age (13). Aging is accompanied by a reduction in T-cell responses *in vivo* and *in vitro* (10) and a reduced frequency of VZV-specific T cells in the blood (14). Existing data regarding *in vitro* T-cell responses to VZV have mostly been obtained by stimulating blood mononuclear cells (MNC) with VZV-infected fibroblasts (21) or with antigens extracted from VZV-infected fibroblasts (25), but precise analysis of these responses has been hindered by uncertainty as to the antigens recognized. Proliferative T-cell responses to individual VZV glycoproteins have been demonstrated (2, 13a), but glycoprotein purification is laborious and it is difficult to prepare sufficient material to test large numbers of subjects. One strategy for the characterization of microbial antigens recognized by T cells is to test peptides either encompassing defined motifs (22) or with helical amphipathicity (4). Information is still accumulating concerning human immune responses to microbial peptides (3, 9, 18, 23, 24), and a T-cell epitope has been reported in residues 1 to 23 of herpes simplex virus gD (8). As a preliminary step toward defining antigenic epitopes on the major VZV glycoproteins and nucleoproteins, we tested peptides from VZV glycoproteins II and IV for T-cell responses as measured by proliferation and cytotoxicity. The criteria for selection of peptides were amphipathicity as identified by the algorithm of Margalit et al. (19) plus the presence of a terminal lysine residue.

MATERIALS AND METHODS

Subjects and separation of MNC. Blood was obtained from 17 consenting healthy adults, defibrinated, and centrifuged to recover serum. Three subjects had no history of VZV infection; their blood MNC did not proliferate in cultures stimulated with VZV antigen and their sera were negative for VZV antibody in an enzyme-linked immunosorbent assay (15). Fourteen VZV-immune subjects were studied. Their

ages ranged from 18 to 30 years, and their serum anti-VZV antibody levels were within 1 standard deviation of a laboratory positive reference serum (their mean was 97% of the reference; range, 89 to 105%). All had had VZV infection in childhood (i.e., more than 10 years before study), and none had had herpes zoster. Blood lymphocytes from each of the 14 VZV-immune individuals proliferated in cultures stimulated with VZV antigen.

The blood MNC were separated by Ficoll-Hypaque centrifugation as described before (14) and suspended at 10⁶/ml in RPMI 1640 medium supplemented with 10% autologous serum. For certain experiments, the responding population was depleted of cells expressing the 180-kilodalton switch variant of the T200 antigen by first incubating 10⁷ cells in 100 μ l of Hanks balanced salt solution with 10 μ l of UCHL1 ascites (20). After 30 min on ice, the cells were washed and 8 $\times$ 10⁸ magnetic anti-mouse immunoglobulin G particles (Advanced Magnetics, Cambridge, Mass.) were added. The mixture was rocked at room temperature for 15 min, and then the beads were precipitated with a magnet. Cells which remained in the supernatant were washed in Hanks balanced salt solution and resuspended in medium as described above. HLA-DR typing was by microcytotoxicity with B-cell-enriched preparations and Terasaki plates.

VZV antigens and peptides. VZV antigen was extracted from human fetal skin fibroblasts which had reached 70% cytopathic effect after infection with VZV as previously described (25). The peptides selected for testing are shown in Table 1. Their sequences are based on the DNA sequence of Davison and Scott (7), and their amphipathic scores are predicted from the algorithm of Margalit et al. (19). These peptides were selected for the presence of a C-terminal lysine and an amphipathic score of >13. They were synthesized by Multiple Peptide Systems (San Diego, Calif.).

Cultures. MNC were cultured at 10⁶ cells per ml in 100- μ l aliquots in Linbro microwell plates at 37°C for 6 days before being pulsed for 6 h and harvested onto glass fiber paper as described before (14). Limiting dilution cultures were at 100,000, 50,000, 25,000, and 12,500 cells per well and were pulsed and harvested after 10 days of culture as previously

TABLE 1. Sequence and amphipathic scores of VZV peptides

Peptide	VZV glycoprotein	Amino acid No.	Sequence	Amphipathic score ^a
P1	II	139-149	EITDTIDKFGK	36.8
P2	II	769-779	LPEGMDPFAEK	38.6
P3	IV	197-207	ARLCDLPATPK	17.8
P4	IV	344-354	PHSVVNPFFVK	14.4

^a Determined by program of Margalit et al. (19).

described (6). To prepare peptide-specific clones, we seeded 96-well plates with 25,000 cells per well and 20 µg of peptide antigen per ml. After 7 days, 100 µl of medium with autologous serum and 20 U of recombinant interleukin-2 (Ala-125 interleukin-2; Amgen, Thousand Oaks, Calif.) per ml was added per well and incubation continued for a further 8 days. Clones visible to the naked eye were recovered and expanded by restimulation with antigen and 2,000-rad-irradiated autologous cells (as a source of antigen-presenting cells).

The restimulation of T cells cultured with peptide or VZV antigen was essentially as previously described (21) and included controls with unrelated donor antigen-presenting cells and an irrelevant antigen. These cultures were pulsed after 68 h and harvested at 72 h.

Cytotoxicity assays. Lysis of uninfected, peptide preincubated, and VZV-superinfected Epstein-Barr virus (EBV)-transformed lymphoblasts was measured as before (16). Briefly, autologous and unrelated lymphoblasts were either preincubated with or without 100 µg of peptide per ml for 18 h or superinfected with VZV for 3 days before they were washed and radiolabeled (100 µCi of [⁵¹Cr]sodium chromate [CJS 1P; Amersham Corp., Arlington Heights, Ill.] per 10⁶ cells) for use as targets in a 4-h chromium release assay. The effector:target cell ratio was 20:1.

RESULTS

T-cell proliferative responses to peptides. Blood MNC from donors of different HLA types were stimulated with VZV antigen and VZV peptides for 6 days and then pulsed and harvested. The results (Table 2) indicated that all MNC from the donors responded to VZV and to at least one of the peptides tested and that MNC from one of the six donors tested responded to all four peptides. The results shown were obtained with peptide concentrations of 2 µg/ml. Higher concentrations (10 to 20 µg/ml) did not increase thymidine uptake, and in most instances, the response did not fall off at concentrations between 20 µg/ml and 2 mg/ml (data not shown).

It was clear from these data that proliferative responses to

FIG. 1. Limiting dilution culture showing mean percentage of nonresponder wells for 24 replicates of MNC from four donors. The MNC were stimulated with VZV antigen for 10 days. The intercept at 37% nonresponder wells is at 18,000 cells per well.

different peptides varied in different donors and that the aggregate response to peptide was smaller than that to VZV antigen. MNC were next cultured at limiting dilution with between 1×10^5 and 1.2×10^4 cells per well with and without peptides or VZV antigens in a preliminary attempt to calculate the frequency of responder T cells (RCF). The results (Fig. 1) indicated that the positive control, VZV antigen, had a mean RCF of 1:18,000, giving the expected straight-line relationship between log percent nonresponder wells and cell number per well. The RCF to the peptide antigens was uniformly below $1:10^5$ in this assay (data not shown). It was not possible to increase the number of cells per well because of an unacceptably high background thymidine uptake from wells containing $>10^5$ cells. Consequently, to obtain an approximation to the frequency of MNC responding to the peptides, 25,000 cells per well in replicates of 100 wells were cultured with and without antigen. Interleukin-2 was added to these cultures after 7 days of incubation, and the frequency of wells with macroscopically visible growth was determined after 18 days. The results (Table 3) suggested that the frequency of cells responding to peptides ranged between 1:83,000 and 1:250,000 of blood MNC between different individuals. Note that this approach does not permit the assumption that a single cell limits the proliferative response to the peptide to be tested by checking a straight-line relationship between log percent nonresponder wells and cell number per well. These data are therefore not directly comparable with standard limiting dilution analyses.

Attempts were made to detect serum antibody to the

TABLE 2. Proliferative responses of MNC to VZV and peptides^a

DR	Mean cpm $\pm$ SE with the following stimulus:					
	Control	VZV	P1	P2	P3	P4
2/4	590 $\pm$ 29	9,780 $\pm$ 120	620 $\pm$ 57	400 $\pm$ 30	2,290 $\pm$ 210	7,260 $\pm$ 420
2/6	1,660 $\pm$ 65	14,700 $\pm$ 400	1,160 $\pm$ 20	4,330 $\pm$ 260	1,600 $\pm$ 42	1,812 $\pm$ 30
3/3	1,770 $\pm$ 70	28,430 $\pm$ 300	1,280 $\pm$ 16	11,510 $\pm$ 610	3,130 $\pm$ 421	9,100 $\pm$ 80
3/4	443 $\pm$ 20	16,220 $\pm$ 380	4,402 $\pm$ 37	2,189 $\pm$ 100	4,090 $\pm$ 330	9,272 $\pm$ 270
3/4	1,220 $\pm$ 270	8,570 $\pm$ 360	1,866 $\pm$ 200	4,240 $\pm$ 540	3,980 $\pm$ 60	1,100 $\pm$ 49
6/7	1,360 $\pm$ 45	17,690 $\pm$ 550	1,640 $\pm$ 60	2,830 $\pm$ 244	1,990 $\pm$ 78	990 $\pm$ 80

^a Cells (250,000) were incubated in triplicate U wells and pulsed with tritiated thymidine for 4 h on day 6 of culture. Results are the mean counts per minute $\pm$ 1 standard error for six unrelated donors. VZV, 1:100 dilution of VZV antigen. P1 to P4, Peptides 1 to 4 shown in Table 1.

TABLE 3. Frequency of responder T cells in individuals responding to peptides^a

Peptide	Frequency of responders	
	Mean	Range
P1	1:260,000	187,000–320,000
P2	1:180,000	147,000–330,000
P3	1:122,000	83,000–370,000
P4	1:370,000	120,000–400,000

^a Six donors were studied whose responses encompassed all four peptides. Frequency of responders (calculated as number of responding wells/number of cells cultured) is derived from responding subjects only.

peptides in individuals with and without T-cell proliferative responses to them in an enzyme-linked immunosorbent assay (15) by using plastic or nitrocellulose as the immobilizing agent for the peptide. These results were uniformly negative (data not shown).

Phenotype and frequency of T cells responding to peptides. The phenotype of proliferating MNC in cultures stimulated by peptides was determined by combined cell surface immunofluorescence with CD4 or UCHL1 followed by fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G and DNA staining with propidium iodide. Of the cells from five stimulated cultures, $4 \pm 2\%$ were in G2, S, or M and their phenotype was CD4⁺ UCHL1⁺. Because UCHL1⁺ cells may be derived from UCHL1⁺ CD45R⁺ precursors, negative depletion experiments were undertaken to determine the phenotype of the precursors of the proliferating cells. Removal of the UCHL1⁺ subset abrogated a subsequent proliferative response to VZV antigen and VZV peptides, while depletion of CD45R⁺ cells was without effect. Note that irradiated MNC were added back to the depleted subsets in these studies to replace antigen-presenting cells which might have been lost during the negative depletion process.

Specificity of T cells responding to VZV peptides. Three donors who lacked immunity to VZV as judged by lack of serum immunoglobulin G antibody or T-cell proliferative response to the virus were identified. None of the lymphocytes from these individuals responded to any of the four peptides tested or to VZV antigen (Table 4, row 1). None of these donors has developed chicken pox, so it was not possible to correlate response to peptide with VZV immunity prospectively. Lack of immunity to VZV is uncommon,

so restimulation assays were used as an alternative test for VZV specificity of cells stimulated with peptide. Blood MNC from five VZV-immune donors were stimulated with VZV antigen or peptides for 6 days, when blasts were enriched on a Percoll gradients. Responder cultures (defined as those from which $>10^6$ T-cell blasts were obtained) were then restimulated with VZV or peptides in the presence of autologous and unrelated antigen-presenting cells. The results (Table 4, row 2) indicated that the VZV antigen-stimulated cultures were restimulated by VZV antigen but not by the peptides. Rows 3 to 6 show that cultures stimulated initially with the peptides are restimulated by the corresponding peptide and respond weakly or not at all to the VZV antigen. Restimulation was not obtained with unrelated donor cells, whether or not peptide or antigen was added, as previously found (21; data not shown).

The restimulation of bulk cultures gives no indication of the frequency of T cells with specificity for a peptide which would respond to VZV antigen. To assess this, we prepared 40 clones (10 for each peptide) by stimulating MNC from three different donors with the peptides. There were seven clones with specificity for peptides 1, 2, or 4 and nine with specificity for peptide 3. Two of the peptide 1 clones, one of the peptide 2 clones, four of the peptide 3 clones, and two of the peptide 4 clones were restimulated by VZV antigen in the presence of autologous antigen-presenting cells. None of the 30 clones tested responded to an irrelevant antigen, tetanus toxoid. For comparison, T-cell clones were also prepared from cultures stimulated with VZV antigen, and these were restimulated with peptide and autologous antigen-presenting cells. Only two positive responses were obtained among 90 clones tested.

Cytotoxicity by peptide-specific T cells. The ability of T-cell clones to lyse targets expressing the homologous or a heterologous peptide was tested by using autologous EBV lymphoblasts as targets. The choice of target cells stemmed from the expression of both class I and class II HLA antigens on EBV lymphoblasts. The target cells were preincubated in medium containing 20 μ g of peptide per ml or with viable VZV for 18 h before testing. Of 30 clones tested, 15 showed specific cytotoxicity for targets preincubated with the homologous peptide. The means and standard errors of isotope release for these cytotoxic clones are given in Table 5. Two of these cytotoxic clones, one specific for peptide 1

TABLE 4. Specificity of T-cell responses to peptides

Cells tested	cpm ($\pm$ SE) of lymphocytes stimulated with:					
	0	VZV antigen	P1	P2	P3	P4
Nonimmune MNC ^a	440 $\pm$ 40	390 $\pm$ 80	450 $\pm$ 40	390 $\pm$ 58	280 $\pm$ 40	502 $\pm$ 120
Immune, VZV antigen-stimulated MNC ^b	380 $\pm$ 220	12,430 $\pm$ 1,200	330 $\pm$ 160	262 $\pm$ 190	420 $\pm$ 270	370 $\pm$ 150
Immune, peptide-stimulated MNC ^c						
P1	1,220 $\pm$ 80	3,870 $\pm$ 50	12,660 $\pm$ 300	1,190 $\pm$ 110	890 $\pm$ 80	1,700 $\pm$ 300
P2	880 $\pm$ 70	4,240 $\pm$ 280	1,100 $\pm$ 90	7,600 $\pm$ 700	650 $\pm$ 60	680 $\pm$ 60
P3	1,800 $\pm$ 100	4,290 $\pm$ 200	1,770 $\pm$ 450	990 $\pm$ 120	5,660 $\pm$ 300	2,000 $\pm$ 312
P4	690 $\pm$ 80	2,990 $\pm$ 70	750 $\pm$ 100	760 $\pm$ 95	580 $\pm$ 120	9,800 $\pm$ 220

^a Blood MNC from three donors without VZV immunity were stimulated as described in Table 1 and pulsed and harvested on day 6.

^b Thymidine uptake by T-cell blasts obtained from cultures of blood MNC from five VZV-immune donors. Cells were stimulated for 6 days in vitro with VZV antigen; then blasts were recovered and restimulated with autologous irradiated MNC as a source of antigen-presenting cells, and with VZV antigen or 20 μ g of peptide per ml.

^c Thymidine uptake by T-cell blasts obtained from cultures initially stimulated with peptide.

TABLE 5. Peptide-specific lysis of autologous EBV lymphoblasts by T-cell clones^a

Effectors stimulated with:	Specific lysis of targets preincubated with:				
	0	P1	P2	P3	P4
P1	3 ± 1	46 ± 12	5 ± 2	5 ± 2	2 ± 1
P2	5 ± 3	7 ± 3	55 ± 17	9 ± 4	7 ± 4
P3	9 ± 5	6 ± 3	10 ± 5	38 ± 3	6 ± 4
P4	4 ± 1	4 ± 2	7 ± 4	8 ± 3	29 ± 5

^a Results shown are mean ± 1 standard error of specific isotope release from targets incubated with peptide-stimulated T-cell clones in a 4-h chromium release cytotoxicity test.

and one for peptide 3, lysed the targets prepared with viable VZV.

DISCUSSION

Peptides with sequences predicted from the DNA sequences of pathogens (including human immunodeficiency virus, herpes simplex virus, and diphtheria and tetanus toxins) have all been reported to stimulate T lymphocytes from immune donors. The rationale for the study of peptide-specific T-cell responses derives from the evidence that antigen-presenting cells employ lysosomal proteolytic enzymes to digest phagocytosed material and then reexpress peptide fragments of the original antigen in association with class II major histocompatibility complex (MHC) antigens. Studies in mice indicate that members of an inbred line respond to some but not all peptides which could be obtained by antigen digestion. Limited binding of a peptide to class II MHC itself is one possible cause of nonresponsiveness (12), but deletion of potentially reactive clones as a consequence of self-tolerance is likely to be as important (17). The potential a peptide has for binding to class II MHC may in part be addressed through an algorithm which identifies potential amphipathic helices, although the extent to which this approach increases the chance of identifying an antigenic peptide is not yet established.

The criterion of potential amphipathic helical structure appears to have been useful for the identification of the four peptides tested here. Nevertheless, it is clear that other structures can elicit T-cell responses. For example, the 1-23 peptide of herpes simplex virus gD reported by DeFreitas et al. (8) (which was identified by virtue of an adjacent antibody epitope) has an amphipathic score of only 12.2 but does have the adjacent hydrophobic residues which contribute to the motif described by Rotbard et al. (22). The 1273-1284 tetanus toxin sequence reported by Demotz et al. (9) has an amphipathic score of 4.1, while the 830-843 tetanus toxin peptide in the same report has no predicted amphipathic segments whether analyzed for 7 or 11 amino acid blocks. Despite these negative observations, the algorithm of Margalit et al. (19) has nevertheless predicted immunogenic peptides in human immunodeficiency virus (3) and, in this study, in VZV glycoproteins II and IV. The failure of the VZV peptides we studied to bind antibody was expected, in that antibodies to glycoproteins are often directed toward conformational epitopes.

In the studies reported here, most of the MNC from VZV-immune donors proliferated in culture with one or two of the peptides of VZV glycoproteins II and IV, but none of MNC from the six donors responded to all of the peptides. The failure of MNC from some donors to respond to some peptides was expected from results in other systems. For

example, Demotz et al. (9) observed variability in restimulation of tetanus toxoid-specific T-cell clones by HLA-DR 3.3-matched antigen-presenting cells. While an immune response gene effect is the most likely explanation for this variability, we were unable to undertake the extended family studies which would be necessary to establish this as the mechanism in the case of VZV peptide responses. Unfortunately, currently available serological typing for HLA-DR does not distinguish all the polymorphisms which contribute to antigen binding, so the dissimilar responses to the peptides of the two individuals who typed as HLA-DR 3/4 might result from genotypic differences or from restriction to DP or DQ antigens.

The amount of thymidine uptake in cultures stimulated with peptides was uniformly smaller than that in cultures stimulated with VZV antigen, as might be expected from the diversity of VZV-encoded products in the antigen preparation. The greater response of peptide-specific T cells to restimulation by homologous peptide than by VZV antigen may reflect a low production of these epitopes by antigen-presenting cells in vitro. An alternative and equally likely possibility is that there may be competition for binding to MHC epitopes between the numerous different peptides which antigen-presenting cells may make from VZV antigen, as has been reported in in vitro models (reviewed in reference 12). Limiting dilution cultures with up to 100,000 cells per well gave a measurable responder cell frequency for only two of eight donors, both for the 107 to 117 region of glycoprotein IV. The low overall RCF for the VZV peptides tested is not surprising, given that multiple epitopes are likely to exist on VZV gene products and that the RCF for VZV antigen is only in the 1:10,000 to 1:20,000 range in young adults. The CD4⁺ phenotype of the peptide-responsive cells suggests that peptide presentation to T cells in the cultures was in association with class II MHC products, in agreement with our previous findings using intact VZV glycoproteins to elicit responses. The UCHL1⁺ phenotype indicates that the responder T cells were derived from the subset associated with immunologic memory (1).

Although the specificity for homologous peptide of T cells cultured from MNC of VZV-immune subjects was readily established by restimulation and proliferation, the potential role of these cells in VZV immunity is less secure. Overall, only 9 of 30 peptide-specific clones proliferated when restimulated by VZV antigen rather than peptide. Of the 15 clones which were cytotoxic for homologous peptide targets, only 2 lysed target lymphoblasts which had been superinfected with VZV. Whether the targets resisted lysis by the other 13 peptide-specific clones because the appropriate peptide was either not expressed or expressed at too low a density on the target cell surface was not determined. Other reports of human peptide-specific lines or clones shed little or no light on the issue of microbial specificity, either because peptide-specific clones were tested only against peptide (rather than the intact pathogen) or because the clones described were selected for study from an unstated number which were screened. The occurrence of VZV peptide-specific T cells among the memory T-cell pool of VZV-immune subjects, but not the three nonimmune subjects would be compatible with their induction by a VZV infection, although prospective immunization studies will be required to confirm this. At least for VZV, it seems clear that about one-half of T cells responding to a peptide can express cytotoxicity for target cells bearing the same determinant and that frequency of cells responding to any one peptide is much smaller than the

frequency of cells responding to the whole viral antigen extract.

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