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Immunology

Cationic lipids direct a viral glycoprotein into the class I major histocompatibility complex antigen-presentation pathway

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ABSTRACT **Recombinant glycoprotein B (gB) of herpes simplex virus (HSV) was processed and presented by class I major histocompatibility complex (MHC) molecules after delivery into cells by using *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methyl sulfate (DOTAP), a commercially available cationic lipid used for DNA transfection. Cells treated with DOTAP-associated gB were susceptible to lysis by class I MHC-restricted, HSV-specific cytotoxic T lymphocytes (CTL), and the treated cells restimulated memory gB-specific CTL activity in spleen cells from HSV-infected mice. gB-specific CTL responses were detected in mice immunized with recombinant gB and DOTAP but not in those receiving gB emulsified in complete Freund's adjuvant. Thus, cationic lipids may facilitate induction of CD8$^+$ T-cell responses in vaccinations with recombinant antigens, and they may serve as readily available reagents for dissecting class I MHC immunity to viruses and other intracellular pathogens.**

Cytotoxic T lymphocytes (CTL) expressing the CD8 antigen recognize short peptides of 7 to 9 amino acids that are associated with class I major histocompatibility (MHC) molecules (1–3). Processing of a foreign protein into peptides for class I MHC presentation occurs when the antigen is synthesized in the cytoplasm or endoplasmic reticulum of antigen-presenting cells (APC) (4, 5) or is introduced into the cytosol by pH-sensitive liposomes (6, 7) or osmotic lysis of pinosomes (8). In contrast, proteins internalized by endocytosis generally do not sensitize cells for CD8$^+$ T-cell recognition (5, 9). These data indicate that priming of CD8$^+$ T-cell responses by recombinant subunit vaccines will require delivery vehicles that direct antigens to the cytoplasm of the APC. To date, priming of CTL responses with intact proteins has been achieved by incorporation of the antigen into immunostimulating complexes (ISCOMS) (10) or liposomes (11–13). In this report, we demonstrate that commercially available cationic lipids deliver glycoprotein B (gB) of herpes simplex virus (HSV) into the class I MHC pathway of APC and that immunization of mice with gB/cationic lipid complexes elicits CD8$^+$ CTL responses.

MATERIALS AND METHODS

Viruses and Viral Proteins. Stocks of HSV-1 strain Patton were prepared and titered by plaque assay in Vero cells (14). Recombinant protein gB-(23–718), which represents amino acids 23–718 of gB of HSV-2 strain 333, was produced in CHO cells and purified as described in detail elsewhere (15). This protein is a soluble glycosylated form of gB that retains native structure as determined by binding of conformation-sensitive neutralizing antibodies (G. Ott, Chiron, personal communication).

A 15-amino acid peptide [gB-(495–509)] representing residues 495–509 of the gB sequence was synthesized by using the *t*-butoxycarbonyl method and an Applied Biosystems 430A synthesizer and was purified by HPLC.

Immunization of Mice and Cytotoxic T-Cell Cultures. Female C57BL/6 (H-2^b) mice were purchased from Charles River Breeding Laboratories and used between 6 and 12 weeks of age. Mice were immunized subcutaneously at the base of the tail with an emulsion containing 25 μg of gB-(23–718) and complete Freund's adjuvant (CFA). The emulsion was created by vigorous mixing of equal volumes of phosphate-buffered saline (PBS) containing the antigen and CFA. Alternatively, 25 μg of gB-(23–718) was mixed with 15 μg of *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methyl sulfate (DOTAP; transfection reagent from Boehringer Mannheim). Priming was followed by two boosts at weekly intervals with 25 μg of gB-(23–718) that was emulsified in incomplete Freund's adjuvant or mixed with DOTAP, respectively. Seven days after the last immunization, 3–4 $\times$ 10^7 spleen cells were cocultured with 2 $\times$ 10^6 thioglycollate (Difco)-induced peritoneal exudate cells (PEC) in 4 ml of culture medium (CM) (RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum and 50 μM 2-mercaptoethanol) containing gB-(495–509) at 10 μg/ml. Virus-specific CTL activity was assessed 5 days later.

Primary HSV-specific CTL were generated as described (16) by subcutaneous injection of mice in the hind footpad with 5 $\times$ 10^5 plaque-forming units of HSV-1. Draining lymph node cells were removed 5 days later, cultured for 3 days in CM, and tested for CTL activity.

Target Cell Preparation. MC57 (H-2^b) and SVBalb (H-2^d) cell lines, which express class I but not class II MHC molecules, were kindly provided by K. Rosenthal (McMaster University, Hamilton, ON). Target cells were infected with HSV-1 at a multiplicity of infection of 10 for 4 hr or were incubated for 1 hr with 10 μg of gB-(495–509).

DOTAP was used according to the manufacturers instructions for transfection of DNA into cells. Briefly, 30 μg of DOTAP in 100 μl of PBS was mixed with gB-(23–718) in an equal volume of PBS. After a 10-min incubation at room temperature, this mixture was diluted to 5 ml with PBS containing 1% fetal calf serum and applied to a 70–80% confluent monolayer of target cells in a 60-mm tissue culture dish. After an overnight incubation, cells were washed once and used as targets in a cytotoxic cell assay.

Brefeldin A (BFA). Where indicated, BFA was added to target cells at the same time as gB-(23–718) and DOTAP, and it was present in the CM at a concentration of 10 μg/ml for the duration of the CTL assay.

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Abbreviations: CTL, cytotoxic T lymphocyte(s); MHC, major histocompatibility complex; APC, antigen-presenting cells; HSV, herpes simplex virus; gB, glycoprotein B; CFA, complete Freund's adjuvant; DOTAP, *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methyl sulfate; PEC, peritoneal exudate cells; BFA, brefeldin A; E:T ratio, ratio of effector to target cells.

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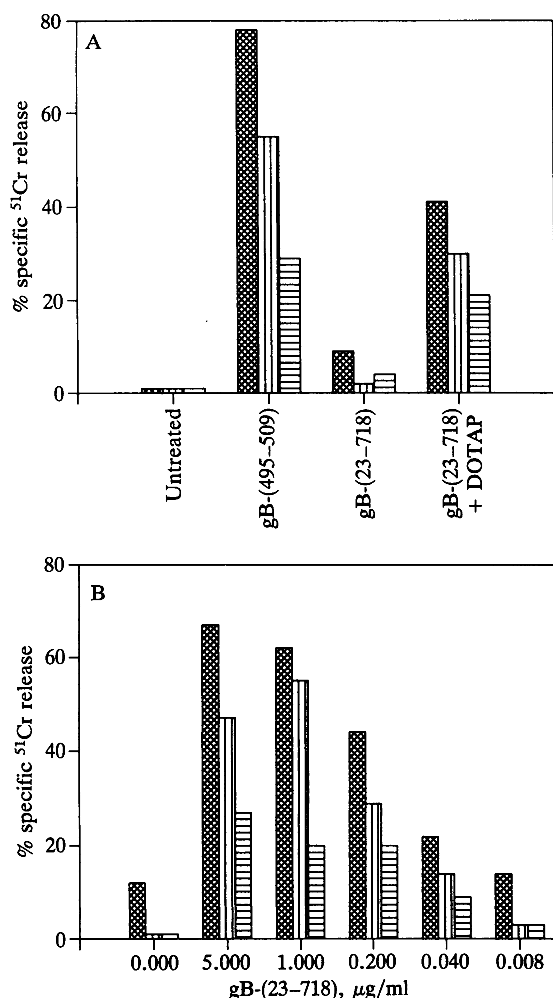

FIG. 1. Cytotoxic activity against target cells sensitized with gB-(23–718) and DOTAP. The ability of HSV-specific effector cells from infected C57BL/6 mice to lyse target cells in a 4-hr ^{51}Cr release assay at E:T ratios of 50:1 (cross-hatched bars), 10:1 (vertically lined bars), or 2:1 (horizontally lined bars) was assessed. (*A*) Target cells were histocompatible MC57 cells that were treated with gB-(495–509) or gB-(23–718) at 25 μg/ml in the presence or absence of DOTAP. (*B*) MC57 cells were treated with 30 μg of DOTAP and the indicated concentration of gB-(23–718) and assessed for their sensitivity to lysis by HSV-specific CTL.

Cytotoxic Cell Assay. Target cells were labeled with 50 μCi (1 Ci = 37 GBq) of $Na^{51}CrO_4$ (^{51}Cr) for 60 min. After washing, 5×10^3 target (T) cells were cultured with effector (E) cells at various E:T ratios in 200 μl of CM in 96-well round-bottom tissue culture plates for 4 hr. The average cpm from duplicate wells was used to calculate percent specific ^{51}Cr release, as described (17).

RESULTS AND DISCUSSION

Target Cell Sensitization with gB-(23–718) and DOTAP. Class I MHC-restricted CTL from HSV-1-infected C57BL/6 (H-2^b) mice lyse autologous target cells expressing the gB protein (18). Epitope mapping studies have revealed that this gB-specific CTL activity is directed against an 11-amino acid sequence (Thr-Ser-Ser-Ile-Glu-Phe-Ala-Arg-Leu-Gln-Phe) (19) that is conserved in most HSV-1 and HSV-2 strains (15). As shown in Fig. 1*A*, HSV-specific CTL from infected C57BL/6 mice recognized histocompatible MC57 (H-2^b) cells sensitized with gB-(495–509), a synthetic 15-amino acid peptide that contains the CTL epitope. This result is consistent with the hypothesis that short peptides associate directly with empty class I MHC molecules on the cell surface (20, 21). MC57 cells were not lysed by anti-HSV CTL after incubation with the recombinant gB-(23–718) protein. However, when gB-(23–718) was mixed with the cationic lipid DOTAP prior to incubation with MC57 cells, significant levels of cytotoxicity were observed. Specific lysis of allogeneic SVBalb cells treated with gB-(23–718) and DOTAP or gB-(23–718) alone was less than 10% at all three E:T ratios tested, indicating that the effector cells were class I MHC-restricted CTL. DOTAP-mediated sensitization of MC57 cells was observed at a range of gB-(23–718) concentrations from 5 to 0.04 μg/ml (Fig. 1*B*). It is noteworthy that treatment of target cells with DOTAP and concentrations of gB-(23–718) greater than 20 μg/ml often resulted in lower levels of lysis (data not shown), suggesting that too much protein could interfere with delivery and/or processing of the antigen. From this dose–response analysis, it is estimated that approximately 0.1–0.2 μg of gB-(23–718) per ml would be required for half-maximal lysis of target cells. Comparison of this estimate with published data for delivery of ovalbumin into the class I MHC pathway by pH-sensitive liposomes (6) and osmotic shock (6, 8) suggests that cationic liposomes are approximately 10- and 200-fold more efficient, respectively. However, direct comparison of these methods will be necessary to control for variables such as the efficiency of processing of different proteins within the cell.

To determine if DOTAP-treated gB-(23–718) was processed via the class I antigen presentation pathway, target cells were treated with BFA, a fungal antibiotic that blocks egress of class I MHC molecules from the endoplasmic reticulum (22). HSV-specific CTL activity was abrogated when the MC57 target cells were exposed to BFA during sensitization with gB-(23–718) and DOTAP (Table 1). Incubation of the BFA-treated targets with peptide gB-(495–509)

Table 1. Effect of BFA on target cell sensitization by gB-(23–718) and DOTAP

Target cell treatment*				% ^{51}Cr release from MC57 targets†		
gB-(495–509)	gB-(23–718)	DOTAP	BFA	50:1	10:1	2:1
–	–	–	–	10	<1	<1
+	–	–	–	57	30	8
–	+	–	–	14	<1	<1
–	+	+	–	49	46	25
–	+	+	+	14	11	6
+	+	+	+	68	48	34

*MC57 target cells were treated with gB-(23–718) at 1 μg/ml in the presence or absence of DOTAP as described in the legend to Fig. 1. gB-(495–509) was added at the time of ^{51}Cr labeling. BFA was added to MC57 cell cultures at the same time as gB-(23–718) and DOTAP and was maintained at 10 μg/ml throughout the CTL assay.

†Assay conditions and methods for generation of HSV-specific CTL were the same as described in the legend to Fig. 1. The three E:T ratios are indicated in the column headings.

Table 2. Restimulation of HSV-immune memory CTL by gB-(23–718)/DOTAP-treated APC

gB-(23–718), μg/ml	DOTAP	% specific ^{51}Cr release from target cells			
		MC57			SVBalb
		None	gB-(495–509)	HSV	gB-(495–509)
10	−	6	12	9	4
2	−	8	8	13	11
0.4	−	4	<1	<1	6
10	+	9	49	60	11
2	+	7	24	16	8
0.4	+	6	10	6	9

Thioglycollate-induced PEC from C57BL/6 mice were incubated overnight in 5 ml of PBS containing the indicated concentration of gB-(23–718) in the presence or absence of DOTAP. PEC were then cocultured for 5 days with spleen cells from mice infected 4–6 weeks earlier with HSV-1 strain Patton. Values represent percent specific release of ^{51}Cr at an E:T ratio of 50:1 in a 4-hr assay.

restored their susceptibility to lysis by CTL from HSV-infected mice. Taken together, these data demonstrate that class I MHC molecules on the target cells were charged with peptides produced by intracellular processing of gB-(23–718).

Restimulation of Memory CTL Activity *in Vitro* with gB-(23–718) and DOTAP. Class I MHC-restricted, virus-specific CTL activity is detected when immune lymphocytes from HSV-infected mice are cultured with HSV for 5–6 days (23). To determine if DOTAP could facilitate the reactivation of memory CTL activity by gB-(23–718), thioglycollate-induced PEC from C57BL/6 mice were treated with various concentrations of the protein in the presence or absence of DOTAP. These APC were then cocultured for 5 days with spleen cells from syngeneic mice infected 4–6 weeks earlier with HSV. As shown in Table 2, significant cytotoxic activity against gB-(495–509)-pulsed MC57 target cells was generated only in those cultures containing gB-(23–718)/DOTAP-treated PEC. Allogeneic SVBalb target cells pulsed with gB-(495–509) were not killed, indicating that lytic activity was class I MHC-restricted. A gB-(23–718) concentration of at least 2 μg/ml was required for restimulation of HSV-specific CTL, a result that is consistent with recent studies using ovalbumin encapsulated in acid-sensitive fusogenic liposomes (7).

Priming of gB-Specific CTL *in Vivo* with gB-(23–718) and DOTAP. The ability of gB-(23–718)/DOTAP to elicit class I MHC-restricted CTL responses in mice was assessed. C57BL/6 mice were immunized three times at weekly intervals with gB-(23–718) and DOTAP. Seven days after the last immunization, lymphocytes from the draining lymph nodes were cocultured for 5 days with gB-(495–509)-pulsed syngeneic PEC and then tested for lytic activity against a panel of target cells. Levels of cytotoxic activity observed against gB-(495–509)-pulsed MC57 cells were significantly greater than those detected against control gB-(495–509)-pulsed SVBalb or unpulsed MC57 target cells at all three E:T ratios tested (Table 3). Similar levels of CTL activity were detected in mice immunized with DOTAP and 1 μg of gB-(23–718), the lowest dose of antigen tested (data not shown). The MC57 target cells do not express class II MHC molecules, thus this lytic activity was most likely mediated by class I MHC-restricted CTL. Lymph node cells from mice immunized with gB-(23–718) in Freund's adjuvant lysed gB-(495–509)-pulsed MC57 cells only slightly more efficiently than unpulsed MC57 targets. This low cytotoxic activity could reflect limited extracellular processing of gB-(23–718) after immunization or generation of a weak primary *in vitro* CTL response, as reported by others after culture of unprimed lymphocytes with synthetic peptides representing CTL epitopes (23–25). Generation of CTL responses by immunization with subunit antigens such as gB-(23–718) may require priming of class II MHC-restricted CD4$^+$ helper cells (26, 27). Whether DOTAP amplifies this class II response in addition to facilitating class I MHC antigen presentation will require further study.

Table 3. HSV gB-specific CTL responses in C57BL/6 mice immunized with gB-(23–718) and DOTAP

Adjuvant	E:T ratio	% specific ^{51}Cr release from target cells		
		MC57		SVBalb
		Untreated	gB-(495–509)	gB-(495–509)
DOTAP	50:1	<1	56	4
	10:1	4	29	<1
	2:1	1	12	<1
CFA/IFA	50:1	9	20	<1
	10:1	7	12	<1
	2:1	6	4	<1

C57BL/6 mice were immunized with gB-(23–718) and DOTAP or CFA and were boosted twice at weekly intervals [after CFA, the boosts were with incomplete Freund's adjuvant (IFA)]. Seven days later, draining lymph node lymphocytes were cocultured with gB-(495–509) pulsed-PEC. ^{51}Cr release was measured in a 4-hr assay.

Other cationic lipids, including Transfectam (Promega), and DOTMA (Bethesda Research Laboratories) also permitted sensitization of target cells with gB-(23–718) (data not shown). They are similar in structure to DOTAP, with two long hydrophobic alkyl chains coupled to one or more positively charged ammonium groups. The mechanism by which these cationic lipids delivered recombinant gB protein into the class I MHC antigen presentation pathway is probably similar to that proposed for transfection of DNA into cells (28). This involves an interaction between the macromolecule–lipid complex carrying an overall positive charge and the negatively charged cell surface, followed by fusion with the cell membrane. In contrast, pH-sensitive liposomes are thought to destabilize upon contact with the acidic environment of the endosome and then rupture and/or fuse with the endosomal membrane to release their contents into the cytoplasm (6).

Formation of complexes between soluble gB-(23–718) and DOTAP was most likely mediated by ionic interactions between negative charges on the protein and the cationic lipid, and thus denaturation or modification of gB-(23–718) was not required. Association required only mixing of the protein and DOTAP solutions prior to application to cells or injection into experimental animals. Thus, cationic lipids are readily available delivery vehicles for study of intracellular events that lead to class I MHC presentation of antigens, and they could serve as an alternative to recombinant viruses for dissecting CD8$^+$ T-cell responses to viruses. Previous studies have demonstrated that the glucocorticoid receptor (29) and chloramphenicol acetyltransferase (M.S., unpublished observation) proteins have biologic activity when introduced into cells by using cationic lipids, indicating that native conformation of the molecules was at least partly retained. These results suggest that vaccination with DOTAP-associated proteins could also elicit antibody responses

against discontinuous B-cell epitopes that are important in the neutralization of viruses.

Priming of anti-viral CD8+ T-cell responses has been accomplished by immunization of mice with short synthetic peptides that contain CTL epitopes (30–32). However, one drawback of peptide vaccination is the selection by class I MHC molecules of epitopes to be presented to T lymphocytes. Immunization with recombinant proteins that contain T- and B-cell epitopes may provide an alternative approach for inducing protective immune responses in vaccinees of diverse MHC haplotypes. The results of this study suggest cationic lipids will effectively elicit CD8+ T-cell responses when complexed with recombinant proteins. In addition, their well-defined chemical structures may permit modifications that enhance association with proteins or reduce toxicity to vaccinees.

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