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Vaccinia Virus-Specific CD8+ Cytotoxic T Lymphocytes in Humans

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Stimulation of human vaccinia virus immune peripheral blood mononuclear cells in vitro from vaccinia virus-immune donors with live vaccinia virus-infected autologous cells generated vaccinia virus-specific cytotoxic T lymphocytes (CTL) capable of lysing vaccinia virus-infected cells. We generated vaccinia virus-specific CD8+ clones and CD4+ CTL lines by limiting dilution from two donors by using peripheral blood mononuclear cells obtained 2 months or 4 years postrevaccination with vaccinia virus. These results demonstrate that vaccinia virus-specific CTL are generated as a result of immunization of humans with vaccinia virus and that both CD8+- and CD4+-specific T cells are maintained as memory cells.

Successful immunization can be defined as induction of immunologic memory specific for defense against microorganisms that can be evoked to protect a host against challenge. Sufficient immunologic memory must be generated to provide long-lasting protection. Such long-lived immunity is possible and has been achieved with smallpox vaccines. Vaccination of humans with vaccinia virus represents the first example of worldwide eradication of a major human disease (9).

Within the last decade, a new and exciting approach to vaccine development has evolved that is based on introduction of genes that encode microbial antigens into a relatively nonpathogenic virus, such as vaccinia virus, and infection of individuals with the recombinant virus to generate immune responses against the foreign protein. Vaccinia virus has been used as a potential viral vector vaccine because of its broad host range, its prior use as the vaccine to eradicate smallpox in humans, and its utility in expression of a variety of foreign antigens which can immunize animals successfully (1, 20, 27). Although vaccinia virus recombinants are being considered as vaccines against diseases of major medical importance, very little is known about human T-cell responses to vaccinia virus.

The importance of the cellular immune response during vaccinia virus infection was demonstrated in 1960 by Kempe, who reported that children with agammaglobulinemia had no adverse reactions to immunization with live vaccinia virus (13). Other clinical studies showed that individuals with deficient T-lymphocyte responses were more likely to have disseminated infections following immunization with vaccinia virus (10, 21). Helper and cytotoxic T-cell responses following infection with poxviruses have been documented in a number of different animal species (2, 5, 12); however, the presence of CD4+ and CD8+ cytotoxic T lymphocytes (CTL) in vaccinia virus-immunized humans had not been demonstrated. Investigators at several laboratories have previously attempted to detect vaccinia virus-specific CTL responses in humans postimmunization but have reported negative results (11, 22, 26). Littaua et al., however, recently demonstrated that vaccinia virus-specific memory CD4+ CTL are present in vivo and could be cloned from peripheral blood mononuclear cells (PBMC) taken from an immune individual (15). It still remained unclear whether CD8+ CTL, which recognize viral antigens in association with class I major histocompatibility complex (MHC) molecules, are generated in humans vaccinated with vaccinia virus.

In this report, we describe the proliferation of human immune PBMC in response to vaccinia virus-infected cells and demonstrate that vaccinia virus-specific memory CD8+ CTL exist in human immune PBMC. In addition, we generated vaccinia virus-specific CD8+ CTL clones and lines from two individuals. The results reported in this communication establish the existence of vaccinia virus-specific CD8+ CTL in humans and provide useful information concerning the potential use of recombinant vaccinia viruses as vaccines in humans.

MATERIALS AND METHODS

Human PBMC. Blood was obtained from three healthy donors who had received the standard New York City Board of Health (NYCBH) strain of vaccinia virus as a smallpox vaccine (Dryvax; Wyeth Laboratories, Philadelphia, Pa.) 2 months or 4 years earlier. PBMC were separated by the Ficoll-Hypaque density gradient centrifugation method. Cells were resuspended in RPMI 1640 medium containing 15% fetal bovine serum (FBS; GIBCO Laboratories, Grand Island, N.Y.) and 10% dimethyl sulfoxide (Fisher Scientific Co., Pittsburgh, Pa.) and cryopreserved until use.

Cell lines. Skin fibroblast cultures were established by using a 3-mm-diameter biopsy sample of skin. The sample was minced, cells were passed several times in 75-cm^2 flasks before being harvested, and aliquots were cryopreserved in liquid nitrogen for subsequent use as target cells in CTL assays. These cells were maintained in minimal essential medium with 10% FBS.

B-lymphoblastoid cell lines (B-LCL) were established from healthy, vaccinia virus-immune donors by infecting PBMC with Epstein-Barr virus obtained from an infected marmoset cell line, B95-8 (25), obtained from the American Type Culture Collection. B-LCL were maintained in RPMI 1640 containing 10% heat-inactivated FBS. In addition, we used HLA-typed allogeneic B-LCL, described below under HLA typing, in the HLA restriction experiments.

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Stimulation of PBMC with live virus. The NYCBH strain of vaccinia virus was used in all experiments, and two methods of virus stimulation were employed. To stimulate cultures with live vaccinia virus, virus was added directly to the culture medium at the desired multiplicity of infection (MOI). Stimulation with vaccinia virus-infected autologous PBMC was carried out by infecting aliquots (10%) of total PBMC with virus at the desired MOI in less than 0.5 ml of RPMI 1640 for 2 h at 37°C. The infected cells were then washed and resuspended in 10% heat-inactivated FBS–10% T-cell growth factor (Cellular Products, Inc., Buffalo, N.Y.)–10% heat-inactivated human AB serum (Hazleton Research Products, Inc., Lenexa, Kans.) in RPMI 1640. In some experiments, the 10% human AB serum was replaced with 10% heat-inactivated human immune sera obtained from donors 2 to 4 months post revaccination. These sera contained high concentrations of anti-vaccinia virus antibodies, as determined by Western blotting (immunoblotting) and by plaque neutralization assays (data not shown). The stimulator cells were mixed with the remaining 90% responder PBMC (for establishment of bulk cultures) or with gamma-irradiated (3,000 R) autologous PBMC (for subsequent stimulation of cells in bulk cultures) at 10^6 total cells per ml before addition to the culture.

Proliferation assays. Proliferation assays were performed in 96-well round-bottom plates by addition of 2×10^5 immune donor PBMC in RPMI 1640–10% human AB serum to triplicate wells in the presence of vaccinia virus antigen for 4 days at 37°C. We then added 1.25 μCi (46 kBq) of [^{3}H]thymidine (Dupont, NEN Research Products, Boston, Mass.) to each well and further incubated the plates for 16 to 18 h. The cells were then harvested by using a Titertek Multiharvester (Skatron, Inc., Sterling, Va.), and [^{3}H]thymidine was counted in a liquid scintillation counter (Packard Instrument Co., Inc., Rockville, Md.).

Cell surface antigen analysis. Anti-Leu4 (CD3), anti-Leu3 (CD4), anti-Leu2 (CD8), and anti-Leu11b (CD16) antibodies directly conjugated to fluorescein isothiocyanate (Becton Dickinson Co., Mountain View, Calif.) were used to quantitate $CD3^+$, $CD4^+$, $CD8^+$, and $CD16^+$ cells in culture. Briefly, 20 μl of antibody was added to 10^5 to 10^6 cells in 50 μl and incubated for 60 min at 4°C. The cells were then washed twice with cold RPMI 1640 and analyzed on a fluorescence-activated cell sorter (FACS 440; Becton Dickinson Co.).

Cytotoxicity assays. B-LCL were infected with vaccinia virus at an MOI of 10 PFU per cell and incubated for 12 to 14 h. Target cells (0.5×10^6) were then labeled with 0.5 mCi (18.5 mBq) of $Na_2{}^{51}CrO_4$ (Dupont) in 0.2 ml of RPMI 1640 containing 10% FBS at 37°C for 1 h. The cells were then washed three times and resuspended in RPMI 1640 containing 10% FBS. Cells (2×10^3/0.1 ml) were added in a 0.1-ml volume to each well of a 96-well round bottom microtiter plate (Costar). Various concentrations of effector cells in 0.1 ml of medium were added to each well to give the described effector/target (E/T) cell ratios. After 5 h of incubation at 37°C, the supernatant was harvested from each well and counted in a gamma counter. The assays were carried out in triplicate wells, and percent specific lysis was calculated by the formula 100 × [(mean experimental release − mean spontaneous release)/(mean maximum release − mean spontaneous release)]. The spontaneous release was between 10 and 30%. Maximum release was determined by treatment of labeled cells with detergent.

Antibody-complement depletion analysis. Anti-OKT3 (CD3), anti-OKT4 (CD4), and anti-OKT8 (CD8) antibodies (Ortho Diagnostic Systems, Inc., Raritan, N.J.) and anti-Leu11b (CD16; Becton Dickinson Co.) antibodies were used in antibody-complement depletion experiments. Vaccinia virus-stimulated effector cells were resuspended in 0.5 ml of RPMI 1640 supplemented with 2% FBS containing a 1:10 dilution of the antibody. Following 30 min of incubation at 4°C, the cells were washed with cold RPMI 1640 and suspended in 0.6 ml to which 0.2 ml of rabbit complement (Cedarlane Laboratories, Hornby, Ontario, Canada) was added. After 1 h of incubation at 37°C, the cells were washed three times and used as effector cells in cytotoxicity assays.

Antibody blocking of cytotoxicity. W6/32 (Accurate Chemical and Scientific Co., Westbury, N.Y.) and OKIa-1 (Ortho) are monoclonal antibodies which recognize framework determinants of HLA A, B, and C, and DR determinants, respectively. A 50-μl volume of antibody diluted 1:20 was added to 50 μl of ^{51}Cr-labeled target cells (10^4 cells per ml) in 96-well round-bottom plates for 30 min; this was followed by addition of 100 μl of effector cells for incubation at 37°C for 5 h.

T-cell cloning by limiting dilution. PBMC from immune donors were stimulated with vaccinia virus by infection of 10% of the cells at an MOI of 75 or 100 PFU per cell. After 7 days in culture, an aliquot of the stimulated cells was used as effector cells in a CTL assay and the remaining cells were restimulated. Autologous PBMC were gamma irradiated (3,000 R), and 10% of the irradiated cells were infected with vaccinia virus for use as stimulators. After infection with vaccinia virus, the 10% infected, gamma-irradiated autologous PBMC and the 90% uninfected, gamma-irradiated autologous feeder PBMC were combined and resuspended to 10^6 cells per ml in RPMI 1640 containing 10% FBS, 10% T-cell growth factor, and 10% heat-inactivated vaccinia virus-immune human serum (source of vaccinia virus antibody) and added to the bulk cultures for restimulation. Preliminary experiments revealed that addition of vaccinia virus-immune serum resulted in improved viability of the cultured cells, presumably by neutralizing residual infectious vaccinia virus.

After 14 days in culture, the bulk effector cells were diluted and placed in 96-well round-bottom plates at a concentration of 1, 3, or 9 cells per well. Every 7 to 11 days, the plates were restimulated with virus-infected, autologous, gamma-irradiated PBMC. In some experiments, as an alternate method of stimulation, the cells were stimulated by 0.1 μg of anti-CD3 monoclonal antibody 12F6 per ml, kindly provided by Johnson Wong, and were placed on gamma-irradiated allogeneic feeder cells in the medium described above containing 25 U of recombinant human interleukin 2 (Collaborative Research, Inc., Bedford, Mass.) per ml. Once weekly, half of the medium was removed and fresh medium was added. After approximately four stimulations, the clones were expanded in flat-bottom 48-well plates (Costar) and tested for cytotoxic activity. For generation of clones JC-FC4 and JC-FD7, $CD8^+$ cells were preferentially separated from bulk cultures by positive selection with anti-$CD8^+$ monoclonal antibodies conjugated to magnetic beads (Becton Dickinson) and then seeded at 1 cells per well by limiting dilution (29).

HLA typing. Donor JC, CB, CP, H12, 63390, and PG B-LCL from the University of Massachusetts Medical Center were HLA typed in the University of Massachusetts Medical Center Tissue Typing Laboratory. GM3106 B-LCL were obtained from the National Institute of General Medical Sciences Human Genetic Mutant Cell Repository, Camden, N.J.

TABLE 1. Proliferative responses of vaccinia virus-immune donor PBMC after stimulation with live virus or virus-infected stimulator cells

Expt	Responder, stimulator	MOI	cpm	SI[a]
1	PBMC[b] vaccinia virus	0.0064	5,225	6.0
	PBMC, vaccinia virus	0.032	10,014	11.5
	PBMC, vaccinia virus	0.16	15,121	17.3
	PBMC, vaccinia virus	0.8	16,441	18.8
	PBMC, vaccinia virus	4.0	14,326	16.4
	PBMC, vaccinia virus	20	442	0.5
	PBMC, vaccinia virus	100	61	0.0
	PBMC, none (control)		874	
2	PBMC[c], PBMC + virus[d]	0.1	1,144	1.3
	PBMC, PBMC + virus	1.0	4,273	4.9
	PBMC, PBMC + virus	10.0	10,488	12.0
	PBMC, PBMC + virus	100	16,706	19.1
	PBMC, none (control)		874	

[a] The stimulation index (SI) was calculated by dividing the amount of stimulation by the control (PBMC alone).
[b] PBMC taken from vaccinia virus-immune donor C were added at 2×10^5 cells per well in triplicate wells.
[c] Donor C PBMC were added at 1.8×10^5 cells per well in triplicate wells.
[d] PBMC (0.2×10^5) were infected with virus at the indicated MOI.

TABLE 2. Specific anti-vaccinia virus cytotoxicity of PBMC restimulated in vitro

Expt	Effector cells (day)	Target	% Specific ^{51}Cr release from target cells at E/T ratio of:		
			100:1	50:1	25:1
1	PBMC (7)[a]	B-LCL	22.5	11.7	
		VAC[b]	77.4	66.0	
2	PBMC (13)[c]	B-LCL		0.1	0.0
		VAC[b]		40.6	25.8
3	PBMC (10)[d]	B-LCL	13.5	0.0	
		VAC[b]	73.9	39.3	
		Fibroblasts	19.8	11.0	
		VAC-fibroblasts	51.6	44.7	
		K562	71.1	49.4	

[a] PBMC were taken from donor C at 4 years post revaccination with vaccinia virus, stimulated in vitro with live-virus-infected cells, and assayed on day 7.
[b] Autologous B-LCL infected with vaccinia virus.
[c] Same PBMC bulk culture assayed on day 13 (after stimulation on day 7).
[d] Same PBMC as used in experiment 1 but maintained in the presence of anti-vaccinia virus antibody.

RESULTS

Proliferative responses of human immune PBMC after stimulation with live virus in vitro. PBMC from immune donors who had been revaccinated with vaccinia virus 2 months or 4 years earlier were stimulated in vitro with live virus or an aliquot of virus-infected, autologous PBMC. In addition, PBMC from a nonimmune donor and two cord blood samples were also stimulated. The proliferative responses of the stimulated PBMC were determined by measuring [^{3}H]thymidine incorporation after 5 days of culture, and the results are shown in Table 1. Stimulation of nonimmune PBMC with live vaccinia virus resulted in no detectable proliferation (data not shown). When infectious virus was added directly to PBMC, maximum proliferation was observed at an MOI of 0.8 (Table 1, experiment 1). As the MOI increased, the proliferation decreased, owing to virus-induced cell death. High levels of proliferation were also observed when 10% of the PBMC were preinfected with virus at an MOI of 100 and added to responder PBMC (Table 1, experiment 2). These results indicated that vaccinia virus-specific T cells proliferate in vitro after stimulation with live vaccinia virus.

Cytotoxic T-cell activity in bulk cultures stimulated by live vaccinia virus. PBMC from the same immune donor were stimulated in vitro with virus-infected PBMC, and a CTL assay was performed on day 7 to determine the cytolytic activity against autologous virus-infected target cells (Table 2, experiment 1). Vaccinia virus-infected B-LCL were lysed to higher levels than uninfected B-LCL. Additional stimulation of the cells was done on day 7, and the cells were tested again for CTL activity on day 13 (Table 2, experiment 2). On day 13, cells in the live-virus-stimulated cultures specifically lysed vaccinia virus-infected targets. Although the cells in the bulk cultures stimulated with live virus maintained virus-specific killing on day 13, we were concerned that eventually residual infectious virus would infect and destroy the cells in culture. We decided, therefore, to supplement the culture medium with anti-vaccinia virus antibodies by replacing the 10% heat-inactivated human AB serum with 10% heat-inactivated human immune sera taken from donors 2 to 4 months post revaccination. These cultures were maintained by stimulation with virus-infected autologous PBMC and cultured in the presence of human immune serum antibodies. On day 10 after stimulation, the virus-specific CTL activity was high and appeared to be specific, with a low level of lysis of uninfected B-LCL targets (Table 2, experiment 3). Autologous fibroblasts were also included as target cells to detect CD8$^+$ CTL activity because fibroblasts express class I but do not constitutively express class II MHC antigens (16). Specific killing of the virus-infected fibroblasts suggested that virus-specific CD8$^+$ CTL were present in this bulk culture.

Characterization of cytotoxic effector cells by using monoclonal antibodies. To determine the identities of the responding vaccinia virus-specific CTL in bulk culture, antibody and complement depletion studies were carried out. PBMC taken from three donors (A, B, and C) were stimulated in vitro with virus-infected autologous PBMC and maintained in the presence of a high titer of anti-vaccinia virus antibody. The results of antibody-complement depletion of cells from 7-day bulk cultures on CTL activity are presented in Table 3, experiments 1 and 2. The bulk-cultured cells were tested for cytotoxic activity against autologous B-LCL; virus-infected, autologous B-LCL (VAC); and natural killer (NK) cell-sensitive tumor cell line K562. The cells generated from donor A efficiently lysed VAC targets. Treatment with anti-CD8 and complement decreased the lytic activity by 25.1%, and anti-CD16 and complement decreased lysis by 62.5%. Donor B's cells exhibited some nonspecific lysis of B-LCL, and treatment with anti-CD16 and complement inhibited the lysis of VAC targets by 32.6%. Treatment with anti-CD4 and complement inhibited lysis of VAC targets by 67.4%, suggesting a dominant role for CD4$^+$ CTL in this culture. Taken together, these results suggest that 7 days after stimulation in vitro, specific anti-vaccinia virus CTL and CD16$^+$ killer cells were activated in bulk cultures, and these responses were heterogeneous and varied between donors.

The same experiment was repeated on two cultures on day 31. Cells generated from donor C had been stimulated four

TABLE 3. Characterization of bulk culture cytotoxic activity

Donor (day), expt, treatment	% Specific ^{51}Cr release from target cells		
	B-LCL	VAC[a]	K562
A (7)[b], 1			
Complement	6.7	59.7	72.4
Anti-CD4+ complement	5.2	56.7	67.5
Anti-CD8+ complement	3.2	44.7	63.9
Anti-CD3+ complement	2.8	45.7	70.0
Anti-CD16+ complement	1.3	22.4[c]	24.2
B (7)[d], 2			
Complement	27.3	47.0	60.3
Anti-CD4+ complement	10.9	15.3	46.5
Anti-CD8+ complement	18.5	37.7	59.3
Anti-CD3+ complement	15.0	11.8	43.8
Anti-CD16+ complement	20.4	31.7	21.1
C (31)[e], 3			
Complement	−3.2	47.1	−1.8
Anti-CD4+ complement	−4.2	47.3	−0.8
Anti-CD8+ complement	−2.7	7.2	−6.8
Anti-CD3+ complement	ND[f]	ND	ND
Anti-CD16+ complement	−1.5	43.2	−3.8
A (31)[g], 4			
Complement	−5.1	18.3	4.7
Anti-CD4+ complement	−5.8	14.8	3.2
Anti-CD8+ complement	−7.6	0.0	2.3
Anti-CD3+ complement	−5.4	1.6	0.0
Anti-CD16+ complement	−7.8	11.6	2.4

[a] Autologous B-LCL infected with vaccinia virus.
[b] PBMC taken from donor A at 2 months post revaccination and stimulated once in vitro with virus. E/T ratio, 60:1.
[c] Underlined values are those which were significantly decreased by treatment.
[d] PBMC taken from donor B at 2 months post revaccination and stimulated once in vitro with virus. E/T ratio, 50:1.
[e] Donor C bulk stimulated four times in vitro with virus. E/T ratio, 20:1.
[f] ND, not done.
[g] Donor A bulk stimulated four times in vitro (twice with virus and then twice with anti-CD3). E:T ratio, 45:1.

TABLE 4. Effect of anti-HLA antibodies on cytotoxic activity of vaccinia virus-specific CTL lines

T-cell line[a]	Phenotype[b]	% Specific ^{51}Cr release from target cells			
		VAC[c]	B-LCL + W6/32[d]	B-LCL + OKIa-1[e]	B-LCL
KT-E3	CD8+	59.9	1.3[f]	30.0	−1.3
KT-C5	CD4+	59.3	41.9	2.7	−0.1
JC-DC7	CD8+	81.2	0.8	49.1	0.0
JC-DD6	CD4+	82.5	72.1	14.9	−8.0
JC-FC4	CD8+	54.9	−7.3	51.9	−4.3
JC-FD7	CD8+	59.0	0.9	61.7	1.4

[a] Lines were used at an E-T ratio of 30:1.
[b] As determined by FACS analysis.
[c] Autologous B-LCL infected with virus.
[d] Anti-class I monoclonal antibody diluted 1:80.
[e] Anti-DR monoclonal antibody diluted 1:80.
[f] Underlined values are those which were significantly decreased by treatment.

times in vitro with virus-infected autologous PBMC, while cells from donor A had been stimulated twice with virus-infected cells and then stimulated twice with anti-CD3 monoclonal antibody. The results are shown in Table 3, experiments 3 and 4. The lytic activity of the cells from donor C was inhibited by 84.7% following treatment with anti-CD8 and complement. No other antibody had a significant effect on lysis by cells in this culture. Similarly, donor A's cells lost all killing activity after treatment with anti-CD8 and complement, and no other antibody had a significant effect on the levels of lysis. Taken together, the results of antibody and complement depletion on CTL activity indicate that stimulation of donor immune PBMC in vitro with virus-infected, autologous PBMC activated $CD8^+$ memory CTL and the levels of $CD8^+$ CTL activity varied between donors.

Establishment of vaccinia virus-specific $CD8^+$ CTL clones. To demonstrate firmly that virus-specific $CD8^+$ memory CTL are generated as a result of vaccination with vaccinia virus, we attempted to establish vaccinia virus-specific T-cell lines. Initially, bulk cultures of PBMC of donors A and C were stimulated twice with virus-infected autologous PBMC. The cells were then diluted and added to 96-well plates at 1, 3, or 9 cells per well. The plates were alternatively stimulated every 10 to 14 days with either virus-infected, autologous PBMC or monoclonal antibody to CD3. When cell numbers were large enough in wells which showed proliferation, the cells were transferred to 48-well plates. Several T-cell lines were generated by using this approach, and six representative lines, two from donor A and four from donor C, will be described here.

Two clones, JC-FC4 and JC-FD7, derived from donor C were generated from plates which had been seeded at one cell per well. Overall 2% of the wells were positive for growth when seeded at one cell per well, and cells from 50% of the growing (or 1% of the total number of wells) killed vaccinia virus-infected autologous target cells. Two additional lines, JC-DC7 and JC-DD6, also derived from donor C, were generated from plates which had been seeded at three cells per well. Overall, 34% of the wells were positive for growth when seeded at three cells per well, and cells from 30% of the growing wells (or 11% of the total number of wells) killed vaccinia virus-infected, autologous target cells. Two other lines, KT-E3 and KT-C5, which were derived from donor A, were generated from wells which had been seeded at nine cells per well. Overall, cells in 56% of these wells grew and were transferred to 48-well plates. A total of 24% of these growing wells (or 14% of the total number of wells) had cells which lysed vaccinia virus-infected target cells.

Cytotoxic activity of T-cell lines. The MHC restriction of lines JC-FC4, JC-FD7, JC-DC7, JC-DD6, KT-E3, and KT-C5 was examined in antibody blocking studies. Each of the lines killed a high level of VAC targets but did not kill uninfected targets (Table 4). Killing of the VAC targets by lines KT-E3 and JC-DC7 and clones JC-FC4 and JC-FD7 was inhibited by >98% by anti-class I monoclonal antibody W6/32. In contrast, incubation of targets with OKIa-1, an anti-HLA-DR monoclonal antibody, inhibited lysis by lines KT-C5 and JC-DD6 by 95 and 82%, respectively.

Cell surface phenotypes of vaccinia virus-specific CTL lines. In addition to the antibody blocking study described above, lines JC-DC7 and KT-E3 and clones JC-FC4 and JC-FD7 were examined by FACS analysis, and their cell surface phenotypes were $CD3^+$, $CD4^-$, $CD8^+$, and $CD16^-$ (Table 4). FACS analysis was also carried out on lines JC-DD6 and KT-C5, and their phenotypes were determined to be $CD3^+$, $CD4^+$, $CD8^-$, and $CD16^-$ (Table 4). Taken together, these results indicate that $CD8^+$, as well as $CD4^+$, CTL are present after revaccination with vaccinia virus and that these

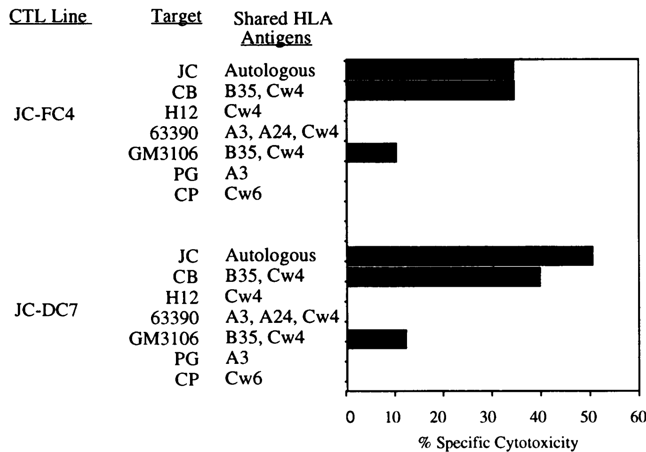

FIG. 1. HLA restriction patterns of vaccinia virus-specific CTL line JC-DC7 and clone JC-FC4. CTL were tested for the ability to lyse autologous and allogeneic, vaccinia virus-infected B-LCL targets matched at specific HLA class I loci, as indicated, with an E/T ratio of 5:1 in a 5-h ^{51}Cr release assay. The HLA class I serotypes of the donors whose cells were used in these experiments were as follows: donor JC, HLA A3, A24, B35, Cw4, Cw6; donor CB, HLA A2, A23, B35, B44, Cw4; donor CP, HLA A2, A28, B51, Bw57, Cw6; donor PG, HLA A2, A3, B7, B27, Cw1, Cw7; donor GM3106, HLA A1, B35, Cw4; donor H12, HLA A2, A23, B44, B53, Bw4, Cw4, Cw7; donor 63390, HLA A3, A24, B7, Bw62, Cw4, Cw7.

CTL are maintained as memory cells for at least 4 years post revaccination.

Cytotoxic activity of CTL lines and clones against vaccinia virus-infected allogeneic target cells. Vaccinia virus-specific CTL line JC-DC7 and clone JC-FC4 were tested for cytotoxic activity against a panel of allogeneic B-LCL that shared HLA class I antigens to analyze MHC class I restriction. Preliminary experiments suggested that HLA B35 was the restricting allele. Clones JC-DC7 and JC-FC4 lysed several vaccinia virus-infected, allogeneic target cells that shared both the HLA B35 and Cw4 allels (Fig. 1 and data not shown); however, allogeneic targets that shared only Cw4 and not B35 were not lysed (Fig. 1). These results strongly suggest that these two vaccinia virus-specific CTL clones are restricted by HLA B35.

DISCUSSION

In this communication, we describe the proliferation of human vaccinia virus-immune PBMC in response to live vaccinia virus-infected cells and characterize the CD4$^+$ and CD8$^+$ CTL activities of these cells in vitro. We also generated vaccinia virus-specific CD4$^+$ and CD8$^+$ CTL lines and clones from two vaccinia virus-immune individuals. The restricting allele for two of these CD8$^+$ lines appears to be HLA B35. This is the first demonstration of vaccinia virus-specific CD8$^+$ CTL in humans. Previously, attempts have been made to identify and characterize CTL responses in vaccinia virus-immune humans, and only CD4$^+$ CTL responses were identified following stimulation with inactivated vaccinia virus antigens (15). In this study, we used live virus-infected cells to stimulate cultures in vitro to induce proliferation of CD8$^+$ CTL.

CTL which recognize virus-infected cells and kill them are thought to be the principal effector cells during viral infection. The importance of CTL in the outcome of viral infections has been demonstrated in several experimental systems (3, 14). The best-defined CTL are CD8$^+$, which recognize viral antigens in association with MHC class I molecules, which are distributed on all cells. The roles that MHC class II-restricted CD4$^+$ CTL play during viral infections are not well understood, but they may be important in controlling infection in cells which express large amounts of class II MHC antigens.

The importance of cell-mediated immune responses associated with infection or immunization with vaccinia virus has been established (6, 8, 23, 31). CTL responses following infection with poxviruses have been documented in a number of species, including mice, rats, rabbits, and sheep (2, 7, 12, 16, 18, 31). However, CTL responses to vaccinia virus have not been well characterized in nonhuman primates or in humans (22, 26).

Specific anti-vaccinia virus CD4$^+$ or CD8$^+$ CTL responses were not detected in studies performed with subhuman primates (26) or with humans (11, 22). Early attempts by Perrin et al. to detect CTL in the PBMC of vaccinia virus-immune humans were unsuccessful (22). Vaccinia virus-specific cytotoxic activity was detected in PBMC by day 5 after vaccination, peaked by days 7 to 8, and subsided by day 12. The researchers found only weak suggestions of participation of classical CTL and concluded that cells bearing Fc receptors and acting in the presence of specific antibodies, not CTL, were primarily responsible for the lysis of virus-infected target cells (22). Stitz et al. searched for specific vaccinia virus CTL in rhesus monkeys that had been immunized by intracutaneous or intravenous injection of 10^8 PFU of vaccinia virus (26). At 5 to 6 days postimmunization, auxiliary lymph node cells, peripheral blood cells, and spleen cells obtained by partial splenectomy were tested for virus-specific cytotoxic activity in vitro. Cytotoxic activity peaked at 5 to 7 days postinfection and was present in T-cell-depleted but not in T-cell-enriched fractions. The investigators concluded that NK-like, not HLA-restricted, T-cell activity was generated in lymphoid organs of rhesus monkeys after vaccinia virus infection (26). Recently, Graham et al. used UV-inactivated vaccinia virus-infected fibroblasts to stimulate immune PBMC in vitro to detect specific CTL responses in revaccinated humans, and no convincing vaccinia virus-specific CTL activity was observed (11). The reasons for the inability to observe vaccinia virus-specific cytotoxicity were not clear (11). In summary, vaccinia virus-specific cytotoxic T-cell activity has been reported in mice (2, 7, 16, 18), but several studies with subhuman primates (26) and with humans (11, 22) have failed to detect vaccinia virus-specific CTL responses.

Recently, Littaua et al. generated several HLA class II-restricted human CD4$^+$ CTL clones by using UV-inactivated vaccinia virus-pulsed PBMC to stimulate immune T cells in vitro (15). This was the first demonstration of vaccinia virus-specific CTL in humans; however, the question of whether CD8$^+$ CTL play any role in vaccinia virus immunity in humans remained unanswered. Morrison et al. reported that influenza virus-specific CD8$^+$ CTL were activated when influenza virus-infected splenocytes were used as stimulators in vitro, but stimulation with UV-inactivated virus-pulsed splenocytes preferentially gave rise to CD4$^+$ CTL (19). In addition, van Binnendijk et al. were able to establish measles virus-specific human CD8$^+$ class I-restricted clones after repeated in vitro simulation of immune T-cell cultures with measles virus-infected autologous B-LCL, whereas immune cultures stimulated with UV-inactivated measles virus-pulsed autologous cells preferen-

tially generated CD4+ CTL clones (28). We therefore chose to stimulate CD8+ CTL in vitro by using vaccinia virus-infected autologous cells. This approach differs from previous attempts to demonstrate CTL in subhuman primates and in humans without in vitro stimulation (22, 26). NK cell activation is an early antiviral host response, and a modest level of specific CTL activity may be masked by NK activity. In vitro stimulation allows for proliferation of antigen-specific CTL after the NK activity subsides. It is possible that the failure of Graham et al. and Littaua et al. to demonstrate human vaccinia virus-specific CD8+ CTL was due to the inactivated nature of the stimulating antigen in their experimental systems (11, 15). We generated vaccinia virus-specific CD8+ and CD4+ CTL lines from two donors, one whose PBMC were obtained 2 months post revaccination and one whose PBMC were obtained 4 years post revaccination. Apparently, the method of in vitro stimulation used to generate T-cell lines from vaccinia virus-immune PBMC is directly related to the phenotypes of the responding T cells.

We have established that both CD4+ and CD8+ vaccinia virus-specific CTL can be generated in humans as a result of vaccination and that both cell types persist as memory cells. We did not determine the precursor frequency of vaccinia virus-specific T cells in this study; however, such information may be useful in light of the recent report by Cooney et al. in which a recombinant vaccinia virus which expresses human immunodeficiency virus type 1 gp160 was used to vaccinate humans against human immunodeficiency virus (4). In this study, individuals who had been immunized many years earlier with vaccinia virus had reduced humoral and cellular immune responses to the novel gp160 expressed in the recombinant vaccine compared with the responses of vaccinia virus-naive individuals. Their results suggest that injection of vaccinia virus-immune individual with a recombinant vaccinia virus activates specific anti-vaccinia virus memory T cells and the virus-infected cells may be eliminated before a sufficient immune response can be induced by the novel antigen. The inability to generate an efficient immune response to a novel antigen expressed in a recombinant vaccinia virus in vaccinia virus-immune persons is likely to be a major obstacle in developing such recombinant vaccines.

Recombinant vaccinia viruses have been successful in generating specific CTL responses against novel antigens expressed in a recombinant vaccinia virus in vaccinia virus-naive subhuman primates (24, 30). In chimpanzees, CD4+ CTL with specificity for human immunodeficiency virus envelope glycoproteins have been demonstrated after immunization with a vaccinia virus recombinant expressing human immunodeficiency virus type 1 glycoproteins (30). Simian immunodeficiency virus gag-specific CD8+ CTL were induced by immunization of rhesus monkeys with a vaccinia virus-simian immunodeficiency virus recombinant (24). These results, in addition to the results reported in this communication, are encouraging and suggest that recombinant vaccinia viruses have potential for vaccination purposes. An improved understanding of vaccinia virus-induced immune responses, including memory T-cell responses, should be helpful in designing experimental recombinant vaccinia viruses which contain novel, medically important antigens.

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