NOTES

Synthetic Glycoprotein D-Related Peptides Protect Mice against Herpes Simplex Virus Challenge

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Glycoprotein D (gD) of herpes simplex virus (HSV) protects mice from a lethal challenge by either HSV type 1 (HSV-1; oral) or HSV-2 (genital). We evaluated whether synthetic peptides representing residues 1 through 23 of gD (mature protein) can be used as a potential synthetic herpesvirus vaccine. The immunogenicity of the peptides was demonstrated by the biological reactivity of antipeptide sera in immunoprecipitation and neutralization assays. All sera which immunoprecipitated gD had neutralizing activity against both HSV-1 and HSV-2. The highest titers were found in animals immunized with the longest peptides. The region of residues 1 through 23 was immunogenic regardless of whether the type 1 or type 2 sequence was presented to the animal. Immunization of mice with gD or synthetic peptides conferred solid protection against a footpad challenge with HSV-2. However, the peptides were not as effective as gD in protection against an intraperitoneal challenge. The results suggested that synthetic vaccines based on gD show promise and should be more rigorously tested in a variety of animal models.

Herpes simplex viruses (HSV) cause a number of human diseases, including cold sores, eye and genital infections, and encephalitis (22). HSV glycoproteins are structural components of the virion envelope and have been implicated in virus-induced alterations of mammalian cells (9, 28, 29). These glycoproteins are expressed on infected cell plasma membranes and act as major antigenic stimuli for cellular and humoral immunity in the host (23, 25). Glycoprotein D (gD) is one of five well-defined and distinct glycoprotein components of the envelope (28, 29). Studies have shown that gD is structurally and antigenically similar though not identical in the two serotypes of HSV (32), HSV type 1 (HSV-1; oral) (gD-1) (16) and HSV-2 (genital) (gD-2) (3). Virus-induced and recombinant-derived gD obtained from a variety of sources protects mice against a lethal virus challenge. For example, gD purified from cells infected with either HSV-1 or HSV-2 protected mice from a lethal intraperitoneal (i.p.) challenge by virus of either serotype (21). In a second study, purified gD-1 protected mice only from HSV-1 challenge (6). The full-length molecule is not necessary for protection since a truncated form of gD-1 (residues 1 through 275 of the mature protein) protected mice challenged i.p. with HSV-1 or HSV-2 (20). In the latter case, the glycoprotein (lacking the transmembrane anchor and cytoplasmic domains) was produced by mammalian cells transformed by a plasmid vector carrying a portion of the gD gene. Finally, the gene for gD-1 has been cloned into vaccinia virus. Immunization with the recombinant virus protected mice from a lethal challenge by HSV-1 and HSV-2

(10, 24). Thus, gD is a reasonable choice for further investigation as a subunit vaccine against HSV infection.

One approach in modern vaccine development utilizes chemically synthesized peptides representing amino acid sequences in proteins of interest (1, 27, 33). For a given viral antigen, the strategy used for the design of a synthetic peptide vaccine includes (i) identification of immunogenic domains of the antigen (criteria for predicting such domains include secondary structure [7] and hydrophilicity [18]; (ii) chemical synthesis of the peptide and attachment to suitable macromolecular carriers; and (iii) in vivo demonstration of the ability of such peptides to confer varying levels of protection against diseases such as serum hepatitis (12), foot and mouth (5), and influenza (2, 26). We used this approach to evaluate whether a portion of the gD amino acid sequence could be used as a potential synthetic herpesvirus vaccine.

Our choice of one particular region of gD was based on previous work (8) in which we described the physical localization and chemical synthesis of a type-common antigenic determinant of gD contained within the N-terminal amino acid residues 8 through 23 of mature gD-1 (8-23[1]). The 8-23[1] peptide is recognized by a type-common gD-specific monoclonal antibody belonging to group VII (14; 15) which neutralizes the infectivity of HSV-1 and HSV-2. The epitope recognized by group VII monoclonal antibody has been mapped and is located between amino acids 11 and 19 (8, 11). Antisera from animals immunized with 8-23[1] coupled to keyhole limpet hemocyanin (KLH) recognized native and denatured gD purified from virus-infected cells and from the 8-23[1] peptide. These sera also neutralized the infectivity of HSV-1 and HSV-2. Thus, the properties of the antipeptide

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FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of rabbit anti-peptide sera and their reactivity against gD-1 and gD-2. Autoradiogram of 10% acrylamide gels cross-linked with *N,N'*-diallyltartardiamide. For metabolic labeling of gD, monolayers (KB cells for HSV-1 and BHK cells for HSV-2) were infected with HSV-1 (strain HF) or HSV-2 (strain Savage) (17). At 2 h postinfection, the monolayers were overlaid with Eagle minimal medium. At 6 h postinfection, the cells were incubated for 15 min in Hanks salts containing 750 μ Ci of [35 S]methionine (Amersham Corp., Arlington Heights, Ill.). The cells were washed with iced saline and lysed, and cytoplasmic extracts were prepared (5, 16, 19, 20), and immunoprecipitated. Odd-numbered lanes contained proteins immunoprecipitated from HSV-1-infected cells. Even-numbered lanes contained proteins from HSV-2-infected cells. The antisera used were as follows. Lanes: 1 and 2, preimmune rabbit serum; 3 and 4, anti-gD-1 serum; 5 and 6, anti-1-23[1] serum; 7 and 8, anti-8-23[2] serum; 9 and 10, anti-1-16[1] serum; 11 and 12, anti-1-16[2] serum; 13 and 14, anti-1-23[H] serum; 15 and 16, anti-1-23[2] serum. The molecular size of pgD-1 is 53,000 daltons, and pgD-2 is 52,000 daltons. The band present in lane 7 does not comigrate with gD, but it does correspond in position to actin. A similar band is also present in lane 15.

sera were similar to those of the group VII monoclonal antibodies.

The sequence of the first 23 amino acids of gD-1 and gD-2 based on nucleic acid (19, 20, 34, 35) and N-terminal amino acid sequencing (13) follows.

1-Lys-Tyr-Ala-Leu-Ala-Asp-(Ala or Pro)-Ser-Leu-Lys-Met-Ala-Asp-Pro-Asn-Arg-Phe-Arg-Gly-Lys-(Asp or Asn)-Leu-Pro-23

In all strains examined to date (13, 19, 20, 34, 35; A. Minson, personal communication), these sequences are identical, except that residue 7 shown above in parentheses is alanine in gD-1 and proline in gD-2 and residue 21 is aspartic acid in gD-1 and asparagine in gD-2. To assess the effects of these sequence differences on antigenicity or immunogenicity, a series of overlapping peptides was synthesized (8, 11), which corresponded to portions of the first 23 amino acids of gD-1 (1-16[1], 8-23[1], 1-23[1]), gD-2 (1-16[2], 8-23[2], 1-23[2]), or a combination (hybrid, 1-23[H]). The hybrid contained proline at residue 7 and aspartic acid at residue 21. The antigenicity of these peptides was tested with polyclonal sera prepared against purified gD-1 or gD-2 (17). The reactivity of these sera suggested that residues 1 to 23 of gD contain two distinct antigenic regions, one within the first 16 residues of gD-2 and the other region contained within residues 8 through 23 of gD-1. In the present report, the immunogenicity of residues 1 through 23 of gD was tested by measuring the biological reactivity of anti-peptide sera in immunoprecipitation and neutralization assays. Second, we tested the ability of these peptides to confer protection in mice challenged in two ways.

Antisera to the three peptides from the region of residues

1 through 23 designated 1-23[1] (Fig. 1, lanes 3 and 4), 1-23[H] (lanes 13 and 14), and 1-23[2] (lanes 15 and 16) immunoprecipitated gD-1 and gD-2 from cytoplasmic extracts of infected cells. These results indicated that the region of residues 1 through 23 was immunogenic regardless of whether the type 1 or type 2 sequence was presented to the animal. However, the immunogenicity of smaller peptides containing gD-1 or gD-2 amino acid residues 1 through 16 or 8 through 23 was sequence dependent. For example, although anti-peptide 1-16[1] sera (with alanine at position 7) failed to immunoprecipitate gD (lanes 9 and 10), anti-peptide 1-16[2] sera (with proline at position 7; lanes 11 and 12) was quite reactive. Anti-peptide 8-23[2] sera (asparagine at position 21; lanes 7 and 8) did not react with gD, but as previously shown (8), anti-8-23[1] sera (aspartic acid at position 21) immunoprecipitated both gD-1 and gD-2. In previous studies, antisera to 1-16[1] and 8-23[2] failed to react with native or denatured gD by the immunoblot assay but did bind to the peptide used for immunization (11).

The immunoprecipitation data correlated with the presence or absence of virus-neutralizing activity in the rabbit anti-peptide sera (Table 1). The two sera (anti-1-16[1] and anti-8-23[2]) which failed to immunoprecipitate gD had no detectable neutralizing activity. The other sera all exhibited varying degrees of neutralizing activity. The results indicated that several epitopes in residues 1 through 23 of gD are capable of inducing a neutralizing antibody response. The highest titers were found in sera from animals immunized with the longest peptides. In a similar manner, human T-cell lines activated by 1-23[H] responded consistently to synthetic peptides of at least 16 amino acids but failed to respond to smaller peptides (10a).

gD, administered i.p. protects BALB/c mice from a lethal i.p. challenge with HSV-1 or HSV-2 (21). We first tested whether immunization with the synthetic peptides would afford protection in this system. One week before HSV-2 challenge, serum samples were obtained and virus neutralization titers were determined (Table 2). Mouse anti-gD-1 sera exhibited consistently high titers of type-common neutralizing activity. Mouse anti-peptide sera exhibited somewhat more variable levels of neutralizing activity. A total of 48 of 58 (83%) sham-immunized mice died from an i.p. challenge with HSV-2 (Table 2). In contrast, 29 of 30 (96.6%)

TABLE 1. Rabbit serum neutralization of HSV infectivity

Rabbit antiserum ^a	Neutralization titer ^b	
	HSV-1	HSV-2
gD-1	1,600	800
gD-2	1,200	1,600
8-23[1]	32	16
8-23[2]	<20	<20
1-16[1]	<20	<20
1-16[2]	20	30
1-23[H]	80	40
1-23[1]	40	80
1-23[2]	40	120

^a For each rabbit, a preimmunization bleeding was tested, and in all cases, the titer against both HSV-1 and HSV-2 was <1:20. All of the rabbit sera were assayed with HSV-1 strain HF or HSV-2 strain Savage (17). For each peptide, two rabbits were immunized and the neutralizing titers of several bleedings from each rabbit were determined. The titers did not vary from those shown by more than one dilution in either direction.

^b The virus neutralization titer is the reciprocal of the highest dilution of antiserum which gave a 50% reduction in plaque number (17).

mice immunized with gD-1 survived with i.p. challenge in three experiments. In two experiments, peptide 8-23[1] was protective because 80% of the animals challenged i.p. survived. The synthetic peptide 8-23[2] appeared not to be protective by this route of immunization and challenge since 70% of the animals died. These results together with those of immunoblot (11), immunoprecipitation (Fig. 1), and neutralization (Tables 1 through 3) assays indicated that peptide 8-23[1] is more immunogenic than peptide 8-23[2]. All of the longer peptides exhibited protective ability, although peptide 1-23[H] gave somewhat variable results. The reason for this variability is not understood, but it does not correlate with the level of neutralizing antibody titers, since in some cases, animals exhibiting high antibody titers succumbed to the virus challenge. In addition, the geometric mean titer induced by peptide 1-23[2] was low, but there was still a significant level of protection. In contrast, peptide 1-23[H] induced high titers of neutralizing antibody but gave variable levels of protection. The absence of a correlation between neutralizing antibody titers and levels of protection may suggest that cellular immune response mechanisms induced by synthetic peptides are involved in protection. Recovery from HSV infections has been shown to involve both humoral and cell-mediated responses (23, 25).

One possible explanation for the variability is that the same site was used for both immunization and challenge. We decided to evaluate the protective capacity of the peptide

TABLE 2. Protection of mice against a lethal i.p. challenge by HSV-2 after immunization with gD-1 or synthetic peptides

Expt. no. ^a	Group	Neutralization titer ^b		No. of mice challenged/ no. dead ^c
		HSV-1	HSV-2	
1 ^d	KLH	6	6	20/16
	gD-1	800	400	10/0
	8-23[1]	40	10	10/2
	1-23[H]	30	10	10/2
2 ^e	KLH	<6	<6	19/13
	gD-1	384	192	10/1
	1-23[H]	384	192	10/2
3	KLH	6	<6	9/9
	8-23[2]	6	<6	10/7
	8-23[1]	384	256	10/2
	1-23[H]	388	239	10/7
	1-23[2]	15	69	10/1
4 ^e	KLH	6	<6	10/10
	gD-1	548	338	10/0
	1-23[H]	388	69	10/6
	1-23[2]	11	26	10/4
	1-23[1]	478	223	10/2

^a All animals were immunized i.p. with the immunogen emulsified in CFA. A total of 6 µg of immunosorbant-purified gD-1 was given in four doses (20). Each of the peptides was coupled to KLH (15, 17), and 50 µg was administered per dosage (total of four doses). Animals were bled 7 days before challenge. Animals were challenged by the i.p. route with HSV-2 strain 186. Fifteen 50% lethal doses were used in experiment 1, 9 in experiment 2, 2 in experiment 3, and 32 in experiment 4.

^b Titers shown for experiments 2 through 6 are geometric mean titers taken from individual serum samples. Geometric mean titers were calculated by assigning a value of 3 to titers <6 and a value of 576 to titers >384. In experiment 1, serum samples were pooled from five mice.

^c Results are reported 40 days postchallenge. Most of the sham-immunized animals died within 7 to 11 days after challenge.

^d gD-1 was purified from HSV-1 strain HF. The strains used for serum neutralization were HSV-1 strain HF and HSV-2 strain Savage.

^e gD-1 was purified from HSV-1 strain MacIntyre. The strains used for serum neutralization were HSV-1 strain MacIntyre and HSV-2 strain 186.

TABLE 3. Protection of mice against paralytic challenge by HSV-2 given by the footpad route after immunization with gD-1 or synthetic peptides

Expt. no.	Group	Neutralization titer ^a		No. of mice challenged/ no. paralyzed or dead ^b
		HSV-1	HSV-2	
1	KLH	<6	<6	10/10
	gD-1	512	181	10/0
	1-23[H]	388	104	10/0
	CFA	<6	<6	10/3 ^c
2 ^d	KLH	6	<6	10/9
	gD-1	534	446	10/0
	1-23[H]	169	128	10/2
	1-23[2]	14	73	10/2
	1-23[1]	512	362	10/0
3	CFA	ND ^e	ND	10/10
	1-23[1] CFA	256	111	5/0
	AP ^f	ND	ND	10/10
	1-23[1] AP	338	158	5/0

^a Animals were challenged with HSV-2 strain 186 by the footpad route (22). The 50% paralytic dose in experiment 1 was 3; in experiment 2, the 50% paralytic dose was 20; in experiment 3, the 50% paralytic dose was 11. Animals were immunized i.p. with the agent shown, emulsified in CFA (20) or aluminum phosphate. The doses of immunogen given were the same as in Table 2, footnote a. Animals were bled 7 days before challenge.

^b The observation period was 40 days.

^c Seven animals showed transient signs of paralysis. Later experiments incorporated a higher 50% paralytic dose, and this effect was not noted.

^d A CFA control was not included in experiment 2.

^e ND, Not determined.

^f AP, Aluminum phosphate.

and gD by using the distal footpad route (31) of viral challenge (Table 3). This route had the additional advantage of allowing assessment of the neurological effects of the virus. All of the animals immunized with gD or peptide 1-23[1] were protected from virus challenge. Second, 80% of the animals immunized with 1-23[2] or 1-23[H] were protected. Moreover, in one experiment (Table 3, experiment 3), aluminum phosphate adjuvant appeared to be as effective as complete Freund adjuvant (CFA) with either gD-1 or 1-23[1] as immunogens.

These studies showed that active immunization of mice either with gD or with synthetic peptides which represent the amino terminus of gD-1 or gD-2 conferred protection against an HSV-2 challenge given by footpad. Since virtually all of the sham-immunized animals that were challenged by the footpad route developed paralysis (or died), it appears that gD-1 or synthetic peptides which represent portions of gD can protect against the development of neurological symptoms in this system.

The question whether subunit or synthetic vaccines will be effective in protection against HSV infections remains open. Clearly, the peptides, particularly 1-23[1], as well as gD, must be evaluated in a variety of animal models by using other routes of immunization and challenge. It is encouraging that gD protects guinea pigs from genital HSV-2 infection (4), since the clinical symptoms in this animal model may be more relevant to human disease (30). The results of the present study demonstrated the potential of a synthetic peptide vaccine to protect against HSV infections. Variations of this approach which merit further investigation include the determination of the efficacy of vaccination with a mixture of types 1 and 2 peptides or with a combination of peptide and protein. Other possibilities to consider would be cyclic versions of the peptides (12) or a mixture of peptides

from the N terminus with those mimicking other regions of gD.

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