Immunogenicity of Herpes Simplex Virus Glycoproteins gC and gB and Their Role in Protective Immunity

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The relative antigenicity of the individual herpes simplex virus type 1 (KOS) glycoproteins gC and gB was analyzed in BALB/c mice by using KOS mutants altered in their ability to present these antigens on cell surface membranes during infection. The mutants employed were as follows: *syn* LD70, a non-temperature-sensitive mutant defective in the synthesis of cell surface membrane gC; *tsF13*, a temperature-sensitive mutant defective in the processing of the precursor form of gB to the mature cell surface form at 39°C; and *ts606*, an immediate early temperature-sensitive mutant defective in the production of all early and late proteins including the glycoproteins. By comparing the relative susceptibility to immunolysis of mouse 3T3 cells infected at 39°C with wild-type virus, presenting the full complement of the glycoprotein antigens, gC, gB, and gD, with target cells infected with mutants presenting only subsets of these antigens, we determined that a major portion of cytolytic antibody contained in hyperimmune anti-herpes simplex virus type 1 (KOS) mouse antiserum was directed against glycoproteins gC and gB. The relative immunogenicity of wild-type and mutant virus-infected cells also was compared in BALB/c mice. Immunogen lacking the mature form of gB induced a cytolytic antibody titer comparable to that of the wild-type virus, whereas that lacking the mature form of gC showed a 70% reduction in titer. The absence of the mature cell surface forms of gB and gC in immunogen preparations resulted in a 4- to 15-fold reduction in virus neutralizing titer. Animals immunized with *ts606*-infected cells (39°C) induced relatively little virus-specific cytolytic and neutralizing antibody. Analysis of the glycoprotein specificities of these antisera by radioimmunoprecipitation showed that the antigens immunoprecipitated reflected the viral plasma membrane glycoprotein profiles of the immunogens. The absence of the mature forms of gC or gB in the immunizing preparation did not appreciably affect the immunoprecipitating antibody response to other antigens. Mice immunized with wild-type and mutant virus-infected cells were tested for their resistance to intracranial and intraperitoneal challenge with the highly virulent WAL strain of herpes simplex virus type 1. Despite the observed alterations in serum virus-specific antibody induced with the individual immunogens, all animals survived an intraperitoneal challenge of 10 50% lethal doses. However, differences in the survival of animals were obtained upon intracranial challenge. Protection could be induced most efficiently with wild-type virus, less well with the gC and gB mutants, and the least with the early mutant *ts606*. The level of protection did not strictly correlate with the relative titers of virus-neutralizing antibodies; however, in general, a reduction in titer was associated with increased susceptibility to virus challenge. These findings suggest that antibody with neutralizing activity *in vitro* may contribute to protection against neurological herpes simplex virus infections.

The herpes simplex virus type 1 (HSV-1)-specified glycoproteins gC, gB, and gD are the major viral antigens on surface membranes of strain KOS-infected cells (14) and cells infected with most other HSV-1 strains (32). The glycoproteins are highly immunogenic and are the principal inducers of virus-specific antibody during HSV-1 infections (33). Their presence on infected cell surfaces early in the infectious cycle before the production of active virus makes them potentially important target antigens for antibody-dependent immune effector reactions that control the spread of virus by destruction of infected cells (13, 31). Antibodies against glycoproteins gC, gB, and gD have been shown to participate in antibody-dependent, complement-mediated and cell-mediated immunolysis of infected cells (14, 21). Moreover, the HSV glycoproteins are essential to recogni-

tion of infected cells by cytolytic T cells (5) and appear to also contain determinants recognized by human natural killer cells (3, 4).

Immunoprecipitating antibody specific for the three major HSV glycoproteins are readily detected in sera from patients with recurrent herpetic lesions and in sera derived from animals infected with live or inactivated virus preparations (8, 9, 11, 35). However, the relative importance of antibodies directed against individual glycoproteins in protective immunity is uncertain. Recent studies with monoclonal antibodies specific for the HSV-1 and HSV-2 glycoproteins have provided evidence that particular epitopes may be involved in different immune effector functions including virus neutralization and immunolysis mediated by complement- or antibody-dependent, cell-mediated mechanisms (1). The use of monoclonal antibodies in passive immune protection studies is suggestive that an immune response to particular epitopes may be important to controlling HSV infections (1, 10, 15, 26).

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The role of the individual HSV glycoproteins in inducing humoral immune responses has not been systematically studied. In this report, we describe experiments aimed at evaluating the importance of glycoproteins gC and gB in the induction of virus-neutralizing and cytolytic antibody. Cells infected with HSV-1 mutants altered in their ability to present glycoproteins gC and gB on the cell surface were used as immunizing preparations to determine whether either of these antigens is essential to the induction of immune antibody populations. The results showed that gC and gB are dominant immunogens and that their presence in immunizing preparations is required for the induction of virus-neutralizing antibody comparable to immunization with wild-type virus. Fluctuations in the magnitude and specificity of these immune responses were correlated with resistance of immunized mice to challenge against the highly virulent WAL strain of HSV-1 (30). A general correlation was observed between virus-neutralizing antibody titers and resistance to intracranial, but not intraperitoneal, challenge.

MATERIALS AND METHODS

Cells and virus. Mouse 3T3 cells were grown and maintained in 150-cm² plastic tissue culture flasks (Corning Glass Works, Corning, N.Y.) containing Eagle minimum essential medium (MEM; GIBCO Laboratories, Grand Island, N.Y.), nonessential amino acids, and 10% heat-inactivated fetal calf serum. Wild-type HSV-1, strain KOS, strain WAL, and KOS mutants were grown in African green monkey kidney (Vero) cells and quantitated as PFU by standard plaque assays in Vero cells (12). The non-temperature-sensitive syncytial mutant *syn* LD70 (14) and the temperature sensitive mutant *ts606* were described elsewhere (12). In mutant *syn* LD70 infections, a truncated form of glycoprotein gC is secreted into the medium, and the cell surface membrane lacks any form of gC (T. Holland, F. Homa, S. Marlin, M. Levine, and J. Glorioso, submitted for publication). Mutant *ts606* fails to produce the late viral proteins including the glycoproteins at nonpermissive temperature, 39°C (12). Also used in these studies was mutant *tsF13*, a temperature-sensitive mutant produced by in vitro mutagenesis of the HSV-1 (KOS) *EcoRI* F fragment and subsequent transfer into the wild-type KOS background (M. Gibson, unpublished data). *tsF13* does not process the major precursor form gA to the mature plasma membrane bound form gB at 39°C (J. Glorioso, unpublished observation).

Antisera. HSV-1 (KOS) antisera were produced in 100 to 200-day-old BALB/c mice (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) against both live virus and UV-inactivated, infected syngeneic cells as previously described (14), except that infections were carried out at 34 and 39°C and immunogen preparations were injected without adjuvant. Each immunogen, consisting of 2×10^7 infected cells, was administered to a group of 10 mice. The mice were bled retroorbitally at 7, 12, 15, and 20 days post-immunization. The serum from each immunization group was pooled (10 mice), heat inactivated at 56°C, diluted 1:5 with MEM, and stored at -70°C in 0.1-ml working samples.

Radioiodination and immunoprecipitation of viral cell surface antigens. Virus-infected 3T3 cell surfaces were radioiodinated by the lactoperoxidase method (14). Radiolabeled cell monolayers were scrapped into phosphate-buffered saline, pelleted, and disrupted in 1 ml of lysis buffer (phosphate-buffered saline [pH 7.4], 1% Nonidet P-40, and 0.1 mM phenylmethylsulfonyl fluoride) containing 5 µg of micrococcal nuclease (Sigma Chemical Co., St. Louis, Mo.) per ml. Viral antigens were precipitated with HSV-specific

antibody, and antigen-antibody complexes were recovered with protein A-Sepharose CL-4B beads (Pharmacia Fine Chemicals, Piscataway, N.J.) as detailed earlier (14). The beads were washed three times with lysis buffer, and the immune complexes were solubilized in electrophoresis sample solution (2% sodium dodecyl sulfate, 5% 2-mercaptoethanol, 10 mM Tris, 10% sucrose, 1 mg of bromophenol blue per ml, pH 7.0) for a final protein concentration of approximately 1 mg/ml and frozen. Before sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the sample was heated to 100°C for 2 min, and the beads were removed by centrifugation at $1,000 \times g$ for 5 min.

Polyacrylamide gel electrophoresis. Viral antigens contained in immunoprecipitates were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with the buffer system of Laemmli (17) in 10% gels as reported earlier (14). Electrophoresis of 30-µl samples was carried out at a constant current of 25 mA/gel. Methanol-acetic acid-fixed gels were treated with 1.0 M sodium salicylate in 0.1 M Tris to fluorographically enhance the autoradiogram. Estimation of molecular weights was made by the method of Weber and Osborn (34), with a Pharmacia protein standards calibration kit.

Virus neutralization and complement-mediated immune cytotoxicity. Mouse antisera were assayed for neutralization of HSV-1 (KOS) in the presence and absence of complement by using a 50% micro-plaque reduction assay as recently described (14). The neutralization titer was expressed as the last dilution of antibody giving a 50% reduction in cytopathic effect. The HSV-specific cytotoxic antibody content of mouse antisera was also determined (14). Titrations were performed with mouse 3T3 cells infected at 34 or 39°C with wild-type KOS or KOS mutants and labeled with 1 mCi of ⁵¹Cr (as sodium chromate; Amersham Corp., Arlington Heights, Ill.). The cells were suspended at a concentration of 2×10^5 cells per ml in MEM containing 2% fetal calf serum and 10% rabbit serum as a complement source. Immune cytotoxicity assays were performed in triplicate in 96-well microtiter trays. Each well contained 100 µl of 2×10^4 ⁵¹Cr-labeled cells and 100 µl of serially diluted antiserum. The plates were incubated at 34 and 39°C for 3 h, and the counts per minute (cpm) released into 100 µl of supernatant was measured with a gamma counter after the plates were centrifuged at $1,000 \times g$ for 10 min. Controls for each experiment included counts of supernatants from wells incubated with normal mouse serum plus complement (minimum release) and counts made from wells treated with 100 µl of 1 N HCl (maximum release). The percent specific chromium release was calculated as (mean test cpm - mean minimum cpm)/(mean maximum cpm - mean minimum cpm) $\times$ 100. The 50% endpoint titers were taken as the dilution giving one-half of the maximum percent ⁵¹Cr release.

Animal protection experiments. Mice immunized with live wild-type or mutant virus or UV-inactivated wild-type or mutant virus-infected cells were challenged with a lethal dose (2×10^4 PFU is 10 50% lethal dose units) of the highly virulent WAL strain of HSV-1 contained in 0.1 ml of MEM at 1, 3, and 7 days post-immunization as described elsewhere (29). The WAL challenge virus was administered intraperitoneally and intracranially by the method of Schröder et al. (28).

Cultivation of virus from mouse tissues. Groups of five BALB/c mice were inoculated intraperitoneally with wild-type KOS and mutants *syn* LD70, *tsF13*, or *ts606* infectious virus at 5×10^6 PFU. Peritoneal fluid was removed by lavage with 1 ml of MEM, and the liver and spleen were

dissected from infected animals killed 1 and 3 days postinoculation. Dissected organs were suspended in 3 ml of MEM containing 0.11 mg of gentamycin and 0.0025 mg of amphotericin B per ml. Dissected organs were subjected to three cycles of freeze-thawing and disrupted by sonication. Virus derived from individual organs was independently titrated at 34°C.

RESULTS

Presentation of viral glycoprotein antigens on the surface membrane of cells infected with wild-type virus and mutants of HSV-1 (KOS). Cells infected with mutants of HSV-1 (KOS) altered in the expression or processing of viral glycoproteins were used in this study both as immunogens and as targets for effector antibody populations. To demonstrate differences in the cell surface display of viral antigens, mouse 3T3 cells were infected at 34 and 39°C with HSV-1 (KOS) and the KOS mutants *syn* LD70, *ts*F13, and *ts*606 and lysates of radioiodinated infected cells were analyzed directly for the presence of viral proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Radioiodination labels only cell surface antigens (13). Even without immunoprecipitation, the major viral glycoproteins gC (130K), gB (120K), and gD (58K) were readily detected in HSV-1 (KOS)-infected cells (Fig. 1). From the intensities of the radioactive bands, gC and gB represented the majority of the glycoprotein antigens on the cell surface of KOS-infected cells. As recently described (14), the non-temperature-sensitive mutant *syn* LD70 infections failed to present gC on the cell surface, but the amount of gB and gD exposed on the plasma membrane were similar to that of KOS-infected cells. Mutant *ts*F13 infections displayed all three glycoproteins on the

FIG. 1. Radiiodinated plasma membrane proteins from mouse cells infected with wild-type HSV-1 (KOS) and KOS mutants. Mouse 3T3 cells infected at 34 and 39°C with HSV-1 (KOS) and the KOS mutants *syn* LD70, *ts*F13, and *ts*606 were radioiodinated by the lactoperoxidase method at 24 h postinfection (12). Radiolabeled cells were solubilized with lysis buffer containing 1% Nonidet P-40, and the lysate was electrophoresed in 10% polyacrylamide gels. Radiiodinated proteins were visualized by fluorography. The positions of molecular weight markers are presented on the left ($\times 10^{-3}$), and the locations of viral glycoproteins gC, gB, and gD are indicated on the right.

FIG. 2. Antibody-dependent complement-mediated cytotoxicity of mouse cells infected with wild-type HSV-1 (KOS) and KOS mutants. Mouse 3T3 cells infected at 39°C with HSV-1 (KOS) and mutants *syn* LD70, *ts*F13, and *ts*606 were treated with serial dilutions of mouse anti-HSV(KOS) antiserum and a standard concentration of rabbit complement by a ^{51}Cr release microassay (10). Immunolysis of cells infected with KOS (○), *syn* LD70 (●), *ts*F13 (△), and *ts*606 (▲) is shown as percent specific ^{51}Cr release.

cell surface at 34°C, whereas at 39°C the amount of gB detected is significantly reduced. In agreement with earlier findings (12), *ts*606 infections displayed normal amounts of antigen on the cell surface at 34°C and essentially no viral glycoproteins at 39°C. Glycoprotein gB, but not gC and gD, was detected in the 39°C mutant *ts*606 lysates after overexposure of autoradiograms (data not shown). Thus, the phenotypes of these mutants are the failure to present gC by *syn* LD70, gB at 39°C by *ts*F13, and all of the glycoproteins at 39°C by *ts*606.

Immune cytotoxicity of mouse cells infected with wild-type KOS and KOS mutants altered in expression of viral cell surface glycoproteins. Since gB and gC represented two major viral cell surface glycoproteins (Fig. 1), removal of either of these antigens from the target cell plasma membrane by infection with mutants might be expected to reduce the susceptibility of these target cells to immunolysis compared with wild-type virus-infected targets. The loss of target antigens might be reflected by a decrease in the effective cytolytic antibody titer. The data in Fig. 2 show the susceptibility of mouse 3T3 cells infected at 39°C with KOS and KOS mutants to immunolysis by mouse anti-HSV-1 (KOS) antiserum. The 50% endpoint titer measured against KOS-infected target cells was 1:1,280, whereas against *syn* LD70 (gC⁻) or *ts*F13 (gB⁻)-infected target cells, the titer was reduced fourfold to 1:320. The 50% endpoint titer measured against *ts*F13- and *ts*606-infected cells (34°C) was similar to that of KOS targets, indicating that the antigenic structure of *ts*F13 and wild-type KOS are similar (data not shown). The removal of single target antigens lowered the effective cytolytic antibody concentration, indicating that a significant portion of the cytolytic antibody was directed against each of these proteins. As reported earlier (12), *ts*606-infected cells were highly resistant to immunolysis, in keeping with the almost complete absence of viral antigens on the infected cell membrane at 39°C. These results confirm that a major portion of cytolytic virus-specific antibody is directed against the gC-gB complex.

Time course of appearance of neutralizing and cytolytic antibody induced by immunization with live HSV-1 (KOS) or inactivated virus-infected cells. The kinetics of production of neutralizing and cytolytic antibody in BALB/c mice were compared by using live KOS virus or KOS-infected syngene-

FIG. 3. Comparison of the kinetics and magnitude of the virus-neutralizing and cytolytic antibody response in mice immunized with live KOS virus and KOS-infected syngeneic cells. Groups of 10 BALB/c mice were each immunized by intraperitoneal inoculation of 2×10^6 PFU live KOS virus (●) or 2×10^6 UV-irradiated KOS-infected mouse 3T3 cells (○). The animals were bled at 7, 12, 15, and 20 days post-immunization, and the sera from each group of animals were pooled for testing. The virus neutralizing antibody titers for all groups were assayed by a microneutralization assay with and without complement as previously described (12) and by our ^{51}Cr release microassay.

ic 3T3 cells as immunogens. The neutralizing antibody titers for both groups were assayed by microneutralization with and without complement, and the titer of the cytolytic antibody was determined by a standard ^{51}Cr release assay. In all cases, the neutralizing and cytolytic antibody titers did not increase appreciably after 7 days post-immunization (Fig. 3). The titer of neutralizing antibody in the presence of complement was uniformly higher (approximately fourfold) than in the absence of complement. The cytolytic antibody titers were comparable to the complement-dependent neutralization titers. The time-course appearance of both neutralizing and cytolytic antibody populations were parallel and similar in magnitude for both methods of immunization, suggesting that comparable antigenic determinants are recognized by the host during both methods of immunization.

Immunization of mice with cells infected with wild-type and mutant viruses. The immunogenicity of the wild-type and mutant viruses was compared to assess the role of these antigens in the induction of virus-neutralizing and cytolytic antibody. All mutant viruses induced the production of antibody titers 10- to 20-fold lower than that of the wild-type virus (Table 1). Although mutants *tsF13* and *ts606* and the non-temperature-sensitive mutant *syn* LD70 grow normally

TABLE 1. Cytolytic and virus-neutralizing antibody titers induced in BALB/c mice after immunization with live wild-type HSV-1 (KOS) or KOS mutant-infected and UV-irradiated 3T3 cells

Antiserum (7 days post-immunization)	50% endpoint titer of anti-KOS antibody ^a		
	Virus neutralization		Immune cytotoxicity
	No complement	Complement	
KOS virus	80	1,280	1,500
<i>syn</i> LD70 virus	5	80	180
<i>ts</i> F13 virus	0	40	180
<i>ts</i> 606 virus	5	40	46
KOS-infected cells (34°C)	160	2,560	3,373
<i>syn</i> LD70-infected cells (34°C)	20	160	1,038
<i>tsF13</i> -infected cells (34°C)	0	1,220	2,560
<i>ts606</i> -infected cells (34°C)	80	1,280	2,937
KOS-infected cells (39°C)	20	640	1,280
<i>syn</i> LD70-infected cells (39°C)	10	160	640
<i>tsF13</i> -infected cells (39°C)	0	160	2,937
<i>ts606</i> -infected cells (39°C)	10	80	320

^a KOS-infected 3T3 cells (37°C) and KOS virus as targets for cytolytic and virus-neutralizing antibody, respectively.

in cell culture at 34°C, these mutants replicated poorly in the mouse peritoneal cavity (Table 2), which may account for their weak immunogenicity. However, since immunization with HSV-infected cells gave antibody titers comparable to those achieved with live virus inoculation (Fig. 3), it was possible to compare the immunizing capacity of mutant virus-infected cells with that of wild-type virus-infected cells. Equal numbers of infected cells were used in the inoculum injected intraperitoneally into mice to provide similar amounts of antigen. The immunogens differed, however, in their antigenic make-up. The results in Table 1 permit the following conclusions. (i) Virus-neutralizing antibody titers were uniformly at least eightfold higher in the presence of complement, demonstrating the importance of complement in neutralization. (ii) The amount of neutralizing antibody induced by wild-type virus-infected cells was always higher than that induced by other immunogens. (iii) Mutant *syn* LD70-infected cells at 34 and 39°C induced 4- to 15-fold lower titers of virus-neutralizing antibody and a 2- to 3-fold lower titer of cytolytic antibody compared with wild-type virus-infected cells at these temperatures, indicating the importance of the mature form of gC to the induction of both virus-neutralizing and cytolytic antibody. (iv) Mutant *tsF13* gave comparably low virus-neutralizing titers at 39°C, suggesting that the mature form of gB is an important immunogen for the induction of neutralizing antibodies. In contrast, cells infected with *tsF13* induced high titers of cytolytic antibody at both temperatures, suggesting that different populations of antibodies are involved in virus neutralization and immunolysis. (v) Mutant *ts606*-infected cells at 34°C were as immunogenic as KOS-infected cells, whereas the immunogen prepared at 39°C was very weakly immunogenic. This confirms the importance of the HSV glycoproteins in the induction of virus-neutralizing and cytolytic antibody.

Specificity of antibodies induced by cells infected with wild-type or mutant viruses. To examine the antigen specificities available in antisera derived from these immunizations, antisera were further tested for their ability to immunoprecipitate detergent soluble wild-type virus glycoprotein antigens. Antiserum from mice immunized with KOS-infected cells at 34°C immunoprecipitated amounts of gC, gB, and gD similar to the amounts of these antigens precipitated with the antiserum from mice immunized with KOS-infected cells at 39°C (Fig. 4). By inspection, the majority of label was associated with gB (Fig. 4A). As expected, immunization with cells infected with *syn* LD70 at either temperature induced very little antibody specific for gC, since this antigen was not present on the surface membrane of the immunogen cell preparations (Fig. 4). The amount of gB and gD immunoprecipitated were similar at both temperatures

TABLE 2. Comparison of the replication of wild-type HSV-1 (KOS) and KOS mutants in BALB/c mice

Infecting virus ^a	Virus titer ^b				
	4 h	24h		48h	
	peritoneal exudate	Peritoneal exudate	Spleen	Peritoneal exudate	Spleen
HSV-1 (KOS)	0	3×10^2	3.5×10^3	4×10^2	1×10^3
<i>syn</i> LD70	0	1×10^2	3.5×10^2	0	7×10^2
<i>tsF13</i>	0	0	0	0	0
<i>ts606</i>	0	0	0	0	0

^a Infecting virus (10^5 PFU) were injected intraperitoneally into nine animals.

^b Average PFU for three animals.

FIG. 4. Comparison of the viral glycoprotein specificity of immunoprecipitating antibodies induced by immunization of mice with cells infected with wild-type KOS and cells infected with KOS mutants. Mouse 3T3 cells infected at 37°C with wild-type HSV-1 (KOS) were radiolabeled and detergent solubilized as in Fig. 1. Soluble viral antigens were immunoprecipitated with antiserum derived from mice immunized with wild-type KOS mouse 3T3 cells infected at 34 or 39°C with mutant *syn* LD70, *ts*F13, or *ts*606. Immune complexes were recovered with Sepharose-protein A beads, and the viral antigens were dispersed by gel electrophoresis of immunoprecipitates in polyacrylamide gels. Radiolabeled viral antigens were visualized by fluorography. (A) Gel exposed to X-ray film for 24 h. (B) Gel exposed to X-ray film for 7 days. Molecular weight markers are indicated on the left (10^{-3}), and viral glycoproteins are designated on the right.

and not apparently different from the amounts of those antigens precipitated by the anti-KOS antiserum (Fig. 1B). The absence of the mature form of gC in the immunogen preparation did not appear to affect the magnitude of the response to glycoproteins B and D. Antisera prepared against *ts*F13-infected cells (39°C) precipitated little gB (Fig. 4A); however, gB was detected in the overexposed autoradiogram (Fig. 4B). C was clearly detected in the overexposed autoradiogram (Fig. 4B). The amount of gD immunoprecipitated by antisera against *ts*F13-infected cells at 34 or 39°C was equivalent and similar to that precipitated by the anti-KOS and the anti-*syn* LD70 antiserum (Fig. 4B). Again, the absence of gB did not affect the response to other viral antigens. Animals immunized with cells infected with *ts*606 at 34°C induced precipitating antibodies similar to wild-type virus-infected cells, but the 39°C immunogen induced no precipitating antibody specific for gC and gD and precipitated only a small amount of gB, which was visible by overexposure of the autoradiogram (Fig. 4B). The general correlation between the absence of immunoprecipitating antibody specific for gC with a reduced neutralizing and cytolytic antibody content of the same serum (Table 1) suggested that anti-gC antibodies are important to both of these responses. The reduction of anti-gB immunoprecipitating antibodies did not adversely affect the overall cytolytic antibody titer. As

demonstrated in Fig. 2, however, immunization with wild-type virus-infected cells induces a strong cytolytic antibody response to gB. These data indicate that gB is immunogenic for the induction of cytolytic antibody, but that the mature form is not required for the cytolytic antibody response to other viral antigens.

Protection of mice immunized with wild-type or mutant virus-infected cells from lethal doses of infectious HSV. To determine whether the presence of antibody populations described above (Table 1 and Fig. 4) showed any relationship with immunity to HSV infections, challenge experiments were performed. Groups of seven BALB/c mice were immunized with 3T3 cells infected at 34 and 39°C with wild-type virus or the virus mutants *syn* LD70 and *ts*F13 and challenged with a lethal dose (10^4 PFU) of WAL virus either intraperitoneally or intracranially at 7 days postimmunization (Table 3). Animals immunized with mock infected cells showed little resistance to challenge virus. All infected cell immunogens were effective in protecting against virus inoculated intraperitoneally. Intracranial challenge revealed that KOS-infected cells (34 and 39°C) gave almost complete protection. However, cells infected with *syn* LD70 and *ts*F13 (34°C) provided about 60% protection. In addition, cells infected at 39°C with either mutant gave little or no protection. The inability of the *ts*F13 (34°C) immunogen to induce

TABLE 3. Resistance of BALB/c mice immunized with 10⁷ 3T3 cells infected with wild-type HSV-1 (KOS) or KOS mutants to challenge with HSV-1 (WAL)^a

Immunogen	Temp (°C)	Intraperitoneal challenge on day 7	Intracranial challenge on day 7
Mock	34	3/7	0/7
KOS	34	7/7	7/7
<i>syn</i> LD70	34	7/7	4/7
<i>ts</i> F13	34	7/7	4/7
KOS	39	7/7	6/7
<i>syn</i> LD70	39	7/7	2/7
<i>ts</i> F13	39	7/7	0/7

^a Fractions indicate proportion of mice surviving experimental virus challenge. The fraction of survivors did not change after 3 additional weeks of observation.

virus-neutralizing antibody titers (Table 1) and protection comparable to that of the wild-type virus immunogen (Table 3) may reflect qualitative as well as quantitative differences in the antibody populations induced by these two immunogens. With the exception of the results obtained with the *ts*F13 (34°C) immunogens, the lack of protective immunity appeared to correlate well with the low neutralizing antibody response and not with the induction of cytolytic antibody (Table 1).

A second group of animals was similarly immunized with a 10-fold increase in the number of immunizing infected cells to test whether protection by the gC⁻ and gB⁻ immunogens could be elevated by increasing the antigenic load. Also, *ts*606-infected cells (34 and 39°C) were tested to assess the importance of the HSV glycoproteins in their induction of protecting immunity. At 24 h after immunization, almost complete protection was achieved with all immunogens against intraperitoneal challenge, except *ts*606 (39°C), demonstrating that the viral glycoproteins are essential to inducing protective immunity (Table 4). By day 6, immunogen *ts*606 (39°C) provided full protection. The low level of synthesis of glycoprotein gB observed in *ts*606 infection (Fig. 4) was likely sufficient and responsible for protection. Protection against intracranial challenge 3 days after immunization was nearly complete for all immunogens, except for *ts*606-infected cells, and even after 6 days several animals died after challenge. These data indicate that differences in immune responsiveness and resistance to experimental virus challenge, induced through use of mutant virus infected cells as immunogens, could be readily obscured by increasing the amount of antigen in immunizing preparations.

DISCUSSION

In this study, we confirmed that immunization of mice with wild-type HSV-1 (KOS) and KOS virus-infected cells induces immunoprecipitating antibody against glycoproteins gC, gB, and gD (14). Antibody specific for gB and gC predominated, which is consistent with the finding that the HSV-1 (KOS) glycoproteins gB and gC are the most prominent viral antigens on the surface membranes of infected cells (12–14).

The relative amount of cytolytic antibody specific for gC and gB in whole anti-HSV-1 (KOS) antiserum was determined by using cells infected with mutants of KOS deficient for the production of the mature plasma membrane forms of these glycoproteins as targets for cytolytic antibody. With cells infected with *ts*606 (39°C), an immediate early mutant defective for the synthesis of early and late viral proteins

including glycoproteins, it was shown that the HSV-specific cell surface glycoproteins were essential target antigens for cytolytic antibody. Mutant *ts*606-infected cells (39°C) were highly resistant to immunolysis by polyclonal mouse anti-KOS antiserum, a finding reported earlier with polyclonal rabbit anti-KOS serum (12). Earlier we reported that the absence of one glycoprotein on the infected cell membrane does not interfere with recognition and subsequent lysis by cytolytic antibodies specific for remaining glycoproteins (14). Therefore, the reduced susceptibility of target cells lacking gC (*syn* LD70 infections) or gB (*ts*F13 infections at 39°C) is interpreted as evidence that cytolytic antibody specific for gC and gB predominated in the anti-KOS antiserum.

Comparisons of virus-neutralizing and cytolytic antibody induction in mice immunized with live KOS virus or virus-infected cells syngeneic with the mouse host showed that the two methods of antigen presentation to the host were comparable in their ability to induce high titers of herpesvirus-specific antibody. This served as a basis for comparing the immunogenicity of the individual glycoproteins, since cells infected with virus mutants defective in glycoprotein synthesis or processing could be used as immunogens.

Having shown that gC and gB are highly immunogenic as part of the full complement of viral cell surface antigens, immunogens lacking the mature forms of gC or gB were compared for their ability to induce immunoprecipitating, virus-neutralizing, and cytolytic antibodies. Immunizations were carried out (i) to examine the immunogenicity of these antigens relative to each other, (ii) to determine whether these antigens differed in their ability to induce the production of antibodies with the several different anti-viral activities, and (iii) to determine whether these antigens behaved independently as immunogens.

With *ts*606-infected cells (39°C) as an immunogen, very low amounts of virus-specific antibody were produced as measured by all three assays of antibody activity. It is clear from these data and from work by others (33) that the viral glycoproteins are the principal inducers of these humoral immune responses. Infected cells lacking the mature form of glycoprotein gC were less immunogenic than were wild-type virus-infected cells, demonstrating the importance of gC as an immunogen. Greatly reduced immunoprecipitating antibody was detected against gC in these immunizations, and the reduction of gC-specific antibody correlated with a 3-fold reduction in cytolytic antibody and a 15-fold reduction in

TABLE 4. Resistance of BALB/c mice immunized with 10⁸ 3T3 cells infected with wild-type KOS or KOS mutants to challenge with HSV-1 (WAL)^a

Immunogen	Temp (°C)	Intraperitoneal challenge on day:		Intracranial challenge on day:	
		1	6	3	6
Mock	34	1/7	0/7	0/7	0/7
KOS	34	7/7	7/7	7/7	7/7
<i>syn</i> LD70	34	7/7	7/7	7/7	7/7
<i>ts</i> F13	34	7/7	7/7	6/7	7/7
<i>ts</i> 606	34	6/7	7/7	7/7	7/7
KOS	39	7/7	7/7	7/7	7/7
<i>syn</i> LD70	39	7/7	7/7	6/7	7/7
<i>ts</i> F13	39	6/7	7/7	5/7	7/7
<i>ts</i> 606	39	2/7	7/7	0/7	5/7

^a Fractions indicate proportion of mice surviving experimental virus challenge. The fraction of survivors did not change after 3 additional weeks of observation.

virus-neutralizing antibody. These results are in general agreement with recently published findings (14); however, the effect of gC in the induction of virus-neutralizing antibody reported here is more dramatic. This difference may reflect the use of adjuvant in immunizing preparations in the earlier paper (14). The absence of gC in the immunogen preparation did not affect the production of immunoprecipitating antibody specific for gB or gD. gB and gD appear to behave independently as immunogens. Immunization with the gB⁻ mutant-infected cell preparation resulted in the production of immunoprecipitating antibody against gC and gD, again showing that these antigens behaved independently as immunogens. Antigenically related precursor forms of gB in the cytoplasm were not as immunogenic as the mature cell surface membrane gB form, indicating the importance of cell surface structures in the induction of antibody responses. The significant reduction in the immunoprecipitating antibody response to gB did not result in a concomitant decrease in the overall cytolytic antibody titer, suggesting that the presence of gB was not essential to achieving cytolytic antibody titers comparable to those obtained with the wild-type virus immunogen. gB was an important inducer of virus-neutralizing antibody, since the absence of the mature form of this antigen in the immunogen preparation resulted in a fourfold reduction in the virus-neutralizing antibody titer.

Antibody specific for the HSV glycoproteins has been reported to represent the majority of immunoprecipitating antibody in human sera from individuals with herpetic infection, and antibody specific for gC was demonstrated in all cases (11, 36). Eberle and Courtney (9) determined that anti-gC antibody was prevalent in human sera from persons with HSV-1 infections, and they suggested that this antibody was diagnostic for HSV-1 infections. These studies indicate that gC is also highly immunogenic in humans. Gilman et al. (11) suggested that gC may represent the major HSV-1 antigen, a finding compatible with our study of the antibody response to HSV in mice.

Having determined that gC and gB are major immunogens in the KOS strain of HSV-1 with our immunization protocol and that antibodies specific for these antigens can be reduced or eliminated in immunization involving mutant-infected cells, we attempted to evaluate the effect of altering the magnitude of the antibody response to individual HSV glycoproteins on protection against virulent virus challenge. Our results indicate that the glycoproteins contribute importantly to the induction of protective immunity. Immunogen preparations that did not contain the glycoproteins were less protective than preparations containing the wild-type glycoproteins. The reduction in the antibody response to gC and gB, in particular, correlated with the lack of protective immunity against intracranial challenge, suggesting that an immune response, possibly antibodies specific for gB or gC, is important to protection against neurological infection. This effect could be overcome, however, by increasing the amount of antigen in the immunizing preparation. Antibodies directed against gC may not be solely involved in protection in these experiments, since Schrier et al. (27) have reported that purified gC of HSV-1 can induce type-specific delayed hypersensitivity that correlates with protective immunity to virus challenge.

It is clear that virus-reactive antibodies can play an important role in immune protection (2, 6, 7, 10, 15, 18–20, 22–25). Kapoor et al. (16) showed that B cell-suppressed mice exhibited a more pronounced infection of the peripheral and central nervous system and showed a higher incidence

of latency than did B cell-competent mice. Dix et al. (10) reported that virus-neutralizing monoclonal antibodies specific for glycoprotein gD prevented the spread of neurological herpesvirus infection in nude mice. Balachandran et al. (1) demonstrated that monoclonal antibodies capable of mediating antibody-dependent cell-mediated lysis of virus-infected cells in vitro were protective in vivo. Methods to reduce active thymocytes in challenge animals receiving protective antibodies resulted in reduced protection against challenge virus, suggesting that protection in vivo also involved antibody-dependent cell-mediated lysis of virus-infected cells (1, 22). No correlation was found between cytolytic antibody activity and immune protection (1). Since protection was as effective in C5 deficient as in C5-sufficient BALB/c mice, complement did not seem to play an important role in protection. In our study, no correlation could be found for cytolytic antibody and immune protection, which supports, in part, the conclusions of Balachandran et al. (1). Complement may play an important role in controlling the spread of HSV in the host, however, through complement dependent virus-neutralization. In this regard, at least one complement-dependent virus-neutralizing monoclonal antibody has been shown to be protective (15).

In our study, a reduction in virus-neutralizing antibody titer was associated with reduced protection against intracerebral challenge. This correlation does not confirm, however that virus neutralization is the protective mechanism. For example, Rector et al. (26) recently reported that monoclonal antibodies reactive with any of the HSV-1 glycoproteins were protective against ocular herpetic infections and that no correlation between in vitro assays of virus neutralization and immune cytotoxicity was observed. One antibody was protective yet nonfunctional in these antiviral assays. In our study, a reduction in immunoprecipitating antibody against gC and gB correlated with increased susceptibility to virus challenge. The relationship between the presence of virus-neutralizing antibody and immune protection is only suggestive evidence for the involvement of virus-neutralizing antibodies in immunity. Further studies comparing the protective capacity of complement-dependent and complement-independent virus-neutralizing antibodies, especially those known to recognize different antigenic sites within the major glycoprotein antigens, should help to clarify the role of virus neutralization in immune protection against viral challenge.

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