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# Vaccinia Virus-Specific Human CD4<sup>+</sup> Cytotoxic T-Lymphocyte Clones

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**Vaccinia virus-specific cytotoxic T-lymphocyte (CTL) clones were established from a healthy donor, who had been immunized with vaccinia virus vaccine, by stimulation of peripheral blood lymphocytes with UV-inactivated vaccinia virus antigen. The phenotype of all of the clones established was CD3<sup>+</sup> CD4<sup>+</sup> CD8<sup>-</sup> Leu11<sup>-</sup>. We used a panel of allogeneic vaccinia virus-infected B-lymphoblastoid cell lines and demonstrated that some of the clones recognized vaccinia virus epitopes presented by human leukocyte antigen (HLA) class II molecules. Monoclonal antibodies specific for either HLA-DP or HLA-DR determinant reduced the cytotoxicity of specific clones. The HLA-restricted cytotoxicity of the clones is vaccinia virus specific, because vaccinia virus-infected but not influenza virus-infected autologous target cells were lysed. Using vaccinia virus deletion mutants, we found that some of the CTL clones recognize an epitope(s) that lies within the *Hind*III KF regions of the vaccinia virus genome. These results indicate that heterogeneous CD4<sup>+</sup> CTL clones specific for vaccinia virus are induced in response to infection and may be important in recovery from and protection against poxvirus infections.**

There is a great deal of interest in the potential use of vaccinia virus as a vector for recombinant vaccines against human and veterinary diseases. Despite the success of vaccination in the eradication of smallpox and much evidence that cellular immune mechanisms are essential for virus elimination, little is known about cellular immune responses to vaccinia virus. In addition, vaccinia virus-specific, human leukocyte antigen (HLA)-restricted cytotoxic T lymphocytes (CTLs) have not been demonstrated in humans (12, 25), although virus-specific CTLs have been shown with many other human viruses. Studies with murine models have demonstrated the presence of vaccinia virus-specific CD8<sup>+</sup> CTL responses that are major histocompatibility complex (MHC) class I restricted (13, 22). Studies with different animal species have demonstrated that these virus-specific CTL responses correlate with recovery from poxvirus infection (1, 3, 5, 10) but have not assessed the potential effector contribution of CD4<sup>+</sup> CTLs as part of the recovery process.

In humans, the significance of vaccinia virus-specific cell-mediated immune responses has been demonstrated by studies of vaccinated children who had thymic aplasia. Some of these children died of progressive vaccinia after accidental immunization despite producing anti-vaccinia virus antibodies and treatment with massive doses of vaccine immunoglobulin (9, 23). Individuals with profound T-cell defects associated with Wiscott-Aldrich syndrome also developed disseminated infection after vaccination with vaccinia virus (14). These and other reports (7) indicate that antibody production alone is not sufficient to protect humans from the complications of poxvirus infection.

In an attempt to identify the nature of the cell-mediated cytotoxic responses in humans against vaccinia virus, we established cytotoxic T-cell clones directed against vaccinia virus-infected cells from a vaccinia virus-immune donor. These clones possess the CD4 surface marker and are vaccinia virus specific and class II restricted. The clones are

heterogeneous because they are restricted by different MHC class II alleles and recognize different regions of vaccinia virus. Our results support the concept that CTL-mediated lysis of vaccinia virus-infected target cells may be significant in restricting the spread of virus infection and in recovery from infection with poxvirus.

## MATERIALS AND METHODS

**Human PBMC.** Blood was obtained from a healthy donor who had received the standard New York City Board of Health (NYCBH) strain of vaccinia virus in smallpox vaccine (Dryvax; Wyeth Laboratories, Philadelphia, Pa.) 8 months earlier. Peripheral blood mononuclear cells (PBMC) were separated by Ficoll-Hypaque density gradient centrifugation method. Cells were resuspended at  $2 \times 10^7$ /ml in RPMI 1640 medium containing 10% fetal calf serum FCS (GIBCO Laboratories, Grand Island, N.Y.) and 10% dimethyl sulfoxide (Fisher Scientific Co., Pittsburgh, Pa.) and were cryopreserved until use.

**Vaccinia viruses.** The NYCBH strain of vaccinia virus and deletion mutants of the virus used in this study were provided by Gail Mazzara and Dennis Panicali of Applied Biotechnology, Inc. The deletion mutants were constructed by Lendon Payne and are shown in Table 1. Seven of these mutants were directly generated by *in vivo* recombination in cells infected with wild-type (NYCBH strain) vaccinia virus and transfected with plasmids containing altered vaccinia virus sequences, by methods previously described (29). vAbT213, which contains a deletion in the thymidine kinase gene, was selected by virtue of its growth in the presence of bromodeoxyuridine (29). The remaining six mutants in this group contained the *Escherichia coli lacZ* gene under the direction of a poxvirus promoter inserted at the site of the disrupted transcription unit. These mutants were selected by virtue of the  $\beta$ -galactosidase activity (24). vAbT342 and vAbT247-6 each arose as a result of intramolecular recombination between duplicated sequences in the genomes of recombinant viruses. vAbT342 was isolated as a small plaque variant of vAbT33, which contains a direct duplica-

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TABLE 1. Mutant vaccinia viruses used in this study

Virus designation	Deletion size (bp)	Transcription units <sup>a</sup> disrupted (gene identity)
vAbT213	420	J2R (thymidine kinase)
vAbT360	180	F4L (small subunit ribonucleotide reductase)
vAbT217	630	A56R (hemagglutinin)
vAbT33	790	M2L (30K) and K1L (29K host range)
vAbT71	3,460	M2L (30K), K1L (29K host range), and K3L
vAbT342	11,000	M2L (30K) through F7L
vAbT247-6	20,000	M2L (30K) through <i>HindIII-C</i> <sup>b</sup>
vAbT396		F7L
vAbT70		(growth factor) <sup>b</sup>

<sup>a</sup> As defined by Goebel et al. (11).

<sup>b</sup> Because these transcription units are located in the highly variable vaccinia virus termini, their identities cannot be defined by the sequence of Goebel et al. (11).

tion of the vaccinia virus BamF promoter separated by 11 kb. Recombination between the duplicated sequences resulted in the deletion of 11-kb intervening sequence. vAbT247-6 was generated from a recombinant virus containing a copy of the vaccinia virus p7.5 promoter inserted in the M2L transcription unit. The inserted p7.5 promoter recombined with the native p7.5 promoter at the left end of the vaccinia virus genome, resulting in the deletion of approximately 20 kb. Deletions were verified by isolation of genomic DNA and digestion with restriction enzymes and comparison with patterns of parental vaccinia virus genomic DNA. Southern blot analysis was also performed with appropriate DNA fragments to confirm the deletion.

**Preparation of vaccinia virus antigen.** CV1 cells grown in plastic flasks (Becton Dickinson Labware, Oxnard, Calif.) were infected with vaccinia virus at a multiplicity of infection of approximately 0.01 PFU per cell and cultured in Eagle's minimal essential medium containing 5% FCS until 50% of the cell monolayer exhibited cytopathic effects. The medium was almost all removed, and the cells were scraped from the flasks with rubber cell scrapers (Costar, Cambridge, Mass.), washed, resuspended in Eagle's minimal essential medium, and subjected to three cycles of rapid freeze-thawing. Cells were then sonicated for 2 to 3 min, trypsinized for 40 min at 37°C, sonicated twice, and centrifuged at 1,600 × g for 10 min. Approximately 1 ml of antigen was obtained from each 175-cm<sup>2</sup> flask of confluent CV1 cells and was stored at -70°C. The virus preparation was later thawed and inactivated by UV irradiation and used as the vaccinia virus antigen. The titer by PFU assay on CV1 cells was 6 × 10<sup>8</sup>/ml, and no plaques were detected after inactivation.

**Induction of proliferative responses of PBMC.** PBMC (2 × 10<sup>5</sup>) were cultured with the vaccinia virus antigens at various concentrations in 0.2 ml of RPMI 1640 medium containing 10% human AB serum (Hazelton Research Products, Inc., Lenexa, Kans.) in 96-well round-bottomed plates (Costar) at 37°C for 4 days. Cells were pulsed with 1.25 μCi of [<sup>3</sup>H]thymidine for 16 h before harvest. Cells were harvested by using a Titertek Multiharvester (Skatron, Inc., Sterling, Va.), and [<sup>3</sup>H]thymidine incorporation was counted in a liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.).

**Establishment of vaccinia virus-specific T-cell clones using a limiting dilution method.** PBMC (2 × 10<sup>7</sup>) were incubated for 2 h with UV-inactivated vaccinia virus at a final dilution of 1:10 in 0.5 ml of RPMI 1640 medium, washed once, and resuspended at 10<sup>6</sup> cells per ml in RPMI 1640 medium containing 10% human AB serum and supplemented with 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml). After 11 days, some of the stimulated cells were used as effectors in a CTL assay and the remaining cells were restimulated by adding gamma-irradiated (3600R) autologous PBMC that had been preincubated for 2 h with UV-inactivated vaccinia virus antigen, and resuspended in the medium described above plus 10% T-cell growth factor (Cellular Products, Inc., Buffalo, N.Y.). Two weeks later, clusters of proliferating blast cells were collected and cultured at concentrations of 1 and 3 cells per well in 96-well round-bottomed plates with 10<sup>5</sup> gamma-irradiated vaccinia virus antigen-pulsed autologous PBMC in 0.2 ml of the same medium. Once weekly, half of the medium was removed and replaced with fresh medium. On day 28, clones were expanded by transferring the cells in wells with positive growth to 48-well flat-bottomed tissue culture plates (Costar) containing 10<sup>6</sup> gamma-irradiated vaccinia virus antigen-pulsed autologous PBMC in 1 ml of RPMI 1640 medium containing 10% human AB serum and 10% T-cell growth factor.

**Preparation of target cells.** B-lymphoblastoid cell lines (B-LCL) were established by Epstein-Barr virus transformation of the PBMC from the vaccinia virus-immune donor (autologous) and from MHC HLA class I- and II-matched or mismatched allogeneic donors. Culture supernatant of B95-8 cells was used as an inoculum of Epstein-Barr virus. The B95-8 cells were provided by T. Sairenji of the University of Massachusetts Medical Center.

The B-LCL were either uninfected (control) or infected with vaccinia viruses (wild-type strain NYC8H and mutant viruses) at a multiplicity of infection of 10 for 12 to 16 h, labeled with sodium [<sup>51</sup>Cr]chromate (New England Nuclear, Boston, Mass.) for 1 h, washed three times, and resuspended at 2 × 10<sup>4</sup>/ml in RPMI 1640 medium containing 10% FCS for use as target cells in cytotoxicity assays.

**Cytotoxicity assays.** Chromium-labeled target cells (2 × 10<sup>3</sup>) in 0.1 ml of RPMI 1640 medium containing 10% FCS were added to each well in round-bottomed microtiter plates (Linbro Chemical Co., Hamden, Conn.). Various concentrations of effector cells in 0.1 ml of medium were added to each well to give the described effector/target (E/T) ratios. After a 6-h incubation at 37°C, the supernatant was harvested from each well and counted in a gamma counter. The assays were performed in triplicate wells, and the percent specific cell lysis was calculated by the formula 100 × (mean experimental release - mean spontaneous release)/(mean total release - mean spontaneous release). The results of an assay were excluded if the average spontaneous release was ≥30% or if the viability prior to <sup>51</sup>Cr labeling was <80%.

**HLA typing.** Most of the donor lymphocytes used in these experiments were typed for HLA-A, HLA-B, HLA-C, and HLA-D antigens on unfractionated PBMC by the Tissue Typing Laboratory at the George Washington University Medical Center. Some of the donors' lymphocytes were typed by using their Epstein-Barr virus-transformed B-cell lines in the Tissue Typing Laboratory at the University of Massachusetts Medical Center.

**Phenotype analysis.** Anti-Leu-2 (CD8) antibody reacts with suppressor-cytotoxic T cells (6). Anti-Leu-3 (CD4) antibody reacts with helper-inducer T cells (6). Anti-Leu-4 (CD3) antibody reacts with pan T cells (20). Anti-Leu-11a (CD16)

antibody reacts with natural killer cells (26). Anti-Leu-19 antibody also reacts with natural killer cells as well as with a subset of cytotoxic T cells that mediate non-MHC-restricted cytotoxicity (19). Anti-Leu-2, anti-Leu-3, anti-Leu-4, anti-Leu-11a, and anti-Leu-19 antibodies were purchased from Becton Dickinson and Co. (Mountain View, Calif.). Clones were stained with monoclonal antibodies (MAbs) conjugated with fluorescein isothiocyanate by direct immunofluorescence methods as described earlier (18). The percentage of antigen-positive cells was determined by using a fluorescence-activated cell sorter (440; Becton Dickinson and Co.).

**Antibody blocking of the lysis of vaccinia virus-infected target cells.** MAbs B7/21.7, S3/4, and OKIa1 recognize HLA-DP, HLA-DQ, and HLA-DR determinants, respectively. MAb W6/32 recognizes a framework determinant of HLA class I antigens. B7/21.7 and S3/4 were kindly provided by Nancy Reinsmoen of the University of Minnesota, Minneapolis. OKIa1 and W6/32 were provided by John Sullivan of the University of Massachusetts Medical Center. A total of  $2 \times 10^3$   $^{51}\text{Cr}$ -labeled target cells in 0.1 ml were incubated for 30 min with 0.05 ml of MAbs diluted 1:20. The effector cells were then added in 0.05 ml and incubated for 6 h. The percent specific cell lysis was determined as described above.

**Antibody complement depletion.** Anti-OKT4 (CD4) and anti-OKT8 (CD8) antibodies (Ortho Diagnostic Systems, Inc., Raritan, N.J.) and anti-Leu11b (CD16; Becton Dickinson Co.) were used in antibody complement depletion experiments. A total of  $2 \times 10^6$  virus-stimulated PBMC were suspended in 50  $\mu\text{l}$  of RPMI 1640 medium containing 1% FCS, 100  $\mu\text{l}$  of antibody diluted 1:15 was added, and the mixture was incubated at 4°C for 30 min. The cells were washed twice in cold RPMI 1640 medium and suspended in 100  $\mu\text{l}$ , to which 200  $\mu\text{l}$  of rabbit complement (Cedarlane Laboratories, Hornby, Ontario, Canada) diluted 1:3 was added, and the resulting solution was incubated for 1 h at 37°C. The cells were then washed three times in RPMI 1640 medium and were used in cytotoxicity assays. This treatment resulted in complete depletion of CD8<sup>+</sup> and Leu11b<sup>+</sup> cells, and 80% decrease in CD4<sup>+</sup> cells as demonstrated by fluorescence-activated sorting.

## RESULTS

**Proliferative response of PBMC to vaccinia virus antigens in bulk culture.** The immune responses to vaccinia virus antigen by a healthy donor, who had been immunized 8 months earlier with live vaccinia virus vaccine supplied by the Centers for Disease Control, were examined by culturing their PBMC with UV-inactivated vaccinia antigen and measuring [<sup>3</sup>H]thymidine incorporation. The PBMC showed a dose-dependent response to vaccinia virus antigen, with maximal responses ranging from 11,000 to 30,000 cpm (data not shown). No responses were detected when the culture supernatant of uninfected CV1 cells was used as a control antigen.

**Induction of cytotoxic effector cells against vaccinia virus-infected autologous target cells.** The donor's PBMC were stimulated in vitro with gamma-irradiated autologous PBMC pulsed with UV-inactivated vaccinia virus and maintained in medium containing 10% heat-inactivated human AB serum and 10% T-cell growth factor. After 11 days of in vitro culture, the proliferating cells were tested for cytotoxic activity against autologous vaccinia virus-infected B-LCL, which had been demonstrated in preliminary experiments to contain vaccinia virus antigens by fluorescent-antibody

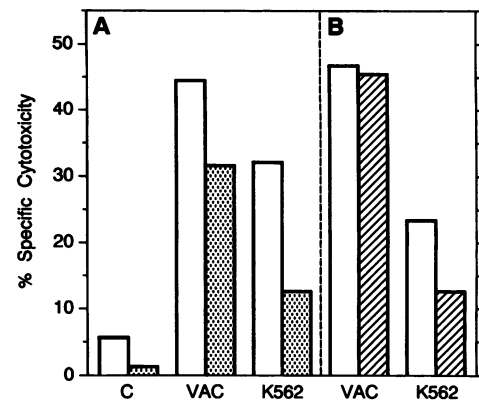


FIG. 1. Cytotoxic activity of the in vitro culture of effector cells against uninfected (C) or vaccinia virus-infected (VAC) autologous B-LCL and K562 cells. (A) CTL response with E/T ratios of 50:1 (□) and 25:1 (▨) on day 11. (B) CTL response after depletion of the Leu11 population on day 21 (□) with an E/T ratio of 50:1. Spontaneous release was 15 to 22%.

staining (data not presented). As shown in Fig. 1A, the stimulated effector cell population exhibited significant lysis of vaccinia virus-infected autologous targets ranging from 30 to 50% but did not kill uninfected targets (E/T ratios, 25:1 and 50:1). The level of cytotoxicity of vaccinia virus-infected targets was much higher than the lysis of the NK-sensitive tumor cell line K562, which suggested that some of the killer cells were vaccinia virus specific. In a repeat experiment (Fig. 1B), depletion of the Leu11 population by treatment with MAb and complement demonstrated that the level of lysis of vaccinia virus-infected target cells was unaffected while the lytic activity to K562 cells was inhibited by 50% after such treatment.

**Determination of the nature of cytotoxic effector cells.** Antibody complement depletion studies were carried out after 22 days of in vitro culture to determine the identity of the killer cells (Table 2). Treatment of the effector cells with anti-CD4 and complement reduced their lytic capacity against vaccinia virus-infected targets by 53%. Similarly, depletion of the CD8<sup>+</sup> population with anti-CD8 and complement resulted in a 51% reduction in cytotoxicity, whereas treatment with anti-Leu11b and complement failed to induce a reduction in the cytotoxic capacity of the effector cells. These results indicate that the cytotoxicity of the bulk culture cell line against vaccinia virus-infected targets reflected killing activity by both CD4<sup>+</sup> and CD8<sup>+</sup> CTLs.

**Establishment of vaccinia virus-specific CD4<sup>+</sup> T-cell clones.** We attempted to clone vaccinia virus-specific CTLs using a

TABLE 2. Characterization of cytotoxic activity<sup>a</sup>

Treatment	% Specific <sup>51</sup> Cr release from target cells		% Inhibition
	C	VAC	
No antibody	12.8	46.3	
Complement	11.2	43.5	
Anti-CD4 + complement	9.5	21.8	53
Anti-CD8 + complement	7.0	22.9	51
Anti-Leu11b + complement	11.7	41.0	11

<sup>a</sup> Antibody complement depletion studies using effector cells on day 22 of in vitro culture, with an E/T ratio of 25:1 in a 5-h assay.

TABLE 3. Cytotoxic activity of clones generated from a vaccinia virus-immune donor

Expt no. and JC clone no.	% Specific <sup>51</sup> Cr release from target cells <sup>a</sup>			
	Uninfected	VAC	K562	Flu
Expt 1 <sup>b</sup>				
1	3.0	47.7	1.1	
11	-2.2	49.4	-2.1	
46	-1.7	32.1	-0.8	
51	-2.3	47.8	-0.6	
88	1.8	24.2	0.7	
31	1.9	6.8	1.2	
36	1.1	0.9	1.0	
Expt 2 <sup>c</sup>				
12	0.3	14.7		2.4
22	0	26.6		-3.2
30	3.6	39.9		6.5
33	1.4	30.5		1.2
32	14.7	NT		35.1
35	5.5	NT		15.4
DG	18.6	NT		57.2

<sup>a</sup> Abbreviations: VAC, vaccinia virus-infected cells; Flu, influenza virus-infected cells; NT, not tested.

<sup>b</sup> Clones established by UV-inactivated vaccinia virus stimulation of PBL from a healthy donor were tested for their ability to lyse uninfected or vaccinia virus-infected autologous B-LCL and K562 cells at an E/T ratio of 3:1.

<sup>c</sup> Clones JC-12, JC-22, JC-30, and JC-33 were tested for their ability to lyse uninfected, vaccinia virus-infected and influenza virus-infected autologous B-LCL at an E/T ratio of 3:1. Influenza virus-specific clones (32, 35, and DG) that had been established by stimulation of PBL with influenza virus (A/PC/1/73) were included to show that influenza virus-infected autologous B-LCL, which were not lysed by the vaccinia virus-specific clones, are susceptible to lysis.

limiting dilution method as detailed in Materials and Methods. A total of 87 clones were generated from 384 wells, with cloning efficiencies of 20% at 1 cell per well and 38% at 3 cells per well. Thirty-six of the clones were not cytotoxic for vaccinia virus-infected or uninfected autologous B-LCL or for K562 cells. The other 51 clones lysed vaccinia virus-infected autologous B-LCL but did not lyse K562 target cells or uninfected B-LCL. In Table 3, experiment 1 shows representative examples of the two broad categories of clones that were detected. Clones such as JC-1, JC-11, JC-46, JC-51, and JC-88 selectively lysed vaccinia virus-infected autologous B-LCL. Experiment 2 in Table 3 demonstrated that vaccinia virus CTL clones such as JC-12, JC-22, JC-30 and JC-33 did not lyse autologous B-LCL infected with influenza virus. Several influenza virus-specific CD4<sup>+</sup> CTL clones that had been derived by in vitro stimulation with influenza virus from the same donor (6a) are included as a control to demonstrate that the influenza virus-infected B-LCL were susceptible to lysis. These results therefore demonstrate that these vaccinia virus CTL clones are virus specific, since they lyse vaccinia virus-infected autologous B-LCL but not influenza virus-infected autologous B-LCL.

**Cell surface phenotypes of the vaccinia virus-specific CTL clones.** Phenotyping of the clones by fluorescence-activated cell sorting analysis (data not shown) showed them all to be CD3<sup>+</sup> CD4<sup>+</sup> CD8<sup>-</sup> Leu11<sup>-</sup>. Of the 21 clones tested for Leu19, 4 were positive and 17 were negative. The Leu19<sup>+</sup> clones are JC-3, JC-9, JC-11, and JC-49.

**HLA class II antigen-restricted lysis of target cells by T-cell clones.** The HLA restriction of lysis of target cells by vaccinia virus-specific T-cell clones was examined by using

TABLE 4. Effect of anti-HLA class I and II antibodies on the lysis of target cells by vaccinia virus-specific CTL clones<sup>a</sup>

JC clone no.	% Specific <sup>51</sup> Cr release for MAb <sup>b</sup> :				
	None	W6/32 (anti-class I)	B7/21.7 (anti-HLA-DP)	OKIa1 (anti-HLA-DR)	S3/4 (anti-HLA-DQ)
18	35.2	38.1	<b>13.7</b>	34.6	37.2
24	38.7	39.0	<b>16.2</b>	38.7	32.9
30	41.4	38.9	<b>20.1</b>	39.5	52.3
34	49.9	49.7	<b>22.4</b>	50.2	40.8
46	19.5	24.0	28.9	<b>0.8</b>	26.0
76	30.5	34.4	16.9	<b>4.1</b>	10.3
87	15.8	22.3	24.4	<b>-1.2</b>	18.2
1	84.4	92.2	<b>25.2</b>	<b>15.1</b>	78.7
9	74.1	68.3	<b>20.9</b>	<b>12.2</b>	68.1
17	48.7	42.9	<b>15.0</b>	<b>13.7</b>	43.4
11	19.5	NT	18.3	26.0	18.7
16	19.1	NT	14.4	25.5	23.7
19	17.3	21.9	20.8	25.7	17.8
33	14.1	NT	13.2	21.8	18.1
49	29.2	37.8	22.6	44.0	36.4
58	43.2	40.0	45.7	56.8	50.2

<sup>a</sup>  $2 \times 10^3$  <sup>51</sup>Cr-labeled target cells were incubated with  $10^4$  effector cells for 5 h in the presence of MAbs, at a final dilution of 1:80.

<sup>b</sup> Boldface type indicate significant decrease or inhibition by each antibody. NT, not tested.

MAbs to HLA antigens. As shown in Table 4, anti-HLA class I MAb W6/32 did not inhibit lysis of target cells by these T-cell clones. Anti-HLA-DP MAb B7/21.7 reduced the lysis of vaccinia virus-infected autologous B-LCL by clones JC-18, JC-24, JC-30, and JC-34. Anti-HLA-DR MAb OKIa1 inhibited the lysis of target cells by clones JC-46, JC-76, and JC-87. Lytic activity by the other clones such as JC-1, JC-9, and JC-17 was also reduced by this MAb and to a lesser degree by anti-HLA-DP. With six clones, JC-11, JC-16, JC-19, JC-33, JC-49, and JC-58, no inhibition of lysis by these MAbs was observed. The results of these MAb blocking experiments demonstrate that these vaccinia virus-specific CTL clones lyse autologous B-LCL targets in an HLA class II-restricted manner. The clones are heterogeneous in their recognition of epitopes presented by class II molecules; 10 of the 16 clones examined are restricted in their cytolytic activity against vaccinia virus-infected autologous target cells by HLA-DP and HLA-DR antigens.

**Cytolytic activity of T-cell clones against allogeneic target cells infected with vaccinia virus.** The vaccinia virus-specific CTL clones were tested for lytic activity using a panel of allogeneic B-LCL that shared two or more HLA class II antigens in order to determine the nature of their HLA restriction. The three clones shown in Fig. 2 demonstrated vaccinia virus-specific lysis in the context of HLA class II determinants. JC-87, a clone with lytic activity that was completely blocked by an anti-HLA-DR MAb, efficiently lysed vaccinia virus-infected allogeneic targets which shared DR4. It failed to lyse virus-infected targets with which it did not share DR4 (WM, UMA, and WT). Lysis by clone JC-41 was reduced by anti-HLA-DQ MAb, and this clone killed vaccinia virus-infected targets which shared DQw1 but not target cells which did not express this allele. JC-19, a clone that was not blocked by any of the MAbs used, lysed two allogeneic target cells sharing DR2 and DQw1, although it did not lyse other target cells sharing these same alleles. The

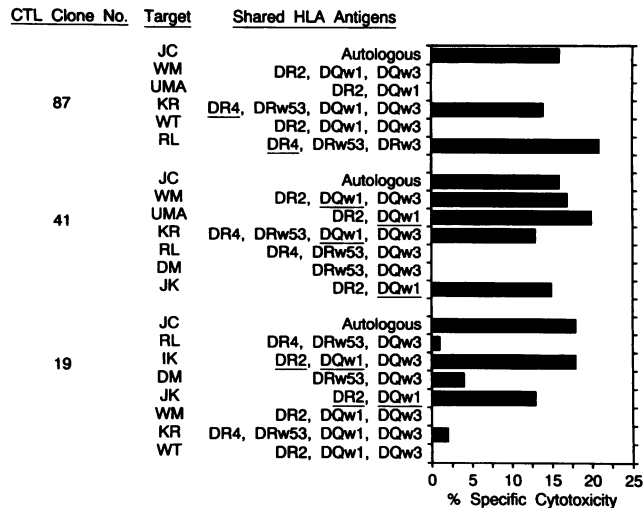


FIG. 2. HLA restriction patterns of vaccinia virus-specific CTL clones. CTL clones were tested for their ability to lyse autologous and allogeneic vaccinia virus-infected B-LCL targets matched at specific HLA class II loci, as indicated, with an E/T ratio of 2.5:1 in a 5-h chromium release assay. Average spontaneous lysis for these assays was always <25%. The alleles which serve as restriction determinants for the clones tested are indicated by underlining. The HLA serotypes of the donors whose cells were used in these experiments were as follows: donor JC, HLA-A3, 24; B35; Cw4; DR2,4; DQw1,3; DRw53; donor WM, HLA-A26,32; B18,55 w6; Cw3; DR2,8; DQw1,3; DRw52; donor UMA, HLA-A11,30; B7,35; Cw4; DR2, w6; DQw1; DRw52; donor KR, HLA-A3,25; B18, w6,62; Cw3; DR4,10; DQw1,3; DRw53; donor WT, HLA-A1,30; B8,14,w6; DR2,5; DQw1,3; DRw52; donor RL, HLA-A1,28; B14,49, w4,6; Cw7; DR4; DQw3; DRw52,53; donor IK, HLA-A24, w33; B44; DR2,5; DQw1,3; donor DM, HLA-A2,23; B44,53, w4; Cw4,7; DR5,7; DQw2,3; DRw52,53; and donor JK, HLA-A2,24; B7,w62; Cw3,7; DR2; DQw1.

rest of the CTL clones failed to lyse any of the allogeneic targets included in this panel (data not shown), which also supports the MHC-restricted nature of the killing by these clones.

**Lysis of target cells infected with deletion mutants of vaccinia virus.** Several recombinant vaccinia viruses containing deletions mainly located at the left end of the vaccinia virus genome, as detailed in the Materials and Methods, were used to begin our efforts to map the regions of vaccinia virus which contain T-cell epitopes (Table 5). Four clones, JC-49, JC-33, JC-35, and JC-20, did not lyse target cells infected with V342 but efficiently lysed target cells infected with the other mutant viruses. This demonstrates that the epitope(s) recognized by these clones reside within the *HindIII* KF regions of the genome which had been deleted from the mutant virus V342. The rest of the clones shown in Table 5 lysed target cells infected with all of the tested recombinant mutant vaccinia viruses, implying that epitopes which reside elsewhere in the vaccinia virus genome are recognized by these clones.

## DISCUSSION

The present study describes CD4<sup>+</sup> CTL clones generated after in vitro stimulation of PBMC from a vaccinia virus-immune donor with UV-inactivated vaccinia virus antigen. These T-cell clones are cytotoxic; they are also virus specific, since vaccinia virus-infected but not influenza virus-

TABLE 5. Lysis of autologous B-LCL infected with deletion mutants of vaccinia virus by CTL clones<sup>a</sup>

Clone no.	% Specific <sup>51</sup> Cr release from target cells									
	VAC	V71	V342	V247-6	V33	V70	V213	V217	V360	V396
49	54	59	12	54	61	54	56	49	56	58
33	13	13	-7	11	14	11	16	13	13	10
35	20	19	0	18	16	19	15	19	21	19
20	14	11	-6	13	13	12	12	12	16	12
19	16	16	15	17	12	14	11	15	18	16
60	14	11	11	14	17	13	14	16	16	12
47	14	12	12	11	16	14	12	16	14	14
34	13	10	10	13	9	12	11	11	14	13
73	20	18	15	23	19	19	14	16	18	21
41	13	NT	12	NT	15	15	12	15	11	13
76	14	NT	13	NT	13	16	14	14	12	13

<sup>a</sup> CTL clones were tested for the ability to lyse autologous B-LCL infected with either wild-type strain NYCBH (VAC) or deletion mutants of vaccinia virus as indicated, at an E/T ratio of 5:1.

infected autologous targets were lysed. The results show a reduction of lysis by specific clones by MAbs directed against either a HLA-DP, HLA-DQ, or HLA-DR framework determinant, but not by an anti-class I MAb (W6/32). Two of the T-cell clones exerted reproducibly strong lytic activity towards vaccinia virus-infected allogeneic targets that shared DR4 or DQw1. A third clone lysed target cells which shared the DR2-associated DQw1 allele, which share a tight linkage disequilibrium (2). It is possible that in the latter case, the cell surface restricting determinant recognized by the CTL clone is not the DR2-associated DQw1 molecule but another product coded by genes that are in a tight linkage disequilibrium with this class II haplotype. Taken together, these results demonstrate that these vaccinia virus-specific CD4<sup>+</sup> CTL clones are HLA class II restricted. Some of the clones exhibited a different pattern of inhibition by available anti-HLA class II MAbs. Killing by five of the clones appears to be restricted by both HLA-DP and HLA-DR antigens in the MAb blocking studies. The reason for this is not known, but there is a high degree of homology between class II molecules (21), and the T-cell epitopes recognized by these clones may be presented by a conserved region of HLA-DP and HLA-DR. Cytotoxicity by seven other clones was not inhibited by MAbs directed to class II determinants. This has been reported earlier by other investigators (16) and has been observed by us with dengue virus CD4<sup>+</sup> CTL clones (17a) and may be explained by differences between the epitopes recognized by antibodies in HLA serological assays versus the restriction element which presents the virus epitope to the T-cell receptor. For instance, three of six MAbs specific for monomorphic determinants on HLA-DR molecules that were used in studies of DR-restricted, CD4<sup>+</sup> influenza virus-specific CTL clones did not inhibit cytotoxicity (16).

The killing of vaccinia virus-infected autologous B-LCL by the T-cell clones was expected after observing cytotoxic responses with the bulk CTL line. Cytotoxic activity was reduced by treatment with an anti-CD4 MAb and complement and by treatment with an anti-CD8 MAb and complement, suggesting that both CD4<sup>+</sup> and CD8<sup>+</sup> T cells contributed to the effector activity. Despite this observation, all 21 of the cytotoxic clones that have been examined are CD4<sup>+</sup>. This suggests that the vaccinia virus-specific CD4<sup>+</sup> CTL

may represent a large proportion of the total vaccinia virus CTL population. Alternatively, we may have selected for CD4<sup>+</sup> clones by our method of cloning and stimulation of the cells through the use of UV-inactivated antigen preparation. We will address this point by stimulating this donor's PBMC with live vaccinia virus and attempt to isolate CD8<sup>+</sup> CTL clones.

Although vaccinia virus-specific proliferative T-cell responses as well as delayed-type hypersensitivity reactions to vaccinia virus have previously been reported (8, 27), this is the first demonstration of vaccinia virus-specific CTL in humans. Poxvirus-specific CTLs have been detected in other animal models (15, 22, 28, 31) using lymph node cells or spleen cells. The inability to detect CTL in the peripheral blood of subhuman primates or humans reported by other researchers (12, 25, 30) may have been due to technical reasons.

It is known that protection and recovery from vaccinia virus infection is T lymphocyte dependent (17). Freed et al. (7) reported that children with Bruton's agammaglobulinemia did not have adverse reactions to smallpox vaccination, suggesting that specific neutralizing antibodies are not necessary for recovery from the vaccinia virus vaccine strains. A child with progressive vaccinia was reported to have prompt improvement and cure after adoptive transfer of immune leukocytes and lymph node material from vaccinia virus-immune donors after failing to respond to hyperimmune gamma globulin (17). Fatal disseminated vaccinia following vaccination, despite the administration of massive doses of vaccine immunoglobulin (9, 23) occurred in individuals who produced viral antibodies but were unable to mount a delayed hypersensitivity reaction to a variety of antigens. In other model systems (1, 3, 5, 10), the cell-mediated immune response also appears to be responsible for recovery and protection from poxvirus infections, since an anamnestic response could be demonstrated upon reexposure to the virus.

Vaccinia vaccines provided potent long-lasting protection against smallpox. Protection correlated with resistance to vaccinia virus challenge intradermally. The long-term nature of the resistance to smallpox or vaccinia virus challenge may be due to the presence of a large number of cross-reactive memory CTLs which would become activated and rapidly eliminate infected cells after challenge. This presents a problem in using vaccinia virus as a vaccine vector to express foreign genes, e.g., human immunodeficiency virus type 1 gp160, because earlier immunization with vaccinia vaccine reduced antibody and cellular immune responses to the recombinant virus vaccine (4). Similarly, it would be expected that a repeat dose of a recombinant vaccinia vaccine may not induce strong immune responses to the novel antigen. Attempts to use alternate animal poxvirus vectors to help avoid this limitation are being considered. Definition of cross-reactive CTL epitopes on poxviruses would be useful in this regard.

In order to understand the immune responses to vaccinia virus in humans, it will be necessary to map the epitopes that are recognized by human T cells. Using deletion mutants of vaccinia virus, we have shown that the *Hind*III KF regions of the viral genome contains potential epitopes recognized by four of the CTL clones. Additional studies are needed to localize the epitope(s) in this region as well as the other epitopes elsewhere in the vaccinia virus genome that are recognized by these vaccinia virus-specific CTL clones. The information gathered from such studies could be important in determining which elements of the CTL response contrib-

ute to the recovery and protection from poxvirus infections and will be useful in designing vaccinia virus vectors for use in recombinant vaccines.

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