

Expression of Human Immunodeficiency Virus Type 1 gp120 from Herpes Simplex Virus Type 1-Derived Amplicons Results in Potent, Specific, and Durable Cellular and Humoral Immune Responses

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Herpes simplex virus type 1 (HSV-1) infects a wide range of cells, including dendritic cells. Consequently, HSV-1 vectors may be capable of eliciting strong immune responses to vectored antigens. To test this hypothesis, an HSV-1 amplicon plasmid encoding human immunodeficiency virus type 1 gp120 was constructed, and murine immune responses to helper virus-free amplicon preparations derived from this construct were evaluated. Initial studies revealed that a single intramuscular (i.m.) injection of 10⁶ infectious units (i.u.) of HSV:gp120 amplicon particles (HSV:gp120) elicited Env-specific cellular and humoral immune responses. A potent, CD8⁺-T-cell-mediated response to an H-2D^d-restricted peptide from gp120 (RGPGRAFVTI) was measured by a gamma interferon ELISPOT and was confirmed by standard cytotoxic-T-lymphocyte assays. Immunoglobulin G enzyme-linked immunosorbent assay analysis showed the induction of a strong, Env-specific antibody response. An i.m. or an intradermal administration of HSV:gp120 at the tail base elicited a more potent cellular immune response than did an intraperitoneal (i.p.) inoculation, although an i.p. introduction generated a stronger humoral response. The immune response to HSV:gp120 was durable, with robust cellular and humoral responses persisting at 171 days after a single 10⁶-i.u. inoculation. The immune response to HSV:gp120 was also found to be dose dependent: as few as 10⁴ i.u. elicited a strong T-cell response. Finally, HSV:gp120 elicited significant Env-specific cellular immune responses even in animals that had been previously infected with wild-type HSV-1. Taken together, these data strongly support the use of helper-free HSV-1 amplicon particles as vaccine delivery vectors.

Genetically engineered herpesviruses have been successfully used for the development of vaccines against important animal diseases, including Aujeszky's disease (pseudorabies virus), infectious bovine rhinotracheitis, and swine fever (hog cholera virus) (21, 56, 57). In addition, attenuated herpesviruses have been used for human vaccination (including the Towne strain of human cytomegalovirus and the Oka strain of varicella-zoster virus) (5, 24, 38, 52). Both herpes simplex virus type 1 (HSV-1) and varicella-zoster virus have been used for the expression of foreign genes, since these viruses can accommodate large segments of exogenous DNA with little effect on virus replication (15, 43). Replication-competent and replication-defective gene replacement vectors based on both viruses are being explored as possible human immunodeficiency virus (HIV) vaccine delivery systems (32, 45). The appeal of this approach lies in part in the ability of herpesviruses to (i) elicit strong cytotoxic-T-lymphocyte (CTL) responses; (ii) infect mucosal surfaces; (iii) infect a broad range of cell types, including dendritic cells (1, 23, 30, 40); and (iv) establish a state of persistence in the infected cell. The latter property may conceivably result in more durable immune responses to herpes-

virus-based vaccines compared to many other vector approaches.

The HSV-1 amplicon is an alternative HSV-1-based gene transfer vector which differs significantly from standard gene replacement vector systems. Most notably, the HSV-1 amplicon is a highly flexible, replication-defective, high-capacity plasmid-based gene transfer vector that contains only the lytic-phase origin of DNA replication and the cleavage and packaging sequences from HSV-1 (13, 46). This plasmid can be replicated and packaged into HSV-1 virions in the presence of either a suitable helper virus or a helper virus genome (13, 46). In this context, ca. 150 kb of concatemeric, replicated amplicon DNA becomes packaged into each virus particle (Fig. 1). Thus, the amplicon particle can contain as many as 15 to 20 copies of the packaged amplicon plasmid. This results in the delivery of multiple gene copies to each individual cell that becomes infected by the amplicon particle.

The amplicon vector system has recently been improved through the development of defective helper-virus genomes which lack viral cleavage and packaging sites of their own (12, 53). These defective helper-virus genomes allow amplicon plasmids to become packaged into HSV-1 particles while failing to become packaged themselves. As a consequence, helper-free amplicon stocks can be readily derived (9, 12, 39, 53). These helper-free amplicon particles express only the exogenous inserted sequence of interest and do not express any open

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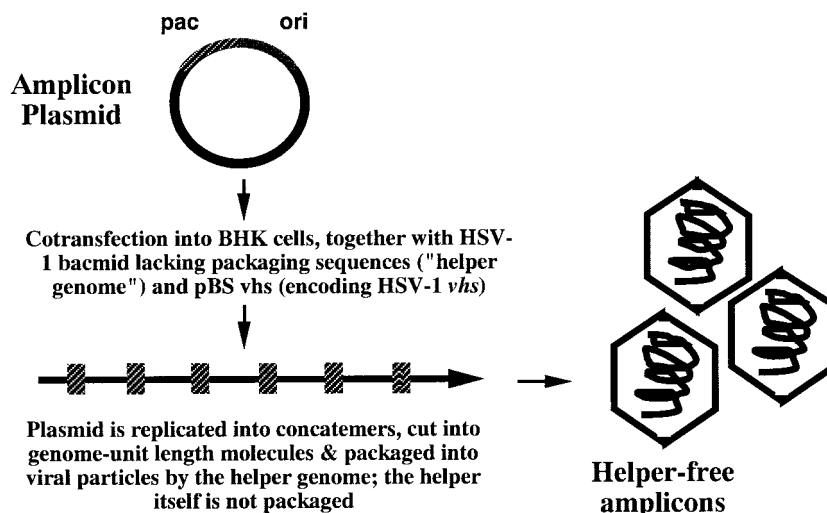


FIG. 1. HSV-1 amplicon vector system. The amplicon plasmid contains the HSV-1 origin of DNA replication, the viral cleavage and packaging site, and a mammalian expression cassette encoding the protein of interest (in this case, gp120). This plasmid is cotransfected into BHK cells, along with pBSvhs (which encodes the HSV-1 virion host shutoff protein), and the defective packaging construct (BAC-HSV), which lacks all viral cleavage and packaging elements. Immediately after transfection, the amplicon plasmid undergoes DNA replication (driven by proteins produced from the helper virus genome); this results in the generation of head-to-tail concatemers of the plasmid. These concatemers are cleaved into genome unit-length molecules and then packaged into virus particles, which can be harvested and concentrated and are ready for use.

reading frame from HSV-1. Thus, helper-free amplicon particles can be considered to be highly safe and devoid of the ability to express any of the immunomodulatory gene products of HSV-1 itself (e.g., ICP47, which downregulates major histocompatibility complex class I [17, 59]). This may in part explain why helper-free amplicon vectors can infect dendritic cells without inhibiting dendritic-cell maturation or immunostimulatory function (58), unlike either wild-type HSV-1 or recombinant HSV-1 vectors (23, 30, 40).

With the pressing need for an effective HIV-1 vaccine, it seems prudent to explore a wide array of viral delivery systems. In the present study, we constructed an HSV-1 amplicon plasmid that encodes a codon-optimized HIV-1 gp120 insert (4), under the transcriptional control of a strong viral promoter (HSV:gp120). We then packaged this plasmid into HSV-1 particles, which were inoculated into mice to evaluate their ability to elicit immune responses against the encoded gp120. Since CD8⁺ CTL activity is considered by many to be critical for controlling both HIV infection and infection with the closely related agent simian immunodeficiency virus (SIV) (6–8, 19, 35, 42), we concentrated our analyses on the ability of the HSV-1 amplicon vector to elicit strong CTL responses to gp120. Among the numerous CD8⁺-CTL epitopes that potentially could be derived from the HIV-1 virion, few are known to be recognized in mice. Among these, an immunodominant H-2D^d-restricted epitope from the V3 loop of HIV-1 gp120 (RGPGRAFTI) has been described in BALB/c mice (2, 3, 25, 33, 37, 44). Our analyses of Env-specific cellular immune responses to HSV:gp120 amplicon particles therefore focused on this particular epitope.

Overall, the findings reported here show that the HSV:gp120 amplicon vector is capable of eliciting strong Env-specific immune responses after a single inoculation of amplicon particles. The intradermal (i.d.) route of immunization appears to result in the generation of maximal cellular immune re-

sponses to gp120, and there is a clear dose effect in terms of the strength of response elicited versus the dose of amplicon delivered. However, even the lowest dose of amplicon that was tested (10,000 infectious particles) was able to generate a strong gp120-specific cellular response. The amplicon-induced immune response was highly durable in mice (lasting >5 months), and strong cellular responses occurred even in animals that had been previously infected with wild-type HSV-1. Overall, these data provide support for further evaluation of this novel approach to HIV-1 vaccine development.

MATERIALS AND METHODS

Cells. All cells were incubated at 37°C in 5% CO₂ under humidified conditions. Culture conditions were as follows: 293 cells were maintained in Dulbecco modified Eagle medium (DMEM; Gibco-BRL, Bethesda, Md.) supplemented with 10% fetal calf serum (FCS; Sigma, St. Louis, Mo.), penicillin, streptomycin, and L-glutamine (Gibco-BRL). P815 (*H-2^d* mastocytoma) cells and EL4 (*H-2^b* thymoma) cells were maintained in RPMI 1640 medium (Gibco-BRL) supplemented with 10% FCS, penicillin, streptomycin, and L-glutamine (Gibco-BRL). Primary splenocytes were prepared and cultured in RPMI 1640 medium supplemented with 10% FCS (HyClone, Logan, Utah), 50 μM β-mercaptoethanol (Gibco-BRL), penicillin, streptomycin, and L-glutamine (Gibco-BRL); this is referred to as CR10-β medium. Baby hamster kidney (BHK) cells were maintained as described previously (27). The NIH 3T3 mouse fibroblast cell line was originally obtained from the American Type Culture Collection and was maintained in DMEM supplemented with 10% FCS, penicillin, and streptomycin.

Amplicon vector construction. A codon-optimized version of HIV-1 gp120 DNA was kindly provided by Jürgen Haas (Max-von-Pettenkofer Institut, Munich, Germany). This gp120 expression vector was derived from HIV-1 MN, except for the V3 loop, which was derived from HIV-1 LAI and contains an epitope recognized by BALB/c mice in the context of H-2D^d (2, 3, 25, 33, 37, 44). The codon-optimized version of gp120 was selected to allow high-level, Rev-independent expression as described previously (4). The HSV-1 amplicon vector pHSVPrPUC (13) contains the ColE1 plasmid origin, an HSV-1 packaging signal and origin, and a multiple cloning site situated downstream from the HSV-1 immediate-early 4/5 promoter (IE4/5) and upstream from the simian virus 40 polyadenylation signal. A *NotI* (blunted)-*XhoI* 1.6-kb fragment encoding the synthetic gp120 sequence was removed from the pSynp120v3LAI plasmid and cloned directionally into a *SalI*-cut, *BamHI*-cut (blunted) pHSVPrPUC vector.

Helper virus-free HSV amplicon packaging. Packaging of virus vectors was performed as described previously (9). Briefly, on the day prior to transfection, 2×10^7 BHK cells were seeded in a T-150 flask and incubated overnight at 37°C in 5% CO₂. On the day of transfection, 1.8 ml of OptiMEM (Gibco-BRL), 25 µg of pBAC-V2 DNA (53), 7 µg of pBSKS (*whs*) (9), and 7 µg of amplicon vector DNA were combined in a sterile polypropylene tube. Then, 70 µl of Lipofectamine Plus reagent (Gibco-BRL) was added over a period of 30 s to the DNA mix, and the mixture was incubated at 22°C for 20 min. In a separate tube, 100 µl of Lipofectamine (Gibco-BRL) was mixed with 1.8 ml of OptiMEM, and this mixture was also incubated at 22°C for 20 min. After the incubations, the contents of the two tubes were combined over a period of 30 s and incubated for an additional 20 min at 22°C. During this second incubation, the medium in the seeded T-150 flask was removed and replaced with 14 ml of OptiMEM. The transfection mix was added to the flask and allowed to incubate at 37°C for 5 h. The transfection mix was diluted with an equal volume of DMEM plus 20% FBS, 2% penicillin-streptomycin, and 2 mM hexamethylene bisacetamide (HMBA) and then incubated overnight at 34°C. The following day the medium was removed and replaced with DMEM plus 10% FBS, 1% penicillin-streptomycin, and 2 mM HMBA. The packaging flask was incubated an additional 3 days before the virus was harvested and stored at -80°C until purification. Viral preparations were subsequently thawed, sonicated, clarified by centrifugation, and concentrated by ultracentrifugation through a 30% sucrose cushion. Viral pellets were resuspended in 100 µl of phosphate-buffered saline (PBS) and stored at -80°C until use. Titters of vectors were determined as described previously by using expression and transduction titrating methods (10). Virus was diluted in sterile Dulbecco PBS (D-PBS; Gibco-BRL) to the appropriate concentration prior to injection. In addition, two separate irrelevant amplicons were prepared as controls by using the same vector system described above; one contained the gene for *Escherichia coli* β-galactosidase (LacZ), and one contained the gene for murine intestinal alkaline phosphatase (MIAP).

Verification of gp120 expression. To verify gp120 expression, 293 cells were first seeded in 12-well plates and allowed to adhere overnight at 37°C in 5% CO₂. Cells were then either infected with HSV:gp120 amplicon particles or with HSV:MIAP amplicon particles as a negative control. On the day of infection, medium was removed, and cells were infected at a multiplicity of infection (MOI) of 0.5 in a volume of 0.5 ml at 37°C in 5% CO₂. After 1 h, 1.5 ml of supplemented DMEM was added to the wells, and the cells were incubated for 48 h. Medium was collected from the wells and concentrated by using the Microcon microconcentrator (M-50; Millipore, Bedford, Mass.). Concentrated supernatants were combined with an equal volume of Laemmli buffer and were separated on 4 to 15% gradient sodium dodecyl sulfate-polyacrylamide gels (Bio-Rad, Hercules, Calif.). Full-range rainbow molecular weight markers (Amersham/Pharmacia, Piscataway, N.J.) were added as a standard. The gels were transferred to nitrocellulose and were blocked in PBST buffer (1× PBS, 0.1% Tween 20) containing 5% nonfat milk. The blots were incubated with the AD3 anti-gp120 monoclonal antibody (AIDS Repository, Rockville, Md.) for 1 h at room temperature. Blots were extensively washed with PBST. The blots were then incubated with sheep anti-mouse horseradish peroxidase secondary antibody (Amersham). Blots were again washed extensively with PBST. Enhanced chemiluminescence (Amersham) was performed for 1 min, and the blots were exposed to X-ray film (Kodak, Rochester, N.Y.).

Peptide. The H-2D^d-restricted 10-mer epitope peptide from the V3 loop (RGPGRFVTI) was synthesized by Alpha Diagnostics (San Antonio, Tex.), purified by high-pressure liquid chromatography and analyzed by mass spectroscopy.

Mice. Female BALB/c mice aged 5 to 6 weeks were obtained from Taconic Laboratories (Germantown, N.Y.) and maintained according to University of Rochester guidelines. Prior to all injections, mice were bled orbitally, and sera were stored at -80°C until testing. For allogeneic CTL cultures, female C57BL/6 mice, aged 5 to 6 weeks, were utilized and handled as indicated above. Mice were injected by using a 28G 0.5-in. 1-ml insulin syringe (Becton Dickinson, Franklin Lakes, N.J.). Injections were given intramuscularly (i.m.) in the right hind hamstring muscle, i.d. at the base of the tail, or intraperitoneally (i.p.) in the body cavity. After inoculation, mice were incubated for 21 to 22 days prior to sacrifice and analysis.

At euthanization, sera were obtained by cardiac puncture, and spleens were removed by using sterile technique with sterile forceps and tweezers and transferred to 60-mm tissue culture dishes containing 5 ml of RPMI 1640 medium (Gibco-BRL). The spleens were aseptically transferred to 100-mm tissue culture dishes containing 10 ml of CR10-β. Autoclaved frosted glass slides (VWR, Media, Pa.) were used to gently grind the spleens to obtain a single cell suspension. Splenocytes were centrifuged, and the cell pellets were resuspended in 10 ml of CR10-β, counted, and diluted to a concentration of 4×10^7 cells/ml.

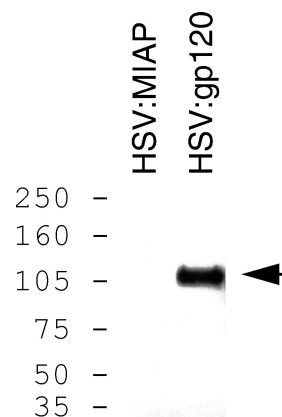


FIG. 2. Amplicon-directed expression of HIV gp120 in 293 cells. 293 cells were infected with either HSV:gp120 amplicon particles or with amplicon particles encoding MIAP (HSV:MIAP) at an MOI of 0.5. At 48 h after infection, cell culture supernatants were collected, concentrated, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and analyzed by immunoblot with the gp120-specific monoclonal antibody AD3 (the position of gp120 is indicated by the arrow). The numbers on the left denote the positions of the prestained molecular mass markers (Amersham-Pharmacia) which were included on the gel; sizes are in kilodaltons.

Two to three days before the CTL cultures were prepared, lipopolysaccharide (LPS) blasts were prepared as stimulator cells for the CTL cultures. Syngeneic BALB/c mice were euthanized, and their spleens were processed as described above. Naive splenocytes were cultured in T-75 flasks at a concentration of 4×10^7 cells/flask in 30 ml of CR10-β supplemented with 150 µl of LPS (1 mg/ml in sterile H₂O, from *E. coli* serotype O55:B5) and 30 µl of dextran sulfate (7 mg/ml in sterile PBS). Flasks were incubated at 37°C in 5% CO₂. After the incubation period, flasks were examined to look for blast-like cells. LPS blasts were washed extensively with RPMI 1640 medium and then pulsed with the RGPGRFVTI peptide for 60 min in a 37°C water bath. Blasts were irradiated in a cesium-137 source for 10 min (ca. 3,000 rads) and then diluted to a concentration of 10^7 cells/ml prior to use.

As an allogeneic control for CTL expansion and killing assessment, one C57BL/6 mouse was euthanized per experiment (as described above), and its spleen processed as described above. The splenocytes were diluted to a concentration of 2×10^7 cells/ml and irradiated along with the LPS blasts.

ELISPOT assay. The rat anti-mouse gamma interferon monoclonal antibody AN18 was produced from the corresponding hybridoma, which was kindly provided by Edith Lord (University of Rochester Medical Center). Hybridoma supernatants were concentrated by using the Cellmax system (Spectrum Laboratories, Laguna Hills, Calif.) according to the manufacturer's instructions. Ninety six-well membrane plates (Millipore) were coated with the AN18 monoclonal antibody diluted in 1× D-PBS (Gibco-BRL), and plates were washed with RPMI 1640 medium (Gibco) and blocked for at least 1 h at room temperature with CR10-β. Splenocytes from immunized animals were diluted in a background of syngeneic splenocytes to allow each enzyme-linked immunospot (ELISPOT) well to contain a fixed cell concentration of 10^6 cells per well. Splenocytes were cultured either in 2 µM (final concentration) of the V3 peptide diluted in CR10-β or in CR10-β alone for 18 h at 37°C in 5% CO₂. Plates were washed extensively with PBST. Biotinylated rat anti-mouse gamma interferon was added to the wells (clone XMG1.2; PharMingen, San Diego, Calif.) and incubated for 2 h at room temperature. Streptavidin-alkaline phosphatase was added (Jackson ImmunoResearch, West Grove, Pa.) to the well, and the mixture was allowed to incubate 1 h at room temperature. Plates were washed as described above. Vector Blue substrate (Vector Laboratories, Burlingame, Calif.) was prepared according to the manufacturer's instructions, added to the plates, and incubated for 10 min at room temperature in the dark. Plates were then washed extensively with distilled H₂O, allowed to dry overnight, and then counted and analyzed by Zellnet Consulting, Inc. (New York, N.Y.).

CTL cultures. To expand the CTL population from the inoculated animals to a level that could be observed by CTL assay, CTL cultures were prepared. Multiple wells were set up per animal to be tested. A 1-ml portion of CR10-β was combined with 0.1 ml of experimental splenocytes (4×10^6 cells) and either 0.1

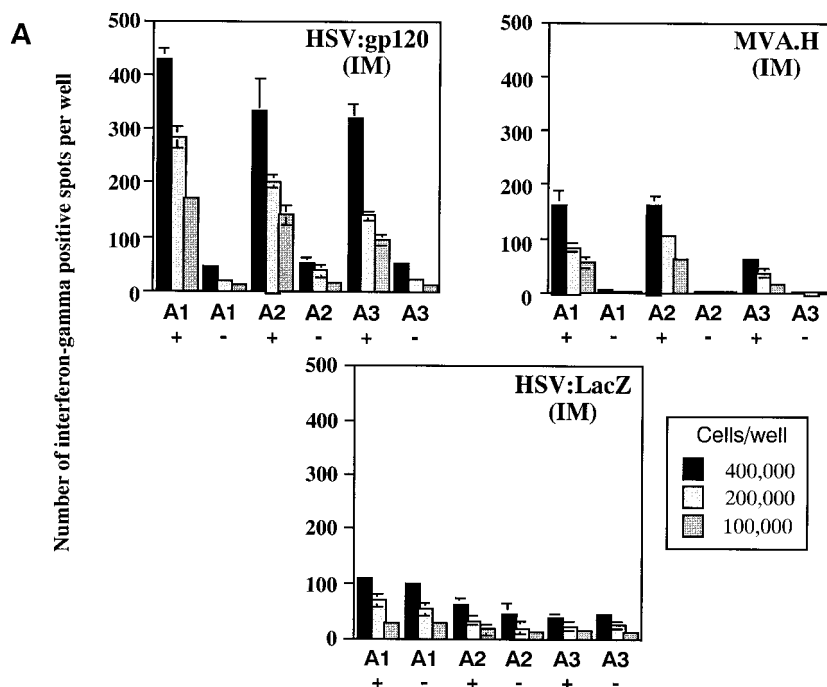


FIG. 3. A single inoculation of HSV:gp120 amplicon particles results in potent and cellular immune responses to gp120. (A) Analysis of gamma interferon release by ELISPOT assay. Mice were inoculated with either 10^6 infectious units of HSV:gp120 or HSV:lacZ amplicon particles or 10^6 PFU of MVA.H. Splenocytes were analyzed 22 days postinfection for gamma interferon release by ELISPOT assay. Results are shown for each individual mouse (A1, A2, and A3) in the presence (+) or absence (-) of the immunodominant V3 peptide from HIV-1 (RGPGRAFVTI). In all cases, three different input levels of splenocytes were analyzed in triplicate (400,000, 200,000, or 100,000 cells as indicated). The results shown represent the mean \pm the standard error of the mean for each datum point. This experiment is representative of two experiments that were performed, both of which yielded similar results. In all cases, relatively few gamma interferon-producing cells were detected in the absence of the specific HIV-1 peptide or in animals immunized with an irrelevant vector (HSV:LacZ). (B) Vaccine-elicited T cells possess potent cytolytic activity. Splenocytes were harvested from immunized mice at 22 days postinfection. The cells were then cultured for 5 days in the presence of irradiated syngeneic LPS blasts pulsed with the class I-restricted epitope. Cytolytic function was determined by incubating various amounts of syngeneic P815 target cells with effector cells, generating different effector-to-target (E:T) ratios. The results are shown for each individual mouse (A1, A2, and A3) in the presence (+; solid lines) or absence (-; shaded lines) of the immunodominant V3 peptide from HIV-1 (RGPGRAFVTI). The results represent the mean values from triplicate samples. This experiment is representative of two experiments that were performed, both of which yielded similar results. (C) Inoculation of mice with a single dose of HSV:gp120 amplicon particles results in a substantial Env-specific humoral response. Estimated endpoint titers are shown; this experiment is representative of two experiments that were performed, both of which yielded similar results.

ml of irradiated, V3-peptide-pulsed LPS blasts (10^6 cells) to determine the peptide-specific response or 0.1 ml of irradiated C57BL/6 cells (2×10^6 cells) to test for the allogeneic response. Cultures were incubated for 5 days at 37°C in 5% CO_2 .

The cytotoxic activity of gp120-specific CTLs was determined by JAM assay (29). Briefly, P815 and EL4 cells were seeded at 2×10^5 cells/ml on the day prior to the assay. On the day of the assay, the cells were centrifuged and resuspended in RPMI 1640 medium supplemented with 10% FCS. The cells were labeled with [^3H]thymidine (5 $\mu\text{Ci}/\text{ml}$; New England Nuclear, Boston, Mass.) for 3 h at 37°C in 5% CO_2 , washed with RPMI 1640 medium, and diluted to a concentration of 10^5 cells/ml in CR10- β . CTL effectors were washed extensively with RPMI 1640 medium and resuspended in a fixed volume of CR10- β . The CTLs were added to 96-well U-bottom plates (Falcon) in triplicate, to give responder/target ratios from 100:1 to 1.6:1. Ten thousand radiolabeled targets were then added either in the presence or in the absence of the V3 peptide. Plates were centrifuged briefly to pellet CTL effectors and targets and then incubated at 37°C in 5% CO_2 . After 3 h, plates were harvested onto glass filters (Perkin-Elmer Wallac, Gaithersburg, Md.) by using a 96-well automated harvester (Tomtec, Hamden, Conn.). After they were dried, the filters were subjected to scintillation counting analysis by using the Microbeta 1450 Trilux counter (Perkin-Elmer Wallac). Cytotoxicity was determined according to the following formula: percent cytotoxicity = $[(S - E) \div S] \times 100$, where E is the experimentally retained DNA in the presence of killers (in counts per minute) and S is the retained DNA in the absence of killers (spontaneous) as described previously (29).

In some experiments, CD8^+ -cell depletion was accomplished with the use of anti- CD8a Ly-2 magnetic beads (Miltenyi-Biotec), according to the manufactur-

er's instructions. To verify successful elimination of the CD8^+ cells, splenocytes were stained with fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD8 (Ly-2; BD PharMingen) and were examined by using a FACScan cytometer (Becton Dickinson).

Tetramer analysis. H-2D d streptavidin-allophycocyanin tetramers containing the V3 peptide were prepared by the NIH Tetramer Facility (Emory University, Atlanta, Ga.). Tetramer staining proceeded as follows: 1×10^6 to 2×10^6 freshly isolated splenocytes were washed once and resuspended in 50 μl of ice-cold fluorescence-activated cell sorting (FACS) buffer ($1 \times \text{PBS}$ [pH 7.2] containing 0.09% sodium azide and 2% fetal bovine serum). For staining, 50 μl of FACS buffer containing 0.1 μg of FITC-conjugated anti-mouse CD8 (Ly-2) and 0.5 μg of tetramer were added to the cells, mixed well, and kept on ice for 1 h in the dark. The cells were subsequently washed with FACS buffer twice, resuspended in 400 μl of FACS buffer, and analyzed on a FACSCalibur (BD PharMingen) by using CellQuest software. A total of 100,000 events were collected, and the histograms shown represent cells gated initially by forward scatter and side scatter.

Anti-gp160 ELISA. ELISA (enzyme-linked immunosorbent assay) plates were coated overnight at 4°C with recombinant soluble HIV-1 MN gp160 (Protein Sciences, Meriden, Conn.), diluted in coating buffer (0.1 M NaHCO_3 [pH 8.6]). Plates were washed with PBS-T buffer and then blocked for 2 h at 37°C by using PBS-T containing 3% bovine serum albumin (Sigma). Plates were washed again in PBS-T, and sera were diluted, added to the ELISA plates in duplicate, and incubated for 1 h at 37°C . Plates were washed as described above. Anti-mouse immunoglobulin G (IgG) Fc-specific secondary antibody (Sigma) was added to the plates, and they were incubated for 1 h at room temperature. After washing,

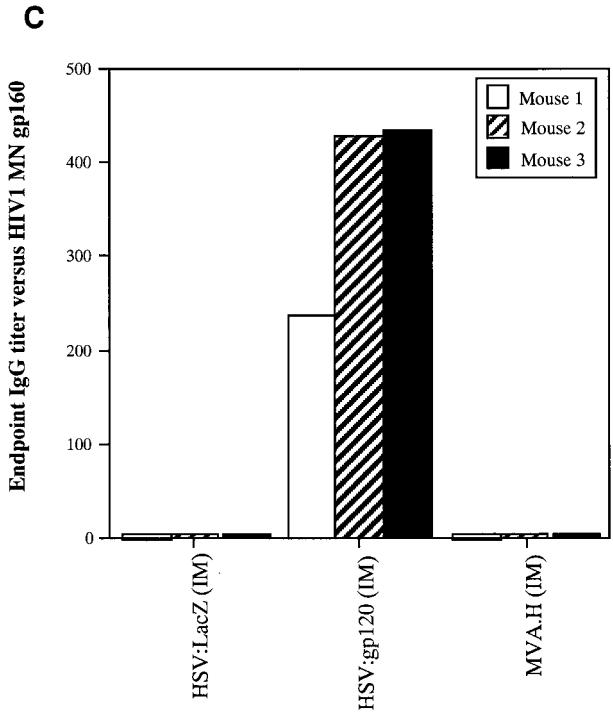
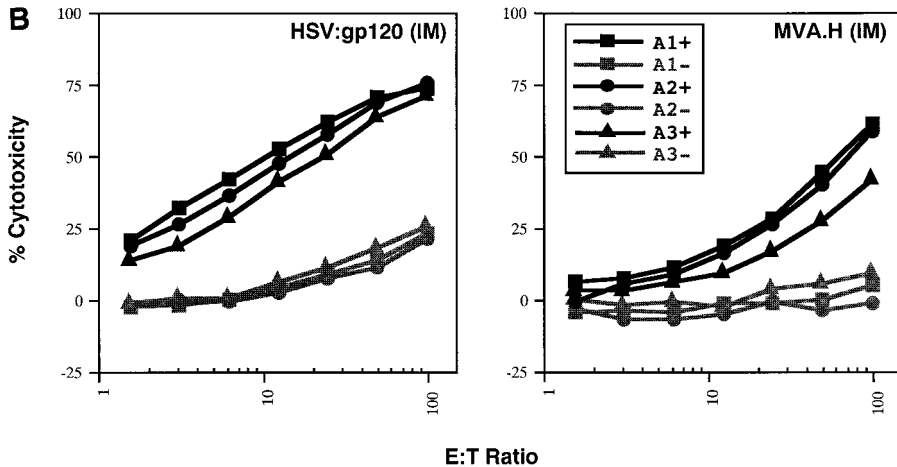


FIG. 3—Continued.

ABTS [2,2'azinobis(3-ethylbenzthiazolinesulfonic acid)] substrate (Sigma) was added to the plates and, after enough time for color development, the absorbance was measured at 405 nm. Endpoint titers were ascertained by using a scatter plot with optical density (OD) values on the y axis and dilution⁻¹ on the x axis, for which the x-axis scale was logarithmic. After the data were plotted, a logarithmic curve fit was applied to each individual dilution series, and the point where the curve fit intersects the positive-negative cutoff value was determined. This cutoff value was calculated as the mean OD of all preimmune sera plus four standard deviations. For the experiment involving the preinfection of mice with wild-type HSV-1, the cutoff value was calculated slightly differently due to the fact that the HSV-1-preinfected mice exhibited an elevation in their background level of serologic reactivity to HIV-1 gp120. The cutoff value was calculated as the mean OD plus four standard deviations of all sera collected from either preinfected or naive mice.

Anti-HSV ELISA. Sera were tested for anti-HSV IgG antibodies by ELISA as described previously (20). Briefly, ELISA plates were coated with UV-inactivated HSV overnight at 4°C. Plates were washed with PBST and blocked with PBS-0.1% bovine serum albumin for 1 h at 37°C. Plates were again washed with PBST. Sera were diluted and incubated in duplicate on the HSV-coated plates

overnight at 4°C. Plates were washed with PBST and treated with alkaline phosphatase-conjugated goat anti-IgG (Cappel, Worthington, Pa.) for 3 h at 37°C. Plates were washed with PBST and incubated with p-nitrophenyl phosphate substrate (Sigma), and the absorbance was read at 405 nm.

Virus neutralization assays. Sera from mice subjected to preinfection with HSV-1 (Patton) strain were analyzed for the presence of virus-neutralizing antibodies as follows. Briefly, sera obtained either prior to or 5 weeks after HSV-1 (Patton) infection were diluted 1:20 and 1:100 in PBS and then combined with an equal volume (1,000 infectious units [i.u.], 100 μl) of HSV:lacZ amplicon particles. After incubation at 37°C for 135 min, this preparation was then added to Vero cells in 24-well tissue culture plates for 60 min at 37°C. DMEM medium containing 5% FCS was then added to each well (1.0 ml), and cells were allowed to incubate at 37°C in 5% CO₂ for 48 h. At the end of the given time period, the cells were fixed with 1% glutaraldehyde, washed with 1× PBS, and analyzed for β-galactosidase activity by a histochemical staining method with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) as the substrate. For each serum and dilution tested, the percent reduction in LacZ-positive cells was calculated relative to cultures that were exposed to untreated HSV:lacZ amplicon particles.

RESULTS

Generation of helper virus-free amplicons. Figure 1 depicts the generation of the helper virus-free amplicons. Briefly, BHK cells were cotransfected with three plasmids: (i) the amplicon plasmid encoding the gp120 expression cassette, as well as critical cis-acting elements necessary for plasmid replication and packaging by the helper virus (i.e., the HSV-1 lytic-phase origin of DNA replication and the viral cleavage/packaging motif); (ii) the defective helper virus genome (an HSV BAC-mid lacking all viral cleavage and packaging motifs); and (iii) a plasmid encoding the HSV *vh*s gene, which allows for the generation of higher-titer amplicon stocks (9). This procedure produces helper-free, replication-defective HSV-1 particles that contain 15 to 20 copies of the amplicon plasmid, arranged in a concatemeric configuration; the particles are devoid of helper virus genomes (<1 helper virus genome per 10⁶ i.u. of amplicon particles; W. J. Bowers and H. J. Federoff, unpublished data) and are incapable of directing the synthesis of any HSV-1 gene products since they lack any coding sequence from HSV-1 (9, 12, 39, 53).

gp120 is expressed and secreted from cells infected with HSV:gp120 amplicon particles. To verify that the amplicon utilized in our immunization studies could lead to transcription and translation of gp120 in infected cells, we tested the purified amplicon particles in vitro. 293 cells were infected with either

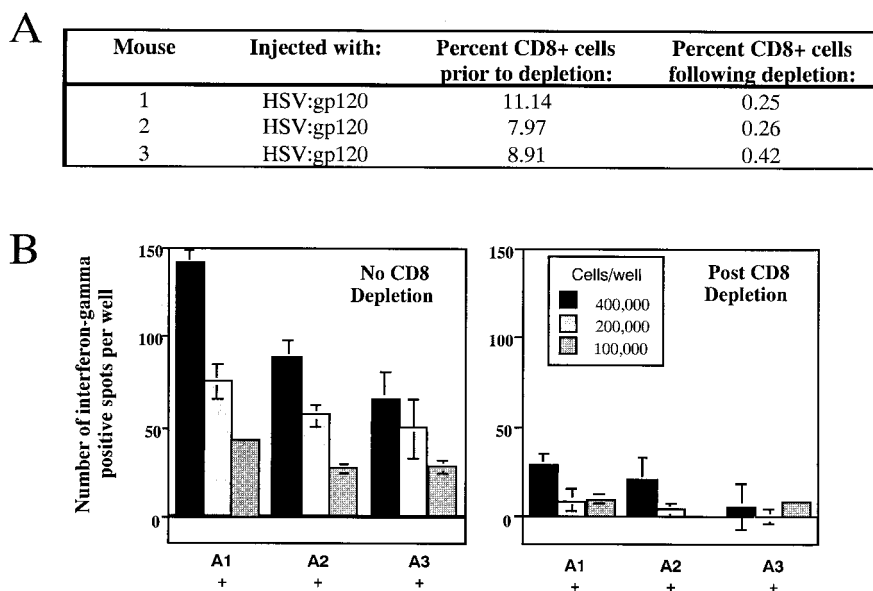


FIG. 4. Gamma interferon-producing cells detected in the ELISPOT assay correspond to CD8⁺ T lymphocytes. Splenocytes were collected from immunized animals at 22 days postinfection, and CD8⁺ T lymphocytes were depleted from this population by using anti-CD8a coupled to magnetic particles. Primary splenocytes were isolated and stained with FITC-conjugated anti-CD8 Ly-2 monoclonal antibody either prior to or after depletion with anti-CD8a magnetic beads. Cells were analyzed by using a FACScan cytometer. (A) Percentage of CD8⁺ cells in the splenocyte population (before and after immunodepletion) for each individual mouse. (B) Splenocytes were subsequently tested for gamma interferon release by ELISPOT assay. The results are shown for each individual mouse (A1, A2, and A3) in the presence of the immunodominant V3 peptide from HIV-1 (RGPGRFVTI). In all cases, three different input levels of splenocytes were analyzed in triplicate (400,000, 200,000 or 100,000 cells as indicated). The results shown represent the mean number of spots \pm the standard error of the mean for each datum point. Note that in this experiment, the mean number of spots was calculated as follows: mean number of spots in the presence of the V3 peptide minus mean number of spots detected in the absence of the V3 peptide. This subtraction of nonspecific background reactivity was performed to facilitate comparison between results from the total and CD8-depleted splenocyte populations.

HSV:gp120 amplicon particles or with HSV:MIAP (as a negative control) at an MOI of 0.5; cells were then incubated for 48 h. At the end of this time, culture supernatant proteins were concentrated, resolved on a polyacrylamide gel, and immunoblotted with an anti-gp120 monoclonal antibody (AD3). As shown in Fig. 2, a band of \sim 120 kDa was detected in culture supernatants from cells infected with the HSV:gp120 amplicon particles but not in supernatants from cells exposed to the HSV:MIAP amplicon particles.

Inoculation with HSV:gp120 amplicon particles results in strong Env-specific cellular and humoral immune responses. Groups of female BALB/c mice were injected i.m. with 10^6 i.u. of either HSV:gp120 or HSV:lacZ amplicon particles or 10^6 PFU of MVA.H; three mice were used per experimental group. At 22 days after injection, mice were sacrificed and bled, and their splenocytes were tested for gamma interferon release by ELISPOT and for cytotoxic function by JAM assay. Figure 3A shows that the frequency of splenocytes secreting gamma interferon in response to V3 peptide-specific stimulation was high in mice immunized with HSV:gp120 (ca. 1,000 spots per 10^6 splenocytes). In contrast, the frequency of these peptide-specific, gamma interferon-producing cells was considerably less in splenocytes harvested from mice that had been immunized with the MVA.H vector, or with HSV:lacZ (as an irrelevant control). It should be noted that the MVA.H vector expresses the same immunodominant HIV-1 V3 loop peptide (RGPGRFVTI) that is contained within the HSV:gp120 construct (14); it was therefore included in these initial experiments as a positive control.

The cytotoxic function of murine splenocytes corresponded well with the ELISPOT data. In particular, splenocytes from the animals inoculated with HSV:gp120 amplicon particles exhibited potent cytotoxic activity, whereas splenocytes from MVA.H-treated mice showed a much lower level of cytotoxicity (Fig. 3B). A strong humoral response was observed only in mice inoculated with the HSV:gp120 amplicon particles, as measured by ELISA (Fig. 3C). The absence of a detectable Env-specific antibody response at a 1:10 dilution in mice immunized with the MVA.H vector was expected, since this vector encodes only a string of CTL epitopes (including the V3 loop peptide) rather than the authentic gp120 protein.

Cells secreting gamma interferon are predominantly CD8⁺ cells. To verify that the gamma interferon-secreting splenocytes were actually CD8⁺ cells and not another cell type capable of secreting this cytokine, we analyzed splenocytes from animals inoculated 22 days earlier with the HSV:gp120 amplicon particles both prior to and following treatment with an anti-CD8a monoclonal antibody coupled to magnetic beads. Both treated and untreated cells were subsequently stained with an anti-CD8 FITC-conjugated antibody and analyzed by flow cytometry. Figure 4A indicates that the overwhelming majority of CD8⁺ cells were eliminated by this treatment. When a gamma interferon ELISPOT assay was performed with these cells, a significant decrease in the frequency of gamma interferon-producing cells was observed in splenocyte cultures that had been depleted of CD8⁺ cells (Fig. 4B). This finding indicates that the splenocytes which secrete gamma

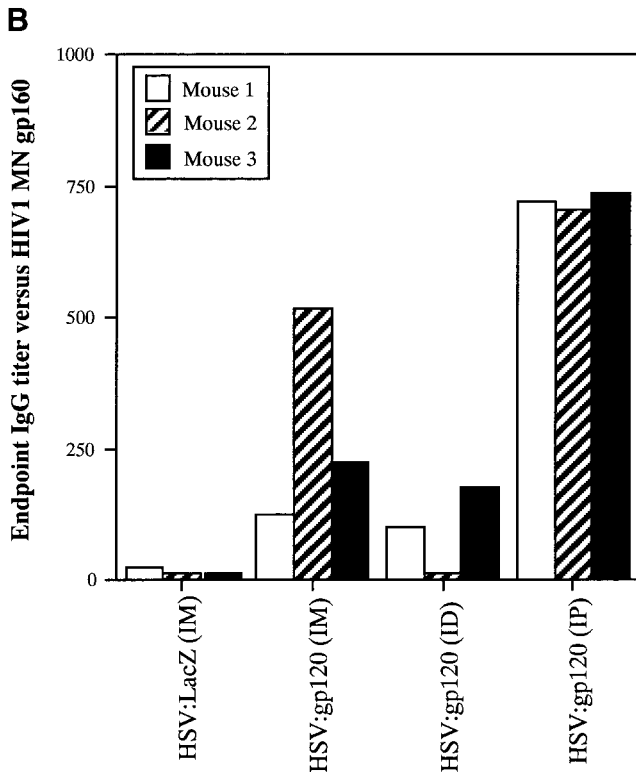
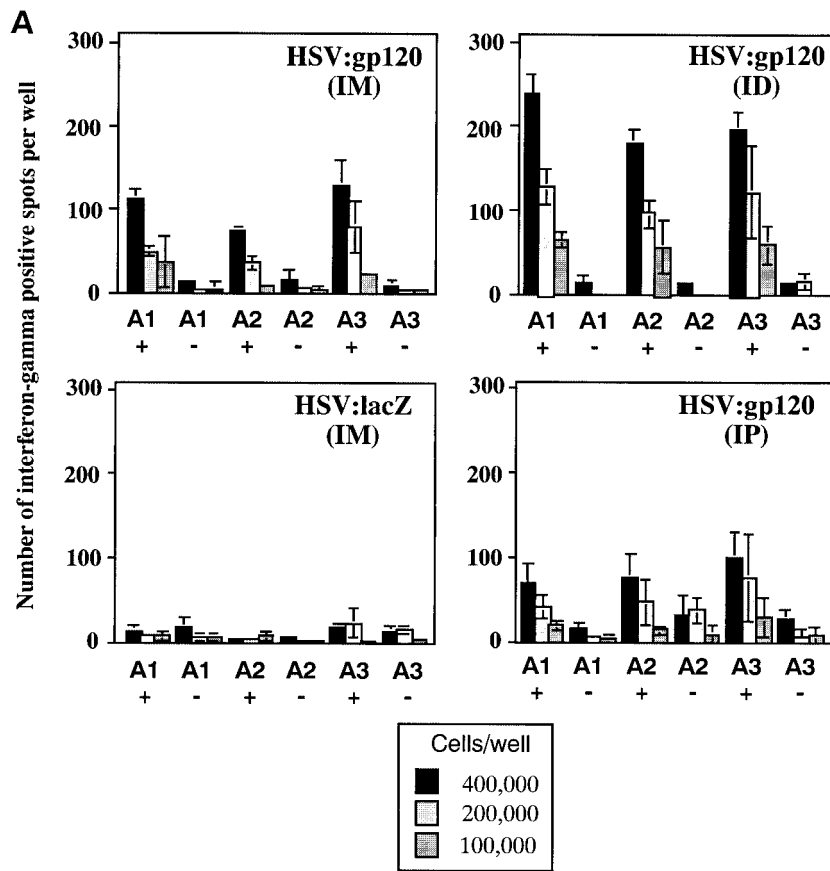


FIG. 5. Effect of the route of delivery on immune responses to HSV:gp120 amplicon particles. (A) i.d. inoculation results in higher frequency of gamma interferon-secreting cells. A single inoculation of 10^6 i.u. was introduced in mice via i.d., i.m., or i.p. routes. At 21 days postinoculation, mice were sacrificed, and splenocytes were tested for gamma interferon release by ELISPOT assay. The results are shown for each individual mouse (A1, A2, and A3) in the presence (+) or absence (-) of the V3 peptide. As noted in the legend to Fig. 3A, three different input dilutions of splenocytes were used, and results are presented as the mean of triplicate datum points \pm the standard error of the mean. The i.d. and i.m. routes of amplicon delivery resulted in higher frequencies of gamma interferon-secreting cells compared to the i.p. route, with the i.d. route resulting in the highest frequency. (B) i.p. inoculation yields the highest level of anti-gp160 antibodies. Of the three routes of inoculation tested, the i.p. route resulted in a stronger anti-gp160 humoral response compared to either the i.m. or i.d. routes.

interferon in response to V3-peptide stimulation in this assay are indeed CD8⁺ cells.

Immune responses to HSV:gp120 amplicon particles are influenced by the route of vector delivery. Since various injection sites can lead to differences in immune responses, we inoculated HSV:gp120 amplicon particles (10^6 i.u.) into groups of 3 mice, by three routes: i.d. at the base of the tail, i.m. in the hamstring muscle, and i.p. One million i.u. of HSV: lacZ was inoculated i.m. in a separate group of three mice as a control. Gamma interferon ELISPOTs and anti-gp160 endpoint serology (IgG ELISA assay) were performed for each mouse 21

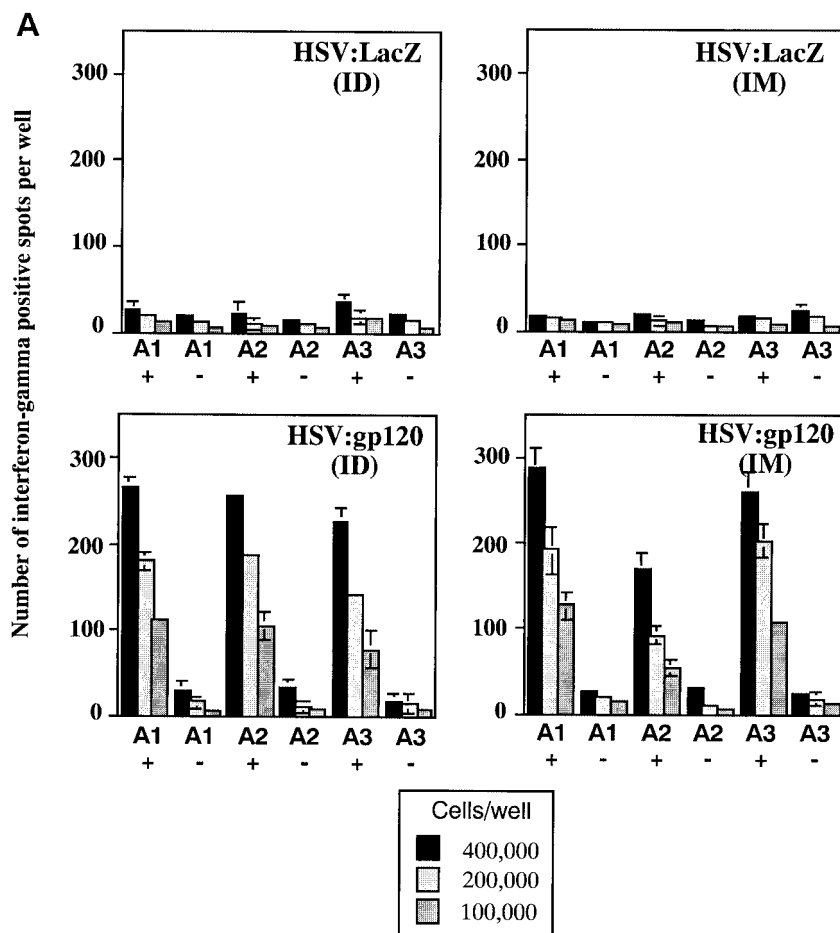


FIG. 6. (A) A single inoculation of HSV:gp120 amplicon particles results in durable cellular immunity, as measured by gamma interferon ELISPOT assay. Mice were inoculated with a single dose of 10^6 i.u. of either HSV:gp120 or HSV:lacZ amplicon particles via either the i.d. or the i.m. routes. Mice were sacrificed, and splenocytes were tested by gamma interferon ELISPOT assay 171 days later. The results are shown for each individual mouse (A1, A2, and A3) in the presence (+) or absence (-) of the V3 peptide. As noted in the legend to Fig. 3A, three different input dilutions of splenocytes were used, and results are presented as the mean of triplicate datum points \pm the standard error of the mean. (B) A single inoculation of HSV:gp120 amplicon particles results in durable cellular immunity, as measured by cytotoxic (JAM) assay. Splenocytes were harvested as described above, and cells were cultured in the presence of irradiated syngeneic LPS blasts pulsed with the class I-restricted epitope as indicated. The results are shown across a range of effector-to-target (E:T) cell ratios for each individual mouse (A1, A2, and A3) in the presence (+; dark lines) or absence (-; light lines) of the HIV-1 V3 peptide. (C) A single inoculation of HSV:gp120 amplicon particles elicits long-lasting Env-specific humoral immunity. Mice were injected with 10^6 i.u. of HSV:gp120 or HSV:lacZ amplicon particles. At 171 days after inoculation, mice were bled, and endpoint anti-gp160 titers were determined by ELISA.

days after the vector administration. Figure 5A shows that administration of the HSV:gp120 amplicon particles via the i.d. and i.m. routes resulted in a higher frequency of gamma interferon-producing cells compared to the i.p. route. The data also showed that the i.d. route of immunization resulted in a stronger cellular immune response than the i.m. route, at least when analyzed at this time point (21 days after immunization).

Analysis of serologic responses to HIV-1 Env in immunized mice revealed a strikingly different hierarchy among the various routes of administration. In this case, the i.p. route resulted in the highest level of Env-specific IgG production (Fig. 5B).

A single inoculation of HSV:gp120 amplicon particles results in durable cellular and humoral immunity. Since herpesvirus vectors may have the potential to elicit long-lived immune responses to encoded antigens, we examined the durability of immune responses elicited by a single inoculation of HSV:gp120 amplicon particles. To accomplish this, groups of

three mice were inoculated with a single 10^6 i.u. dose of either HSV:gp120 or HSV:lacZ via either the i.d. or the i.m. route, since these routes gave the strongest levels of cellular immune responses. Approximately 5 months (171 days) after this single inoculation, mice were sacrificed, and their splenocytes were assayed for cytokine production (gamma interferon ELISPOT) and cytolytic function (JAM assay); the level of Env-specific IgG in sera from these mice was also quantitated by using anti-gp160 endpoint serology. This analysis revealed that a single administration of the replication-defective HSV:gp120 amplicon vector resulted in the production of a high level of peptide-specific gamma interferon secreting cells that persisted for >5 months (Fig. 6A). The potent nature of this durable T-cell response was confirmed by analysis of functional cytotoxicity by the JAM assay (Fig. 6B). In addition, endpoint antibody titers calculated from ELISA analysis of sera collected at the 171 day time point also showed the persistence of

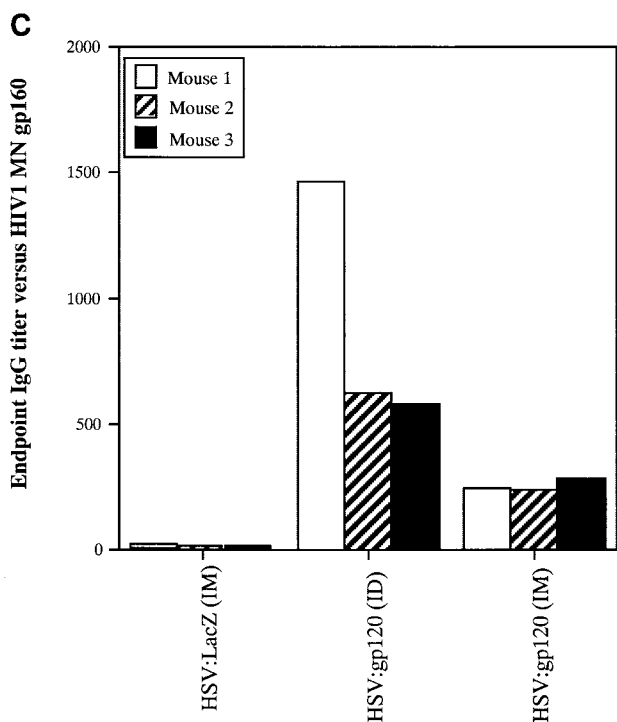
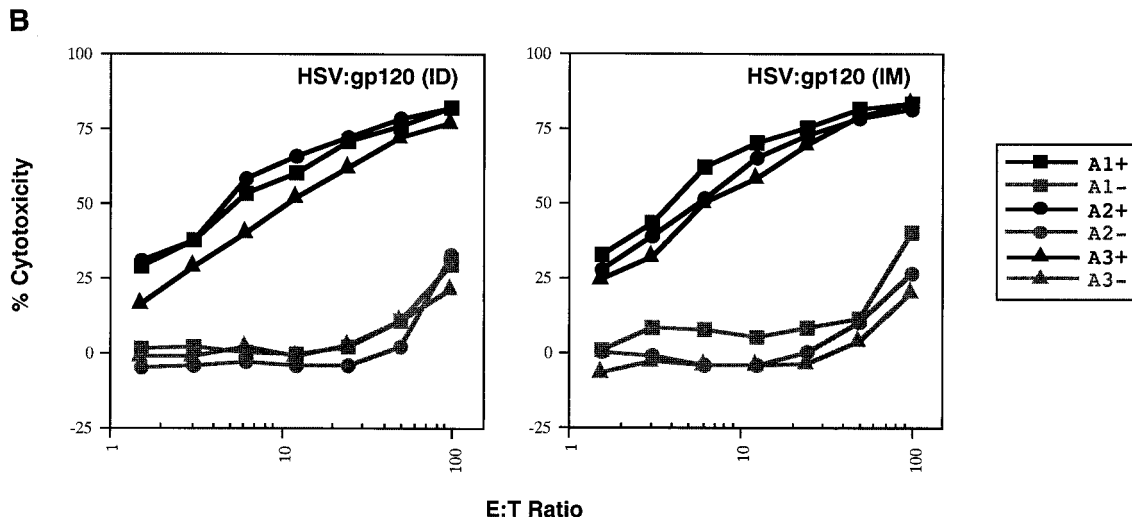


FIG. 6—Continued.

a strong, Env-specific humoral response (Fig. 6C). The i.d. route of amplicon delivery appeared to elicit higher antibody titers than the i.m. route, although both routes performed similarly in terms of their ability to elicit a cellular immune response to gp120. Overall, it is notable that the long-lived cellular and humoral response to gp120 was induced by a vector that is not capable of replication. It remains unclear whether or not there may be prolonged expression of gp120 in vivo.

Cellular and humoral immune responses are generated in a dose-dependent fashion. To determine the minimal dose of the HSV:gp120 amplicon that would result in a measurable immune response, we performed a dose-response experiment.

Groups of three mice were immunized via the i.d. route with the HSV:gp120 amplicon particles in decreasing dosage levels, which declined in half-log increments from 10^6 to 10^4 i.u.; animals were sacrificed and analyzed 21 days later. One group of three mice was inoculated with 10^6 i.u. of HSV:MIAP amplicon particles as a control. Quantitation of the response was determined by gamma interferon ELISPOT and major histocompatibility complex type I tetramer analyses.

Figure 7A reveals that the frequency of peptide-specific gamma interferon-secreting cells decreases in a continuous fashion as a function of the dose of HSV:gp120 amplicon particles that was administered. Similarly, tetramer staining analysis revealed an association between the dose of vector that was administered and the frequency of H-2D^d peptide-specific CD8⁺ T lymphocytes (Fig. 7B), as did analysis of cytotoxic activity by splenocytes from the immunized animals (Fig. 7C). In contrast, the humoral response to HSV:gp120 exhibited a more discontinuous relationship with the dose of amplicon particles that was administered. Strong Env-specific antibody responses were detected at the highest dose tested (10^6 i.u.), but this response became undetectable at doses of 10^5 i.u. or below (Fig. 7D).

Prior infection with HSV-1 results in a modest reduction in Env-specific immune responses after administration of HSV:gp120 amplicon particles. Due to the high level of seroprevalence to HSV-1 in the United States and the possible effect that HSV-specific antibodies might exert on amplicon-elicited immune responses, we conducted an experiment to determine whether prior infection with HSV-1 might reduce immune responses to the vectored HIV-1 gp120 in mice. To accomplish this, mice were either injected with a sublethal dose of the wild-type HSV-1 Patton strain (4×10^7 PFU, administered by the i.p. route) (20) or left untreated (naive). Approximately 5 weeks later, sera were collected from the mice and analyzed for the presence of anti-HSV antibodies by ELISA. Figure 8A indicates that all mice responded as predicted; that is, naive mice did not generate a measurable humoral immune response to HSV, while the animals that had been infected with HSV-1 Patton produced an obvious antibody response. This humoral

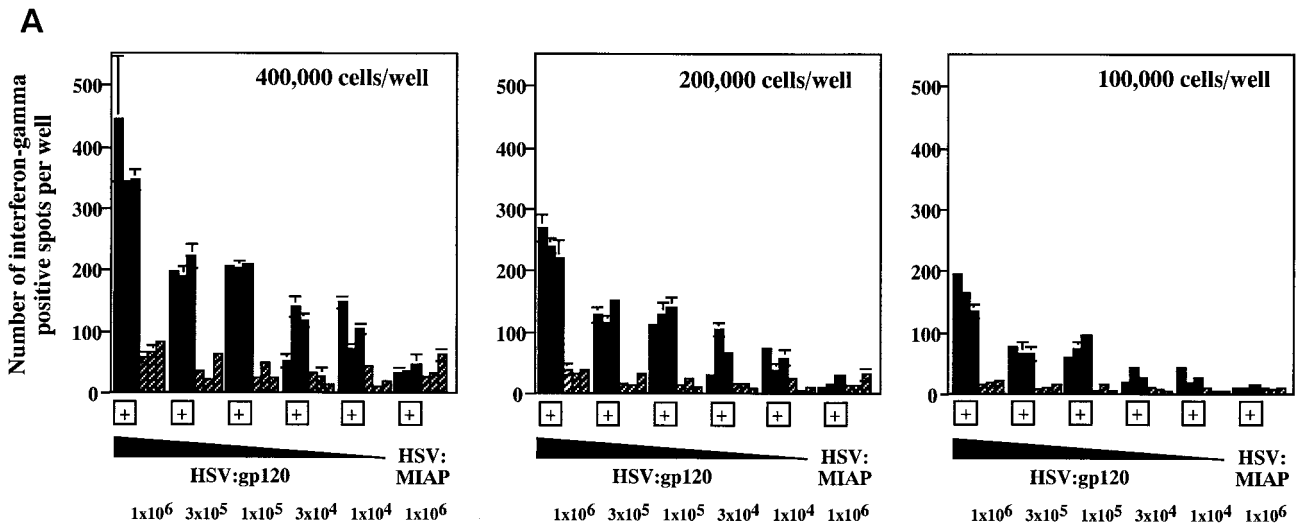
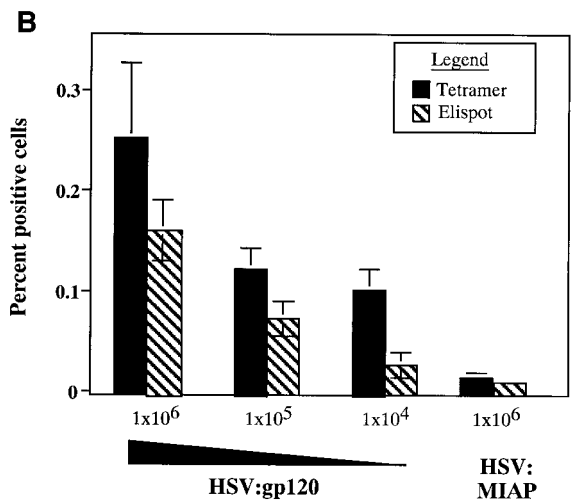


FIG. 7. The immune response to HSV:gp120 amplicon particles is dose dependent, but strong responses are detected even at very low amplicon doses. (A) Dose dependence of the cellular response to HSV:gp120 amplicon particles, as determined by the gamma interferon ELISPOT assay. Decreasing 0.5-log increments of HSV:gp120 amplicon particles, ranging from 10^6 to 10^4 i.u. (as indicated), were inoculated into BALB/c mice via the i.d. route; as a negative control, an additional group of animals received an irrelevant amplicon construct (HSV:MIAP) at the high-dose level (10^6 i.u.) via the i.d. route. At 21 days postinfection, mice were sacrificed and splenocytes were tested for gamma interferon release by ELISPOT assay. Results are shown for each individual mouse (each bar reflects one mouse), in the presence (+; solid bars) or absence (hatched bars) of the HIV-1 V3 peptide. Three different input levels of splenocytes were analyzed in triplicate (400,000, 200,000, or 100,000 cells as denoted in the three panels). The results shown represent the mean \pm the standard error of the mean for each datum point; the experiment shown is representative of two experiments that yielded similar results. (B) Comparison of data from the gamma interferon ELISPOT assay and the tetramer assay. Freshly isolated splenocytes from immunized mice (see above) were also evaluated by staining with an *H-2D^d* tetramer folded with the V3 peptide (RGPGRAFVTI; see Materials and Methods). The results of tetramer staining (solid bars) and gamma interferon ELISPOT analysis (hatched bars) are expressed as percentages of positive splenocytes; the data show represent mean values from the three mice that were analyzed in each group \pm the standard error of the mean. (C) Dose dependence of the cellular response to HSV:gp120 amplicon particles, as determined by functional cytolytic (JAM) assay. Splenocytes were isolated from immunized animals, as described in panel A, and then analyzed for cytolytic activity using the JAM assay (see Materials and Methods) after a 5-day period of in vitro expansion. The results are shown across a range of effector-to-target (E:T) cell ratios for each individual mouse (A1, A2, and A3) in the presence (+; solid lines) or absence (-; shaded lines) of the HIV-1 V3 peptide. (D) Dose dependence of the humoral response to HSV:gp120 amplicon particles. Endpoint antibody titers were calculated by the gp160 IgG ELISA assay.

response included a strong component of neutralizing antibodies (data not shown). Briefly, sera were tested at dilutions of 1:20 and 1:100 for their ability to neutralize the infectivity of HSV:lacZ amplicon particles. This was achieved by determining the number of LacZ-positive cells generated after exposure of Vero cells to HSV:lacZ amplicon particles, either in the absence or in the presence of the various sera. Results from this experiment revealed that the sera from animals that were preinfected with wild-type HSV-1 were able to neutralize 99%



of the infectivity of HSV:lacZ amplicon particles, even at a 1:100 dilution; control (preimmune) sera had no effect on amplicon infectivity (data not shown). These data confirm that preinfection with wild-type HSV-1 (Patton) generated a virus-neutralizing serologic response, with activity against a heterologous viral strain (strain 17 [the strain used to generate the amplicon particles]).

After this 5-week time point, the mice were inoculated with a single dose of 10^6 i.u. of either HSV:gp120 or HSV:lacZ amplicon particles by the i.d. route. Three mice were tested for each condition. At 21 days after the amplicon administration, mice were sacrificed, and their splenocytes assayed for gamma interferon release and cytolytic function. Sera were tested for anti-gp160 endpoint serology as performed in previous experiments. The data presented in Fig. 8B and C show that HSV-naive mice generated a somewhat stronger cellular immune response to HIV-1 gp120, as measured by the gamma interferon ELISPOT assay (Fig. 8B) or by the cytotoxicity assay (Fig. 8C). The overall extent of the decrease in cellular immune responses to the vectored HIV-1 antigen varied but was found to be ca. 40 to 60% in both the ELISPOT and the JAM assays in mice previously infected with HSV-1. In contrast, the

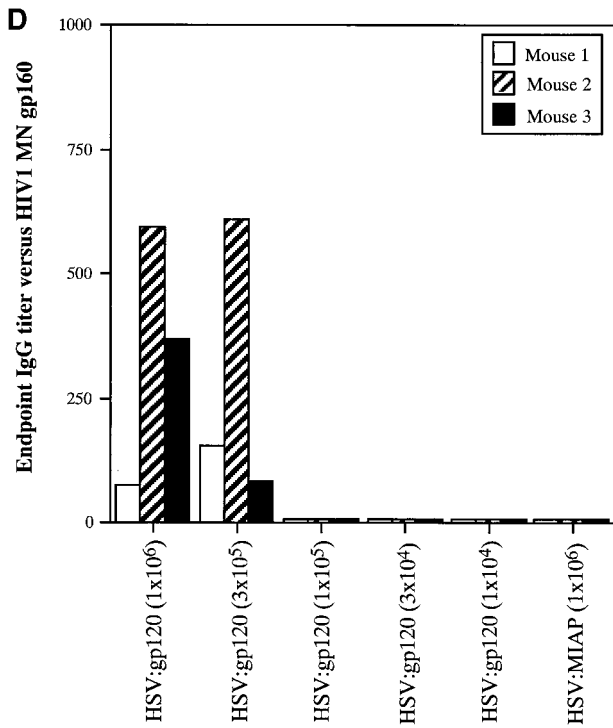
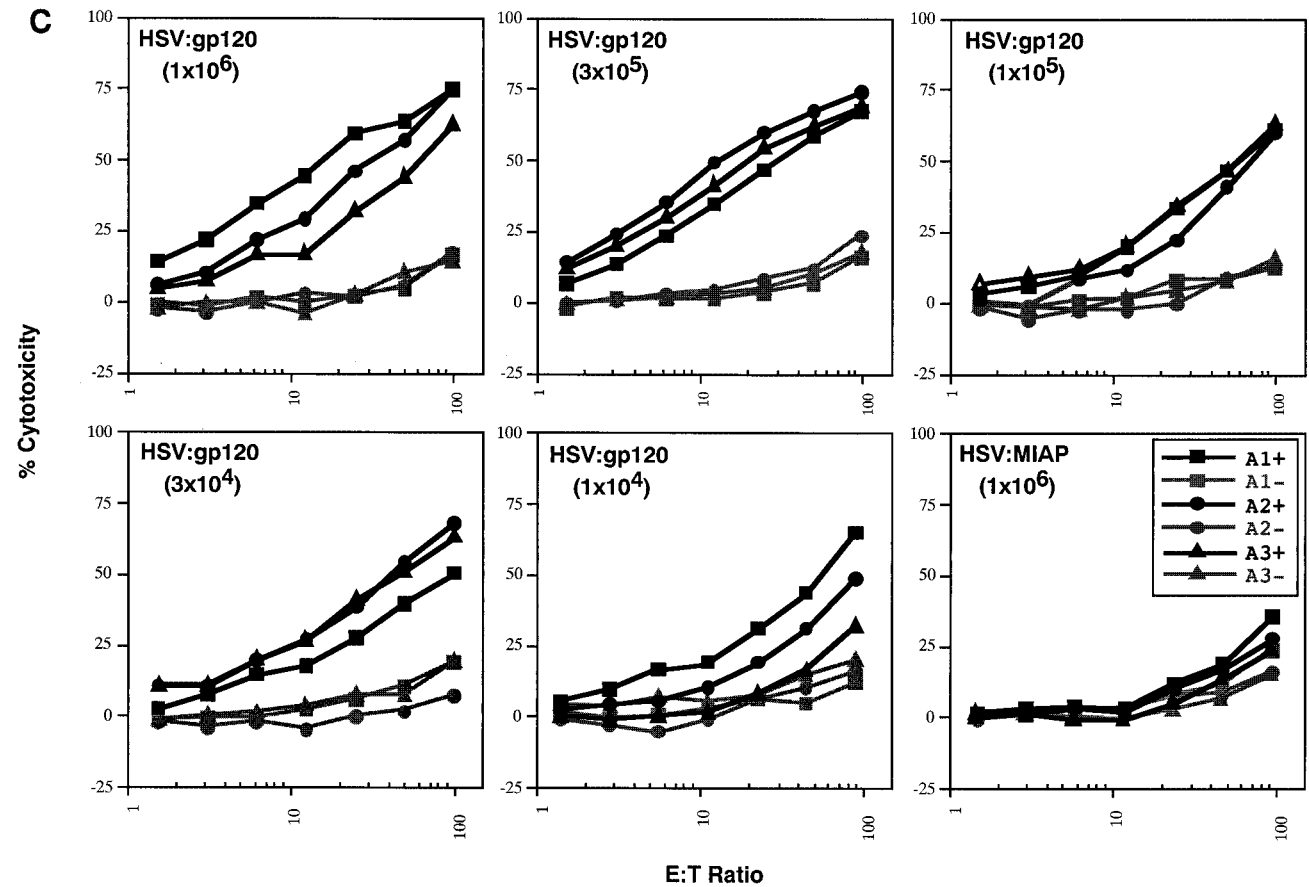


FIG. 7—Continued.

Env-specific antibody response was greatly reduced in preinfected animals compared to naive mice (Fig. 8D). Thus, prior infection with HSV-1 appears to exert a substantial inhibitory effect on Env-specific antibody responses to HSV:gp120 amplicon particles, whereas it exerts only a modest effect on cellular responses to the same antigen.

DISCUSSION

In the search for an effective vaccine for HIV (AIDS), many diverse approaches are being considered. Included among these are virus-derived platforms employing a wide variety of vector systems. Due to its favorable safety profile, convenience, broad tropism, and unique properties, we chose to examine the helper virus-free HSV-1 amplicon vector system with respect to its potential utility for HIV-1 vaccine development. Engineering this vector to allow for robust, Rev-independent expression of gp120 by using a codon-optimized construct ensured that sufficient protein would be present in the targeted cells in vivo.

Our initial studies focused on analyzing immune responses to a single i.m. inoculation of HSV:gp120 amplicon particles, whereas followup experiments examined other routes of inoculation. These experiments revealed that the i.d. route of vector administration resulted in a somewhat higher level of cellular immunity, at least at the 21-day time point. This is not surprising, since it has been reported that Langerhans cells,

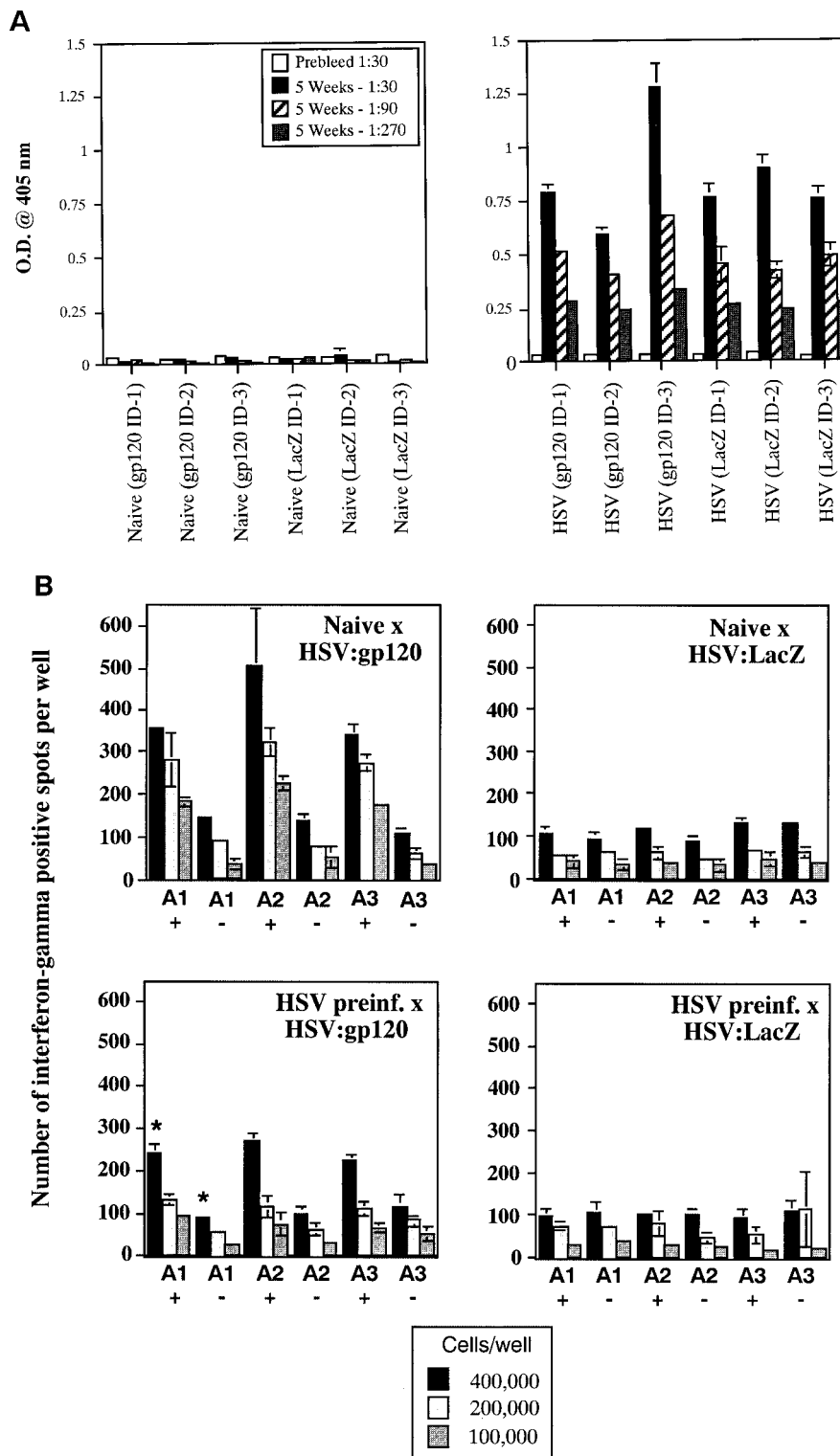


FIG. 8. (A) Inoculation of mice with wild-type HSV-1 Patton elicits a strong humoral response against HSV-1. Mice were infected with 4×10^7 PFU of HSV-1 Patton via the i.p. route, and 5 weeks later, sera were collected and analyzed by anti-HSV-1 IgG ELISA; as a control, HSV-1-naive mice were also analyzed. Results are expressed in terms of OD readings at 405 nm for each individual mouse; the results from naive animals are shown in the left panel, while the results from the Patton strain-infected animals are shown in the right panel. All animals are identified with respect to the experimental group to which they were assigned (for example, mice which subsequently received HSV:gp120 amplicon particles via the i.d. route are denoted as “gp120 ID,” whereas mice which subsequently received HSV:lacZ amplicon particles are denoted as “LacZ ID”). (B) Mice preinfected with wild-type HSV still mount a strong Env-specific cellular response after inoculation with HSV:gp120 amplicon particles, as determined by the ELISPOT assay. Mice were either left untreated (naive) or preinfected with wild-type HSV-1, as outlined above. All mice were then inoculated with either HSV:gp120 or HSV:lacZ amplicon particles (10^6 i.u. via the i.d. route). Splenocytes were harvested at 21 days after inoculation with amplicon particles and were analyzed for gamma interferon release by ELISPOT assay. The results are shown for each individual mouse (each bar reflects one mouse), in the presence (+ peptide) or absence of the HIV-1 V3 peptide. Three different input levels of

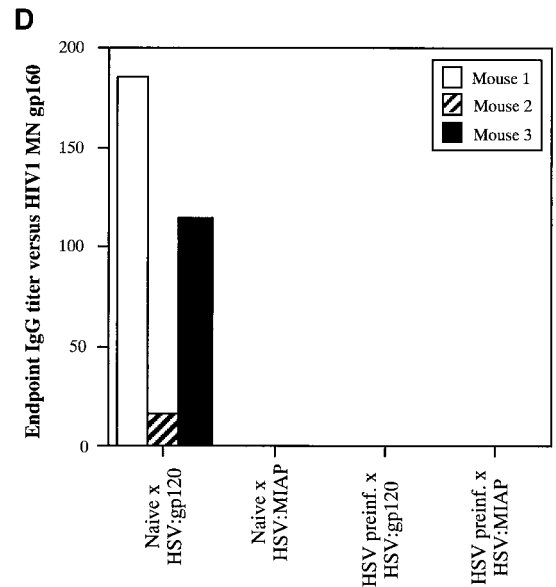
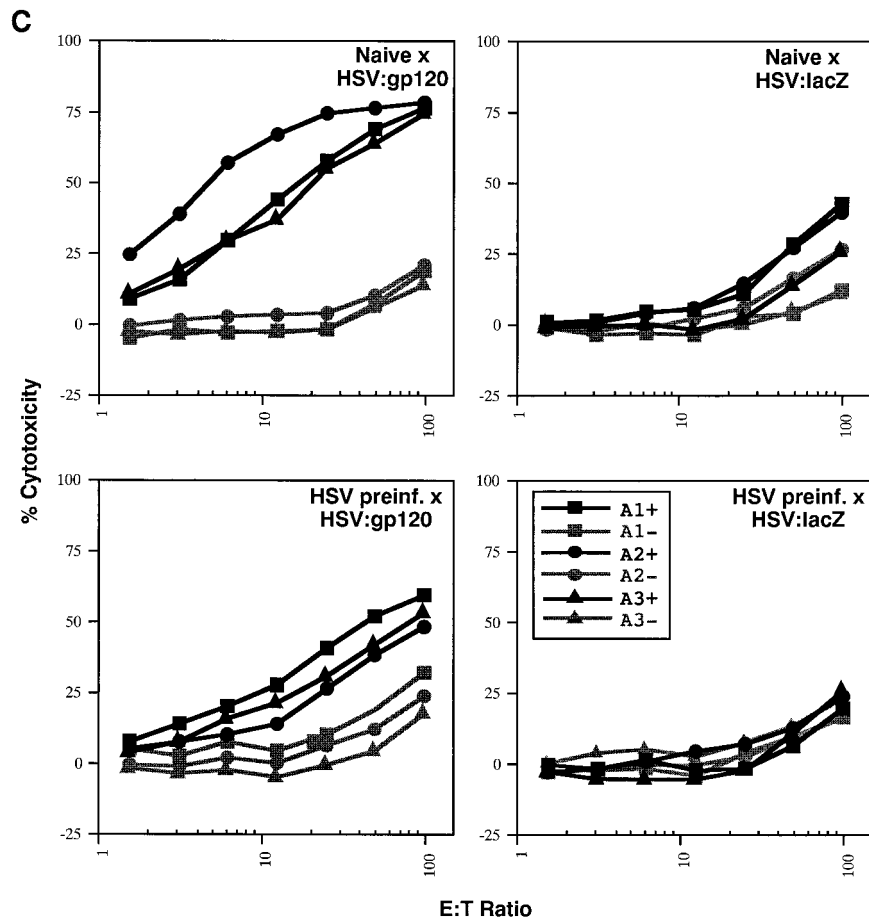


FIG. 8—Continued.

splenocytes were analyzed in triplicate (400,000, 200,000, or 100,000 cells), and the results shown represent the mean \pm the standard error of the mean for each datum point. Data denoted with an asterisk correspond to mean of only two values, with the corresponding standard deviation; one outlier datum point was discarded. The four panels represent data from naive mice treated with HSV:gp120 or HSV:lacZ (top panels, left and right, respectively) and from HSV-preinfected mice treated with HSV:gp120 or HSV:lacZ (lower panels, left and right, respectively). (C) Mice preinfected with wild-type HSV still mount a strong Env-specific cellular response after inoculation with HSV:gp120 amplicon particles, as determined by functional cytolytic (JAM) assay. Mice were either left untreated (naive) or preinfected with wild-type HSV-1; they were then inoculated with either HSV:gp120 or HSV:lacZ amplicon particles, as outlined above. Splenocytes were harvested at 21 days after immunization with amplicon and were analyzed for functional cytotoxicity by the JAM assay after a 5-day period of in vitro restimulation. The results are shown across a range of effector-to-target (E:T) cell ratios for each individual mouse (A1, A2, and A3) in the presence (+; dark lines) or absence (-; light lines) of the HIV-1 V3 peptide. The four panels represent data from naive mice treated with HSV:gp120 or HSV:lacZ (top panels, left and right, respectively) and from HSV-preinfected mice treated with HSV:gp120 or HSV:lacZ (lower panels, left and right, respectively). (D) Mice preinfected with wild-type HSV mount a weak or undetectable Env-specific humoral response after immunization with HSV:gp120 amplicon particles. Endpoint antibody titers were calculated by a gp160 IgG ELISA. Note that the data shown in this panel are representative of three independent experiments that yielded similar results; this particular data set derives from a different experiment than the datasets shown in panels A to C, as reflected by the use of a different negative control amplicon (HSV:MIAP).

found widely in the skin, may play an important role in HSV-1 infection (47–50); such cells might also become infected by the amplicon particles. More intriguing to us was the very strong nature of the durable immune response to gp120. Responses at the 171-day time point, which corresponds to approximately one-quarter of the mouse's normal life span, were in fact stronger than those measured at the 21-day time point. It is note-

worthy that this strong and durable response was elicited by a nonreplicating vector system that quite likely does not allow for sustained expression of gp120 over an extended period. Broadly similar findings have been reported by Morrison and Knipe in their immunization studies with replication-defective mutants of HSV-1 (31).

These two vector systems (HSV-1 amplicons and replication-defective HSV-1 mutants) may elicit long-lasting immunity in different ways. Morrison and Knipe have shown that replication-defective mutants remain infectious *in vivo* for several days, and these authors proposed that infection of target cells may proceed over an extended time period, resulting in immunologic boosting. In the present study, we did not examine whether amplicon particles may remain in an infectious form for an extended period at local sites of inoculation. Rather, our preferred hypothesis is that the durable immune response may be the result of a brief exposure to antigen, followed by the production of specific cytokines which can enhance expansion and survival of memory T cells (51). Further experimentation will be necessary to test this hypothesis, but in any event, the fact that the response to the HSV:gp120 amplicon was both potent and durable is certainly encouraging.

Important considerations with any vaccine delivery approach are affordability and scalability. We therefore examined the relationship between amplicon dose and antigen-specific immunity elicited in response to administration of amplicon particles. These studies revealed that the Env-specific cellular immune response varied in a continuous fashion with amplicon dose over a range of from 10^6 to 10^4 i.u. of amplicon particles. Furthermore, a strong cellular response was detected by both the tetramer staining and ELISPOT assays after a single administration of as few as 10^4 i.u. of amplicon particles. In contrast, the relationship between the Env-specific antibody response and the HSV:gp120 amplicon dose was found to be more complex. Strong humoral responses were detected at high amplicon doses (10^6 i.u.), but doses of $\leq 10^5$ i.u. failed to elicit detectable Env-specific antibody responses. This might perhaps be related to the sensitivity of our gp160 ELISA assay, but it more likely reflects an inherent Th1 bias in the immune response elicited by the amplicon particles. Further studies will be required to determine whether this is indeed the case. If this prediction is confirmed by subsequent work, it may suggest that the amplicon vector system could be particularly useful when deployed in prime-boost vaccine approaches in combination with (for example) protein immunogens capable of eliciting strong humoral responses. Studies to investigate the utility of prime-boost strategies that incorporate the amplicon vector system are under way, as are experiments designed to determine whether the immunogenicity of the amplicon vector system extends from mice to (nonhuman) primates.

The fact that even a low dose inoculum of amplicon particles (10^4 i.u.) can elicit strong cellular immune responses is certainly encouraging with respect to possible future use of these vectors in humans. Nonetheless, the technology employed here relies upon transfection and therefore presents some potential concerns with respect to scalability. Two of our coauthors (W. J. Bowers and H. J. Federoff) were able to generate amplicon stocks with titers as high as 1×10^9 to 10×10^9 i.u. by using this technology, but further scale-up will require additional improvements. In fact, such improvements are within reach. An

instructive analogy can be found in studies that relate to the production of high-titer stocks of helper-dependent (HD) adenovirus vectors. Initial efforts to generate such stocks, in the absence of significant contamination with helper virus, were rather unsatisfactory and poorly scalable. However, scalable methods for the production of such HD vector stocks have since been developed through the use of helper viruses which contain recombination sequences (loxP, *frt*) flanking their packaging sites (34, 36, 41, 55). Passage of HD vector stocks in cells which express the corresponding recombinases (Cre, FLP) results in almost complete elimination of helper virus contamination and has permitted the development of very high-titer, clinical-grade HD adenovirus vector preparations. This same technology can readily be applied to the generation of HSV-1 amplicon vectors and is being developed by Logvinoff and Epstein (26) and others.

When considering a virally based vaccine delivery platform, the presence of preexisting immunity to the vector can pose a problem due to elimination of the vector by such factors as circulating antibodies. Since the rate of seroprevalence to HSV-1 in the United States is as high as 70% or more (28), the use of an HSV-1-derived vector for the purpose of vaccine delivery could pose a problem. However, HSV-1 vectors have been shown to successfully mediate efficient gene transfer, even in HSV-1-seropositive animals (11). Furthermore, reinfections with HSV-1 have previously been documented in normal, healthy individuals (22), and preexisting anti-HSV-1 immunity has been found to reduce but not abolish gene transfer by an HSV-1 vector (levels of gene expression were 15 to 20% of normal in immune mice compared to control animals [16]). These observations suggest that preexisting anti-HSV-1 immunity may not preclude the success of the vaccination (gene delivery) method described here (11). To address this question head-on, we examined the effect of prior infection with wild-type HSV-1 on the immune response elicited by HSV:gp120.

In this experiment, the wild-type HSV-1 strain used to generate an anti-HSV-1 immune response in the mice was a heterologous strain (Patton) compared to the strain used to produce the amplicon particles (strain 17). We believe that the choice of a heterologous preinfecting strain is justified because humans who harbor HSV-1 infection or anti-HSV-1 antibodies will not have been infected with strain 17; rather, they will likewise have been infected by any one of a multitude of heterologous strains circulating in the human population.

We also examined the issue of serologic cross-reactivity between strains Patton and 17 (amplicon) of HSV-1. To accomplish this, HSV:lacZ amplicon particles were coated onto an ELISA plate, and sera from both a naive and an HSV-1 (Patton)-infected mouse were assayed to determine whether antibodies elicited by HSV-1 Patton could interact with the strain 17-derived amplicon particles. Cross-reactive antibody binding was indeed observed with serum from the animal that had preexisting anti-HSV-1 immunity but not with serum from the naive mouse (data not shown). This result was further supported by our ability to demonstrate the generation of antibodies capable of neutralizing the infectivity of strain 17-derived amplicon particles in mice that were preimmunized with wild-type HSV-1 (Patton).

Analysis of the Env-specific immune response in mice that were previously infected with HSV-1 Patton revealed a rela-

tively modest (ca. 40 to 60%) reduction in cellular immune responses to gp120 after inoculation with HSV:gp120 amplicon particles. In contrast, the Env-specific humoral response elicited by HSV:gp120 amplicon particles was dramatically inhibited in preinfected mice. One possible explanation for this is that anti-HSV-1 antibodies may neutralize the infectivity of some, but not all, of the administered amplicon particles. Thus, prior infection with HSV-1 may reduce the effective amplicon dose from 10^6 i.u. in naive animals to perhaps 1×10^5 to 3×10^5 i.u. in preinfected animals. This in turn would result in a major drop in the Env-specific antibody response but only a modest decline in the cellular immune responses (as indicated by the results from the dose-response study).

The question as to why prior infection with HSV-1 did not prevent the induction of strong Env-specific cellular immune responses by HSV:gp120 amplicon particles requires further analysis and is not directly addressed here. However, several possible explanations exist. First, the local concentration of virus-neutralizing antibodies may be low at the site of amplicon inoculation, and amplicon particles may undergo rapid intracellular internalization. Second, amplicon particles may selectively target local antigen-presenting cells that express high levels of the gD-binding HSV-1 receptor, HveC. Recent studies have shown that human keratinocytes represent one such cell type and that high levels of gD on the virion surface may allow HSV-1 to escape neutralization by anti-gD antibodies, at least insofar as infection of these cells is concerned (18). Third, the presumptive presence of HSV-reactive T cells in the preinfected mice may result in enhanced T-cell help after introduction of HSV:gp120 amplicon particles. This could partially compensate for an antibody-mediated reduction in effective amplicon titers.

The appealing properties of HSV and other herpesviruses have made vectors based on these agents attractive as potential platforms for HIV vaccine delivery. Knipe and coworkers have developed and tested recombinant HSV-1 vectors that express SIV Env and Nef (32). When introduced into rhesus macaques, these vectors elicited both cellular and humoral immune responses, which were sufficient to protect three of seven animals either fully or partially against a rectal challenge with pathogenic SIVmac239 (32). It is not possible to make a direct comparison between Knipe's studies (which were performed in nonhuman primates) and the experiments described here (which were performed in mice). However, it is possible that gene replacement HSV-1 vectors and amplicon vectors may have different properties. For example, amplicon vectors able been shown to infect cultured dendritic cells without inhibiting their maturation or functional activity (58), in contrast to recombinant HSV-1 vectors (40).

In summary, the experiments reported here show that replication-defective, helper virus-free, HSV-1 amplicon particles can elicit potent cellular and humoral immune responses to a vectored HIV-1 antigen in BALB/c mice and that these responses can persist for at least 5 months. Furthermore, even very low doses of the amplicon preparation can elicit strong cellular immune responses, and amplicon-induced cellular immune responses are only modestly reduced in animals previously infected with wild-type HSV-1. This findings provide support for the future development of the HSV amplicon concept for HIV (AIDS) vaccine design.

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