Evidence for Antibody-Mediated Enhancement of Simian Immunodeficiency Virus (SIV) Gag Antigen Processing and Cross Presentation in SIV-Infected Rhesus Macaques

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Received 25 July 2002/Accepted 30 September 2002

By using the dominant simian immunodeficiency virus (SIV) Gag Mamu-A01 restricted major histocompatibility complex (MHC) class I epitope p11CM, we demonstrate antibody-mediated enhanced MHC class I cross presentation of SIV Gag. In vitro restimulation of peripheral blood mononuclear cells from SIV-infected rhesus macaques with recombinant full-length SIV Gag p55 plus p55 affinity-purified immunoglobulin G (p55 Gag/p55-IgG) led to the generation of markedly higher frequencies of p11CM specific precursor cytotoxic T lymphocytes (p-CTLs) compared with restimulation with (i) SIV Gag p55 alone or (ii) optimal concentrations of the p11CM peptide alone. These results, along with the finding that CD4 depletion abrogated the enhancement, suggest a prominent role for CD4+ T cells. Testing for p-CTLs against other Mamu-A01-restricted SIV Gag epitopes suggested that this mechanism favored recognition of the dominant p11CM peptide, potentially further skewing of the CTL response. The p-CTL enhancing effect was also decreased or abrogated by pepsin digestion of the p55-specific IgG or by the addition of monoclonal antibodies to Fc receptor (FcR) II/III, suggesting that the effect was dependent on FcR-mediated uptake of the immune-complexed antigen. Finally, incubation of antigen-presenting cells with SIV Gag p55 immune complexes in the presence of lactacystin or of bafilomycin indicated that the mechanism of antibody-mediated enhancement of cross presentation required both the proteasomal and the endosomal pathways. These data demonstrate for the first time the cross presentation of antigens via immune complexes in lentiviral infection and indicate a heretofore-unrecognized role for antibodies in modulating the magnitude and potentially also the breadth of MHC class I-restricted antigen processing and presentation and CTL responses.

The compartmentalization of antigen-specific humoral and cellular responses mediated by CD4+ and CD8+ T-cell subsets, respectively, has provided a convenient framework for the analysis of the mechanisms by which such responses are initiated and regulated (44). This lineage specific division of immunological labor is further supported by the fact that whereas CD4+ T cells recognize predominantly exogenously presented antigens in the form of peptides in association with major histocompatibility complex (MHC) class II antigens, CD8+ T cells predominantly recognize endogenous antigens in the form of peptides associated with MHC class I molecules (51). However, several lines of investigation suggest that exogenous antigens can also be processed for presentation by MHC class I molecules utilizing several alternate mechanisms of MHC class I processing (14, 15, 17, 27, 29, 46, 50, 56, 58). These include both cytosolic and partially or fully TAP-independent vacuolar alternate MHC class I antigen-processing mechanisms (9, 16, 35, 57–59, 71, 74). The precise role and importance of such cross presentation in vivo remains to be elucidated.

Our laboratory has been involved in the characterization of disease-protective virus-specific humoral and cellular responses of nonhuman primates experimentally infected with the simian immunodeficiency virus (SIV) (7, 8, 43, 65–68). During the course of these studies, an important role for virus-specific cytotoxic T lymphocytes (CTLs) in disease-protective immune responses has been defined by a number of laboratories, including ours (1, 7, 8, 10, 21, 24, 32, 34, 43, 64, 66, 68). Such CTL activity has been ascribed to effector CD8+ T cells, since depletion of this lineage in vivo led to the elimination of CTL activity, increase in viral loads and eventually disease progression (53). In a more recent study, it was noted that SIV-specific CTL activity required the presence of CD4+ T-helper cells in nonhuman primates (66), similar to studies reported in human human immunodeficiency virus type 1 (HIV-1) infection and in the murine model of lymphocytic choriomeningitis virus infection (30, 36, 69). However, the precise mechanism(s) and pathways by which such CD4+ T-helper-cell responses facilitate the generation and maintenance of virus antigen-specific CD8+ T-cell responses remain unclear. In parallel, antiviral antibodies in the context of lentiviral infection have so far predominantly been considered for
their virus neutralizing capacity, whereas the contribution of nonneutralizing antibodies has been hypothesized to promote primarily antibody-dependent cellular cytotoxicity, antigen uptake by phagocytes and MHC class II presentation. A potential role for antibodies in the generation and/or maintenance of virus-specific CD8+ T-cell responses, particularly in lentiviral infection, has not so far been elucidated. Immune complexes have, however, been successfully utilized for vaccines against a number of antigens such as hepatitis (37, 70), Newcastle disease virus (47), prostate-specific antigens (13), etc., and their role in antigen uptake by dendritic cells have been studied (60). Thus, in the normal physiological in vivo conditions, virus and viral antigens are present not only in soluble forms but to a large extent as viral antigen-antibody complexes and, as such, these must be internalized for eventual elimination. Their role in antigen processing and presentation has received little attention in the context of lentiviral infection and specific immunity.

The present study was therefore carried out to delineate the role of virus-specific antibody in the processing and presentation of SIV G antigen. These studies were facilitated by the previous demonstration of a dominant SIV Gag peptide (p11CM) that is the target of CTLs in Mamu-A01+ monkeys. Our results demonstrate that antibody-mediated antigen presentation in the form of immune complexes markedly enhances the MHC class I cross presentation of the virus-specific dominant p11CM Gag peptide to effector CD8+ T cells from Mamu-A01+ SIV-infected rhesus macaques. In addition, this enhancement of CTL function requires CD4+ T helper cells. We document here an important additional role for virus-specific antibody in addition to virus-specific neutralization.

MATERIALS AND METHODS

Animals. The six adult rhesus macaques utilized herein as a source of peripheral blood mononuclear cells (PBMC) were part of a separate study but were chosen because they were positive for the Mamu-A01 MHC class I allele (RPu-3, RU4-3, RE4-4, RD3-3, RWP-2, and RH-2). The monkeys were housed at the Yerkes Regional Primate Research Center in accordance with the instructions of the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council and the U.S. Public Health Service Guidelines, Guide for the Care and Use of Laboratory Animals. Prior to SIV infection, all monkeys were hyper-immunized with KLH, tetanus toxoid (TT) and live attenuated influenza virus as detailed elsewhere (8). Each of these six macaques was infected with 200 50% tissue culture infective doses of uncloned highly virulent SIVmac251 intravenously; one of these six macaques was infected with 200 50% tissue culture infective doses of recombinant Mamu interleukin-12 (IL-12) during the acute infection phase. The remaining animal (R3-2) served as a non-cytokine-treated control (8). rMamu IL-12 treatment of the five monkeys induced a significant decrease in viral load set points and delay in appearance (>24 months) of symptomatic immunodeficiency compared to untreated control monkeys (8).

Isolation of PBMCs and preparation of transformed cell lines. Heparinized whole blood was centrifuged at 450 g for 10 min, and the plasma was removed and stored at −70°C until it was used for the isolation of total immunoglobulin G (IgG) and affinity-purified antibodies as described below. The cells were separated by using Ficol-Hypaque gradients, washed free of platelets, and resuspended to the desired cell concentration for the appropriate assay. Prior to the initiation of the study, aliquots of such PBMC from each monkey were incubated in vitro with herpesvirus papio for the derivation of predominantly B-cell-lineage lymphocytoblasts (B-LCB). These cultures were monitored, and the emerging transformed cells were expanded, divided into aliquots, and cryopreserved for future use.

Derivation of APCs. The procedure for the derivation of in vitro mature dendritic cells from rhesus macaques has been studied in detail by Melhop et al. (39). Our laboratory has basically derived similar data and has utilized slight modifications of this protocol for the studies described here. Briefly, freshly prepared PBMC were depleted of CD3+, CD19+, and CD68+ cells by incubation with a cocktail of monoclonal antibodies (clone FN 18 [Biosource International, Camarillo, Calif.] and clone anti-4G7 [Caltag Laboratories, South San Francisco, Calif., Mountain View, Calif.]) for 30 min with gentle rotation at 4°C. This cell suspension was then incubated with anti-mouse immunoglobulin-coated magnetic beads (Dynal Corp, New York, N.Y.), and the antibody-coated cells were depleted by using a magnetic field. The depleted cells were then incubated at 106/ml in a 250-ml flask in RPMI 1640 complete medium (RPMI 1640 supplemented with 100 U of penicillin/ml, 100 μg of streptomycin/ml, 5 x 10−3 M 2-mercaptoethanol, and 5% normal human plasma) containing 1,600 U of granulocyte-macrophage colony-stimulating factor and 100 U of a prescreened functional lot of IL-4 for 6 to 7 days at 37°C in a 7% CO2 humidified atmosphere. The cultures were fed fresh complete medium containing the same concentration of cytokines every other day. On day 7, the medium was removed, and fresh complete medium containing tumor necrosis factor alpha (5 ng/ml), IL-1β (25 ng/ml), and IL-6 (25 ng/ml) was added, and the cultures were incubated for 48 h. The resulting cells were then gently removed from the flasks and used as a source of antigen-presenting cells (APCs). These APCs were positive for the following markers: CD1a, HLA-DR, CD11b, CD11c, CD40, CD80, CD86, and CD80, suggesting that these cells had evolved into mature dendritic cells. Alternatively, select experiments used B-LCB as APCs as indicated in the text.

EIA s. Plasma samples from the monkeys were assayed for the presence and quantitation of SIVmac251-specific antibodies by using standard whole inactivated SIVmac251 as an antigen in an enzyme immunoassay (EIA) established by our lab (43). The titer was defined as the reciprocal of the highest dilution of the plasma that showed an optical density value three standard deviations above the background (the value was obtained in parallel on a 1:20 dilution of plasma from the same monkey prior to SIV infection). The virus stock utilized as antigen was prepared utilizing day 3 phytomagglutinin blasts from infected blood donor rhesus macaques.

Preparation of affinity-purified antibodies. A stock of recombinant SIV G protein was prepared utilizing the baculovirus expression system as described elsewhere (45). The resulting protein was analyzed for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and shown to contain a single band of 55 kDa, which showed reactivity against a monoclonal antibody against SIV Gag. Aliquots of recombinant SIV Gag p55 were also obtained from the National Institute of Health (NIH) AIDS Research and Reference Reagent Program (National Institute of Allergy and Infectious Disease, NIH, Rockville, Md.) and utilized similarly for the isolation of affinity-purified antibodies. The procedure utilized to prepare the affinity-purified antibodies was performed as outlined (6). Briefly, the procedure consisted of covalently conjugating the SIV Gag p55 protein to CNBr-Sepharose CL4B and after extensive washes to remove unconjugated protein, blocking of the remaining uncoupled sites was performed. Small aliquots of the CNBr-Sepharose CL4B-Sepharose CL4B were prepared and utilized to obtain individual preparations of affinity-purified IgG from the plasma of the SIV-infected rhesus macaques. The IgG was first isolated from a pool of preinfection plasma from individual monkeys (preinfection IgG) or individual postinfection (p.i.) plasma samples utilizing protein A-Sepharose columns. The eluted IgG was dialyzed against phosphate-buffere d saline (PBS; pH 7.4), concentrated by using Amicon YM30 filters and an aliquot utilized for the quantitation of protein (the total yields of IgG varied between 5.7 and 10 mg/ml of original plasma). The purified preinfection IgG was divided into aliquots and stored at −70°C until use. The p.i. IgG was subjected to affinity purification as follows. The SIV Gag p55-conjugated columns were washed several times with binding buffer (0.05 M Tris-HCl, 0.15 M NaCl, 0.02% NaN3). The amount of IgG applied in the binding buffer to individual columns varied depending on the yield of affinity-purified antibody desired. Columns were regenerated and utilized several times for aliquots of each individual plasma sample until the desired quantity of affinity-purified antibody was achieved. The columns were eluted with stepwise elution buffers (0.05 M sodium phosphate, 0.15 M NaCl, 0.02% NaN3; pH 7.0), (0.05 M sodium citrate, 0.15 M NaCl, 0.02% NaN3; pH 5.5), (0.05 M sodium citrate, 0.15 M NaCl, 0.02% NaN3; pH 4.3) and (0.5 M glycine, 0.15 M NaCl, 0.02% NaN3; pH 2.3), and the eluted antibody was collected in tubes containing Tris buffer (pH 8.0) to immediately neutralize the acidity. The columns were then washed with 10 volumes of binding buffer and kept at 4°C until further use. The eluted affinity-purified IgG was dialyzed extensively in PBS (pH 7.4) to remove NaN3, concentrated by using Amicon YM30 filters, and stored at −70°C until use (the total yield of affinity-purified antibodies varied from 26 to 112 μg/ml of the original plasma sample). For select experiments, the affinity-purified IgG were
subjected to pepsin digestion in efforts to prepare F(ab)2 preparations, utilizing immobilized pepsin and the manufacturer’s instructions (Pierce Biochemicals, Rockford, Ill.). The pepsin-digested antibody preparation was passed over an immobilized protein A column (Pierce) to remove any undigested antibody and the Fc portion. The F(ab)2 preparation was diazotized, concentrated by using Amicon YM30 minilters to 100 μg/ml, and stored at 4°C until use. In select experiments, ovalbumin (OVA; Sigma Chemicals, St. Louis, Mo.) and an IgG monoclonal anti-OVA antibody (clone OVA 14; Accurate Chemicals/ Sigma) were utilized at 10 and 5 μM, respectively, as nonspecific immune complex controls.

Mouse monoclonal antibodies. Purified mouse anti-Fc receptor (FcR) IIII monoclonal antibody (clone 2-4G2) was obtained from the Emory CFAR Immunology Core Laboratory. Murine monoclonal antibodies against monomorphic human MHC class I (clone W6/32) and MHC class II (clone L243) were prepared in our laboratory from hybridoma cell lines obtained from the American Type Culture Collection (Rockville, Md.). These antibodies have been previously shown by our lab to cross-react with Mamu MHC class I and II molecules, respectively (2). The amounts of these antibodies utilized was in 10-fold excess of the amount required for maximal staining of rhesus PBMC as determined by flow microfluorometry (0.25 μg of each of these antibodies was sufficient to stain 106 cells, and 2.5 μg per 106 cells were utilized in the experiments described here). Monoclonal antibodies specific for SIV Gag (clones KK59 and KK64) were obtained by courtesy of the NIH AIDS Research and Reference Reagent Program. The other monoclonal antibodies utilized were purchased either commercially from BD Pharmingen (San Diego, Calif.) or Coulter Immunotech (Hialeah, Fla.).

p-CTL and CTL analysis. The precursor CTL (p-CTL) assay for SIV Gag p11CM (CTPDYDINQ) or other Mamu-A01-restricted Gag peptides LF8 (LA PVPFPFA), Q19 (QNPVPVGNQ), or LW9 (LSPRTLNAW) was performed by limiting dilution assay (LDA) as standardized by our laboratory (7, 8, 66, 68). Briefly, aliquots of APCs (2 × 105 cells/ml) from the monkey to be assayed were cultured in vitro for 6 to 8 h with a predetermined optimum concentration of 10 μM of the recombinant SIV Gag p55 protein alone, a 10 μM concentration of SIV Gag p11CM peptide alone, a 10 μM concentration of the SIV Gag p55 protein plus autologous or heterologous SIV Gag p55 affinity-purified antibodies at a 1:1 molar ratio (p55/p55-IgG) or, as a control, 10 μM SIV Gag p55 protein plus 5 μM autologous preinfec tion IgG (p55/preinfec tion IgG). These cells were then irradiated (8,000 rads), washed, and resuspended at 2 × 105 cells/ml of complete medium, and 0.1 ml was dispensed into each well of a 96-well microtiter plate. Ficol-Hyapaque-purified autologous PBMC to be evaluated for p-CTL were adjusted to various concentrations, and each dilution tested in 24 replicate wells. The cultures were incubated at 37°C in a 5% CO2 humidified atmosphere for a total of 10 to 14 days, and the medium was replenished as required. The medium was supplemented on days 3, 7, and 10 with recombinant IL-2 (20 U/ml). Cultures were washed and then subjected to CTL assays as described elsewhere (7, 8, 66, 68). Target cells consisted of autologous B-LCB previously pulsed with a 10 μM concentration of the p11CM peptide and labeled with 51Cr. For select experiments, the effector PBMC were depleted of CD4+ or CD8+ T cells by incubation with anti-CD4 (1 μg/106 cells, clone Leu3a; Becton Dickinson) or anti-CD8 monoclonal antibody (clone 2ST8.5H7; Coulter Immunotech). PBMC were then subjected to LDA restimulation in the presence of p55/p55-IgG for 8 h, either in the presence or absence of 5 μM lactacystin or 40 μM bafilomycin. For restimulation in the presence of bafilomycin, the APCs were first pretreated for 4 h with 100 μM bafilomycin to effectively block the endosomal pathway prior to pulsing with the antigens. The cells were then matured for 3 days with tumor necrosis factor alpha, IL-1β, and IL-6 before we set up restimulation cocultures with autologous PBMC as described above. To establish the specificity of the pathways being utilized for the loading of antigen, aliquots of the same PBMC were cocultured with vaccinia virus constructs expressing the influenza virus matrix protein (Vacc-Flu-MP)-infected autologous B-LCB as described elsewhere (8) in the presence of the same concentration of lactacystin or bafilomycin and according to the same protocol. The restimulated PBMC cultures were then assayed for levels of p-CTL against the promiscuous Flu-MP 58-66 peptide-pulsed autologous B-LCB target cells. In a separate experiment, aliquots of PBMCs were also assayed for their ability to proliferate when cocultured with autologous B-LCB, which were previously pulsed with TT in the presence or absence of lactacystin or bafilomycin at the same concentration and according to the same protocol. The proliferation assay was performed as described elsewhere (43).

Statistical analyses. Statistically significant differences in the p-CTL values were determined by one-way analysis of variance with contrast by using the Analyze-It statistical package for Microsoft Excel.

RESULTS

Addition of antibody to SIVp55 Gag antigen enhances the generation of p11CM peptide-specific p-CTL in vitro. PBMC from the Mamu-A01+ SIV-infected monkeys included in the present study have been previously shown by our lab to contain readily detectable levels of SIV Gag- and Env-specific CTLs as early as 6 to 8 weeks p.i., and these CTL levels were maintained for a long period of time with a predominance for Mamu-A01-restricted SIV Gag p11CM-specific responses (8). These data served as a platform for the studies reported here, which were designed to elucidate the role of SIV-specific antibodies in the generation of SIV Gag-specific CTL responses. In a preliminary study, PBMC from three Mamu-A01+ SIV-infected monkeys were cocultured in vitro with autologous APCs that were previously pulsed with p55 Gag alone, p11CM peptide alone, or p55 Gag plus 10% autologous plasma as a source of SIV Gag-specific antibody. The results of this assay demonstrated marked augmentation of levels of p11CM peptide-specific p-CTL levels by the inclusion of autologous plasma (data not shown). However, in efforts to have a more precise measurement of the amount of antigen specific antibody that was being utilized for unit amount of antigen in the pulsing of the APCs and the expected variability in the levels of SIV Gag-specific antibody in the various monkeys and at various times p.i., prompted us to perform all subsequent studies with SIV p55 Gag affinity-purified IgG (subsequently referred to as p55-IgG) and, as a control, IgG purified from the plasma of the same monkey prior to SIV infection (subsequently termed preinfection IgG). Aliquots of APCs were pulsed with either p55 Gag, p55-IgG alone, p11CM peptide, preinfec tion IgG, p55 Gag/p55-IgG, or p55 Gag-preinfection IgG. As seen in Fig. 1, PBMC (3 months p.i.) from 4/5 of these Mamu-A01+ monkeys, as expected, showed significant number of p-CTLs when they were restimulated with p11CM peptide alone. However, aliquots of the same PBMC from each of these four SIV-infected but asymptomatic Mamu-A01+ monkeys demon-
strated marked augmentation of p-CTL values when they were restimulated with p55 Gag/p55-IgG compared to restimulation with p55 Gag-preinfection IgG \((P < 0.0001\) in each case). The data shown are representative of three separate experiments. When similar studies were carried out on PBMC from the SIVmac251-infected Mamu-A01 \(+/H11001\) rapid progressor monkey RJt-2 at 3 months p.i. (who died at 34 weeks p.i.), the PBMC samples showed little or no p11CM-specific p-CTLs with the use of the p11CM peptide during restimulation. The addition of p55 Gag/p55-IgG during the restimulation period from this animal did not rescue detectable levels of p11CM CTLs in spite of a significant anti-SIV EIA titer (1:50,000). The augmentation thus appeared to require functional CTLs and both the affinity-purified p55 Gag antibodies and the viral protein during restimulation, presumably as immune complexes.

Sensitivity and specificity of the enhancement of p-CTLs by affinity-purified antibodies. To more precisely define the sensitivity of the antibody-mediated enhancement of the cross presentation of the antigenic SIV peptide and to compare the relative efficiency of antibody-mediated enhancement with the loading of the APCs with the dominant peptide, two experiments were performed. In the first experiment, aliquots of PBMC from two SIV-infected asymptomatic Mamu-A01 \(+/H11001\) monkeys (RPu-3 and REn-4 were collected at 5 months p.i.) were cultured with a 0.1, 1, 10, or 20 \(\mu M\) concentration of p11CM Gag peptide-pulsed autologous APCs. These were then tested for CTL activity at an effector/target ratio of 20:1 or 40:1 against autologous B-LCB pulsed with either 0.01, 0.1, or 1 \(\mu M\) p11CM peptide. As seen in Fig. 2A, although the peak cytotoxicity appeared to require a \(\geq 10 \mu M\) concentration of the p11CM Gag peptide for the restimulation period, as little as 0.1 \(\mu M\) of the p11CM peptide was sufficient for recognition and lysis of the target cells (the data shown are for PBMC from REn-4; the levels for RPu-3 were lower but showed similar trends). A similar experiment was subsequently performed with PBMC from two additional SIV-infected asymptomatic Mamu-A01 \(+/H11001\) monkeys (RUq-4 and RId-3 at 5 months p.i.; only data from RUq-4 are shown). In this case the autologous APCs were pulsed with a 0.1, 1.0, 10, or 20 \(\mu M\) concentration of the recombinant p55 Gag protein in the presence of a 2:1 ratio of p55-IgG (5-month-p.i. sample). After the standard restimulation coculture, each of these effector cell cultures was analyzed for cytotoxicity against autologous target cells pulsed with 0.01, 0.1, or 1.0 \(\mu M\) p11CM peptide. As seen in Fig. 2B, as little as 0.1 \(\mu M\) p55 Gag/p55-IgG induced readily detectable levels of cytotoxicity against autologous target cells pulsed with 0.01, 0.1, or 1.0 \(\mu M\) p11CM peptide. The percent net cytotoxicity in general appeared to be higher with the use of the p55 Gag/p55-IgG compared to that seen with the use of the p55 Gag alone (control). The antibody-mediated enhancement of p-CTL generation was not due to nonspecific activation by immune complexes since use of OVA plus anti-OVA in the presence of SIVp55 Gag during the restimulation period failed to show any enhancement of p11CM peptide-specific p-CTL.
In addition, the incubation of APCs with 10 μM p11CM peptide in the presence of 5 μM p55-IgG failed to show any enhancement of p-CTL frequencies versus the frequencies obtained with p11CM alone (data not shown). These data demonstrate the sensitivity and specificity of the enhanced p-CTL generation with the use of presumably SIV Gag immune complexes.

Enhancement of p-CTL requires FcR, MHC class I and
MHC class II molecules. PBMC from three Mamu-A01+ monkeys were restimulated with p55 Gag alone or with p55 Gag/p55-IgG in the presence or absence of saturating amounts of anti-MHC class I, anti-class II, or anti-FcRII/III monoclonal antibodies in our standard LDA. Representative data obtained on PBMC sample from one of these monkeys is presented here. As seen, the frequencies of p11CM-specific p-CTL after restimulation showed marked inhibition of restimulation in the presence of anti-MHC class I and, to a lower extent, anti-MHC class II and anti-FcRII/III antibodies (Fig. 3). These data suggest various degrees of involvement of the MHC class II and FcR pathways in addition to the MHC class I pathway for the enhanced restimulation of p11CM-specific p-CTLs. In a separate experiment, bulk CTLs were generated with p55 Gag or p55 Gag/p55-IgG. After restimulation, such effector cells were assayed for CTL activity against p11CM peptide-pulsed autologous B-LCB in the presence or absence of saturating amounts of anti-MHC class I or anti-MHC class II antibodies. Whereas the addition of anti-MHC class II antibodies at this effector stage had a minimal effect, anti-MHC class I antibodies led to >80% blocking of CTL effector activity (data not shown), suggesting that the effector cells were indeed mediating their CTL function via MHC class I-restricted mechanisms.

Enhancement of CTL responses by antibodies is not animal specific. Although it is difficult to conceive that the affinity-purified antibodies would only function to enhance the generation of autologous but not heterologous p-CTLs, this aspect still had to be confirmed. Thus, PBMC from four SIVmac251-infected clinically asymptomatic Mamu-A01+ monkeys collected at 6 to 7 months p.i. were restimulated with autologous APCs pulsed with 10 μM p55 Gag and either 5 μM autologous or heterologous p55-IgG isolated from the plasma of the same blood samples prior to testing for p11CM p-CTL levels (Fig. 4). As seen previously, each aliquot of PBMC showed augmentation of p-CTL levels when the restimulation was carried out in the presence of autologous p55-IgG, albeit differences among the individual PBMC samples were noted in terms of the degree of augmentation. Not surprisingly, each of the four p55-IgG samples were also able to promote relatively equivalent enhancement of p-CTL frequencies, suggesting similar qualitative properties in the SIV Gag-specific IgG responses of these four monkeys. In sharp contrast, the addition of a 5 μM concentration of monoclonal antibodies directed against SIV Gag p17 and p27 (KK59 and KK64) with p55 Gag protein during the restimulation phase failed to demonstrate any significant enhancement of p-CTL activity in aliquots of the same PBMC samples (Fig. 4). It is unclear at present whether this failure was secondary to the murine origin of the monoclonal
antibody reagents, potentially the wrong epitope being recognized, the need for binding to multiple Gag epitopes, the relative affinity of the antibodies, or some other as-yet-undefined required IgG quality.

SIV p55-IgG F(αβ)2 fragments are unable to enhance MHC class I-restricted CTL responses. PBMC from two SIVmac251-infected clinically asymptomatic Mamu-A01+ monkeys (REn-4 and RPu-3) were restimulated in the presence of autologous APCs pulsed with various doses of the p11CM peptide, p55 Gag, p55 Gag/p55-IgG, or p55 Gag/p55-IgG F(αβ)2 derived from the same affinity-purified IgG. As seen in Fig. 5, the addition of intact p55 Gag/p55-IgG to APCs led to stimulation of markedly enhanced p-CTLs over similar APCs pulsed with p55 alone or with p11CM. However, when similar concentrations of the F(αβ)2 fraction of the same IgG were substituted for the p55-specific IgG, the enhancement of p11CM-specific p-CTLs over restimulation with p55 only was abrogated. A single repeat of the same assay utilizing twice the F(αβ)2 concentration also failed to show significant enhancement of p-CTL generation (data not shown), suggesting that this failure is not secondary to the concentration of the antibody. These data suggest that this enhanced generation of p-CTL requires the Fc-FcR interaction to occur.

CD4+ T cells play a prominent role in the immune complex-mediated MHC class I cross presentation. Unfractionated or CD4-depleted PBMC samples from three SIV-infected asymptomatic monkeys collected at 8 months p.i. were cocultured in our standard LDA with autologous APCs, which were previously pulsed with either p55 Gag/p55-IgG or p55 Gag/preinfection IgG. The resulting restimulated cells were then assessed for levels of p-CTL against p11CM peptide-pulsed target cells. As illustrated in Fig. 6, the frequencies of p11CM-specific p-CTLs generated from the CD4+ T-cell-depleted cultures were markedly lower than p-CTLs obtained from the same sample of unfractionated PBMC. These data support the view that the augmented generation of p-CTL by the p55 Gag affinity-purified antibodies require CD4+ T-cell help during the restimulation period. In efforts to determine whether the CD4+ T-cell help required for this augmentation of p-CTL generation needed to be primed against SIV (i.e., is it antigen specific or non-antigen-specific CD4+ T-cell help), a separate subtract and add back restimulation p-CTL assay was performed as described in Materials and Methods with autologous CD4+ T cells enriched from cryopreserved PBMC obtained pre-SIV infection versus those isolated from the same monkey at 8 months p.i. As seen in Fig. 6B, whereas unfractionated PBMCs when restimulated in the presence of p55 Gag/p55-IgG showed significant frequencies of p11CM peptide-specific
p-CTLs, depletion of CD4+ T cells markedly reduced the levels of p-CTL generation. Although the addition of p.i. (primed) autologous CD4+ T cells showed significant reconstitution of the levels of p-CTL generated, there was also reconstitution mediated by pre-SIV infection (unprimed) CD4+ T cells, albeit the values obtained with the preinfection CD4+ T cells were modest compared to the p.i. CD4+ T cells. In addition, restimulation of aliquots of the CD8+ T-cell-depleted PBMC from two of the Mamu-A01+ SIV-infected monkeys in the presence of p55 Gag alone, p11CM peptide alone, or p55/p55 IgG F(ab')2 did not demonstrate any significant p11CM peptide-specific p-CTL activity (data not shown).

Both lactacystin- and bafilomycin-sensitive pathways are involved in antibody-mediated enhanced antigen loading with p55 Gag. In order to address the respective requirements for endosomal and endocytic processing pathways in the antibody-mediated enhancement of p11CM peptide MHC class I presentation, APCs were pulsed with p55 Gag/p55-IgG in the presence or absence of 5 μM lactacystin, an inhibitor of proteasome degradation or bafilomycin, an inhibitor of endosomal acidification. These APCs were then cocultured with autologous PBMC from three SIV-infected Mamu-A01+ monkeys as described in Materials and Methods. After restimulation, the cultures were assayed for levels of p-CTL with autologous p11CM peptide-pulsed target cells.

Does antibody-mediated enhancement of class I presentation also enhance the response to subdominant CTL epitopes?
D. Watkins et al. has identified several Mamu-A01-restricted epitopes that not only bind to class I but also elicit a CTL response in SIV-infected monkeys (3). In order to determine whether the p11CM CTL enhancing mechanism also enhances CTL recognition of subdominant epitopes, aliquots of the APCs were loaded with either 1 or 10 μM SIV Gag p55 alone, a 10 μM concentration of the p55 Gag/p55-IgG complex, or a 10 μM concentration of each individual peptide. Autologous PBMC from two Mamu-A01− SIV-infected monkeys were then restimulated with such APCs, which were then assayed for p-CTLs against the dominant and the subdominant SIV Gag peptide-pulsed target cells. As illustrated in Fig. 8A, restimulation of PBMC with the optimal concentration of individual SIV Gag peptides provided clearly detectable frequencies of p-CTLs directed against each epitope. These levels conformed to a hierarchy, with p11CM clearly dominant over the other three Gag epitopes. Restimulation of APCs loaded with SIV Gag p55 alone also induced detectable p-CTL levels against each of the Mamu-A01-restricted SIV Gag epitopes, although the hierarchy of the peptides differed from the one observed after restimulation with the peptides, suggesting differences in the efficiency of antigen processing and cross presentation. The LF8 peptide, in particular, appeared the least well recognized despite a higher binding affinity to Mamu-A01 (3). As seen

![Graph](http://jvi.asm.org/content/18/1/18.full.png)
FIG. 7. Antibody-mediated enhancement of MHC class I-mediated p11CM processing or presentation requires both the endocytic and the lysosomal pathways. (A) APCs from three Mamu-A01 monkeys collected at 9 months p.i. were pulsed with either p55 Gag-preinfection IgG (pre IgG) or with p55 Gag/p55-IgG in the presence or absence of 5 μM lactacystin or 40 μM bafilomycin before being used to restimulate autologous PBMC. After restimulation, the cultures were assayed for p-CTL by using autologous p11CM-pulsed target cells. (B) Control p-CTL restimulation and quantitation with vaccinia virus expressing Flu-MP-stimulated autologous B-LCB with or without lactacystin or bafilomycin. (C) Control TT-specific proliferation in the presence of lactacystin or bafilomycin.
above, restimulation with APCs loaded with p55 Gag/p55-IgG markedly enhanced p11CM p-CTL levels but only marginally enhanced p-CTL levels to the other epitopes, especially in PBMC from monkey REn-4 (Fig. 8A). In addition, this marginal enhancement of p-CTLs against subdominant epitopes was further diminished when APCs were loaded with a suboptimal (1 μM) dose of p55 Gag/p55-IgG (Fig. 8B), whereas enhancement of p11CM-specific p-CTLs appeared to be magnified even under such conditions (8.7- and 4.5-fold with 1 μM versus 4.2- and 2.4-fold with 10 μM p55 Gag/p55-IgG, respectively). These data suggest that this antibody-mediated MHC class I cross presentation may contribute to the dominance of p11CM CTL responses in Mamu-A01+ rhesus macaques infected with SIV.
DISCUSSION

The orchestration of an optimal immune response against an invading pathogen involves multiple pathways, including the cellular elements that comprise the innate and adaptive humoral and cellular immune system, whose interaction provides an efficient immune defense against the pathogen. Although the role of antigen specific antibodies, particularly in human HIV-1 infection and the nonhuman primate SIV model has so far primarily been focused on the presence or absence of virus-neutralizing antibodies (41, 42, 52, 72) and, if present, the breadth of such virus-neutralizing antibodies (11), the bulk of virus-specific antibodies made during lentiviral infections appear to be nonneutralizing (5, 11, 18, 28, 63). Whereas their (18, 63) role in assays such as antibody-dependent cellular cytotoxicity and complement-mediated cytotoxicity assays has been considered (52), relatively less attention as been paid to their role in immune complex formation and in facilitating antigen processing and presentation. The results of the studies reported here for the first time document an important, heretofore unappreciated, potential role for antiviral antibodies that is most likely distinct from their virus-neutralizing capability since the latter are primarily directed against epitopes of the viral envelope (20, 38, 73). Antibodies that may promote MHC class I cross presentation of exogenous SIV viral proteins are most likely not restricted to Gag, but the availability of recombinant SIV p55 Gag combined with the knowledge that there is an immunodominant p11CM epitope of p55 Gag that is recognized by effector CD8+ CTLs in Mamu-A01+ macaques (19, 22, 40) provided a convenient model for investigating the potential utilization of such a pathway. It appears likely that this mechanism is functional for other SIV gene products and most likely also for other viral infections. In fact, several studies have addressed the role of immune complexes and BCR-mediated antigen uptake in antigen cross presentation in murine models (31, 48, 55).

There are, however, salient differences between the murine studies and our monkey study, including the role of CD4+ T cells, the lineage of APCs capable of promoting such cross-presentation, and the fact that the studies reported here were performed in the context of a fully pathogenic lentiviral infection. Thus, whereas the murine studies suggested that cross presentation was CD4+ T cell independent, our results underscore the critical contribution of CD4+ T cells in mediating this enhancing effect in the lentiviral model, suggesting that one major component in the enhancing effect is the presentation of epitopes via MHC class II leading to enhanced help, an effect similar to the role of immune complexes highlighted by Hamao et al. (26). The data presented here clearly suggest that the antibody-mediated class I processing/presentation of Gag p55 is a multistep mechanism that includes both lysosomal and proteasomal digestion. Several findings converge to highlight the importance of enhanced class II-mediated presentation leading to enhanced MHC class I-specific CTL responses, most likely via licensing of the APCs by CD4+ T cells (12, 33, 49, 54). First, p-CTL levels obtained with p55 Gag/p55-IgG were markedly higher than the p-CTL levels obtained during restimulation with an optimal concentration of the p11CM peptide, suggesting that the augmentation observed was most likely not secondary to the density of Mamu-A01 presenting p11CM on the surface of APCs but likely depended on costimulation and/or a more efficient cytokine milieu. Preliminary studies with the use of the p11CM peptide bearing Mamu-A01 tetramer reagent has provided support for this view. Thus, as seen in Table 1, whereas the frequency of tetramer-positive cells ex vivo in the PBMC of three SIV-infected Mamu-A01+ monkeys ranged from 3.56 to 7.32% of the CD8+ T cells; after coculture with p55 Gag-pulsed APCs, such frequencies increased to 8.77 to 12.2%. However, coculture of an aliquot of the same PBMC with APCs pulsed with p55 Gag/p55-IgG led to a further increase of from 12.4 to 18.9%. The fact that such enhancement of p-CTL generation requires CD4+ T-cell help (see Fig. 6) suggests that such CD4+ T cells synthesize cytokines and/or antiapoptotic factors that lead to the better induction and/or survival of the CD8+ CTLs. Second, although such enhancement of viral peptide-specific p-CTL generation was most optimal with SIV-primed CD4+ T cells, there clearly also was significant (P < 0.01) enhancement of p-CTL generation by autologous CD4+ T cells collected prior to SIV infection (non-antigen primed). These latter data support our previous documentation of the ability of non-antigen-primed but in vitro-activated autologous CD4+ T cells to provide rescue of virus-specific effector function in SIV-infected rhesus macaques in vivo (66). Whether the mechanisms by which unprimed compared to SIV-primed CD4+ T cells mediate such enhancement are identical or different (quantitative or qualitative differences) remains to be established. In this context, it is also important to note that the enhancement was not due to nonspecific activation of APCs by SIV p55 Gag immune complexes, since the addition of OVA plus anti-OVA antibodies at the same concentrations or the addition of p55-IgG to p11CM peptide failed to demonstrate any significant augmentation (Fig. 2). It would be of interest to determine the nature of the p55 Gag MHC class II epitope(s) that provide such helper function. Analysis of reactive Gag epitopes is currently ongoing with a large battery of overlapping peptides encompassing the entire SIV Gag protein.

Although this enhancing mechanism may rely mostly on the induction of CD4+ help in licensing APCs and/or CD8+ effectors (12, 33, 49, 54), the data also provide evidence for enhanced class I presentation on the surface of APCs similar to the murine studies with OVA plus polyclonal anti-OVA (55). Delineation of these various pathways in outbred primates is more difficult than in well-defined murine models, but three

<table>
<thead>
<tr>
<th>Culture point</th>
<th>Frequency of p11CM tetramer+ CD8+ cells (%)</th>
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<tbody>
<tr>
<td>RPU-3</td>
<td>3.56</td>
</tr>
<tr>
<td>RUq-4</td>
<td>5.11</td>
</tr>
<tr>
<td>REN-4</td>
<td>7.32</td>
</tr>
<tr>
<td>After culture with SIV p55 Gag</td>
<td>8.77</td>
</tr>
<tr>
<td>After culture with p55 Gag/p55-IgG</td>
<td>12.40</td>
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a PBMC from three Mamu-A01+ monkeys were subjected to analysis to determine the frequencies of p11CM peptide-bearing Mamu-A01 by using the p11CM-MamuA01 tetramer either ex vivo or after in vitro expansion in the presence of SIV Gag p55 alone or p55 Gag/p55-IgG for 10 to 14 days. The analysis was gated on CD8+ T cells, and the frequency of the tetramer+ cells within this gated population was evaluated. A total of 200,000 cells were analyzed.
sets of findings support the notion that enhanced p11CM presentation in the context of class I was achieved on the surface of APCs. First, CD4-depleted PBMC still demonstrated modest but significant ($P < 0.01$) enhancement of CTL activity (Fig. 6), suggesting a more efficient loading of the antigenic peptide within the MHC class I molecules. Second, blocking of MHC class II determinants also demonstrated modest but clearly identifiable enhancement of p11CM pCTL levels over restimulation with p55 only (Fig. 3). Finally, the discrepant enhancement of p-CTL recognition among various Mamu-A01-restricted SIV Gag epitopes (Fig. 8) suggests that differences in the efficiency of peptide processing via this pathway do exist. Thus, compared to the enhancing effect of p55 Gag/p55-IgG on p11CM responses, such mechanisms did not significantly improve p-CTL values for the other three Gag epitopes over restimulation with the peptides alone (Fig. 8), suggesting that p11CM may be more efficiently processed and presented after FcR-mediated uptake. Such preferential presentation cannot also be solely ascribed to the affinity of peptide binding, since peptide LF8 binds with markedly higher affinity to Mamu-A01 than p11CM (31). Clearly, if active in vivo, such mechanism may exert a profound influence on the dynamics of the overall immune response, not only in terms of enhancing or maintaining CTL responses but potentially also in terms of narrowing the response to dominant epitopes, a rather undesirable effect for a chronic viral infection highly prone to mutation and immune evasion (4). This observation is especially true for Mamu-A01+ rhesus macaques, which upon SIV infection develop high frequencies of p11CM-specific CTLs at the expense of a larger breadth of epitopes recognized (5). Clearly, such implications need to be investigated in more detail by using other models that provide a better control over the type of response and the choice of immunogens to be used than the outbred macaque monkeys.

Among other relevant clinical issues are (i) the kinetics of such antibodies and effector cells, (ii) the point at which they appear, (iii) how long are they maintained, and (iv) the nature of the role of viral loads on such enhancing antibodies and effector cells. Preliminary studies by our lab to address this issue suggest that such antibodies arise within 4 to 6 weeks p.i., prior to the achievement of viral load set point, and are present throughout the asymptomatic period but clearly decrease during the disease stage even though significant titers against SIV proteins, including Gag, remain (data not shown). We have not as yet specifically addressed the issue of the effector cell population that is amenable to augmentation by immune complexes, nor the effect of the viral load. The fact that CD4$^+$-T-cell helper function is compromised quite early post-SIV infection raised questions about the importance of the findings reported here. However, the data for this enhancement seen at least during the clinically asymptomatic period suggest a role for such enhanced CTL function during the asymptomatic period.

Several additional issues need to be addressed. Thus, while on the one hand, this mechanism is likely to expand antigen-specific CTL responses in vivo, it is hypothesized that this mechanism promotes sufficient exposure of class I-restricted viral epitopes on any given cell and hence is likely to contribute to bystander killing of FcR-bearing uninfected cells. This concern is borne from the observation that, unlike the murine studies of Schuurhuis et al. (55) and Regnault et al. (48), enhancement of class I cross presentation, while optimal with APCs, was also demonstrated when B-LCB were used as APCs (data not shown). It remains to be seen whether such presentation differs between species or antigen model utilized, since Ke and Kapp also reported receptor-mediated class I cross presentation in B cells (31).

In summary, data are presented here that provide support for an important role for virus-specific antibodies in the generation and/or maintenance of virus-specific CTLs. Such activity appears to require the presence of CD4$^+$-T-helper function that is more efficient if it is antigen primed. The ability to generate such virus-specific CTLs appears to be inhibited to various degrees by both lactacytin and bafilomycin treatment of the APCs, suggesting the involvement of both the proteasomal and the vacuolar pathways in the processing or presentation of the antigenic peptides and the development of CTL responses. Although the in vivo significance of these findings remains to be established, they do provide support the view that the generation of such antibodies by potential vaccines against SIV and, by implication HIV, could play a role in virus-specific protective mechanisms and need to be examined. In addition, a similar role for virus-specific IgA in mucosal specific cellular response may be of importance in containing viral loads locally and needs to be examined.

ACKNOWLEDGMENTS

This work was supported by a grant from the NIH (1RO1-27057) and an AIDS research grant from the Health Sciences Foundation, Ministry of Health, Japan.

We are deeply grateful to the staff of the Yerkes National Primate Research Center for handling and care of the animals, to Jeffrey Bloom for providing the protocol for the preparation of the affinity-purified antibodies, and to the NIH AIDS Research and Reference Reagent Program (J. Altman and M. Hulsey) for supplying reagents utilized in these studies.

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