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Specific Lysis of Targets Expressing Varicella-Zoster Virus gpI or gpIV by CD4$^+$ Human T-Cell Clones

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Varicella-zoster virus (VZV)-specific CD4-positive T cells are known to lyse targets expressing VZV antigen, but little is known of the glycoprotein specificity or phenotype of these cells. To test the ability of T cells to distinguish between gpI and gpIV (which share an antibody-defined epitope), we prepared clones from blood from four healthy individuals by limiting dilution. Among 68 T-cell clones from four donors which were VZV specific in tests of proliferation, 30 lysed autologous Epstein-Barr virus-transformed lymphoblasts which had been superinfected with a recombinant vaccinia virus which included the whole VZV gpI sequence. These clones were characterized as major histocompatibility complex class II restricted by inhibition of their cytotoxicity with HLA-DR and CD4 monoclonal antibodies. Twenty-one clones lysed targets expressing gpIV. Fifteen of these clones lysed targets expressing gpI and gpIV. Four clones with gpI-gpIV specificity were examined in detail, and their dual specificity was confirmed by cold target inhibition. These four clones failed to kill target cells infected with a mutant gpIV recombinant vaccinia virus from which amino acid residues 212 to 354 had been deleted. This region includes one of the two gpIV decapeptides which have 50% homology with amino acids 111 to 121 of gpI. Our data confirm that T-cell-receptor-associated structures are required for specific lysis of VZV targets and indicate that (i) gpI-specific CD4 cytotoxic T cells outnumber gpIV-specific T cells in blood and (ii) 50% of gpI-specific T-cell clones also lyse gpIV-expressing targets.

Cell-mediated immune responses, including specific cell-mediated cytotoxicity and cytokine production by activated T cells, are thought to be important for the clearing of viruses following infection. Natural infection with varicella-zoster virus (VZV) is followed by the appearance of VZV-specific T cells in blood (1, 6, 8) as well as VZV-specific antibodies (15). The temporal association between VZV reactivation in the elderly and a reduction of T-cell but not antibody levels with aging (12) has drawn attention to VZV-specific T-cell responses. Previous studies indicate that about 1 in 14,000 blood mononuclear cells (MNC) proliferates in cultures stimulated by VZV antigen (5), that most of these cells are CD4$^+$ (11), and that they mediate class II-restricted cytotoxicity as well as antigen-specific help for B cells (13).

The role of class II-restricted cytotoxicity by VZV-specific T cells in vivo is uncertain, and little is known of the use these cells make of accessory molecules for target cell lysis or their VZV antigen specificity. We previously showed that T-cell clones specific for glycoprotein I (gpI), gpII, and gpIII could be cultured from blood (9, 11), but the limited availability of purified glycoproteins prevented our testing these clones for glycoprotein-specific cytotoxicity. The use of vaccinia virus recombinants overcomes problems of limited glycoprotein availability, and Arvin et al. (1) have recently shown that gpI and the IE62 gene product can both serve as targets for cytotoxicity. The studies reported here were undertaken to look for additional target glycoproteins and to determine whether such cross-reactions could occur between different gene products. We selected gpI and gpIV for this purpose, as the reading frames for these glycoproteins are adjacent in the VZV genome and they are known to share at least one antibody-defined epitope (18, 19).

MATERIALS AND METHODS

Viruses. The vaccinia virus-VZV gpI construct was prepared by Cabirac et al. (4). It employs the WR strain of vaccinia virus into which the *BglI* fragment of VZV, which includes the entire gpI sequence, has been cloned. The recombinant vaccinia virus (rvv) expressing VZV gpIV (designated rvv-gpIV) was prepared as described in reference 20. Briefly, the *AvaI* DNA fragment (spanning nucleotides 114385 to 115760 of the VZV genome [7]) containing the coding region of the gpIV gene was cloned into vaccinia virus by using the pSC11 plasmid. The gpIV rvv mutant was prepared by excising DNA from the pSC11 recombinant plasmid between the *KpnI* and *NcoI* sites (from 115128 to 115741) and then blunt ending and religating (16). The vaccinia virus recombinants were grown in human fetal skin fibroblasts, and the titers of the stock viruses obtained were determined by plaque assay. Their titers were 1.2×10^7, 1.3×10^7, and 0.7×10^7 PFU/ml for rvv-gpI, rvv-gpIV, and vaccinia virus WR strain, respectively. The rvvs were stored at −20°C.

Preparation of VZV-specific T-cell clones. The frequency of donors' blood MNC with specificity for VZV was determined by limiting dilution culture with an extracted VZV antigen (22) as previously described (5). Linearity of the slope of log (percent nonresponder wells) against cell number per well, with extrapolation of the line to 1, was verified for each donor. To prepare clones, MNC were cultured at densities to allow one responder cell per well in 96-well plates and a 1:200 dilution of extracted VZV antigen. After 7 days, 100 μl of medium with autologous serum and 20 U of recombinant interleukin-2 (Ala-125 interleukin-2; Amgen,

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TABLE 1. HLA type of donors and source of clones

Donor no.	HLA type	Total no. of clones with specificity for:			Clone no. (Table 3)
		gpI-gpIV	gpI only	gpIV only	
1	A29, B8 Bw58, DRw15, DRw17	6	10	0	1, 2, 5, 6
2	A3A31, B18 Bw61, DRw15 DRw14	3	4	3	3
3	A1 A32, B17 Bw52, DR2, DR3	5	0	1	4, 7, 8
4	A2 A3, B7 B44, DR3, DR4	1	1	2	9

Thousand Oaks, Calif.) was added per well, and incubation continued for a further 2 weeks. Clones visible to the naked eye were recovered and expanded by restimulation with antigen and 2,000-rad-irradiated autologous blood MNC as previously described (13). The clones used are numbered, and the HLA type of their donors is shown in Table 1.

Monoclonal antibodies and immunofluorescent staining. The antibodies used were as follows: CD4 = OKT4; CD8 = OKT8; CD45R0 = UCHL1; T-cell receptor Vβ5 = 1C1; T-cell receptor Vβ8 = Mx6. All were grown as ascites and diluted for use. Antibodies to Vβ6 and Vβ12 were purchased from T Cell Sciences (Cambridge, Mass.). For immunofluorescence, CD4, CD8, and 1C1 were used as fluorescein isothiocyanate conjugates and Mx6 was used as a phycoerythrin conjugate. The remaining antibodies were localized by fluorescein isothiocyanate goat anti-mouse immunoglobulin G. Stained cells were washed in Hanks balanced salt solution and fixed in 1% paraformaldehyde in phosphate-buffered saline, and their fluorescence was quantitated on an EPICS C (Coulter Instruments, Hialeah, Fla.) or by fluorescence microscopy. The anti-VZV monoclonal antibodies were 79.7 and 100.4, both previously reported to bind to VZV gpI and gpIV (19) and to be of the immunoglobulin G2 isotype.

Preparation of autologous targets expressing VZV gpI or gpIV. Epstein-Barr virus (EBV)-transformed B-cell lines were prepared by infecting blood MNC with B95-8 in the presence of 1 μg of cyclosporine A per ml. After 2 to 3 weeks of culture, the lines were frozen in medium with 10% dimethyl sulfoxide and were stored in liquid N_2. To prepare targets, we infected B lymphoblasts with rvv (gpI or gpIV) or with the wild WR strain of vaccinia virus by adding the cell-free virus at a multiplicity of infection of 5 to 10. The rvv-superinfected EBV-transformed B lymphoblasts were permissive for replication of rvv as judged by an increase in rvv titer after inoculation. Overnight infection with rvv did not affect the expression of HLA-DR on B-lymphoblast target cells as judged by staining with fluorescein isothiocyanate anti-HLA-DR antibody and fluorescence-activated cell sorter analysis. Time course studies indicated that rvv-infected targets were optimal for cytotoxicity tests after overnight infection.

Cytotoxicity assay and inhibition by monoclonal antibodies or cold targets. Lysis of targets was determined in a standard chromium (^{51}Cr) release assay. rvv (gpI or gpIV)- or vaccinia virus (WR strain)-infected and uninfected EBV-transformed lymphoblasts were radiolabeled with 100 μCi of sodium [^{51}Cr]chromate (CJS 1P; Amersham Corp., Arlington Heights, Ill.) per 10^6 cells at 37°C for 60 min. The targets were washed, mixed with the cloned effector T cells, and briefly centrifuged (50 × *g*, 4 min) before incubation at 37°C. After 6 h, 100 μl of supernatant was harvested and counted. The percent specific lysis was determined as 100 × [(experimental release − spontaneous release)/(maximal release − spontaneous release)]. In certain experiments, the cloned T effector cells were incubated with monoclonal antibodies against HLA-DR, CD4, T-cell receptor Vβ5, or T-cell receptor Vβ8 for 30 min on ice before incubation with targets.

Cold target inhibition tests of double-specific clones used a 4- to 16-fold excess of unlabeled gpI and gpIV rvv- and wild-type strain vaccinia virus (WR)-infected autologous EBV-transformed B lymphoblasts as cold targets. The cold targets were mixed with labeled targets before cloned T cells were added.

RESULTS

Expression of VZV glycoprotein on rvv-infected cells. Expression of VZV gpI and gpIV following infection with the rvvs was tested by immunofluorescent staining of fixed fibroblasts in which the virus had been grown. The results (Table 2) indicate that the gpI-gpIV monoclonal antibodies, 100.4 and 79.7, stain cells infected with recombinant gpI and gpIV virus. However, the gpIV recombinant from which bp 115128 to 115741 had been excised was not stained by the 79.7 monoclonal antibody. Comparable staining for VZV with the monoclonal antibodies was obtained by using EBV-transformed B lymphoblasts cultured for 48 h with the rvvs before fixation and staining (Fig. 1 shows rvv-gpIV-infected lymphoblasts stained with monoclonal antibody 79.7). Trypan blue exclusion by lymphoblasts infected with wild-type vaccinia virus or rvv was >80% after 16 h (overnight) of culture and fell to 50% after >48 h of culture with the rvvs, so overnight culture was routinely used for the preparation of rvv targets for cytotoxicity experiments.

Phenotype of VZV-specific T-cell clones. Sixty-eight T-cell lines which were VZV specific in tests of proliferation (data not shown) were obtained from the limiting dilution cultures. All the lines were $CD4^+$ and all expressed the CD45R0 splice variant of CD45, which characterizes memory T cells. Analysis of the confidence interval for the regression lines from which the responder cell frequency was estimated indicated that there was only a 2% of chance that the wells which yielded these lines were derived from two or more responder

TABLE 2. Monoclonal antibody staining of fibroblasts infected with rvv[a]

Monoclonal antibody	Staining of cells infected with[b]:			
	rvv-gpI	rvv-gpIV	rvv-mgpIV	vv
79.7	+	+	−	−
100.4	+	+	+	−

[a] Fibroblasts were infected overnight with the rvv shown and then stained with one of two gpI-gpIV-specific monoclonal antibodies and fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G. Fibroblast fluorescence was determined by fluorescence microscopy and is recorded as positive (+) or negative (−).

[b] rvv-mgpIV, truncated VZV gpIV-rvv from which bases 115128 to 115741 had been excised; vv, wild-type vaccinia virus, WR strain.

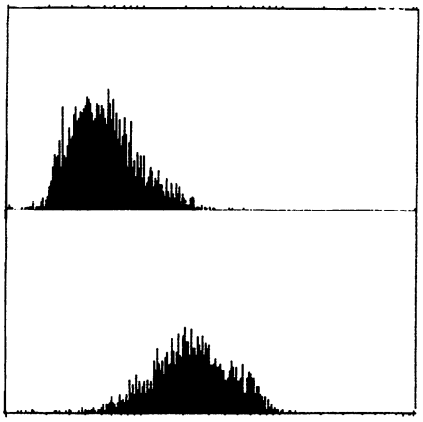

FIG. 1. Staining of EBV lymphoblasts infected with wild-type vaccinia virus (upper panel) or with rvv-gpI (lower panel) by monoclonal antibody 79.7. The lymphoblasts were cultured for 48 h with wild-type or recombinant virus and then fixed in methanol before staining. LGFL, log green fluorescence.

cells. For this reason, the lines derived from limiting dilution are described as clones. When the T-cell receptor Vβ usage of the clones was examined with the limited available range of human Vβ reagents, only one clone was positive (staining for the Vβ8 family with Mx6 approached 100% of cells). Low frequencies of cells stained for any of the other Vβ tested (Vβ5, Vβ6, or Vβ12), suggesting contamination of the clones, were not found.

Specificity of T-cell clones for HLA-DR and VZV. The T-cell clones lysed autologous targets expressing VZV antigens, and this lysis of target cells was inhibited by CD4 and HLA-DR antibodies but not by CD8 antibody (Table 3 shows results from five representative clones; clones 3, 4, and 5 were tested at two effector cell/target cell ratios). The reduction in target cell lysis in the presence of CD4 and anti-HLA-DR is statistically significant by the Wilcoxon matched-pairs test ($P = 0.05$) and as expected for $CD4^+$ effectors, there was no inhibition by the anti-CD8 monoclonal antibody. At a 20:1 effector cell/target cell ratio, the mean lysis (±1 standard error) of these five clones for autologous rvv-gpI targets was 33 ± 5%, for autologous rvv targets it was 4 ± 3%, for major histocompatibility complex-mismatched rvv-gpI targets it was 7 ± 1.4%, and for uninfected targets it was 5 ± 0.8%. The restriction of lysis to autologous targets and the pattern of monoclonal antibody inhibition are those expected for major histocompatibility complex class II-restricted T cells. Participation of the T-cell receptor in target cell lysis was additionally confirmed for the single clone which expressed a defined Vβ family (Vβ8) by inhibition with the anti-Vβ8 antibody, Mx6 (Fig. 2).

TABLE 3. Inhibition of cytotoxicity by CD4 and HLA-DR monoclonal antibodies[a]

Clone	% Specific lysis of targets (mean ± 1 SE) in presence of:				
	Anti-CD4	Anti-CD8	Anti-HLA-DR	No antibody	E:T[b]
1	46 ± 3.6	ND[c]	30 ± 0.1	90 ± 1.7	40:1
2	63 ± 1.9	ND	66 ± 4.2	87 ± 2.4	40:1
3	37 ± 2.7	55 ± 5.5	29 ± 1.4	48 ± 4.8	20:1
3	1 ± 0	14 ± 0	0 ± 0	14 ± 0	10:1
4	24 ± 1.5	36 ± 2.0	21 ± 1.8	39 ± 1.8	20:1
4	11 ± 0.6	27 ± 1.3	8 ± 2.9	26 ± 2.2	10:1
5	25 ± 1.0	38 ± 2.9	20 ± 2.3	40 ± 2.0	20:1
5	9 ± 1.0	23 ± 2.1	7 ± 1.1	21 ± 0.6	10:1

[a] Effector cells were preincubated with monoclonal antibodies for 30 min before testing on autologous lymphoblasts infected with rvv-gpI.
[b] E:T, effector cell/target cell ratio.
[c] ND, not determined.

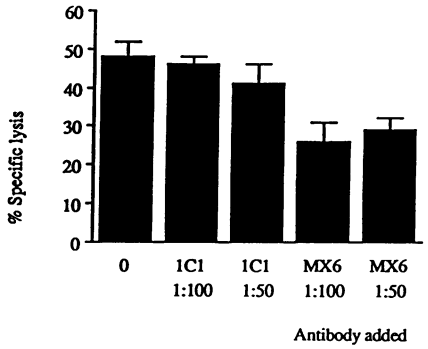

FIG. 2. Antibody to Vβ8 inhibits cytotoxicity by the Vβ8-positive gpI-specific clone 2. The effectors were preincubated with Mx6 (Vβ8) or 1C1 (Vβ5) at the dilutions shown for 30 min on ice before being mixed with the target cells at a 20:1 effector cell/target cell ratio.

Sharing of an epitope for cytotoxicity between gpI and gpIV. Thirty of the 68 VZV-specific T-cell clones tested lysed autologous lymphoblast targets superinfected with rvv-gpI. Twenty-one clones lysed targets superinfected with rvv-gpIV. Fifteen (22%) of the clones lysed targets expressing either gpI or gpIV (Table 4). The frequency with which T-cell clones lysed gpIV-infected targets as well as gpI targets was unexpectedly high. The absence of lysis of control targets infected with vaccinia virus alone made it unlikely that the effectors with dual specificity were responding to the vaccinia virus or to an irrelevant antigen such as fetal calf serum or that they were merely nonspecifically cytotoxic. There was a weak but statistically significant positive correlation between the killing of gpI and gpIV targets ($r = 0.47$; $P < 0.05$; Fig. 3) by the clones with dual specificity.

All nine of the gpI-gpIV-specific T-cell clones tested failed to lyse the mutant gpIV from which bp 115128 to 115741 had been removed. Of the six clones with specificity for gpIV, two lysed the mutant gpIV and four did not.

To obtain more definitive evidence for epitope sharing between gpI and gpIV, we maintained five clones showing dual specificity in culture for cold target inhibition studies. Representative results from one of these clones show that cold gpI targets compete for the lysis of hot gpIV targets

TABLE 4. Specificity for gpI and gpIV of T-cell clones

Result for rvv-gpIV targets[a]	No. of clones with result for rvv-gpI targets[b]		Total
	Lysis	No lysis	
Lysis	15	6	21
No lysis	15	32	47
Total	30	38	68

[a] rvv-gpIV target; VZV gpIV-rvv-infected autologous EBV-transformed B cell.
[b] gpI target; VZV gpI-rvv-infected autologous EBV-transformed B cell.

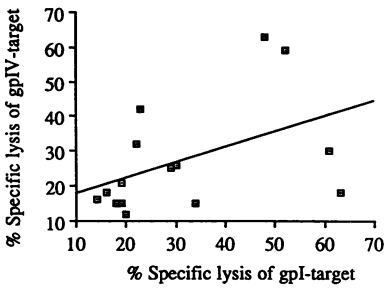

FIG. 3. Correlation between the lysis of rvv-gpI and rvv-gpIV targets by 15 gpI-gpIV-specific clones at 20:1 effector cell/target cell ratios.

TABLE 5. Inhibition of lysis of ^{51}Cr-labeled gpI or gpIV targets by gpI and gpIV cold targets

Hot target	Cold target[a]		
	vv	gpI	gpIV
gpI	22.6 ± 2.7	9.6 ± 2.5	9.2 ± 2.5
gpIV	30.8 ± 4.2	18.2 ± 4	16.6 ± 3.4

[a] Results are the mean percent specific lysis (±1 standard error) of gpI or gpIV targets by five clones in the presence of a four- to fivefold excess of autologous cold targets superinfected as follows: vv, rvv; gpI, gpI-rvv; gpIV, gpIV-rvv.

(Fig. 4a). In contrast, the cold gpI targets did not compete for the lysis of the hot gpIV targets by a gpIV-specific T-cell clone (Fig. 4b). The mean values for target cell lysis by the five clones in cold target inhibition studies employing a 10:1 effector cell/target cell ratio and a four- to fivefold excess of cold targets are shown in Table 5. Whether gpI- or gpIV-expressing cells were used as hot targets, their lysis was inhibited by cold targets expressing the reciprocal gpI or gpIV but not by targets expressing the wild-type vaccinia virus alone. This difference is statistically significant ($P < 0.005$ by the Wilcoxon paired sign rank test).

DISCUSSION

VZV causes a clinically trivial infection in most healthy children but is clinically important as a cause of herpes zoster (and postherpetic neuralgia) in the elderly and of lethal chicken pox in severely immunosuppressed subjects. Significant progress has been made in immunization against VZV with the Oka strain vaccine (3, 8, 17, 21), but the occasional occurrence of clinical symptoms in vaccine recipients has stimulated further studies of mechanisms of VZV immunity and the immunogenicity of VZV-encoded products. Our previous studies indicated that all the T-cell clones grown from blood under limiting dilution culture conditions were CD4$^+$ T cells and that they lysed VZV-bearing targets with major histocompatibility complex class II restriction. Arvin et al. (1) have lately shown that CD8 T cells with specificity for VZV glycoproteins can be cultured from blood depleted of CD4$^+$ cells, and we found that class I-restricted VZV-specific cytotoxic T cells could be cultured from guinea pigs by cross priming (10). In other studies using proliferation by blood T cells as an end point, we found that more T cells proliferated in response to gpI than to gpII or gpIII, but the limited amounts of glycoprotein which could be purified by immunoaffinity techniques prevented our testing these cells for glycoprotein-specific cytotoxicity (11). Major histocompatibility complex class II restriction of the effector cells studied here was shown by inhibition of cytotoxicity by monoclonal antibodies to CD4 and HLA-DR but not by CD8. The inhibition of the single clone expressing Vβ8 by the monoclonal antibody Mx6 is consistent with participation of the T-cell receptor in antigen binding to VZV-expressing targets.

In the present study, we used vaccinia virus-VZV-gpI recombinants to obtain expression of selected viral glycoproteins on the target cells. This approach has previously been successfully used to locate influenza virus epitopes (2) and show cross-reactivity by influenza virus-specific cytotoxic T lymphocytes. Thirty of the 68 VZV-specific T-cell clones studied here lysed targets expressing the rvv-gpI recombinant. The absence of lysis of targets infected with the vaccinia virus alone argues against their cytotoxicity being nonspecific or specific for confounding antigens (such as might derive from the EBV or from fetal calf serum). The high proportion of clones lysing gpI targets in the present study is consistent with our previous finding that gpI was the commonest specificity of clones characterized by proliferative responses to purified glycoproteins (11).

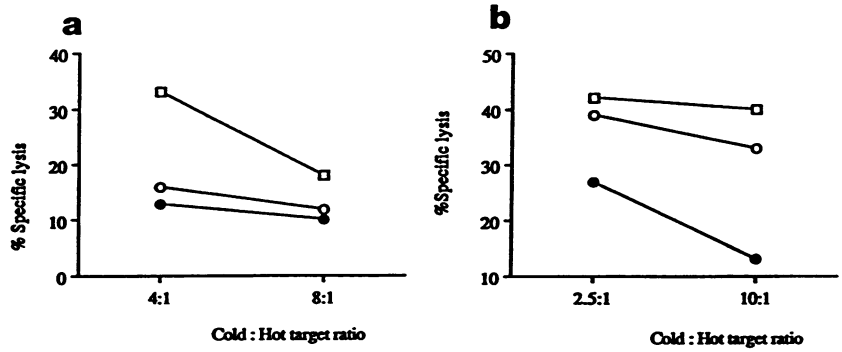

FIG. 4. (a) Lysis of hot rvv-gpIV targets by a gpI-gpIV-specific T-cell clone is equally reduced by cold rvv-gpI and rvv-gpIV targets, while the control (rvv) cold targets give less inhibition of lysis. (b) Only cold rvv-gpIV targets inhibit the lysis of rvv-gpIV targets by a gpIV-specific T-cell clone. The effector cell/hot target cell ratio in both these experiments was 20:1. ○, gpI; ●, gpIV; □, vaccinia virus.

Serological cross-reactivity between VZV gpI and gpIV by the 79.0 monoclonal antibody was described by Vafai et al. (18, 19). The gpI epitope recognized by this antibody was localized to the 14 amino acids between residues 107 and 121 of gpI. Although the gpIV epitope identified by this antibody was not defined, two regions displaying up to 36% sequence homology were identified between gpIV residues 55 to 69 and 245 to 259. Our present finding that cells infected with the rvv-gpIV mutant from which residues 212 to 354 had been excised did not stain suggests that these residues contribute to the gpIV epitope recognized by the 79 monoclonal antibody. The other gpI-gpIV-specific monoclonal antibody we tested, 100.4, did not require residues 212 to 354 of gpIV to bind to gpIV. The residues in the 55 to 69 region of gpIV may contribute to the epitope seen by this antibody.

In addition to serological cross-reactivity, our present report indicates that T-cell epitopes are shared between gpI and gpIV. Nonspecific cytotoxicity was clearly not the explanation for the lysis of the two targets since control targets infected with wild-type vaccinia virus were not lysed. Other possible explanations for the dual specificity observed could include the inadvertent culture of polyclonal lines or the genuine sharing of epitopes. The limiting dilution cultures from which the T-cell clones were obtained provide at least a statistical argument against polyclonality. This is because the logarithm of the percentage of nonresponding wells for the limiting dilution cultures from which the T-cell clones were obtained was linearly related to the cell number per well and the line extrapolated to 1, indicating that a single species of cell limits the response (14). The T-cell clones we studied were harvested at responder cell frequencies ranging from 1:9,000 to 1:25,000, at which only 2% of cultures would be expected to be polyclonal. The absence of low numbers of cells staining with any of the four Vβ family antibodies tested from all the clones we tested provides additional support for the clonal nature of the effector populations. The frequency with which the gpI-specific T-cell clones lysed gpIV targets is clearly far above the 2% chance of a polyclonal line which would be estimated from the Poisson distribution.

Aside from the statistical probability that the T cells we studied were clonal, the most secure evidence for the sharing of an epitope between gpI and gpIV comes from the cold target inhibition studies. When clones with single glycoprotein specificity were tested, the lysis of the hot target was more inhibited by addition of the relevant than an irrelevant cold target (Fig. 4b illustrates this). For the dual gpI-gpIV-specific clones, the lysis of gpI and gpIV targets was inhibited by addition of cold targets expressing the reciprocal glycoprotein (Fig. 4a). The failure of clones with dual specificity to lyse the rvv-mutant gpIV target suggests that amino acids 212 to 354 of gpIV include the epitope of gpIV seen by these clones. Amino acids 245 to 259 are therefore a candidate peptide for the gpIV epitope.

The T-cell receptor for antigen is thought to bind to a peptide derived from the antigen which is held in the antigen-binding groove on a histocompatibility antigen, so individual differences in VZV peptide binding and epitope recognition are to be expected. Nevertheless, T-cell clones identifying an epitope shared between gpI and gpIV were obtained from all four donors tested. It is therefore unlikely that our data arise from a chance association restricted to a single HLA type. Whether other shared VZV glycoprotein epitopes exist and whether a response to these epitopes will be influenced by the HLA antigens of the individual remain to be determined.

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