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Protection Against Lethal Challenge of BALB/c Mice by Passive Transfer of Monoclonal Antibodies to Five Glycoproteins of Herpes Simplex Virus Type 2

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Monoclonal antibodies secreted by six hybridomas and recognizing antigenic sites on glycoproteins gC, gAB, gD, gE, and gF of herpes simplex virus type 2 were examined for their ability to protect BALB/c mice from lethal infection by the virus. Administration of monoclonal antibodies to individual glycoproteins intraperitoneally 3 h before footpad challenge with 10 times the 50% lethal dose of virus protected between 35 and 75% of the mice, except for one of two monoclonal antibodies recognizing antigens on gC. The antibodies did not neutralize virus in vitro and protected A/J mice deficient in the fifth component of complement as efficiently as complement-sufficient BALB/c mice. A good correlation was found between protection and titers of monoclonal antibodies assessed by antibody-dependent cell-mediated cytolysis. The results indicate that any of the glycoproteins can serve as antigens for a protective immune response. In addition, the data are compatible with protection being mediated by an antibody-dependent cell-mediated cytolysis mechanism.

Subcutaneous infection of mice with herpes simplex virus (HSV) type 1 (HSV-1) or 2 (HSV-2) usually results in ascending neurological illness and death (3, 9). Passive immunization with antiviral antibody has been shown to prevent this lethal outcome (1, 4, 10, 12, 13, 18–20), and the protective ability of the antibody depends upon the time of administration after infection and the presence of immunocompetent thymus-derived lymphocytes. Effective protection was seen when antibody was given within 48 h of infection (12, 20), and animals immunosuppressed by irradiation, cyclophosphamide, or antithymocyte serum were not protected (17, 18, 20). The exact mechanism(s) by which antibodies mediate protection is not clear, but it appears that antibody acts in cooperation with cellular and other host cell factors (19). In vitro antibodies to the viruses participate in the destruction of infected cells by antibody-dependent cell-mediated cytolysis (ADCC) (8, 25–27) and by complement-mediated cell cytolysis (11, 16, 21). However, the role of these mechanisms as well as virus neutralization in the in vivo protection of mice has not been precisely determined.

All of the mechanisms mediated by antibodies depend upon recognition of HSV-specific glycoproteins present on the surface of the infected cells and on the envelope of virions. There appear to be at least five HSV-2 glycoproteins which can be designated gC, gAB, gD, gE, and gF. (N. Balachandran et al., submitted for publication). For developing a subunit vaccine effective in controlling primary HSV infection, it is pertinent to understand the role of these individual glycoproteins in the induction of various components of the immune reaction. As a first step, we examined the ability of the monoclonal antibodies directed against the individual glycoproteins to protect BALB/c mice against lethal HSV-2 challenge. The data show that antibodies against each glycoprotein were protective.

MATERIALS AND METHODS

Virus. Strain 333 of HSV-2 (24) was grown and titrated in Vero cells. Monolayers of Vero cells were propagated in Eagle minimal essential medium (MEM) containing 5% calf serum, antibiotics, and $NaHCO_3$. Stock virus was prepared by infecting the cells at a multiplicity of 0.1 PFU per cell, and when cytopathogenic effect was extensive, the cells were collected, washed, and suspended in phosphate-buffered saline. Virus was released by sonication, and the lysate was clarified by centrifugation at 1,500 × *g* for 20 min. Aliquots of the virus were stored at −70°C, and a single batch was used throughout the study. Virus was assayed by plaque formation in Vero cells (23).

Monoclonal antibodies. The production and characterization of mouse hybridomas secreting monoclonal antibodies to HSV-2 antigens have been described before (7). Pristane-primed BALB/c mice were injected intraperitoneally with the hybridomas, and the resulting ascitic fluids containing monoclonal antibodies were used in this study. Monoclonal antibodies

(ascitic fluid) to an adenovirus protein were kindly provided by Frank Graham, McMaster University, Hamilton, Ontario, Canada. The isotypes of the immunoglobulins secreted by the hybridomas were determined by immunodiffusion using rabbit anti-mouse immunoglobulin subclasses obtained from Bionetics Laboratories, Toronto.

Mice. Male BALB/c mice, 4 to 6 weeks old, were used in all of the studies except one experiment where 4- to 6-week-old male A/J mice, which were deficient in the fifth component of complement (15), were used. All of the mice were purchased from Jackson Laboratories, Bar Harbor, Maine.

Inoculation of mice. Mice were anaesthetized with ether, and a volume of 0.05 ml containing 10 times the 50% lethal dose of virus was injected in the right hind footpad. Titration of the virus stock revealed that about 2×10^5 PFU were equivalent to 1 50% lethal dose. For passive immunization studies, anaesthetized mice were injected intraperitoneally with 0.1 to 0.6 ml of the appropriate antibodies, followed by injection of the virus either at the same time or 3 h after injection of the antibody. Mice were observed daily for signs of neurological illness and death for a period of 6 weeks.

Antibody assays. Neutralizing activity of the monoclonal antibodies was assayed by a microneutralization test (23). Briefly, the virus was diluted to contain 100 PFU/0.025 ml in MEM with 2% fetal bovine serum (FBS) (pH 6.9), and the ascitic fluids were twofold diluted in the same medium beginning at 1:2.5. A volume of 0.025 ml of diluted ascitic fluid was mixed with 0.025 ml of virus and incubated for 1 h at room temperature in 96-well microtiter plates. A suspension of 2.5×10^4 Vero cells in 0.05 ml of MEM with 2% FBS (pH 7.2) was added to each well, and the cultures were incubated for 4 days at 37°C, after which the cells were fixed in Carnoy fixative and stained with 15% crystal violet. The absence of viral cytopathic effect was taken as evidence of neutralizing antibodies.

The lysis of HSV-2-infected cells by antibody and complement was tested by a technique detailed elsewhere (11). Briefly, BHK cells grown in 75-cm^2 tissue culture flasks were infected at a multiplicity of 3 to 5 PFU per cell, and 8 h after infection, the cells were labeled by adding 200 Ci of ^{51}Cr (as sodium chromate; New England Nuclear Corp., Dorval, Quebec) to the culture medium. The cells were monodispersed about 20 h after infection with 0.025% trypsin and 0.02% EDTA. After washing, the cells were suspended in MEM with 10% FBS at a concentration of 10^5 viable cells per ml. Ascitic fluids, which had been heated for 30 min at 56°C, were serially twofold diluted beginning at 1:10. To 0.1 ml of target cells was added 0.1 ml of diluted ascitic fluid, and the mixture was incubated for 1 h at 37°C. Then 0.2 ml of a dilution of guinea pig complement containing 10 U of activity (11) was added, and the mixture was incubated for an additional 2 h. The cell-free and cell-associated radioactivity was determined, and the percent specific ^{51}Cr release was calculated as previously defined (11). The highest dilution of antibody that yielded more than 10% specific ^{51}Cr release was taken as the antibody titer.

The assay used to measure antibody-dependent cellular cytotoxicity (ADCC) has been detailed elsewhere (29). Target cells consisted of BHK cells prepared as described above. Effector cells were obtained from the peritoneal cavity of adult golden Syrian hamsters which had received 5 ml of sterile paraffin oil intraperitoneally 3 days before harvest. The peritoneal exudate cells were harvested in Hanks buffered saline, washed twice, and resuspended in MEM with 10% FBS. An effector-to-target cell ratio of 200:1 was used, and the highest antibody dilution which yielded 30% specific ^{51}Cr release was taken as the antibody titer.

To determine the antibody titer by immunofluorescence, ascitic fluids were diluted in phosphate-buffered saline and reacted with Vero cells which had been infected with HSV-2 and fixed with acetone when viral cytopathic effect was extensive. The antibody fixed to antigen was detected using fluorescein-conjugated goat anti-mouse immunoglobulin G (IgG) (Cappel Laboratories, Cochranville, Pa.). The highest dilution producing definite fluorescence in the majority of the cells was taken as the antibody titer.

Statistical methods. Differences in survival of different groups of mice were analyzed for statistical significance by the chi-square test. Correlation coefficients were calculated for survival in relation to antibody titers.

RESULTS

Properties of monoclonal antibodies. Monoclonal antibodies secreted by seven different hybridomas were used in this study, and their properties are summarized in Table 1. The hybridomas were derived from independent fusions. Two secreted IgG2a antibodies, while the other five secreted IgG1 antibodies. The glycoproteins recognized by the monoclonal antibodies were determined by cross adsorption experiments as well as by immunoprecipitation and polyacrylamide gel electrophoresis of the precipitates (Balachandran et al., submitted for publication). Glycoprotein C was precipitated by antibodies from hybridomas 13αC5 and 18αA5; however, 13αC5 antibodies reacted only with the fully glycosylated form of the molecule, while 18αA5 antibodies also reacted with precursor molecules. Antibodies from all of the other hybridomas shown in the table except 13αA5 reacted with both partially and fully glycosylated glycoproteins. None of the monoclonal antibodies neutralized HSV-2, and indirect immunofluorescence titers of all of the monoclonal antibody preparations were within fourfold of each other. In the presence of guinea pig complement, the monoclonal antibodies lysed HSV-2-infected cells to varying degrees, and the titers at which the antibodies lysed infected cells in the ADCC assay also varied (Table 1).

Passive transfer of monoclonal antibodies to the glycoproteins. An initial experiment was carried out to assess the ability of monoclonal antibodies to protect against HSV-2 lethality. Ascitic fluids containing monoclonal antibodies reacting with HSV-2 glycoproteins gC, gAB, gD, gF, and gE (18αA5, 20αD4, 17βA3, 17αA2, 17βC2) were diluted 1:5 in phosphate-buffered saline, and

TABLE 1. Properties of monoclonal antibodies

Monoclone designation	Antibody isotype	Reacting glycoprotein	Titers determined by:			
			Complement-dependent cytolysis[a]	ADCC[b]	Neutralization[c]	Indirect immunofluorescence[d]
13αC5	IgG1	gC	>1:5,260	1:30	<1:5	1:1,600
18αA5	IgG1	gC	1:30	1:1,000	<1:5	1:800
20αD4	IgG1	gAB	>1:5,260	1:10,000	<1:5	1:800
17αA2	IgG2a	gF	>1:160	1:4,400	<1:5	1:6,400
17βA3	IgG1	gD	1:1,000	1:3,000	<1:5	1:1,600
17βC2	IgG2a	gE	1:40	1:1,000	<1:5	1:1,600
13αA5	IgG1	None[e]	<1:10	<1:30	<1:5	1:3,200

[a] Antibody titers showing more than 10% specific lysis of BHK-21 cells infected with HSV-2 in the presence of guinea pig complement.
[b] Antibody titers showing more than 30% specific lysis of BHK-21 cells infected with HSV-2 in the presence of peritoneal exudate cells from golden Syrian hamsters.
[c] Neutralization of 100 PFU of HSV-2.
[d] Antibody titers by indirect immunofluorescence using acetone-fixed HSV-2-infected Vero cells.
[e] Antibodies to 13αA5 reacted to a non-glycosylated protein (7).

equal volumes of each were mixed. A 0.5-ml amount of the antibody mixture per mouse was injected intraperitoneally to a group of 20 mice which were simultaneously challenged with a dose of 2×10^6 PFU of HSV-2. Another group of 20 mice was injected only with virus, and as a control for specificity, 20 mice were injected with ascitic fluid (diluted 1:5) of monoclonal antibodies against an adenovirus protein.

The inoculation with 2×10^6 PFU of virus alone resulted in initial induration and erythema of the foot which was followed by a flaccid paralysis of the ipsilateral leg. This was followed by ascending myelitis, encephalitis, and death. Death occurred at as early as 6 days, and 100% mortality was seen within 21 days (Table 2). Morbidity and mortality were similar in the group of mice injected with adenovirus monoclonal antibodies. In contrast, only 6 out of 20 (30%) mice died in the group injected with the mixture of monoclonal antibodies to HSV-2 glycoproteins, and in the observation period of 6 weeks, no other mortality was seen. The data showed that a single administration of a mixture of diluted monoclonal antibodies against HSV-2 glycoproteins was able to afford a significant level of protection against lethal virus challenge.

TABLE 2. Effect of passive transfer of a mixture of monoclonal antibodies reacting with HSV-2 glycoproteins on HSV-2 lethality

Mice injected with HSV-2[a] plus:	No. that survived/ no. tested	% Protection	P^b
Nothing	0/20	0	
Mixture of HSV-2 monoclonal antibodies[c]	14/20	70	<0.01
Monoclonal antibodies against adenovirus protein	0/20	0	>0.05

[a] A total of 2×10^6 PFU (10 times the 50% lethal dose) of HSV-2 were injected into the footpad.
[b] P value for the difference in survival between the test group and the group injected with virus only.
[c] A 0.5-ml amount of a mixture of monoclonal antibody preparations (diluted 1:5) reacting with gC, gAB, gD, gE, and gF was injected intraperitoneally into each mouse at the same time virus was injected into the footpad.

Passive immunization with monoclonal antibodies to individual glycoproteins. To determine which of the glycoprotein antibodies participate in the protection against HSV-2 deaths, further experiments were carried out in which mice were injected with monoclonal antibodies against individual glycoproteins. Groups of 10 mice were injected intraperitoneally with undiluted monoclonal antibodies (ascitic fluid, 0.1 ml per mouse) against HSV-2 glycoproteins gC, gAB, gD, gE, and gF. As controls, groups of 10 mice were injected with either monoclonal antibodies directed against a non-glycosylated HSV-2 protein or against an adenovirus protein. In addition, equal volumes of the six monoclonal antibodies to HSV-2 glycoproteins were mixed together, and 0.6 ml of the mixture was given to another group of mice. Three hours after the injection of antibody, HSV-2 was injected into the footpad at a dose of 2×10^6 PFU per mouse. The mice were observed for 6 weeks. The experiment was repeated again with an additional 10 mice in each treatment group.

The combined mortality results of the two experiments are given in Table 3. The groups injected with virus only developed the classic neurological symptoms, and all mice died within 21 days. Similarly, 100% mortality was seen in the groups injected with monoclonal antibodies

TABLE 3. Protection mediated by monoclonal antibodies against individual glycoproteins

Mice injected with HSV-2[a] plus monoclonal antibodies against:	No. that survived/ no. tested	% Protection	P[b]
Nothing	0/20	0	
Adenovirus protein	0/20	0	>0.05
Non-glycosylated HSV-2 protein[c]	0/20	0	>0.05
gC(13αC5)	0/20	0	>0.05
gC(18αA5)	11/20	55	<0.01
gE	7/20	35	<0.025
gF	14/20	70	<0.01
gD	15/20	75	<0.01
gAB	15/20	75	<0.01
Mixture of gC, gAB, gD, gE, and gF[d]	18/20	90	<0.01

[a] A total of 2×10^6 PFU of HSV-2 were injected into the footpad 3 h after the administration of antibody intraperitoneally.

[b] *P* value for the difference in survival between the test group and the group injected with virus only.

[c] Monoclonal antibodies to a non-glycosylated protein of HSV-2 which has been only partially characterized (7).

[d] Each mouse received 0.1 ml of a mixture of equal parts of undiluted ascitic fluids.

against a non-glycosylated protein of HSV-2 and with antibodies to an adenovirus protein. Varying degrees of protection were observed in mice receiving monoclonal antibodies against HSV-2 glycoproteins except for antibodies from 13αC5 which recognize only the fully glycosylated molecule. Thus, antibodies against gE protected 35% of the mice, while antibodies against gC (18αA5) protected 55% of the mice. Antibodies against gF, gD, and gAB were all able to protect 70 to 75% of the mice. The greatest protection (90%) was afforded by the mixture of monoclonal antibodies to the glycoproteins, and in this group, death was only observed at late times after infection (days 18 and 20).

Effect of passive transfer in A/J mice. Since protection was seen with the monoclonal antibodies which did not neutralize virus but which did lyse cells in vitro in the presence of complement, the possible role of the lytic pathway of complement in the in vivo protection was investigated by passive transfer studies in A/J mice. These mice are deficient in the fifth component (C5) of complement (15). Groups of mice were injected intraperitoneally with undiluted monoclonal antibody preparations (0.1 ml per mouse) directed against gAB, gD, and gC (13αC5), and the mice were injected with virus in the footpad 3 h later. Monoclonal antibodies against both gAB and gD protected the C5-deficient mice as efficiently as BALB/c mice (Table 4), whereas 13αC5 antibodies again did not afford protection. Results of this study suggested that the C5 component of complement was not needed for the in vivo protection mediated by the passively transferred antibodies.

DISCUSSION

Virus-specified glycoproteins inserted into the membrane of cells infected with HSVs are thought to serve as major antigenic sites recognized by the infected host (28). Where examined, each glycoprotein so far defined appears capable of eliciting an immune response which can alter the integrity of virus-infected cells. For example, polyvalent antisera to purified gAB, gC, and gD were shown to lyse infected cells in vitro in the presence of complement or peripheral blood mononuclear cells (16). More recently, gC has been shown to possess an antigen(s) recognized by cytotoxic T-lymphocytes (2, 6). The data presented in this paper confirm the lysis of cells by antibodies directed against antigens on gAB, gC, and gD in the presence of complement or mononuclear cells. Polyvalent antibodies to purified gE can neutralize virions (21), and we found that monoclonal antibodies to gE can lyse cells in the presence of complement or by ADCC. In addition, antibodies to gF can lyse cells under similar conditions. Thus, no one glycoprotein appears to be the sole source of antigens relevant to immune reactivity to the HSVs.

We found disagreements between the titers of the monoclonal antibodies in the different assays used. While all preparations had titers between 1:800 and 1:6,400 as determined by indirect immunofluorescence, none of the preparations neutralized virus. Antibodies from hybridomas 20αD4 and 17βA3 lysed cells in the presence of complement and by ADCC at comparable titers, while these two assays yielded discordant titers for ascitic fluids induced by the other four hybridomas. The differences in titers by the different assays did not correlate with the

TABLE 4. Effect of passive transfer in C5-deficient A/J mice of monoclonal antibodies against HSV-2 glycoproteins

Mice injected with HSV-2[a] plus monoclonal antibodies against:	No. that survived/ no. tested	% Protection	P[b]
Nothing	0/10	0	
gC(13αC5)	1/15	6.7	>0.05
gD	10/15	66.7	<0.01
gAB	11/15	73.4	<0.01

[a] See footnote *a* of Table 3.

[b] See footnote *b* of Table 3.

immunoglobulin isotype. Antibodies from 13αC5, which were directed against an epitope on gC, lysed cells at high titers in the presence of complement and at low titers by ADCC, while the reverse was observed for 18αA5 antibodies which also recognized an epitope on gC. These observations suggest that the efficiency by which infected cells are lysed by the different mechanisms is dependent upon the location of the epitope on the glycoprotein rather than reaction of a specific isotype of immunoglobulin or reaction of the antibody to a specific glycoprotein.

Polyvalent antisera reacting with membrane antigens of cells infected with virus have been shown to protect mice infected with HSV-1 or HSV-2 from neurological illness and death (1, 4, 10, 12, 18–20, 22). We found that monoclonal antibodies from all of the hybridomas tested, except 13αC5, protected mice when passively transferred. This suggests that antibodies to any of the glycoproteins may be involved in protecting mice from virus replication and spread. Our findings confirm the recent report of Dix and coworkers (5) who demonstrated protection of mice with monoclonal antibodies against gC and gD of HSV-1. These monoclonal antibodies differed from those used in our study in that they neutralized virus in vitro.

Dix et al. (5) attributed protection to neutralization of virus in vivo; however, the demonstration of protection by monoclonal antibodies which did not neutralize virus in vitro raises doubts regarding this mechanism. A number of pathways by which antibodies could produce their effect in vivo have been postulated. These included neutralization of extracellular virus, aggregation or opsonization of viruses leading to phagocytosis, lysis of virus-infected cells in the presence of complement, activation of complement by antigen-antibody complexes with resulting enhanced chemotactic activity for phagocytic cells, and cell-dependent lysis of infected cells by binding of the Fc portion of antibody with effector cells bearing Fc receptors (14). While the monoclonal antibodies used in our study did not neutralize virus in vitro, we cannot conclude that neutralization in the presence of various serum factors did not occur in vivo. A/J mice, which were C5 deficient, were protected by the monoclonal antibodies as efficiently as were C5-sufficient BALB/c mice, which suggests that the lytic pathway of complement was not critical for protection. These findings support conclusions of a recent report in which hyperimmune serum was passively transferred (19). In addition, no correlation was found between antibody titers assayed by complement-dependent cytolysis and protection in our experiments.

Cellular elements of recipient mice appear to participate in the protection from lethal herpetic infections afforded by passively transferred antibodies; however, the importance of these elements may depend upon the strain of mice used. CFW mice are relatively resistant to lethal infections by HSV-1, and treatment of the animals with cyclophosphamide, antithymocyte serum, or X-irradiation enhanced mortality. This enhanced mortality could be reversed by passively transferring antibodies directed against the virus (12). Similarly, the susceptibility of BALB/c mice to lethal infection by HSV-1 or HSV-2 could be reduced by passively transferred antibodies (4, 13, 18–20). However, treatments which reduced thymic-dependent lymphocyte reactivity abolished the protective effects of antiviral antibody in BALB/c mice (18). We found BALB/c mice to be susceptible to lethal HSV-2 infections, and passive protection with monoclonal antibodies was observed in untreated animals. We also found that X-irradiation reduced the protective effect of the monoclonal antibodies (N. Balachandran, unpublished data). Thus, the monoclonal antibodies probably mediated protection by mechanisms similar to those of hyperimmune polyvalent antisera. A good correlation was found between protection and titers of monoclonal antibodies as measured in vitro by ADCC ($r = 0.9$, $P < 0.05$). This observation is compatible with an antibody-mediated cellular cytolysis in which the effector cells in vivo may be either a T-lymphocyte subset, a cell population controlled by T-lymphocytes, or a cell population influenced by treatments directed at T-lymphocytes. However, the number of monoclonal antibodies which we used was small, and the correlation between ADCC titers and protection could be spurious. Additional studies with monoclonal antibodies to the same, as well as to additional, epitopes on the glycoproteins of the virus should allow resolution of the mechanisms involved in the protection mediated by antibodies in the mouse model.

ADDENDUM IN PROOF

The designations of the glycoproteins reacting with antibodies secreted by hybridomas 17αA2 and 17βC2 have been altered from those previously reported (N. Balachandran et al., J. Virol. 39:438–446, 1981). Analysis with reference antiserum to the glycoprotein of HSV-2, which binds the Fc portion of immunoglobulins, revealed that antibody from 17βC2 was directed to the glycoprotein gE.

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