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Herpes Simplex Virus Glycoproteins: Isolation of Mutants Resistant to Immune Cytolysis

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Immune cytolysis mediated by antibody and complement is directed against components of the major herpes simplex virus (HSV) glycoprotein complex (molecular weight, 115,000 to 130,000), comprised of gA, gB, and gC, and against glycoprotein gD—all present on the surfaces of infected cells. Tests with a temperature-sensitive (*ts*) mutant of HSV-1 (*ts*A1) defective in glycoprotein synthesis at the nonpermissive temperature (39°C) demonstrated that over 90% of mutant-infected cells maintained at 39°C and treated with antibody and complement were not lysed, presumably due to the absence of viral glycoproteins on the surface of infected cells at this temperature. Furthermore, a small number of *ts*A1-infected cells could be detected among a large excess of wild-type virus-infected cells by virtue of their failure to be lysed at 39°C by antibody and complement. Making use of the involvement of viral glycoproteins in immune cytolysis and the ability of cells infected with glycoprotein-defective mutants to escape cytolysis, we sought mutants defective in the expression of individual viral glycoproteins. For this purpose, antisera directed against the VP123 complex and against the gC and combined gA and gB glycoprotein subcomponents of this complex were first tested for their ability to lyse wild-type virus-infected cells in the presence of complement. Wild-type virus-infected cells were lysed after treatment with each of the three antisera, demonstrating that the gC glycoprotein and the combined gA and gB glycoproteins can act as targets in the immune cytolysis reaction. Next, these antisera were used to select for mutants which were resistant to immune cytolysis. Cells infected with wild-type virus which had been mutagenized with 2-aminopurine and incubated at 39°C were treated with one of the three types of antisera (anti-VP123 complex, anti-gC, or anti-gAgB) and lysed by the addition of complement. Cells which survived immune cytolysis were plated, and virus in the resulting plaques was isolated. Plaque isolates were tested for temperature sensitivity of growth and altered cytopathic effects in cell culture at 34°C (the permissive temperature) and 39°C. A total of 73 mutants was isolated in this manner. Selection with glycoprotein-specific antisera resulted in a 2- to 16-fold enrichment for mutants compared with "mock"-selected mutants using normal rabbit serum. Phenotypically, 24 mutants were temperature sensitive for growth, 27 were partially temperature sensitive, and 22 were not temperature sensitive but exhibited markedly altered cytopathic effects at both permissive and nonpermissive temperatures. Nine mutants of each phenotype (temperature sensitive, partially temperature sensitive, and non-temperature sensitive) were selected at random for confirmatory immune cytolysis tests with the antisera used in their selection. Cells infected with eight of the nine mutants were shown to be significantly more resistant to immune cytolysis at the nonpermissive temperature than were the mock-selected mutants or the wild-type virus from which they were derived.

Infection of cells with herpes simplex virus (HSV) results in the synthesis of new, virus-specific glycoproteins (22, 25). These new glycoproteins, which can readily be demonstrated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of [^{14}C]glucosamine-labeled infected cell extracts, are found in purified membrane fractions of virus-infected cells and in the envelopes of progeny virions (7, 25, 26).

Although the precise functions of these glycoproteins in the biology of the virus have yet to

be elucidated, one glycoprotein (gB) has recently been shown to be essential for virion infectivity (19), whereas virus replication can apparently proceed in the absence of synthesis of another (gC) (10). The expression of the syncytial (*syn*) phenotype characteristic of cells infected with some strains of HSV appears to involve at least two viral glycoproteins (10) and three or more distinct genetic loci (18; S. P. Little and P. A. Schaffer, manuscript in preparation). Glycoprotein gE has been identified as the virus-induced Fc receptor on infected cell surfaces (1). In addition, viral glycoproteins have been implicated as the antigens which stimulate the humoral immune response in the infected host (15). Virus-infected cells are susceptible to lysis either by sensitized lymphocytes (14) or complement-dependent antibody (17). Glorioso and Smith and Glorioso et al. (5, 6) have shown that antibody-dependent, complement-mediated cytotoxicity is directed against components of the major viral glycoprotein complex (VP123; average molecular weight, 123,000), observed in SDS polyacrylamide gels. Recently, Norrild et al. confirmed these findings and showed that gD (molecular weight, 50,000 to 59,000) also functions as a target for cytolysis (12). Additionally, these investigators have demonstrated that all four glycoprotein antigens (gA+gB, gC, gD) serve as targets for cell-mediated immune cytolysis in vitro (12). In addition to their role in the HSV replicative cycle and in the induction of the immune response, viral glycoproteins have been detected on the surfaces of HSV-1 and HSV-2-transformed cells (2, 15, 16; R. J. Courtney, manuscript in preparation). That these glycoproteins may play a role in modulating tumorigenicity is suggested by the observation that animals bearing HSV-induced tumors develop neutralizing antibody to the virus (4, 9, 14).

As major virion structural components, glycoproteins reach maximal rates of synthesis late in infection, consistent with their classification in the γ class of viral proteins (8). Five major glycoproteins, gA, gB, gC, gD, and gE, derived from four antigenically distinct polypeptides, are synthesized (1, 24). Three of these polypeptides, gB, gC, and gD, are glycosylated in two stages yielding partially glycosylated intermediates and fully glycosylated products. The final products are found in the envelope of mature virions and in infected cell membranes. Glycopeptide gA, which is antigenically related to gB (R. Eberle and R. J. Courtney, personal communication; P. Spear, personal communication), is not extensively glycosylated, and little of it is incorporated into virions relative to glycoproteins gB, gC, and gD (24).

To investigate the roles of individual glycoproteins in the biology of HSV, we made use of the involvement of viral glycoproteins in antibody-dependent complement-mediated cytotoxicity.

We describe here a procedure for the selective enrichment of HSV mutants defective in the expression of specific glycoproteins at the cell surface, using antisera directed against individual viral glycoproteins.

MATERIALS AND METHODS

Cells and cell culture. Monolayer cultures of human embryonic lung (HEL) fibroblasts (passages 8 to 17) were used as target cells in immune cytolysis experiments. Vero cell cultures were used for the preparation of virus stocks, for virus assay, and for the assay of infectious centers after treatment of infected HEL cells with antibody and complement. Both HEL and Vero cells were grown at 37°C in Eagle medium (Autopow, Flow Laboratories, Inc., Rockville, Md.), supplemented with 10% fetal bovine serum containing 0.075% $NaHCO_3$ (for cultures in closed vessels) or 0.225% $NaHCO_3$ (for cultures in open vessels in a 5% CO_2 atmosphere) (13).

Viruses and virus assays. Properties of the HSV-1 wild-type strain, KOS, and of the temperature-sensitive (*ts*) mutants, *ts*A1, *ts*E6, and *ts*J12, have been described (20, 22). Mutants *ts*A1, *ts*E6, and *ts*J12 are members of complementation groups 1.-1, 1.-5, 1.-9, respectively, in the standard set of HSV-1 complementation groups (21). Virus stocks were prepared as described previously (13). Virus assays and infectious center assays were performed in Vero cells with a 2% methylcellulose overlay in CO_2 (5%) incubators (Wedco, Silver Spring, Md.) with temperature variations of 0.2°C. The permissive temperature was 34°C, and the nonpermissive temperatures were 39°C and 40°C.

Immune cytolysis assay. The protocol employed for the measurement of antibody-dependent, complement-mediated cytotoxicity of virus-infected cells is summarized in Fig. 1. Confluent monolayers of HEL cells (3×10^6 cells in 25-cm^2 flasks) were infected with HSV-1 at a multiplicity of 2.5 to 5 PFU/cell. After adsorption for 1 h at 34°C, inocula were removed, and monolayers were rinsed twice with 2 ml of Tris-buffered saline (pH 7.4) and overlaid with prewarmed medium. Infected cultures were then incubated for the appropriate interval at either 34°C or 39°C. After incubation, monolayers were rinsed, and cells were detached from the support medium by treatment with 0.5% EDTA. Cells were pelleted by centrifugation at $800 \times g$ for 5 min and were suspended and diluted to 10^5 cells per ml in serum-free medium. This cell suspension was then dispensed in 1-ml volumes, and cells were pelleted a second time. Cells in paired tubes were then suspended in either 0.1 ml of antiserum or 0.1 ml of control serum at the indicated dilutions and incubated for 60 min at 34°C in a shaking water bath. An equal volume of a 1:2 dilution of guinea pig complement (GIBCO Laboratories, Grand Island, N.Y.) was added, and the suspension was incubated for an additional 90 min in a shaking water bath at 34°C. After the second incubation, infected cells were diluted in

medium containing 0.2% pooled human gamma globulin (Merck Sharp & Dohme, West Point, Pa.) and plated with 3×10^6 indicator Vero cells at 34°C. (0.2% gamma globulin was sufficient to reduce the titer of wild-type virus by greater than 99.9%.) When cells had attached to petri dishes, they were overlaid with medium containing 2% methylcellulose. After 5 days of incubation, neutral red was added to the overlay, and the plaques, representing infectious centers derived from cells which survived lysis by antibody and complement, were counted. The relative sensitivity of a population of infected cells to immune cytolysis was determined by comparing the number of surviving infectious centers in the treated population with that in the population treated with normal serum.

This procedure was developed to measure the reduction in infectious centers when a population of virus-infected cells was treated with antibody and complement before plating on indicator cells. Cells having viral antigens on their surfaces were lysed with antibody and complement under the conditions of assay, and such cells did not form infectious centers. Cells not so treated or cells treated with antibody and complement before the appearance of viral antigens on their cell surface were not lysed and subsequently gave rise to infectious centers upon plating. The plating overlay contained immune gamma globulin to prevent plaque formation by cell-free, progeny, or unadsorbed virus. Thus, the plaques which appeared probably originated from virus in infected cells.

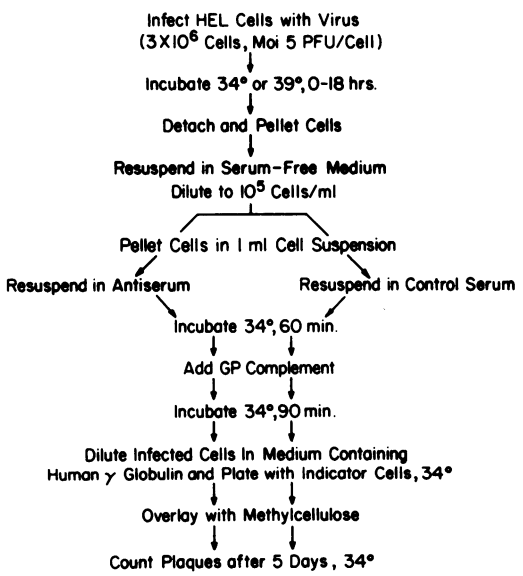

FIG. 1. *Immune cytolysis assay.*

Sensitivity of the immune cytolysis assay: reconstruction experiments. Cells infected with mutants which do not express HSV-specific glycoproteins or which express antigenically altered glycoproteins should be insensitive to immune cytolysis and, therefore, should be recoverable from mixtures of cells infected with wild-type virus after treatment of the total population with antibody and complement. The efficiency of recovery of mutant-infected cells was tested in the following way. Cells were infected either with wild-type virus or with *ts*A1, a mutant defective in glycoprotein synthesis at 39°C (22). The two types of cells were incubated at 39°C for 18 h, mixed, treated with antibody and complement, plated at 34°C with indicator cells, and overlaid with medium containing 2% methylcellulose (Fig. 2). Such cultures should yield a population of plaques enriched for those initiated by the mutant.

Antisera. (i) Human antiserum to HSV. Serum was obtained from an individual who experienced frequent, recurrent facial lesions caused by HSV-1. This serum, obtained 1 week after the appearance of the most recent recurrence, had a 50% plaque-reduction neutralization titer to HSV-1 of 1:512.

(ii) Antiserum to HSV-infected cells. The procedure used for the preparation of antiserum to HSV-1-infected cells was that described by Sim and Watson (23). Extracts of HSV-1-infected rabbit kidney cells maintained in rabbit serum were used as the immunogen.

(iii) Antiserum to the major viral glycoprotein complex, VP123 (average molecular weight, 123,000). The preparation of polypeptide-specific antisera was carried out essentially as described by Courtney and Benyesh-Melnick (3). The viral glycoprotein complex (VP123) was purified from HSV-1-infected HEp-2 cells as described by R. Eberle and R. J. Courtney (manuscript in preparation). Briefly, cells were infected at a multiplicity of 10 PFU/cell with HSV-1, strain KOS. Cells were harvested after 24 h, and glycoproteins were solubilized with 1% sodium deoxycholate and 1% Tween 40 for 1 h at 37°C. The resulting extract was centrifuged at 100,000 × *g* for 1 h, and the glycoprotein-containing supernatant fluids were removed. The glycoprotein complex was separated by two cycles of preparative SDS-PAGE, and fractions containing the complex were pooled, concentrated, and electrophoresed on cylindrical tube gels. The section of the tube gel containing glycoproteins of molecular weight 115,000 to 130,000 was cut from the gel and frozen. Gel sections were later thawed and emulsified in Freund complete adjuvant. The emulsion was used to immunize rabbits by the schedule described previously (3).

(iv) Antisera to viral glycoproteins gAgB and gC. The techniques used for the fractionation of virus-infected cells by preparative SDS-PAGE and SDS-

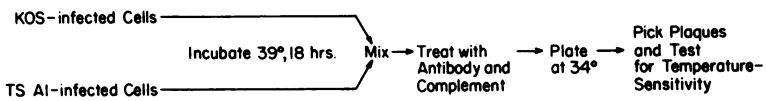

FIG. 2. *Scheme for recovering tsA1-infected cells from an excess of wild-type virus-infected cells by immune cytolysis using anti-HSV serum.*

hydroxylapatite column chromatography and the preparation of antisera using the glycoproteins purified in this way have been described (3; Eberle and Courtney, in preparation). Briefly, glycoproteins were solubilized as described above and fractionated by SDS-hydroxylapatite chromatography by the procedure of Moss and Rosenblum (11). The two major bands resolved were repurified twice by SDS-hydroxylapatite chromatography and were used for the preparation of antisera as described above. One band contained viral glycoprotein gC, and the other contained glycoproteins gA and gB. Thus, anti-gC antiserum precipitated a single protein, and anti-gAgB precipitated two proteins, when immunoprecipitates of HSV-infected cells were examined by SDS-PAGE. No cross-reaction was demonstrated between anti-gC and anti-gAgB antisera (Eberle and Courtney, in preparation). Recent studies have demonstrated that the gA and gB components are antigenically related (R. Eberle and R. J. Courtney, unpublished data; P. Spear, personal communication).

Mutagenesis. Four HEL cell cultures were infected at a multiplicity of 1 PFU/cell with HSV-1 strain KOS, using a virus stock which had been plaque purified three times (Fig. 3). After adsorption, monolayers were overlaid with 2 ml of maintenance medium containing 700 μg of 2-aminopurine per ml. This concentration of 2-aminopurine had been shown previously to reduce the yield of HSV-1 by 80% after 48 h (J. Jofre, R. J. Courtney, and P. A. Schaffer, manuscript in preparation). Infected cultures were incubated for 48 h, at which time pronounced cytopathic effects (CPE) were observed. Cultures were then frozen at −90°C. Later they were thawed, subjected to two additional cycles of freezing and thawing, and clarified by low-speed centrifugation. Virus in supernatant fluids from individual tubes was then used in the mutant selection step. Multiple mutagenized stocks were employed to avoid the selection of clonally related mutants.

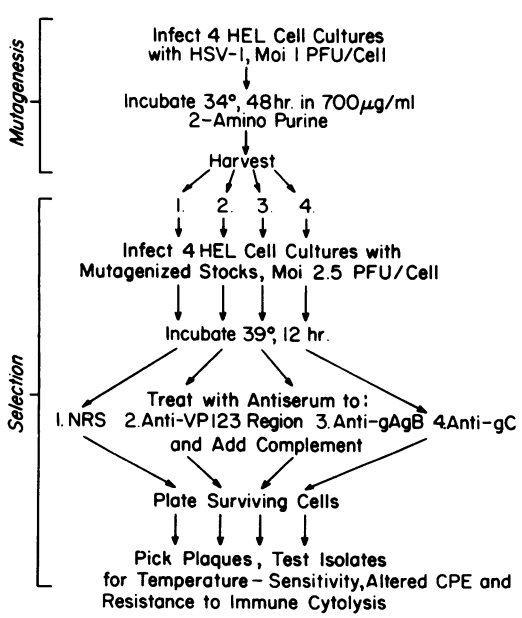

FIG. 3. *Mutagenesis and selection of HSV-1 mutants resistant to immune cytolysis.*

Mutant selection. Cultures of HEL cells (2×10^6 to 3×10^6) were infected at a multiplicity of 2.5 PFU/cell with 2-aminopurine-mutagenized virus stocks (Fig. 3). Virus was adsorbed for 1 h at 34°C. Inocula were then removed, and monolayers were rinsed three times with 2 ml of Tris-buffered saline, pH 7.4. Monolayers were overlaid with maintenance medium which had been prewarmed to 39°C, and cultures were incubated for an additional 11 h at 39°C in a water bath. After incubation, monolayers were rinsed twice, and cells were detached with EDTA. Cells were washed, pelleted, and treated with antiserum as described above. The dilutions of antisera employed had been determined previously to yield an 80 to 90% decrease in infectious centers in cells infected with the wild-type virus. After incubation of the serum-cell mixtures for 60 min at 34°C in a shaking water bath, an equal volume of complement was added to each tube, and incubation was continued for 90 min. The cells were then diluted and plated with indicator Vero cells so that plates received approximately 10 to 1,000 infected cells per dish. After 6 h of incubation at 34°C in immune gamma globulin-containing medium, medium was removed from monolayers and replaced with medium containing immune globulin and methylcellulose. After 5 days of incubation at 34°C, neutral red was added, and plaques were counted and picked the following day. Plaque isolates were transferred to test tube cultures of HEL cells, and cultures were incubated until complete CPE was observed. The cultures were then frozen and thawed three times to release virus; these virus stocks were frozen at −90°C. The plaques isolated were then screened for temperature sensitivity and altered CPE in the following manner. Duplicate 24-well cluster dishes were seeded with HEL cells, and individual wells were infected with a 1:1,000 dilution of each plaque isolate. After adsorption of virus, paired dishes were incubated at 34°C and 39°C, respectively, and observed daily for temperature sensitivity of growth and for the appearance of altered CPE. Plaque isolates which exhibited markedly reduced CPE at 39°C when maximal CPE had been achieved in the well maintained at 34°C were considered to be presumptive temperature-sensitive mutants. The temperature-sensitive phenotype was confirmed by determining the efficiency of plating (EOP) of mutants at 39°C and 34°C. Those mutants demonstrating EOPs of 3 logs or greater were considered to have been confirmed as temperature sensitive. Isolates with EOPs of less than 3 logs as well as those which exhibited equal EOPs at both temperatures but significantly smaller plaques at 39°C compared with 34°C were designated partially temperature-sensitive (p*ts*) mutants. Because temperature-sensitive and partially temperature-sensitive mutants exhibited extensive leak at 39°C, the nonpermissive temperature used in subsequent tests was 40°C. Isolates which exhibited equal EOPs and plaque sizes at 34°C and 39°C but morphologically altered CPE were designated non-temperature-sensitive (n*ts*) mutants. Altered CPE was most commonly evident as the tendency of cells to

form syncytia or any other readily recognizable alteration in social behavior, e.g., failure to induce "ballooning" or lysis of cells. Mutants were plaque purified three times, and stocks were prepared in Vero cells.

RESULTS

The immune cytolysis assay. The number of infectious centers produced in a population of wild-type virus-infected HEL cells treated with anti-HSV serum and complement 18 h postinfection at 34°C was a linear function of serum dilution at a fixed number of input infected cells (10^5) (Fig. 4, large graph). The inset graph shows the reduction in infectious center formation as a function of serum dilution. In later experiments, we used 10^5 cells and a 1:8 dilution of human anti-HSV serum, which resulted in a reduction of approximately 80% of the number of surviving infectious centers at 18 h postinfection.

Kinetics of antigen appearance. We next examined the time at which cells infected with the wild-type virus or with the glycoprotein-defective mutant, *ts*A1, were maximally susceptible to immune cytolysis (Fig. 5). Multiple HEL cell cultures were infected either with wild-type virus or *ts*A1. After virus adsorption and washing, each set of cultures was divided into two groups. One group was incubated at 34°C, and the other was incubated at 39°C. At 2-h intervals, one culture incubated at each temperature was harvested, and the sensitivity to immune cytolysis of 10^5 cells in the culture was measured using a 1:8 dilution of human anti-HSV serum.

In cells infected with the wild-type virus, antigens involved in immune cytolysis appeared earlier at 39°C (4 h) than at 34°C (6 h), but a slightly greater total number of cells was lysed when infected cells were maintained at 34°C than when they were maintained at 39°C. These differences in the kinetics of cytolysis are analogous to differences in the growth curves of wild-type virus at 34°C and 39°C: replication at 39°C precedes that at 34°C by approximately 2 h, through 12 h postinfection.

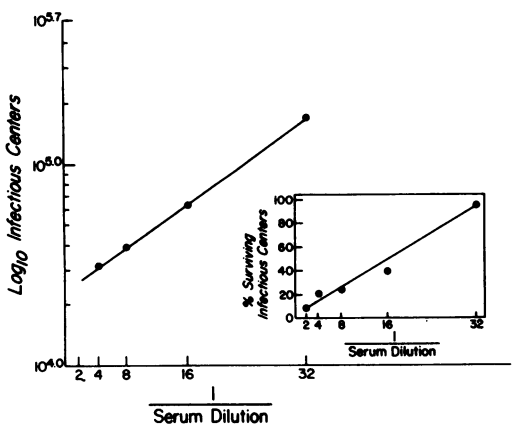

FIG. 4. *Effect of serum dilution on the survival of HSV-1-infected cells after 18 h of incubation at 34°C in immune cytolysis tests using anti-HSV serum.*

Virus-specific antigen synthesis as determined by the chromium 51 (^{51}Cr) release assay (5) was included for comparison (Fig. 5, dashed line). The maximum number of cells lysed as detected by ^{51}Cr release was slightly greater than that measured by reduction in infectious center formation. Although the ^{51}Cr release experiments were conducted at 37°C, the kinetics of antigen appearance as measured by either technique are strikingly similar. In both cases, the time of first lysis of infected cells was between 2 and 4 h postinfection, and maximum lysis was observed at 10 to 12 h postinfection.

At 34°C, *ts*A1-infected cells were not lysed at the same rate as wild-type virus-infected cells. Hence, the target antigens for immune cytolysis were not expressed as fully as in wild-type virus-infected cells at this temperature. Such an observation was not unexpected as temperature sensitivity is a continuously variable function of temperature, rather than an all-or-none phenomenon. At 39°C, little or no cytolysis of *ts*A1-infected cells was evident during the 18-h incubation period. This observation is consistent with the absence of glycoprotein synthesis characteristic of *ts*A1 at 39°C (22). When plated at 34°C after treatment, these cells did produce infectious centers; therefore, the inhibition of glycoprotein synthesis at 39°C with this mutant is apparently reversible.

Rescue of tsA1-infected cells. Experiments were then conducted to determine whether cells infected with *ts*A1 could be recovered from a mixture of cells containing an excess of wild-type virus-infected cells using the immune cytolysis test. The procedure for this experiment is illustrated in Fig. 2, and the results obtained are summarized in Table 1. We began with two populations of virus-infected cells, both of which were maintained at 39°C for 18 h (Fig. 2). Small numbers of *ts*A1-infected cells were mixed with a 100-fold excess or greater of wild-type virus-infected cells. The mixed suspension was then treated with human anti-HSV antibody and complement and plated. Plaques were picked, and plaque isolates were tested for temperature sensitivity. All temperature-sensitive isolates were screened for complementation with *ts*A1 and an unrelated mutant, *ts*E6 (20).

The expected number of temperature-sensitive isolates could be calculated from (i) the titer of the control after treatment with antibody and

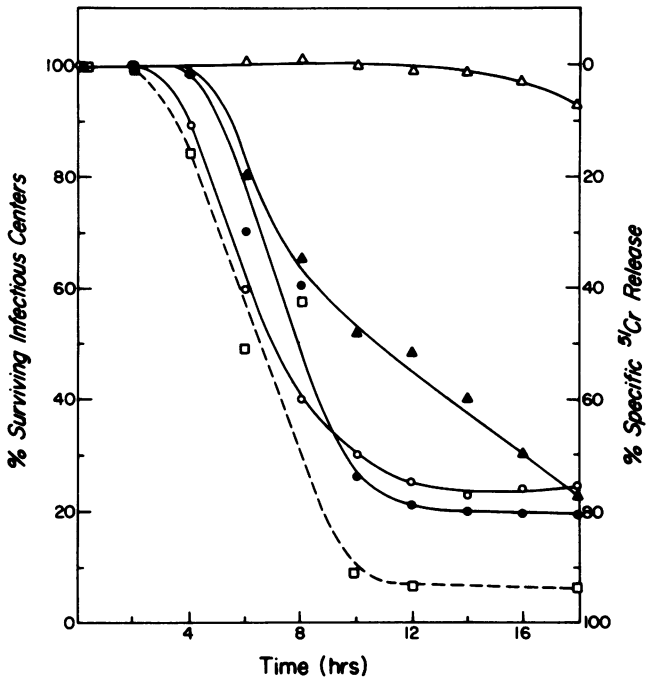

FIG. 5. *Kinetics of immune cytolysis of virus-infected cells using human anti-HSV serum. Symbols: ● and ○, the wild-type virus at 34°C and 39°C; ▲ and △, glycoprotein-defective mutant tsA1 at 34°C and 39°C; □, from Glorioso and Smith (5). This curve was derived from a ^{51}Cr release assay after growth of HSV-1-infected cells at 37°C.*

complement and (ii) the number of input *ts*A1-infected cells, knowing that the latter cells should not be sensitive to immune cytolysis. The results of the reconstruction experiment are summarized in Table 1. As seen in the last two columns, temperature-sensitive isolates were recovered from all three suspensions to which mutant-infected cells had been added. Moreover, all six temperature-sensitive mutant isolates failed to complement *ts*A1, but complemented *ts*E6 efficiently (Table 2), thus confirming their identity as *ts*A1.

Comparison of the actual percent recovery with the expected percent recovery demonstrates that a two- to fivefold-higher percentage of infectious centers was initiated by the temperature-sensitive mutants than had been anticipated. This discrepancy may have been due to error in the actual number of *ts*A1-infected cells added to the mixed suspension or to failure to inactivate all infectious progeny virus with human gamma globulin during the plating procedure.

Nonetheless, these results demonstrate that *ts*A1-infected cells were preferentially recovered from a large excess of wild-type virus-infected cells after immune cytolysis.

Cytolysis mediated by glycoprotein-specific antisera. To isolate new glycoprotein-defective mutants, we next attempted to capitalize upon the reduced sensitivity to cytolysis of cells infected by glycoprotein-defective mutants. Antisera directed against combined HSV-specific glycoproteins gA and gB, and against gC, as well as antiserum directed against the VP123 complex, were available for these studies. It was reasoned that these antisera might facilitate the selection of mutants defective in the expression of the glycoprotein antigens against which the antisera were prepared. It was first necessary to demonstrate that such antisera would indeed mediate antibody-dependent, complement-mediated cytotoxicity (Fig. 6). Unlike normal rabbit serum, which lysed approximately 10% of wild-type virus-infected cells at a 1:2 dilution, rabbit

TABLE 1. *Reconstruction experiment: recovery of tsA1-infected cells from a mixture of KOS- and tsA1-infected cells*

Input cell no. in mixture:		No. of infectious centers	Expected % *ts*A1 infectious centers	No. of temperature-sensitive infectious centers recovered/ no. tested (%)
KOS	*ts*A1			
10^5	0	2.8×10^3	0.0	0/100 (<1.0)
10^5	8	2.6×10^3	0.3	1/70 (1.4)
10^5	20	2.4×10^3	0.8	2/100 (2.0)
10^5	40	2.8×10^3	1.4	3/100 (3.0)

TABLE 2. *Complementation tests between tsA1, tsE6, and six putative reisolates of tsA1*

Virus	Complementation index[a] in tests with:	
	*ts*A1	*ts*E6
Isolate 1	1.2	93
Isolate 2	1.1	31
Isolate 3	0.8	45
Isolate 4	0.5	26
Isolate 5	1.9	54
Isolate 6	1.3	82
*ts*A1	0.6	75
*ts*E6	47	1.6

[a] Complementation tests were performed as previously described (19); indices of 2 or greater were considered positive.

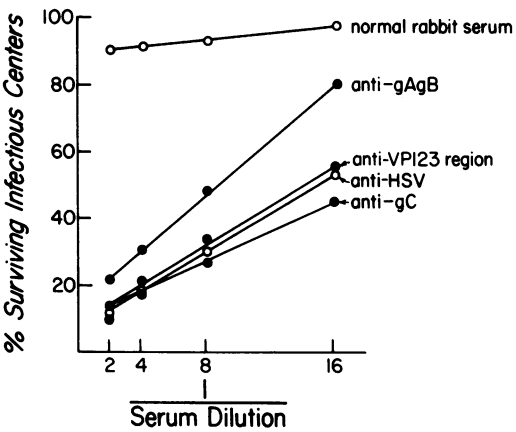

FIG. 6. *Cytolysis mediated by antisera prepared against specific HSV-1 glycoproteins. HSV-1-infected HEL cells were incubated at 39°C for 18 h and subjected to immune cytolysis using various dilutions of normal rabbit serum, anti-VP123, anti-gC, or anti-gAgB.*

anti-HSV serum and antisera to glycoproteins gAgB and gC and to the VP123 complex lysed 80% or more of wild-type virus-infected cells at this dilution. For all sera, the percent surviving infectious centers increased as a function of serum dilution throughout the 1:2 to 1:16 range of dilutions tested. The cytolytic activities of anti-gC and anti-VP123 complex sera (anti-gAgBgC) were similar to that of anti-HSV serum, whereas anti-gAgB serum was less active, particularly at higher dilutions. The ability of glycoprotein-specific antisera to induce lysis of HSV-infected cells confirmed earlier observations (5, 6) that cytolytic antibody is directed against glycoproteins in the VP123 complex and demonstrated the potential usefulness of these sera for mutant selection.

Mutagenesis and selection of mutants resistant to immune cytolysis. Since all three glycoprotein-specific antisera were active in our immune cytolysis assay, these antisera were used for the selection of cytolysis-resistant mutants derived from 2-aminopurine-mutagenized stocks of HSV-1 strain KOS. Cells infected with each of four mutagenized stocks and maintained at 39°C were treated with one of the following: normal rabbit serum, anti-gAgB serum, anti-gC serum, or anti-VP123 serum (Fig. 3). Cells which escaped immune cytolysis were plated at 34°C, and plaques were picked. Selection by immune cytolysis was conducted at 39°C in an attempt to isolate temperature-sensitive mutants; such mutants might express viral glycoproteins on cell surfaces at 34°C but not at 39°C. In addition to classical temperature-sensitive mutants with defects in essential replicative functions, mutants able to replicate at both temperatures but unable to express glycoproteins at 34°C, 39°C, or both temperatures might be selected. Such mutants would be defective, by definition, in functions not essential for replication or would synthesize antigenically altered glycoproteins. Because glycoprotein-defective mutants might be expected to induce atypical CPE in cell culture (e.g., syncytia formation), all plaque isolates were screened for altered CPE, as well as for temperature sensitivity. A total of 532 plaques were picked, and 73 mutants were isolated (Table 3). During plaque purification and stock preparation, potential temperature-sensitive mutants exhibited extensive leak (3 to 5 logs) at 39°C. Consequently, in subsequent tests 40°C was used as the nonpermissive temperature. At 40°C, leak was reduced to 2 to 4 logs. Three distinct classes of mutants were identified: (i) mutants which produced plaques at 34°C but not at 40°C (classical temperature-sensitive mutants with EOPs 40°/34° ≥3 logs), (ii) mutants which plated less well at 40°C than at 34°C (EOP 40°/34° <3 logs) or those which exhibited equal titers at 34°C and 40°C but which produced significantly smaller plaques at 40°C, and (iii) mutants which plated equally well and produced plaques of wild-type size at both temperatures but which exhibited altered CPE. All mutants in the latter class and some mutants in the former two classes exhibited altered CPE at one or both temperatures. Mutants in the first class were designated temperature sensitive, those in the second class were designated partially temperature sensitive, and those in the third class were designated non-temperature sensitive.

Five mutants were identified from among 100 plaque isolates derived from cells infected with

TABLE 3. *Isolation of mutants resistant to immune cytolysis*

Antiserum used for selection	% Survivors[a]	No. of mutant isolates/no. of plaques picked (%)	No. of mutants		
			Temperature sensitive	Partially temperature sensitive	Non-temperature sensitive
Normal rabbit serum	90.0	5/100 (5.0)	0	2	3
Anti-VP123 region	3.0	11/148 (7.4)	3	7	1
Anti-gAgB	0.7	30/192 (15.6)	12	10	8
Anti-gC	0.5	32/192 (16.6)	9	10	13

[a] The quotient of the number of plaques after treatment with antiserum divided by the number of plaques after treatment with normal rabbit serum multiplied by 100.

mutagenized virus and treated with normal rabbit serum. Mutants of two distinct types, partially temperature sensitive and non-temperature sensitive, were identified. Because mutants of each type could be clonally related, the frequency of occurrence of mutants in 2-aminopurine-mutagenized stocks was at least 2% but no greater than 5%. No mutants were observed among 100 plaques isolated from unmutagenized, unselected KOS (unpublished observations).

Although treatment of infected cells with antiserum to the VP123 complex reduced the number of surviving cells to only 3%, a higher percentage of surviving cells contained mutant progeny: compare 5% (normal rabbit serum) and 7.4% (VP123 complex serum). Treatment of infected cells with anti-gAgB or anti-gC serum resulted in an even greater reduction in the number of infected cells. In both cases, approximately 16% of the virus from surviving cells was mutant. Thus, treatment of cells infected with mutagenized virus with all three glycoprotein-specific antisera resulted in enrichment for mutant progeny.

Sensitivity of cells infected with new mutants to immune cytolysis. To examine the extent to which mock-selected and antiserum-selected mutants expressed antigens involved in immune cytolysis compared with wild-type virus, four mutants mock selected with normal rabbit serum (termed O mutants), one representative of each type of mutant (AB, C, and R, selected with anti-gAgB, anti-gC, and anti-VP123 complex sera, respectively, and which had been characterized as temperature sensitive, partially temperature sensitive, and non-temperature sensitive), and wild-type virus were tested in the immune cytolysis assay at 34°C and 40°C. The antisera used in these tests were anti-VP123 complex serum for mock-selected mutants and anti-gAgB, anti-gC, and anti-VP123 complex sera for mutants selected with these sera, respectively. The results of immune cytolysis tests are shown in Fig. 7. The four mutants isolated after treatment of mutagenized virus-infected cells with normal antiserum were tested with anti-VP123 complex antiserum. This antiserum was selected because, theoretically, it contained antibodies to all precursors, intermediates, and fully glycosylated products of the gA, gB, and gC glycoprotein species. Cells infected with each of the four mock-selected mutants were approximately as sensitive to lysis as wild-type virus-infected cells at 40°C, demonstrating no detectable alteration in the expression of glycoproteins gA, gB, and gC by these mutants (Fig. 7A). In contrast, cells infected with nine antiserum-selected mutants demonstrated moderate (n*ts*130-AB, Fig. 7C) to greatly enhanced (p*ts*63-R, Fig. 7B; *ts*108-AB, Fig. 7C; n*ts*79-C, Fig. 7D) resistance to immune cytolysis at 40°C when tests were conducted with the same antisera used for mutant selection. *ts*A1 and *ts*J12, two glycoprotein-defective mutants (20, 22; J. Jofre and S. Little, personal communication), also exhibited enhanced resistance to immune cytolysis at 40°C in tests with all three antisera. These results thus demonstrate the effectiveness of the antiserum selection procedure for the isolation of mutants defective in immune cytolysis and, potentially, in the expression of viral glycoproteins.

Because the immune cytolysis procedure used in mutant selection was conducted at the nonpermissive temperature, mutants might be expected to exhibit either reduced or equal sensitivity to cytolysis at the permissive temperature. In fact, cells infected with some mutants were partially temperature sensitive in the immune cytolysis test (e.g., p*ts*63-R and *ts*112-R, Fig. 7B; *ts*134-C and n*ts*79-C, Fig. 7D) whereas others were equally resistant to cytolysis at 34°C and 40°C (p*ts*110-R, Fig. 7B; *ts*108-AB and p*ts*61-AB, Fig. 7C). Similarly, the glycoprotein-defec-

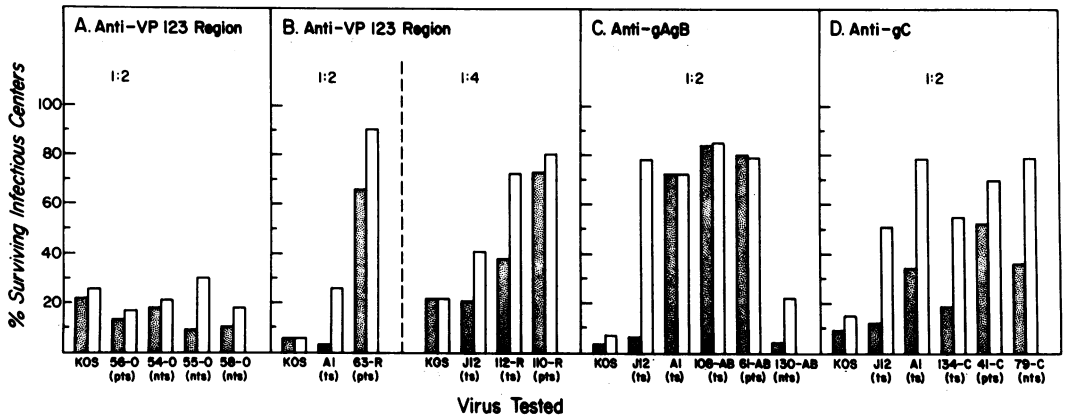

FIG. 7. *Immune cytolysis of cells infected with mutants selected for resistance to cytolysis. 10^5 HEL cells were infected with the glycoprotein-defective mutants tsA1 and tsJ12, wild-type virus, or 13 newly isolated mutants, randomly selected for testing. Infected cells were incubated for 18 h at 34°C (■) and 40°C (□) and subjected to immune cytolysis using the antisera at the dilutions indicated.*

tive mutants *ts*A1 and *ts*J12 exhibited temperature-sensitive behavior in immune cytolysis tests with glycoprotein-specific antisera.

DISCUSSION

In an effort to isolate mutants defective in viral glycoprotein synthesis, we have developed a simple and effective procedure for the selection of mutants which are resistant to immune cytolysis. The procedure is based upon the assumption that cells infected with mutants which exhibit decreased expression or aberrant synthesis of viral glycoproteins will survive treatment with anti-HSV serum and complement. This assumption was first tested and proven with a mutant, *ts*A1, which is both temperature-sensitive for glycoprotein synthesis and resistant to immune cytolysis at 39°C.

All viral glycoprotein-specific antisera used in mutant selection were able to mediate cytolysis, indicating that the gAgB, gC, and possibly other components of the major viral glycoprotein complex (VP123) can serve as targets for immune cytolysis and are inserted into the cell membrane. These observations are in agreement with the findings of Glorioso and Smith and Glorioso et al. (5, 6) and Norrild et al. (12) that viral glycoproteins within the VP123 complex serve as target antigens for immune cytolysis. We do not know the contribution of each HSV glycoprotein to complement-mediated cell lysis or whether less extensively glycosylated precursors can also serve as targets. Moreover, whether gE is involved in immune cytolysis remains to be determined. However, the isolation of mutants resistant to immune cytolysis yet able to replicate as efficiently as the wild-type virus (non-temperature-sensitive mutants) demonstrates that normal expression of target antigens is not essential for virus replication. This finding is consistent with the observation that at least one glycoprotein, the gC glycoprotein, is not essential for virus replication (10). Thus, the density of a given target antigen on the cell surface may be too low to permit efficient immune cytolysis, but sufficiently high on the nuclear membrane to permit budding of particles which are able to initiate infection. In this regard, accurate quantitation of viral glycoprotein synthesis will be essential to an understanding of the roles of glycoproteins in the viral replicative cycle.

The 2- to 16-fold increase in mutant isolation observed after selection with glycoprotein-specific antisera is significant, demonstrating that the selection procedure enriches for progeny which exhibit the desired phenotype. In the future, it should be possible to include a second cycle of antibody treatment in the selection procedure which may further increase the proportion of lysis-resistant mutants.

In the present study, it is possible that some or all mutants within a given group (i.e., all mutants selected with a particular antiserum) could be clonally related because each group was selected from only one mutagenized stock. By the same token, however, mutants in different groups cannot be clonally related. Clonal relationships should be detected by complementation tests now in progress.

Although the nature of the defects in individual mutants remains to be determined, one can postulate a variety of mutations which could result in resistance to immune cytolysis: mutations in structural or regulatory genes for individual glycoproteins, mutations in viral genes involved in glycosylation (if such genes exist),

and mutations in genes encoding other viral membrane proteins which can affect the ability of normal glycoproteins to be inserted, to name several.

Interestingly, preliminary tests of several lysis-resistant temperature-sensitive mutants indicate that they synthesize significant amounts of virus-specific glycoproteins at the nonpermissive temperature as assayed by SDS-PAGE. The localization of these glycoproteins is presently being investigated. It is possible that these mutants are either defective in the insertion of glycoproteins into the cellular membrane or that the antigenic specificity of individual glycoproteins has been altered so that glycoprotein-specific antisera no longer recognize these target antigens. In any event, the defects exhibited by these temperature-sensitive mutants differ markedly from that of *ts*A1, which exhibits drastically reduced expression of all viral glycoproteins at the nonpermissive temperature, resulting in decreased expression of target antigens at the cell surface.

Having isolated mutants which are resistant to immune cytolysis and, thus, putatively altered in the expression of individual viral glycoproteins, we believe these mutants should prove useful in defining (i) the kinetics of appearance of virus-specific glycoproteins in cellular membranes; (ii) the mosaic of viral antigens on the cell surface; and (iii) the function of viral glycoproteins in the HSV replicative cycle, in transformation by the virus, and in the induction of humoral and cellular immunity to HSV.

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