A SYNTHETIC PEPTIDE INDUCES LONG-TERM PROTECTION FROM LETHAL INFECTION WITH HERPES SIMPLEX VIRUS 2

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Herpes simplex virus (HSV) is one of the most common infectious agents of man. One important feature of this virus is its ability to produce a latent infection. The reactivation of this infection, however, generally does not result in a viremia but rather spreads between cells. Thus, even though an antibody response is induced, it might be expected that a cellular mechanism of protection is required (1). Clinical data supports the notion that high anti-HSV virus-neutralizing antibody (VNA)[1] titers do not protect from reinfection and reactivation in man (2, 3), and may in fact play a negative role in protection from HSV (4, 5).

In considering mechanisms of protection other than antibody, we have been studying the T cell response in mice to glycoprotein D (gD), a viral encoded molecule found on the surface of infected cells. We found that immunization with a 23-amino-acid synthetic peptide having a sequence corresponding to the NH_3-terminus of gD from either HSV-1 or HSV-2 induces a T cell response in vitro against related peptides (6) and HSV itself. This peptide will also induce antibody that can neutralize viral infectivity (7, 8). We were, of course, interested in determining the ability of these peptides to protect against an HSV challenge in vivo. In an attempt to construct an antigen capable of enhancing the T cell response (9, 10), we coupled the peptides to palmitic acid side chains (11) and inserted these acylated peptides into a liposome structure (12). This report describes the protection achieved and its ability to be transferred by T cells and not serum.

Materials and Methods

Antigen Preparation. Peptides were synthesized and purified as previously described (6). To add the palmitic acid side chains onto these peptides, a tripeptide linker, GGK, was added to the NH_3 terminus; the NH_3-terminal lysine was coupled as the *bis-t-*butyloxycarbonyl derivative, and deprotected using trifluoroacetic acid. The palmitic acid moieties were coupled by the symmetric anhydride methods (11). This molecule was then mixed with lipids as follows (12). Phosphatyl choline, cholesterol, and lysolecithin were each dissolved in methanol/chloroform (1:3) and then mixed in the ratio of 16:2:1, respectively.
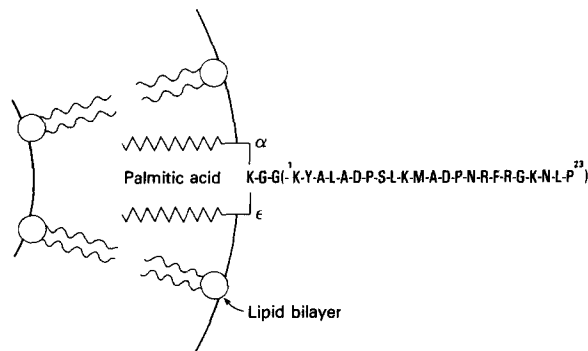
FIGURE 1.   The first 23 NH_3-terminal amino acids of the gD of HSV-2 are shown attached to a spacer, Gly-Gly-Lys, and then covalently coupled to two palmitic acid molecules.

This mixture was then dried with a nitrogen stream, rotating the vial in warm water to get an even film over the entire vial. 5 mg of the peptide–palmitic acid conjugate was dissolved in 2 ml of a 1% octylglycoside in PBS and then added to 10 mg of the lipid mixture. The mixture was dialyzed against PBS using a 3.5-kD cutoff Spectropore dialysis membrane for 24 h. The liposomes were probe-sonicated for 5 min. The peptide-containing liposome preparation (see Fig. 1 for diagram of this molecular structure) was then mixed with CFA in a ratio of 1:1 into an emulsion and injected into the hind footpads of mice. The volume given was ∼0.2 ml/ animal, and the amount of peptide was 150 μg/animal or 10 μg/gm body weight.

The HSV-1 and HSV-2 NH_3-terminal 1–23 sequences differ at two residues: at residue 7, where the HSV-1 sequence is Ala and the HSV-2 sequence is Pro, and at residue 21, where the HSV-1 sequence is Asp and the HSV-2 sequence is Asn (13). As demonstrated in Fig. 2A, ^{14}C-labeled acylated 1–23(1)-peptide liposomes with the gD sequence of HSV-1 are homogeneous in regard to their buoyant density; moreover, the buoyant density of these peptide-liposomes correlate with the amount of peptide incorporated. Such purified liposomes were examined by electronmicroscopy (Fig. 2, B and C) and found to be an average of 250 nm in diameter, and multilammelar. The membrane of the peptide-liposomes exhibited a higher electron density compared with the lipid membranes, which do not contain peptide. This may indicate that the peptide is incorporated, at least in part, into the lipid membrane.

Virus Preparation and Challenge.   HSV-1 strain F was obtained from Nigel Fraser (Wistar Institute), and HSV-2 strain 186 was obtained from Gary Cohen (University of Pennsylvania School of Dental Medicine, Philadelphia, PA). Both viruses were grown in baby hamster kidney (BHK) fibroblast cells. Infected cells (10^7) were resuspended in 1 ml of medium, frozen and thawed three times, and virus titer was determined by counting PFU on BHK monolayers. Challenge with infectious virus was carried out by injection of both footpads with a given dose of HSV-2 in culture supernatant.

Virus Neutralization.   In 96-well flat bottom tissue culture plates, 100 PFU of virus in 25 μl was added to serum antibody, in the same volume, with the serum being diluted in twofold dilutions. This mixture was incubated for 1 h at 37°C, and BHK cells were added to the wells at a concentration of 5 × 10^4 cells/ml in 50 μl. 3–4 d later, the cells were stained with crystal violet dissolved in 10% phosphate buffered formalin. The titer is reciprocal dilution of the geometric mean of the number of wells of a twofold dilution.

T Cell Preparation and Responses.   Cell suspensions obtained from the popliteal and inguinal lymph nodes of animals immunized with antigen 2 wk previously were passed over a nylon wool column (14), the T cells were purified, and then cultured with x-irradiated spleen (2,500 rad) plus antigen. These cultures were tested for responsiveness to antigen by cell proliferation measured by the incorporation of [3H]thymidine after 3 d in culture (15).
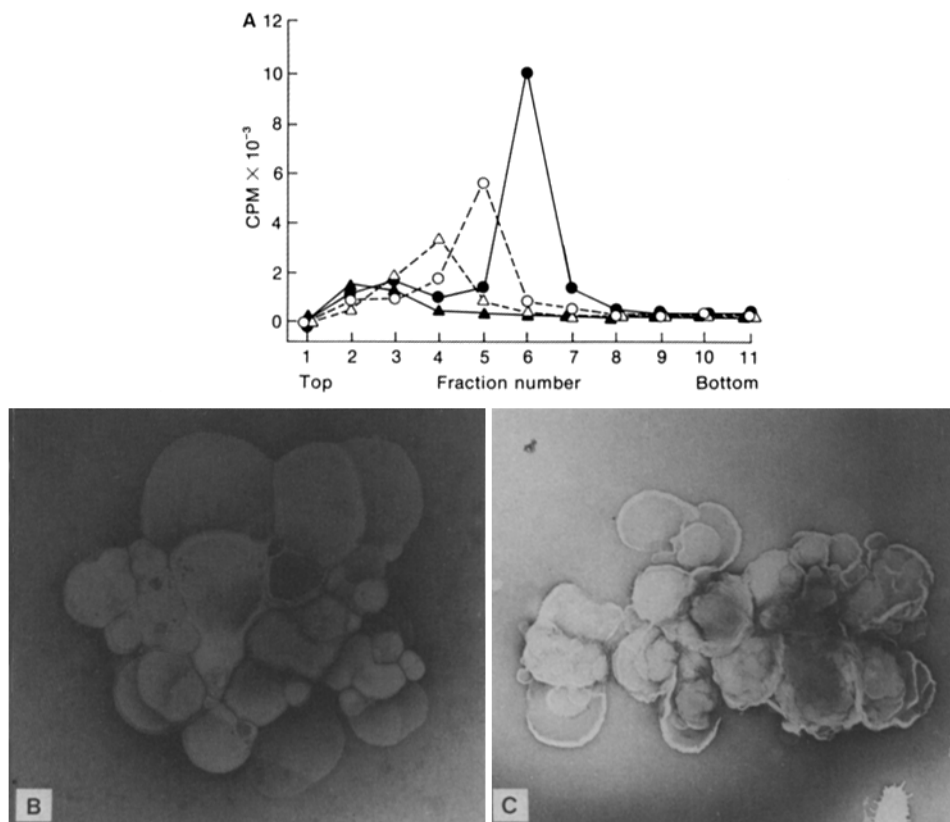
FIGURE 2. (A) ^{14}C-Labeled palmitic acid–peptide–liposomes were layered on a continuous 5–15% sucrose gradient and centrifuged to 35,000 rpm in a Beckmann SW 50.1 rotor at 10°C. Fractions were collected and counted for radioactivity and density was determined for the peak fractions: (open circles), a density of 1.020, with a peptide/lipid ratio of 4:10; (filled circles), a density of 1.017, with a ratio of 3:10; (open triangles), a density of 1.014, with a ratio of 2:10; and (filled triangles), a density of 1.010, with a ratio of 1:10. Electronmicrographs of liposomes (B) or peptide–liposomes at a peptide/lipid ratio of 4:10 (C).

For adoptive transfer experiments, cell suspensions from spleen and lymph nodes were treated with both 14.4.4, an anti-I-Eα monoclonal antibody (16), and J11D, an anti–B cell monoclonal antibody (17), and in some cases 3.168, an anti-Lyt-2 antibody (18), plus C' for 75 min at 37°C. These cells were then tested for their response to LPS and Con A in the presence of x-irradiated normal splenocytes.

Results

BALB/c female mice at 6 wk of age were given a single dose of the acylated 1–23(2) peptide liposome with the NH$_3$-terminal sequence of gD from HSV-2 and then tested for protection by challenging with a lethal dose of HSV-2, after intervals of up to 7 mo.

It is clear from Fig. 3 that HSV-2 infection leads to pathogenic effects (a characteristic paralysis) in unprotected mice by day 8, that death ensues ~6–10 d later, and that a single dose of acylated peptide–liposome is protective. We have shown both the percentage of mice without symptoms and the percentage
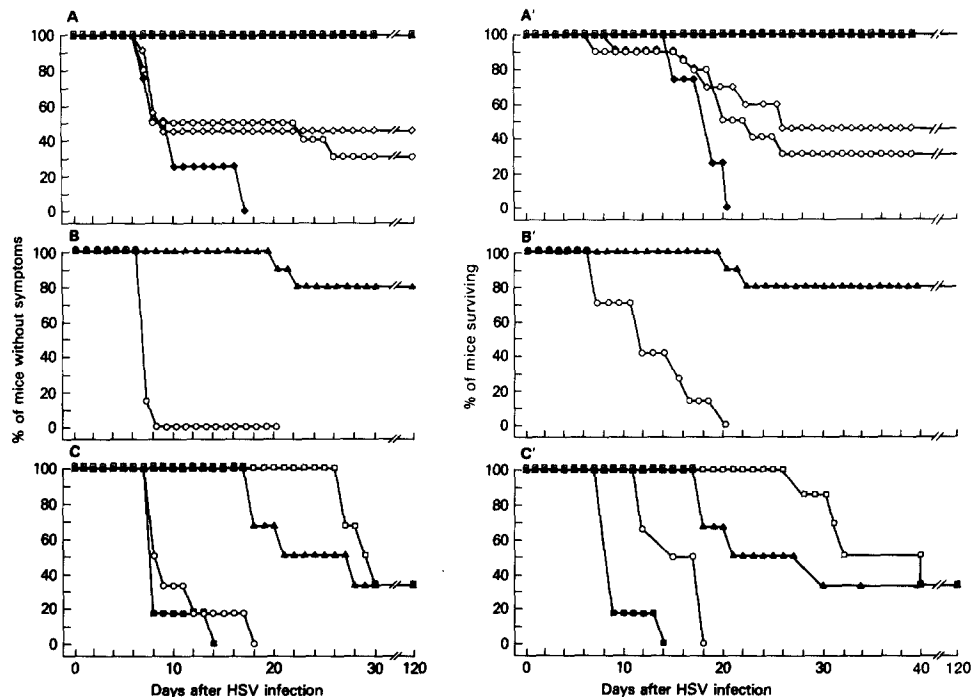
FIGURE 3.   BALB/c female mice at 6 wk of age were immunized with a single dose of antigen and then challenged with lethal dose of HSV-2. The data is presented as both the percentage of mice without symptoms (A, B, C) and the percentage of mice surviving (A', B', C') versus the number of days after HSV-2 challenge. Symptoms include: ruffled fur, shaking, paralysis, and death. The antigens used for immunization include: UV-inactivated HSV-1 in CFA (□); acylated peptide–liposome in CFA μg (▲); CFA alone (○); acylated peptide–liposome in saline (◆); liposome in CFA (◇); acylated peptide in CFA (■); and saline (●). (A and A') Mice were immunized once, 2.5 mo before challenge, and received 1.2×10^6 PFU of challenge virus. (B and B') Mice were immunized 2.5 mo before challenge with 2.2×10^6 PFU of HSV-2. (C and C') Mice were immunized once, 7 mo before challenge with 3.1×10^6 PFU of virus.

of mice surviving up to 120 d after virus challenge, which essentially describes the time of onset and the endpoint of HSV disease in these mice. Fig. 3,A and A' (Exp. 1) illustrate the results when animals were challenged with a 1.2×10^6 PFU dose of HSV-2 (4-LD_{50}), 2.5 mo after immunization. If the mice were immunized with either UV-inactivated HSV-1 (10 mice) or acylated peptide–liposome (14 mice) in CFA, 100% of them were protected. When the controls were immunized with either saline (10 mice), or liposome (in the absence of peptide) in CFA (11 mice), 30–45% of the animals survived the challenge infection.

Interestingly, a fifth experimental group receiving the acylated peptide–liposome in saline (5 mice) did far worse than the controls. This result, not just a lack of protection but rather an enhanced frequency of disease, points to the importance of considering the different forms of immunogen and their effects, both positive and negative.

To better determine the effectiveness of antigen priming, we increased the challenge dose of virus. In Fig. 3,B and B' (Exp. 2), animals (10 per group) were

TABLE I

Crossreactive Protection with Acylated 1–23 Peptide–liposomes

Immunized animals*	Mice surviving (day 30)
	%
CFA control	30
Acylated 1–23(1) liposome in CFA	100
Acylated 1–23(2) liposome in CFA	100

* 8-wk-old BALB/c mice were immunized once intrafootpad 3 mo before HSV challenge. Animals were then challenged with 1.6×10^6 PFU of HSV-2 and followed for 30 d after challenge. 10 mice were used in each group.

challenged with a 2.2×10^6 PFU dose of HSV-2 (7-LD_{50}), 2.5 mo after immunization. In this case, 100% of the control animals (CFA alone) died by day 20. 80% of the animals receiving acylated peptide–liposome in CFA, however, remained healthy.

A third experiment was carried out (Fig. 3,C and C'), in which mice (six per group) were challenged with a 3.1×10^6 PFU dose of HSV-2 (10-LD_{50}), 7 mo after priming. The mice that had been immunized with CFA or acylated 1–23(2) peptide without liposomes but in CFA were diseased by day 8 after virus challenge, with the acylated peptide–primed group dying most rapidly and the CFA control group dying by day 18. In contrast, 70% of the animals immunized with acylated peptide–liposomes in CFA showed no HSV-specific symptoms by day 20. By day 40, mice immunized with either UV-irradiated HSV-1 or acylated peptide–liposome had survived the challenge in equal numbers (30%). Thus, acylated peptide–liposome in CFA appeared approximately as effective as UV-inactivated HSV-1 under the conditions of this study.

The difference in the two ways the data is expressed, the percentage of mice without symptoms (interpreted as the first appearance of the disease) and percentage of mice surviving (interpreted as the endpoint of the disease or death) is seen as the displacement of curves to the right by ~6–10 d, which is the time the animals are sick but have not died.

We also examined whether the acylated 1–23(1) peptide liposome with the gD sequence of HSV-1 would protect mice against an HSV-2 challenge. As seen in Table I, animals immunized with 1–23(1) peptide liposomes 3 mo before HSV-2 challenge were protected, and furthermore, the 1–23(1) peptide liposome was comparable to the 1–23(2) peptide liposome in inducing protection.

It has been reported that, in mice, passive administration of anti-HSV VNA at the time of HSV challenge can protect mice from a lethal infection (19–22). Because this was a possible explanation for the protection seen here, we checked the sera of mice that had been immunized 7 mo previously (from Exp. 3, Fig. 3,C and C'), 9 d after HSV-2 challenge to determine the VNA titer. As shown in Table II, neutralizing antibody titers are consistent with the protection in the HSV-1-primed group but could not explain the protection seen with the acylated peptide–liposome–primed group.

We examined the animals at later times after virus challenge to determine any evidence of antigen priming for antibody. As seen in Table III, mice immunized

TABLE II

Anti-HSV Antibody

Pooled serum from each group	Neutralization titers for:	
	HSV-1	HSV-2
CFA control	12	6
UV-irradiated HSV-1 in CFA	389	97
Acylated peptide in CFA	6	8
Acylated peptide–liposome in CFA	5	5

BALB/c mice were challenged with HSV-2 in the footpads 7 mo after a single immunization of antigen in CFA. Bleedings were done 9 d after challenge with HSV-2. The peptide was the 1–23(2) sequence. See also Exp. 3, Fig. 4C.

TABLE III

Kinetics of Antibody Production in Immunized Mice

Week	CFA-primed		Acylated peptide–lipo-some–primed	
	Anti-HSV-1	Anti-HSV-2	Anti-HSV-1	Anti-HSV-2
−2	<2	<2*	<2	<2
2	32	64‡	32	32‡
3	—	—§	16	8

* See Materials and Methods for determination of virus neutralizing titer.
‡ Sera from wk 2 after infection was examined for isotypes of anti-HSV antibody. In a binding assay with HSV-1- and HSV-2-infected cells, antibody was determined to be IgM-positive, IgG (γ_1, γ_{2a}, γ_{2b}, γ_3)-negative, whereas an anti-HSV secondary response serum control was found to be IgM- and IgG-positive.
§ All control animals had died.

2.5 mo previously (from Exp. 2, Fig. 3,B and B') were bled before and after virus challenge to look for an anti-HSV antibody response. It was clear that at 2 wk, there was no difference in the antibody titer between the CFA controls and the antigen-primed mice. By the third week, the controls had died and the already low antibody titer in the antigen-primed group fell. Furthermore, the antibody produced reflected a primary response to the virus, because only IgM was detected, supporting the conclusion that antibody is not important in protection induced with the acylated peptide–liposome.

We next considered the T cell response, though as something other than help for virus-specific antibody, and its correlation with protection from HSV. In vitro antigen-specific T cell proliferation was determined 2 wk after animals were immunized. As seen in Table IV, T cells from mice primed to various forms of peptide, whether protective or not, responded to peptide, gD, and to virus.

The lack of antibody suggested a role for T cells in protection, but we could not show a unique T cell function in these protected mice. It was important, however, to show that even with a proliferative T cell response in all groups, evidence for T cell protection was present. Thus, we examined both the serum and lymphocytes from animals primed to the acylated 1–23(2) peptide–liposomes by adoptive transfer into normal mice and then challenge with HSV-2. It is clear

TABLE IV
Activation of T Cells by Peptide and Viral Antigens

Priming antigen	Antigen in culture	[^{3}H]Thymidine incorporation (cpm × 10^{-3})	Antibody titer*
1-23(2) in CFA (nonprotective)	1-23(2), 50 μg/ml	16.1	<100
	UV HSV-1‡	9.2	
	UV HSV-2‡	13.2	
	gD-1, 1 μg/ml	11.0	
	OVA, 50 μg/ml	−5.3	
Acylated peptide§ in CFA (nonprotective)	1-23(2)	41.4	<100
	UV HSV-1	69.6	
	UV HSV-2	54.8	
	gD	64.8	
	OVA	−4.5	
Acylated peptide-liposome§ in CFA (protective)	1-23(2)	50.8	<100
	UV HSV-1	20.9	
	UV HSV-2	14.9	
	gD	18.6	
	OVA	−1.1	
UV-irradiated HSV-1 in CFA (protective)	1-23(2)	3.8	>3,200
	UV HSV-1	64.2	
	UV HSV-2	31.8	
	gD	25.8	
	OVA	0.3	

* ELISA assay titers are reciprocal dilutions. OD considered positive if >0.2 when OD (infected cell minus control cell) was read at 405 nm.
‡ Infected cell lysates.
§ The peptide is the 1-23(2) sequence.

from Table V, Exp. 1 that animals injected with T cells from acylated peptide liposome immunized mice were protected, while animals injected with serum from such mice were completely unprotected. Furthermore, this T cell protection was abolished with anti-Lyt-2 treatment (Table V, Exp. 2).

Discussion

We have shown that a synthetic acylated peptide corresponding to either an HSV-1 or HSV-2 glycoprotein D (gD) sequence and incorporated into liposomes induces striking protection from a lethal HSV-2 infection. It is significant that this synthetic antigen construct is approximately as potent and effective an immunogen as is UV-inactivated HSV-1 under the conditions of this study. Both give long-term protection (at least 7 mo) with only a single dose of antigen. It is important to note that the mixture of three components, acylated peptide, liposome, and an adjuvant is essential for a protective response.

Though acylated peptide–liposome and virus appear similar in their ability to confer protection, they are clearly different in their effect on the immune system. Thus, before virus challenge, no virus-specific antibody is detectable in the

TABLE V

*Adoptive Transfer from Animals Immunized with
Acylated Peptide–liposomes*

Exp.	Donors	Cell or serum dose	Mice surviving (day 30)
			%
1	CFA-primed splenic T cells*	4×10^7	0
	Peptide-primed spleen cells[‡]	8×10^7	100
	Peptide-primed splenic T cells[‡]	4×10^7	100
	Peptide-primed LN T cells[‡]	3.4×10^7	60
	Peptide-primed serum[‡]	0.2 ml	0
	HSV-primed serum[§]	0.2 ml	100
2	CFA-primed splenic T cells[‖]	4×10^7	10
	Peptide-primed splenic T cells[‡]	4×10^7	70
	Peptide-primed splenic T cells treated with anti-Lyt 2 + C′	4×10^7	0
	Peptide-primed LN T cells	4×10^7	80

* Cells from mice injected with CFA alone or antigen plus CFA 1 mo previously were injected into 8-wk-old BALB/c recipients (five mice per group) intravenously. 24 h later, recipients were challenged with a 7-LD_{50} dose (7.5 × 10^6 PFU, lot 2) of HSV-2.

[‡] BALB/c mice (7-wk-old) were injected with acylated 1–23(2) peptide–liposome in CFA intrafootpad. 1 mo later, spleen and lymph nodes were removed and the T cells were prepared. Sera from these animals were collected at the same time and shown to have no binding or neutralization activity for HSV-1 or HSV-2.

[§] Sera were collected from animals injected with HSV-2 and used at a binding titer for HSV-2 of 1:64.

[‖] Cells from primed mice were injected into 8-wk-old BALB/c recipients (10 mice per group) intravenously. 24 h later, recipients were challenged with a 7-LD_{50} dose (2.1 × 10^6 PFU, lot 6) of HSV-2.

acylated peptide–liposome–primed group, but it is present in the virus-primed group. After HSV-2 challenge, the peptide-immune group, like the unimmunized controls, responds with only a weak primary antibody response to virus, seen after 2 wk, while the virus-primed group gives a strong secondary response within a week. Upon adoptive transfer of sera from peptide-primed animals, no protection was seen, although sera from HSV-primed animals were completely protective. We conclude that antibody is unlikely to play a significant role in protection with the acylated peptide–liposome, though we cannot eliminate the possibility that undetected antibody is responsible (23).

T cells, however, do seem to be important. Adoptive transfer of T cells from either spleen or lymph node of acylated peptide–liposome–primed mice confers protection to normal mice and this protection is eliminated by pretreatment of the T cells with anti-Lyt-2 antibody plus complement. An obvious candidate for this protection is the cytotoxic T cell, which has been shown previously to be induced in vitro by an antigen construct of viral antigens incorporated into liposomes (24–28). However, there is evidence against this type of effector cell, because gD does not induce an $Lyt-2^+$ class I–restricted CTL response (J. Bennick, personal communication). Furthermore, the induction of a CTL re-

sponse does not easily explain the results in which animals receiving certain forms of the peptide (Fig. 3 A) do worse than the controls.

On the other hand, the involvement of suppressor T cells in protection might be suggested from the data presented in Fig. 3 A, where animals immunized with acylated peptide–liposome in saline did worse than animals immunized with saline alone. One possible mechanism for this effect is that proliferating T cells that are harmful (29, 30) are induced in all the immunized groups, but are checked only in the protected group, which can generate a suppressor T cell population. The lack of suppressors leads to no protection or enhanced viral pathogenicity. This explanation is not so unusual when one considers the pathology induced in an autoimmune disease such as experimental allergic encephalomyelitis (EAE). In this case, proliferating class II–restricted T cells can induce the disease, and the suppression of these cells results in refractoriness to EAE and lack of disease (31).

In light of the Lyt-2 (suppressor/cytotoxic T cell) nature of protection, it is not surprising that there is no correlation with the ability of T cells to proliferate specifically (a helper T cell function) to the 1–23(2) peptide, gD, or HSV-1 cell lysates (Table IV) in vitro. That is, T cell proliferation in vitro is not a criterion for a protective response even when the protection can be shown to be conferred by T cells.

It is interesting that T cells could be primed with acylated peptide–liposome in vivo and respond to virus in the absence of a detectable B cell response. The same 1–23 peptide has been shown to induce anti-HSV antibody responses when attached to keyhole limpet hemocyanin (KLH), and also to induce short-term protection (32). However, in our case, after acylated peptide–liposome priming and then challenge with HSV-2, the antibody response induced was IgM not IgG; a primary response. Whether the T cells provide help for such a primary antivirus response is difficult to assess; these T cells might be responding to antigen only in association with certain antigen-presenting cells such as macrophages or dendritic cells, but not others, such as B cells, yielding no antibody response (33–35).

The phenomenon of a T cell response with no antibody has been previously described (9, 10) for a protein antigen that was covalently coupled to a lipid. In this case, the appearance of delayed-type hypersensitivity (DTH) without antibody was attributed to localization of antigen in a T cell region of the lymph node.

Whatever the mechanism, it is interesting to note that the way antigen is presented to the animal and its immune system has drastic effects on the outcome of immunization, either positive or negative. Thus, peptides in this system do not always confer protection and in some cases seem to enhance viral pathogenicity.

Summary

Immunization against viral pathogens is generally directed toward the induction of virus neutralizing antibody (VNA) and the maintenance of the potential for a second-set (IgG) response. Indeed, an elevated level of specific antibody is considered a reliable clinical indicator that a state of immunity exists in the host.

However, in the case of herpes simplex virus (HSV), the presence of circulating VNA does not necessarily correlate with protection. Thus, it has been found that secondary infections occur in individuals even with high neutralizing titers to HSV, suggesting that antibody to the virus may be useless or even deleterious. In consideration of these facts, we were interested in inducing a T cell response to HSV. We had already shown that synthetic peptides corresponding to the NH$_3$-terminal region of the glycoprotein D (gD) molecule of HSV could induce a strong T cell response when injected into mice, but did not, by themselves, confer protection. In this report, we examined the ability of peptides, covalently coupled to palmitic acid and incorporated into liposomes, to induce virus-specific T cell responses that confer protection against a lethal challenge of HSV-2. We have demonstrated that long-term protective immunity is achieved with a single immunization in the absence of neutralizing antibody when antigen is presented in this form. Furthermore, T cells but not serum from such immune mice can adoptively transfer this protection.

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