

The Enhanced Immune Response to the HIV gp160/LAMP Chimeric Gene Product Targeted to the Lysosome Membrane Protein Trafficking Pathway*

(Received for publication, November 12, 1996)

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The lysosome-associated membrane proteins (LAMP), found in the outer membrane of lysosomes and also in a multilaminar compartment that contains major histocompatibility complex class II (MHC II) proteins, are directed to their localization by a cytoplasmic carboxyl-terminal sequence. Our studies of the immune response to LAMP-targeted proteins has led to the application of a HIV-1 gp160/LAMP chimeric gene as a novel means to enhance the MHC II presentation of gp160. Immunofluorescence microscopy confirmed that the gp160/LAMP protein had a cellular localization corresponding to that of lysosomes. Pulse-chase analysis confirmed that the rates of synthesis of gp160/LAMP and wild type gp160 were comparable and that both proteins were processed to gp120 at similar rates. However, the gp160/LAMP was degraded more rapidly than the wild type gp160. MHC II-mediated T cell proliferation assays performed with cloned human cell lines showed that gp160/LAMP stimulated greater responses than did the wild type gp160. Moreover, mice vaccinated with recombinant vaccinia expressing gp160/LAMP had greater gp160-specific lymphoproliferation responses and higher titers of anti-V3 loop antibodies than mice vaccinated with recombinant vaccinia expressing wild type gp160.

antigen processing and presentation pathway, which conventionally operates only in professional antigen presenting cells (APC) that express MHC II molecules. This pathway is believed to be accessed primarily by the endocytosis or phagocytosis of extracellular proteins, with transport of the protein to endosomal/lysosomal compartments where it is proteolytically degraded, and the antigenic peptides loaded onto MHC II molecules for transport to the cell surface and presentation to CD4⁺ T cells (18–20). Some endogenously synthesized membrane proteins, including the influenza hemagglutinin and HIV envelope protein, are also presented by MHC II molecules, presumably by endocytosis from the cell surface or other suggested pathways (21–24).

We have hypothesized that the targeting of recombinant antigens to the lysosome-associated membrane protein (LAMP) trafficking pathway will enhance the loading of endogenously synthesized antigen onto MHC II molecules and consequently elicit an enhanced CD4⁺ helper T cell response with the resulting increase in both humoral and cell-mediated immunity. The basis of this approach is to create a chimeric antigen containing the endosomal/lysosomal localization signal from the LAMP family of lysosomal membrane proteins (25, 26). The LAMP molecules are type I transmembrane proteins that contain a 24-amino acid transmembrane domain and an 11-amino acid cytoplasmic tail with a carboxyl-terminal YQTI sequence that, in the context of the LAMP cytoplasmic domain, is necessary and sufficient to target recombinant proteins through a vesicular pathway to lysosomes (27). Recent evidence has indicated that at some point in the trafficking of the LAMP protein it enters a compartment that also contains MHC II molecules. Several electron microscopy and cell fractionation studies have shown colocalization of LAMP with MHC II molecules (28–31), and a multilaminar prelysosomal compartment containing LAMP and MHC II molecules, termed MIIC by some, has been implicated as the site of antigen processing and peptide loading onto MHC II molecules (32–35). We propose that the co-localization of LAMP with MHC II proteins, or possibly the more efficient processing and delivery of antigen peptides to MHC II as a result of LAMP-mediated targeting, may provide the basis for an enhanced immune response to the LAMP-targeted as compared with the wild type antigen. The enhancement of both cell-mediated and humoral immune responses by LAMP targeting of antigen has been demonstrated by Wu *et al.* (36), with a recombinant vaccinia

Much of the effort in developing an effective vaccine for HIV-1 has focused on the envelope protein. In addition to protein vaccines, some vaccine strategies have featured recombinant viruses that express the envelope protein (1–11), and other more recent studies have employed DNA immunization (12–17). Envelope-specific humoral and cell-mediated responses have been demonstrated by use of these approaches. However, one problem with DNA vaccines in general is that the vaccine antigen made in cells taking up the vector may not enter the major histocompatibility complex class II (MHC II)¹

* This work was supported in part by National Institutes of Health Grant AI28108, the National Science Foundation, and NICHD Grant NP1-HD-6–2915. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Supported in part by National Institutes of Health NIGMS Graduate Training Grant (5.T32.GM07626).

¶ Supported in part by the Gustavus and Louise Pfeiffer Research Foundation.

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¹ The abbreviations used are: MHC II, major histocompatibility complex class II antigens; LAMP, lysosome-associated membrane protein; APC, antigen presenting cells; m.o.i., multiplicity of infection; B-LCL,

EBV-transformed B lymphoblastoid cell lines; PBS, phosphate buffered saline solution; BCM, B cell medium; TCR, T cell receptor; HA, hemagglutinin; pfu, plaque-forming units; MEM, minimal essential medium; HIV, human immunodeficiency virus; EBV, Epstein-Barr virus.

virus-expressed HPV-16 E7 cytoplasmic/nuclear antigen. In this study, LAMP targeting was shown to enhance CD4⁺ T cell responses to the E7 antigen in *in vitro* systems and to increase both humoral and cell mediated anti-E7 responses in vaccinated mice. In addition, it was shown that vaccination of mice with LAMP-targeted E7 antigen elicited a much stronger immune response to tumor cells bearing the E7 antigen than did vaccination with wild type E7 (37).

In a previous report, we described the LAMP-directed sorting of the HIV gp120 envelope protein to lysosomes through the synthesis of a CD4/LAMP chimeric protein containing the LAMP lysosomal targeting sequence (38). The hypothesis underlying this study was that, when both CD4/LAMP and soluble gp120 are expressed in the same cell, the CD4/LAMP molecule would bind gp120 and direct the viral protein to the MHC II processing pathway. The feasibility of direct targeting was also explored. In this present study, we have further characterized the response to LAMP targeting of the HIV gp160 antigen by placing the LAMP targeting sequence directly into the gp160 envelope protein. We have demonstrated the lysosomal localization of this chimeric gp160/LAMP and shown that this localization is associated with a more rapid degradation of the protein. Cytolytic and proliferation assays were performed using several lines of cloned human CD4⁺ cytotoxic T cells. The gp160/LAMP construct was shown to stimulate greater proliferation responses of the T cell clones *versus* the wild type gp160. Moreover, immunization of mice with recombinant vaccinia expressing the gp160/LAMP chimeric protein resulted in an enhanced induction of helper T cell responses as evidenced by increases in antigen-specific lymphoproliferation responses and antibody levels than did vaccination with recombinant vaccinia expressing the wild type gp160.

EXPERIMENTAL PROCEDURES

HIV gp160 and LAMP Gene Constructs and Recombinant Vaccinia—The HIV-1 gp160 envelope gene used in these studies was derived from HIV-1 LAI (39). The vaccinia vector vPE16 and plasmid pPE15 both contain the HIV-1 LAI gp160 gene and have been described previously (40, 41). The gp160 gene encoded by both the vPE16 and pPE15 was engineered to contain silent mutations that remove two cryptic vaccinia early transcription termination signals. These constructs express full-length envelope protein, and the silent mutations result in increased expression of gp160 protein in vaccinia-infected cells. The gp160/LAMP chimeric gene was constructed by first amplifying DNA encoding the transmembrane and cytoplasmic domains of murine LAMP-1 by polymerase chain reaction. The product was engineered to contain *EcoRI* and *XhoI* sites at the 5' and 3' ends. The sense primer, 5'-GGGGGAAT-TCTTGTATCCCCATTGCTGTGGGC-3' (*EcoRI* site in bold), was annealed to mLAMP-1 nucleotides encoding Leu³⁴⁸-Gly³⁵⁴. The antisense primer, 5'-AAAACCTCGAGCTAGATGGTCTGATAGCCGGC-3' (*XhoI* site in bold, stop code underlined), was annealed to LAMP-1 nucleotides encoding amino acids Ala³⁷⁷-Ile³⁸². The 120-base pair product was cloned into the *EcoRI* and *XhoI* sites of the vector pCDNA1 (Invitrogen, San Diego, CA) to form pCDNA1/L1 and confirmed by DNA sequencing. The plasmid pPE15 was cut with *HindIII* and *EcoRI* yielding a 1.2-kilobase pair fragment encoding the amino-terminal extracellular portion of HIV gp160. This fragment was then cloned into pCDNA1/L1 at *HindIII* and *EcoRI* sites to form pCDNA1/GP160(5')/L1. This plasmid was cut with *BamHI* and *XhoI* and the 1.32-kilobase pair fragment was cloned into the *BamHI* and *XhoI* sites of pGEM7Z (Promega, Madison, WI) to form pGEM7Z/GP160(5')/L1. The DNA encoding the remainder of the extracellular portion of HIV gp160 was amplified by polymerase chain reaction using the *Pfu* polymerase (Stratagene, La Jolla, CA) from just upstream of the internal *EcoRI* site and was engineered to contain an additional *EcoRI* site at the 3' end just 5' to the start of its transmembrane domain. The sense primer, 5'-GGAGGGGACCCAGAAATTGTAAGCG-3' annealed to nucleotides 1095 to 1120. The antisense primer, 5'-TTTTTGAATTCTTTTATATACACAGCCAATT-TGT-3' annealed to nucleotides 2011 to 2035 (*EcoRI* site in bold). This fragment was cloned into the *EcoRI* site of pGEM7Z/GP160(5')/L1 to form pGEM7Z/160/L1. This HIV gp160/LAMP chimera was cloned into the vaccinia recombination vector pSC11MCS1 (41) using the *SalI* and

SphI restriction enzymes to form the recombinant vaccinia virus vTA160/L1 as described (42–44). The recombinant vaccinia vHA/LAMP expressing the influenza hemagglutinin antigen/LAMP chimera has been described elsewhere,² and was used in these studies for a negative control of a vaccinia-expressed LAMP-targeted irrelevant antigen. All of the recombinant vaccinia used in this study were derived from the WR strain.

Immunofluorescence—Human embryonic kidney 293S cells (2 ml of 2.5×10^5 cells/ml) in MEM-10 (MEM, 10% HyClone Fetal Clone III serum product, 100 units/ml penicillin G, and 100 μ g/ml streptomycin) were plated in a six-well tissue culture plate containing sterile glass coverslips. The cells were incubated overnight at 37 °C in a 5% CO₂ incubator. The medium was aspirated and 0.5 ml of complete MEM-2.5 (same as MEM-10 except it contains 2.5% serum product) was added containing the recombinant vaccinia vTA160/L1 or vPE16 at a multiplicity of infection (m.o.i.) of 1. The cells were incubated for 1 h, then overlaid with 1.5 ml of complete MEM-2.5 and incubated overnight. Subsequently, the cells were fixed in 2% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min. After washing with PBS, PBS containing 4% normal goat serum and 0.1% saponin was added to block and permeabilize the cells. The cells were then incubated for 45 min with monoclonal antibody 902 (45) supernatant medium containing 0.1% saponin and 4% normal goat serum. After washing twice with PBS, the cells were incubated with 1 ml of PBS containing 0.1% saponin and 10 μ g/ml Texas Red-conjugated goat anti mouse IgG (Jackson, West Grove, PA). The cells were then washed twice in PBS and the coverslip inverted onto a microscope slide with a drop of mounting medium. The slides were observed under a Zeiss Axiophot and exposures were adjusted manually using Kodak TMAX 400 film.

Pulse-Chase and Immunoprecipitation—Separate culture tubes for each time point of the pulse-chase protocol contained 3×10^6 human EBV-transformed B lymphoblastoid cells, clone 81 EBV, at log phase growth in 1 ml of B cell medium (BCM) (RPMI 1640, 10% fetal bovine serum, 200 units/ml penicillin G, 200 μ g/ml streptomycin, and 4 mM L-glutamine). The cells were infected with the recombinant vaccinia vPE16, vTA160/L1, or vHA/LAMP at a m.o.i. of 5, and after 2 h, washed three times with 5 ml of methionine-free RPMI 1640 and then incubated in 1 ml of methionine-free BCM for 20 min. The cells were then pulsed with 150 μ Ci of [³⁵S]methionine (Amersham Corp.) for 10 min. BCM (10 ml) was immediately added as chase medium, and the cells were washed twice with 5 ml of BCM. The cells were resuspended in 5 ml of BCM and placed into a T-25 flask for the chase period incubation. For chase times shorter than 30 min, the wash steps were omitted and the cells were incubated in the 10 ml of BCM. At the end of each chase time, the cells were pelleted by centrifugation, washed twice with ice-cold PBS, and resuspended in 0.5 ml of ice-cold lysis buffer (90 mM KCl, 5 mM MgCl₂, 1 mM benzamide, 10 mM HEPES, 1 mM dithioerythritol, 0.5% Nonidet P-40, 100 μ g/ml phenylmethylsulfonyl fluoride, 1 μ g/ml pepstatin A, 2 μ g/ml leupeptin, and 1 μ g/ml aprotinin). After incubation on ice for 30 min, nuclei were removed by centrifugation for 10 min in a microcentrifuge. The cell lysates were precleared overnight with 3 μ l of normal human serum (Jackson) and 150 μ l of protein G-Sepharose (Pharmacia Biotech Inc.) suspended in an equal volume of Sepharose wash buffer (PBS, 1% Nonidet P-40, 0.1% SDS, 0.5% deoxycholic acid, 1 mM dithioerythritol). The envelope protein was precipitated from the precleared lysates with 3 μ l of heat-inactivated human antisera from HIV-positive donors and 30 μ l of the protein G-Sepharose suspension. The antigen-antibody-Sepharose pellet was boiled for 5 min in 2 \times reducing sample buffer. The samples were resolved on SDS 9% polyacrylamide gels, and the dried gels were exposed to a Fuji BAS-III phosphor image screen for 20 h. The screen images were captured with a Fuji BAS1000 phosphor image scanner and MACBAS version 2.0 software.

T Cell and B Cell Lines—The human CD4⁺ T cell lines and autologous EBV-transformed B lymphoblastoid cell lines (B-LCL) were previously described (46–48). All T cell clones were isolated from HIV-1 seronegative individuals who participated in various HIV-1 candidate vaccine programs or by *in vitro* stimulation of T cells with gp120-pulsed monocytes.

Cloned Human T Cell Cytolytic Assays—B-LCL target cells at a concentration of 1×10^6 cells/ml in BCM, were infected overnight with the recombinant vaccinia vPE16, vTA160/L1, or vHA/LAMP at a m.o.i. of 5. [⁵¹Cr]Na₂CrO₄ (Amersham; 50 μ Ci/ml) was added to the target

² K. Staveley-O'Carroll, B. Purow, Y. Chen, A. L. Ruff, S. R. Schell, H. Levitsky, D. Pardoll, J. T. August, and F. G. Guarnieri, unpublished observations.

cells and incubated for 2 h. The cells were then washed three times with wash medium (PBS with 1% Fetal Clone III serum product), resuspended in 1 ml of BCM, counted for viable cells, and diluted to 1×10^5 cells/ml in BCM. Effector cells (autologous non-transformed human T cell clones) were counted for viability and resuspended at 1×10^6 , 3×10^5 , and 1×10^5 cells/ml in BCM. In V-bottom 96-well plates, 100 μ l of the target cell suspension was plated in triplicate with the addition of: 1) 100 μ l of each effector T cell dilution (effector/target ratios of 1:1, 3:1, and 10:1), or 2) 100 μ l of 1% Nonidet P-40 (maximum lysis), or 3) 100 μ l of BCM (nonspecific lysis). The plates were centrifuged for 5 min at 1200 RPM then incubated for 4 h at 37 °C in 5% CO₂. The plates were centrifuged again for 5 min, and 100 μ l of supernatant was collected. Supernatant total counts were measured in a γ counter. Specific lysis was measured as % specific lysis = (experimental lysis - nonspecific lysis / maximum lysis - nonspecific lysis) \times 100.

Cloned Human T Cell Proliferation Assays—B-LCL stimulator cells were pulsed with antigen by overnight infection with the recombinant vaccinia vPE16, vTA160/L1, or vHA/LAMP at a m.o.i. of 5 in 1×10^6 cells/ml in BCM. Proliferation of stimulator cells and vaccinia was blocked by treatment with either 5000-rad γ irradiation or psoralen treatment (incubation in 10 μ g/ml of psoralen for 15 min followed by long wave UV light for 5 min). These infected stimulator cells were washed three times with wash medium and resuspended to 5×10^4 cells/ml in BCM. Infected cells were combined with non-infected cells at ratios of 1:0, 1:2, 1:4, and 1:10. Responder autologous T cells were resuspended to 5×10^5 cells/ml in BCM. The T cell suspension, 100 μ l, was combined with 100 μ l of each stimulator cell dilution in triplicate in U-bottom 96-well plates. After incubation for 72 h, 1 μ Ci of [³H]thymidine (Amersham) was added to each well and cells were harvested 24 h later with a Packard Micromate cell harvester. [³H]Thymidine incorporation into DNA was measured on a Packard Matrix 96 direct β counter.

Lymphoproliferation Assay of T Cells from Vaccinated Mice—BALB/c mice (Charles River Laboratories, Wilmington, MA), 6–8 weeks old, were immunized by footpad injection with 1×10^6 plaque-forming units (pfu)/mouse of purified recombinant vaccinia vPE16, vTA160/L1, or vHA/LAMP diluted in PBS. Two mice were used for each recombinant vaccinia group. The mice were boosted 2 weeks later using the same immunization protocol. After 3 weeks, the mice were euthanized by CO₂ asphyxiation, and the regional lymph nodes from both mice in each group were removed, pooled, crushed to form a single cell suspension, and passed through a 200- μ m nylon screen. T cells were enriched from this suspension by non-adherence to nylon wool as described (49). Peptides HP19 (AA 322–336, FVTIGKIGNMRQAHC) and HP33 (AA 485–499, KYKVVVKIEPLGVAPT) (Johns Hopkins University Protein Laboratory, Baltimore, MD), BALB/c MHC II gp160 peptide epitopes (50), were diluted in C-RPMI (RPMI 1640, 10% fetal bovine serum, 100 units/ml penicillin, 100 μ g/ml streptomycin, and 50 μ M β -mercaptoethanol) and dispensed into the wells of a 96-well U-bottom plate so that the final concentration after the addition of cells was 10, 3.15, and 0.99 μ g/ml. The plate also contained negative and positive control wells with no peptide antigen or 2 μ g/ml phytohemagglutinin (Sigma), respectively. Psoralen-treated, naive syngeneic splenocytes were added at 1×10^5 cells/well in C-RPMI. The previously enriched responder T cells were suspended in C-RPMI and plated at 3×10^5 cells/well in triplicate. The plate was incubated for 5 days in a humidified 37 °C incubator with 5% CO₂. After the incubation period, 1 μ Ci of [³H]thymidine (Amersham) was added to each well and cells were harvested 24 h later with a Packard Micromate cell harvester. [³H]Thymidine incorporation into DNA was measured on a Packard Matrix 96 direct β counter.

Antibody Responses of Vaccinated Mice—BALB/c mice (Charles Rivers, Wilmington, MA), 6–8 weeks old, were immunized by tail vein injection with 5×10^5 pfu/mouse of purified recombinant vaccinia vPE16, vTA160/L1, or vHA/LAMP diluted in PBS. Four mice were used for each recombinant vaccinia group. The mice were boosted twice at 2-week intervals by the same immunization protocol. Blood (200 μ l) was collected by tail bleed 13 days after each immunization and centrifuged to remove cells, and the serum collected and stored at 4 °C for immediate use. V-3 loop peptide (AA 305–329, CNNTRKSIRIQRG-PGRAFTIGKIG) was diluted in enzyme-linked immunosorbent assay protein dilution buffer (50 mM Tris, pH 9.5) at a concentration of 38 μ g/ml, and 100 μ l of the solution was added to each well of an enzyme immunoassay/radioimmunoassay high binding 96-well plate (Costar, Cambridge, MA). After overnight incubation at 4 °C, the peptide solution was removed and 200 μ l of blocking buffer (PBS with 0.05% Tween 20, 4% bovine serum albumin, and 4% normal goat serum) was added. The plates were incubated overnight at 4 °C and then washed with PBS containing 0.05% Tween 20 wash buffer. Serum samples were prepared in five serial dilutions from 10⁻¹ to 10⁻⁵ in blocking buffer, and 100 μ l

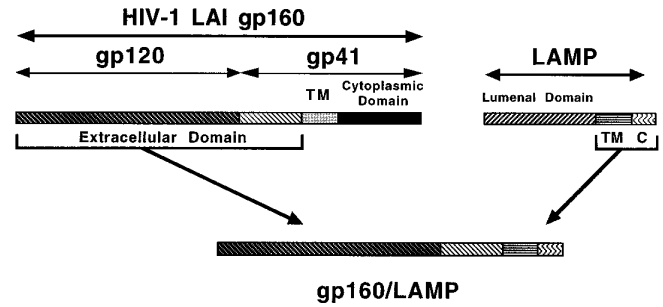


FIG. 1. Construction of the gp160/LAMP chimera. The HIV-1 LAI gp160 gene was engineered to contain silent mutations that remove two cryptic vaccinia early transcription termination signals. The gene sequence encoding all of gp120 and the entire extracellular domain of gp41 was linked to the gene sequence encoding the transmembrane (TM)/cytoplasmic (C) domains of the murine lysosomal membrane glycoprotein, LAMP-1. The chimeric gene, gp160/LAMP, was cloned into vaccinia to form the recombinant vaccinia vTA160/L1.

of each dilution was added to the blocked plate. The plate was incubated overnight at 4 °C. Horseradish peroxidase-conjugated goat anti-mouse IgG (Jackson) was diluted in blocking buffer to 0.5 μ g/ml, and 100 μ l was added to each well after washing the plate. The plates were incubated at room temperature for 4 h and then washed. Turbo TMB substrate solution (Pierce, 100 μ l/well) was added to each well and incubated for 10 min at room temperature. The reaction was stopped by adding 100 μ l of 1 M sulfuric acid, and absorbance at 450 nm was measured in a Cambridge Technology model 750 plate reader.

RESULTS

Construction of the gp160/LAMP Chimera—The gp160/LAMP chimeric gene construct was prepared by ligating the sequence encoding the entire extracellular domain of HIV-1 LAI gp160 to the sequence encoding the transmembrane/cytoplasmic domains of the murine lysosomal membrane glycoprotein, LAMP-1, as described in the methods (Fig. 1). DNA sequence analysis was performed to verify gene fidelity. The chimeric gene, gp160/LAMP, was then cloned into vaccinia to form the recombinant vaccinia vTA160/L1.

Protein Expression and Lysosomal Targeting—Other reports have shown the localization of LAMP-targeted chimeric proteins to the lysosomal membrane (27). We have used a similar immunofluorescence experiment in this study to verify the expression and lysosomal targeting of the chimeric gp160/LAMP protein (Fig. 2). Human embryonic kidney 293S cells were infected overnight with recombinant vaccinia vTA160/L1 or vPE16 expressing the chimeric or wild type protein, respectively, and stained the next day with a monoclonal antibody specific for gp120. The LAMP-targeted and wild type proteins were found to have distinctly different cellular staining patterns. Cells that expressed the wild type gp160 (*panel B*) had the expected reticular staining pattern. In contrast, cells that expressed the gp160/LAMP chimera (*panel A*) had a vesicular staining pattern characteristic of the lysosomal localization of LAMP and LAMP chimeras (25, 27).

Pulse-Chase Analysis of Protein Synthesis and Degradation—The biochemical properties of gp160/LAMP were further characterized by a kinetic pulse-chase labeling analysis of the synthesis and degradation of the LAMP-targeted and wild type gp160 (Fig. 3). B-LCL cells were infected with recombinant vaccinia, vTA160/L1, vPE16, or vHA/LAMP, labeled with [³⁵S]methionine for 10 min, and chased with excess unlabeled methionine for the times indicated in the figure. The HIV envelope proteins were immunoprecipitated with human HIV antiserum and resolved by SDS-polyacrylamide gel electrophoresis. The wild type gp160 and gp160/LAMP can be distinguished because the gp160/LAMP has a lower molecular weight than the wild type protein; the LAMP transmembrane/

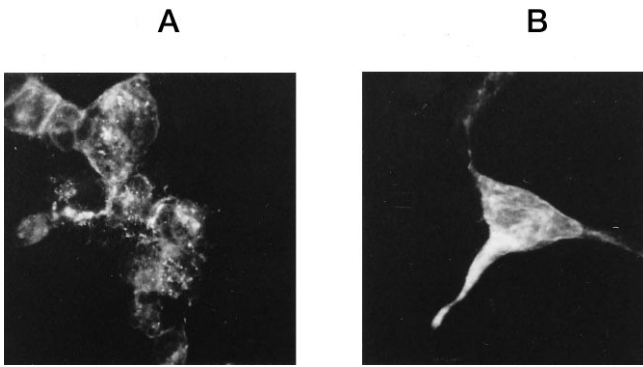


FIG. 2. Immunofluorescence localization of gp160 and gp160/LAMP. Human embryonic kidney 293S fibroblast cells were infected with recombinant vaccinia vTA160/L1 or vPE16 overnight and stained with mouse anti-gp120 monoclonal antibody 902 (45). Cells infected with vTA160/L1 have a granular staining pattern consistent with lysosomal localization of the gp160/LAMP protein (A). Cells infected with vPE16 have the expected reticular staining pattern of the wild type gp160 (B).

cytoplasmic domains of the gp160/LAMP being 101 amino acids smaller than the transmembrane/cytoplasmic domains of the gp41 subunit. At 15 min, the intensities of the wild type gp160 and the gp160/LAMP protein bands was equivalent, as confirmed by densitometry, indicating that the rate of synthesis of these two proteins was essentially equal. Processing of the proteins to yield gp120 was first apparent at 60 min and more so at 90 min. The gp160 protein band was apparent at every time point throughout the experiment consistent with a previous study showing that only a fraction of the gp160 protein is processed to the constituent gp120 and gp41 subunits (51). Beginning at 120 min, the intensities of the LAMP-targeted gp160 and gp120 protein bands were decreased relative to the 15-min time point and to the wild type proteins, and at 240 min, the LAMP-targeted gp160 and gp120 proteins had almost completely disappeared. In contrast, there was no obvious degradation of the wild type gp160 and gp120 proteins. No protein of similar molecular weight to the wild type gp160, gp160/LAMP, or gp120 was apparent in any of the lanes from cells infected with the negative control vHA/LAMP construct.

Cloned T Cell Cytolytic and Proliferation Assays of MHC II Presentation—MHC II presentation was analyzed by use of a cytolytic assay, which takes advantage of the fact that some CD4⁺ T cells have cytolytic activity (46–48). Autologous target B-LCL cells were infected overnight with the recombinant vaccinia vPE16, vTA160/L1, or vHA/LAMP, labeled with [⁵¹Cr]sodium chromate, and mixed with a CD4⁺ cytotoxic T cell clone. Because the effector cells are CD4⁺, they are MHC II-restricted and lyse only those target cells presenting gp120 peptides in MHC II molecules. Two different T cell clones, Ee 217 and 11.8, were tested. These clones were derived from different donors and recognize different gp120 peptides presented by distinct MHC II alleles. In each case, lysis of target cells expressing gp160/LAMP was greater than or equal to lysis of target cells expressing wild type gp160 (Fig. 4). These results indicate that gp160/LAMP is processed for MHC II-restricted recognition at least as well as the wild type gp160. The difference in specific lysis was small owing to the high sensitivity of this assay and the end point being limited to 100% specific lysis. It is therefore possible that the cytolytic assay underestimates the real differences in processing of the wild type and LAMP-targeted antigens.

A more relevant measure of the ability of the LAMP-targeted antigen construct to enhance MHC II presentation involves the ability of APC to induce proliferation of CD4⁺ T cells. MHC II-restricted CD4⁺ T cell proliferation responses were meas-

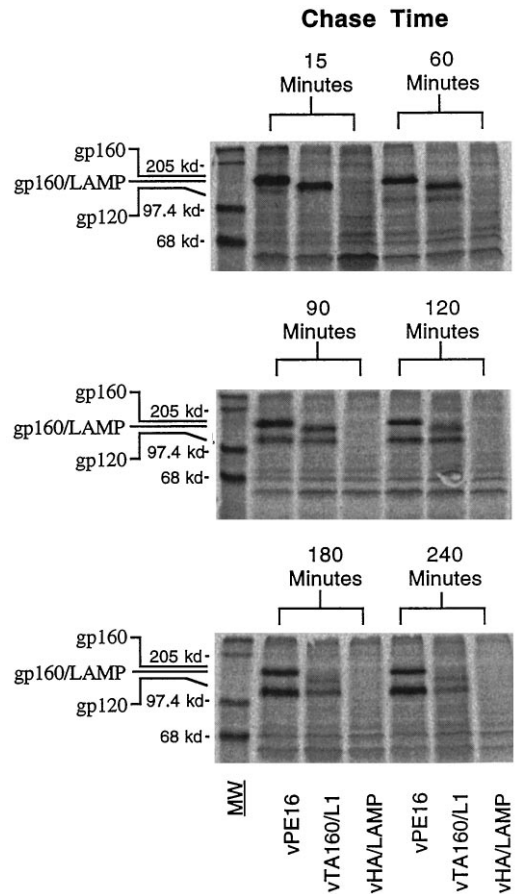


FIG. 3. Pulse-chase, [³⁵S]methionine labeling, immunoprecipitation, and phosphor image of HIV envelope proteins expressed by recombinant vaccinia-infected cells. 81 EBV B-LCL cells were infected with the recombinant vaccinia virus vPE16, vTA160/L1, or vHA/LAMP for 2 h. These cells were then pulsed for 10 min with [³⁵S]methionine and chased with excess unlabeled methionine for the time indicated. After the chase time, lysates were prepared and the gp160 proteins immunoprecipitated with human antisera from HIV-positive donors and protein G-Sepharose. The samples were resolved by SDS-polyacrylamide gel electrophoresis, and the dried gels were exposed to a phosphor image plate for 20 h. The chase times and recombinant vaccinia for each lane are given in the figure, and the location of the wild type gp160, gp160/LAMP, and gp120 protein bands are indicated at the left of each panel. Molecular weight markers (MW) are as shown. The rate of synthesis of the wild type gp160 and gp160/LAMP proteins was similar as indicated by the similar intensities of the wild type gp160 (160 kDa) and gp160/LAMP (149 kDa) protein bands at the 15-min chase point. Processing of gp160 to yield gp120 (120 kDa) was apparent at 60 min. At 90 min the intensities of the LAMP-targeted gp160 and gp120 proteins began to fade, and by 240 min, the LAMP-targeted proteins had almost completely disappeared. In contrast, the intensity of wild type gp160 and gp120 protein bands appeared unchanged throughout the 240-min experiment.

ured by overnight infection of antigen presenting stimulator cells, autologous B-LCL, with the recombinant vaccinia vPE16, vTA160/L1, or vHA/LAMP, blocking the proliferation of the cells and vaccinia with psoralen treatment or γ irradiation, and mixing them with a responding CD4⁺ T cell clone specific for a gp120 epitope. Three T cell clones derived from different donors were tested. These clones each had a different MHC II restriction and gp120 epitope specificity. All showed a marked increase in their proliferation response to the lysosome-targeted gp160/LAMP antigen versus the wild type gp160 (Fig. 5).

Mouse Vaccination and Immune Responses—The potential utility of the LAMP targeting of gp160 to enhance helper T cell responses in a vaccine was assessed by analysis of the humoral and lymphoproliferative response of vaccinated mice. For assay

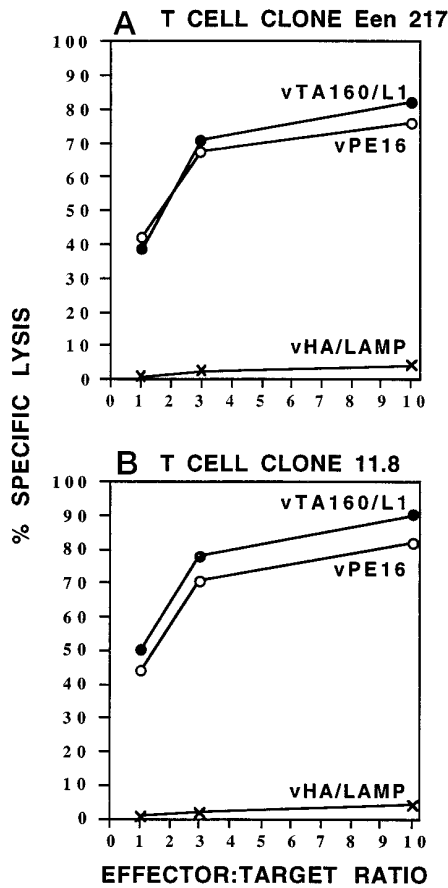


FIG. 4. Lysis of recombinant vaccinia-infected cells by CD4⁺ cytolytic T cells. These studies utilized cloned human CD4⁺ cytotoxic T cells specific for gp120 epitopes as effector cells and autologous EBV-transformed B-LCL cells as target cells. Target cells were infected overnight with vPE16, vTA160/L1, or vHA/LAMP and labeled with ⁵¹Cr for 2 h. These cells were mixed with the effector T cells at effector to target ratios of 1:1, 3:1, and 10:1. After incubating for 4 h, 100 μ l of the culture supernatants was removed and [⁵¹Cr] release measured by a γ counter. The data are plotted as % specific lysis versus effector:target cell ratio. Two effector cell lines were tested: T cell clone Een 217 with autologous target cell population B-LCL Laz 509 (A) and T cell clone 11.8 with autologous target cell population B-LCL 11EBV (B).

of the humoral response, BALB/c mice were vaccinated three times at 2-week intervals by tail vein injection with purified recombinant vaccinia vTA160/L1, vPE16, vHA/LAMP, or PBS (vehicle control). Serum samples were collected 13 days after each immunization, and antibodies to the V3 loop were measured by peptide enzyme-linked immunosorbent assay (Fig. 6). The anti-V3 loop antibody responses of mice vaccinated with vPE16 were at or below the background levels of the PBS and vHA/LAMP-negative controls. In contrast, in mice vaccinated with vTA160/L1, the LAMP targeted gp160 generated robust anti-V3 loop antibody titers.

The lymphoproliferation response was measured with T cells from BALB/c mice vaccinated twice with a 2-week interval by footpad injection with the recombinant vaccinia vTA160/L1, vPE16, or vHA/LAMP. Three weeks after the last immunization, T cells were isolated from the regional lymph nodes pooled from the two mice in each group. These cells were mixed with stimulator cells (naive splenocytes) and two peptides, HP19 and HP33, which are BALB/c MHC II gp160 peptide epitopes. The proliferation of the T cells from mice that received the LAMP-targeted gp160 was several times greater than that of T cells from mice that received the wild type gp160 (Fig. 7); the response to the wild type gp160 remained at background level.

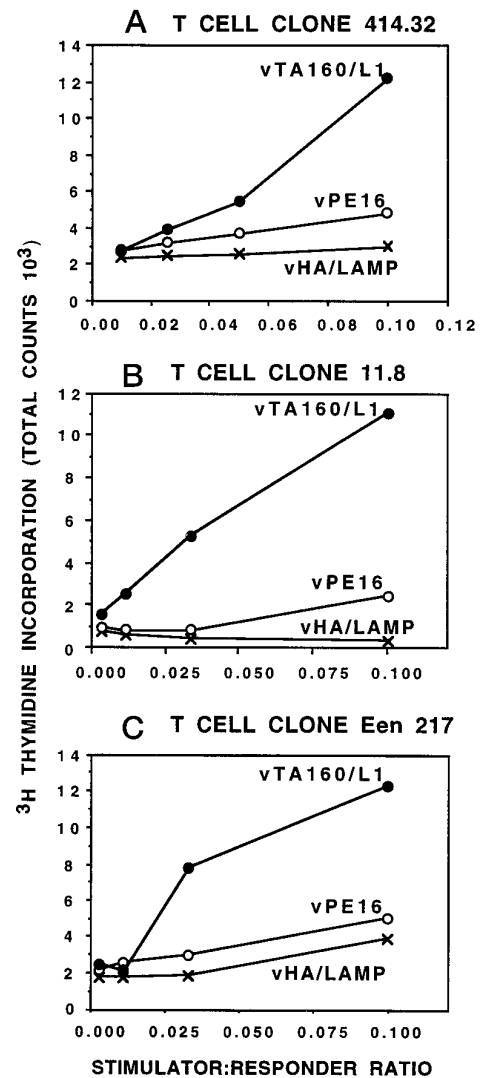


FIG. 5. T cell proliferation induced by recombinant vaccinia-infected stimulator cells. These studies utilized cloned human CD4⁺ T cells specific for gp120 epitopes as responder and autologous EBV-transformed B cells as antigen presenting stimulator cells. Stimulator cells were infected overnight with vPE16, vTA160/L1, or vHA/LAMP, and proliferation was blocked by psoralen treatment or γ irradiation. The stimulator cells were then mixed with the responding T cell clone at stimulator to responder ratios indicated in the figure. After incubation for 72 h, the cells were pulsed with [³H]thymidine and harvested 24 h later. Proliferation was measured by [³H]thymidine incorporation into DNA. The data are plotted as total counts incorporated versus stimulator cell:responder cell ratio. Three responder cell lines were tested: T cell clone 414.32 with autologous stimulator cell population B-LCL 414EBV (A), T cell clone 11.8 with autologous stimulator cell population B-LCL 11EBV (B), and T cell clone Een 217 with autologous stimulator cell population B-LCL Laz 509 (C).

DISCUSSION

Infection of antigen presenting cells or mice with the recombinant vaccinia virus, vTA160/L1, expressing HIV gp160 as a chimeric protein containing the LAMP lysosomal targeting sequence, was found to elicit increased responses of cloned CD4⁺ T cells *in vitro* and increased lymphoproliferation and anti-V3 loop antibody titers *in vivo*, as compared with the recombinant vaccinia virus vPE16 expressing wild type gp160. The cytolytic and proliferation responses of the cloned human CD4⁺ T cells suggest that these effects result from an enhanced MHC II processing and presentation of the chimeric gp160/LAMP, evidently as a consequence of its trafficking through the LAMP protein pathway. This model was dependent upon the demon-

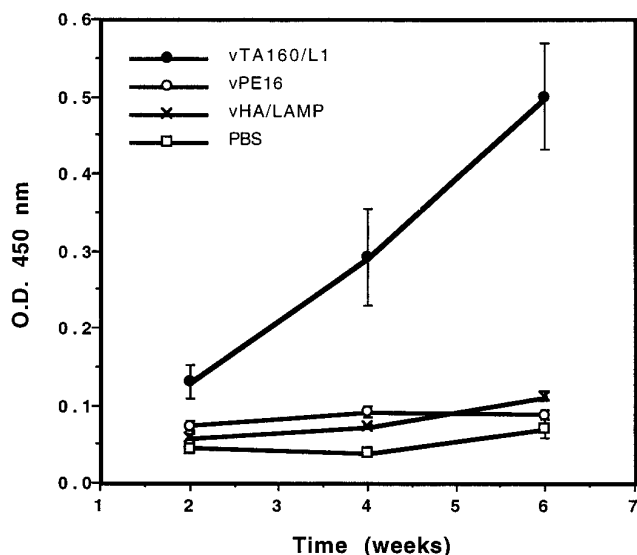


FIG. 6. Anti-V3 loop antibody responses of vaccinated BALB/c mice. Mice were vaccinated by tail vein injection with 5×10^5 pfu of the recombinant vaccinia vTA160/L1, vPE16, vHA/LAMP, or PBS control three times at 2-week intervals. Serum samples were collected 13 days after each immunization. The serum dilution in the experiment for this figure was 1:1000, which was the lowest serum dilution at which the mean of the absorbances of the vehicle controls was less than 0.1 at all time points. The value for each group is the mean of the absorbances from four mice. The data are plotted as $A_{450 \text{ nm}}$ versus time in weeks \pm standard error.

stration that the protein was indeed delivered to lysosomes and that the increased response to the LAMP-targeted protein was not due to a greater production of the chimeric protein. The steady-state cellular localization of the gp160/LAMP chimera in lysosomes was demonstrated by immunofluorescence microscopy, as described previously for LAMP proteins themselves (25) and other LAMP chimeras (27). Kinetic pulse-chase experiments confirmed that the gp160/LAMP protein was synthesized at a rate similar to that of the wild type gp160, thus indicating that the increased immune response to the LAMP-targeted protein cannot be attributed to an increased rate of synthesis of the protein. Most importantly, the pulse-chase analysis demonstrated that the chimeric protein was more rapidly degraded than the wild type gp160, as would be expected for a molecule delivered to lysosomes. The degradation of LAMP targeted gp160 (and gp120) became evident after 90 min, consistent with the results of a previous kinetic analysis of the biosynthesis and localization of LAMP-1 in mouse embryo 3T3 cells, which showed that approximately 1 h was required for delivery of newly synthesized protein to lysosomes (52). In contrast, the apparent concentration of the wild type gp160 (and gp120), remained constant throughout the 4-h experiment. It is also noteworthy that despite its difference in structure and vesicular trafficking, the gp160/LAMP protein was processed to yield the gp120 subunit at a rate similar to the wild type gp160 and comparable to that shown by others for the gp160 in HIV-1 pNL4-3-infected human lymphocytic leukemia cells (51). It is intriguing, however, that there are otherwise significant differences between the fate of gp160 synthesized in the HIV virus-infected lymphocytic leukemia cells as compared with that synthesized in vaccinia-infected B-LCL cells. Specifically, the gp160 made in HIV-infected cells was rapidly degraded during the first 4 h of synthesis in a process inhibited by NH_4Cl , suggesting that the degradation occurred in an acidic compartment with other evidence that this occurred in the lysosome (51). In contrast, the wild type gp160 made in vaccinia virus-infected cells was stable through 4 h. These findings

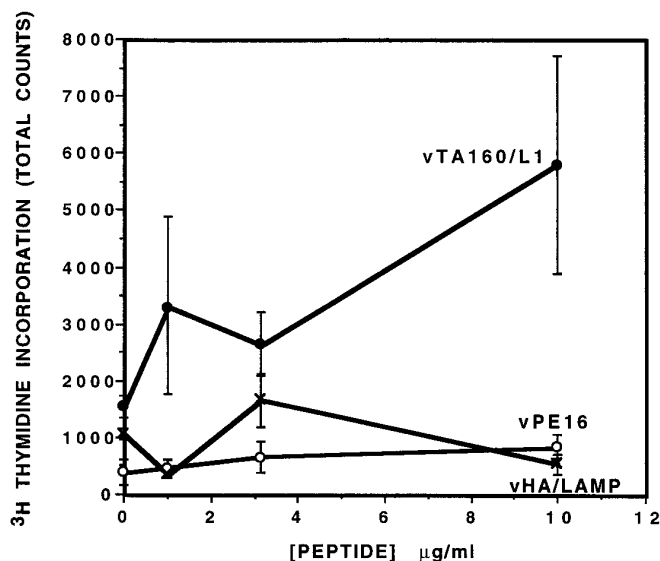


FIG. 7. Lymphoproliferation responses of T cells from vaccinated BALB/c mice. Mice were vaccinated by footpad injection with 1×10^6 pfu of the recombinant vaccinia vTA160/L1, vPE16, or vHA/LAMP two times with a 2-week interval. Lymph nodes were removed 3 weeks after the second immunization, and T cells were isolated from the pooled lymph nodes of each group. These cells were mixed with naive splenocytes as stimulator cells and peptides HP19 and HP33, BALB/c MHC II gp160 peptide epitopes, as indicated in the figure. After incubation for 5 days, the cells were pulsed with [^3H]thymidine and harvested 24 h later. Proliferation was measured by [^3H]thymidine incorporation into DNA. The data are plotted as total counts incorporated versus peptide concentration.

raise the possibility that the vesicular trafficking and processing of HIV virus-expressed gp160 and the vaccinia-expressed gp160 differ or that the differences are a function of the host cells.

The *in vitro* assays, target cell cytolysis and T cell proliferation, used in this study to analyze MHC II presentation of antigen both utilized cloned human CD4^+ cytotoxic T cells specific for gp120 epitopes. The data showed that LAMP targeting of the envelope protein enhanced both of these MHC II-mediated responses. The most significant response occurred with the T cell proliferation assays, which compared with the cytolytic assays, have the advantage of not having a limited end point. Here, stimulator cells expressing the LAMP-targeted gp160 elicited proliferation responses that were severalfold greater than the responses elicited by the cells expressing wild type gp160. This is despite the fact that wild type gp160 is presented in MHC II (21, 22), and elicits a CD4^+ T cell proliferation response. Thus, LAMP targeting appears to be superior to the normal gp160 processing pathway for MHC II presentation. In this context it should also be noted that the direct targeting of the gp160 to the lysosomal pathway is more efficient than the use of a CD4/LAMP chimeric molecule to direct the associated gp120 to the MHC II antigen processing pathway (38). It may be that the CD4/LAMP molecule itself is not as efficiently directed to the lysosome or that the kinetics of the binding of gp120 to CD4/LAMP limit the processing or presentation of gp120 for MHC II by this indirect mechanism. In the cytolytic assays of this study, although the difference in specific lysis between the gp160/LAMP and wild type gp160 groups was small, approximately 9%, the LAMP-targeted antigen elicited consistently greater cytolytic responses. The lower difference in response between the targeted and wild type antigen in the cytolytic as compared with the proliferation assays is attributed to the high sensitivity and limited end point of the cytolytic assay.

The extent of the immune response of mice vaccinated with

the LAMP-targeted gp160 as compared with the absence of response to the wild type gp160 was remarkable. The anti-V3 loop antibody titers of the vPE16 group were no greater than background after three immunizations with 5×10^5 pfu of recombinant vaccinia, and the lymphoproliferation responses of this group were also undetectable after two immunizations with a higher dose (1×10^6 pfu) of recombinant vaccinia. The vTA160/L1 vaccination group showed a lymphoproliferation response that was approximately 7 times greater than the vPE16 group at the maximum response level of the assay. The antibody response was also markedly enhanced; at a dilution of 1:1000, serum from mice vaccinated with vTA160/L1 showed an increasing amount of V3 loop antibodies during the course of the experiment. This relatively high titer antibody response, despite the targeting of the protein to lysosomes and the ensuing rapid degradation, suggests that some of the protein escaped to the extracellular milieu to interact with B cells, possibly by gp120 subunit dissociation from the gp41/LAMP component and subsequent secretion, or the presence of a fraction of the gp160/LAMP protein at the cell surface. It is known that a small fraction of the native LAMP proteins are expressed on the plasma membrane (53, 54).

There have been several studies that have shown the generation of anti-gp160 immune responses in mice that had been vaccinated with recombinant vaccinia expressing the HIV-1 envelope protein (10, 55, 56). These studies utilized a variety of immunization protocols, some of which included boosting with recombinant protein. The difference in protocols between those reports and our studies precludes direct comparison. In general, one difference is the amount of recombinant vaccinia inoculated. We have initiated a quantitative dose-response analysis of vaccinated mice comparing the LAMP-targeted *versus* wild type gp160.

Our model for the enhanced immune response mediated by LAMP targeting of endogenous antigen proposes that a limiting element in the immune response is the quantity of antigen processed and presented in MHC II molecules by APC. The LAMP targeting of antigen can be considered to act as a CD4⁺ T cell-specific molecular adjuvant, enhancing the activation of CD4⁺ T cells and increasing the population of helper T cells responsive to a specific viral or tumor antigen. A recent investigation of the relationship between the number of T cell receptors triggered and T cell activation suggests a possible mechanism by which LAMP targeting of antigen enhances the CD4⁺ T cell response. That study showed that T cells became activated, irrespective of the ligand, when a threshold of approximately 8000 T cell receptors (TCR) were triggered, and a reduction in the number of the TCR expressed severely compromised the capacity of the T cell to reach the activation threshold. Cells with a low number of TCR required up to 1000 times greater concentration of antigen for activation (57). These results support the model that an increase in antigenic peptide-loaded MHC II resulting from LAMP-targeted antigen may more efficiently reach the TCR triggering threshold and activate T cells that express too few TCR to be effectively triggered by an equivalent concentration of non-targeted antigen.

Several studies have recently shown that MHC II molecules co-localize with LAMP in a unique multilaminar compartment, termed MIIC by some (32–35). This compartment appears to be another vesicular compartment accessed by the LAMP molecules or an intermediate compartment in the pathway of LAMP trafficking to lysosomes, and is potentially the site of antigen processing and peptide loading onto MHC II molecules (31–35, 58). Our results of an enhanced CD4⁺ T cell proliferation stimulated by APC transfected with the gene encoding a

LAMP-targeted antigen as compared with wild type antigen is consistent with the presence of a processing compartment containing both MHC II and LAMP proteins. However, the site of antigen processing and peptide loading onto MHC II molecules is controversial, and some studies have reported that antigen processing and peptide loading onto MHC II molecules occur in a compartment that is largely devoid of LAMP molecules (59, 60). It should be noted that our results could theoretically result without the co-localization of MHC II and LAMP molecules, as the data do not rule out the possibility that proteolytic processing of antigen occurs in a LAMP-related compartment, that is devoid of MHC II, but facilitates peptide loading of the MHC II molecule in another compartment.

The LAMP targeting system has been studied with several antigens. Extensive *in vitro* studies of the influenza virus hemagglutinin (HA) protein have shown an increase in the lymphoproliferative responses to the LAMP-targeted HA, both of a single epitope included in a recombinant LAMP-1 construct and of the HA protein containing the LAMP transmembrane and cytoplasmic domains.² In another study, the indirect targeting of soluble gp120 to lysosomes by a CD4/LAMP chimera resulted in enhanced MHC II presentation of gp120 peptides as demonstrated by an increase in both the cytolytic and proliferative responses of the gp120-specific CD4⁺ T cells (38). Further data have come from the HPV-16 E7 studies. Mice that received the LAMP-targeted E7 vaccine had higher titers of anti-E7 antibodies and greater CTL and lymphoproliferative responses to E7 peptides (36). Other studies with this system demonstrated that 80% of mice vaccinated with the recombinant vaccinia expressing E7/LAMP then challenged with tumor, remained tumor-free after 3 months, whereas 100% mice receiving the vaccinia expressing wild type E7 developed tumors within 2 weeks of challenge. Vaccination with E7/LAMP was also shown to result in the cure of mice with small established tumors. *In vivo* antibody depletion of lymphocyte subsets showed that this tumor rejection required both CD4⁺ and CD8⁺ T cells (37).

This procedure for antigen targeting to the MHC II antigen processing pathway by the LAMP system may have human applications. Foremost is the use of LAMP targeting with DNA vaccines to enhance helper T cell response. This novel process acting on an endogenously synthesized protein is in contrast to the normal endocytic pathway for MHC II processing and presentation of foreign or non-endogenous antigens. The LAMP strategy would have the greatest impact in the case of DNA immunization, where the endogenously synthesized antigens have limited access to the MHC II processing and presentation pathway.

Acknowledgments—We gratefully acknowledge Dr. James Hildreth for providing human antisera from HIV⁺ donors and many helpful suggestions. We also thank Dr. Fred R. Vogel, and Quality Biological, Inc., Gaithersburg, MD, for providing V3 loop peptide. We are also grateful to Dr. Jay Berzsofsky for many helpful suggestions.

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