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Cytotoxic T-Lymphocyte Responses to Cytomegalovirus in Normal and Simian Immunodeficiency Virus-Infected Rhesus Macaques

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Disseminated cytomegalovirus (CMV) infection is a frequent occurrence in human immunodeficiency virus-infected humans and in simian immunodeficiency virus (SIV)-infected rhesus macaques. Rhesus macaques are a suitable animal model with which to study in vivo interactions between CMV and AIDS-associated retroviruses. Since cytotoxic T lymphocytes (CTL) play a major role in control of viral infections, we have characterized CMV-specific CTL responses in SIV-infected and uninfected rhesus macaques. Autologous fibroblasts infected with rhesus CMV were used to stimulate freshly isolated peripheral blood mononuclear cells from CMV-seropositive animals. Following in vitro stimulation, specific CTL activity against CMV-infected autologous fibroblasts was detected in CMV-seropositive but not in CMV-seronegative normal macaques. CMV-specific CTL activity comparable to that in normal animals was also detected in two CMV-seropositive macaques infected with a live attenuated SIV strain (SIVΔ3) and in two of three macaques infected with pathogenic SIV strains. The CMV-specific CTL response was class I major histocompatibility complex restricted and mediated by $CD8^+$ cells. An early CMV protein(s) was the dominant target recognized by bulk CTL, although the pattern of CTL recognition of CMV proteins varied among animals. Analysis of CMV-specific CTL responses in macaques should serve as a valuable model for CMV immunopathogenesis and will facilitate prospective in vivo studies of immune interactions between CMV and SIV in AIDS.

Disseminated cytomegalovirus (CMV) infection is a common cause of morbidity and mortality in immunosuppressed humans, particularly in recipients of organ transplants and individuals with AIDS (15, 23, 24). CMV disease is also seen in nonhuman primates with simian immunodeficiency virus (SIV) infection and AIDS (4, 5, 38). The pathogenesis of CMV disease in AIDS is thought to reflect reactivation of latent CMV infection following human immunodeficiency virus (HIV)- or SIV-induced immunosuppression (4, 11), and a number of different mechanisms have been proposed to explain this interaction. In vitro studies suggest that CMV can enhance HIV replication and vice versa (24, 39). CMV immediate-early (IE) gene products can transactivate transcription of the HIV or SIV long terminal repeat (2, 3, 11). Similarly, the HIV Tat protein can transactivate transcription of CMV IE genes (16, 39). However, these observations are derived from cells coexpressing CMV and HIV genes, probably an uncommon event in vivo, and their biological significance is unknown. CMV gene products in monocytes may act also as superantigens in HIV-infected CMV-seropositive individuals, leading to preferential HIV replication in $CD4^+$ cells with the T-cell receptor phenotype Vβ12 (10). There is also evidence in HIV-infected individuals that CMV seropositivity, CMV viremia, and acute CMV infection are associated with an increased risk of progressive HIV infection (23). However, despite the expanding body of evidence regarding the interactions between CMV and HIV, the factors determining CMV dissemination in HIV infection and its role in accelerating progression to AIDS remain incompletely elucidated.

CMV infection is widely prevalent in captive nonhuman primates such as African green monkeys and rhesus macaques (41–43). The clinical course of CMV infection in these animals appears to be similar to that in humans in that immunocompetent animals become latently infected and may have intermittent asymptomatic viruria (1, 41). Immunocompromised animals, including rhesus macaques with SIV infection, can develop disseminated CMV disease akin to that occurring in late-stage HIV infection in humans (4, 5, 27, 28, 38). In fact, CMV is the most common viral opportunistic infection occurring in SIV-infected macaques (21, 38). Thus, the simian model, particularly the rhesus macaque, should be a useful model with which to longitudinally study the effects of different stages of SIV infection on CMV viral load and anti-CMV immune responses.

The role of host immune responses to CMV has been well characterized in murine CMV infection. In BALB/c mice, $CD8^+$ cytotoxic T lymphocytes (CTL) directed against a nonapeptide of the IE protein pp89 protect against lethal CMV infection (9, 19, 30). The role of $CD4^+$ cells is limited to clearance of virus from the salivary glands, and this function is independent of B cells (20). Antibodies do not play a major role in containment of primary murine CMV infection but do limit dissemination following reactivation (20). In human CMV infection, there is evidence that CTL protect against CMV disease (29, 31, 36). Bone marrow and renal transplant recipients without CMV-specific CTL have a higher rate of CMV pneumonitis and CMV-related mortality than do recipients with detectable CTL responses (22, 29, 31, 36). Adoptive

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transfer of $CD8^+$ CMV-specific clones derived from donors into bone marrow allograft recipients may reduce CMV-related disease (32, 34, 44).

Since host immune responses to simian CMV infection may mimic those of humans, a study of simian CMV-specific CTL may shed light on the nature of CMV-HIV/SIV interactions in vivo. In this report, we characterize CMV-specific CTL responses in rhesus macaques. Healthy CMV-seropositive animals mounted a strong CMV-specific CTL response which was mediated by $CD8^+$ cells and was class I major histocompatibility complex (MHC) restricted. A similar response was detectable in four SIV-infected macaques that had not progressed to AIDS, two of which were infected with a pathogenic SIV strain and two of which were infected with an attenuated strain of SIV.

MATERIALS AND METHODS

Animals. Rhesus macaques used in the study were housed in the conventional or in the specific-pathogen-free (SPF) colony at the New England Regional Primate Research Center (NERPRC). SPF animals are free of type D retrovirus, simian T-cell lymphotropic virus type 1, SIV, and herpes B virus; animals from the conventional colony were seronegative for SIV unless otherwise noted. In addition, the following groups of SIV-infected macaques were studied: two infected with an attenuated virus, SIVΔ3 (deficient in *nef*, *vpr*, and the negative regulatory elements of the long terminal repeat (46), and three infected with a pathogenic molecular clone of SIV (SIVmac239 or SIVmac316).

Animals were maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and the "Guide for the Care and Use of Laboratory Animals" (DHHS publication no. [NIH] 85-23, revised 1985).

Virus stocks. CMV isolated from the lungs and gastrointestinal tract of a rhesus macaque (Mm 180.92) that died of AIDS was serially passaged on a primary rhesus fibroblast cell line to titers of $10^{6.0}$ to $10^{6.6}$ 50% tissue culture infectious doses ($TCID_{50}$)/ml. The virus was propagated by scraping infected fibroblasts off the culture flask after 100% cytopathic effect (CPE) was reached, sonicating the scraped cells, and resuspending them in medium containing 10% sorbitol. Virus aliquots were stored at −80°C and used for subsequent CTL experiments. Virus stocks were titered on 24-well plates by endpoint dilution using primary rhesus fibroblasts. Plates were monitored for CPE, and the number of wells with CPE at each virus dilution was determined after 3 weeks. The $TCID_{50}$ was calculated by using the formula of Reed and Muench as described elsewhere (18). Virus from passages 11 to 16 was used for infection in CTL assays at a multiplicity of infection of 5 to 10 $TCID_{50}$/cell.

Strain 68-1 of rhesus CMV (ATCC VR-677) was obtained from the American Type Culture Collection and propagated on a human embryonal lung fibroblast line (MRC-5 cells [ATCC CCL-171]) to titers of $10^{5.4}$ $TCID_{50}$/ml. Monolayers of MRC-5 cells infected with this virus and showing 75 to 100% CPE were used to make protein lysates for Western blotting (immunoblotting; WB) analysis.

Cell lines. Autologous rhesus fibroblast lines were established from 5-mm skin punch biopsies. Skin biopsies were dissected into 1- to 2-mm pieces, placed under a sterile glass slide on a scored 100-mm-diameter petri dish, and cultured at 37°C in Dulbecco's modified Eagle's medium (Cellgro, Mediatech, Herndon, Va.) with 20% fetal bovine serum (FBS; Sigma Chemical Co., St. Louis, Mo.), 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; Cellgro), 2 mM L-glutamine (Cellgro), 50 IU of penicillin (Cellgro) per ml, 50 μg of streptomycin (Cellgro) per ml, 30 μg of vancomycin (Sigma) per ml, and 10 μg of gentamicin (Sigma) per ml. Fibroblasts appearing in 4 to 7 days were maintained with twice-a-week medium exchanges and trypsinized when confluent. Established fibroblast lines could be propagated for a median of 18 to 20 passages and were usually used in CTL assays between passages 3 and 15.

Autologous transformed B-cell lines (B-LCL) of study animals were established for characterization of class I MHC alleles by isoelectric focusing (IEF) of immunoprecipitates of B-LCL. B cells were transformed by incubating peripheral blood mononuclear cells (PBMC) at 37°C in a 5% CO_2 incubator with herpesvirus papio derived from the supernatant of S594 cells (provided by Norman Letvin, Beth Israel Hospital, Boston, Mass.), and cyclosporin (0.5 μg/ml) in RPMI with 20% FBS. B-LCL were propagated in RPMI 1640 (Cellgro) supplemented with 20% FBS, 10 mM HEPES, 2 mM L-glutamine, 50 IU of penicillin per ml, and 50 μg of streptomycin per ml.

MRC-5 cells were used for propagation of rhesus CMV strain 68-1. Protein lysates made from infected and uninfected MRC-5 cells were used for immunoblotting.

Generation of CMV-specific CTL. PBMC were isolated from fresh heparinized blood by centrifugation over a Ficoll-Hypaque (Sigma) gradient and suspended at 2×10^6 cells per ml in RPMI 1640 medium (Sigma) supplemented with 10% FBS, 10 mM HEPES, 2 mM L-glutamine, penicillin (50 IU/ml), and streptomycin (50 μg/ml) (R-10 medium). Stimulator cells consisted of CMV-infected autologous fibroblasts. Fibroblasts were grown to 70% confluence on 6- or 24-well plates (Costar, Cambridge, Mass.) in the presence of recombinant human gamma interferon (800 IU/10^6 cells; Genzyme Diagnostics, Cambridge, Mass.) to upregulate class I MHC expression and then infected with rhesus CMV at a multiplicity of infection of 5 $TCID_{50}$ per cell. Following 16 to 24 h of infection, virus was inactivated by using long-wave (400-nm) UV irradiation (Fisher model UV 350) in the presence of psoralen (10 μg/ml; HRI Associates). Cells were UV irradiated for 10 min at a distance of 3.5 cm from the light source, washed once, and then used as stimulators. PBMC were cultured on stimulator cells at a responder-to-stimulator ratio of 16:1 to 30:1 and incubated at 37°C in a 5% CO_2 incubator. After 7 days, stimulated PBMC were harvested and restimulated with fresh stimulator cells prepared as before, irradiated (3,000 rads or 30 Gy) autologous feeder PBMC, and recombinant human interleukin-2 (kindly donated by M. Gately, Hoffman-LaRoche) at 20 IU/ml. PBMC were subsequently fed with R-10 medium and interleukin-2 (20 IU/ml) every 48 to 72 h. CTL assays were performed 7 to 9 days after restimulation.

Chromium release assay. Target cells consisted of autologous adherent fibroblasts pretreated for 24 to 48 h with recombinant human gamma interferon (800 IU/10^6 cells; Genzyme Diagnostics) and infected overnight with rhesus CMV at a multiplicity of infection of 5 to 10 $TCID_{50}$ per cell. Uninfected autologous fibroblasts served as control targets. Target cells were labeled overnight with ^{51}chromium (100 μCi/10^6 cells; DuPont NEN, Wilmington, Del.). On the day of the assay, the adherent target cells were dispersed with a nonenzymatic cell dissociation solution (Sigma), washed thrice in cold R-10, resuspended at 10^5/ml, and kept on ice until use. Target cells (10^4 per well) were dispensed in duplicate for each effector/target (E:T) ratio into 96-well U-bottom plates (Costar). Chromium release was assayed after 5 h incubation at 37°C in a 5% CO_2 incubator. Plates were spun at 1,000 rpm for 10 min at 4°C, after which 30 μl of supernatant was harvested from each well onto wells of a LumaPlate-96 (Packard) and allowed to dry overnight. Emitted radioactivity was measured in a 1450 MicroBeta Plus liquid scintillation counter (Wallac, Turku, Finland). Spontaneous release was measured from wells containing only target cells and medium. Maximum release was measured from wells containing target cells and 0.1% Triton X-100 (Sigma). The percent specific cytotoxicity was calculated as follows: [(test release − spontaneous release)/(maximum release − spontaneous release)] × 100. Spontaneous release of target cells was <25% in all assays.

Sequential blockade of CMV replication. Target cells expressing selectively only CMV IE or both IE and early (E) proteins were prepared by using sequential blockade with metabolic inhibitors. For selective IE protein expression, targets were treated with an inhibitor of protein synthesis, cycloheximide (100 μg/ml; Sigma) 30 min prior to and during 2 h of infection with rhesus CMV. Actinomycin D (Act D; Sigma), an inhibitor of transcription, was then added at a concentration of 20 μg/ml for 30 min. Cells were then washed and incubated in medium containing Act D alone. Target cells expressing only IE and E proteins were prepared by incubating them with an inhibitor of viral DNA replication, phosphonoformic acid (200 μg/ml; Sigma), 30 min before and throughout the period of infection. Finally, to obtain target cells in which viral transcription was blocked, fibroblasts were treated with only Act D (20 μg/ml) 30 min prior to and during rhesus CMV infection. All metabolic inhibitors were maintained at the given concentrations throughout the period of infection, which varied from 4 to 20 h in different experiments, as well as during cell dispersion, washes, and the 5-h incubation period of the chromium release assay.

Blockade of MHC class I-restricted antigen presentation. In selected experiments, brefeldin A (BFA) was used to determine whether CMV-specific CTL killing was class I MHC restricted. Targets were preincubated with BFA (6 μg/ml; Sigma) 30 min prior to and during CMV infection. BFA (6 μg/ml) was maintained during target washes and throughout the chromium release assay.

$CD8^+$ and $CD4^+$ lymphocyte separation. $CD8^+$ lymphocytes were prepared from stimulated PBMC by depletion of $CD4^+$ cells, using magnetic beads (CD4 Dynabeads; Dynal, Oslo, Norway) at a bead/cell ratio of 20:1 for 60 min at 4°C. The supernatant enriched for $CD8^+$ cells was collected by using a magnetic separation device (Dynal). $CD4^+$ cells attached to the magnetic beads were released from the beads by incubation with a commercial polyclonal antibody against papain-digested Fab fragments of mouse immunoglobulin (DETACHaBEADS; Dynal) with continuous shaking at 37°C for 1 h. Detached $CD4^+$ cells were separated from the beads over a magnetic separation device (Dynal) and were >98% pure as assessed by flow cytometry. Similarly negatively selected $CD8^+$ cell populations contained <7% $CD4^+$ cells. Fractionated cells were suspended in R-10 medium and used the same day in the CTL assay.

IEF of class I MHC immunoprecipitates. A modified one-dimensional IEF technique (45, 47) was used for the characterization of class I MHC alleles in cultured B-LCL. Cultured cells (3×10^6) were incubated in methionine-free medium for 30 min and subsequently labeled with [^{35}S]methionine for 6 h. Labeled cells were washed, lysed, and immunoprecipitated with monoclonal mouse anti-human HLA class I antibody W6/32 (Dako A/S, Copenhagen, Denmark) bound to protein A-Sepharose beads (Sigma). The labeled lysates were run on a 0.75-mm polyacrylamide gel (Sigma) containing 9 M urea, 1.6% (pH 5.0 to 7.0) ampholine, 0.4% (pH 3.5 to 10.0) ampholine, and 0.16% (pH 7.0 to 9.0) ampholine (Pharmacia Biotech, Piscataway, N.J.). Fixed and dried gels were visualized by autoradiography.

WB analysis of CMV-specific antibodies. MRC-5 cells infected with rhesus CMV strain 68-1 and showing 75 to 100% CPE were treated with lysis buffer (50 mM Tris [pH 7.8], 1% sodium dodecyl sulfate, 0.02% Nonidet P-40, 1 mM

dithiothreitol) for 20 min on ice. The cells were then scraped into Eppendorf tubes, heated for 3 min at 100°C, sonicated, and spun at 10,000 × g for 10 min. The supernatant fluid containing the protein lysate was stored at −20°C. Lysates of uninfected MRC-5 cells were used as controls.

Protein lysates were run on a 10% resolving–4% stacking polyacrylamide gel and transferred onto a 0.2-μm-pore-size polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, Calif.). WB was carried out by successive incubations with serum at 1:1,000 to 1:5,000 dilution for 3 h at room temperature, rabbit anti-monkey immunoglobulin G (Nordic Immunological Laboratories, Tilburg, The Netherlands) at 1:4,000 dilution for 20 min, and goat anti-rabbit horseradish peroxidase (Bio-Rad) at 1:4,000 dilution for 20 min. Target proteins were detected by enhanced chemiluminescence using a commercial kit containing the substrate luminol and an enhancer (Amersham Life Science Inc., Arlington Heights, Ill.). Positive bands were visualized on a photosensitive film (Hyperfilm-ECL; Amersham).

RESULTS

CMV infection in captive rhesus macaques at NERPRC. To establish experimental conditions for detection of CMV-specific CTL, we first identified CMV-seropositive animals. Serum from rhesus macaques housed at NERPRC was screened for antibodies to rhesus CMV, initially by indirect immunofluorescence assay and later by WB analysis, using MRC-5 cells infected with rhesus CMV strain 68-1 as the source of test antigen and uninfected MRC-5 cells as controls. All of 28 adult macaques (24 of them from the SPF colony) screened by indirect immunofluorescence assay and/or WB had antibodies reactive to rhesus CMV. A representative WB with the characteristic band pattern is shown in Fig. 1a. Sera showing at least three of five specific bands on WB (Fig. 1a) were considered to be CMV seropositive. Since all adult macaques tested were CMV seropositive, sera from juvenile and newborn macaques were also obtained. Six of six juvenile macaques (4 to 6 months old) residing with adult macaques in the SPF colony showed only a single weak positive band on WB. Since their mothers were all seropositive, this finding could represent waning maternal antibodies or early seroconversion. Eleven newborns delivered by cesarian section and separated from their mothers at birth were followed up for 9 months to 1 year with sequential CMV WB. All showed a strong antibody response at birth which waned with time, suggesting that the initial positive bands were due to passively transferred maternal antibodies (Fig. 1b). By 1 year, CMV-specific antibodies were weak (2 of 11) or undetectable (9 of 11) by WB. Isolation of these macaques from CMV-seropositive animals has prevented horizontal transmission of CMV.

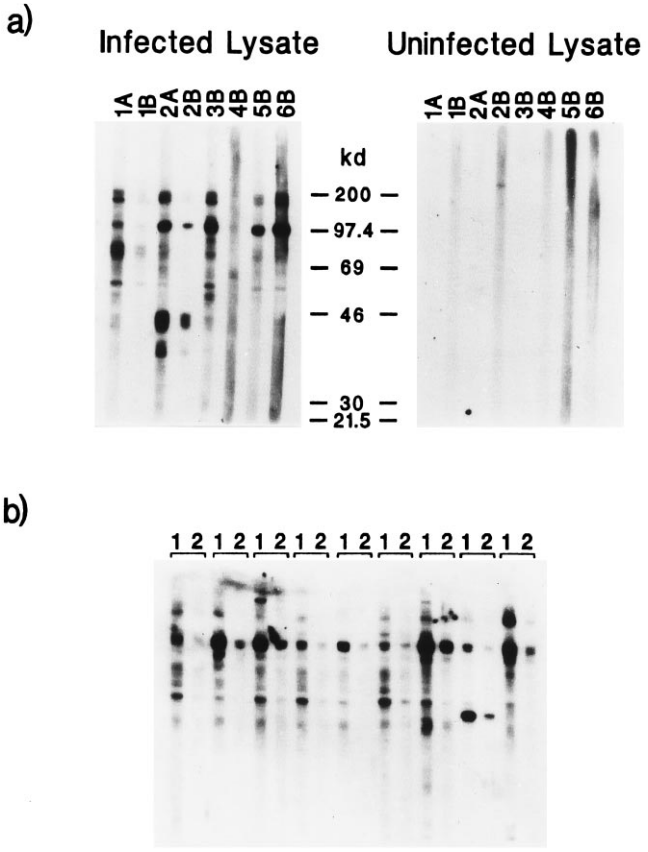

FIG. 1. WB analysis of CMV-specific antibody responses. (a) A representative WB analysis using sera from six rhesus macaques screened for antibodies to CMV. The characteristic bands are a doublet running below the 200-kDa marker, a prominent band of approximately 97 kDa, and single bands of approximately 69 and 55 kDa. Lanes 1A and 2A show results at 1:1,000 dilution, while lanes 1B to 6B depict sera tested at 1:3,000 dilution. Serum in lane 4B is from a CMV-seronegative animal. Sera were screened against lysates of MRC-5 cells infected with rhesus CMV. Lysates of uninfected MRC-5 cells were used as controls. (b) Sequential Western blots of sera from nine newborn macaques delivered by cesarian section and kept separate from their mothers following birth. Paired sera (numbered 1 and 2) from each animal, taken 5 weeks apart, were tested at the same dilution. The ages of the animals at first testing ranged from 8 to 48 days.

Rhesus CMV-specific CTL activity is present in CMV-seropositive and SIV-uninfected rhesus macaques. Detection of virus-specific CTL generally requires in vitro stimulation with autologous or MHC-matched cells expressing processed viral peptides. To detect CMV-specific CTL responses in rhesus macaques, we adapted techniques for detection of CMV-specific CTL in humans that employ autologous CMV-infected cells as stimulator cells (6, 33). Bulk PBMC were stimulated in vitro with rhesus CMV-infected autologous fibroblasts. Initial studies using stimulation of PBMC with CMV-infected fibroblasts resulted in relatively poor growth of effector cells, limiting the ability to perform CTL assays. We subsequently carried out in vitro stimulation with CMV-infected fibroblasts that were treated with psoralen and UV light irradiation to block viral replication (14). Compared with PBMC stimulated with CMV-infected fibroblasts not treated with psoralen and UV light, incubation with psoralen-treated, UV-irradiated stimulator cells resulted in greater number of stimulated PBMC at the end of 2 weeks (data not shown). Another modification from earlier protocols was that of pretreating not only target cells but also stimulator cells with gamma interferon prior to infection, the rationale being that gamma interferon would upregulate surface expression of class I MHC on the fibroblasts and enhance their antigen-presenting function.

Using this modified stimulation protocol, we analyzed CMV-specific CTL responses in four healthy SIV-uninfected and CMV-seropositive adult rhesus macaques. A vigorous CTL response, as evidenced by killing of CMV-infected autologous target cells but not uninfected cells, was detected in all four macaques (Fig. 2a). These results were reproducible on at least three separate occasions for each macaque over a 1-year period (data not shown). Induction of the lytic activity was observed only following antigen-specific stimulation, not when PBMC of CMV-seropositive animals were stimulated with uninfected autologous fibroblasts (Fig. 2b), nor was it evident when PBMC from a CMV-seropositive animal were stimulated with CMV-infected heterologous fibroblasts (data not shown). Further, no CTL activity was detected in either of two CMV-seronegative animals following antigen-specific stimulation of PBMC with CMV-infected autologous fibroblasts (Fig. 2c).

Rhesus CMV-specific CTL activity is mediated by $CD8^+$ cells and is class I MHC restricted. The requirements of antigen-specific stimulation and autologous antigen-presenting

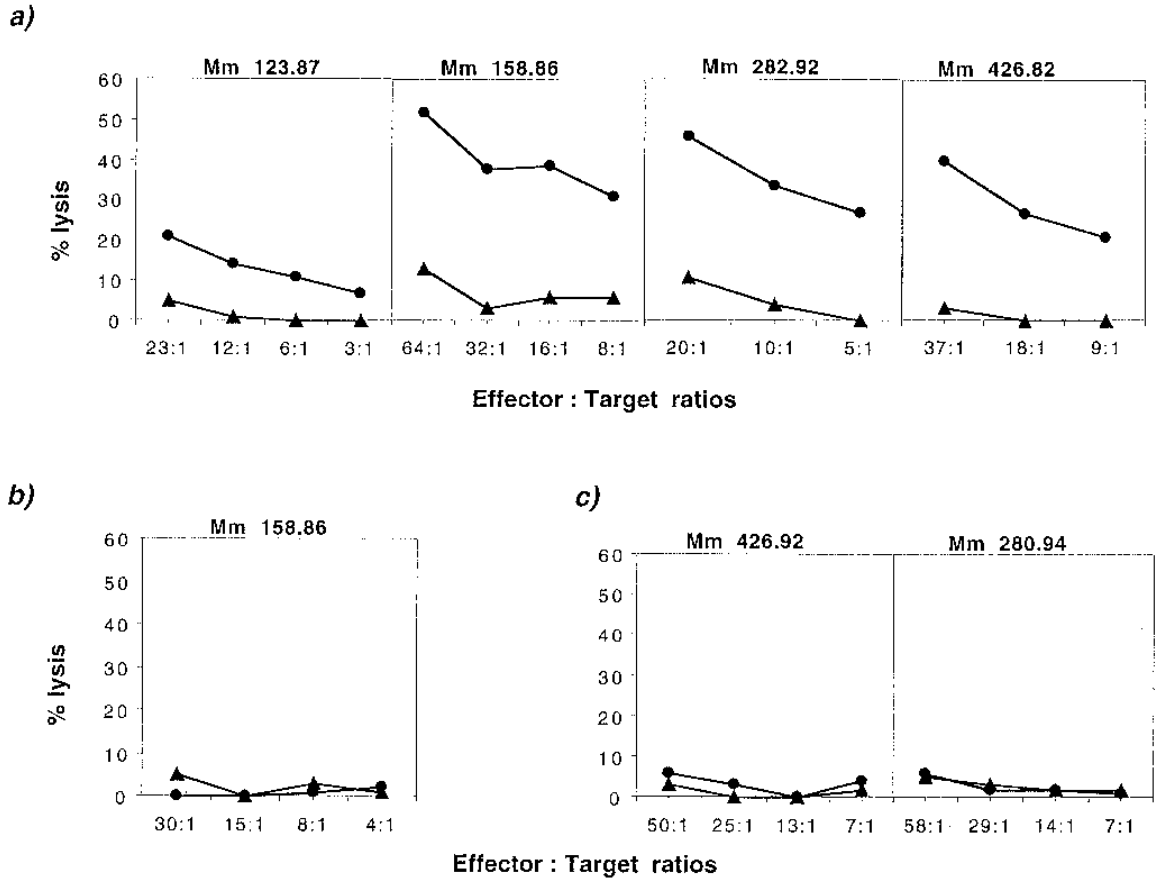

FIG. 2. CMV-specific CTL activity in normal rhesus macaques not infected with SIV. (a) CMV-specific CTL activity following antigen-specific stimulation of PBMC from CMV-seropositive macaques. Representative data from one of three experiments for each animal are shown. (b) Absence of CMV-specific CTL activity following stimulation of PBMC from CMV-seropositive animals with uninfected autologous fibroblasts. (c) Absence of CMV-specific CTL activity after antigen-specific stimulation of PBMC from two CMV-seronegative macaques. ▲, uninfected targets; ●, infected targets.

cells for in vitro generation of CMV-specific CTL activity in macaques suggested that this was an MHC-restricted phenomenon, likely to be mediated by classic $CD8^+$ CTL. To determine the phenotype of lymphocytes mediating lysis, we separated stimulated effector cells into $CD4^+$ and $CD8^+$ populations by using monoclonal antibodies conjugated to magnetic beads. $CD8^+$ lymphocytes obtained by depletion of $CD4^+$ cells (>80% $CD8^+$, <7% residual $CD4^+$ cells) lysed CMV-infected target cells at levels comparable to those observed in assays using unfractionated effector cells (Fig. 3). Comparison of CTL activity in $CD8^+$ and $CD4^+$ (<2% residual $CD8^+$ cells) cells at equal E:T ratios showed CMV-specific CTL activity only in the $CD8^+$ cells, not the $CD4^+$ cells (Fig. 3). Attempts to compare CMV-specific CTL activity in unfractionated, $CD4^+$, and $CD8^+$ cells from the same animal were complicated by the relatively small number of cells available following in vitro stimulation.

To establish whether CTL activity was MHC restricted, we examined the ability of stimulated effector cells to lyse allogeneic CMV-infected fibroblasts. Stimulated PBMC from animals Mm 123.87 and 282.92 did not kill CMV-infected allogeneic target cells (Fig. 4a). However, although CTL from animal Mm 158.86 did not kill allogeneic target cells from animals Mm 358.91 and 426.82, they did lyse those from animals Mm 123.87 and 301.91 (Fig. 4a), suggesting the possibility of shared MHC alleles among these animals. Class I MHC molecules of these animals were characterized by immunoprecipitation using a monoclonal anti-human class I HLA antibody followed by one-dimensional IEF. This analysis showed a similar pattern of a subset of class I MHC alleles among the animals, with two shared bands identified in animals whose fibroblasts were lysed by CTL from Mm 158.86 (Fig. 4b). These bands may represent a class I MHC allele responsible for presentation of CMV antigens.

Since fibroblasts do not normally express class II MHC proteins, killing by CMV-specific CTL was likely to be restricted by class I MHC molecules. To confirm restriction by class I MHC molecules, we analyzed the ability of BFA to inhibit lysis of CMV-infected fibroblasts. BFA blocks egress of proteins, including class I molecules, from the endoplasmic reticulum to the Golgi complex and in this way interferes with class I but not class II MHC-associated antigen presentation (26, 48). BFA completely blocked the lytic activity mediated by both the unfractionated and $CD8^+$ cell fractions of stimulated PBMC (Fig. 4c), thus demonstrating that infected fibroblasts require association of intracellularly processed rhesus CMV antigens with class I MHC molecules before they are recognized by CTL.

An E protein of rhesus CMV is a dominant target for bulk rhesus CMV-specific CTL. Following infection of cells by herpesviruses, viral transcription and protein synthesis proceed in a cascade fashion (13). Metabolic inhibitors were used to generate CMV-infected fibroblasts selectively expressing IE or IE and E proteins or to block viral protein synthesis. Such cells

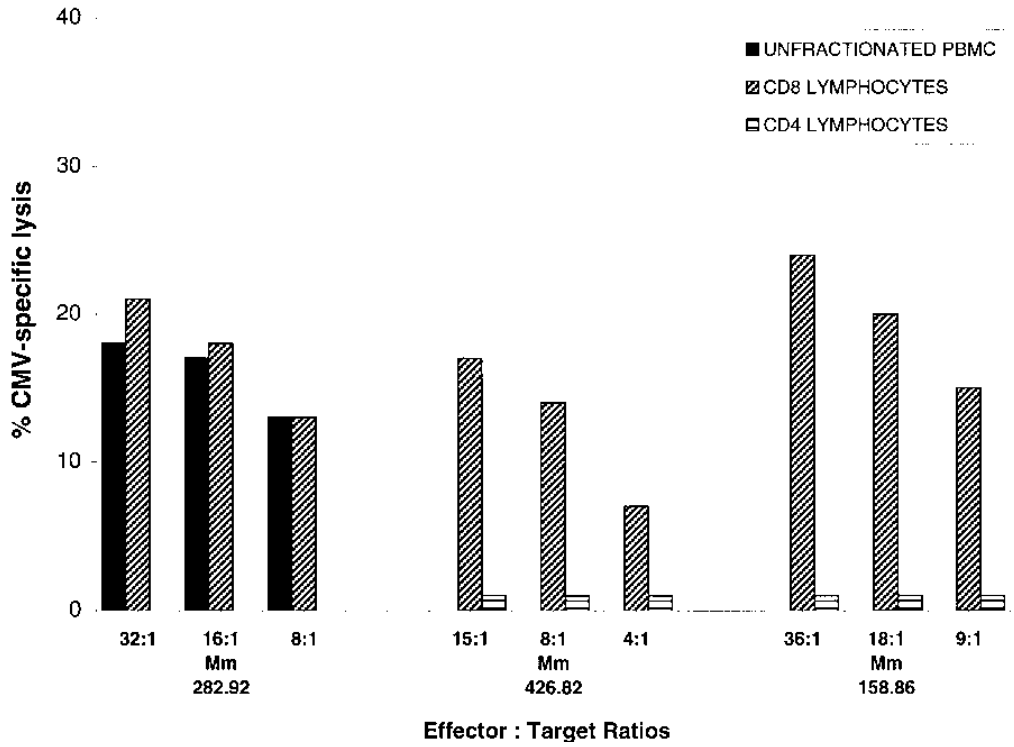

FIG. 3. CMV-specific CTL in rhesus macaques is mediated by $CD8^+$ cells. Results were reproducible on at least two occasions for each animal studied. Percent CMV-specific lysis was calculated as the difference in lysis between infected and uninfected targets. Lysis of uninfected targets in all experiments was <8%. E:T ratios are means of those used for unfractionated, $CD8^+$, or $CD4^+$ lymphocytes (range ± 20%).

when used as targets in CTL assays helped to define the immunodominant target proteins recognized by rhesus CMV-specific CTL.

The pattern of CTL recognition of target cells infected with CMV in the presence of metabolic inhibitors is shown in Fig. 5. Unless otherwise stated, target cells were infected for 16 to 20 h in the presence of metabolic inhibitors. Bulk CTL from animals Mm 158.86, 426.82, and 123.87 lysed targets infected in the presence of phosphonoformic acid to the same extent as target cells infected for 16 to 20 h in the absence of metabolic inhibitors (Fig. 5a). The concentration of phosphonoformic acid (200 μM) used for these experiments is able to inhibit rhesus CMV replication by 4 logs units (data not shown) and therefore quite likely to effectively block late protein synthesis in these target cells. In contrast, for most animals studied, target cells expressing IE proteins or infected with CMV for only 4 to 6 h were not efficiently lysed, with levels of CMV-specific lysis generally ranging from 0 to 5%. Thus, in these three animals, an E CMV protein or proteins expressed later than 4 h after CMV infection were the dominant protein(s) recognized by bulk CTL. In one animal, bulk CTL lysed target cells infected with CMV for 4 h but did not lyse cells expressing only IE proteins (data not shown). An E protein is the likely immunodominant protein in this animal. In addition, in animal Mm 158.86, low levels (9 to 10%) of CMV-specific lysis were reproducibly observed ($n = 3$) at high E:T ratios in target cells infected with rhesus CMV in the presence of Act D (Fig. 5b). Comparable levels of lysis were observed in target cells infected for 4 to 6 h or those expressing only IE CMV proteins (Fig. 5b). These findings suggest that CTL from this animal also recognize a CMV protein that does not require endogenous protein synthesis in order to be processed. This is likely to be a viral structural protein, such as an envelope, capsid, or matrix protein, introduced into the cytosol following viral entry. Thus, the CMV-specific CTL response in these animals is heterogenous, directed primarily against E proteins that require endogenous synthesis for target cell recognition but also against CMV structural proteins that may be recognized without endogenous synthesis.

Rhesus CMV-specific CTL in SIV-infected macaques. To determine whether SIV-infected animals maintained cellular immune responses to CMV, CMV-specific CTL activity was analyzed in CMV-seropositive macaques with established SIV infection. In two macaques infected with an attenuated SIV strain (SIVΔ3) that does not lead to immunodeficiency (46), CMV-specific CTL activity was detected at levels comparable to those observed in normal animals (Table 1). It was also detectable, at similar levels, in two of three animals infected with pathogenic SIV strains. However, in one SIV-infected animal (Mm 418.93), CMV-specific CTL activity was not detected on multiple occasions, even when tested at high E:T ratios (Table 1). This animal, while maintaining a normal $CD4^+$ T-lymphocyte count (1,328 cells per mm^3), had SIV viral loads ranging from 41 to 729 infectious units/10^6 PBMC at the time of the CTL assays (data not shown). In contrast, animal Mm 377.90, with intact CMV-specific CTL activity, had a low SIV viral load (<1 infectious unit/10^6 PBMC).

DISCUSSION

CTL play a critical role in controlling CMV replication and protecting against disseminated CMV disease. Analysis of the effects of HIV and SIV infection on CMV-specific CTL should elucidate mechanisms underlying the pathogenesis of CMV infection in AIDS. In this report, we have characterized CMV-specific CTL in rhesus macaques, an animal model appropriate

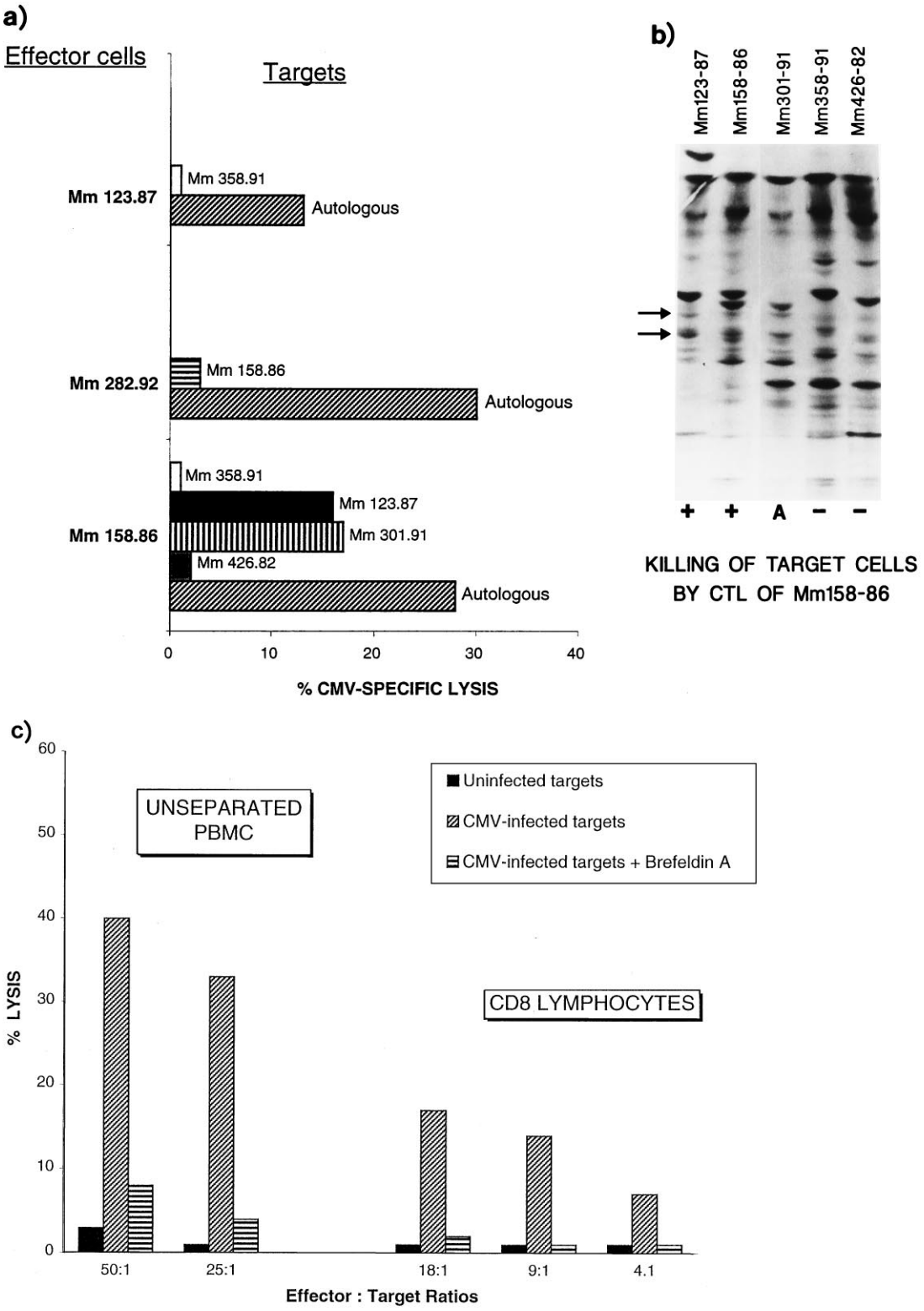

FIG. 4. CMV-specific CTL in rhesus macaques is MHC class I restricted. (a) CMV-specific CTL activity against allogeneic CMV-infected fibroblasts (E:T ratio of 12:1 for the data shown). (b) IEF gel of MHC class I molecules of rhesus macaques. Arrows indicate bands shared by animals whose target cells are lysed by CTL of animal Mm 158-86. (c) BFA blocks killing of rhesus CMV-infected autologous fibroblasts by both unfractionated and $CD8^+$ lymphocytes.

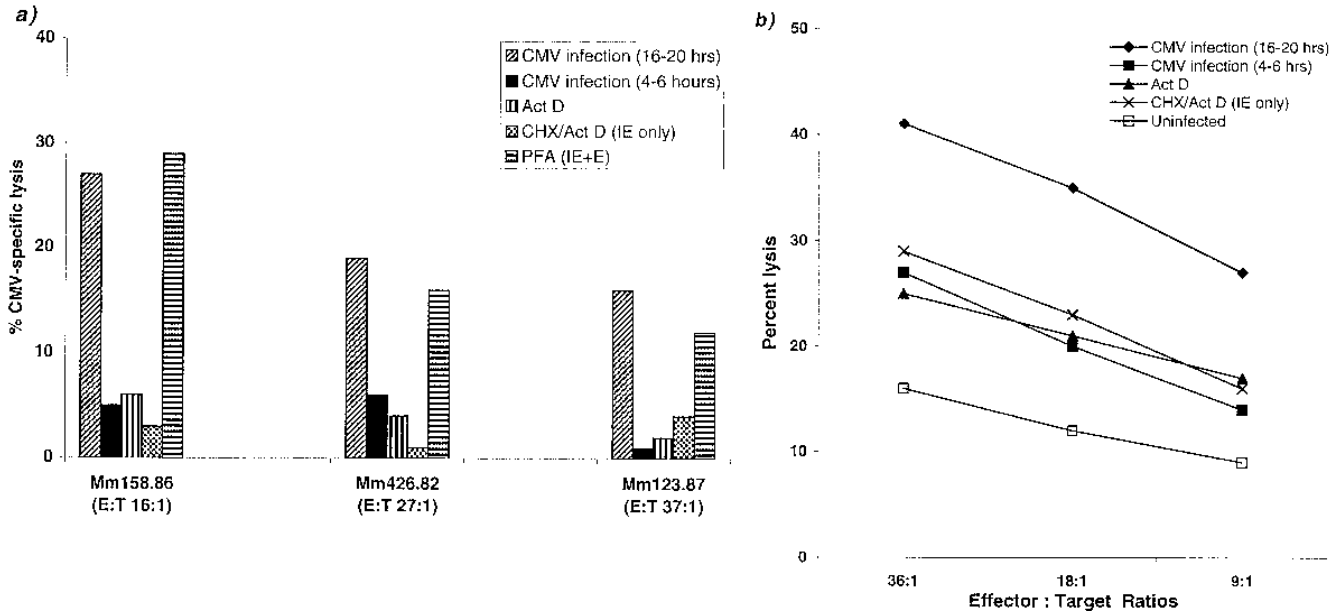

FIG. 5. CMV-specific CTL against CMV-infected target cells blocked with metabolic inhibitors. (a) Recognition of early proteins by CMV-specific CTL. Representative results from duplicate experiments are shown. CHX, cycloheximide; PFA, phosphonoformic acid. (b) Low levels of CMV-specific lysis by CTL of Mm 158-86 in target cells blocked with Act D.

for studying in vivo interactions between CMV and pathogenic retroviruses. Using in vitro stimulation techniques adapted from those used to detect CMV-specific CTL in humans (6, 33), we have demonstrated that healthy CMV-seropositive rhesus macaques possess vigorous CMV-specific CTL activity which is class I MHC restricted and mediated by $CD8^+$ T lymphocytes. These findings are similar to those observed in asymptomatic CMV-seropositive humans (6).

On the basis of both epidemiological evidence and laboratory studies, CMV has long been thought to be a significant cofactor modulating progression to AIDS. Prospective in vivo studies to identify mechanisms underlying this interaction have not been carried out in humans. Cross-sectional studies on changes in CMV-specific immunity in HIV infection have focused chiefly on humoral and proliferative T-cell responses (8, 12, 37). Asymptomatic HIV-infected people with normal $CD4^+$ T-cell counts had decreased proliferative responses to specific CMV antigens (12). Decreased T-cell proliferative responses to CMV in patients with AIDS was a predictor for developing CMV retinitis, particularly in those with HLA DR7, B44, or B51 haplotypes (37). The only published studies on CMV-specific CTL activity in HIV infection used an insensitive technique for CTL detection. Using fresh PBMC without in vitro stimulation, CTL activity was not detected in AIDS patients with CMV viremia but was detected in a proportion of renal transplant recipients with active CMV infection (35, 36). The rhesus model is ideally suited for in vivo prospective studies, and the similarities between simian and human CMV, as well as SIV and HIV, support the validity of this experimental system as a model for the study of CMV pathogenesis in AIDS. Characterization of CMV-specific CTL responses in rhesus macaques and the ability to maintain CMV-seronegative animals free of CMV infection have provided us with powerful tools for such studies.

TABLE 1. Analysis of CMV-specific CTL in SIV-infected rhesus macaques

Infection	Animal	% CMV-specific lysis[a]		
None		23:1[b]	12:1	6:1
	123.87	16	13	11
		64:1	32:1	16:1
	158.86	39	35	33
		20:1	10:1	5:1
	282.92	35	30	27
		37:1	18:1	9:1
	426.82	37	27	21
SIVΔ3		26:1	13:1	6:1
	358.91	20	10	9
		24:1	12:1	6:1
	301.91	13	9	5
SIVmac239		82:1	41:1	20:1
	418.93	7	7	3
		45:1	23:1	11:1
	377.90	29	22	14
		24:1	12:1	6:1
SIVmac316	155.85	40	33	26

[a] Difference in percent lysis between infected and uninfected targets. A difference of ≥10% CMV-specific lysis observed at one or more E:T ratios was considered significant.

[b] E:T ratio.

In this study, we present cross-sectional data on CMV-specific CTL activity in rhesus macaques with established SIV infection. CMV-specific CTL activity was not detected in one of three animals infected with a pathogenic strain of SIV. Even though the $CD4^+$ T-cell counts of this animal were normal, it had higher SIV viral loads than the other two SIV-infected animals with intact CMV-specific activity. It is possible that depressed $CD4^+$ helper function consequent to SIV infection may contributes to the absence of CTL responses. In HIV infection, antigen-specific responses generally are lost before $CD4^+$ T-cell depletion (7). In bone marrow transplant recipients, recovery of $CD8^+$ CMV-specific CTL was always preceded by the appearance of $CD4^+$ proliferative responses to CMV (31). Thus, loss of $CD4^+$ cell number and/or function

could potentially affect T-cell proliferative responses to pathogens including CMV and in this way abolish virus-specific CTL activity.

By examining lysis of CMV-infected target cells treated with metabolic inhibitors, we identified an E CMV protein (or proteins) as the major target recognized by rhesus CTL. The specific identity of this protein or proteins remains to be determined but may differ among animals. While CTL from one animal recognized an E protein present within the first 6 h following infection, in three other animals, the targeted E viral protein appeared to be one synthesized at a later time point. An alternative explanation is that the same E protein is immunodominant for all these animals and that the differences in target cell recognition reflect quantitative differences in antigen processing or CTL recognition of the same viral antigen. CTL from one animal also recognized a viral antigen not blocked by Act D treatment of target cells, a finding consistent with CTL recognition of a viral protein introduced into the cell cytoplasm following viral entry. This situation is analogous to that seen in human CMV infection, in which case the CTL response to pp65 lyses CMV-infected target cells before the onset of viral protein synthesis in the cell (25). Our in vitro stimulation techniques were optimized to yield maximal CMV-specific CTL activity in rhesus macaques. Since in our assays, PBMC were stimulated with CMV-infected fibroblasts that were inactivated by psoralen and UV irradiation 16 to 20 h following CMV infection, it is possible that we failed to stimulate CTL precursors for late CMV proteins. Differences in stimulation methods, including the virus isolates used for generating CMV-specific CTL, could also contribute to the observed species-specific differences. The amount of human CMV protein pp65 present in cells infected with the laboratory strain AD169 is greater than that present in cells infected with low-passage-number clinical isolates (17, 40). It is significant that the stimulator cells used to generate rhesus CMV-specific CTL in these experiments were infected with a clinical isolate of rhesus CMV, in contrast to most human studies, which have used a laboratory isolate of CMV (6, 33). Further delineation of the specificity of CMV-specific CTL in rhesus macaques awaits studies using target cells expressing recombinant rhesus CMV proteins.

In conclusion, this study has laid the groundwork for future in vivo prospective studies on immune interactions between CMV and SIV in AIDS. Such studies are likely to help elucidate the mechanisms underlying the interactions of CMV with HIV and SIV in AIDS.

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