JOURNAL OF VIROLOGY, Feb. 1989, p. 584–590
0022-538X/89/020584-07$02.00/0

Vol. 63, No. 2

Antigenic Specificity of Antibody-Dependent Cell-Mediated Cytotoxicity Directed against Human Immunodeficiency Virus in Antibody-Positive Sera

RICHARD A. KOUP,[1]* JOHN L. SULLIVAN,[1] PETER H. LEVINE,[1] FRANK BREWSTER,[1] ANNA MAHR,[2] GAIL MAZZARA,[2] SARA MCKENZIE,[2] AND DENNIS PANICALI[2]

Department of Pediatrics and Medicine, University of Massachusetts Medical School, Worcester, Massachusetts 01655,[1] and Applied bioTechnology, Inc., Cambridge, Massachusetts 02142[2]

Received 7 September 1988/Accepted 26 October 1988

Antibody-dependent cell-mediated cytotoxicity (ADCC) specific for human immunodeficiency virus (HIV) has been described for HIV-infected individuals. To determine the antigenic specificity of this immune response and to define its relationship to the disease state, an ADCC assay was developed using Epstein-Barr virus-transformed lymphoblastoid cell line targets infected with vaccinia virus vectors expressing HIV proteins. The vaccinia virus vectors induced appropriate HIV proteins (envelope glycoproteins gp160, gp120, and gp41 or *gag* proteins p55, p40, p24, and p17) in infected lymphoblastoid cell lines as demonstrated by radioimmunoprecipitation and syncytia formation with c8166 cells. Killer cell-mediated, HIV-specific ADCC was found in sera from HIV-seropositive but not HIV-seronegative hemophiliacs. This HIV-specific response was directed against envelope glycoprotein but was completely absent against target cells expressing the HIV *gag* proteins. The ADCC directed against gp160 was present at serum dilutions up to 1/316,000. There was no correlation between serum ADCC titer and the stage of HIV-related illness as determined by T-helper-cell numbers. These experiments clearly implicated gp160 as the target antigen of HIV-specific ADCC activity following natural infection. Vaccines which stimulate antibodies directed against gp160, which are capable of mediating ADCC against infected cells, could be important for protection against infection by cell-associated virus.

The acquired immunodeficiency syndrome is the ultimate result of infection with the human retrovirus human immunodeficiency virus (HIV) (15). Natural infection with this virus usually occurs after contact with infected blood or genital secretions (15), both of which are lymphocyte-containing fluids. Recent evidence suggests that semen contains both monocytes/macrophages and $CD4^+$ lymphocytes which could be infected with HIV (29), indicating the possible importance of cell-associated virus in HIV transmission.

One hypothesized method by which the body can protect itself against cell-associated virus invasion is by the action of antibodies which mediate antibody-dependent cell-mediated cytotoxicity (ADCC), in which cells expressing appropriate viral antigens are lysed, in a non-major histocompatibility complex-restricted manner, in the presence of antibodies and killer cells (8). Considering the probable contribution of cell-associated HIV to disease transmission, potential vaccines should be able to stimulate antibodies capable of mediating ADCC against infected cells, in order to offer maximum host protection.

It has recently been shown that high titers of HIV-specific ADCC antibodies can be found in sera from HIV-antibody-positive individuals (2, 10, 16, 21), but whether this antibody is predominantly directed against p55/p24/p17 (*gag*) or gp120/gp41 (envelope) determinants remains controversial. Vaccinia virus (VV) vectors have been used successfully to express foreign viral proteins in target cells and have been instrumental in delineating target antigens of virus-specific cytotoxic T lymphocytes in HIV (26) and other virus (12) infections. A recent report indicates that VV vectors can also be used successfully in HIV-specific ADCC assays (23). In the present study we used a series of VV vectors to express proteins from the envelope and *gag* genes of HIV to delineate the target antigens of HIV-specific ADCC antibodies in the sera of HIV-infected hemophiliacs. In addition, we attempted to correlate these ADCC responses with other known prognostic indicators of HIV disease progression.

MATERIALS AND METHODS

Sera. Samples were obtained from the extensive serum bank which is maintained for sera of approximately 150 hemophilia patients as part of an ongoing study of immunoregulatory defects in hemophilia (25). All samples were maintained at −80°C and were heat inactivated for 30 min at 56°C prior to assay. HIV serology was determined by Western blot (immunoblot).

Lymphocyte separation. Peripheral blood mononuclear cells (PBMCs) from healthy HIV-seronegative laboratory controls (for ADCC assay) or hemophilia patients (for surface marker analysis) were isolated from freshly drawn heparinized venous blood by centrifugation on Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, N.J.) density gradients (25). PBMC preparations contained 70 to 80% lymphocytes, 10 to 20% monocytes, and <20% polymorphonuclear leukocytes as determined by cell morphology.

Lymphocyte surface marker studies. The relative percentage of the CD4 lymphocyte population was determined by direct immunofluorescence with phycoerythrin-conjugated mouse monoclonal antibody Leu3a (Becton Dickinson and Co., Mountain View, Calif.). Samples were analyzed with a FACS 440 fluorescence-activated cell sorter (Becton Dickinson). Absolute numbers of CD4 lymphocytes per microliter of blood were determined by multiplying the relative percentage by the absolute number of PBMCs as determined from complete blood counts.

VV vectors. Molecular clones containing sequences encod-

* Corresponding author.

ing envelope, *gag*, or *pol* genes from HIV strain BH10 (vAbT 140, 141, or 228) or HXB2 (vAbT 200) (20) were inserted into VV strain NYCBH (ATCC VR-325) by methods previously described (11, 14). Briefly, plasmid vectors that direct the insertion and expression of both the HIV gene of interest and the *Escherichia coli lacZ* gene into the thymidine kinase (*tk*) locus of VV were constructed. These plasmids, which contain the HIV gene under the control of the VV 7.5K (vAbT 140, 141, or 200) or 40K (vAbT 228) promoter (30), the *E. coli lacZ* gene under the control of the VV *Hin*d-F promoter (17), and flanking sequences from the VV *tk* gene (17) were introduced into BSC-40 cells (3) previously infected with VV. Homologous recombination between plasmid and virus in the *tk* region resulted in recombinant viruses which were selected on the basis of their TK^- phenotype (27) and identified as blue plaques, due to expression of β-galactosidase, in the presence of the chromogenic substrate halogenated indolyl-β-D-galactoside (Bluo-Gal; Bethesda Research Laboratories, Gaithersburg, Md.) (17). Expression of the desired HIV antigen was confirmed by an in situ enzyme-linked immunosorbent assay (ELISA) (black-plaque assay) as described below. Recombinant vAbT 140 contains the entire HIV envelope-coding sequence and 96 base pairs (bp) of 5′-proximal and 107 bp of 3′-proximal untranslated sequences. Recombinant vAbT 141 contains the entire HIV *gag* coding sequence beginning at the translation initiation codon and extending approximately 200 bp beyond the *gag* translation termination codon. Recombinant vAbT 200 contains the entire HIV *gag* and *pol* genes beginning at the initiation codon of *gag* and extending to the *Nde*I site 26 bp beyond the *pol* translation termination codon. Recombinant vAbT 228 contains *gag* sequences from the initiation codon of *gag* through the *Pvu*II site, 38 bp upstream of the end of p17 coding sequences. A synthetic linker was added to restore the terminal sequences of p17, along with a stop codon and a VV transcription termination sequence. The predicted molecular structures of the recombinant viruses were confirmed by restriction endonuclease analysis and DNA hybridization of viral genomic DNA. VV strain NYCBH was used as the control virus in all ADCC studies.

Expression analysis. Expression of the HIV antigens by the recombinant VV vectors was confirmed in BSC-40 cells by an in situ ELISA performed directly on viral plaques (24). Incubation with 4D12.1 (monoclonal anti-p55; Epitope, Beaverton, Oreg.), NEA-9303 (monoclonal anti-gp41; Du Pont Co., Wilmington, Del.), or p17 Monobody (Cellular Products, Buffalo, N.Y.) was followed by incubation with alkaline phosphatase-labeled goat anti-mouse immunoglobulin G (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) and the precipitating substrates 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (Kirkegaard & Perry).

Radioimmunoprecipitations were done by infecting 10^6 target cells at a multiplicity of infection of 10 for 4 h (BSC-40) or 16 h (RH-LCL) in the presence of 100 μCi of [^{3}H]glucosamine (vAbT 140) or [^{3}H]leucine (vAbT 141, 200, and 228). Harvesting and precipitation were carried out as previously described (28) by using mouse monoclonal antibodies against HIV antigens, pooled HIV-positive VV-negative human sera, or specific rabbit antisera from previously vaccinated animals.

Cell culture supernatants were tested for HIV *gag* antigen in an antigen capture ELISA, using a monoclonal antibody system (Du Pont).

Surface expression of HIV envelope glycoprotein was assessed by the ability of VV vector-infected cells to form syncytia with c8166 cells in a 4-h coincubation (26).

ADCC assay. An Epstein-Barr virus (EBV)-transformed B lymphoblastoid cell line (RH-LCL), created by coincubation of a B95.8 (ATCC CRL-1612) cell culture supernatant with the lymphocytes of an 8-year-old HIV-seronegative individual, was used as the target cell line in these experiments.

RH-LCL cells were infected with VV vectors (NYCBH and vAbT 140, 141, 200, and 228) at a multiplicity of infection of 10 by the methods of McMichael et al. (12). Sixteen hours later the cells were washed, incubated with 100 μCi of $Na_2{}^{51}CrO_4$ (New England Nuclear Corp., Boston, Mass.) at 37°C for 60 min, washed twice with phosphate-buffered saline, and suspended, after viability determination by trypan blue exclusion, to an appropriate concentration in RPMI 1640 with 10% heat-inactivated fetal calf serum (FCS). Ninety microliters of target cell suspension containing 10^4 target cells was dispersed into wells of 96-well round-bottom microdilution plates containing 20 μl of serum appropriately diluted in RPMI 1640 with 10% FCS. After 20 min at room temperature, 90 μl of effector cell suspension containing 2.5×10^5 freshly isolated PBMCs from a healthy HIV-seronegative laboratory donor was added to the test wells (effector cell-to-target cell ratio, 25:1). Control wells with RPMI 1640 with 10% FCS in place of effector cells, serum, or both, were included in all assays. The test plates were centrifuged at $200 \times g$ for 5 min and incubated for 8 h at 37°C in a humidified, 5% CO_2 environment. The plates were then recentrifuged at $200 \times g$ for 5 min, 100 μl of assay supernatant was collected from each well, and the radioactivity was counted in a gamma counter (Packard Instrument Co., Inc., Downers Grove, Ill.). Maximal incorporation was established by determining the counts per minute (cpm) in a 45-μl sample of the target cell suspension. All tests were performed in triplicate.

Percent ADCC was calculated as percent cytolysis in the presence of serum minus percent cytolysis in the absence of serum, where percent cytolysis was calculated by the following formula: $100 \times$ [(test cpm − spontaneous cpm)/(maximal cpm − spontaneous cpm)].

Effector cell depletions. Effector PBMCs were depleted of cells expressing a natural killer (NK) phenotype by using monoclonal antibody and rabbit complement. Briefly, freshly isolated PBMCs were incubated with monoclonal antibody Leu11b (Becton Dickinson) for 30 min at 4°C in RPMI 1640 with 2% FCS. After being washed, the cells were lysed by incubation with rabbit complement (Cederlane Laboratories Ltd., Hornby, Ontario, Canada) for 60 min at 37°C. The cytotoxic activity of the depleted cells was assessed immediately after depletions. PBMCs incubated with complement alone were included in all depletion assays as background controls, and the adequacy of the depletions was assessed by inhibition of killing of the NK cell-sensitive erythroleukemia cell line K-562 (ATCC CCL243) in parallel assays.

Statistical analysis. The data shown in figures represent means ± standard deviations, and *n* refers to the number of serum samples tested from separate individuals. Significance was determined when appropriate by analysis of variance, and statistical significance was defined as $P < 0.05$.

RESULTS

The production and processing of HIV proteins in RH-LCL cells infected with recombinant VV vectors was as-

TABLE 1. HIV protein expression in RH-LCL cells infected with recombinant VV vectors[a]

Vector	RIP	Supernatant *gag*-antigen ELISA	Syncytia with c8166 cells
None (uninfected RH-LCL cells)	–	–	–
NYCBH	–	–	–
vAbT 140	gp41, gp120, gp160	–	+
vAbT 141	p55	+	–
vAbT 200	p24, p40, p55	+	–
vAbT 228	p17	–	–

[a] Expression of HIV proteins in RH-LCL cells 16 h after infection with recombinant VV vectors, determined by radioimmunoprecipitation (RIP) using polyclonal human HIV-seropositive, VV-seronegative serum; HIV *gag*-antigen ELISA, using a monoclonal antibody assay system (Du Pont); and ability of infected cells to form syncytia with c8166 cells.

sessed by radioimmunoprecipitation. The envelope gene product (gp160) was found to be produced and processed to surface protein gp120 and transmembrane protein (9) gp41 in vAbT 140-infected cells. The *gag* recombinant vectors produced unprocessed p55 protein (vAbT 141), processed *gag* products p55, p40, and capsid protein p24 (vAbT 200), or matrix protein p17 (vAbT 228) (Table 1). Surface expression of envelope glycoprotein was demonstrated in vAbT 140-infected RH-LCL cells by their ability to form syncytia with c8166 cells during a 4-h cocultivation, and immunologically active extracellular *gag* protein was demonstrated in vAbT 141- and vAbT 200-infected RH-LCL cells by ELISA of culture supernatant (Table 1). This production of extracellular HIV p55 occurred without loss of cell viability over a 24-h period and therefore did not reflect release of p55 related to cell death. The presence of *gag* proteins on the surface of vAbT 141-, 200-, or 228-infected RH-LCL cells could not, however, be demonstrated by indirect immunofluorescence. This was true despite the use of several HIV-specific mouse monoclonal antibodies and several different second antibody-fluorescein isothiocyanate conjugates.

Having demonstrated appropriate HIV protein production in VV vector-infected RH-LCL targets, we assayed multiple serum samples from pediatric and adult hemophilia patients for their ability to mediate ADCC against NYCBH-infected and vAbT 140-infected RH-LCL targets. In all HIV-seropositive sera tested, ADCC reacting against targets expressing HIV surface protein and transmembrane protein was present at greater serum dilutions than ADCC reacting against NYCBH-infected targets, allowing for a clear delineation of these two activities. Indeed, in more than 80% of these sera, the difference was present at a serum dilution of 1/1,000. No ADCC against gp160 above that observed against VV could be demonstrated in HIV-seronegative sera. Representative data for three sera are presented in Fig. 1 and demonstrate HIV gp160-specific ADCC at a serum dilution of 1/1,000 or greater, whereas VV- or EBV-specific ADCC was absent at similar serum dilutions. In all further experiments, sera with greater than 20% ADCC against NYCBH-infected targets at a serum dilution of 1/1,000 were discarded (<15% of sera tested).

NK cells bearing a CD16 phenotype are known to mediate in vitro ADCC responses (8). The HIV envelope glycoprotein-specific ADCC demonstrated by the results in Fig. 1 was shown to be K-cell mediated, as evidenced by inhibition of killing after Leu11b-directed complement-mediated depletion of effector cells (Table 2).

Having determined that gp160-specific ADCC was detectable with VV vector-infected targets and that the optimum serum dilution was approximately 1/1,000, we assayed sera from 19 hemophilia patients (6 HIV seronegative, 7 HIV seropositive with normal numbers of T helper cells, and 6 HIV seropositive with decreased numbers of T helper cells)

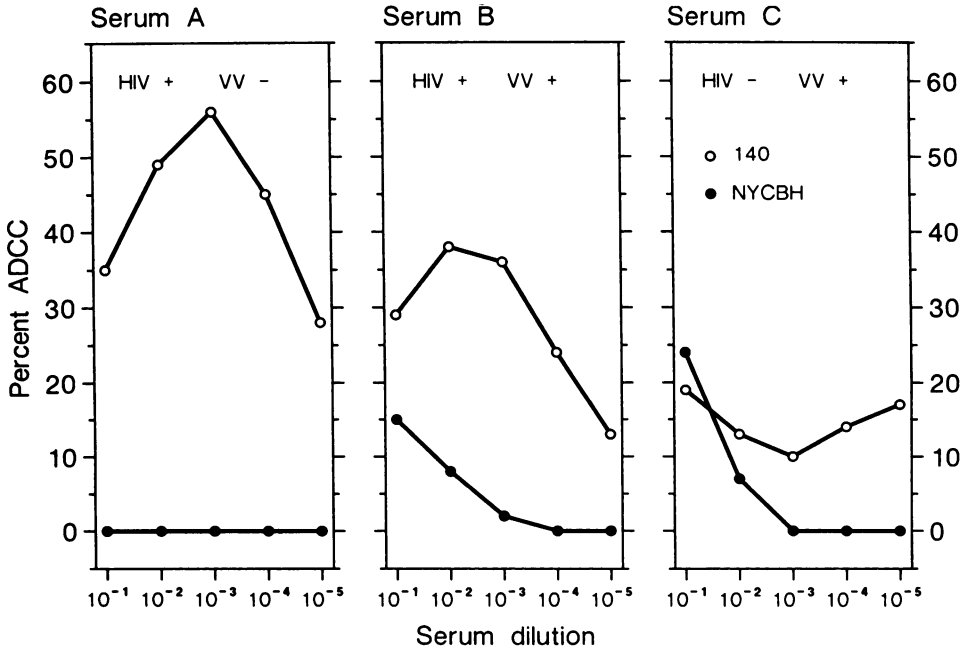

FIG. 1. Percent ADCC for three representative sera assayed at various dilutions against RH-LCL targets infected with control VV NYCBH or with vAbT 140, which expresses HIV envelope gp160. The ability to differentiate VV- from HIV-specific ADCC at a serum dilution of 1/1,000 (10^{-3}) is evident.

TABLE 2. NK cell dependence of measured ADCC responses[a]

Effectors	% ADCC against RH-LCL cells infected with:		% Cytolysis of NK-cell control K562
	NYCBH	vAbT 140	
PBMCs	0	46	80
PBMCs + complement	2	36	70
PBMCs + anti-Leu11b + complement	0	11 (69)	17 (76)

[a] NK cells were depleted from effector PBMCs by using anti-Leu11b and rabbit complement and then used in an ADCC assay with a known HIV-seropositive serum diluted 1/1,000. The numbers in parentheses indicate percent decreases in ADCC compared with that in the complement control. Cytolysis of the NK-sensitive cell line K562 was measured in the absence of added serum and was included as a measure of NK cell depletion.

for their ability to mediate HIV envelope- or *gag*-specific ADCC at a serum dilution of 1/1,000. Of the 13 HIV-seropositive sera tested, 12 had antibodies to p55, 10 had antibodies to p24, 5 had antibodies to p17, and all 13 had antibodies to gp41 and gp120 by Western blot. HIV envelope-specific ADCC activity could be demonstrated at a serum dilution of 1/1,000 in all 13 HIV-seropositive sera tested. None of these sera, however, were capable of mediating HIV *gag*-specific ADCC against targets expressing processed or unprocessed *gag* antigens at the same dilution. The six HIV-seronegative sera failed to mediate ADCC against targets expressing envelope or *gag* antigens. Cumulative data for these 19 sera are shown in Fig. 2 and clearly demonstrate that the HIV-seropositive sera contained a statistically significant ($P < 0.01$) ADCC activity against envelope-expressing targets compared with that against VV- or *gag*-expressing targets. The level of HIV envelope-specific ADCC noted in these HIV-seropositive sera did not differ significantly between individuals with

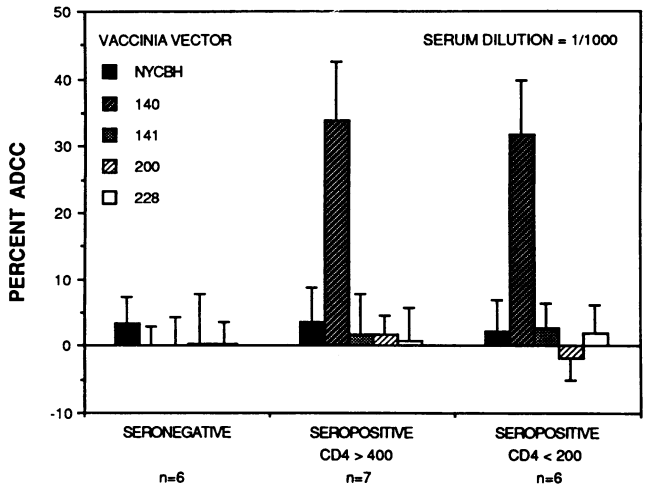

FIG. 2. Cumulative ADCC results for 19 HIV-seropositive and -seronegative sera assayed at a dilution of 1/1,000. Target cell lines were RH-LCL infected with vectors NYCBH (control), vAbT 140 (gp41, gp120, and gp160), vAbT 141 (p55), vAbT 200 (p24, p40, and p55), or vAbT 228 (p17). Error bars represent 1 standard deviation of the mean. RH-LCL cells infected with vector vAbT 140 were lysed in the presence of HIV-seropositive sera from individuals with low or mean-normal numbers of CD4 lymphocytes to a significantly ($P < 0.01$) greater degree than were other vector-infected RH-LCL cells or RH-LCL cells in the presence of HIV-seronegative sera.

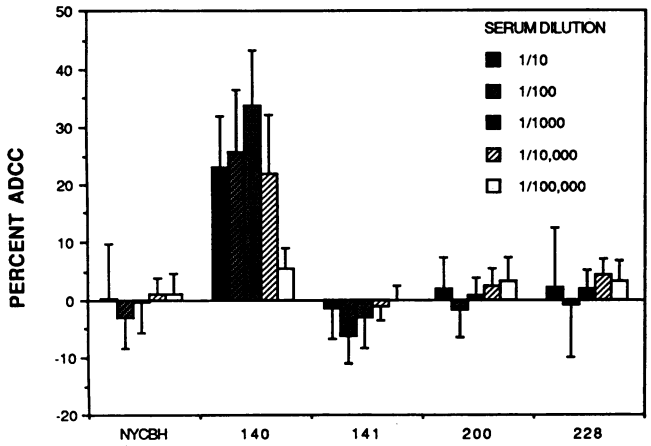

FIG. 3. Cumulative ADCC results for eight HIV-seropositive sera assayed over a range of dilutions against RH-LCL cells infected with NYCBH (control), vAbT 140 (gp41, gp120, and gp160), vAbT 141 (p55), vAbT 200 (p24, p40, and p55), or vAbT 228 (p17). Error bars represent 1 standard deviation of the mean. While ADCC against vAbT 140-infected RH-LCL cells is apparent to a serum dilution of 1/10,000 or greater, no ADCC is apparent against *gag*-expressing targets at any serum dilution.

near-normal (>400/μl) numbers of T helper cells and those with severely decreased (<200/μl) numbers of T helper cells.

To determine whether the absence of HIV *gag*-directed ADCC in HIV-seropositive sera was a result of assaying nonoptimal serum dilutions, eight HIV-seropositive sera were assayed over a range of serum dilutions. All eight sera tested, regardless of maximum cytolysis, showed optimum envelope-specific ADCC at a serum dilution of 1/1,000. No HIV *gag*-specific ADCC was demonstrable in any of the eight sera tested over a range of serum dilutions from 1/10 through 1/100,000 (Fig. 3). Subsequent experiments performed at higher effector cell-to-target cell ratios (up to 100 to 1) and longer incubation times (up to 12 h) also failed to demonstrate *gag*-specific ADCC in any HIV-seropositive serum tested (data not shown).

The HIV envelope-specific ADCC titers of four sera from HIV-seropositive hemophiliacs with near-normal numbers of T helper cells and four sera from HIV-seropositive hemophiliacs with very low numbers of T helper cells were determined at half-log dilutions to establish whether ADCC titer varied between these two groups of patients. HIV ADCC titer was defined as the greatest serum dilution which was capable of mediating ADCC against vAbT 140-infected targets greater than two standard deviations above the mean ADCC against NYCBH-infected targets for all sera assayed at that dilution on a given day. By using this system, it was found that even patients with severe T-helper-cell depletions maintained high-titer serum HIV-specific ADCC activity and no difference in titer could be demonstrated between the two groups (Table 3).

DISCUSSION

ADCC has been shown to be of prognostic importance in certain viral systems, particularly in chronic or transforming virus infections. Antibodies to EBV antigens which mediate ADCC have been shown to be of prognostic importance in Burkitt's lymphoma and nasopharyngeal carcinoma (18, 19).

TABLE 3. Serum titer of HIV envelope-specific ADCC

Patient	T helper cells/ μl of blood	ADCC titer[a]
A	9	1/10,000
B	33	1/100,000
C	72	1/31,600
D	138	1/100,000
E	677	1/10,000
F	705	1/100,000
G	792	1/100,000
H	1,163	1/316,000

[a] Serum envelope-specific ADCC titers in the sera of four HIV-seropositive hemophiliacs with near-normal numbers of T helper cells (patients E through H) and four HIV-seropositive hemophiliacs with very low numbers of T helper cells (patients A through D). The difference in geometric mean titers (1/75,000 and 1/42,200, respectively) between the two groups is not statistically significant.

In addition, human T-cell lymphotropic virus type I (HTLV-I)-specific ADCC antibodies have been demonstrated in HTLV-I-infected individuals and have been correlated with the presence or absence of adult T-cell leukemia (13).

HIV-specific ADCC activity in the sera of infected individuals has been described (2, 10, 16, 21), and the present study was undertaken to determine the antigenic specificity of these ADCC antibodies. Recombinant VV vectors expressing foreign viral genes have been valuable tools in defining immune responses in HIV and other viral infections (12, 26) and were chosen for use in this study for four reasons. First, HIV is known to infect B cells and EBV-transformed B-lymphoblastoid cell lines (B-LCLs), allowing the assay results to have some relevance to in vivo infection (6). Second, the use of recombinant VVs expressing HIV genes allows us to present individual HIV proteins in infected cells. Third, B-LCLs infected with VV vectors expressing foreign viral proteins have been used with great success in defining the antigenic specificity of virus-specific cytotoxic T-lymphocyte responses after HIV (26) and other viral (12) infections, and as a result these cells are known to be relatively resistant to NK cell lysis in the absence of antibody. Fourth, HIV envelope-specific ADCC is known to be measurable with VV vectors expressing this glycoprotein (23).

Since the majority of the hemophilia patients tested had antibodies to EBV or VV or both and these viral antigens were present in the recombinant VV vector-infected B-LCL target cells, the possibility existed that serum HIV-specific ADCC activity would be obscured by VV- or EBV-specific responses. In our screening, however, we found that serum HIV-specific ADCC was always present at higher titers than ADCC activity against uninfected or VV-infected B-LCL targets. In more than 85% of the sera tested a clear difference in ADCC against NYCBH-infected targets compared with that against vAbT 140-infected targets was demonstrable at a serum dilution of 1/1,000. The low titer of VV-specific ADCC noted in this hemophiliac population was not unexpected since smallpox vaccination represents a remote viral infection and as a result ADCC titers to this virus have declined, whereas HIV represents an ongoing infection with stimulation of high-titer HIV-specific ADCC antibody responses. In addition, it is known that EBV-transformed B-LCLs are latently infected with EBV and do not have surface expression of membrane antigens responsible for EBV-specific ADCC (1).

We were able to screen multiple serum samples at a dilution of 1/1,000 in the ADCC assay and demonstrated K-cell-dependent, HIV-specific responses in all 13 HIV-seropositive sera and in none of the 6 HIV-seronegative sera. Other ADCC assay systems have been unable to detect HIV-specific responses in all HIV-seropositive sera (2, 10, 21). Our ability to more easily detect these responses probably relates to the high infection rate and HIV protein presentation in recombinant VV vector-infected targets compared with HIV-infected targets.

All of the measured ADCC activity in these assays was directed against targets expressing HIV envelope glycoproteins and not *gag* antigens. When the titers of the HIV envelope-specific ADCC were determined for a small population of HIV-seropositive hemophiliacs, no correlation between ADCC titer and absolute numbers of T helper cells could be identified. Indeed, one patient with acquired immunodeficiency syndrome with only nine T helper cells per microliter of blood and no p24 serum antibody still had a serum HIV ADCC titer of 1/10,000. Other investigators have failed to correlate HIV-specific ADCC activity with disease markers such as T helper/T suppressor cell ratios (10), but some studies have shown a decrease in ADCC activity after the development of acquired immunodeficiency syndrome (2, 10, 21). The failure to corroborate the latter observation in our study may simply relate to the low number of sera tested.

One previous study of HIV-specific ADCC responses was able to correlate decrease or absence of ADCC activity with the disappearance of Western blot reactivity with p24 antigen (22), whereas others have not reported similar findings and indeed have reported a correlation between ADCC activity and antibody to gp120 and gp41 (2, 10, 16). In our study we were unable to detect any serum ADCC activity against targets expressing *gag* antigens. This inability to detect serum *gag*-specific ADCC occurred despite the fact that Western blots confirmed the presence of *gag*-specific immunoglobulin G in the sera of 12 of the 13 HIV-seropositive individuals tested.

The absence of serum ADCC activity against *gag* antigens probably relates to the fact that these proteins are not known to be present on the cell surface. We were able to detect intracellular *gag* antigens in vAbT 141-, 200-, and 228-infected target cells and extracellular *gag* antigens in vAbT 141- and 200-infected cells but could not determine their presence on the cell surface by indirect immunofluorescent staining. This is probably not unique to the way *gag* antigens are produced in VV vectors, as other groups have also failed to detect surface expression of *gag* antigens in HIV-infected cells by using *gag*-specific monoclonal antibodies (5, 7). In addition, Evans et al. (7) have recently shown that a human immunoglobulin G4 monoclonal antibody which reacts with HIV p55 and p25 is incapable of mediating ADCC against HIV-infected targets.

One must also consider the possibility that individual antigens as expressed by vector-infected cells may be presented differently than they are in naturally infected cells. The envelope glycoprotein of HIV, as expressed in recombinant VV vectors, is known to be processed and glycosylated normally (4), and the end products become situated in the outer cell membrane in a manner which allows for spontaneous syncytia formation in the presence of CD4-bearing cells (26). As such, this viral product appears to be presented in much the same manner as it would be in HIV-infected cells. Less is known, however, about the natural presentation of *gag* antigens in HIV-infected cells. It has been suggested that the p17 product of the *gag* gene may

actually protrude through the surface of the intact virion (22), and therefore it could be postulated that p17 may protrude through the surface of the cell membrane during the process of viral budding and as a result be a target antigen of ADCC. Our inability to detect ADCC activity against cells infected with a vector expressing p17 does not support this theory, although it is possible that envelope glycoproteins are required for the correct orientation and anchoring of the p17 molecule in the budding virion. Studies in our laboratory and those of others have been able to show that recombinant VV vector-infected B-LCL targets present *gag* antigens on their surfaces which are recognized by cytotoxic T lymphocytes from HIV-infected patients (26; R. A. Koup, J. L. Sullivan, P. H. Levine, D. Brettler, S. McKenzie, and D. Panicali, Clin. Res. **36**:575A, 1988), but these antigens probably represent small amphipathic peptides presented in the context of major histocompatibility complex molecules which are recognized by T cells and not antibodies.

In conclusion, the present study demonstrates that VV vectors expressing foreign viral proteins, which have been used with great success in elucidating cellular immune responses to virus infection, can also be used to delineate combined humoral and cellular immune responses to virus infection in the form of ADCC. This study clearly identified the envelope glycoprotein complex gp120/gp41 as a major target antigen of HIV-specific ADCC antibodies and established that these antibodies were present at high titer and remained at high titer even in advanced stages of disease. Although we were unable to demonstrate *gag* antigens as targets for ADCC antibodies in our system, the differences between antigen presentation in vector-infected and HIV-infected cells does not allow us to rule these out as possible target antigens of HIV-specific ADCC in some HIV-infected cells.

ACKNOWLEDGMENTS

We thank B. Q. Ferguson and S. R. Petteway, Experimental Station, E. I. du Pont de Nemours & Co., Inc., Wilmington, Del., for the gift of molecular clones containing sequences encoding envelope and *gag* genes of HIV strain BH10. In addition, we thank Antonia Destree, Nancy Cormier, and Eric Day for technical assistance in vector preparation and expression analysis and Kathy Beauregard and Teresa Reed for manuscript preparation.

This work was supported in part by Public Health Service grant AI 21161 from the National Institutes of Health. J.L.S. is an established investigator of the American Heart Association.

LITERATURE CITED

1. **Aya, T., F. Mizuno, and T. Osato.** 1980. Immunologic cytotoxicity against autologous human lymphocytes transformed or infected by Epstein-Barr virus: role of antibody-dependent cellular cytotoxicity in healthy individuals. J. Natl. Cancer Inst. **65**:265–271.
2. **Blumberg, R. S., T. Paradis, K. L. Hartshorn, M. Vogt, D. D. Ho, M. S. Hirsch, J. Leban, V. L. Sato, and R. T. Schooley.** 1987. Antibody-dependent cell-mediated cytotoxicity against cells infected with the human immunodeficiency virus. J. Infect. Dis. **156**:870–877.
3. **Brockman, W. W., and D. Nathans.** 1974. The isolation of simian virus 40 variants with specifically altered genomes. Proc. Natl. Acad. Sci. USA **71**:942–946.
4. **Chakrabarti, S., M. Robert-Guroff, F. Wong-Staal, R. C. Gallo, and B. Moss.** 1986. Expression of the HTLV-III envelope gene by a recombinant vaccinia virus. Nature (London) **320**:535–537.
5. **Chassagne, J., P. Verrelle, C. Dionet, F. Clavel, F. Barre-Sinoussi, J. C. Chermann, L. Montagnier, J. C. Gluckman, and D. Klatzmann.** 1986. A monoclonal antibody against LAV GAG precursor: use for viral protein analysis and antigenic expression in infected cells. J. Immunol. **136**:1442–1445.
6. **Dalgleish, A. G., P. C. L. Beverley, P. R. Clapham, D. H. Crawford, M. F. Greaves, and R. A. Weiss.** 1984. The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. Nature (London) **312**:763–767.
7. **Evans, L. A., J. M. Homsy, W. J. W. Morrow, I. Gaston, C. D. Sooy, and J. A. Levy.** 1988. Human monoclonal antibody against gag gene products of the human immunodeficiency virus. J. Immunol. **140**:941–943.
8. **Lanier, L. L., and J. H. Phillips.** 1986. Evidence for three types of human cytotoxic lymphocyte. Immunol. Today **7**:132–134.
9. **Leis, J., D. Baltimore, J. M. Bishop, J. Coffin, E. Fleissner, S. P. Goff, S. Oroszlan, H. Robinson, A. M. Skalka, H. M. Terniu, and V. Vogt.** 1988. Standardized and simplified nomenclature for proteins common to all retroviruses. J. Virol. **62**:1808–1809.
10. **Ljunggren, K., B. Bottiger, G. Biberfeld, A. Karlson, E. M. Fenyo, and M. Jondal.** 1987. Antibody-dependent cellular cytotoxicity-inducing antibodies against human immunodeficiency virus. J. Immunol. **139**:2263–2267.
11. **Mackett, M., G. L. Smith, and B. Moss.** 1984. General method for production and selection of infectious vaccinia virus recombinants expressing foreign genes. J. Virol. **49**:857–864.
12. **McMichael, A. J., C. A. Michie, F. M. Gotch, G. L. Smith, and B. Moss.** 1986. Recognition of influenza A virus nucleoprotein by human cytotoxic T lymphocytes. J. Gen. Virol. **67**:719–726.
13. **Miyakoshi, H., H. Koide, and T. Aoki.** 1984. In vitro antibody-dependent cellular cytotoxicity against human T-cell leukemia/lymphoma virus (HTLV)-producing cells. Int. J. Cancer **33**: 287–291.
14. **Nakano, E., D. Panicali, and E. Paoletti.** 1982. Molecular genetics of vaccinia virus: demonstration of marker rescue. Proc. Natl. Acad. Sci. USA **79**:1593–1596.
15. **Nicholson, J. K. A., J. S. McDougal, H. W. Jaffe, T. J. Spira, S. M. Kennedy, B. M. Jones, W. W. Darrow, M. Morgan, and M. Hubbard.** 1985. Exposure to human T-lymphotropic virus type III/lymphadenopathy-associated virus and immunologic abnormalities in asymptomatic homosexual men. Ann. Intern. Med. **103**:37–42.
16. **Ojo-Amaize, E. A., P. Nishanian, D. E. Keith, R. L. Houghton, D. F. Heitjan, J. L. Fahey, and J. V. Giorgi.** 1987. Antibodies to human immunodeficiency virus in human sera induce cell-mediated lysis of human immunodeficiency virus-infected cells. J. Immunol. **139**:2458–2463.
17. **Panicali, D., A. Grzelecki, and C. Huang.** 1986. Vaccinia virus vectors utilizing the β-galactosidase assay for rapid selection of recombinant viruses and measurement of gene expression. Gene **47**:193–199.
18. **Pearson, G. P., B. Johanson, and G. Klein.** 1978. Antibody-dependent cellular cytotoxicity against Epstein-Barr virus-associated antigens in African patients with nasopharyngeal carcinoma. Int. J. Cancer. **22**:120–125.
19. **Pearson, G. R., L. F. Qualtiere, G. Klein, T. Norin, and I. S. Bal.** 1979. Epstein-Barr virus-specific antibody-dependent cellular cytotoxicity in patients with Burkitt's lymphoma. Int. J. Cancer **24**:402–406.
20. **Ratner, L., W. Haseltine, R. Patarca, K. J. Livak, B. Starcich, S. F. Josephs, E. R. Doran, J. A. Rafalski, E. A. Whitehorn, K. Baumeister, L. Ivanoff, S. R. Petteway, Jr., M. L. Pearson, J. A. Lautenberger, T. S. Papas, J. Ghrayeb, N. T. Chang, R. C. Gallo, and F. Wong-Staal.** 1985. Complete nucleotide sequence of the AIDS virus, HTLV-III. Nature (London) **313**:277–284.
21. **Rook, A. H., H. C. Lane, T. Folks, S. McCoy, H. Alter, and A. S. Fauci.** 1987. Sera from HTLV-III/LAV antibody-positive individuals mediate antibody-dependent cellular cytotoxicity against HTLV-III/LAV-infected T-cells. J. Immunol. **138**:1064–1067.
22. **Sarin, P. S., D. K. Sun, A. H. Thornton, P. H. Naylor, and A. L. Goldstein.** 1986. Neutralization of HTLV-III/LAV replication by antiserum to thymosin α_1. Science **232**:1135–1137.
23. **Shepp, D. H., S. Chakrabarti, B. Moss, and G. V. Quinnan, Jr.** 1988. Antibody-dependent cellular cytotoxicity specific for the envelope antigens of human immunodeficiency virus. J. Infect. Dis. **157**:1260–1264.

24. **Smith, G., B. Murphy, and B. Moss.** 1983. Construction and characterization of an infectious vaccinia virus recombinant that expresses the influenza hemagglutinin gene and induces resistance to influenza virus infection in hamsters. Proc. Natl. Acad. Sci. USA **80**:7155–7159.
25. **Sullivan, J. L., F. E. Brewster, D. B. Brettler, A. D. Forsberg, S. H. Cheeseman, K. S. Byron, S. M. Baker, D. L. Willitts, R. A. Lew, and P. H. Levine.** 1986. Hemophiliac immunodeficiency: influence of exposure to factor VIII concentrate, LAV/HTLV III and herpesviruses. J. Pediatr. **108**:504–510.
26. **Walker, B. D., S. Chakrabarti, B. Moss, T. J. Paradis, T. Flynn, A. G. Durno, R. S. Blumberg, J. C. Kaplan, M. S. Hirsch, and R. T. Schooley.** 1987. HIV-specific cytotoxic T lymphocytes in seropositive individuals. Nature (London) **328**:345–348.
27. **Weir, J. P., and B. Moss.** 1983. Nucleotide sequence of the vaccinia virus thymidine kinase gene and the nature of spontaneous frameshift mutations. J. Virol. **46**:530–537.
28. **Wittek, R., M. Hanggl, and G. Hiller.** 1984. Mapping of a gene coding for a major late structural polypeptide on the vaccinia virus genome. J. Virol. **49**:371–378.
29. **Wolf, H., and D. J. Anderson.** 1988. Potential human immunodeficiency virus-host cells in human semen. AIDS Res. Hum. Retroviruses **4**:1–2.
30. **Yenkatesan, S., B. M. Baroudy, and B. Moss.** 1981. Distinctive nucleotide sequences adjacent to multiple initiation and termination sites of an early vaccinia virus gene. Cell **125**:805–813.