Induction of Primary, Antiviral Cytotoxic, and Proliferative Responses with Antigens Administered via Dendritic Cells

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Cytotoxic T lymphocytes (CTL) play an essential role in recovery from viral infections, but induction of CTL responses with nonreplicating antigens is difficult to achieve. Exogenous antigens, such as viral proteins and peptides, normally induce CD4$^+$ T-cell responses unless appropriately delivered to the major histocompatibility complex class I antigen presentation pathway. In vitro studies performed to address this issue revealed a similar scenario, and primary CTL induction with nonreplicating antigens has rarely been reported. This study demonstrated primary antiviral CTL induction in vitro with exogenous antigens delivered in vivo to dendritic cells. This study also evaluated the efficacy of glycoprotein B peptide (free or encapsulated in liposomes), peptide–tripalmitoyl-S-glyceryl cysteinyl conjugate (acylpeptide), and glycoprotein B protein encapsulated in pH-sensitive liposomes as antigen delivery vehicles. Our results show that higher levels of cytotoxicity against herpes simplex virus type 1 resulted from exposure of dendritic cells to peptide–tripalmitoyl-S-glyceryl cysteinyl in liposomes. Macrophages treated in a similar manner were not effective stimulators for primary CTL induction. Our data have relevance to the understanding of mechanisms of antigen processing and presentation and the design of antiviral vaccines.

Class I-restricted CD8$^+$ cytotoxic T lymphocytes (CTL) are major components of antiviral immunity, particularly for recovery from infection (21, 25). In consequence, understanding how to best induce and control CTL is relevant in vaccine design. CTL usually recognize peptides derived from viral proteins synthesized within the cell in the context of major histocompatibility complex class I molecules (5, 13, 18, 34). In many cases, a few specific peptide fragments derived from only a single viral protein appear to dominate the response (4). Immunodominance of specific amino acid residues or epitopes in induction of CTL responses may depend upon the affinity of a peptide for major histocompatibility complex molecules, as well as the T-cell repertoire in the responding strain (3, 8). Identification of CTL epitopes and understanding how to induce CTL responses with them are, therefore, important aspects of vaccine development.

A limiting factor in the use of viral proteins or peptides as vaccines is that following administration they readily induce CD4$^+$ T-cell and antibody responses but usually fail to induce CD8$^+$ CTL (25). The endogenous synthesis requirement for CD8$^+$ CTL induction could be bypassed if antigen were delivered to an appropriate cytosolic compartment of antigen-presenting cells (10, 17, 20, 23, 26, 29, 30). One such approach is osmotic loading, found useful with ovalbumin and other soluble proteins (17). Delivery of proteins by means of immunostimulating complexes may also effect CTL priming (29). Certain peptides may be rendered CTL inducing if given with powerful adjuvants, such as Freund's or following modification by covalently coupling them to acyl groups (1, 10, 12, 26, 27). Recently, we have shown that nontoxic liposomes can be used to achieve CTL recognition

of soluble proteins (23). Moreover, if liposomes designed to disrupt under acid conditions (pH-sensitive liposomes) were used to deliver antigen to dendritic cells (DC), a delivery system capable even of primary CTL induction in vitro was obtained (19). In this study, we used the in vitro primary CTL induction approach to explore optimal and potentially practical means of inducing CTL responses to herpes simplex virus type 1 (HSV-1). Glycoprotein B (gB) of HSV was chosen for this study since this protein was shown recently to act as a major CTL target in H-2^b mice and a region (amino acids [aa] 489 to 515) that represents an immunodominant epitope has been identified (14, 32). Our results show that delivery of gB protein or gB peptide to DC, but not to macrophages (MØ), results in primary HSV-specific CTL responses. Moreover, optimal responses occurred when soluble proteins were delivered via liposomes and when the peptide was presented as the tripalmitoyl-S-glyceryl cysteinyl (PAM$_3$Cys) derivative. Our results set the stage for practical means of inducing antiviral CTL responses and are based on an approach useful for rapid evaluation of the CTL-inducing activities of proteins and peptides.

MATERIALS AND METHODS

Mice. Seven- to eight-week-old, retired breeder female C57BL/6 mice were obtained from Harlan Sprague Dawley, Indianapolis, Ind., or from Sasco, Omaha, Nebr. In conducting the research described here, the investigators adhered to the *Guide for the Care and Use of Laboratory Animals* as proposed by the Committee on Care of Laboratory Animal Resources Commission on Life Sciences, National Research Council. The facilities used are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

Antigens. HSV-1 strain KOS was propagated on Vero cell

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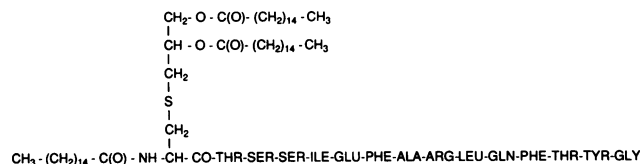CH₃ - (CH₂)₁₄ - C(O) - NH -CH - CO-THR-SER-SER-ILE-GLU-PHE-ALA-ARG-LEU-GLN-PHE-THR-TYR-GLY

FIG. 1. Structure of PAM₃Cys gB peptide (497–510).

monolayers and stored as infectious cell preparations at −70°C. Viral titers for HSV-1 KOS were expressed as 50% tissue culture infective doses. A recombinant vaccinia virus that expresses gB protein and a thymidine kinase-negative vaccinia virus (Vtk⁻) which does not contain the gB protein gene were grown on CV1 cell monolayers and titrated (9). Recombinant protein gB, which represents amino acids 1 to 696 of mature gB of HSV-2 strain 333, was generously supplied by Chiron Corporation, Emeryville, Calif. HSV-1 gB peptide 497–510 has been previously described as a potent CTL epitope in the H-2^b haplotype and was used in either free or tripalmitoylated form (Fig. 1). Peptides encompassing CTL epitopes in HSV-1 gB protein were synthesized on the basis of the T-cell epitope prediction schemes reported by Falk et al. (11, 24). The H-2^d specific peptide (aa 280 to 288; VYPYDEFVL) and the H-2^b-specific peptide (aa 498 to 505; SSIEFARL) were synthesized on resin to have unblocked (free) amino and carboxyl ends (Research Genetics, Birmingham, Ala.).

Mass spectroscopy (MS). The molecular weights of synthetic peptide and lipid products and intermediates were determined by MS with a JEOL HX-110 double-focussing mass spectrometer. A sample solution (usually about 1 nmol/μl) of the material to be analyzed was mixed with a few microliters of 3-nitrobenzyl alcohol. Ions were produced by fast atom bombardment with a 6-keV beam of Xe⁰ atoms. The acceleration voltage was 10 kV.

Synthesis of PAM₃Cys. S-[2,3-Bis-palmitoyloxy-(2RS)-propyl]-N-palmitoyl-(R)-cysteine (rac-PAM₃Cys), as a diastereomeric mixture of the R- and S-propyl configurations, was synthesized essentially as described by Weismüller et al. (31). Certain protocols were modified since synthetic intermediates of the desired purity could not be obtained solely by recrystallization. Thus, acylation of cystine di-*tert* butyl ester was done with palmitic anhydride with 4-pyrrolidino pyridine as a catalyst in place of 4-dimethylamino pyridine. Purification of the N,N'-dipalmitoyl-cystine di-*tert* butyl ester product was effected by flash chromatography over neutral alumina to remove excess palmitic acid, following an initial precipitation with methanol (−20°C). The N,N'-dipalmitoyl-cystine di-*tert* butyl ester was eluted from this column with chloroform (palmitic acid retained). The final yield of purified material was 48% from starting cystine di-*tert* butyl ester (mp, 78°C).

The intermediate S-[2,3-dihydroxy-(2RS)-propyl]-N-palmitoyl-(R)-cysteine *tert*-butyl ester was initially recrystallized by petroleum ether at −20°C and then purified by flash chromatography over neutral alumina by elution with a mixture of chloroform-ethyl acetate-acetic acid (3:3:1 [vol/vol/vol]; mp, 44°C; observed MH + 490.6 [by fast atom bombardment − MS]).

The intermediate S-[2,3-bis-palmitoyloxy-(2RS)-propyl]-N-palmitoyl-(R)-cysteine *tert*-butyl ester was purified by flash chromatography over neutral alumina, with elution first with hexane-ether (1:1) to remove impurities (higher R_f on silica gel 60 thin-layer chromatography) and then with chlo-

roform to elute the product. Any remaining palmitic acid-containing impurities were removed by flash chromatography over silica gel 60 using hexane-ether (1:1) to elute the rac-PAM₃Cys *tert*-butyl ester.

The above-described products and intermediates gave single spots by thin-layer chromatography and satisfactory melting points, proton magnetic resonance spectra, and MS data.

Peptide synthesis. The HSV-1 gB peptide (aa 497 to 510) was synthesized by solid-phase methodology on a glycyl-4-(hydroxymethyl)phenoxyacetyl polystyrene resin with an automated Applied Biosystems 431A peptide synthesizer with 9-fluorenylmethoxycarbonyl α-amino protection-based chemistry. The core sequence was Thr-Ser-Ser-Ile-Glu-Phe-Ala-Arg-Leu-Gln-Phe-Thr-Tyr.

The amino acid side chain protection included *t*-butyl for threonine, tyrosine, and serine hydroxyls; *t*-butyl for glutamic γ-carboxyl; 2,2,5,7,8-pentamethyl-chroman-6-sulfonyl for the guanidino group of arginine; and *t*-butyl for the cysteine sulfhydryl.

For the PAM₃Cys–HSV-1 peptide derivative (Fig. 1), the amino-terminal threonine was deprotected with a 20% solution of piperidine in N-methylpyrrolidinone. The peptidyl resin (100 mg), containing 24 μmol of peptide, was treated with 2 ml of an N-methylpyrrolidinone solution containing 32 mg (34.5 μmol) of PAM₃Cys, 15.93 mg (36 μmol) of benzotriazol-1-yloxytris-(dimethylamino)-phosphonium hexafluorophosphate, 5.5 mg (36 μmol) of 1-hydroxybenzotriazole, and 5.7 μl (52 μmol) of N-methylmorpholine. The mixture was shaken overnight, and the extent of coupling was found to be 95%, as determined by ninhydrin analysis of residual free amino groups. The peptidyl resin was filtered, washed with N-methylpyrrolidinone and dichloromethane, and dried. The PAM₃Cys-HSV peptide was cleaved from the resin by treatment with a mixture of 5 ml of trifluoroacetic acid (TFA), 0.25 ml of thioanisole, and 0.125 ml of water for 2 h at room temperature. The mixture was filtered, evaporated to dryness, and dissolved in 5 ml of dichloromethane. A 25-ml volume of acetone was added, and the mixture was kept at −20°C overnight. The precipitate was collected, dissolved in a minimal amount of N-methylpyrrolidinone, and purified by reversed-phase chromatography with a Vy-Dac C4 column with a mobile phase consisting of 20 to 80% isopropanol (containing 0.05% TFA) in 0.05% aqueous TFA buffer. The purified HSV peptide gave the correct amino acid analysis and the predicted parent ion by MS analysis (predicted mass, 2,512.6; observed mass, 2,512.1).

For the N-palmitoylated derivative, S-(*t*-butyl)-cysteine was added to the amino-terminal threonine of the peptidyl resin, followed by acylation with palmitic anhydride (2 equivalents). The N-palmitoylated peptide was cleaved from the resin (100 mg) by treatment with a solution of TFA (5 ml) containing phenol (6.3%), ethanedithiol (2%), thioanisole (4%), and water (4%). After 1.5 h at room temperature, the mixture was filtered, the resin was washed with a few milliliters of TFA, and the acyl peptide was precipitated by addition of a mixture of methyl *tert*-butyl ether (30 ml) and hexane (10 ml) prechilled to −20°C. The peptide was collected by centrifugation, and the precipitate was triturated twice with 40 ml of a chilled methyl *tert*-butyl ether–hexane (3:1) solution to remove scavengers. The acyl peptide was dried under high vacuum and used without further purification. It gave the expected amino acid composition and parent ion by MS analysis.

Lipids. Dioleoyl phosphatidylethanolamine, dioleoyl-Sn-3-succinyl glycerol, phosphatidylcholine, cholesterol, and

phosphatidylserine, used to prepare liposomes, were obtained from Avanti Polar Lipids, Birmingham, Ala. Labeling of antigen with ^{125}I was done with Iodogen (Pierce Chemical Co., Rockford, Ill.).

Preparation of liposomes. Dioleoyl phosphatidylethanolamine and dioleoyl-Sn-3-succinyl glycerol (10 μmol of total lipid) were used to make pH-sensitive liposomes containing gB protein as described elsewhere (33). Briefly, the lipid mixture was dried with N_2 gas and vacuum desiccated. The lipid film was resuspended in 50 μl of 50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer, pH 8.0, and 200 μl of 0.25 M sucrose and incubated at 4°C for 48 to 72 h. The hydrated suspension was sonicated to form small unilamellar vesicles. Unlabeled gB protein and ^{125}I-labeled gB protein were added to the liposomes, and the mixture was subjected to three cycles of freezing (1 min in liquid N_2) and thawing (3 h at 4°C). The free, unentrapped protein was separated by being passed over a Bio-Gel A 1.5 M column equilibrated with phosphate-buffered saline (PBS), pH 8.0.

Phosphatidylcholine, cholesterol, and phosphatidylserine, at a molar ratio of 7:3:1, were used to make multilamellar vesicles (MLV) containing tripalmitoylated gB peptide and free gB peptide (11 μmol of total lipid). The final lipid-to-protein ratio of the liposomes was 40:1. The lipid mixture was dried to a thin film with N_2 gas and vacuum desiccated for 60 min. MLV were prepared by hydrating the lipid film in PBS, pH 7.0, containing gB peptide and vortexing it for 5 min. Rehydration was continued for an additional 2 h with intermittent vortexing every 15 min. The unincorporated gB peptide was removed by washing the liposomal preparation three times with PBS (pH 7.0) at 30,000 $\times$ g for 15 min, and the pellet was resuspended in 500 μl of PBS. Efficiency of incorporation was determined with ^{125}I-labeled tracer gB peptide, and the entrapment efficiency routinely obtained was 45 to 50%. From 75 to 100 μg of peptide was injected per mouse.

Target cells for cytotoxicity assays. The tumor cell lines used were EL4 (C57BL/6 *H-2^b* lymphoma), EMT6 (BALB/c *H-2^d* mammary adenocarcinoma cells kindly provided by Ed Cantin, City of Hope National Medical Center, Duarte, Calif.), and YAC-1 (mouse *H-2^d* lymphoma). All cell lines were cultured in Dulbecco modified Eagle medium (GIBCO, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal calf serum (GIBCO).

Antigen-presenting cells (APC) and responder T cells. Splenocytes obtained from naive C57BL/6 female retired breeders were treated with ammonium chloride-containing Tris buffer for 3 min to deplete erythrocytes. Splenocytes (3 ml), at 2 $\times$ 10^7/ml, were layered over a 2-ml metrizamide gradient column (analytical grade; 14.5 g added to 100 ml of PBS, pH 7.0; Nycomed Pharma AS, Oslo, Norway) and centrifuged at 600 $\times$ g for 10 min. Cells from the interface were collected, and fluorescence-activated cell sorter (FACS) analysis showed 57% DC (monoclonal antibody [MAb] 33D1, kindly provided by Ralph Steinman, The Rockefeller University) (22), 1% MØ (MAb F4/80) (2), 22% T cells, and 16% B cells (data not shown). The pellet was resuspended and allowed to adhere for 1 h. More than 75% of the adherent population was identified as MØ by FACS analysis, with 5% lymphocytes and <5% DC. B cells were separated from the nonadherent population by panning on anti-immunoglobulin-coated plates. The separated cell population, which consisted of >80% T lymphocytes (as determined by FACS analysis), was used as responder naive T cells.

Pulsing of APC. For primary in vitro assays, DC or MØ (5

$\times$ 10^6) were pulsed in vitro with HSV-1 (multiplicity of infection [MOI], 5) or vaccinia virus gB or Vtk$^-$ at an MOI of 10 in 3 ml of RPMI 1640 for 3 h at 37°C. After 3 h, APC infected with virus were washed and used as stimulators. For in vivo pulsing of APC, groups of five young mice were intravenously injected with antigen (50 to 100 μg of gB protein in pH-sensitive liposomes, 75 to 100 μg of gB peptide in MLV, 75 to 100 μg of tripalmitoylated gB peptide in MLV, 100 μg of free gB protein, or 100 μg of gB peptide or empty liposomes) and 3 h later their spleens removed for isolation of DC and MØ, washed, and used as stimulators for primary in vitro CTL induction.

Induction of in vitro proliferative responses. DC and MØ (10^5/ml) pulsed with virus, protein, or peptide (as described in the previous paragraph) were added to naive responder T cells in 96-well U-bottom plates at responder-stimulator (R/S) ratios ranging from 100:1 to 0.78:1. The total volume per well was 200 μl of NCTC 109 and RPMI 1640 (1:1 [vol/vol] GIBCO) supplemented with 10% heat-inactivated fetal calf serum, 10 mM L-glutamine (GIBCO), 1 mM oxaloacetic acid (Sigma), 0.2 U of bovine insulin (Sigma) per ml, and 5 $\times$ 10^{-5} M 2-mercaptoethanol. After 3 days at 37°C, cultures were pulsed with 1 μCi of [^{3}H]thymidine (ICN Pharmaceuticals). Incorporation of [^{3}H]thymidine into cellular DNA was determined after 18 h by β-scintillation (INOTECH Trace 96 counter) and used as a measurement of lymphocyte proliferation.

Induction of HSV-1-specific CTL in vitro. Purified T cells (2.5 $\times$ 10^6/ml) from naive C57BL/6 mice were cultured with DC or MØ (10^5/ml) to give an R/S ratio of 25:1 in 20 μl of NCTC 109 and RPMI 1640 (1:1 [vol/vol]; GIBCO) supplemented with 10% heat-inactivated fetal calf serum, 10 mM L-glutamine, 1 mM oxaloacetic acid, 0.2 U of bovine insulin per ml, and 50 μl of 2-mercaptoethanol. Cultures were in 10-μl wells of Terasaki plates (Nunc, Naperville, Ill.), and the plates were incubated upside down, to form hanging drops, in a well-humidified and gassed incubator at 37°C. After 5 days, the hanging drops were pooled and used as effectors in a standard 4-h ^{51}Cr release assay.

Cytotoxicity assay. For HSV-1-specific lysis, 2 $\times$ 10^6 target cells in 500 μl of RPMI 1640 were labeled with 100 μCi of ^{51}Cr and simultaneously infected with HSV-1 at an MOI of 5 or Vtk$^-$ at an MOI of 10 for 3 h. For peptide-specific lysis, 2 $\times$ 10^6 target cells were labeled in 500 μl of RPMI 1640 with 100 μCi of ^{51}Cr for 90 min. After being washed, 10^4 labeled target cells and serial dilutions of effector cells were incubated in 200 μl of RPMI 1640 with 10% heat-inactivated fetal calf serum in 96-well V-bottom plates with appropriate peptides at concentrations of 7.5 to 10 μg/ml. The plates were centrifuged at 500 $\times$ g for 3 min and incubated at 37°C in 5% CO_2 for 4 h. A 100-μl volume of the supernatant fluid was collected to measure radioactivity, and specific cytotoxic activity was determined with the following formula: % specific release = [(experimental release − spontaneous release)/(total release − spontaneous release)] $\times$ 100. Each assay was performed in triplicate, and the spontaneous release was less than 25% of the total release by detergent in all assays.

Flow cytometry. Antigen-pulsed DC and uninfected DC were incubated with naive responder T cells in 96-well U-bottom plates and incubated for 4 days at 37°C. T cells were pooled on day 4, washed with PBS containing 3% bovine serum albumin, and used for FACS analysis. Cells (10^6) were left unstained or stained with a combination of either fluorescein isothiocyanate-conjugated rat anti-mouse L3T4 (CD4) MAb and R-phycoerythrin-conjugated rat anti-

TABLE 1. Induction of in vitro primary proliferative responses

Group[a]	Mean [³H]thymidine uptake (cpm) ± SD (SI)[b] at R/S ratio of:	
	12.5:1	6.25:1
DC-uninfected	294 ± 79	115 ± 35
DC–HSV-1	1,677 ± 522 (5.7)	702 ± 235 (6.1)
DC-gB peptide (497–510)	1,117 ± 358 (3.8)	467 ± 189 (4.1)
DC-empty liposomes	334 ± 103	202 ± 68
DC-gB peptide (PAM₃Cys) liposomes (497–510)	3,219 ± 481 (9.6)	2,104 ± 305 (10.4)
DC-gB peptide liposomes (497–510)	2,010 ± 345 (6.0)	1,802 ± 371 (8.9)
DC-gD peptide liposomes (1–23)	1,985 ± 420 (5.9)	1,865 ± 287 (9.2)
MØ–uninfected	344 ± 129	143 ± 29
MØ–HSV-1	594 ± 70 (1.7)	179 ± 42 (1.2)
MØ-empty liposomes	477 ± 183	185 ± 47
MØ-gB peptide (PAM₃Cys) liposomes (497–510)	1,141 ± 323 (2.3)	558 ± 73 (3.1)

[a] Naive mouse (C57BL/6) spleens were used to isolate T cells and APC. APC were treated with peptide in vivo or with virus in vitro for 3 h as described in Materials and Methods. Responder T cells (10⁷/ml) were incubated with APC at various R/S ratios in 96-well U-bottom plates. [³H]thymidine incorporation was measured in a dry β-scintillation counter. Results are means of 10 replicate cultures plus or minus 1 standard deviation of the mean at R/S ratios of 12.5:1 and 6.25:1. The data shown are representative of three independent experiments performed with similar results.

[b] SI (stimulation index) = [responders + treated APC (virus, peptide or liposomal peptide)]/[responders + untreated APC (mock-infected or empty liposomes)].

mouse Lyt-2 (CD8) MAb (Pharmingen, San Diego, Calif.) or fluorescein isothiocyanate-conjugated rat anti-mouse PgP-1 (CD44) MAb and R-phycoerythrin–CD8 (Pharmingen) MAb for 1 h on ice. Cells were washed and fixed with 2% paraformaldehyde, and double-color fluorescence analysis was performed by using FACS flow cytometry (Becton Dickinson, Mountain View, Calif.) and a 9153C computer system Hewlett-Packard, Palo Alto, Calif.).

RESULTS

Induction of in vitro primary proliferative responses. As shown in Table 1, splenic DC, when pulsed with antigen in vitro (HSV-1) or in vivo (peptides), stimulated potent in vitro proliferative responses in naive responder T cells within 3 days. Whereas antigen administered with DC regularly provided responses, when the same antigen was presented with MØ insignificant proliferative responses were observed. Positive responses (stimulation index, ≥2) were achieved with all forms of peptide tested at R/S ratios of 50:1 to 6.25:1, but maximal effects occurred when peptides were acylated with PAM₃Cys and incorporated into liposomes (Table 1). Not shown is the fact that in separate experiments DC pulsed in vivo with gB protein in liposomes induced primary proliferative responses.

To determine the nature of cells proliferating in the cultures, cells were harvested and FACS analysis was performed to identify cells that expressed the CD4⁺ or CD8⁺ phenotype, as well as PgP-1, a marker for activated cells (7). As shown in Fig. 2A and B, unstimulated cells contained both CD4⁺ and CD8⁺ T cells but none additionally expressed PgP-1. However, after antigen stimulation, both CD4⁺ PgP⁺ and CD8⁺ PgP⁺ cells were present (Fig. 2C, D, and E). Of the two populations, CD4⁺ PgP⁺ cells predominated, but with all of the systems studied, CD8⁺ PgP⁺ cells were also present. Although the antigen specificity characteristics of proliferating cells were not analyzed, the CD8⁺ PgP⁺ population presumably represented the precursors of the CTL described below.

Induction of in vitro primary CTL responses. Microcultures of antigen-pulsed APC and naive responder T cells were established in Terasaki plates, and cultures were harvested after 5 days for measurement of HSV-specific and peptide-specific CTL responses. Optimal responses were obtained at an R/S ratio of 25:1, and all of the data in Table 2 reflect these conditions. As shown in Table 2, CTL responses were generated but only when DC were used as APC. This was further established by treating DC pulsed with HSV-1 with MAb 33D1 and complement in vitro to deplete DC, which effectively removed the CTL-inducing activity of the cell population (data not shown). Major histocompatibility complex class I-restricted anti-HSV-1 responses were induced in T cells stimulated with DC pulsed with HSV-1, gB protein, or H-2ᵇ-recognized gB peptide 497–510. Cytotoxicity did not occur in cultures with DC pulsed with control gD peptide 1–23. DC pulsed with gD peptide in MLV, however, did induce proliferative responses (Table 1). Cytotoxicity was also detected in the HSV-positive cultures by using targets pulsed with H-2ᵇ-recognizable minimal peptide 498–505, although not by using targets pulsed with H-2ᵈ-recognizable peptide 280–288. In separate experiments, peptides 498–505 and 280–288 were also used to induce CTL responses. Both HSV-specific and peptide-specific responses were induced by gB peptide 498–505 but not by 280–288 (Table 3). In these experiments, DC were pulsed with peptides in vitro. Also shown in Table 3 is the fact that DC pulsed with vaccinia virus expressing gB protein in vitro stimulated potent anti-HSV-1 and peptide-specific cytotoxic responses.

Liposome incorporation was necessary to obtain CTL responses with the gB protein antigen (Table 2). In this instance, liposomes were of the pH-sensitive type since previous studies with ovalbumin had shown that pH-sensitive liposomes are superior to insensitive MLV for CTL induction (19). With peptide 497–510, primary CTL induction occurred with DC from animals exposed to either free peptide or liposome-delivered peptide. The latter form was, however, marginally superior. Consistently superior CTL responses were obtained in cultures with DC from animals pulsed with PAM₃Cys-peptide in liposomes. Since this form of the peptide was insoluble in aqueous buffers, it was not administered to animals in the nonliposomal form. In three separate experiments, in addition to that whose results are given in Table 2, PAM₃Cys-peptide routinely induced greater HSV-1-specific and peptide-specific responses than did the other immunogens investigated.
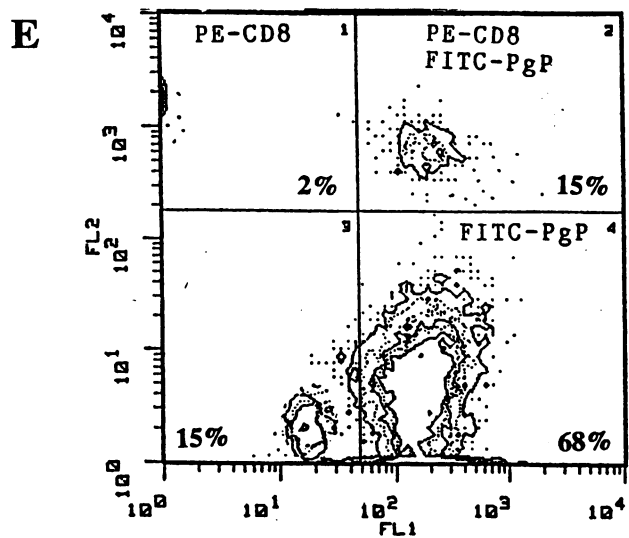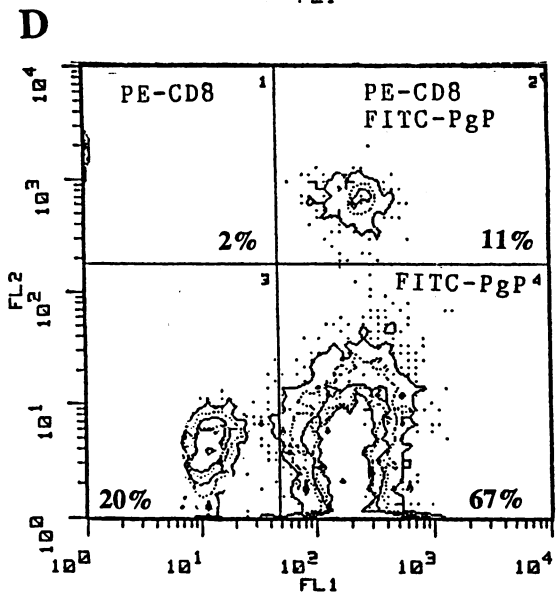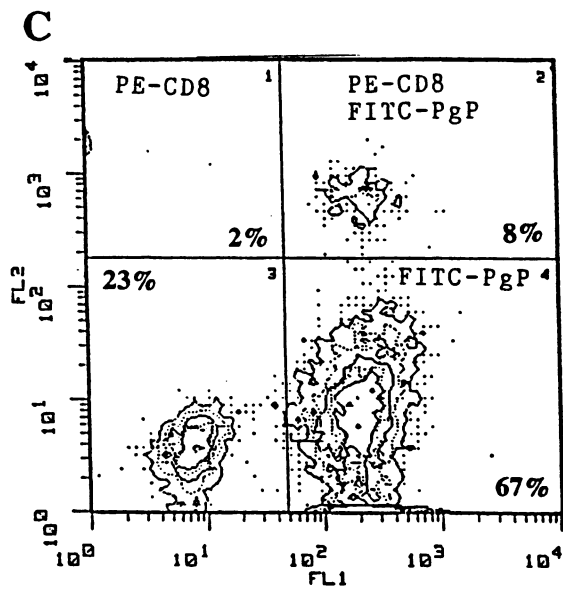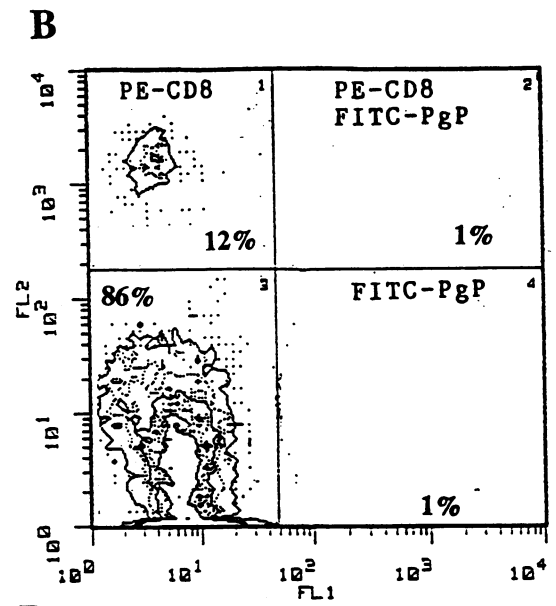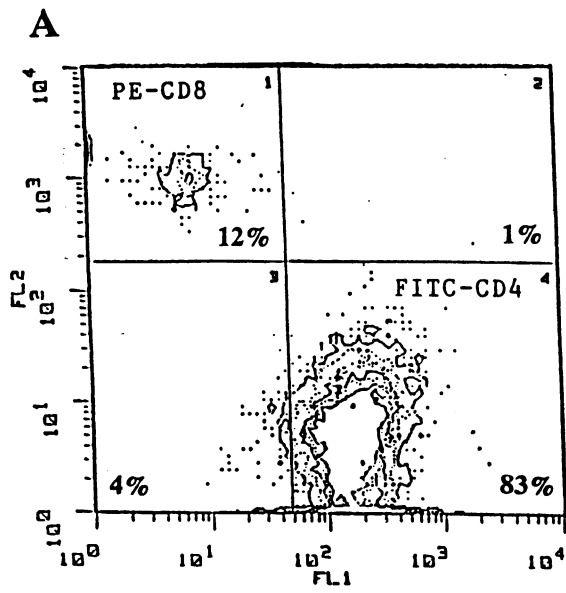
FIG. 2. Representative profiles of CD4, CD8, and PgP-1 expression on proliferating T cells poststimulation with APC. Panels: A, uninfected DC, (percentage of CD4 and CD8 T cells by dual-color fluorescence analysis with fluorescein isothiocyanate-CD4 and phycoerythrin-CD8); B, C, D, and E, percentage of activated CD4 and CD8 T cells following stimulation with fluorescein isothiocyanate–PgP-1 and phycoerythrin-CD8. Activated CD8 T cells which are both CD8$^+$ and PgP-1$^+$ are in quadrant 2. Unstimulated CD8$^+$ cells are in quadrant 1. Cells in quadrant 4 represent cells which are presumably activated CD4$^+$ T cells. Panels: B, uninfected DC; C, HSV-1-infected DC; D, DC treated with gB peptide (497–510) in liposomes; E, DC treated with PAM$_3$Cys gB peptide (497–510) in liposomes.

DISCUSSION

In this study, we confirmed the identity of a major CTL-recognizable epitope on gB of HSV-1 in H-2^b mice and showed the minimal peptide capable of inducing a primary CTL response in vitro (Table 3). Accordingly, by using an in vitro culture technique that successfully generates CTL, cells specific to HSV and the H-2^b-recognizable gB peptide 498–505 were obtained. Optimal responses to the gB peptide occurred with a PAM$_3$Cys version incorporated into liposomes. In addition, primary CTL responses to gB protein occurred only following its delivery within pH-sensitive liposomes.

Achievement of CTL induction depended on the use of DC as APC and a culture system, devised originally by Macatonia et al. (15, 16), that permitted dense cell concentrations. In most instances, DC were primed by injection of antigen in vivo, followed after 3 h by isolation of DC from the spleens of such antigen-pulsed animals. This approach was used since previous studies with ovalbumin had indicated that antigen pulsing of DC in vivo is more effective than antigen pulsing in vitro (19), but the reasons for the greater efficacy of in vivo exposure were not elucidated.

It is becoming increasingly evident that DC represent superior antigen-presenting cells not only for CTL induction but also for other types of T-cell responses (15, 16, 19). This is true for in vivo and in vitro CTL induction, although the present report represents one of the few which have successfully achieved in vitro CTL induction. Satisfactory molecular explanations for the superior APC activity of DC still have not been provided. However, possibilities include high levels of major histocompatibility complex class I and II

expression, lower levels of sialic acid residues, and high levels of adhesion molecules (reviewed in reference 28).

It is now well established that exogenously applied non-replicating antigen becomes capable of inducing CD8$^+$ CTL responses only if the antigen gains access to appropriate compartments within the cytoplasm, where processing may occur (6). This usually means that the antigen must escape from the endosome, where either complete digestion or processing for class II-restricted T-cell recognition occurs. For protein delivery, several systems which achieve CTL recognition have been described. These include osmotic loading (17) and delivery via immunostimulating complexes (29), cationic lipids (20, 30), and pH-sensitive liposomes (19). The latter approach was presumed to function because such liposomes disrupt and become fusion active upon exposure to the acidifying conditions of the endosomes (33). Interestingly, primary CTL responses to gB occurred only when antigen was delivered to DC via pH-sensitive liposomes and were absent when DC were merely exposed to soluble gB. This was true both in vivo (Table 2) and in vitro (data not shown).

Whereas protein delivery to DC appears to require a specialized delivery system to become CTL inducing, this was not the case for simple peptides. Thus, the 14-aa gB peptide readily induced CTL upon delivery to DC in soluble or liposomal form. However, optimal immunogenicity was obtained when the peptide was derivatized with a triacyl tail and administered via liposomes. Exactly why such peptides effect greater CTL-inducing activity was not determined. However, likely possibilities include more effective use of peptide since the triacylated liposome-associated form may

TABLE 2. Induction of primary CTL responses in vitro

Group[a]	Mean % specific lysis at E/T ratio of 50:1 ± SD (LU$_{10}$)[b]				
	HSV-infected EL4	EL4 gB peptide (498–505; H-2^b)	Mock-infected EL4	EL4 gB peptide (280–288; H-2^d)	HSV-infected EMT6
DC–HSV-1	19 ± 2 (3.7)	38 ± 1 (7.1)	0 ± 1	1 ± 4	3 ± 3
DC-gB peptide (497–510)	15 ± 3 (3.0)	25 ± 1 (4.0)	2 ± 2	2 ± 1	4 ± 2
DC-gB peptide in liposomes (497–510)	15 ± 6 (5.0)	26 ± 4 (8.0)	2 ± 1	1 ± 3	2 ± 3
DC-gD peptide in liposomes (1–23)	3 ± 1	2 ± 3	1 ± 2	0 ± 4	1 ± 3
DC-gB peptide (PAM$_3$Cys) (497–510) in liposomes	56 ± 7 (75.0)	38 ± 4 (20.0)	0 ± 1	1 ± 3	0 ± 2
DC-gB protein in pH-sensitive liposomes	16 ± 1 (3.2)	39 ± 2 (10.0)	0 ± 1	2 ± 2	4 ± 1
MØ–HSV-1	7 ± 4 (1.2)	3 ± 2 (1.0)	1 ± 2	2 ± 2	3 ± 1
MØ-gB peptide (497–510; H-2^b)	2 ± 1	1 ± 2	1 ± 1	2 ± 1	2 ± 2
MØ-gB peptide in liposomes (497–510)	5 ± 2	0 ± 2	2 ± 3	3 ± 2	0 ± 4
MØ-gB peptide (PAM$_3$Cys) (497–510) in liposomes	6 ± 1 (1.0)	0 ± 3	1 ± 2	4 ± 1	10 ± 4
MØ-gB protein in pH-sensitive liposomes	5 ± 1	1 ± 2	0 ± 2	2 ± 3	2 ± 2

[a] DC or MØ pulsed with HSV-1 in vitro for 3 h at 37°C or pulsed in vivo with gB peptide (free or encapsulated in liposomes), gD peptide in liposomes, PAM$_3$Cys gB peptide in liposomes, gB protein (free or in pH-sensitive liposomes) or empty liposomes for 3 h and added to naive T cells in 20-μl hanging-drop cultures in vitro. A standard ^{51}Cr release CTL assay was done on day 5 as described in Materials and Methods. DC and MØ treated with soluble gB protein or empty liposomes failed to induce levels of CTL above 3%, and those were considered negative.

[b] The lytic unit (LU) defined as the number of cells required for 10% target cell lysis. LU$_{10}$ was calculated by using linear regression analysis, and the coefficient r was significant (0.9 to 1.0) for all groups. The value is expressed as lytic units per 10^6 lymphocytes.

TABLE 3. In vitro primary CTL induction with DC pulsed with peptide in vitro

Group[a]	Mean % specific lysis at E/T ratio of 50:1 ± SD			
	HSV-1-infected EL4	EL4 gB peptide (498–505)	EL4 gB peptide (280–288)	Vtk⁻-infected EL4
DC–HSV-1	23 ± 2	34 ± 4	0 ± 1	1 ± 1
DC-vaccinia virus gB	58 ± 1	44 ± 1	1 ± 2	19 ± 2
DC-Vtk⁻	1 ± 1	2 ± 2	2 ± 1	23 ± 3
DC-gB peptide (498–505)	40 ± 1	35 ± 2	2 ± 1	0 ± 1
DC-gB peptide (280–288)	1 ± 4	1 ± 5	1 ± 3	1 ± 1

[a] DC were pulsed in vitro with gB peptide (3.6×10^{-5} M) for 90 min at 37°C or HSV-1 (MOI, 5) or vaccinia virus gB or Vtk⁻ (MOI, 10) for 3 h at 37°C as described in Materials and Methods. Pulsed DC were added to naive T cells in 20-μl cultures at an R/S ratio of 25:1 in Terasaki plates. A standard ^{51}Cr release assay was done on day 5. Control target cells, i.e., mock-infected EL4 cells and EMT6 cells infected with HSV-1, showed negative lysis.

be more stable and may better attach to the DC membrane and trigger uptake. The form of antigen may also cause additional effects in DC that are important for antigen presentation. These could include induction of certain cytokines or enhanced expression of proteins involved in processing, such as proteases and peptide transporters. Whether these latter effects do in fact occur in DC is currently under investigation.

In conclusion, our results demonstrate the necessity of delivering antigen to DC to achieve optimal CTL responses in vitro. Studies are currently under way to establish the phenotype of the CTLs generated and determine the requirement of CD4⁺ T cells for CTL induction. The culture system we have described promises to be a useful one to guide the selection of peptides for use in vivo to induce a relevant CTL response. It is also evident that if an antigen delivery system is to achieve satisfactory CTL induction in vivo, it should be designed to maximally deliver antigen to DC.

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