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Passive Immune Protection by Herpes Simplex Virus-Specific Monoclonal Antibodies and Monoclonal Antibody-Resistant Mutants Altered in Pathogenicity

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Virus-neutralizing monoclonal antibodies specific for 13 different genetically defined epitopes of glycoproteins gC, gB, and gD of herpes simplex virus type 1, strain KOS-321, were compared for their ability to provide passive immunity to DBA-2 mice challenged intracranially. Protection was highly specific, since individual monoclonal antibodies failed to protect against infection with monoclonal antibody-resistant (*mar*) mutants altered in the single epitope recognized by the injected antibody. The dose-response kinetics of passive immunity paralleled the in vitro neutralization titers for each antibody. No correlation was observed between immune protection and antibody isotype or complement-dependent in vitro neutralization titers. This suggests that virus neutralization was not the protective mechanism. In general, antibodies reactive with epitopes of gC were protective at the lowest antibody doses, antibodies specific for gB were less efficient in providing immunity, and antibodies against gD were the least effective. *mar* mutants with single epitope changes in gC and multiple epitope changes in gB showed highly reduced pathogenicity, requiring up to 5×10^6 PFU to kill 50% of infected animals. These findings indicated that antigenic variation affects virus growth and spread in the central nervous system. Thus, mutations which affect antigenic structure also can alter virus pathogenicity. The alteration of these epitopes does not, however, appreciably reduce the development of resistance to infection. Infection of mice with these mutants or inoculation of mice with UV-inactivated, mutant-infected cells before challenge rendered the animals resistant to infection with wild-type herpes simplex virus type 1.

Immunization with purified herpes simplex virus (HSV) glycoproteins or with mutant virus-infected cells lacking one or more of the mature forms of the HSV glycoproteins has demonstrated that these antigens are essential for the induction of humoral and cell-mediated antiviral immune responses which, in turn, provide protective immunity (3, 4, 10, 17, 22). To explore in greater detail the role of antibodies in immune protection, researchers have used monoclonal antibodies reactive with these antigens to protect animals against lethal virus challenge (1, 7, 15, 21). The mechanism of immune protection is unclear but may involve virus neutralization and monoclonal antibody-dependent complement- or cell-mediated lysis of virus-infected cells. This presumption is supported by in vitro studies which have shown that monoclonal antibodies specific for viral glycoproteins can neutralize virus and participate in complement-mediated and cell-mediated immune cytolysis reactions (1, 5, 7, 9, 11, 13, 15, 16, 18).

Although these reports clearly showed that single monoclonal antibodies protect against virus challenge, most of the antibodies used were of undefined epitope specificities. Consequently, the question of whether antibodies which recognize different epitopes on the same or different glycoproteins are equivalent in their protective capacity remains unanswered. The purpose of this study was to determine whether several virus-neutralizing antibodies, each reactive with different epitopes on one of the HSV glycoproteins (gC, gB, and gD), differed in their ability to protect mice against lethal doses of challenge virus inoculated directly into the mouse cerebrum. The epitope specificities of these antibodies were genetically defined by analyzing the reactivity patterns of monoclonal antibody-resistant (*mar*) mutants with the panel of monoclonal antibodies used in the mutant selections (13, 14). In addition, the *mar* mutants were used to determine whether specific epitope alterations were associated with either a reduced viral pathogenicity for the mouse central nervous system (CNS) or a reduced capacity to induce resistance to virus challenge.

MATERIALS AND METHODS

Mice. Six- to eight-week-old adult male DBA-2 mice were purchased from Deutsche Gesellschaft für Versuchstierkunde, Hannover, Federal Republic of Germany.

Monoclonal antibodies. The procedures used for the production of hybridomas secreting neutralizing monoclonal antibodies specific for HSV type 1 (HSV-1) strain KOS-321 have been described in detail elsewhere (13). The antibodies were determined to be specific for HSV-1(KOS-321) glycoprotein gC, gB, or gD by immunoprecipitation of [^{35}S]methionine-labeled HSV-1 viral antigens from Nonidet P-40 extracts of infected cells (13). Descriptions of the monoclonal antibodies used in this study, including their designations, immunoglobulin isotypes, glycoprotein specificities, and neutralization titers against the immunizing virus, HSV-1(KOS-321), and the heterologous serotype, HSV-2(186-111) are given in Table 1. The methods used for the production of ascites fluids, immunoglobulin isotyping, and quantitative virus neutralization titrations have been described elsewhere (18). All of the gC-reactive antibodies

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TABLE 1. Characteristics of HSV glycoprotein-specific monoclonal antibodies

HSV-1(KOS-321) glycoprotein	Monoclonal antibody	Immunoglobulin isotype[a]	Neutralization titer[b] for:		
			HSV-1 (KOS-321)		HSV-2 (186-111)
			+	−	+
gC	C3[c]	IgG2a	6,400	<40	<40
	C4[c]	IgG2a	6,400	<40	<40
	C5	IgG2a	6,400	<40	<40
	C7	IgG3	640	<10	<10
	C8	IgG2a	20,480	<10	<10
	C9	IgG2a	20,480	<10	<10
	C10	IgG2a	20,480	<10	<10
	C11[c]	IgG2a	40,960	<10	<10
	C13[c]	IgG2a	81,920	<320	<320
	C14[c]	IgG2b	10,480	<160	<160
	C15[c]	IgG2a	1,280	<5	<5
	C16[c]	IgG2a	5,120	<5	<5
gB	B1	IgG3	2,560	<10	<10
	B2	IgG2b	163,840	<40	40,960
	B3[c]	IgG3	2,560	<40	<40
	B4[c]	IgG3	10,240	5,120	640
	B5[c]	IgG3	163,840	<80	5,120
	B6[c]	IgG2b	10,240	<160	<160
	B7	IgG2a	655,360	<160	5,120
	B8	IgG2a	655,360	<160	2,560
	B9	IgG2a	655,360	<160	40,960
gD	D1	IgG1	102,400	12,800	<10
	D2[c]	IgG3	81,920	20,480	25,600
	D3[c]	IgG2a	64,084	<100	20,480
	D4	IgG2b	10,240	<10	10,240

[a] IgG2a, Immunoglobulin G2a.
[b] +, The neutralization mixture contained normal rabbit serum as a complement source at a final concentration of 10%: −, no normal rabbit serum was present. The values are the 50% endpoint titers determined by a quantitative virus neutralization assay (18).
[c] Monoclonal antibody used in passive immune protection against i.c. challenge with HSV-1(KOS-321).

used in this study were type specific for HSV-1(KOS-321) and were therefore only weakly neutralizing for HSV-2(186-111). In contrast, about half of the gB- and gD-reactive antibodies were cross-reactive and neutralized both serotypes. In addition, all required complement for virus neutralization, except for B4, D1, and D2.

Antigen preparations for immunizations. Monolayers of 3T3 cells in 150-mm petri dishes were infected with wild-type HSV-1(KOS-321) or antigenic variants of KOS-321 at a multiplicity of two. At 20 h postinfection, the medium was replaced with 5 ml of phosphate-buffered saline (PBS), and the cells were irradiated with UV light (8 Gy/m^2 per min) for 25 min to inactivate infectious virus particles (10). The irradiated cells were scraped off the plastic surface and suspended in PBS. Titration showed that the antigen suspension contained less than 5 PFU of live virus per ml.

Cells and viruses. Wild-type HSV-1(KOS-321) and antigenic variants of KOS-321 were routinely passaged at a low multiplicity (0.03 PFU per cell) in an African green monkey cell line (RC-37 Rita; Italdiagnostics, Rome, Italy) at 37°C. The cells were maintained in Eagle minimum essential medium containing 7% fetal calf serum. Virus titers were determined by a plaque assay in the RC-37 cell line (23). Mouse 3T3 cells (CRL 163; American Type Culture Collection, Rockville, Md.) were grown in Dulbecco modified Eagle minimum essential medium containing 7% fetal calf serum. Antigenic variant strains were derived from a plaque isolate of HSV-1(KOS), termed KOS-321, on the basis of resistance to neutralization with glycoprotein-specific monoclonal antibodies as described previously (13, 18; S. D. Marlin, S. L. Highlander, T. C. Holland, M. Levine, and J. C. Glorioso, submitted for publication; Sutherland et al., manuscript in preparation) and were designated *mar* mutants. The *mar* mutants were altered in distinct epitopes of viral glycoproteins. The *mar* mutants used in this study were grouped according to their epitope alterations. These epitope changes were operationally defined by the pattern of resistance and sensitivity resulting from neutralization with the panel of monoclonal antibodies (Table 2). Each unique reactivity pattern identified a distinct epitope. The *mar* mutants were named for the selecting antibody. For example, *mar*C11.1 was the first *mar* mutant selected with antibody C11. Table 2 lists the monoclonal antibodies used in the selection of individual *mar* mutants, the epitope designations, and the corresponding *mar* mutants in which the monoclonal antibody-defined epitopes were altered. The antibodies and *mar* mutants in each row of Table 2 define distinct epitopes.

Animal protection experiments. We compared the protective capacities of similar doses of individual preparations of monoclonal antibodies against HSV-1(KOS-321)-induced encephalitis. The dose of protecting antibody was defined as the product of the reciprocal of the in vitro 50% endpoint virus neutralization antibody titer in the presence of rabbit complement (18) and the volume used. Thus, all antibody titers are given in virus neutralization antibody titer units; 10 units (the lowest dose used) equals a titer of 1:10, and 2×10^4 units (the highest dose used) equals a titer of 1:20,000. The virus neutralization titer of some of the monoclonal antibody ascites fluids was below 10^4 (Table 1), and therefore it was necessary to concentrate the antibodies by ammonium sulfate precipitation as described previously (18). The individ-

TABLE 2. *mar* mutants used to define distinct epitopes on the HSV-1 (KOS-321) glycoproteins gC, gB, and gD

HSV-1(KOS-321) glycoprotein	Monoclonal antibodies defining distinct epitopes	*mar* mutants variant in a defined epitope[a]
gC	C4	*mar*C4.4[b]
	C11, C14	*mar*C11.1, *mar*C14.1
	C15	*mar*C15.1
	C16	*mar*C16.1
	C3	*mar*C3.1, *mar*C3.2
	C8, C9	*mar*C9.6, *mar*C9.1
	C13	*mar*C13.1, *mar*C13.2
	C7, C10	*mar*C7.1, *mar*C7.2, *mar*C10.1
	C5	*mar*C5.4
gB	B1, B3	*mar*B1.1, *mar*B1.2, *mar*B1.3, *mar*B1.4
	B2, B5, B7, B8, B9	*mar*B2.1, *mar*B2.2, *mar*B5.1, *mar*B5.2
	B4	*mar*B4.1, *mar*B4.2
	B6	*mar*B6.1, *mar*B6.2
	B3 + B4	*mar*B3/4.1, *mar*B3/4.2
	B2 + B4 + B6	*mar*B2/4/6.1, *mar*B2/4/6.3
	B2 + B4 + B6 + B3	*mar*B2/4/6/3.1, *mar*B2/4/6/3.3
gC + gB	C pool[c] + B2 + B4 + B6 + B3	gC$^-$*mar*B2/4/6/3.1
gD	D1	*mar*D1.1, *mar*D1.2
	D2	*mar*D2.1
	D3	*mar*D3.1
	D4	*mar*D4.1

[a] Epitopes were assigned by identifying unique reactivity patterns of monoclonal antibodies with a series of *mar* mutants described elsewhere (13, 18; Marlin et al., submitted; Sutherland et al., in preparation).

[b] *mar* mutants were isolated and characterized by methods described elsewhere (18).

[c] A gC$^-$–multi-gB *mar* mutant was produced by recombination of a gC deletion mutant (gC$^-$39) (12) with the multi-gB *mar* mutant. The resulting mutant was resistant to neutralization with a pool of gC- and gB-specific monoclonal antibodies consisting of C4, C11, C13, C7, B2, B4, B6, and B3.

ual antibody protein concentrations differed and ranged from 0.3 μg/ml to 5 μg/ml for 10 units.

Mice were passively immunized by intraperitoneal (i.p.) injection with glycoprotein-specific monoclonal antibody preparations or were actively immunized by i.p. injection with virus-infected cellular immunogen preparations containing wild-type or antigenically altered viral glycoproteins. Immunized mice were challenged by intracerebral (i.c.) injection of a lethal dose of either HSV-1(KOS-321) or *mar* mutants in 20 μl of PBS 24 h postimmunization as described elsewhere (23). Animal survival was recorded daily, and the experiments were terminated after 30 days. Mice which did not die within that time survived for a least 3 months. The 50% lethal dose (LD_{50}) was calculated by the method of Spearman-Karber (8). Control mice, which received an i.c. injection of 20 μl of PBS, survived without clinical signs of illness or neurological disorders.

RESULTS

Epitope-specific passive immunity. Passive immunity was first demonstrated with monoclonal antibody C14, which is directed against a previously defined epitope of gC (18). Groups of eight mice injected with this antibody were challenged 24 h postimmunization by i.c. injection of 400 LD_{50}s of (i) wild-type HSV-1(KOS-321), (ii) a *mar* mutant of KOS-321 that is altered in the epitope against which monoclonal antibody C14 is directed (*mar*C14.1), (iii) a *mar* mutant that is altered in another distinctly different epitope of gC (*mar*C13.2), or (iv) a *mar* mutant that is altered in an epitope of gB (*mar*B6.1). The wild-type virus dose which resulted in lethal encephalitis in 50% of DBA-2 mice (i.c. LD_{50}) was 20 PFU. The antibody dose was sufficient to provide full protection against 400 LD_{50}s of wild-type HSV-1(KOS-321) challenge virus (Table 3). The experiment further demonstrated that antibody C14 was highly protective against challenge with those *mar* mutants which preserved the epitope recognized by antibody C14. In contrast, antibody C14 completely failed to protect against challenge with *mar*C14.1, which is resistant to in vitro neutralization with antibody C14 and altered in the epitope recognized by antibody C14. Similarly, passive immunization with monoclonal antibodies B6, C13, and D2 also protected against challenge with the wild-type and all *mar* mutant viruses except those lacking the epitope recognized by the protecting antibody (data not shown). Thus, the specificity of passive immunity paralleled the specificity of in vitro neutralization reactions, indicating that protection involves antibody binding to defined glycoprotein epitopes.

Dependence of the protective capacity of monoclonal antibodies used in passive imunizations on both antibody dose and challenge virus dose. To establish the most revealing test conditions for comparing the protective capacity of different

TABLE 3. Passive immune protection with monoclonal antibody C14 against lethal challenge with wild-type HSV-1(KOS-321) and *mar* mutants of KOS-321

Challenge virus[a]	Protective capacity (LD_{50} units[b])
HSV-1(KOS-321)	400
*mar*C14.1	<1
*mar*C13.2	400
*mar*B6.1	500

[a] The LD_{50} for each challenge virus was determined to be 20 PFU as described in Materials and Methods.

[b] Data represent the maximum number of LD_{50} units of challenge virus against which 10^4 units of monoclonal antibody C14 provided complete protection, i.e., 100% of mice surviving the infection. The units of protecting antibody were derived from the complement-enhanced 50% endpoint virus-neutralization antibody titer.

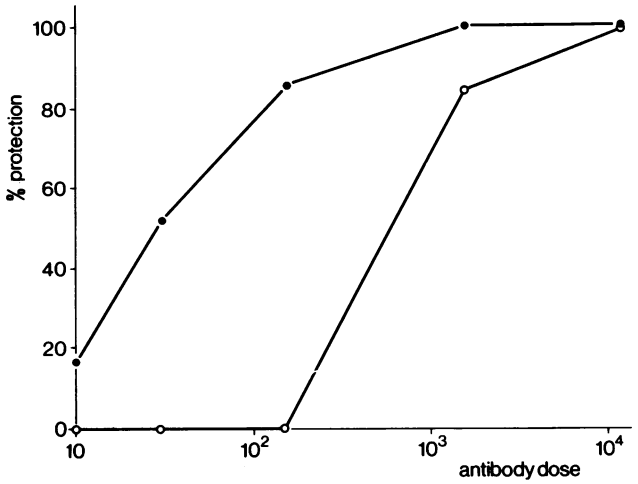

FIG. 1. Dose-response kinetics of passive immunity afforded by monoclonal antibody C14 (gC specific) to i.c. challenge with wild-type HSV-1(KOS-321). Eight DBA-2 mice were passively immunized by i.p. administration of different doses of antibody C14 24 h before i.c. challenge with 15 (●) or 150 (○) LD_{50}s of wild-type HSV-1(KOS-321) as described in Materials and Methods. The antibody dose was derived from the complement-enhanced 50% endpoint virus neutralization antibody titer. The percent protection was based on the fraction of animals surviving i.c. infection for at least 30 days postchallenge.

monoclonal antibodies in immune protection experiments, we used monoclonal antibody C14 to determine the dose-response kinetics of passive immunity. The fraction of surviving mice per group (percent protection) was compared at different protecting antibody doses. The data in Fig. 1 show the protection provided against 15 LD_{50}s (3×10^2 PFU) and 150 LD_{50}s (3×10^3 PFU) of HSV-1(KOS-321). Immune protection of DBA-2 mice was dependent on both the dose of antibody and that of the challenge virus. The protective capacity of the antibody paralleled the in vitro neutralization titer. Partial protection against 15 LD_{50}s was observed with only 10 units of antibody, whereas 10^3 units provided complete protection. As expected, the antibody was less effective against 150 LD_{50}s of virus, requiring 10 times the amount to provide complete protection. Based on these data, 15 LD_{50}s of challenge virus were used in subsequent experiments to compare the protective capacity of 10^1 to 10^4 units of virus-neutralizing monoclonal antibodies.

Comparison of the protective capacity of monoclonal antibodies directed against different epitopes of HSV glycoproteins gC, gB, and gD. The specificity of passive immune protection provided by the monoclonal antibodies led us to compare the protective capacity of different monoclonal antibodies directed against 13 different epitopes of glycoproteins gC, gB, and gD (Tables 1 and 2). Figure 2 shows the results of these comparisons. The antibody protection titration curves demonstrated that, in general, monoclonal antibodies specific for gC provided protection at low doses, antibodies specific for gB were less protective, requiring higher doses, and antibodies reactive with gD were the least protective. There were, however, some exceptions to this generalization. Antibody C13 behaved similarly to antibodies B6 and B4 and was less protective than antibody B3. Antibody B3 was as protective as several of the gC-specific antibodies.

It should be noted that the dose of antibody was defined on the basis of in vitro neutralization tests and, thus, the antibody titers over the range of doses were functionally

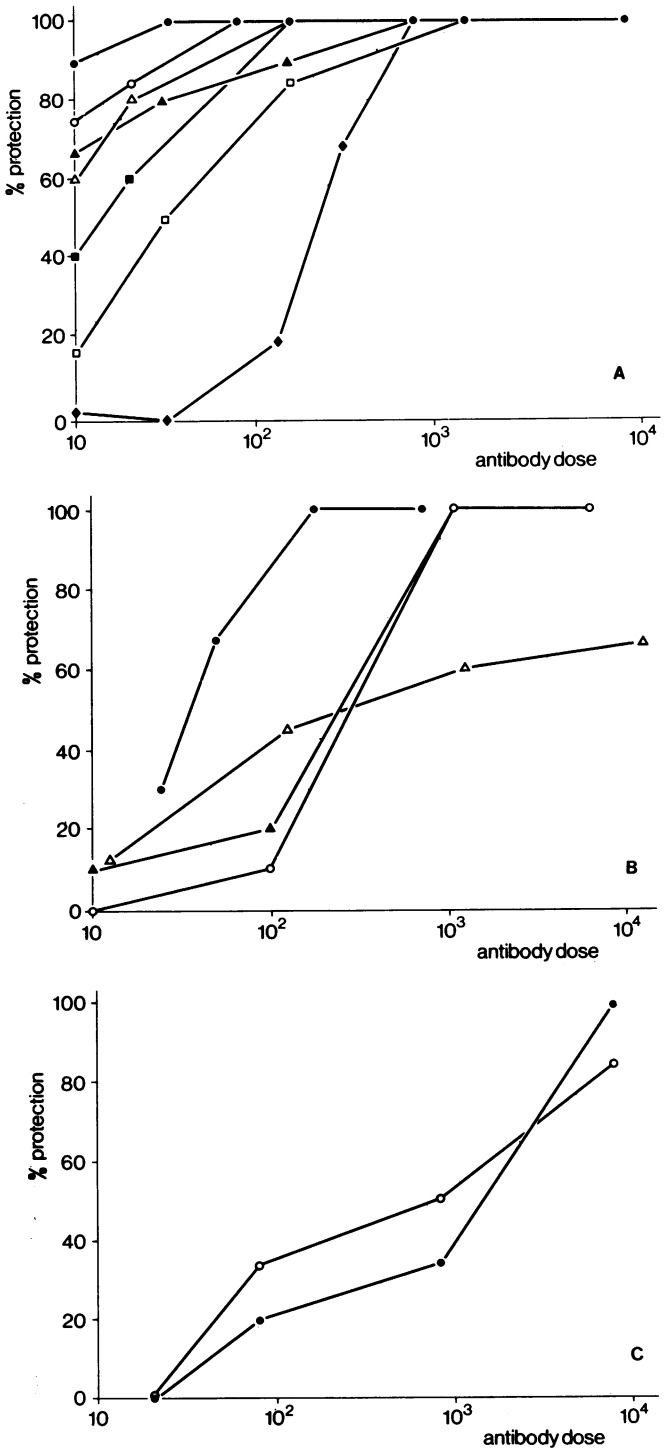

FIG. 2. Comparison of the protective capacity of monoclonal antibodies specific for different epitopes of glycoproteins gC, gB, and gD against i.c. challenge with wild-type HSV-1(KOS-321). Eight DBA-2 mice were passively immunized by i.p. administration of different doses of antibodies specific for gC (panel A: C3, ●; C4, ○; C11, △; C15, ▲; C16, ■; C14, □; and C13, ◆), gB (panel B: B3, ●; B5, △; B6, ▲; and B4, ○), and gD (panel C: D2, ○; and D3, ●) 24 h before i.c. challenge with 15 LD_{50}s of wild-type HSV-1(KOS-321) as described in Materials and Methods. The antibody doses were derived as described in the legend to Fig. 1. The percent protection was based on the fraction of animals surviving i.c. infection for at least 30 days postchallenge.

similar for all antibodies. Despite their functional similarities in vitro, however, they behaved dissimilarly in providing immune protection in the animals. No correlation could be drawn between their in vitro neutralization titers and their protective capacity in vivo. In addition, no correlations were found between antibody concentrations and immune protection. For example, 10 units (1.2 μg/ml) of antibody C11 afforded 60% protection, whereas 10 units (5 μg/ml) of antibody C14 afforded only 17% protection. The only correlation with antibody-mediated protection was with epitope specificity and, presumably, individual antibody avidity for the different epitopes.

Comparison of the titration curves for antibodies C11 and C14 showed that antibodies recognizing the same epitope can vary in their protective capacity. This may reflect differences in antibody avidity in vivo. Full protection could not be reached with antibody B5 even at doses as high as 2×10^4. This incomplete protection may be a reflection of the antigenic diversity of the wild-type challenge virus, the low avidity of the antibody in vivo, or differences in epitope expression in vivo and in vitro.

Monoclonal antibodies B4, D1, and D2 neutralized virus in the absence of complement (Table 1). Since antibody binding directly inactivated virus, the epitopes defined by these antibodies are presumably contained in regions of gD and gB which are essential to the functions of these glycoproteins in infections. The finding that these antibodies were no more efficient in immune protection than other antibodies which required complement for neutralization suggests that virus neutralization is not the major protective mechanism. Grouping the antibodies according to immunoglobulin isotype (Table 1) also did not distinguish among monoclonal antibodies with respect to their patterns of passive immune protection.

Antigenic variation in epitopes of glycoproteins gC and gB resulting in reduced pathogenicity for the CNS. Epitopes which provide particularly effective targets for protecting antibodies may be located in glycoprotein domains which are important in the infection process and in virus spreading in the CNS of the mouse. Thus, alterations of particular epitopes may interfere with the ability of the virus to grow in nerve tissue, despite the fact that these same epitope changes are neutral for virus production in vitro. As a consequence, these variants may be less pathogenic. To test this possibility, we compared the i.c. LD_{50}s of 39 independently selected *mar* mutants (Table 2) in search of antigenic variants altered in their ability to induce encephalitis. i.c. injection was chosen for these experiments because mice are very sensitive to virus introduced by this route and because of the importance of virus replication in CNS tissue for viral neuropathogenesis.

TABLE 4. i.c. LD_{50}s of glycoprotein mutants of HSV-1(KOS-321) altered in pathogenicity

Virus	i.c. LD_{50}[a]
HSV-1(KOS-321)	2×10^1
gC⁻39	5×10^5
*mar*C5.4	4×10^5
*mar*C4.4	5×10^4
*mar*C10.1	5×10^6
*mar*C9.6	7×10^4
*mar*B2/4/6.1	8×10^4
*mar*B2/4/6/3.1	5×10^6
gC⁻*mar*B2/4/6/3.1	5×10^6

[a] Determined after i.c. injection of wild-type virus or virus mutants as described in Materials and Methods.

TABLE 5. Protection by ic. injection of active *mar* mutant viruses against challenge with wild-type HSV-1(KOS-321)

Protecting *mar* mutant[a]	Challenge virus[b]	No. of dead mice per group[c]
*mar*C10.1	None	0
*mar*C5.4	None	0
*mar*B2/4/6.1	None	0
*mar*B2/4/6/3.1	None	0
None	20 LD_{50} units	8
*mar*C10.1	20 LD_{50} units	2
*mar*C5.4	20 LD_{50} units	3
*mar*B2/4/6.1	20 LD_{50} units	1
*mar*B2/4/6/3.1	20 LD_{50} units	1

[a] Protecting virus mutants were injected i.c. at a concentration of 6×10^4 PFU/20 μl of PBS (23). The percent animal survival was compared with a modified *t* test (24).
[b] Challenge virus was given 24 h postinjection with protecting virus.
[c] Eight mice per group were tested.

Table 4 shows the i.c. LD_{50}s of eight antigenic variants that differed from wild-type HSV-1(KOS-321) in their ability to induce encephalitis. These mutants showed a considerable reduction in virulence, requiring up to 5×10^6 PFU to kill 50% of infected mice. Some single epitope alterations in gC were associated with a significant reduction in virulence. However, multiple epitope changes in gB were required to interfere with the capacity to cause CNS disease. The LD_{50}s of single *mar*B mutants did not differ from the LD_{50}s of wild-type virus. Surprisingly, one *mar*C mutant, *mar*C10.1, was less virulent than gC⁻39, which does not produce any detectable form of gC (12). None of our gD variants showed reduced neurovirulence. Taken together, these findings demonstrated that the alteration of certain glycoprotein epitopes can significantly affect the ability of HSV to produce CNS disease.

Protection of mice against challenge virus after inoculation with *mar* mutants and *mar* mutant-infected cells. As shown above, *mar* mutants altered in particular epitopes of gC and gB were also affected in their ability to induce encephalitis. The question arose as to whether these epitopes were also essential to the induction of resistance to infection. Table 5 shows that i.c. injection of antigenic variants protected mice against i.c. injection of challenge virus. The differences in the mortality rates when different *mar* mutants were used as protective agents were not significant. The data support the general conclusion that epitope alterations which affect neurovirulence do not significantly affect the ability to induce a state of resistance to lethal infection.

We further analyzed the ability of antigenic variants to induce protection against i.c. challenge virus by inoculating mice i.p. with UV-irradiated *mar* mutant-infected cells containing large amounts of viral antigens, including membrane-associated viral glycoproteins. Using this approach, we could determine whether in the i.c. protection-challenge experiments, the presence of live virus at the challenge site, the CNS, was necessary to induce the resistant state. We infected mouse 3T3 cells with HSV-1(KOS-321), *mar*C10.1, or gC⁻*mar*B2/4/6/3.1. *mar*C10.1 appears to differ from the wild type in a single epitope of gC, and gC⁻*mar*B2/4/6/3.1 lacks gC and carries four independent variant epitopes of gB (Table 2). Virus was inactivated by UV irradiation of the infected mouse 3T3 cells. These antigen preparations were injected into DBA-2 mice without any further treatment. A

TABLE 6. Protection by i.p. injection of UV-inactivated HSV-1-infected mouse cells

Immunogen (infectious agent)[a]	Challenge virus[b] (LD_{50} units)	No. of dead mice per group/no. tested
None	5	5/5
None	1	2/5
3T3 cells (none)	5	5/5
3T3 cells (none)	1	3/5
3T3 cells (KOS-321)	500	2/8
3T3 cells (KOS-321)	50	4/8
3T3 cells (KOS-321)	5	0/8
3T3 cells (*mar*C10.1)	500	8/8
3T3 cells (*mar*C10.1)	50	4/8
3T3 cells (*mar*C10.1)	5	1/8
3T3 cells (gC$^-$*mar*B2/4/6/3.1)	500	8/8
3T3 cells (gC$^-$*mar*B2/4/6/3.1)	50	5/8
3T3 cells (gC$^-$*mar*B2/4/6/3.1)	5	2/8

[a] Monolayers of mouse 3T3 cells were mock infected or infected for 24 h with wild-type or mutant HSV-1 (KOS-321), and live virus was inactivated by UV irradiation. The cells were scraped off the culture dishes and injected i.p. into DBA-2 mice (2×10^6 cells per mouse) as described in Materials and Methods.

[b] HSV-1(KOS-321) in 120 μl of physiological saline was injected i.c. 24 h after administration of the immunogen.

control group of mice received similarly treated, uninfected mouse 3T3 cells. The effect of viral antigen priming of DBA-2 mice on animal survival after i.c. challenge with different doses of HSV-1(KOS-321) was tested.

Table 6 shows that uninfected cells had no influence on the rate of mortality. All three viral antigen preparations conferred comparable protection to the animals at challenge virus doses of 5 and 50 LD_{50}s. Even when gC was totally absent from the immunogen and there were multiple antigenic changes in gB, resistance to challenge virus was still induced. These experiments indicated that mutants with variations in antigenic structure are capable of inducing a protective response against wild-type virus and that live virus is not required for protection.

DISCUSSION

Monoclonal antibodies directed against each of the HSV-1 glycoproteins gB, gC, gD, and gE provide protection against viral encephalitis after peripheral challenge with virulent strains of HSV-1 (reviewed in reference 11a). In the study reported here, virus-neutralizing, glycoprotein-specific monoclonal antibodies were used to protect mice against encephalitogenic HSV-1(KOS-321) administered directly into the cerebrum. We sought to determine whether monoclonal antibody-dependent protection is epitope specific and whether antibodies with different distinct epitope specificities are equal in their protective capacity. In addition, we examined whether antigenic variation in glycoprotein epitopes affects either viral pathogenicity for the CNS or the induction of protective immunity.

To carry out these studies, we used monoclonal antibodies with different epitope specificities. The antibody specificities were defined by analysis of their reactivity patterns with panels of *mar* mutant viruses selected for their resistance to neutralization with individual monoclonal antibodies (13). *mar* mutants express an antigenically altered envelope form of an HSV glycoprotein in amounts similar to those in wild-type parent strain, HSV-1(KOS-321), from which all the *mar* mutants were derived (13). All *mar* mutations so far studied genetically have been localized to their respective glycoprotein structural genes and are point mutations leading to single amino acid changes, presumably in an epitope-encoding sequence (14; F. Homa, D. J. Dorney, T. B. Wu, M. Levine, and J. C. Glorioso, unpublished data).

The use of *mar* mutants as challenge viruses demonstrated that passive immune protection with monoclonal antibodies is epitope specific and requires binding of the antibody to a precise site on the glycoprotein molecule. Antigenic variation in the epitope defined by the protective monoclonal antibody resulted in escape from passive immunity. This was shown for four different monoclonal antibodies which recognized epitopes on gC, gB, or gD. This finding extends those of Dix et al. (7), who showed that a gC-specific monoclonal antibody failed to protect against a virulent challenge virus strain lacking gC. In addition, escape from passive immunity by the *mar* mutants demonstrate that the *mar* phenotype is expressed in the animal in a manner similar to that observed in vitro and suggests that such mutants arising in natural infections would be resistant to epitope-directed immune responses.

Passive immune protection was shown to depend on the doses of both the protecting antibody and challenge virus. By quantitating the antibody dose-protection response for 13 monoclonal antibodies, we were able to make a comparison of the protective capacity of individual antibodies. Antibodies recognizing different epitopes on a single glycoprotein were not equally protective, despite the fact that the antibody doses were functionally equivalent on the basis of in vitro neutralization tests. That is, two antibodies at similar titers often behaved differently in providing protection, one protecting more effectively than the other. We interpret these findings to mean that immune protection likely involves an antiviral mechanism other than virus neutralization.

Two lines of evidence bear on this suggestion. First, DBA-2 mice are C5 deficient and are thus defective in carrying out the complement cascade (2). Since the majority of protecting antibodies were dependent on complement for virus neutralization in vitro, it is unlikely that the neutralization of virus could occur in the animal without active complement. Second, antibodies which yielded complement-independent neutralization in vitro were no more effective in providing protective immunity than complement-dependent neutralizing antibodies. Complement-independent neutralizing antibodies should inhibit virus replication by binding to or interfering with the domains of the glycoprotein molecules which are essential for the process of virus infectivity. Thus, if virus neutralization were the protective mechanism, it might be expected that these antibodies would be highly effective in providing protection, particularly in a complement-deficient animal.

The mechanism of passive immune protection by monoclonal antibodies is unknown, although antibody-dependent, cell-mediated immune cytolysis could arrest the production of active virus by destroying infected cells. This speculation is supported by the experiments of Rector et al. (21), who reported effective passive immunization with virus-specific monoclonal antibodies that lack in vitro neutralization activity. Balachandran et al. (1) showed that complement-deficient and complement-competent animals are equally protected by monoclonal antibodies, indicating that complement is not essential for the protective mechanism. These

antibodies were poorly protective in animals in which the active thymocyte population was reduced, a finding compatible with the hypothesis that protection is largely mediated by antibody-dependent cell-mediated immune cytolysis.

It was generally true that antibodies reactive with gC were protective at lower doses than antibodies reactive with gB and at much lower doses than antibodies reactive with gD. Some antibodies reactive with gB and gD failed to provide complete protection even at high concentrations. These data suggest that gC may be an important target antigen for protective antibodies. It should be pointed out, however, that immune protection was relative, depending on the antibody and epitope concentrations, and that most antibodies were fully protective if applied at sufficiently high doses.

Recently, Marlin et al. (18) reported that gC contains at least nine epitopes which are clustered in two antigenic sites, designated CI and CII. Antibody competition experiments showed that these two antigenic sites lay in topographically separated regions of the external domain of gC. Although in general epitopes on gC were the most effective target sites for virus inactivation in mice, no correlation between immune protection by gC-reactive antibodies and their specificity for epitopes or antigenic sites CI or CII could be drawn. Thus, it appears that although these sites are localized in different antigenic domains of gC, they are both available in the animal and provide good targets for binding protective antibodies.

Davis et al. (6) and Dix et al. (7) suggested that HSV-1-specific antibodies might not only restrict the transmission of the virus from the peripheral tissues to the CNS but even inhibit virus replication and spread within the CNS itself. We have confirmed this in part by showing that antibody-dependent protection is effective against direct i.c. application of the virus in a dose-dependent manner. The experiments demonstrated that the immunospecific recognition of viral antigen in the brain by the antibody can be the primary and essential step in the clearance of the virus from the CNS.

Several laboratories have now reported that antigenic variants of HSV are readily selected by antibodies in vitro (13, 16, 18) and, as might be expected, antigenic variation occurs in the human host, since variants have been found among fresh natural human isolates (19, 20). Antigenic variation plays an important role in the epidemiology of other viruses, such as influenza virus (25). However, antigenic variation may not contribute significantly to the epidemiology of HSV infections, since HSV is antigenically complex, encoding at least four glycoprotein antigens, which together contain multiple antigenic sites (11a). For example, a polyclonal antibody response directed against wild-type HSV-1(KOS) neutralized a multi-gB*mar* mutant altered in four distinct epitopes of gB to the same extent as wild-type virus did (Marlin et al., submitted). Further, a gC$^-$–multi-gB*mar* mutant was neutralized even more efficiently by the same antiserum. We have not yet produced antigenically altered mutants of HSV-1 which escape a polyclonal protective response. Further immunization with *mar* mutants altered in multiple epitopes of gB and even lacking gC induced significant protective immunity against challenge with wild-type virus. Again, this may be a consequence of the antigenic complexity of HSV. The presence of unaltered forms of the other glycoproteins in these particles apparently accounts for the residual antigenicity and immunogenicity.

An important and related question is whether changes in antigenic structure also affect other aspects of the biology of the virus. Although *mar* mutants grow to titers comparable to those of wild-type virus in cell cultures, these mutants might replicate poorly in animals. Accordingly, we screened 39 *mar* mutants altered in epitopes of gC, gB, or gD for reduced pathogenicity for the CNS. Four *mar*C mutants, as well as a gC$^-$ variant, showed significantly reduced neurovirulence. This raises the possibility that gC may play a role in virus infectivity in vivo that is not observed in cell cultures. Although the gC$^-$ variant showed reduced pathogenicity for the CNS of DBA-2 mice, this phenomenon may be limited to this particular model of HSV-induced encephalitis. It is clear that gC is not essential for infection of the CNS, since other gC$^-$ variants of HSV-1 and HSV-2 cause encephalitis through the peripheral route of virus infection (8; D. Johnson, personal communication; C. H. Schröder, G. Kümel, J. C. Glorioso, and H. C. Kaerner, unpublished data). Our strain of HSV-1(KOS-321) does not induce encephalitis when injected at doses as high as 10^8 PFU into the peritoneal cavity (J. C. Glorioso and H. C. Kaerner, unpublished data).

None of the single *mar*B or *mar*D mutants were apathogenic. However, multiple epitope changes in gB resulted in a reduced pathogenicity for the CNS. Multi-gB*mar* mutants have been shown to be temperature sensitive in cell cultures at 39°C (Marlin et al., submitted). Since the body temperature of the mouse is 38.5°C, the temperature-sensitive phenotype of these mutants might account for their highly reduced pathogenicity. The possibility has not been ruled out that *mar* mutants showing reduced pathogenicity carry otherwise silent mutations that account for the reduced virulence phenotype. However, we have recently shown that about two-thirds of gC$^-$39 mutants rescued to the gC$^+$ phenotype are similar to wild-type virus in pathogenicity (J. Sunstrum, C. Chrisp, and J. C. Glorioso, unpublished data). This observation supports a role for gC in contributing to the neuropathogenic phenotype. Experiments are in progress to confirm that *mar* mutations are responsible for altered pathogenicity.

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