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Vaccination of mice with herpes simplex virus type 1 glycoprotein D DNA produces low levels of protection against lethal HSV-1 challenge

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Abstract

The herpes simplex virus type 1 (HSV-1) glycoprotein D (gD) gene was inserted into vectors pSVL or pRc/CMV under control of the SV40 late promoter or the human cytomegalovirus major immediate–early promoter, respectively. Intramuscular injection of mice with these gD-containing plasmids appeared to induce low levels of serum anti-gD antibody, as judged by the appearance of low levels of anti-HSV-1-neutralizing antibody and anti-gD ELISA responses in the serum of gD-DNA-vaccinated mice. As previously reported in other virus systems, vaccination with vector DNA also induced ELISA and neutralizing antibody titers. However, these titers were lower than those induced by the gD-containing plasmids. The ELISA and neutralization titers induced by the vectors appeared to be non-specific rather than directed at specific HSV-1 proteins, since serum from mice vaccinated with plasmid-gD immunoprecipitated significant amounts of gD from extracts of HSV-1-infected cells, while serum from mice vaccinated with vectors was unable to immunoprecipitate gD or any other obvious HSV-1 proteins. Neither pSVL-gD nor pRc/CMV-gD induced detectable lymphocyte proliferative or CTL responses. Vaccination with pSVL-gD provided a significant ($P = 0.04$, Fisher's exact test), but low level of protection against lethal challenge with HSV-1. Vaccination with pRc/CMV-gD also appeared to provide a low level of protection against challenge, that was statistically significance at the 10% level ($P = 0.054$, Fisher's exact test). Reports from numerous laboratories (including ours) have shown that

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vaccination with recombinantly expressed gD can provide very high levels of protection against HSV-1 lethal challenge. Thus, the results reported here suggest that vaccination with HSV-1 gD-DNA is not yet a useful alternative to a gD subunit vaccine.

Keywords: DNA vaccine; glycoprotein D (gD); Herpes simplex virus type 1 (HSV-1)

Recently, injection of mice with plasmid DNA encoding the influenza virus nucleoprotein was reported to provide protection against lethal influenza challenge, induce antibody to the nucleoprotein, and produce cytotoxic T-lymphocyte (CTL) responses against influenza (Ulmer et al., 1993). More recently, it was shown that injection of mice and cattle with plasmids encoding bovine herpes virus 1 glycoproteins induced neutralizing antibody responses (Cox et al., 1993).

To determine if this novel approach would also provide protection against herpes simplex virus type 1 (HSV-1) challenge, we constructed two plasmids containing the DNA coding region for glycoprotein D (gD) of HSV-1. gD was used because our comparative studies with 7 HSV-1 glycoproteins expressed in a baculovirus expression system indicated that when used as a vaccine in mice, gD provided more effective protection against lethal HSV-1 challenge than any of the other 6 HSV-1 glycoproteins (gB, gC, gE, gG, gH, gI) (Ghiasi et al., 1994). Other work has also shown that vaccination with purified virion gD (Mishkin et al., 1991) or gD expressed in a variety of systems (Berman et al., 1983; Lasky et al., 1984; Cremer et al., 1985; Ghiasi et al., 1991), induces high titer neutralizing antibodies to HSV-1 and provides protection against lethal challenge of animals. Furthermore, monoclonal antibodies against gD can protect mice against lethal challenge with HSV (Metcalf et al., 1988), indicating that neutralizing antibody alone is sufficient to provide protection against lethal HSV-1 challenge. We report here that injection of mice with gD-DNA resulted in low levels of neutralizing antibody to HSV-1 and low anti-gD ELISA titers. Despite the apparent inability to induce observable lymphocyte proliferative or CTL responses, vaccination with gD-DNA did provide significant, but low levels of, protection against lethal HSV-1 challenge.

We constructed two plasmids expressing gD. Plasmid pSVL-gD contains gD under control of the SV40 late promoter (Lee et al., 1981; Southern and Berg, 1982). Plasmid pRc/CMV-gD contains gD under control of the human cytomegalovirus (HCMV) major immediate–early promoter (Boshart et al., 1985). To construct pSVL-gD, the plasmid pAc-gD1, containing the gD region from HSV-1 strain KOS (Ghiasi et al., 1991), was digested with *Bam*HI. A 1.2-kb fragment containing the complete coding region of gD was isolated and ligated into the unique *Bam*HI site of the vector pSVL (Clontech, Palo Alto, CA) just downstream from the SV40 late promoter. The construct was confirmed by restriction enzyme analysis and partial sequencing, and shown to contain the entire coding sequence of the gD gene in the correct orientation to be expressed from the SV40 late promoter. There are only 6 HSV-1 nucleotides in front of the first ATG of gD and no HSV-1 nucleotides downstream from the gD termination codon (TAG). To construct pRc/CMV-gD, the complete gD open reading frame was isolated from plasmid pAc-gD1 as above and after addition of *Hind*III linkers the

linearized gD was ligated into the unique *Hind*III site of vector pRc/CMV (Invitrogen, San Diego, CA) just downstream from the HCMV major immediate–early promoter.

Two independent experiments were done to determine if gD-DNA could induce antibody titers. In each experiment, 20 BALB/c mice (6–8 weeks old) were injected in both quadriceps with 100 μg of DNA/leg of cesium chloride gradient purified gD-plasmid DNA as previously described (Ulmer et al., 1993). pSVL-gD was used in Expt. 1 and pRc/CMV-gD in Expt. 2. The mice were injected 3 times at 3-week intervals. Mock control mice (20 per group) were identically vaccinated with either pSVL or pRc/CMV vectors containing no gD-DNA. In Expt. 1, an additional 10 mice were vaccinated as above with PBS and a positive control group of 10 mice was vaccinated i.p. according to the same time schedule with 2×10^5 PFU of live KOS (a non-virulent strain of HSV-1). In Expt. 2 (pRc/CMV-gD), a positive control group of 20 mice was immunized i.p. according to the same time schedule with 2×10^6 PFU of live vaccinia-gD (VgD52) (Cremer et al., 1985).

Five mice from each group were bled prior to any vaccinations (prebleed) and again 3 weeks after the final vaccination. Sera were assayed for HSV-1-neutralizing antibody titers by 50% plaque reduction assays and for anti-gD antibody titers (Ghiasi et al., 1991). In Expt. 1, no neutralizing antibody (titer $< 1:10$) was detected in any mice prior to vaccination (Fig. 1, left side, Pre). Following vaccination, mice vaccinated with PBS had no detectable neutralizing antibody (Fig. 1, PBS, titer $< 1:10$). Surprisingly, mice vaccinated with pSVL vector alone showed a neutralizing antibody titer of 1:28 (Fig. 1, pSV). However, mice injected with pSVL-gD-DNA induced a titer of 1:67, that was significantly higher than the average prebleed neutralizing antibody titer of $< 1:10$ and the average neutralizing titer of 1:28 in the pSVL-vaccinated mice ($P < 0.0001$, Student's t-test). Mice vaccinated with live HSV-1 (Fig. 1, KOS) had significantly higher neutralizing antibody titer ($> 1:640$) than pSVL-gD ($P = 0.0001$, Student's t-test). This was not unexpected, since unlike gD-DNA, KOS is able to induce neutralizing antibodies against epitopes in glycoproteins other than just gD.

In Expt. 2, the neutralizing antibody titer of 1:91 induced by pRc/CMV-gD-DNA was similar to that induced by pSVL-gD-DNA in Expt. 1 (Fig. 1, compare pCgD to pSgD) ($P = 0.3$, Student's t-test). Again, the neutralizing antibody titer induced by the pSVL vector in Expt. 1 and the pRc/CMV vector in Expt. 2 were similar (1:28 for pSV; 1:26 for pCM) ($P = 0.3$, Student's t-test). As in Expt. 1, the average gD-DNA induced titer was significantly higher than that seen in the vector DNA (Fig. 1, pCM) ($P = 0.017$, Student's t-test) and in the prebleed sera ($< 1:10$, $P = 0.0001$, Student's t-test). In addition, the neutralizing antibody titers induced by injection of pRc/CMV-gD (Fig. 1, pCgD; 1:91) were similar to the neutralizing antibody titers induced by vaccination with vaccinia expressed gD (Fig. 1, v52; 1:105) ($P = 0.3$, Student's t-test).

The above sera were also analyzed by ELISA using gD as the capture antigen as we described previously (Ghiasi et al., 1991). Sera from mice vaccinated with pSVL-gD had a titer of approximately 1:640 (Fig. 2, Expt. 1). This was significantly higher than the average ELISA titer of approximately 1:10 seen in pSVL-vaccinated mice ($P < 0.05$, Student's t-test). The average ELISA titer in KOS-vaccinated mice was $> 1:1280$, which was significantly higher than the ELISA titer induced by pSVL-gD ($P < 0.05$, Student's t-test). pRc/CMV-gD-vaccinated mice had an average ELISA titer of approx-

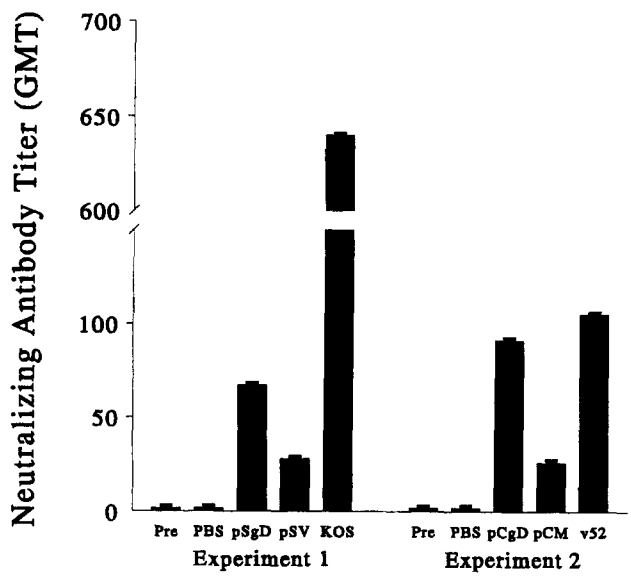

Fig. 1. Neutralizing antibody titers induced by vaccination with gD-DNA. Five mice/group were vaccinated 3 times at 3-week intervals as described in Materials and methods. Serum was obtained prior to the first vaccination (prebleeds) and 3 weeks after the final vaccination. HSV-1 neutralizing antibody titers were determined individually for each serum by plaque-reduction assays as we previously described (Ghiasi et al., 1991). For each bar, the neutralizing antibody titer represents the geometric mean of the titers from 5 sera. The range of neutralizing antibody titers were 10–40 for pSVL, 40–80 for pSVL-gD, 10–80 for pRc-CMV, 40–160 for pRc-CMV-gD and VgD52. The error bars show the standard errors. pre, prebleed; PBS, phosphate-buffered saline; pSgD, pSVL-gD; pSV, pSVL; KOS, HSV-1 strain KOS; pCgD, pRc/CMV-gD; pCM, pRc/CMV; VgD52, vaccinia-gD.

imately 1 : 1280, which was significantly higher than the titer of approximately 1 : 320 for mice vaccinated with the corresponding vector ($P < 0.05$, Student's t-test) (Fig. 2, Expt. 2). The positive control VgD52 vaccinated mice had an ELISA titer of $> 1 : 1280$, which was significantly higher than that of the pRc/CMV-gD vaccinated mice ($P < 0.05$, Student's t-test).

None of the prebleed sera had any significant ELISA titers in these assays (not shown). However, as seen with the above neutralization antibody titers, mice vaccinated with the vectors had higher ELISA titers than expected (1 : 10 for pSVL; 1 : 320 for pRc/CMV). These high ELISA and neutralizing antibody titers induced in response to the plasmid control may be due to an adjuvant effect of the DNA (Rabinovich et al., 1994) or to the expression of cross reacting protein(s).

The induction of neutralizing and ELISA titers in mice vaccinated with vector DNAs made it important to confirm that the gD-DNAs had induced antibodies that react with authentic gD. Sera raised in mice against pRc/CMV-gD and pRc/CMV DNA were therefore tested for their ability to recognize authentic HSV-1-gD by immunoprecipitation of extracts of HSV-1-infected rabbit skin (RS) cells (Fig. 3). The gD band was readily observed by SDS–PAGE using sera from pRc/CMV-gD vaccinated mice (Fig.

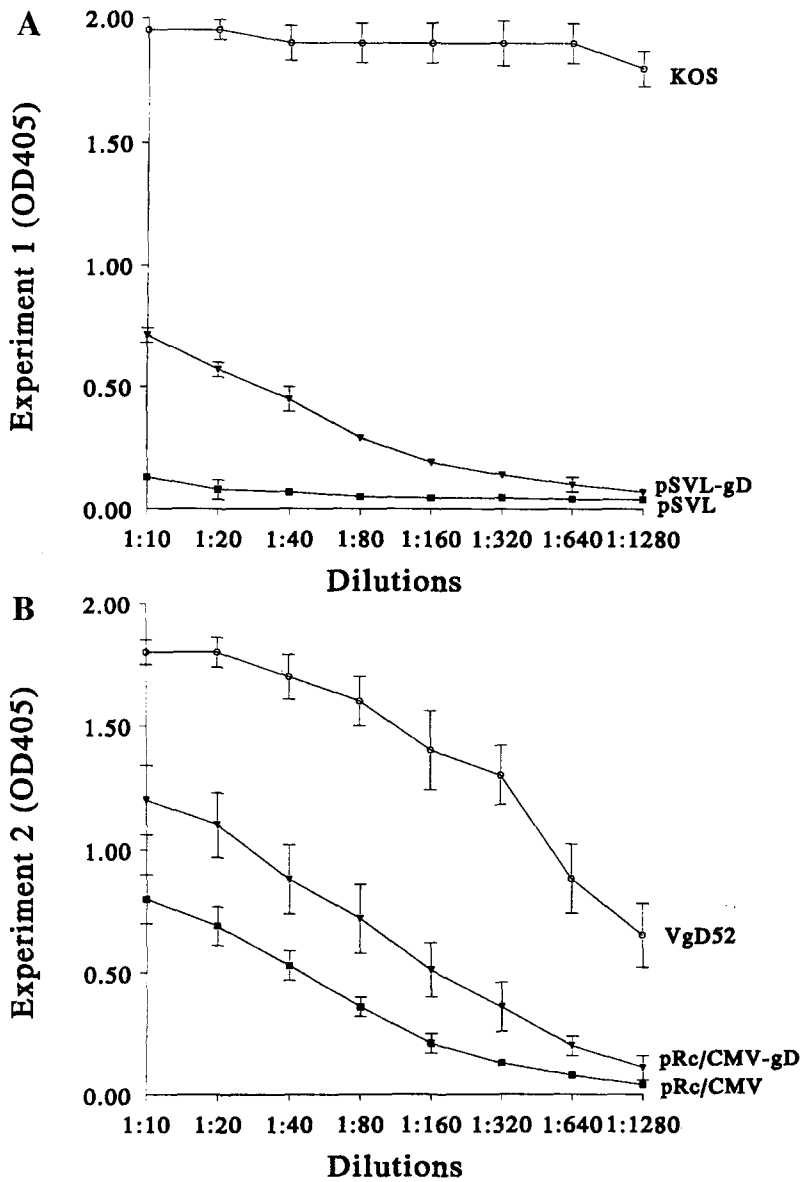

Fig. 2. ELISA anti-gD antibody titers in sera of mice vaccinated with gD-DNA. A solid-phase, indirect micro-ELISA was used for detection of gD antibody as described previously (Inummaru et al., 1987). The wells were coated with 3 μg/100 μl of vAc-gD recombinant protein (Ghiasi et al., 1991). Individual sera were used for ELISA assay. The results are plotted as the geometric mean of the readings for 5 sera per group. The error bars show the standard errors. Expt. 1 with pSVL-gD. Expt. 2 with pRc/CMV-gD.

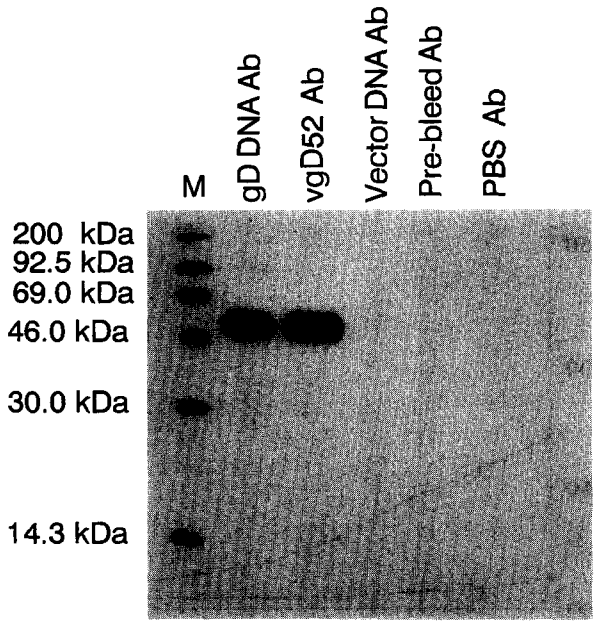

Fig. 3. Immunoprecipitation of ^{35}S-labeled gD. RS cells were infected with the HSV-1 strain KOS at a multiplicity of infection of 10 PFU/cell for 12 h. The cells were labeled 8 h postinfection with 100 μCi of translabeled [^{35}S]methionine (ICN Co.) per ml of methionine-free media for 4 h, harvested, lysed in RIPA buffer, and gD-related proteins were immunoprecipitated using pRc/CMV-gD, vgD52, pRc/CMV, prebleed, and PBS sera at 1 : 100 dilution as described previously (Inummaru et l., 1987). The proteins were separated by 12% SDS–PAGE. The gels were treated with Amplify (Amersham Co.), dried, and exposed for autoradiography. Lanes: M, molecular weight markers; (1) gD DNA Ab, KOS-infected cells immunoprecipitated with pRc/CMV-gD vaccinated antibody; (2) vgD52 Ab, KOS-infected cells immunoprecipitated with vaccinia-gD vaccinated antibody; (3) Vector DNA Ab, KOS-infected cells immunoprecipitated with pRc/CMV vector vaccinated antibody; (4) Pre-bleed Ab, KOS-infected cells immunoprecipitated with prebleed antibody; and (5) PBS Ab, KOS-infected cells immunoprecipitated with PBS-vaccinated antibody.

3, gD DNA Ab). This band appears identical to the gD band precipitated with antibodies raised against the recombinant vaccinia virus expressing gD (Fig. 3, vgD52 Ab). In contrast, no gD-specific band was immunoprecipitated by sera from mice vaccinated with the vector (Fig. 3, vector DNA Ab). In addition, no band similar to gD was immunoprecipitated from extracts of KOS-infected RS cells (Fig. 3, lane 4, prebleed Ab; lane 5, PBS Ab). Thus, it appears that the neutralization and ELISA titers induced by the vectors are not due to cross reactivity to gD.

To determine if gD-DNA can protect against lethal challenge with HSV-1, mice in both experiments were challenged i.p. with HSV-1 3 weeks after the final vaccination. In Expt. 1, mice were challenged with 1×10^7 PFU (20 LD_{50}) of the virulent McKrae strain of HSV-1 (Table 1). Ten of 10 PBS-vaccinated mock control mice died following HSV-1 challenge. Likewise all 15 (100%) of the pSVL-vaccinated negative control mice died. In contrast 5 of the 15 pSVL-gD-vaccinated mice (33%) survived the 2-week observation period. This increased survival was statistically significant ($P = 0.04$,

Table 1
Protection against lethal HSV-1 challenge in mice vaccinated with gD-DNA[a]

Immunization	Survival/total	% Survival
Experiment 1		
pSVL-gD	5/15	33
pSVL	0/15	0
PBS (mock)	0/10	0
KOS (HSV-1)	5/5	100
Experiment 2		
pRc/CMV-gD	13/15	87
pRc/CMV	8/15	53
Vaccinia-gD (VgD52)	13/15	87
PBS (mock)	2/10	20

[a] Mice were vaccinated with pSVL-gD, pSVL (vector), PBS (mock) live non-lethal HSV-1 (KOS) (Expt. 1), or with pRc/CMV-gD, pRc/CMV (vector), vaccinia virus expressing gD (VgD52), or PBS (mock) (Expt. 2), as described in Materials and methods. Three weeks after the final vaccination, mice were challenged i.p. with 1×10^7 PFU (Expt. 1) or 2×10^6 PFU (Expt. 2) of HSV-1 McKrae. Survival was determined 14 days postchallenge.

Fisher's exact test). In the KOS-positive control group, 5 of 5 mice (100%) survived the challenge. This was significantly higher than the pSVL-gD-DNA group ($P = 0.03$, Fisher's exact test), suggesting that vaccination with KOS provided enhanced protection compared to vaccination with gD-DNA (KOS, Expt. 1).

Because of the low, but statistically significant, survival rate in Expt. 1, in Expt. 2, the mice were challenged with 5-fold less virus (2×10^6; $LD_{50} = 4$) (Table 1, Expt. 2). Thirteen of the 15 mice (87%) vaccinated with pRc/CMV-gD survived, compared to 8/15 mice (53%) in the vector group. This difference was statistically significant at the 10% level ($P = 0.053$, Fisher's exact single sided). Compared to the PBS control (2/10 survivors) the protection provided by pRc/CMV-gD was highly significant ($P = 0.002$, Fisher's exact test). The results of the above experiments were similar enough to allow the data to be combined to take advantage of the power that might be gained by the increased sample size. This produced a combined 8/30 survivors in the vectors and 18/30 survivors in the gD-DNA-vaccinated groups. This difference was statistically significant ($P = 0.02$, Fisher's exact test). Martins et al. (1995) and Yokoyama et al. (1995) have also shown that mice vaccinated with LCMV-NP-DNA induced partial protection against LCMV infection. Interestingly, although vaccination with gD-DNA induced lower neutralizing and ELISA titers than vaccination with vaccinia expressed gD, gD-DNA's ability to protect against lethal challenge appeared similar to that of vaccinia gD.

To determine if induction of a cell-mediated immune response might be involved in the protection induced by gD-DNA, we looked for the ability of pSVL-gD-DNA to induce either HSV-1-specific lymphocyte proliferation or cytotoxic T-lymphocytes. The results for lymphocyte proliferation are shown on the left side of Table 2. Controls included unstimulated lymphocytes, lymphocytes from vector-vaccinated mice (negative), and lymphocytes from KOS (positive)-vaccinated mice. Following in vitro stimu-

Table 2
Lymphocyte proliferation and CTL responses in mice vaccinated with gD-DNA [a]

Immunization	Lymphocyte proliferation stimulation index [b]		CTL (killer activity) ^{51}Cr release assay [c]		
	In vitro stimulation using		E:T ratio		
	KOS	Purified gD	100:1	50:1	25:1
pSVL-gD	0.9	1.2	2	3	< 1
pSVL	1.1	1.0	5	5	4
KOS (HSV-1)	5.5 [d]	3.9 [d]	59	31	19

[a] Five mice per group were vaccinated as described in Materials and methods and spleens were harvested 3 weeks after the third vaccination. On day 1, single-cell spleen cell suspensions pooled from 5 mice were prepared and stimulated in vitro with 1.5 PFU/cell of UV-inactivated HSV-1 strain KOS (Expt. 1, and CTL assay) (Lawman et al., 1980; Zarling et al., 1986) or 1 μg of purified gD2 per 1×10^6 spleen cells (Expt. 2) for 5 days as described (Ishizaka and Mishkin, 1991; Deres et al., 1989).

[b] On day 5, 2×10^5 lymphocytes were labeled with 1 μCi of [^{3}H]thymidine and incorporation determined 18 h later as described previously (Horohov et al., 1985). The stimulation index is the average [^{3}H]thymidine incorporation of the stimulated spleen cells divided by the average [^{3}H]thymidine incorporation of the unstimulated spleen cells ($n = 8$).

[c] On day 5, CL7 target cells were infected with HSV-1 strain KOS at an m.o.i. of 10 PFU per cell for 4 h and labeled for 45 min with ^{51}Cr (300 μCi per 2×10^6 cells). After labeling, the target cells were washed and 2×10^4 target cells were incubated with different ratios of effector cells (E:T ratios) for 4 h at 37°C. The specific ^{51}Cr release was calculated from 6 replicates as described (Lawman et al., 1980). The spontaneous release was < 20% of the total incorporation.

[d] Significantly different from pSVL-gD- or pSVL-injected mice.

lation with KOS, the positive control KOS-infected mice had a stimulation index of 5.5. In contrast, the stimulation index for spleen cells from pSVL-gD-injected mice and vector (pSVL)-vaccinated mice were both close to 1 (0.9 and 1.1, respectively). Following in vitro stimulation with purified gD, the KOS-infected mice had a stimulation index of 3.9. Again, the stimulation index for spleen cells from pSVL-gD-injected mice and vector (pSVL)-vaccinated mice were both close to 1 (1.2 and 1.0, respectively). Similar results were also obtained in a repeat experiment using a colorimetric rather than a radioactive assay and with spleen cells from mice vaccinated with pRc/pCMV-gD (not shown). Thus, no lymphocyte proliferation response was detected following vaccination of mice with gD-DNA. This is similar to the results reported by Martins et al. (1995) and Yokoyama et al., (1995) following vaccination with LCMV-NP DNA.

To detect total CTL responses, lymphocytes were pooled from 5 mice/group 21 days after the third injection and stimulated in vitro with UV-inactivated HSV-1 (Lawman et al., 1980; Zarling et al., 1986) (Table 2, right side). No HSV-1-specific CTL activity was detected in mice injected with pSVL-gD-DNA. The pSVL-gD- and the pSVL-injected mice had similar levels of ^{51}Cr release at all effector to target cell ratios. In contrast, the KOS-vaccinated group had significant CTL activity at all effector to target ratios tested. Similar results were obtained with lymphocytes stimulated in vitro with gD, and with lymphocytes from mice vaccinated with pRc/CMV-gD-DNA (not shown). Thus, we were unable to detect induction of CTL activity in mice vaccinated with gD-DNA. Similarly, Martins et al. (1995) and Yokoyama et al. (1995) found that

LCMV-specific CTL responses were below detectable levels in DNA-vaccinated mice. However, when the DNA-vaccinated mice were infected with LCMV, memory CTL were seen.

The immune response to gD appears to play a major role in viral clearance and protection against lethal HSV-1 challenge (Berman et al., 1983; Lasky et al., 1984; Mishkin et al., 1991; Ghiasi et al., 1994). The results reported here suggest that injection of gD-DNA in the form of a plasmid in which gD expression is controlled by a strong promoter (either SV40 late promoter or HCMV immediate–early promoter), can produce HSV-1-neutralizing antibody titers. Although these titers were much lower than those induced by vaccination with HSV-1, they were only slightly lower than those induced by vaccination with vaccinia gD. We also found significant induction of anti-gD ELISA titers by gD-DNA. The ELISA titers were much lower than those induced by vaccination with HSV-1 or vaccinia gD. gD-DNA also produced protection against lethal HSV-1 challenge. This protection was much lower than that provided by vaccination with HSV-1, but was similar to that provided by vaccination with vaccinia gD. One of the potential advantages of using DNA as a vaccine is the possibility of induction of strong CTL responses (Ulmer et al., 1993). However, although gD can induce CTL responses (Johnson et al., 1990), in this study, no CTL responses were detected. Thus, the protection induced by gD-DNA immunization against HSV-1 challenge was likely due to neutralizing antibody or antibody-dependent cell cytotoxicity (ADCC) (Bernstein et al., 1988; Kohl, 1991).

In this study, both vectors (pSVL and pRc-CMV) induced higher than expected neutralizing and ELISA backgrounds. How vaccination with the DNA vectors caused neutralizing antibody and ELISA titers is not known. However, it did not appear to be due to induction of antibody with gD reactivity, since sera from vector vaccinated mice were unable to immunoprecipitate gD. Similar induction of neutralizing and ELISA titers by vectors has previously been reported in studies with BHV (Cox et al., 1993), influenza (Fynan et al., 1993), and HIV (Wang et al., 1993). In all these vectors, only the gene for neomycin is under the control of a mammalian promoter (Southern and Berg, 1982). It is possible that the product of the neomycin gene expressed by these vectors may be involved in the ELISA and neutralizing antibody backgrounds.

In summary, our results suggest that vaccination with gD-DNA can produce a small amount of protection against lethal HSV-1 challenge. This protection was similar to that induced by vaccination with a vaccinia gD plasmid, but lower than that induced by vaccination with HSV-1. We have previously shown that a gD subunit vaccine produced higher neutralization and ELISA titers and similar or more efficient protection against HSV-1 challenge than the vaccinia-gD used here (Ghiasi et al., 1991, 1994). Thus, at present, work still remains to make these gD-DNA plasmids a useful alternative to a gD subunit vaccine against HSV-1.

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