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PROTECTION OF MICE FROM LETHAL HERPES SIMPLEX VIRUS INFECTION BY VACCINATION WITH A SECRETED FORM OF CLONED GLYCOPROTEIN D

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Mammalian cell lines have been constructed which synthesize a truncated form of the HSV 1 glycoprotein D (gD). The truncated protein lacks the carboxy-terminal 93 amino acids, is glycosylated, and is constitutively secreted to the culture medium. Radioimmunoprecipitation experiments demonstrate that truncation of the protein did not drastically alter its antigenic structure. Vaccination of mice with the secreted HSV 1 gD resulted in the induction of antibodies able to neutralize both HSV 1 and HSV 2 *in vitro*. Mice vaccinated with this antigen were protected from a lethal challenge with either HSV 1 or HSV 2.

Herpes Simplex Virus Types 1 and 2 (HSV 1 and HSV 2) are responsible for a variety of diseases including oral and genital lesions, aseptic meningitis, disseminated viral infections in neonates, and, possibly, cervical carcinoma[1]. Although immunotherapies are not currently available, it is possible that a vaccine against HSV 1 and HSV 2 infection could provide an important prophylactic measure. Because of concern regarding the oncogenic potential of the viral DNA[2], vaccination with either a killed virus or an attenuated live virus has not gained widespread acceptance. A less objectionable alternative to the virus-based vaccine would be one containing one or more of the HSV envelope glycoproteins. A purified HSV glycoprotein, free of HSV DNA, could provide the basis of a subunit vaccine which would be free of any oncogenic risk.

Although the HSV genome encodes at least 4 glycoproteins (gA/B, gC, gD, and gE[3]), several lines of evidence suggest that the gD glycoprotein would be the best candidate for inclusion in an HSV subunit vaccine. Previous

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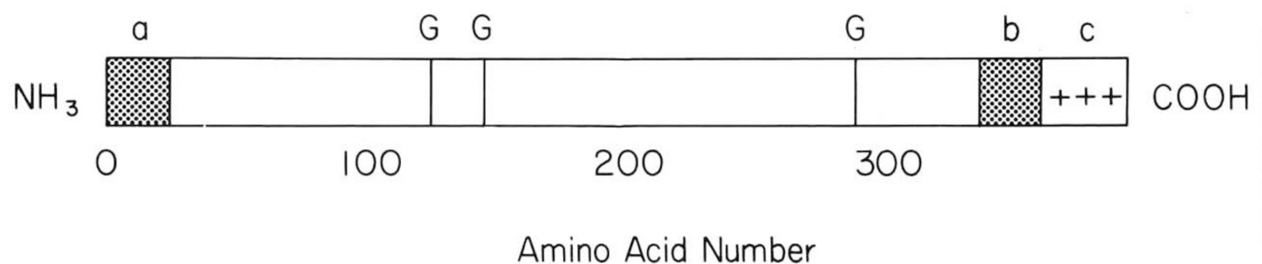
studies have shown that gD from HSV 1 and HSV 2 share a number of antigenic determinants such that antibody induced by one type of gD will cross-neutralize both viruses[4–6]. Passive transfer studies have shown that anti-gD polyclonal and monoclonal antibodies protect mice against lethal HSV 1 and HSV 2 challenges[7]. Finally, purified gD isolated from HSV 1 infected cells was found to induce protective immunity in mice vaccinated with the protein[8–10]. Together, these observations suggest that the purified gD protein possesses sufficient antigenic structure to generate a protective response and that gD from either HSV 1 or HSV 2 may be a sufficient subunit vaccine against HSV infection.

One method which has been used to purify gD has been to fractionate a lysate of virus infected cells using anti gD monoclonal antibody affinity columns[11]. A second method for the production of gD protein entailed the expression of gD in *Escherichia coli* as a chimaeric fusion protein, where the amino- and carboxy-termini of the cloned gD were fused to bacterial protein sequences[12]. In this paper, we describe a third procedure which involves the use of mammalian cell lines which have been genetically altered to produce high levels of a secreted form of HSV 1 gD. Cell lines producing this secreted antigen provide a novel approach to subunit vaccine production because the product can be directly harvested from the culture medium. This scheme considerably simplifies the isolation of the viral protein since it obviates the solubilization of cells to extract membrane-bound viral antigens and results in a preparation substantially free of contaminating cellular debris. Mice immunized with this secreted antigen generate neutralizing antibodies to both HSV 1 and HSV 2 and are protected from a lethal challenge with both types of virus.

RESULTS AND DISCUSSION

Construction of a Mammalian Cell Line Expressing Secreted HSV 1 gD. We have previously demonstrated that the HSV 1 gD gene could be transfected into mammalian cells and expressed under the control of an SV 40 early promoter[22]. Ligation of the gD gene into a vector containing a cDNA copy of the murine DHFR gene[28], allowed us to select cells in which the transfected gD gene was translated, glycosylated, and transported to the cell surface in a manner similar to that which occurs in virus-infected cells[22]. Because a variety of both monoclonal and polyclonal antibodies, including human, were found to react with this membrane-bound form of gD, we concluded that the antigenic structure of the transfected gene product closely resembled that of the native protein.

Analysis of the hydrophobic and hydrophilic properties of the protein sequence derived from the gD gene sequence revealed that the protein contained features typical of membrane-bound glycoproteins[15, 29]. The deduced amino acid sequence[14, 15] predicted a hydrophobic signal sequence[30] at the amino terminus, three potential N-linked glycosylation sites[31] and a hydrophobic transmembrane domain followed by a hydrophilic cytoplasmic sequence at the carboxy terminus[32]. Previous work on the Vesicular Stomatitis Virus G protein[33] and the Influenza



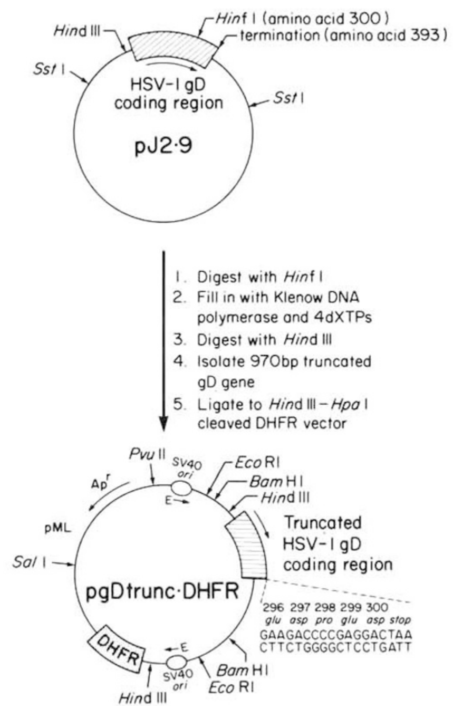


FIGURE 1 A. Schematic diagram showing regions of the HSV 1 glycoprotein D (gD) thought to be important for export and binding to the plasma and viral membranes. A hydrophobic signal sequence consisting of amino acids 1–25 and a hydrophobic membrane binding domain consisting of residues 340–360 (shaded regions) were identified from hydropathy analysis[28] of the gD amino acid sequence predicted from the DNA sequence[14, 15]. Also shown are three potential N-linked glycosylation sites (marked G) at asparagine residues 119, 146, and 287[31], and a carboxy-terminal hydrophilic domain (marked +) consisting of residues 361–393 predicted to extend from the inner surface of the plasma membrane or viral membrane. **B.** Diagram of the plasmid pgDtrunc. DHFR constructed for the expression of a secreted form of gD. pJ2.9 contains a 2.9 kilobase pair SstI subclone containing the entire HSV 1 gD gene cloned in pBR322. The expression plasmid, pgDtunc.DHFR, consisted of the pBR322 bacterial origin of replication and ampicillin resistance gene[37], a cDNA encoding murine dihydrofolate reductase (DHFR) under the transcriptional control of the SV40 early promoter[16] and a HindIII-HinfI fragment encoding the first 300 amino acids of the HSV 1 gD gene under the transcriptional control of a second SV40 early promoter[22]. The HindIII site of the gD gene fragment lies 74 base pairs to the 5′ side of the gD initiator methionine[14, 15]. The HindIII site to which it was ligated is 230 base pairs to the 3′ side of the SV40 early promoter Goldberg-Hogness box[16]. The HinfI site, blunted with DNA polymerase I Klenow fragment and 4 deoxynucleotide triphosphates, was ligated to the HpaI site in the 3′ nontranslated region of the Hepatitis B virus surface antigen gene,[35] creating an in-phase TAA stop codon.

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hemagglutinin protein[34] revealed that removal of the carboxy-terminal membrane binding domain resulted in the secretion of these proteins when transiently expressed in mammalian cells. We reasoned that deletion of the carboxy-terminal coding region of the gD gene might similarly result in a gene product which would be secreted from the cell. Moreover, the transfection of this truncated gene into cells using a vector encoding the selectable dihydrofolate reductase (DHFR), marker would allow the isolation of continuous cell lines producing the secreted antigen[22].

Figure 1A shows a schematic illustration of the HSV 1 gD protein as derived from the DNA sequence[14, 15]. It can be seen that truncation of the protein after residue 300 results in a polypeptide which lacks the presumed carboxy-terminal membrane binding domain, but which contains approximately 75% of the protein. The resultant protein, which contains the amino-terminal signal sequence[30] but which lacks the carboxy-terminal membrane binding domain[32] should be translated on membrane-bound ribosomes, pass through the lumen of the rough endoplasmic reticulum, and be directed through the cell's constitutive secretory pathway. Figure 1B illustrates the scheme for the construction of a plasmid expressing this truncated gD. The HinfI site located in the region encoding amino acid 300 is approximately 40 codons upstream from the putative transmembrane domain and 93 codons from the gD translational termination codon. A HindIII-HinfI gene fragment corresponding to gD residues 1–300 was isolated and was fused by blunt end ligation to a HpaI site derived from the 3 prime nontranslated region of the Hepatitis B virus surface antigen gene[35]. This fusion resulted in the creation of an in-phase TAA stop codon immediately after amino acid 300 of the gD gene. The resultant plasmid, pgD trunc.DHFR, contained the truncated gD gene under the transcriptional control of the SV40 early promoter and the polyadenylation signals of the Hepatitis B virus surface antigen gene[36]. In addition, the plasmid contained a cDNA fragment encoding the murine DHFR gene[28] under the transcriptional control of a second SV40 early promoter adjacent to the ampicillin resistance gene and bacterial origin of replication derived from the plasmid pBR322[37]. The messenger RNA, transcribed from the SV40 early promoter, includes 5′ nontranslated sequences derived from the SV40 T antigen and the HSV gD gene, the region coding for the truncated HSV gD gene, and the 3′ nontranslated region of the Hepatitis B surface antigen gene.

The plasmid described in Figure 1B was transfected into a DHFR deficient Chinese Hamster Ovary (CHO) cell line as described in Experimental Protocol. After transfection, cells were selected for growth in media which lacked hypoxanthine, glycine, and thymidine[16]. Several thousand colonies were obtained per microgram of plasmid DNA. The expression of the truncated HSV 1 gD gene in two independently isolated colonies was analyzed by immunoprecipitation of both ^{35}S-methionine labeled intracellular proteins as well as the proteins secreted into the medium conditioned by these cells. Figure 2 shows that a discrete band of 37,000 daltons was specifically immunoprecipitated from the intracellular lysates using rabbit HSV 1 antiserum. When media conditioned by the cell lines was similarly analyzed, a larger, more diffuse band of approximately 45,000 daltons could be specifically immunoprecipitated. The extracellular polypeptide was approximately 15,000 daltons smaller than the full-length, membrane-bound form of gD shown in Figure 2[22]. The difference in molecular weights found for the intracellular form of the truncated protein and the extracellular secreted protein appeared to represent a precursor-product relationship

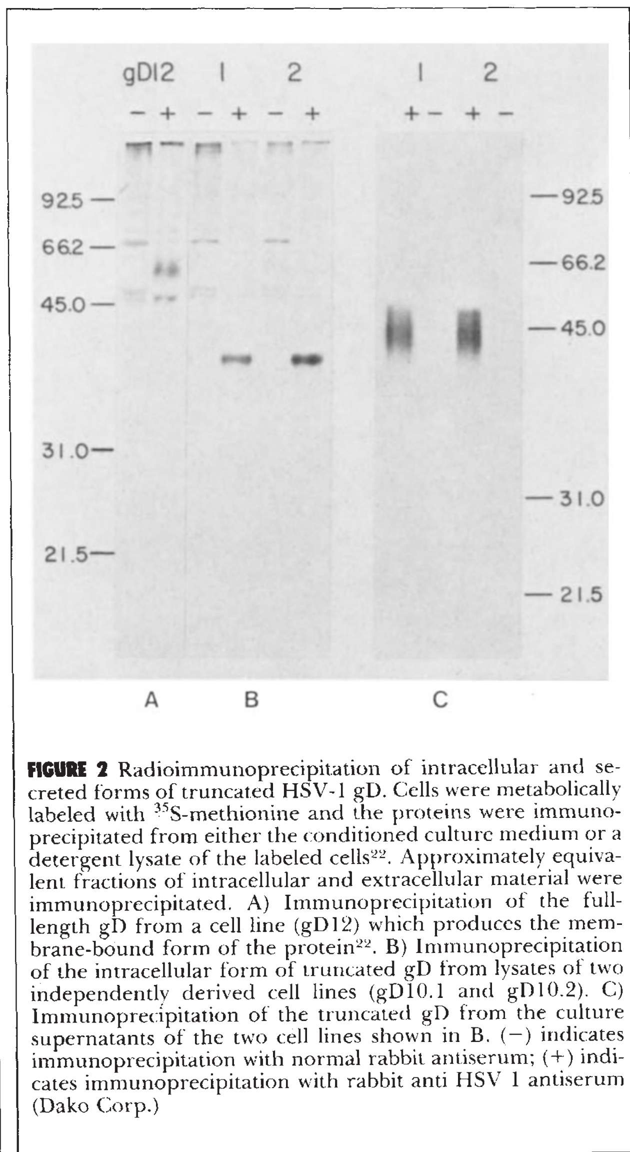

FIGURE 2 Radioimmunoprecipitation of intracellular and secreted forms of truncated HSV-1 gD. Cells were metabolically labeled with ^{35}S-methionine and the proteins were immunoprecipitated from either the conditioned culture medium or a detergent lysate of the labeled cells[22]. Approximately equivalent fractions of intracellular and extracellular material were immunoprecipitated. A) Immunoprecipitation of the full-length gD from a cell line (gD12) which produces the membrane-bound form of the protein[22]. B) Immunoprecipitation of the intracellular form of truncated gD from lysates of two independently derived cell lines (gD10.1 and gD10.2). C) Immunoprecipitation of the truncated gD from the culture supernatants of the two cell lines shown in B. (−) indicates immunoprecipitation with normal rabbit antiserum; (+) indicates immunoprecipitation with rabbit anti HSV 1 antiserum (Dako Corp.)

between the intracellular gD and a more highly glycosylated form of the secreted material[31]. We have previously demonstrated a similar precursor-product relationship for the membrane-bound form of cloned gD when expressed in CHO cells[22]. In addition, subsequent analysis (P. Berman, unpublished observations) has shown that the intracellular form of the protein represents a high mannose (i.e., endoglycosidase H sensitive) precursor to the more extensively glycosylated secreted protein. These results demonstrate that the truncated gD is processed, glycosylated, and secreted from these cell lines. In addition, these results, in conjunction with previous work[20], prove that at least one function of the carboxy-terminal 93 amino acids of gD is to bind the protein to either the cell plasma membrane or the viral envelope.

The antigenic structure of the truncated gD was explored by testing the ability of various polyclonal and monoclonal antibodies raised against the native protein to immunoprecipitate the truncated molecule. Results of this type of study are shown in Figure 3. Rabbit anti-HSV 1 antibodies, mouse anti-HSV-1 antibodies, human anti-HSV serum, and several mouse monoclonal anti-gD antibodies all immunoprecipitated the secreted gD. Normal (pre-immune) mouse and rabbit serum failed to immunoprecipitate the truncated viral protein as did human

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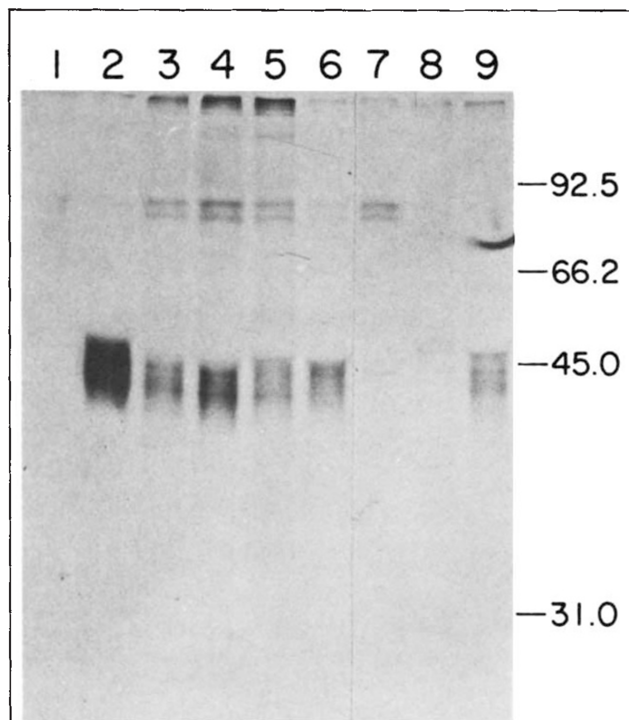

FIGURE 3 Radioimmunoprecipitation of truncated HSV-1 gD with monoclonal and polyclonal antibodies raised against native full-length gD. gD10.2 cells were metabolically labeled with ^{35}S-methionine and the growth conditioned culture medium was harvested after an overnight incubation. Antisera (2–5 μl) was incubated with 0.5 ml of the conditioned medium, immunoprecipitated, and run on a 10% polyacrylamide gel as described in Experimental Protocol. Immunoprecipitations shown above were carried out with the following antisera: normal rabbit serum, lane 1; rabbit anti-HSV-1 (Dako), lane 2; anti-gD monoclonal antibody D4.7, lane 3; anti-gD monoclonal antibody D4.11, lane 4; anti-gD monoclonal antibody 4S, lane 5; polyclonal mouse anti-HSV-1 antibodies, lane 6; preimmune mouse serum, lane 7; HSV seronegative human serum, lane 8; HSV seropositive human serum, lane 9.

serum from individuals known to be seronegative for HSV-1 and HSV-2. In other studies (data not shown) 16 independently isolated anti-gD monoclonal antibodies were tested for binding to truncated gD using a dot-blot assay[38]. All of the monoclonals tested, with the exception of those known to bind to the carboxyterminus of full-length gD, bound to the truncated molecule (R. Eisenberg and G. Cohen, personal communication). These results strongly suggest that the truncated gD expressed in these cells is antigenically similar to the full-length gD protein synthesized in virus infected cells and that the deletion of the carboxy-terminal membrane binding domain does not drastically alter the conformation of the protein.

The Immunogenicity of Secreted HSV 1 gD. Previous work[8–10] demonstrated that vaccination of mice with gD purified from virus infected cells could generate a protective neutralizing response against subsequent HSV infection. In order to address the question of whether the truncated gD secreted from mammalian cells would be useful as an HSV subunit vaccine, mouse vaccination experiments were conducted. Eight-week-old BALB/c mice were immunized with one of two partially purified preparations of secreted gD. Each mouse received approximately 3 μg of gD per injection incorporated in either complete or incomplete Freund's adjuvant. Control mice were immunized on the same schedule with human serum albumin. Three weeks after the second immunization, mouse sera were assayed for their ability to neutralize HSV 1 and HSV 2 *in vitro*. Table 1 shows that all mice vaccinated with secreted gD showed significant neutralizing titres to both HSV 1 and HSV 2. Thus, secreted HSV 1 gD is able to induce type common neutralizing antibodies against both virus types. The titers against HSV 1 were significantly higher than those against HSV 2, suggesting that the amino acid sequence differences between HSV 1 and HSV 2 gD[15], and previously described antigenic differences[39] between the gD proteins from the type 1 and type 2 viruses have an effect on the relative levels of neutralizing antibody. It can also be seen (Table I) that the group of mice vaccinated with a preparation of gD which was 50% pure (Experimental group 4), had higher average HSV 2 neutralizing titers than the group which was vaccinated with a preparation containing only 10% gD (Experimental group 2).

We next determined if mice immunized with secreted gD were protected from a lethal challenge with HSV. Intraperitoneal injection of mice with either HSV 1 or HSV 2 results in an ascending neurological infection which induces either death or paralysis[40]. This system thus represents a convenient model with which to test the efficacy of the secreted gD antigen as a vaccine. In the first experiment, mice which had been vaccinated with either the secreted gD (Experimental group 2) or with human serum albumin (HSA) (Experimental group 1) were challenged with approximately 1×10^7 plaque-forming units of HSV 1. Dose response experiments showed that this virus dose was approximately two-fold higher than that which was necessary to kill 50% of the non-vaccinated mice ($LD_{50} = 5 \times 10^6$ plaque forming units). At the end of the three week observation period, we found that 70% of the mice in the control (HSA) group either died or showed signs of HSV 1 illness, including weight loss, lack of grooming, and hind-limb paralysis. The mice vaccinated with secreted gD showed no visible signs of viral infection. From this experiment we conclude that the secreted gD was able to induce a type-specific protective immunity in mice against HSV 1 infection.

The next set of experiments were performed to determine if the type common anti-HSV activity induced by HSV 1 secreted gD was sufficient to protect mice from a lethal challenge with HSV 2. Dose response experiments with HSV 2 infection of mice revealed that the virus LD_{50} was approximately 5×10^2 plaque forming units (P. Berman and L. Lasky, unpublished observation). Mice in the group vaccinated with secreted gD (Experimental group 4) and control group (Experimental group 3) were challenged with 1×10^5 plaque forming units of HSV 2, a dose which corresponds to approximately 200 times greater than the LD_{50} for HSV 2. Table 1 shows that all 25 of the mice in the control group were dead by the end of the three week observation period. In contrast, mice vaccinated with secreted gD (Experimental group 4) were completely protected from HSV 2 infection and showed no signs of illness. This result demonstrates that HSV 1 secreted gD was able to induce a protective immune response in mice against a challenge with HSV 2 which is far in excess of that necessary to result in a lethal HSV 2 infection.

The efficacy of killed virus (subunit vaccines) to protect against viral infections has been debated for many years. While it is known that subunit vaccines generate good humoral immune responses, their role in the generation of cell mediated immunity is still unclear. The availability of virtually unlimited quantities of secreted gD as well as cell lines expressing individual viral surface antigens[22] will

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TABLE 1 Immunogenicity of Secreted HSV-1 gD in Mice.

Expt'l. Group	Immunogen[1]	No. of Mice	Average *In vitro* Neutralization Titer[2] (log_2) HSV-1	HSV-2	Challenge Virus	Asymptomatic	Symptomatic	Dead
1.	HSA	13	<3	<3	HSV-1	3	3	7
2.	Secreted gD	11	7.0 ± 1.6	3.4 ± 0.5	HSV-1	11	0	0
3.	HSA	25	<3	<3	HSV-2	0	0	25
4.	Secreted gD	15	7.3 ± 2.8	5.4 ± 2.9	HSV-2	15	0	0

1. Eight week old female BALB/c mice were injected at multiple intradermal and subcutaneous sites, and were boosted 4 to 5 weeks later as described in Experimental Protocol. Each mouse received approximately 3 μg of secreted HSV-1 gD or Human Serum Albumin (HSA) per injection.
2. For *in vitro* neutralization studies, serially diluted mouse serum (log_2 dilution range 3–14) were incubated with 40 pfu of HSV-1 (MacIntyre strain) or HSV-2 (MS strain) for 1 hour at 37°C. Each dilution was then applied to 4×10^4 Vero cells contained in each well of a 96 well microtiter plate. After 4 days, the cells were stained with 0.5% crystal violet. Neutralization titers were calculated by determining the highest serum dilution which prevented virus growth. Values indicated represent the average neutralization titers (log_2) ± standard deviation. The serum tested in experiments 1 and 2 was harvested 19 days after boosting. The serum tested in experiments 3 and 4 was harvested 24 days after boosting.
3. Mice were challenged by intraperitoneal injection of virus and then monitored daily for a period of 3 weeks for signs of virus induced wasting, paralysis, or death. The animals in experiments 1 and 2 were challenged with 1×10^7 pfu of HSV-1 (MacIntyre strain) 36 days after boosting. All lethally infected animals expired by day 14 after the virus challenge. The animals in experiments 2 and 4 were challenged with 2×10^5 pfu of HSV-2 (MS strain) 31 days after boosting. All lethally infected animals expired by day 10 after the virus challenge.

permit us to define the extent of protection conferred by various HSV subunit vaccines in the generation of both cell mediated and humoral responses.

The results presented here demonstrate that the gene for HSV 1 gD can be engineered to a secreted form which retains many of the antigenic properties of the native glycoprotein when expressed in permanent mammalian cell lines. In addition, this protein functions as an effective subunit vaccine which induces type-common neutralizing antibody and which protects mice against lethal HSV 1 and HSV 2 infections. Although this vaccine has not yet been formulated (i.e., adjuvanted) into a preparation acceptable for testing in humans, preparations suitable for human use are now being developed. The results described above demonstrate the potential for the production of subunit vaccines using genetically engineered mammalian cell lines.

EXPERIMENTAL PROTOCOL

Cells and Viruses. DHFR-deficient Chinese Hamster Ovary Cells (CHO) K1 DUX-B11 were obtained from L. Chasin (Columbia University) and propagated as described[13]. VERO cells were grown in Dulbeccos Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum (Gibco), penicillin (100 μ/ml) and streptomycin (100 μ/ml). HSV 1 (MacIntyre strain) and HSV 2 (MS strain) were obtained from MA Bioproducts and were propagated on VERO cells.

Nucleic Acid Manipulations. Restriction enzymes and T4 DNA ligase were obtained from New England Biolabs and were used according to the manufacturer's recommendations. The HSV 1 gD gene was isolated as described[15]. The vectors containing the murine DHFR cDNA have been previously described[16]. Plasmid DNA was isolated using the method of Birnboim and Doly[17]. Transformations were performed as described[16] using *E. coli* strain MM294[17].

Cell Transfections and Selections. Transfections were performed using the calcium phosphate precipitation procedure[20] and the cells were treated with 20% glycerol 3 hr after the addition of DNA[21]. Medium was replaced after the glycerol shock, and the cells were allowed to grow for two days in the absence of selective media. Cells were then passaged into selective media [Ham's F-12 medium lacking hypoxanthine, glycine, and thymidine (HGT^- media) supplemented with extensively dialyzed fetal bovine serum (Gibco)]. Medium was changed every 3–4 days. After 7–14 days, visible clones were isolated using a cloning cylinder and passaged.

Radioimmunoprecipitation of Intra- and Extracellular Extracts. Cells were grown to confluence under selective conditions, metabolically labeled with ^{35}S-methionine, harvested, and solubilized in non-ionic detergent as described previously[22]. Cell lysates or growth conditioned culture media (typically 500 μl) were mixed with 2–5 microlitres of the appropriate antiserum and were incubated at 4°C for 30 minutes[22]. Immune complexes were prepared by adsorbtion to fixed *S. aureus* cells[23] and analyzed on 10% polyacrylamide slab gels according to the method of Laemmli[24]. ^{14}C-labeled molecular weight markers (phosphorylase B, bovine serum albumin, ovalbumin, and carbonic anhydrase) were included in the gels. Polyclonal rabbit anti-HSV-1 and HSV-2 antisera were obtained from Dako, Inc. Anti-gD monoclonal antibodies 4S and 55S[25] were kindly provided by M. Zweig. Anti-gD monoclonal antibodies D4.7 and D11 were kindly provided by D. Wiley and M. Trusdale. A monoclonal anti-gD antibody prepared by C. Benton was purchased from Bethesda Research Laboratories. Human sera known to be seropositive or seronegative for HSV were kindly provided by Dr. John Stewart of the Centers for Disease Control.

Preparation of Antigen and Immunization of Mice. Cells secreting truncated HSV-1 gD were grown to confluence in polystyrene roller culture bottles (850 cm^2) in selective medium containing 7% dialyzed fetal bovine serum. The growth medium was then removed, the cells were washed 2–3 times with PBS, and the cells were cultured in serum-free harvest medium (DMEM containing 25mM Hepes). After 3–5 days, the conditioned medium was collected, filtered through a 0.2 micron sterilization filter (Sybron-Nalge), and concentrated by ultrafiltration (Amicon). The protein concentration in different preparations was determined by the Lowry method[26] using bovine serum albumin as a standard. The method used for the purification and quantitation of secreted gD

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will be described in detail elsewhere. Two preparations of secreted gD were used to immunize the mice in these studies. The preparations differed in that the first preparation tested was approximately 10% pure while the second preparation was approximately 50% pure. The protein concentrations were adjusted such that each mouse received approximately 3 μg of secreted gD per injection (6 μg or 30 μg of total protein).

Eight week old BALB/c mice (Simonsen Laboratories or Charles River Laboratories) were injected at multiple intradermal or subcutaneous sites with the secreted protein emulsified in complete Freund's adjuvant[27]. Four to five weeks after the primary immunization, the mice were boosted with the same amount of antigen incorporated in incomplete Freund's adjuvant. Control mice, matched to the experimentals with regard to age, sex, and strain, were immunized according to the same protocol with the exception that 3 μg of human serum albumin (HSA) replaced the secreted gD. Serum was harvested by tail-bleeding 19 to 24 days after boosting and was used for *in vitro* virus neutralizing studies.

***In Vitro* Serum Neutralization Assays.** Mouse sera were serially diluted ($\log_2$ dilution range of 3–14) with DMEM containing 10% fetal bovine serum. Serum dilutions were subsequently incubated with 40 plaque-forming-units of either HSV 1 or HSV 2 for 1 hour at 37°C in a volume of 200 microlitres. 175 microlitre aliquots of antibody-treated virus were applied to 4×10^4 VERO cells in each well of a 96 well microtitre tissue culture plate. After 4 days, the culture medium was removed and the cells were stained with 0.5% crystal violet. Neutralization titres were calculated by determining the highest serum dilution which prevented virus growth.

Virus Challenges. Mice were challenged by intraperitoneal injection of 10^7 plaque-forming-units of HSV 1 or 10^5 plaque-forming-units of HSV 2 in 100 μl of DMEM containing 10% fetal bovine serum. Challenged mice were observed for a period of three weeks and were scored for wasting, paralysis and mortality.

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