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Immunization with a Replication-Deficient Mutant of Herpes Simplex Virus Type 1 (HSV-1) Induces a CD8$^+$ Cytotoxic T-Lymphocyte Response and Confers a Level of Protection Comparable to That of Wild-Type HSV-1

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Replication-deficient viruses provide an attractive alternative to conventional approaches used in the induction of antiviral immunity. We have quantitatively evaluated both the primary and memory cytotoxic T-lymphocyte (CTL) responses elicited by immunization with a replication-deficient mutant of herpes simplex virus type 1 (HSV-1). In addition, we have examined the potential role of these CTL in protection against HSV infection. Using bulk culture analysis and limiting-dilution analysis, we have shown that a replication-deficient virus, *d*301, generates a strong primary CTL response that is comparable to the response induced by the wild type-strain, KOS1.1. Furthermore, the CTL induced by *d*301 immunization recognized the immunodominant, H-2K^b-restricted, CTL recognition epitope gB498–505 to a level similar to that for CTL from KOS1.1-immunized mice. The memory CTL response evoked by *d*301 was strong and persistent, even though the frequencies of CTL were slightly lower than the frequencies of CTL induced by KOS1.1. Adoptive transfer studies indicated that both the CD8$^+$ and the CD4$^+$ T-cell responses generated by immunization with *d*301 and KOS1.1 were able to limit the extent of a cutaneous HSV infection to comparable levels. Overall, these results indicate that viral replication is not necessary to elicit a potent and durable HSV-specific immune response and suggest that replication-deficient viruses may be effective in eliciting protection against viral pathogens.

Infection with herpes simplex virus (HSV) initiates at a peripheral site and is followed by the establishment of a latent infection in the local, sensory ganglia. HSV elicits both cellular and humoral immune responses that are important for the resolution of infection (56, 66). To prevent the progression of an HSV infection, an effective immune response must limit both the primary infection and the subsequent establishment of latency. A variety of methods (12) have been utilized to evoke HSV-specific immune responses and protection in vivo, including the use of attenuated HSV (49, 52) and the expression of HSV proteins by recombinant vaccinia viruses (4, 14, 20, 44, 47, 48, 50), adenoviruses (21, 49), and baculoviruses (24–26, 39). Furthermore, immunization with cell lines expressing HSV-encoded proteins or cytotoxic T-lymphocyte (CTL) recognition epitopes (3, 5, 7, 67), as well as direct immunization with either purified HSV-encoded proteins (13) or synthetic peptides (10, 28), has been shown to elicit HSV-specific immune responses. More recently, immunization with DNA vectors encoding HSV proteins has shown promise as a potential delivery system to induce anti-HSV immunity (23, 46). Together, these studies illustrate the effectiveness of multiple approaches in eliciting an HSV-specific immune response.

An alternative approach for the induction of anti-HSV immunity is to use replication-defective viruses. Replication-defective viruses are appealing as immunogens because they offer, in many cases, substantial gene expression characteristic of wild-type virus yet lack the production of infectious virus. Previous studies have shown that immunization with either of two replication-defective mutants of HSV, an ICP8 deletion mutant (*d*301) (54, 55, 60) or a gH deletion mutant (SC16ΔgH) (18, 51), was effective in eliciting immune responses and in protecting against challenge with HSV. The *d*301 virus contains a large deletion in the gene encoding ICP8, the HSV major DNA binding protein, which results in the inability of this virus to replicate its DNA. During the course of infection with *d*301, the pattern of gene expression is characterized by the expression of the α, β, and γ1 genes but not γ2 genes (22). Whereas infection with SC16ΔgH results in the production of wild-type levels of noninfectious progeny virus (19), *d*301 is unable to produce any progeny virus. Despite this property of *d*301, the magnitude of the T-cell response evoked by immunization with *d*301, as measured by antigen-specific in vitro proliferation, is similar to that of wild-type HSV (54). In addition, mice immunized with *d*301 have been shown to be protected against a lethal challenge with wild-type HSV and protected against the development of keratitis and encephalitis following ocular infection (54, 55, 60).

To precisely quantitate and compare the efficiencies of *d*301 and a wild-type HSV-1 strain, KOS1.1, to induce T-cell immunity to HSV, we have used limiting-dilution analysis (LDA) to determine the frequencies of both primary and memory CD8$^+$ CTL specific for an immunodominant, H-2K^b-restricted CTL recognition epitope in HSV-1 glycoprotein B (gB498–505) (10, 28). The gB498–505 epitope sequence is identical to the sequence in HSV type 2 (HSV-2), and CTL induced by gB498–505 cross-react with HSV-2-infected cells (10). Furthermore, the gB498–505 epitope, cloned into T antigen of simian virus 40 (SV40), is processed from T antigen and presented in the

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context of H-2K^b on the surface of a cell expressing the recombinant protein (7). Therefore, the gB498–505 epitope provides a well-defined target for the comparison of *d*301- and KOS1.1-induced CD8$^+$ responses in vivo. To extend this comparison and to identify the cellular components of the *d*301 immune response involved in the protection of a host against an HSV infection, we performed adoptive transfer studies. The persistence of the memory CTL (CTLm) response elicited by immunization with *d*301 was also evaluated. The results of our studies suggest that immunization with a replication-deficient mutant is as effective as immunization with wild-type HSV in inducing an HSV-specific CTL response and indicate that both CD8$^+$ and CD4$^+$ T lymphocytes from mice immunized with *d*301 are effective in conferring protection against primary, local HSV infection.

MATERIALS AND METHODS

Mice. Male C57BL/6 mice were obtained from the Jackson Laboratory at 5 to 6 weeks of age and were housed in metal cages at four or five mice per cage. All mice were maintained on a 12-h light/dark cycle (lights off 1800 to 0600) and were allowed to acclimate to such conditions for a 1-week period prior to any experimental manipulations. Food and water were provided ad libitum.

Cell lines and media. The B6/WT-3 (65) and B6/K-1,4,5 (75, 76) cell lines were maintained in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 5% (vol/vol) heat-inactivated fetal bovine serum (FBS), 20 mM HEPES buffer, and 0.075% (wt/vol) $NaHCO_3$. Lymphocyte cultures were grown in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FBS, 0.225% $NaHCO_3$, 25 mM HEPES buffer, and 5×10^{-5} M 2-mercaptoethanol. The H-2K^b-restricted CTL clone 2D5 has been described previously and specifically recognizes the gB498–505 epitope (10). 2D5 cells were grown in 12-well cluster dishes and maintained on supplemented IMDM containing 10% (vol/vol) Rat T-Stim culture supplement (Collaborative Biomedical Research Products, Bedford, Mass.) and 0.05 M α-methyl-D-mannoside (Sigma, St. Louis, Mo.). To each well were also added 5×10^5 HSV-infected B6/WT-3 cells that had been treated with mitomycin as described previously (9). Vero cells (a continuous line of African green monkey kidney cells) were maintained in medium 199 supplemented with 4% heat-inactivated FBS, 4% heat-inactivated newborn calf serum, 8% tryptose phosphate broth, and 5% (closed vessel) or 15% (open vessel) $NaHCO_3$. S2 cells (22) were derived from Vero cells and express HSV ICP8 upon infection with HSV. The S2 cell line was maintained in supplemented medium 199 containing neomycin (500 μg/ml). All media also contained 2 mM glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin sulfate per ml.

Viruses. HSV-1 KOS1.1 and HSV-1 *d*301 were used in these studies. The *d*301 virus is derived from the KOS1.1 strain and contains a 2,001-bp deletion in the gene encoding ICP8 (22). KOS1.1 and *d*301 stocks were prepared in Vero and S2 cells, respectively, by infection at a multiplicity of infection of 0.01. Virus titers were determined by plaque assay on Vero cells (KOS1.1) or S2 cells (*d*301). SV40 strain VA-45-54 was obtained from Mary Judith Tevethia, Hershey Medical Center.

Synthetic peptides. A synthetic peptide corresponding to the HSV-1 CTL recognition epitope gB498–505 (SSIEFARL [10]) was synthesized at the Macromolecular Core Facility of the M. S. Hershey Medical Center by 9-fluorenylmethoxycarbonyl chemistry, using an automated peptide synthesizer (model 9050) MilliGen PepSynthesizer. The purity and amino acid composition of each peptide was determined by high-pressure liquid chromatography tracing (Waters, a division of MilliGen). Peptide stock solutions were made by solubilizing the lyophilized peptides in dimethyl sulfoxide and adjusting the volume with unsupplemented RPMI 1640 medium.

Generation of primary and memory HSV-specific CTL. A primary HSV-specific CTL response was induced in C57BL/6 mice as described previously (15, 62, 63). To induce a primary CTL response in the popliteal lymph nodes, mice were infected in both rear footpads by an intraplantar injection of 10^6 PFU of HSV-1 in a volume of 50 μl. An HSV-specific immune response was induced in the inguinal lymph nodes by immunization with 10^6 PFU of HSV-1 subcutaneously in each side at the base of the tail. In some experiments, the immunizing dose of virus used to infect the footpads was varied as indicated in the text. Five days later, the popliteal and inguinal lymph nodes were removed and single-cell suspensions were prepared. These lymph node-derived cells were cultured in 60-mm-diameter plates (2×10^7 cells/plate) in 5 ml of supplemented IMDM at 37°C and 5% CO_2 for 3 days. The procedure used to induce an HSV-specific CTLm response has been described previously (32, 43). Briefly, mice were immunized with 10^7 PFU of HSV-1 intraperitoneally. At least 4 weeks later, spleen-derived lymphocytes were cultured in 12-well cluster dishes (10^7 cells/well) in 4 ml of supplemented IMDM at 37°C and 5% CO_2 for 5 days. To each well were also added 5×10^5 HSV-infected B6/WT-3 cells that had been treated with mitomycin.

Enrichment of T cells and T-cell subsets by using affinity chromatography columns. Cellect immunocolumns (Biotex Laboratories, Inc., Edmonton, Alberta, Canada) were used to enrich for populations of T cells and T-cell subsets within the lymph nodes of HSV-immunized mice. To enrich for T cells, single-cell suspensions of lymph node-derived cells were passed through a glass bead immunocolumn to which was bound both polyclonal goat anti-mouse immunoglobulin G (IgG) and polyclonal goat anti-rat IgG. To obtain an enriched population of T cells, the column was washed with phosphate-buffered saline (PBS) supplemented with 2% FBS; both B cells and macrophages were specifically retained in the column. To specifically enrich CD8$^+$ T cells, a single-cell suspension of lymph node cells was first incubated with the rat anti-mouse L3T4 (CD4; clone YTS 191.1) antibody, and following the incubation, the cells were passed through the immunocolumn to which was bound both polyclonal goat anti-mouse IgG and polyclonal goat anti-rat IgG. Following a wash with 2% FBS, an enriched population of CD8$^+$ T cells was eluted. The enrichment of CD4$^+$ cells was accomplished by incubating the lymph node cells with rat anti-mouse Ly2 (CD8a; clone YTS 169.4) antibody. The CD4$^+$ cells were recovered by the protocol used for the CD8$^+$ cells.

Adoptive transfer model in sublethally irradiated recipient mice. For adoptive transfer experiments, C57BL/6 mice were subjected to sublethal, whole-body irradiation (600 rads) and were subsequently challenged with 2×10^6 PFU (in a volume of 4 μl) of HSV-1 KOS1.1 in both rear footpads, using the multiple piercing method as described previously (8). Three days postinfection, lymphocytes were adoptively transferred intravenously (i.v.) into these HSV-infected mice. The lymphocytes were derived from the popliteal or a combination of popliteal and inguinal lymph nodes obtained from HSV-immunized, syngeneic mice as described above. In some experiments, enriched populations of CD4$^+$ and CD8$^+$ lymphocytes were prepared as described above. Cells (2×10^7 unfractionated, 7.5×10^6 T-cell enriched, 4×10^6 CD8 and CD4 enriched) were transferred in Hanks' balanced salt solution in a volume of 0.2 ml. Four days following the adoptive transfer, the rear footpads were removed and frozen at −80°C in 1 ml of supplemented medium 199 until time of virus titration (73).

Quantitation of infectious HSV-1 levels in footpads of HSV-1-challenged mice. Footpads from C57BL/6 mice infected with HSV-1 KOS1.1 were thawed at 37°C and then homogenized in a 1-ml Tenbroeck glass tissue homogenizer (Wheaton Industries, Millville, N.J.). Homogenized samples were centrifuged at $1,000 \times g$ for 5 min, and 10-fold serial dilutions were prepared in PBS supplemented with 1% FBS and titrated on Vero cells grown in 60-mm-diameter tissue culture plates. Virus was absorbed onto these cells for 1 h followed by the addition of 4 ml of methylcellulose overlay medium (15) supplemented with 3.5 μg amphotericin B (Fungizone; E. R. Squibb & Sons, Inc., Princeton, N.J.) per ml. After 4 or 5 days of incubation at 37°C and 5% CO_2, the overlay medium was removed, and the plates were fixed with 5% formaldehyde and stained with 0.5% crystal violet for visualization of plaques. The lower limit of detection for infectious virus was 10 PFU/footpad.

Assay for cell-mediated cytotoxicity. The ^{51}Cr release assay for quantitation of HSV-specific CTL was based on previously published methods (15) and has been described in detail elsewhere (6). Briefly, HSV-infected target cells were prepared by allowing HSV-1 to adsorb to the cells at a multiplicity of infection of 10 for 1 h in PBS–1% FBS followed by an incubation of 3 to 12 h, during which time the cells were labeled with ^{51}Cr. Following the incubation, cells were harvested and washed three times with supplemented DMEM. To prepare peptide-pulsed target cells, uninfected cells were resuspended at a concentration of no greater than 10^6/ml. These cells were then incubated with 1 μM synthetic peptide for 30 min at 37°C and 5% CO_2 and then washed twice with supplemented DMEM to remove excess peptide. Target cells (10^4) were added in a volume of 100 μl to triplicate wells in 96-well, V-bottom microtiter tissue culture plates (Costar, Cambridge, Mass.). Lymph node cells were then added in a volume of 100 μl to achieve graded effector-to-target ratios as indicated in the figures. The plates were centrifuged at $100 \times g$ for 3 min and incubated at 37°C and 5% CO_2 for 4 to 5 h. After the incubation, the plates were centrifuged at $200 \times g$ for 3 min, and 100 μl of the supernatant was removed from each well. The amount of radioactivity released in the supernatants was quantitated in a gamma counter. Percent specific lysis was calculated as $[(E - S)/(M - S)] \times 100$, where E equals the counts per minute released from targets incubated with lymphocytes, S equals the counts per minutes released from target cells incubated with no lymphocytes, and M equals the counts per minute released from cells following lysis with 5% sodium dodecyl sulfate.

Determination of HSV-specific CTL frequencies by LDA. The procedure for LDA has been described previously (33). Briefly, draining lymph nodes or spleens from HSV-1-immunized mice were collected, and single-cell suspensions were prepared. Graded numbers of lymphocytes were cultured in 96-well, U-bottom microtiter tissue culture plates (Costar) in replicates of 18 in a volume of 200 μl of supplemented IMDM. To each well were also added 10^5 gamma-irradiated (200 rads) splenocytes from naive C57BL/6 mice, 0.2 U of human recombinant interleukin-2 (Amgen, Thousand Oaks, Calif.), 10 μl of Rat T-Stim culture supplement, and 0.1 M α-methyl-D-mannoside. To activate HSV-specific CTLm, 2×10^3 HSV-1-infected syngeneic fibroblasts (B6/WT-3 cells) were added to each well. The plates were then incubated for 7 days at 37°C and 5% CO_2. Following the incubation, cells from individual wells were assayed for lytic activity against HSV-1-infected, gB498–505 peptide-pulsed, and mock-infected target cells as described above. A well was considered to contain at least one CTL

precursor (CTLp) if the percentage of specific lysis in that well was greater than 10%. The CTLp frequency was estimated by the minimal χ^2 method (77).

Fluorescent flow cytometric analysis. The percentages of $CD8^+$, $CD4^+$, and $CD2^+$ populations within the draining popliteal lymph nodes of C57BL/6 mice immunized with HSV-1 were determined by fluorescent flow cytometric analysis. Briefly, 10^6 lymph node cells were washed once in PBS containing 2% FBS and 0.1% NaN_3 (fluorescence-activating cell sorting [FACS] buffer), resuspended in 100 μl of the appropriate fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated monoclonal antibodies (PharMingen) (diluted in FACS buffer), and incubated at 4°C for 1 h. The monoclonal antibodies used included PE-conjugated hamster anti-mouse T-cell receptor α/β (TCR) (PharMingen 01305), PE-conjugated rat anti-mouse CD2 (PharMingen 01175), PE-conjugated rat anti-mouse L3T4 (CD4) (PharMingen 01075), and FITC-conjugated rat anti-mouse Ly-2 (CD8a) (PharMingen 01044). Following the incubation period, the cells were washed free of excess antibody and resuspended in 1 volume of FACS buffer and 2 vol of 2% (wt/vol) paraformaldehyde. Cells were analyzed on an EPICS V flow cytometer (Coulter Electronics, Inc., Hialeah, Fla.), using single-color analysis at an excitation wavelength of 488 nm. The percentages of each lymphocyte subset were based on events that were visualized on the extent of their forward angle light scatter and 90° light scatter. To determine these percentages, events exhibiting a typical 90° versus forward angle light scatter pattern of lymphocytes as well as those cells having a light scatter typical of cells in an activated or blast-like state were selected for analysis.

Western blot analysis. Western blot analysis was used to detect the expression of HSV-1 gB during infection with either KOS1.1 or *d*301. B6/WT-3 cells were infected with either strain of HSV-1 or were mock infected. At various times after infection, cells were lysed and then immunoprecipitated with a mouse monoclonal anti-HSV-1 gB antibody, I-144-2B (36), as previously described (38). Prior to immunoprecipitation, samples were normalized for protein concentration (300 μg) by using the Bradford assay (Bio-Rad, Hercules, Calif.). The immunoprecipitated samples were subjected to electrophoresis on a sodium dodecyl sulfate–7.5% acrylamide gel. Proteins were transferred to nitrocellulose and blotted under conditions described previously (38). The primary antibody used for immunodetection was a 1:3,000 dilution of a rabbit polyclonal antibody (1161) specific to HSV-1 gB, kindly provided by Richard Courtney; a goat anti-rabbit antibody was used as the secondary antibody. An ECL kit (Amersham, Arlington Heights, Ill.) was used for protein detection as described previously (38).

Statistical analysis. The Mann-Whitney test was used to compare the levels of protection afforded by adoptive transfer of lymphocytes. All analyses were done using the InStat program (GraphPad Software, Inc., San Diego, Calif.).

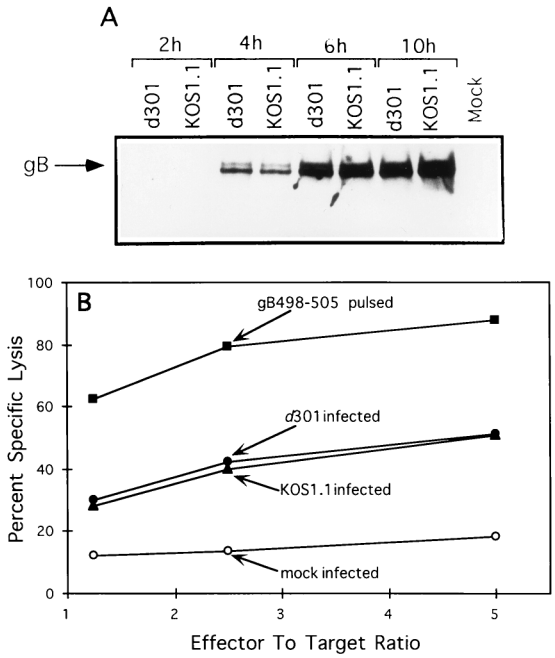

FIG. 1. Comparison of gB expression during infection with *d*301 and KOS1.1. Western blot analysis was used to determine the kinetics of gB expression in *d*301-infected B6/WT-3 cells (A). Cells were infected with either *d*301 or KOS1.1 for 2, 4, 6, and 10 h. Cell lysates were immunoprecipitated with an anti-gB monoclonal antibody (I-144-2B) and subjected to Western blot analysis using a polyclonal antibody specific for gB (1161) as described in Materials and Methods. To quantitate the level of gB expressed during infection with *d*301, the CTL clone 2D5 was used to confirm the expression of the CTL recognition epitope, gB498–505, presented on the cell surface (B). The susceptibility of target cells to lysis by 2D5 was determined in a standard ^{51}Cr release assay.

RESULTS

Analysis of gB expression in cells infected with *d*301 or KOS1.1. To use the gB498–505 CTL recognition epitope as a standard for comparing the CTL responses induced in mice immunized with either *d*301 or KOS1.1, it was necessary to examine the kinetics and levels of gB expression during infection of B6/WT-3 cells with these viruses. B6/WT-3 cells were infected with either *d*301 or KOS1.1 for 2, 4, 6, and 10 h, and cell lysates were then subjected to Western blot analysis as described in Materials and Methods. At each of the time points tested, *d*301-infected cells expressed a significant level of gB that was similar to that observed in KOS1.1-infected cells (Fig. 1A). To examine the efficiency with which the gB498–505 epitope is processed and presented in the context of $H\text{-}2K^b$ during infection, the gB498–505-specific CTL clone, 2D5, was used in a standard ^{51}Cr release assay. The 2D5 clone was shown to recognize and lyse *d*301-infected B6/WT-3 cells to the same extent as KOS1.1-infected cells, while the recognition of mock-infected B6/WT-3 cells was at background levels (Fig. 1B). Together, these results indicate that cells infected with *d*301 are able to express gB and present the gB498–505 epitope in the context of class I major histocompatibility complex.

Comparison of the local, primary CTL response induced by immunization with *d*301 and KOS1.1. C57BL/6 mice were immunized with 10^6 PFU of either HSV-1 KOS1.1 or *d*301. Five days later, the popliteal lymph nodes were removed and bulk cultures were established. The lytic activity of the cells from these cultures was determined as described in Materials and Methods. Lymphocytes obtained from KOS1.1 (Fig. 2A)- and *d*301 (Fig. 2B)-immunized mice exhibited similar levels of lytic activity against both KOS1.1- and *d*301-infected target cells. These findings demonstrate that although *d*301 expresses a somewhat limited array of viral genes during infection, there is expression of at least one CTL recognition epitope that is able to induce an HSV-specific CTL response. We suspected that the gB498–505 epitope contributed to the induction of the overall CTL response because the lymph node cells from both of these groups of mice also lysed target cells pulsed with the synthetic peptide, gB498–505, to a significant extent. Together, these results indicate that although *d*301 does not replicate in the footpads of mice (55), it is sufficient to drive the induction of a CTL response that is similar to the CTL response elicited by immunization with the replication-competent virus, KOS1.1.

These results demonstrate that immunization with *d*301 generates a CTL response; however, these experiments did not examine the components of the local immune response following footpad immunization with HSV. To further characterize the immune response elicited by immunization with KOS1.1 and *d*301, we determined the percentages of the cell types within the draining lymph nodes of these mice by fluorescent flow cytometry. The percentages of $CD8^+$ and TCR^+ lymphocytes in mice immunized with *d*301 were statistically greater than in those mice immunized with KOS1.1 (data not shown). In contrast, the percentage of $CD4^+$ T lymphocytes within the lymph nodes of mice immunized with *d*301 was greater than in mice immunized with KOS1.1, although these differences were not statistically significant (data not shown). The percentages of $CD2^+$ cells were similar in both *d*301- and KOS1.1-immunized mice (data not shown).

LDA of the primary CTL response in mice immunized with HSV-1 KOS1.1 and *d*301. The foregoing results demonstrated

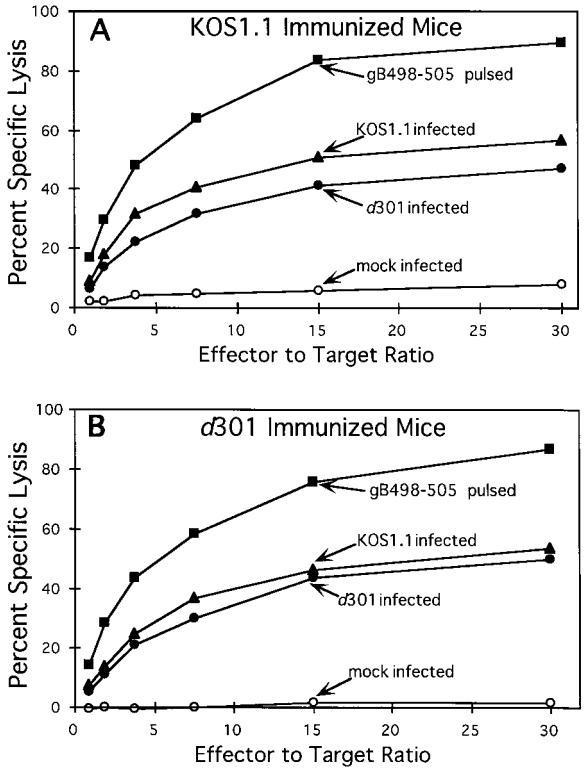

FIG. 2. HSV-specific CTL response induced by immunization with KOS1.1 and *d*301. C57BL/6 mice were immunized with 10^6 PFU of either KOS1.1 (A) or *d*301 (B) in both rear footpads via an intraplantar route. After 5 days, the draining popliteal lymph nodes were removed and single-cell suspensions were prepared. Lymph node cells were cultured in vitro for 3 days and then tested for lytic activity in a standard ^{51}Cr release assay.

that *d*301 can induce a CTL response comparable to that induced by immunization with KOS1.1 as measured by the level of lytic activity generated under bulk culture conditions. To quantitate this response, we determined, by LDA, the frequency of HSV-specific CTL in mice that were immunized with 10^6 PFU of either *d*301 or KOS1.1. Five days after infection, lymph node cells from these mice were cultured under limiting-dilution culture conditions as described in Materials and Methods. Cells from each of these wells were tested for lytic activity against B6/WT-3 cells that were either KOS1.1 infected, gB498–505 peptide pulsed, or mock infected. As shown in Table 1, immunization with *d*301 induces a CTL response comparable to that in mice immunized with KOS1.1, as determined by lysis of KOS1.1-infected target cells. Likewise, CTL specific for the gB498–505 epitope were generated comparably in mice immunized with either of these viruses. Despite the lack of *d*301 replication, the degree of CTLp activation and subsequent lymphoproliferation in vivo were comparable in the two groups of mice, as indicated by the number of HSV-specific CTL recovered from the lymph nodes. It should be noted that the frequencies of CTLp specific for gB498–505 are higher than the frequencies of CTLp specific for KOS1.1-infected cells. This difference in frequencies may be attributed to the down regulation of H-2K^b expression on surface of B6/WT-3 cells infected with KOS1.1 (34). This lower level of peptide-major histocompatibility complex complexes may not be optimal for recognition by CTL, while cells pulsed with gB498–505 maintain levels of class I expression that allow enhanced recognition. The results indicate that immunization with *d*301 is effective in inducing a potent CTL response in the absence of virus replication and that this response is quantitatively comparable to that induced by immunization with KOS1.1.

To further quantitate the efficacy of CTL generation, we immunized mice with 10^6, 10^5, or 10^4 PFU of either *d*301 or KOS1.1. Limiting-dilution culture conditions were established, and lytic activity was determined against gB498–505 peptide-pulsed target cells. As shown in Table 2, immunization with all three doses of KOS1.1 elicited a strong anti-gB498–505-specific CTL response. At immunizing doses of 10^6 and 10^5 PFU of *d*301, the CTLp frequency was similar to that of KOS1.1. However, immunization with the lowest dose of *d*301, while capable of inducing a significant CTL response, was somewhat lower in magnitude than that elicited by KOS1.1. Also, as indicated by the yield of total CTL, the lymph nodes from mice receiving the lowest dose of *d*301 were significantly smaller than in mice immunized with the same dose of KOS1.1. Together, these results suggest that the HSV-specific CTL response to *d*301 is quantitatively comparable to the response for KOS1.1, except at the immunizing dose of 10^4 PFU. Immunization with this dose of *d*301 may not attain the minimal threshold of antigenic stimulation necessary for the induction of CTL. In contrast, the replication potential of KOS1.1 may increase the degree of viral antigenic load to a level that is sufficient to fulfill this minimal requirement.

Evaluation of the protective capacity of lymph node cells obtained from mice immunized with HSV-1 *d*301 and KOS1.1. To assess the ability of lymph node cells from both *d*301- and KOS1.1-immunized mice to limit the extent of HSV replication in vivo, we performed adoptive transfer studies. Mice were immunized with 10^6 PFU of either HSV-1 KOS1.1 or *d*301. Five days later, unselected lymphocytes obtained from the pop-

TABLE 1. Frequencies of CTLp induced by immunization with KOS1.1 and *d*301

Immunization[a]	Target cells[b]	CTLp frequency (95% confidence limits)	Total CTLp/lymph node (range)[c]
KOS1.1	KOS1.1 infected	1/1,064 (1/808–1/1,556)	8,459 (5,784–11,138)
*d*301	KOS1.1 infected	1/2,273 (1/1,676–1/3,529)	3,079 (1,983–4,176)
KOS1.1	gB498–505 pulsed	1/389 (1/284–1/619)	23,136 (14,539–31,690)
*d*301	gB498–505 pulsed	1/710 (1/530–1/1,075)	9,859 (6,511–13,207)
KOS1.1	Mock infected	1/1,062,070 (1/626,945–1/3,471,293)	8 (3–14)
*d*301	Mock infected	1/1,095,423 (1/646,856–1/3,573,469)	6 (2–10)

[a] Popliteal lymph nodes were removed from C57BL/6 mice 5 days after footpad immunization with HSV-1 KOS1.1 or *d*301.
[b] B6/WT-3 cells were either infected with KOS1.1, pulsed with the synthetic peptide gB498–505, or mock infected. The target cells were labeled with ^{51}Cr, and lytic activity was determined by a standard ^{51} Cr release assay.
[c] Calculated by using the following formula: (CTLp frequency) × (viable cell yield/lymph node).

TABLE 2. Frequencies of gB498–505-specific CTLp induced by various doses of KOS1.1 and *d*301

Immunization[a]	Dose (PFU)	Target cells[b]	CTLp frequency (95% confidence limits)	Total CTLp/lymph node (range)[c]
KOS1.1	10^6	gB498–505 pulsed	1/550 (1/408–1/841)	15,455 (10,107–20,833)
*d*301	10^6	gB498–505 pulsed	1/796 (1/591–1/1,217)	5,863 (3,835–7,896)
KOS1.1	10^5	gB498–505 pulsed	1/357 (1/264–1/551)	23,459 (15,200–31,723)
*d*301	10^5	gB498–505 pulsed	1/560 (1/418–1/845)	8,482 (5,621–11,634)
KOS1.1	10^4	gB498–505 pulsed	1/424 (1/315–1/648)	18,868 (12,346–25,397)
*d*301	10^4	gB498–505 pulsed	1/1,568 (1/1,172–1/2,368)	1,156 (765–1,547)
KOS1.1	10^6	Mock	1/295,502 (1/211,514–1/490,115)	29 (17–40)
*d*301	10^6	Mock	1/97,769 (1/74,306–1/142,887)	48 (33–63)
KOS1.1	10^5	Mock	1/75,177 (1/57,905–1/107,135)	111 (78–145)
*d*301	10^5	Mock	1/144,445 (1/108,840–1/214,671)	33 (22–44)
KOS1.1	10^4	Mock	1/389,723 (1/272,542–1/683,671)	21 (12–29)
*d*301	10^4	Mock	1/55,790 (1/42,957–1/79,555)	32 (23–42)

[a] Popliteal lymph nodes were removed from C57BL/6 mice 5 days after footpad immunization with HSV-1 KOS1.1 or *d*301.
[b] B6/WT-3 cells were either pulsed with the synthetic peptide gB498–505 or mock infected. The target cells were labeled with ^{51}Cr, and lytic activity was determined by a standard ^{51}Cr release assay.
[c] Calculated by using the following formula: (CTLp frequency) × (viable cell yield/lymph node).

liteal lymph nodes of these mice were transferred i.v. into syngeneic C57BL/6 mice that 3 days earlier had been sublethally irradiated (600 rads) and subsequently challenged with KOS1.1 in each hind footpad by the multiple piercing method. After 4 days, the level of infectious HSV was determined in each of the footpads. The results presented in Fig. 3 show that the transfer of total lymph node cells from mice immunized with either *d*301 or KOS1.1 was able to reduce the level of detectable virus within infected tissue greater than 100-fold from the virus level in control animals ($P < 0.001$ and $P < 0.001$, respectively). The extents of protection afforded by the *d*301- and KOS1.1-derived lymphocytes were statistically equivalent ($P > 0.05$). In contrast, lymph node cells from SV40-immunized mice provided no significant reduction in viral titers ($P > 0.05$). Together, these results indicate that immunization with *d*301 elicits an immune response that is as effective as immunization with KOS1.1 in controlling the extent of HSV replication at a cutaneous site of infection.

To determine the lymphocyte subsets involved in controlling virus replication, we enriched lymph node cells for TCR^+, $CD8^+$, or $CD4^+$ populations, which were subsequently transferred into HSV-challenged mice as outlined above. Lymph node cells obtained from *d*301- or KOS1.1-immunized mice were fractionated by affinity chromatography as described in Materials and Methods. The transfer of enriched T cells (Fig. 4) from mice immunized with either *d*301 or KOS1.1 resulted in at least a 100-fold decrease in the level of virus compared to the control ($P < 0.001$ and $P < 0.001$, respectively). The extents of reduction in virus titers mediated by the transfer of T cells induced by KOS1.1 and by *d*301 were statistically equivalent ($P > 0.05$). The transfer of enriched $CD8^+$ T cells (Fig. 5A) from mice immunized with either *d*301 or KOS1.1 reduced

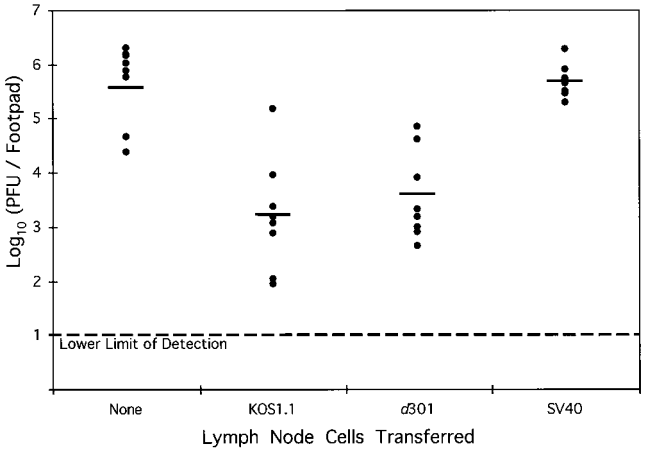

FIG. 3. Protection afforded by the adoptive transfer of lymph node cells from C57BL/6 mice immunized with HSV-1 KOS1.1 or *d*301. C57BL/6 mice were immunized in each of the rear footpads with 10^6 PFU of *d*301 or KOS1.1. Five days later, the draining popliteal lymph nodes were recovered, and single-cell suspensions were prepared. Lymph node cells (2×10^7 cells/mouse) were transferred i.v. into syngeneic mice ($n = 4$) that had been sublethally irradiated (600 rads) and subsequently challenged with KOS1.1 (2×10^6 PFU) by the multiple piercing method as described in Materials and Methods. Four days following the transfer, the footpads were removed, and the HSV levels were determined by plaque assay. As a negative control, popliteal lymph node cells from C57BL/6 mice immunized with 3×10^6 PFU of SV40 were transferred into recipient mice as described above. The geometric mean for each group is represented by the solid horizontal line. Statistical analysis was performed by the nonparametric Mann-Whitney test.

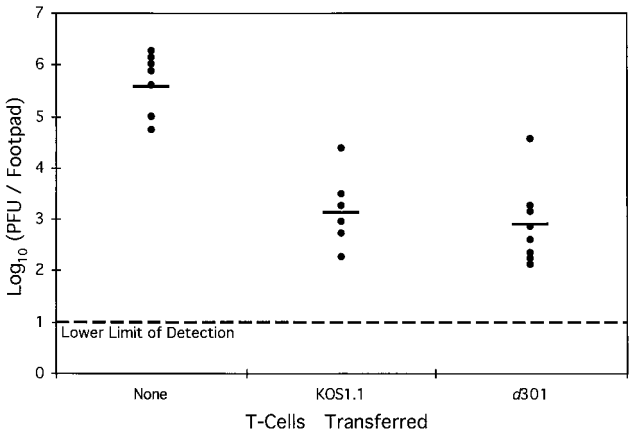

FIG. 4. Protection afforded by the adoptive transfer of T lymphocytes from C57BL/6 mice immunized in each of the rear footpads with HSV-1 KOS1.1 or *d*301. T cells were enriched from popliteal lymph nodes of mice immunized with 10^6 PFU of either KOS1.1 or *d*301 and were subsequently transferred (7.5×10^6 cell/mouse) into HSV-challenged mice as described in the legend to Fig. 3 ($n =$ 3 to 4 per group). The resulting enriched cell population was determined to be 87% TCR^+ by fluorescent flow cytometry. The geometric mean for each group is represented by the solid horizontal line. Statistical analysis was performed by the nonparametric Mann-Whitney test.

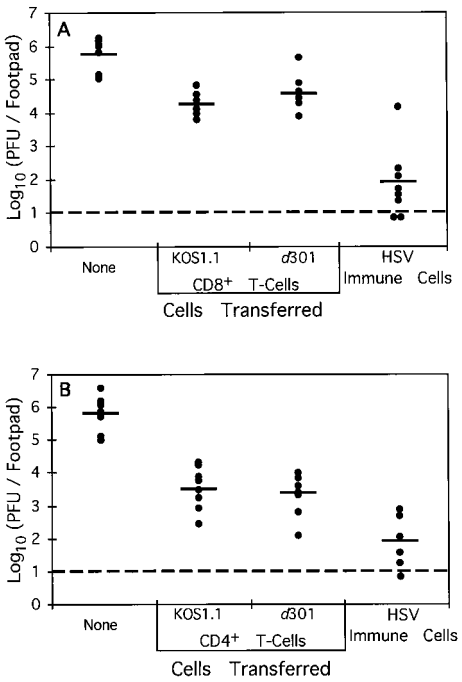

FIG. 5. Protection afforded by the adoptive transfer of $CD4^+$ and $CD8^+$ T lymphocytes from C57BL/6 mice immunized in each of the rear footpads and at the base of the tail with HSV-1 KOS1.1 or *d*301. C57BL/6 mice were immunized with 10^6 PFU of either KOS1.1 or *d*301. After 5 days, the draining popliteal and inguinal lymph nodes were recovered, and single-cell suspensions were prepared. Enriched $CD8^+$ (A) and $CD4^+$ (B) T cells were prepared and transferred i.v. (4×10^6 cells/mouse) into KOS1.1-challenged mice ($n = 3$ to 4 per group). For both enriched populations, purity was approximately 85% as determined by fluorescent flow cytometry. As a positive control, unfractionated lymph node cells from KOS1.1-immunized mice were transferred into KOS1.1-challenged mice as above. The geometric mean for each group is represented by the solid horizontal line. Statistical analysis was performed by the nonparametric Mann-Whitney test.

the amount of infectious virus greater than 10-fold ($P = 0.0012$ and $P = 0.0012$, respectively), and the levels of reduction were statistically equivalent ($P > 0.05$). Likewise, the transfer of enriched $CD4^+$ T cells (Fig. 5B) from mice immunized with either virus decreased viral titers greater than 100-fold ($P < 0.001$ and $P < 0.001$, respectively), and as above, the levels of protection were equivalent ($P > 0.05$). As in Fig. 3, the transfer of the unfractionated lymph node cells from KOS1.1-immunized mice (HSV-immune cells) resulted in a greater than 100-fold reduction in viral titers compared to the control animals in both experiments ($P = 0.0043$ and $P = 0.0027$, respectively). These results support a role for both $CD4^+$ and $CD8^+$ T lymphocytes in conferring protection against a cutaneous HSV infection and indicate that immunization with a replication-deficient virus is able to elicit HSV-specific T lymphocytes that are effective in conferring protection.

Evaluation of the magnitude and duration of the CTLm response elicited by immunization with HSV-1 *d*301 and KOS1.1. An important consideration in assessing the ability of a vector to induce a virus-specific immune response is the strength of the memory response that is elicited and maintained as a consequence of immunization (17). Previous studies have indicated that immunization with *d*301 confers long-term protection against challenge with wild-type HSV (55). However, the magnitude and persistence of the CTLm response to immunization with *d*301 have not been determined.

Because CTL are an important component of the memory immune response to HSV infection (69), the HSV-specific CTLm response induced by immunization with *d*301 was evaluated. Mice were immunized intraperitoneally with 10^7 PFU of either *d*301 or KOS1.1, and spleens were recovered 1 month postimmunization. Splenic lymphocytes were cultured in vitro with HSV-infected syngeneic target cells as described in Materials and Methods. Cells from these cultures were tested for the ability to lyse target cells infected with either KOS1.1 (Fig. 6A) or *d*301 (Fig. 6B) or pulsed with the synthetic peptide, gB498–505 (Fig. 6C). The levels of lytic activity against HSV-1-infected and gB498–505 peptide-pulsed target cells were sim-

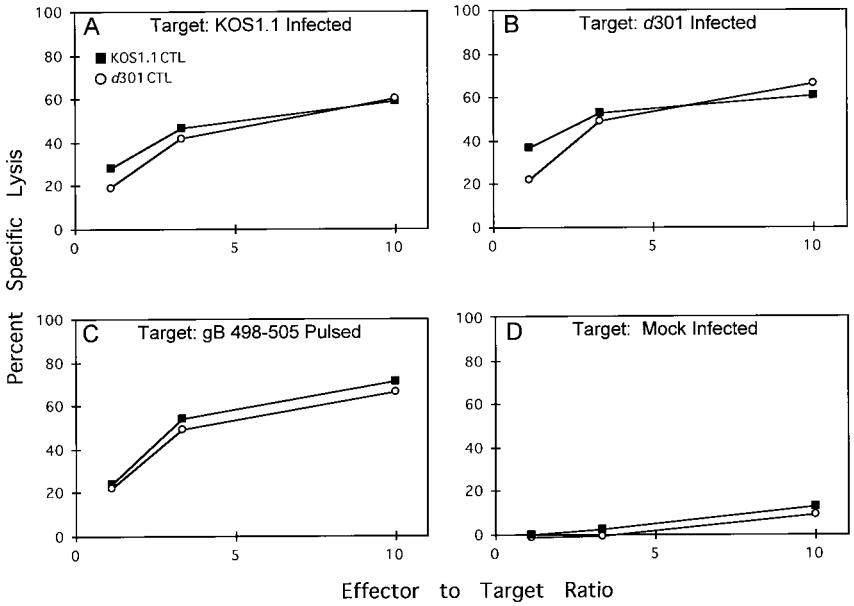

FIG. 6. Memory CTL response induced by immunization with HSV-1 KOS1.1 or *d*301. C57BL/6 mice were immunized with 10^7 PFU of KOS1.1 or *d*301 intraperitoneally. After 4 weeks, the spleens were removed and single-cell suspensions were prepared. Splenocytes were cultured in vitro for 5 days in the presence of HSV-infected, mitomycin-treated B6/WT-3 cells. Cells from these cultures were assayed for lytic activity in a standard ^{51}Cr release assay.

TABLE 3. Frequencies of CTLm induced by immunization with KOS1.1 and *d*301

Immunization[a]	Target cells[b]	CTLm frequency (95% confidence limits)	Total CTLm/spleen (range)[c]
KOS1.1	KOS1.1 infected	1/5,258 (1/3,942–1/7,893)	9,224 (6,145–12,303)
*d*301	KOS1.1 infected	1/19,960 (1/15,254–1/28,864)	2,255 (1,559–2,950)
KOS1.1	gB498–505 pulsed	1/9,561 (1/7,228–1/14,116)	5,073 (3,436–6,710)
*d*301	gB498–505 pulsed	1/27,394 (1/21,225–1/40,842)	1,611 (769–2,120)
KOS1.1	Mock	1/4,507,730 (1/1,522,840–ND[d])	11 (1–32)
*d*301	Mock	1/239,167 (1/160,129–1/472,285)	188 (95–281)

[a] Spleens were removed from C57BL/6 mice 1 month following intraperitoneal immunization with HSV-1 KOS1.1 or *d*301. Splenocytes were cultured with KOS1.1-infected B6/WT-3 stimulator cells.
[b] B6/WT-3 cells were either infected with KOS1.1, pulsed with the synthetic peptide gB498–505, or mock infected. The target cells were labeled with ^{51}Cr, and lytic activity was determined by a standard ^{51}Cr release assay.
[c] Calculated by using the following formula: (CTLm frequency) × (viable cell yield/lymph node).
[d] ND, not determined.

ilar for both groups of mice. The recognition of mock-infected targets by HSV-specific CTL was at background levels (Fig. 6D). In addition, mice immunized with PBS showed no HSV-specific cytotoxic activity (data not shown). These results indicate that *d*301 can induce a CTLm response comparable to that induced by KOS1.1 as measured by bulk culture analysis.

LDA was used to quantitatively examine the CTLm response induced by *d*301. Splenocytes from mice immunized with either KOS1.1 or *d*301 1 month earlier were cultured under limiting-dilution culture conditions. Lytic activity was determined against B6/WT-3 cells that were either KOS1.1 infected, gB498–505 peptide pulsed, or mock infected. While immunization with *d*301 was capable of inducing high frequencies of CTL specific for HSV and gB498–505 (relative to frequencies against mock-infected target cells), they were three- to fourfold lower than in mice immunized with KOS1.1 (Table 3). The results from the bulk culture analysis (Fig. 6) had indicated that the population of CTLm induced by immunization with *d*301 and activated in vitro are as effective in lysing targets as are CTLm from KOS1.1-immunized mice. However, the results from the LDA suggest that the generation of HSV-specific CTLm in *d*301-immunized mice, although substantial, is less efficient than in those mice immunized with KOS1.1.

Although the foregoing results suggested that immunization with a replication-deficient virus is able to efficiently generate HSV-specific CTLm, the persistence of such a response over an extended period of time was unknown. To investigate this issue, mice were immunized as described above, and spleens recovered at 1, 9, and 22 months following immunization with either *d*301 or KOS1.1 were used to establish bulk cultures. Cultured splenocytes were examined for cytotoxicity against B6/K-1,4,5 cells that were infected with KOS1.1, peptide pulsed with gB498–505, or mock infected. The results show that both the HSV-specific and gB498–505-specific CTLm responses in KOS1.1-immunized mice were detectable for at least 22 months postimmunization and were comparable to the CTLm response induced by *d*301 (data not shown).

To further quantitate the durability of the CTLm response, LDA was used to determine the frequency of gB498–505-specific CTLm persisting at the time points noted above. As in Table 3, the frequency of CTLm induced by *d*301, while substantial (compared to the lysis of mock-infected target cells), is lower than is seen for immunization with KOS1.1 (Table 4). However, immunization with *d*301 induces frequencies of gB498–505 CTLm that are similar for each time point, indicating that the number of these CTLm in *d*301-immunized mice is maintained over time. Together, these results indicate that the CTLm response following *d*301 immunization is potent and long-lived, although the overall magnitude of the CTL

TABLE 4. Long-term frequencies of CTLm induced by immunization with KOS1.1 and *d*301

Immunization[a]	Mo postimmunization	Target cells[b]	CTLm frequency (95% confidence limits)	Total CTLm/spleen (range)[c]
KOS1.1	1	gB498–505 pulsed	1/14,114 (1/10,694–1/20,752)	3,330 (2,265–4,395)
*d*301	1	gB498–505 pulsed	1/24,251 (1/17,960–1/37,324)	2,062 (1,340–2,784)
KOS1.1	9	gB498–505 pulsed	1/6,104 (1/4,649–1/8,884)	8,919 (5,628–10,755)
*d*301	9	gB498–505 pulsed	1/23,662 (1/18,180–1/33,714)	2,029 (1,424–2,640)
KOS1.1	22	gB498–505 pulsed	1/6,393 (1/4,853–1/9,366)	7,117 (4,858–9,367)
*d*301	22	gB498–505 pulsed	1/14,337 (1/10,880–1/21,018)	2,964 (2,022–3,906)
KOS1.1	1	Mock	1/4,539,230 (1/1,533,483–ND[d])	11 (1–31)
*d*301	1	Mock	1/4,539,230 (1/1,533,483–ND)	11 (1–33)
KOS1.1	9	Mock	1/4,603,026 (1/1,555,070–ND)	11 (1–32)
*d*301	9	Mock	1/4,539,230 (1/1,533,483–ND)	11 (1–33)
KOS1.1	22	Mock	1/4,571,454 (1/1,544,395–ND)	10 (1–29)
*d*301	22	Mock	1/299,544 (1/196,404–1/630,802)	142 (67–216)

[a] Spleens were removed from C57BL/6 mice 1 month, 9 months, or 22 months following intraperitoneal immunization with HSV-1 KOS1.1 or *d*301. Splenocytes were cultured with KOS1.1-infected B6/WT-3 stimulator cells.
[b] B6/K-1,4,5 cells were either pulsed with the synthetic peptide gB498–505 or mock infected. The target cells were labeled with ^{51}Cr, and lytic activity was determined by a standard ^{51}Cr release assay.
[c] Calculated by using the following formula: (CTLm frequency) × (viable cell yield/lymph node).
[d] ND, not determined.

response is somewhat lower than that induced by immunization with KOS1.1.

DISCUSSION

The results of the studies presented here illustrate that an anti-HSV CTL response can be elicited by immunization with a replication-defective mutant of HSV-1, *d*301. The results demonstrate that immunization with *d*301 is effective for the induction of both primary and memory CTL responses and that these responses are comparable in magnitude to those resulting from immunization with wild-type HSV (KOS1.1). Furthermore, the response induced by *d*301 limits viral replication in vivo, and both $CD4^+$ and $CD8^+$ T cells contribute to this protection. These findings imply that the generation of an effective CTL response is not dependent on production of progeny virus and that the limited expression of HSV genes during the truncated infection cycle of *d*301 is sufficient for the induction of CTL in vivo.

Immunization with HSV-1 has been demonstrated to induce both a primary (62, 63) and memory (43) CTL response. The predicted importance of the CTL response in limiting an HSV infection has led to an interest in the development of safe vaccine vectors that elicit CTL. To date, a variety immunogenic vectors have been shown to elicit HSV-specific CTL responses in murine models. For example, cell lines expressing either entire HSV glycoproteins (3, 5) or HSV-specific CTL recognition epitopes (7) have been shown to induce CTL activity. Recombinant viruses have also been shown to function as efficient vectors in generating CTL responses. As with the cell lines noted above, both intact HSV proteins and CTL recognition epitopes have been expressed in the context of recombinant vaccinia virus (2, 20, 50), adenovirus (21, 28), and baculovirus (25). Last, immunization with an HSV glycoprotein emulsified in a cationic lipid (78) and synthetic peptides representing CTL recognition epitopes (2, 10, 28) was shown to induce HSV-specific CTL. Live viruses are very effective in inducing significant CTL responses; however, the virulence associated with live virus immunization has raised concern about the safety of such approaches (80). An alternative to immunization with live viruses is the use of replication-defective mutants of HSV, such as the *d*301 virus, to evoke CTL responses.

A number of approaches have been used to induce effective immune responses. Early studies indicated that inactivated virus and extract from HSV-infected cells may be effective as therapeutic agents (12). The use of attenuated viruses, such as the R7020 virus (52), as vaccines has also shown promise. Recent clinical trials have evaluated the use of recombinant HSV glycoproteins as vaccines. Immunization with a vaccine containing recombinant gD from HSV-2 has been shown to induce immunity in recipients and to lessen the severity of HSV reactivation in seropositive patients (74). Initial phase I and II trials for the evaluation of a vaccine containing both gB and gD from HSV-2 demonstrated that immunization with this vaccine is effective in inducing both humoral and cellular immune responses (40).

The attraction of immunization with replication-deficient viruses, such as *d*301, is illustrated by the ability of these viruses to express a defined array of viral proteins, some of which may be immunogenic, during infection and by their inherent, limited pathogenic potential. Infection with *d*301 is characterized by a distinct pattern of viral gene expression. During the course of the abortive infection, α, β, and γ1 genes are expressed, but γ2 genes are not (22). Because *d*301 does not replicate, the input virus alone must express levels of immunogenic HSV proteins sufficient to evoke a CTL response. The level of gB expression, a γ1 gene product, during a *d*301 infection is of importance for these studies because this glycoprotein contains an immunodominant, H-$2K^b$-restricted CTL recognition epitope spanning residues 498 to 505 (10, 21, 28). Immunization with gB498–505, either as a synthetic peptide (10) or expressed within a recombinant vector (7, 20), results in the induction of an epitope-specific CTL response which is able to recognize and lyse HSV-infected cells. We confirmed that during a *d*301 infection, gB498–505 is efficiently expressed, processed, and presented in the context of H-$2K^b$ by demonstrating that cells infected with *d*301 are recognized by a gB498–505-specific CTL clone to the same extent as cells infected with KOS1.1 (Fig. 1B). Utilizing Western blot analysis, we showed that during a *d*301 infection, the kinetics and levels of gB expression are similar to that seen following infection with KOS1.1 (Fig. 1A). Taken together, these results verify that *d*301 expresses a significant quantity of gB during infection and support our finding that *d*301 immunization elicits a strong CTL response specific for gB498–505 (Fig. 2 and Table 1).

The evaluation of the immunogenicity of a replication-deficient virus, such *d*301, is of significant interest for the study of a virus-induced immune response. The ability of *d*301 virus to elicit an immune response has been addressed previously. In these studies, using a BALB/c model system, immunization with *d*301 had been shown to elicit the generation of HSV-specific antibody as well as to evoke a population of T cells that proliferate in vitro in response to HSV-specific stimulation (54, 55, 60). Overall, these results indicated that *d*301 is effective in inducing an anti-HSV immune response. However, the CTL response resulting from immunization with *d*301 was not specifically evaluated. The CTL component of the immune response has been indicated to be important for the resolution of the HSV infection in both mice (72, 73) and humans (64).

As in previous studies to determine the presence and magnitude of the HSV-specific CTL response, we used both bulk culture analysis (15, 62, 63) and LDA (27, 29, 35, 68). The results presented in this study demonstrate that immunization of C57BL/6 mice with *d*301 induces a primary CTL response with frequencies of CTLp that are approximately twofold lower than the frequencies for KOS1.1 despite the fact that the replication potential of KOS1.1 is significantly greater than that of *d*301. More importantly, immunization with *d*301 was able to induce a gB498–505-specific CTL response that was similar to that induced by KOS1.1. Our in vitro studies indicate that during an infection with *d*301, gB is expressed to nearly wild-type levels, suggesting that in vivo, a substantial quantity of gB may be expressed in the cells initially infected with *d*301. However, from a previous study (55), it is known that progeny HSV is not produced. Therefore, the antigenic load of gB resulting from infection with *d*301 is thought to be substantially less than that for KOS1.1. The ability of *d*301 to induce a strong CTL response specific for gB498–505 could be explained by the persistence of the virus in the infected tissue. Following footpad infection, *d*301 is detectable for 6 days in the tissue despite the lack of replication. From these studies, it had been suggested that this retention of virus results in a prolonged series of primary infection events leading to an effective induction of an immune response (55). To explore this hypothesis, LDA was used to measure frequencies of gB498–505-specific CTLp in mice immunized with various doses of either *d*301 or KOS1.1. KOS1.1 is able to replicate, producing substantial amounts of infectious virus, and therefore exceeding, even at the lowest immunizing dose (10^4 PFU), the threshold level of virus necessary to generate a significant CTL response as shown in Table 2. In contrast, immunization

with *d*301 introduces a small and finite source of antigen, which at the two higher doses used (10^6 and 10^5 PFU) is able to attain this threshold. However, at the lowest dose of *d*301 (10^4 PFU), this threshold may not be achieved, thus resulting in a substantially lower CTL response.

Immunization with *d*301 readily evokes an HSV-specific immune response, and the protection against subsequent HSV challenge mediated by this immune response is of significant interest. Previous studies have shown that BALB/c mice immunized with *d*301 are protected against a lethal challenge with HSV (60) and do not develop keratitis following ocular infection (54, 55). In these latter studies, immunization was also shown to reduce the establishment of latent infection within the trigeminal ganglia (54, 55). The components of the immune response necessary to control an HSV infection has been an area of debate. Innate immunity, including natural killer cells, interferons α and β, and macrophages, have all been implicated in controlling the initial spread of HSV (56, 81). The humoral response against HSV has also been demonstrated to diminish the neurovirulence by the passive transfer of HSV-specific antiserum; however, this response appears to have no effect on the cutaneous phase of the infection (16, 37, 41, 53, 71).

Significant interest has focused on the role of T cells in the protection against HSV infection. The T-cell response induced by HSV challenge is critical for the resolution of the infection, and both $CD8^+$ cells (8, 31, 41, 42, 53, 70, 72, 73) and $CD4^+$ cells (37, 44–46, 57–59, 73) have been shown to mediate anti-HSV effects. To evaluate the ability of the T-cell response generated in mice immunized with *d*301 to confer protection against HSV challenge, we used an adoptive transfer model. In this model, lymphocytes generated by immunization with *d*301 or KOS1.1 were transferred into C57BL/6 mice that had been immunosuppressed and infected with KOS1.1 (73). The results of these experiments indicate that immunization with *d*301 induces both $CD8^+$ and $CD4^+$ T cells that are effective in reducing the replication of HSV to an extent equivalent to the reduction afforded by the $CD8^+$ and $CD4^+$ T cells from KOS1.1-immunized mice. Furthermore, in this model, the $CD4^+$ T cells from *d*301-infected mice were shown to be more effective in reducing the level of infectious HSV (10-fold-lower titers) than were $CD8^+$ T cells. The ability of both $CD8^+$ and $CD4^+$ T cells induced by immunization with HSV-1 to reduce viral titers is in agreement with the findings of previous transfer experiments in this model system (73). The antiviral activities of the *d*301-induced CTL may be mediated by gamma interferon (IFN-γ), which has been previously demonstrated by studies in which in vivo depletion of IFN-γ resulted in the inability of otherwise immunocompetent C57BL/6 mice to control a cutaneous HSV infection (73). The importance of IFN-γ was further indicated by the demonstration that IFN-γ knockout mice were not effective in the limiting the replication of HSV following a corneal challenge (11). The adoptive transfer studies described herein clearly indicate a role for both $CD8^+$ and $CD4^+$ populations in mediating protection conferred by immunization with *d*301. The ability of $CD8^+$ and $CD4^+$ cells inherent to HSV-immunized mice to migrate to the site of infection and be activated to a functional phenotype has yet to be determined.

An important aspect in the evaluation of a vector to elicit antiviral immunity is the efficiency of immunization to generate a potent and durable memory response that is activated quickly following challenge. The immune responses generated and the protection conferred by immunization with *d*301 was found to be present up to 7 months (55). It had also been shown that 4 weeks following ocular immunization with either *d*301 or KOS1.1, the frequency of proliferating T cells in BALB/c mice immunized with *d*301 was significantly lower than that seen for KOS1.1 (1). Our studies, using bulk culture analysis and LDA, show that *d*301 induces a CTLm response and that this response is maintained for at least 22 months. The level of cytotoxicity exhibited by these CTLm is similar to the level for KOS1.1-generated CTLm; however, the frequency of *d*301 induced CTLm is lower than in KOS1.1-immunized mice. The CTLm frequencies resulting from immunization with KOS1.1 are consistent with previous studies (61, 68). The apparent discrepancy between the bulk culture data and the LDA data can be explained by the fact that lymphocyte numbers are normalized for bulk culture analysis but not for LDA. Normalization equalizes the number of lymphocytes between each experimental sample and allows a direct analysis of cytotoxicity. Our results suggest that while *d*301 is able to evoke a strong CTLm response, the overall magnitude of the response is lower than that induced by KOS1.1. A mechanism that may explain the induction of higher frequencies of CTLm by immunization with KOS1.1 is the capacity for wild-type HSV to establish a latent infection (79) and potentially provide a long-lived source of antigen. Previous evidence has indicated that restimulation of CTLm with viral antigen, while not required to maintain CTLm, increases the clonal burst size of the memory response, thus facilitating the detection of the CTLm by LDA (30). In contrast to wild-type HSV, latency is not readily established by *d*301 (55), and thus infection with *d*301 may not provide a source of viral antigen after clearance.

We have demonstrated that a replication-deficient mutant of HSV is able to elicit both primary and memory CTL responses. In addition, the T-lymphocyte component of the primary immune response was shown to limit the extent of primary HSV infection. Although the overall magnitude of the primary and memory CTL responses induced by immunization with *d*301 was slightly lower than the response induced by wild-type HSV, the protection afforded by *d*301 immunization is as effective as that for KOS1.1 immunization. This finding may reflect the effectiveness of $CD4^+$ cells to provide a level of protection and suggests that the $CD4^+$ response may play a critical role in controlling a cutaneous HSV infection. Together, these studies support the potential importance of replication-deficient viruses as immunogens for mediating protection against HSV and other viral infections.

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