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Mutant Analysis of Herpes Simplex Virus-Induced Cell Surface Antigens: Resistance to Complement-Mediated Immune Cytolysis

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BHK-21 cells infected with temperature-sensitive mutants of herpes simplex virus type 1 strain KOS representing 16 complementation groups were tested for susceptibility to complement-mediated immune cytolysis at permissive (34°C) and nonpermissive (39°C) temperatures. Only cells infected by mutants in complementation group E were resistant to immune cytolysis in a temperature-sensitive manner compared with wild-type infections. The expression of group E mutant cell surface antigens during infections at 34 and 39°C was characterized by a combination of cell surface radioiodination, specific immunoprecipitation, and gel electrophoretic analysis of immunoprecipitates. Resistance to immune lysis at 39°C correlated with the absence of viral antigens exposed at the cell surface. Intrinsic radiolabeling of group E mutant infections with [^{14}C]glucosamine revealed that normal glycoproteins were produced at 34°C but none were synthesized at 39°C. The effect of 2-deoxy-D-glucose on glycosylation of group E mutants at 39°C suggested that the viral glycoprotein precursors were not synthesized. The complementation group E mutants failed to complement herpes simplex virus type 1 mutants isolated by other workers. These included the group B mutants of strain KOS, the temperature-sensitive group D mutants of strain 17, and the LB2 mutant of strain HFEM. These mutants should be considered members of herpes simplex virus type 1 complementation group 1.2, in keeping with the new herpes simplex virus type 1 nomenclature.

It is well established that the surface membranes of cells infected with herpes simplex virus (HSV) contain virus-specified proteins, the majority of which are glycosylated (2, 8-10, 12, 28). Currently, five terminal glycoprotein species have been identified in both virion envelopes and in infected cell plasma membranes (2, 28). These proteins have been designated gC, gB, gA, gE, and gD (molecular weights, 129,000, 126,000, 119,000, 83,000, and 59,000, respectively). These glycoproteins appear to be exposed at the surfaces of HSV-infected cells and can be immunoprecipitated with specific antisera (2, 9, 10, 28). They contain antigenic determinants which are common to the two serotypes of HSV (HSV type 1 [HSV-1] and HSV-2) and determinants which are specific for each serotype (10, 27, 31). These are referred to as type-common and type-specific antigens and are reactive with cross-reactive and type-specific antibodies.

During active infections, herpesvirus-specified cell surface antigens are thought to play a central role in stimulating an immune response in hosts and, in turn, are involved in humoral and cellular immune reactions which destroy infected cells (13, 31). In vitro studies have shown that HSV cell surface antigens participate in complement-mediated (9, 19, 27) and cell-mediated cytotoxicity reactions (18, 19, 25, 26) through specific recognition by cytolytic antibody or immune effector cells. Using a combination of cell surface radioiodination, immunoprecipitation, and polyacrylamide gel electrophoresis, Glorioso and Smith (9) showed that a particular gel region, designated *a*, contains the majority of immunoprecipitable radioactivity. This region contains HSV glycoproteins having molecular weights in the range 130,000 to 115,000 and appears to contain the glycopeptide species gC, gB, and gA. Both HSV type-specific and cross-reactive antibodies react with antigenic determinants associated with region *a* plasma membrane glycoproteins and sensitize infected cells to complement-mediated lysis (10). Recent studies have shown that antisera specific for either gC, gA and gB or gD are cytolytic for HSV-infected cells in both complement-mediated and antibody-dependent cell-mediated immune lysis re-

actions (19). Thus, a number of viral proteins may react with cytolytic antibody or immune effector cells. The role of any single viral plasma membrane protein in inducing an immune response or as target antigen in immune lysis reactions remains to be elucidated fully.

Studies were undertaken in our laboratory to characterize further the role of virus-specified cell surface antigens in immune lysis reactions through the use of a combination of genetic and immunological techniques. Our aim was to identify and characterize temperature-sensitive (*ts*) mutants of HSV-1 strain KOS which are resistant to immune cytolysis in a *ts* manner and to correlate resistance with viral cell surface antigen expression. To this end, cells infected with *ts* mutants of KOS representing 16 complementation groups were tested for susceptibility to complement-mediated immune cytolysis and compared with cells infected with wild-type virus. Mutant-infected cells that are resistant to immune lysis at a nonpermissive temperature would be expected to fall into one of the following three categories: (i) infections which fail to produce or produce highly reduced amounts of late viral proteins, including the glycoproteins that are normally expressed on cell surfaces; (ii) infections that produce late proteins but fail to insert the glycoproteins into cell surface membranes; and (iii) infections which fail to produce one or several glycoproteins that are essential target antigens for cytolytic antibody.

The mutant-infected cells showed a wide range in susceptibility to immune cytolysis. However, only cells infected with our complementation group E mutants were found to be *ts* for immune cytolysis. These cells were almost completely resistant to immune lysis at 39°C and did not express the major viral glycopeptides in their plasma membranes. These polypeptides were clearly produced at the permissive temperature (34°C). The group E mutants are representatives of the first category described above. Mutants inducing infections belonging to the other two categories will be discussed in a subsequent paper.

MATERIALS AND METHODS

Cells and virus. A continuous line of baby hamster kidney cells (BHK-21) was grown and maintained in 32-ounce (960-ml) glass prescription bottles containing Eagle minimum essential medium (GIBCO Laboratories, Grand Island, N.Y.), nonessential amino acids, and 10% heat-inactivated fetal calf serum (GIBCO), as described previously (1). Stocks of HSV-1 strain KOS and mutants of strain KOS *ts* for growth were grown in African green monkey kidney (Vero) cells and titrated by plaque assay on Vero cells (1). Nine *ts* mutants, which fell into seven complementation groups (groups A through G [14]), were kindly supplied by W. Munyon and R. Hughes, Roswell Park Memorial Institute. Also utilized were representative *ts* mutants from nine other complementation groups (groups H through P), which were isolated in this laboratory. These latter *ts* mutants were isolated after 5-bromo-2'-deoxyuridine mutagenesis by the method of Hughes and Munyon (14). These mutants were complemented among themselves and with the mutants in groups A through G by a procedure similar to the quantitative complementation test of Schaffer et al. (22, 23). Monolayers of Vero cells were inoculated at a multiplicity of 3 PFU of one virus and 3 PFU of another virus per cell (a combined multiplicity of 6 PFU/cell), and singly infected control cultures were inoculated with 5 PFU of each virus alone per cell. Infected cultures were incubated for 18 to 24 h at the nonpermissive temperature (39°C) in CO_2 incubators, and viruses were titrated on Vero cell monolayers. Complementation indices (CI) were calculated from the following formula: CI = (yield of A + B at 39°C)/(yield of A at 39°C + yield of B at 39°C), where A and B are two mutants. Total virus yields were assayed at the permissive temperature (34°C). A value of 2 or greater was usually taken as indicating positive complementation (22). For the mutants used in this study, a CI of 10 or greater was accepted as evidence for complementation. All mutants utilized plated at frequencies 10^3- to 10^5-fold lower at 39°C than at 34°C, indicating very low reversion and leakage rates.

Antisera. HSV-1 antisera were obtained by intramuscular injection of New Zealand white rabbits with KOS-infected primary rabbit kidney cells (UV inactivated) in complete Freund adjuvant (GIBCO), as described previously (27). The antisera were heat inactivated at 56°C for 30 min and tested for their ability to lyse KOS-infected cells with added complement. By using the method described by Smith and Glorioso (27), only sera capable of lysing nearly 100% of the infected cells were used in immune cytolysis assays and for immunoprecipitation of radiolabeled viral antigens. Before use, the antiserum was centrifuged at 50,000 × *g* for 30 min in a Beckman SW50.1 rotor to remove aggregated antibody molecules.

^{51}Cr release assay for immune cytolysis. The ^{51}Cr release assay involving infected cell suspensions (27) was not satisfactory for testing large numbers of mutant infections. Therefore, a monolayer cell assay was devised. Each well of 96-well microtiter plates (Linbro, Hamden, Conn.) was seeded with 10^4 BHK cells. After 24 h, the medium was removed, and 10^6 PFU of wild-type or *ts* mutant virus in 50 μl of minimum essential medium containing 2% fetal calf serum (MEMM) was placed in each appropriate well. After absorption for 1 h at the appropriate temperature, 5 μCi of ^{51}Cr (as sodium chromate; Amersham/Searle, Arlington Heights, Ill.) in 100 μl of MEMM was added to each well. Infected monolayers were incubated at either the permissive temperature (34°C) or the nonpermissive temperature (39°C). After 18 h, 100 μl of medium was removed from each well and discarded. The infected cells then were washed four times with 200 μl of MEMM containing 20 mM HEPES (*N*-2-

hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer (MEMH) (Sigma Chemical Co., St. Louis, Mo.), pH 7.0. Subsequently, the cells were incubated for 2 h with 200 μl of appropriate dilutions of HSV-1 antiserum and guinea pig serum (GIBCO) diluted 1:20 in MEMH as a complement source. Controls for each experiment included wells incubated with 200 μl of similarly diluted guinea pig serum without antiserum (minimum release) and wells incubated with 200 μl of 0.1% Nonidet P-40 (Shell Chemicals) (maximum release). At the end of 2 h, a 125-μl sample of medium was removed for determination of the specific ^{51}Cr release. The ^{51}Cr was counted by using a Biogamma 4000 gamma counter (Beckman Instruments, Inc., Palo Alto, Calif.). Six duplicate wells were used for each determination, and the mean counts per minute was determined for each six-well set. The following formula was used to calculate the percent specific ^{51}Cr release: percent release = [(mean test counts per minute − mean minimum counts per minute)/(mean maximum counts per minute − mean minimum counts per minute)] × 100.

Radioiodination of cell surface proteins. Infected cell monolayers were harvested with trypsin and washed three times in Dulbecco phosphate-buffered saline (PBS) (pH 7.0) containing 10 μM KI (PBS-KI); 1 mCi of $Na^{125}I$ in a solution containing 0.25 ml of 5 μM Na_2SO_3, 0.125 ml of lactoperoxidase (2 mg/ml), and 0.025 ml of H_2O_2 (1.3 mM) in PBS was added to 1.5 ml of PBS-KI containing 2×10^7 cells. Additional H_2O_2 was added to the reaction mixture at 2-min intervals, and specific labeling was inhibited after a total of 15 min by the addition of 10 ml of cold PBS-KI. Labeled cells were washed three times with PBS-KI and solubilized in 1 ml of 2% Nonidet P-40 in PBS. After 30 min, the insoluble material was removed by centrifugation for 1 h at 30,000 × *g*. The supernatant was precipitated with 0.2 ml of heat-inactivated HSV antiserum for 1 h at 37°C, followed by an overnight incubation at 4°C. Immune complexes were pelleted by centrifugation through 20% sucrose containing 0.1% Nonidet P-40 at 50,000 × *g* for 1 h in a Beckman SW50.1 rotor. The pellet was prepared for electrophoresis by suspension in 0.1 ml of electrophoresis sample solution (ESS) (37.5 mM Tris, pH 7, 5% β-mercaptoethanol, 0.25 mg of bromophenol blue per ml, 2% sodium dodecyl sulfate, 20% sucrose) at a final protein concentration of approximately 1 mg/ml; this suspension was placed in a boiling water bath for 5 min.

Intrinsic radiolabeling of viral proteins. BHK cell monolayers in 24-well trays (Costar, Cambridge, Mass.) (2×10^4 cells per well) were infected (multiplicity of infection, 10) with wild-type virus or KOS mutants, and the virus was allowed to absorb for 1 h at the appropriate temperature. Subsequently, the monolayer was overlaid with MEMM, and infected cells were incubated at either 34 or 39°C. At 4 h postinfection, the medium was replaced with MEMM containing either [^{14}C]glucosamine (3 μCi/ml) or [^{35}S]methionine (3 μCi/ml) (both from New England Nuclear Corp., Boston, Mass.). Infected cells were harvested with a rubber policeman at 24 h postinfection and pelleted by centrifugation at 100 × *g* for 10 min. The pellet was suspended in 200 μl of ESS.

Polyacrylamide gel electrophoresis. Immune precipitates or extracts of radiolabeled whole cells were solubilized in ESS and subjected to sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis. Gels (100 by 140 by 1.5 mm) were cast in a model SE 500 slab gel apparatus (Hoefer Scientific Instruments, San Francisco, Calif.). The resolving gel contained 8.5% acrylamide and 0.47% N,N'-diallyltartardiamide, and the stacking gel contained 4% acrylamide and 0.2% N,N'-diallyltartardiamide. Electrophoresis of 20-μl samples was carried out at a constant current of 23 mA/gel by the method of Laemmli (17). After electrophoresis, gels were fixed for 1 h in an aqueous solution containing 10% acetic acid and 45% methanol (fixer) and stained for 1 h with 0.125% Coomassie brilliant blue dissolved in fixer. Gels were destained with two changes of fixer for 1 h each, followed by two changes of a solution containing 7.5% acetic acid and 5% methanol for 2 h each.

Gels containing electrophoretically separated ^{35}S- or ^{14}C-labeled proteins were prepared for fluorography by the method of Bonner and Laskey (3). Gels were dried on a Hoefer SE 540 slab gel dryer. Dried gels were exposed to Kodak X-Omat R film at −70°C. Gels containing proteins labeled only with ^{125}I were dried directly after destaining and autoradiographed at −70°C on Cronex Xtra Life intensifying screens (DuPont Co., Wilmington, Del.). In each experiment, purified protein standards of known molecular weights (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) were electrophoresed in parallel with labeled viral proteins for estimation of molecular weights (32). These standards included ferritin (subunit molecular weight, 220,000), phosphorylase b (94,000), albumin (67,000), catalase (60,000), ovalbumin (43,000), lactate dehydrogenase (36,000), and carbonic anhydrase (30,000).

RESULTS

Immune cytolysis of mutant-infected cells. Mutant-infected cells were screened for susceptibility to complement-mediated immune cytolysis at permissive (34°C) and nonpermissive (39°C) temperatures. Monolayers of BHK-21 cells contained in individual wells of microtiter plates were infected with representative *ts* mutants of HSV-1 KOS from each of 16 complementation groups. Duplicate infected cultures were labeled with ^{51}Cr and incubated for 18 h at either 34 or 39°C. HSV-specific antiserum and complement were added to individual wells for immune cytolysis. After 2 h, samples were removed from each well and counted for the amount of ^{51}Cr released.

The results from a representative experiment are shown in Fig. 1. The observed immune lysis was specific for viral antigens because mock-infected cells failed to release ^{51}Cr at either temperature in the presence of cytolytic antibody. Normal serum was not cytolytic for either mock-infected or virus-infected cells (data not shown). When this micro ^{51}Cr release assay was used, the percent lysis for wild-type-infected

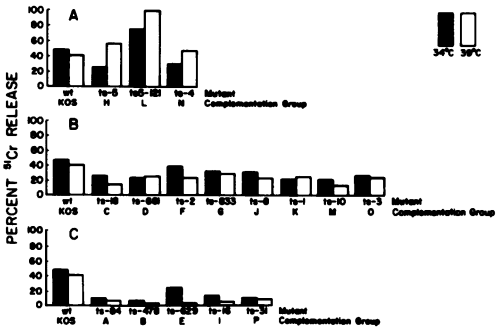

FIG. 1. *Complement-mediated immune cytolysis of BHK-21 cells infected with ts mutants of HSV-1 strain KOS. Mutants representing 16 complementation groups were used to infect cells at 34 and 39°C. Mutant-infected cells were placed into one of three groups based on susceptibility to immune lysis compared with wild-type-infected cells. (A) Mutants showing high levels of specific ^{51}Cr release at 34 and 39°C. (B) Mutants showing intermediate levels of specific ^{51}Cr release at 34 and 39°C. (C) Mutants showing very low levels of specific ^{51}Cr release at 34 and 39°C. wt, Wild type.*

cells never reached 100%. However, the amount of specific release from infected cells was consistent among experiments and nearly equivalent at the two temperatures (48 and 45%).

The mutants could be divided into three groups on the basis of the response of infected cells to cytolytic antibody. The mutants shown in Fig. 1A released a high percentage of ^{51}Cr at both 34 and 39°C. Therefore, these mutants were not *ts* for complement-mediated cytolysis. In fact, the cells infected with this group released more ^{51}Cr at 39°C than did wild-type-infected cells. *ts*5-121 exhibited a syncytia-forming phenotype. This phenotype may be the consequence of a second mutation different from the mutation causing growth temperature sensitivity. The fused cells resulting from infection with this mutant appeared to be more fragile than cells infected with a non-syncytia-forming virus. This may account for the increased susceptibility of *ts*5-121 to cytolytic antibody and complement. The mutants in Fig. 1B were somewhat less sensitive to immune cytolysis than wild type. They also were not *ts* for this character since they gave nearly equivalent amounts of lysis at the two temperatures. The mutants in Fig. 1C gave the most anomalous response to immune cytolysis. All were very resistant at 39°C. With the exception of *ts*-629, they were also resistant at 34°C. *ts*-629 apparently was the only mutant which was *ts* for immune cytolysis. The susceptibility to lysis of cells infected with this mutant approached that of KOS-infected cells at 34°C, but they were not susceptible to lysis at 39°C. Cells infected with *ts*-606 and *ts*-756, two other independently isolated mutants in complementation group E, behaved like *ts*-629-infected cells in immunolysis assays (Fig. 2).

The level of sensitivity to immune cytolysis could be quantitated more precisely by measuring the effect of antiserum dilution on the amount of immune killing. The serum dilution curves in Fig. 2 also provide data for calculation of 25% killing endpoints, a more quantitative measure of sensitivity to cytolytic antibody and complement (Table 1). The 25% endpoint was the serum dilution required to give 25% ^{51}Cr release from a standard concentration of infected cells.

The percentages of ^{51}Cr release at 34 and 39°C for wild-type-infected cells were about equal at each serum dilution (Fig. 2A), and as expected, the 25% endpoints for wild-type-infected cells were similar at permissive (1:388) and nonpermissive (1:294) temperatures (Table 1). This suggests that any differences in lysis of mutant-infected cells are a consequence of the *ts* mutations and not of the effects of temperature on normal viral polypeptides. Mutant *ts*-8 and *ts*-18 infections, which are *ts* for virus production but not for immune cytolysis, showed wild-type or higher levels of cytolysis at 34°C for each dilution of antiserum, giving 25% killing endpoints of 1:446 and 1:388, respectively (Fig. 2B and C; Table 1). At 39°C, *ts*-8-infected cells gave a serum dilution curve similar to that of wild-type-

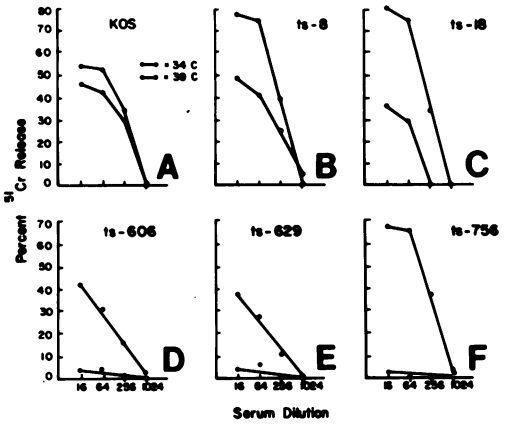

FIG. 2. *Effect of serum dilution on ^{51}Cr release from infected BHK-21 cells. Cells infected with wild type (A) and ts mutants (B through F) at 34 and 39°C were tested for susceptibility to complement-mediated immune cytolysis at varying dilutions of antiserum. Mutant-infected cells showing intermediate levels of immune lysis (B and C) were compared with mutant-infected cells showing ts resistance to lysis (D through F). The resistant mutants all belonged to complementation group E.*

TABLE 1. *Titrations for 25% endpoints for HSV-1 KOS-infected BHK-21 cells*

Virus	Serum dilution which gave 25% immune cytolysis at:	
	34°C	39°C
Wild type	388	294
ts-8 (J)	446	194
ts-18 (C)	388	64
ts-606 (E)	96	<16[a]
ts-629 (E)	64	<16[a]
ts-756 (E)	388	<16[a]

[a] Less than 5% ^{51}Cr release.

infected cells, whereas *ts*-18-infected cells showed less lysis than cells infected with wild type. This was reflected in a 25% endpoint of 1:194 for *ts*-8 infections, compared with 1:64 for *ts*-18 infections (Table 1). Mutants in complementation group E (*ts*-606, *ts*-629, and *ts*-756 [Fig. 2D through F, respectively]) showed a striking absence of immune lysis at 39°C. Less than 5% ^{51}Cr release was observed with a 1:16 dilution of rabbit antiserum. Undiluted serum gave similar results (data not shown). At 34°C, cells infected with *ts*-756 behaved like wild-type-infected cells (25% endpoint, 1:388), whereas *ts*-606- and *ts*-629-infected cells required concentrated antiserum to achieve appreciable amounts of ^{51}Cr release.

Virus-specific plasma membrane antigens present in wild-type- and mutant-infected cells. Since the complementation group E mutants were very resistant to immune cytolysis at 39°C, it was anticipated that at the nonpermissive temperature these mutants would fail to express those viral glycopeptides which are required to combine with cytolytic antibody at the cell surface. Mutant *ts*-8, which behaved like wild type, and mutant *ts*-18, which was moderately resistant to cytolysis at 39°C, were also examined for their expression of viral plasma membrane antigens.

Infected cells were radioiodinated enzymatically by using a lactoperoxidase technique in which only cell surface polypeptides were radiolabeled. Radiolabeled cell surface antigens were solubilized with Nonidet P-40, reacted with HSV-specific antiserum, and pelleted through sucrose. The immunoprecipitates were solubilized in ESS and analyzed by polyacrylamide gel electrophoresis. Figure 3 shows the autoradiogram profiles for ^{125}I-labeled antigens from wild-type- and mutant-infected cells.

Wild-type- and mutant-infected cells showed similar profiles at 34°C. At least nine precipitable viral antigens were observed. These ranged in molecular weight from 50,000 to 125,000. At 39°C, wild-type-infected cells showed reduced amounts of antigens ICP 125 and ICP 119, whereas ICP 104 and ICP 83 were not detected. In contrast, ICP 77, ICP 68, ICP 58, and ICP 56 all showed more intense labeling in the gel profiles at 39°C. As might be expected from the immune cytolysis data, the *ts*-8 profile at 39°C resembled that of wild type. The profile for the *ts*-18 infection at 39°C also resembled that of wild type, with the exception that ICP 77 was reduced to nondetectable levels. *ts*-18 infections were moderately resistant to immune lysis at 39°C compared with wild-type infections. It is not clear at this time whether reduced amounts of any particular viral antigen were responsible for this variation in the immune lysis reaction. Most strikingly, the three group E mutants

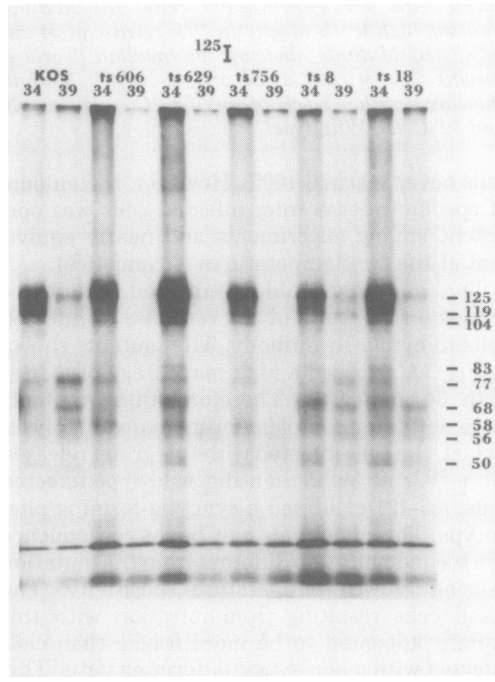

FIG. 3. *Comparison of viral radioiodinated cell surface antigens induced by wild type and ts mutants at 34 and 39°C. Infected cell surface membranes were radioiodinated by using the lactoperoxidase technique and solubilized with Nonidet P-40. The lysates were clarified by centrifugation and reacted with anti-HSV-1 strain KOS serum. Immune complexes were sedimented through 20% sucrose and solubilized with ESS. Radiolabeled viral antigens in immunoprecipitates were subjected to analytical sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis (N,N'-diallytartardiamide-cross-linked 8.5% sodium dodecyl sulfate–polyacrylamide gels) by the method of Laemmli (17) and labeled protein bands were visualized by autoradiography. Molecular weights ($\times 10^3$) are shown on the right.*

failed to express any viral peptides on the cell surface at 39°C. This finding must certainly account for the highly resistant nature of group E mutant infections to immune cytolysis, as described above.

Virus-specific glycoproteins in wild-type- and mutant-infected cells. Most surface antigens in virus-infected cells are known to be glycoproteins. Accordingly, we performed experiments to examine glycoprotein synthesis in these mutant infections.

Figure 4 shows a comparison between the ^{125}I-labeled viral surface antigen profiles and the [^{14}C]glucosamine-labeled viral glycoprotein profiles of cells infected with wild-type virus and *ts*-629 (group E). The 34°C profiles for both infections were similar. With the exception of ICP 104 and ICP 50, there were corresponding glycosylated bands for the ^{125}I-labeled viral cell surface proteins. Viral glycopeptides ICP 99 and ICP 53 did not appear in the ^{125}I-labeled viral cell surface antigen profiles, indicating that not all viral glycoprotein species were exposed at the cell surface during infection. The profile for KOS-infected cells at 39°C was similar to that at 34°C. Although showing a profile similar to the profile of KOS-infected cells at 34°C, cells infected with the group E mutant *ts*-629 produced only small amounts of glycoproteins at the nonpermissive temperature.

In similar experiments, the glycoprotein profiles of *ts*-18- and *ts*-8-infected cells were compared with those of the three group E mutants. At 34°C, the major viral glycoproteins were labeled in wild-type and all mutant infections (Fig. 5). At 39°C, however, the three group E mutant infections showed a marked reduction in the synthesis of glycoproteins. At 39°C, *ts*-8 and *ts*-18 infections showed all of the glycoprotein bands observed at the lower temperature. However, the intensity of the banding with *ts*-18 was much reduced compared with the intensities with *ts*-8 and wild type. This decrease in glycoprotein synthesis correlated with a reduction in corresponding viral cell surface antigens and reduced susceptibility to immune cytolysis.

The absence of glycoproteins in the nonpermissive infections with the group E mutants could have been due to a failure to synthesize the glycoprotein precursors or to a defect in the glycosylation of these precursors. The mannose analog 2-deoxy-D-glucose blocks glycosylation of HSV-1 proteins, resulting in an accumulation of lower-molecular-weight polypeptides and an absence of the terminal glycoprotein species (5, 16). These accumulating polypeptides are presumably precursor forms of the mature viral glycoproteins. To determine whether the group

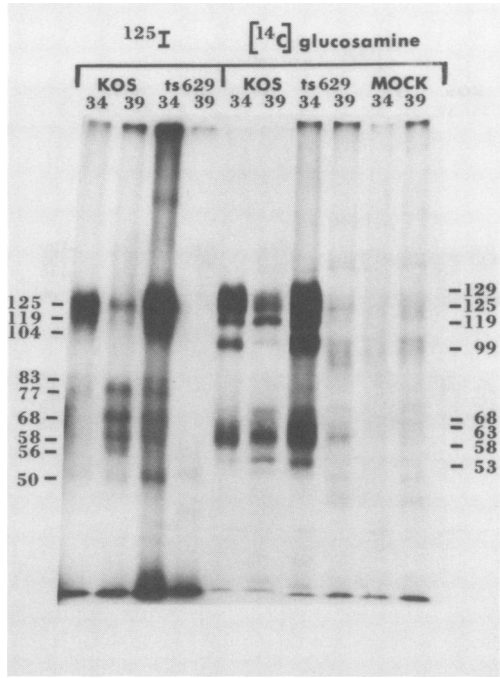

FIG. 4. *Comparison of radioiodinated viral cell surface antigens and [^{14}C]glucosamine-labeled viral glycoproteins induced by wild-type and ts mutant viruses at 34 and 39°C. Cell surface proteins were radioiodinated and analyzed as described in the legend to Fig. 3. Glycoproteins were radiolabeled with [^{14}C]glucosamine from 4 to 24 h after infection. Whole cell extracts were prepared in ESS and electrophoresed in parallel with ^{125}I-labeled proteins. ^{125}I-labeled and ^{14}C-labeled proteins separated in sodium dodecyl sulfate-polyacrylamide slab gels were visualized by fluorography. Molecular weights (×10^3) are shown on the right and left.*

E mutants were defective in glycosylation or in the production of glycoprotein precursors, mock-infected, KOS-infected, and *ts*-606-infected cells were labeled with [^{35}S]methionine in the presence and absence of 10 mM 2-deoxy-D-glucose at 34 and 39°C and examined by polyacrylamide gel electrophoresis for the synthesis of precursor and fully glycosylated polypeptides. The analog was present from 5 to 24 h after infection.

As Fig. 6 shows, 2-deoxy-D-glucose resulted in the accumulation of a 92-kilodalton (kdal) polypeptide in KOS-infected cells at both temperatures. Concomitantly, no labeled 123-kdal terminal glycoprotein was found. The 92-kdal polypeptide band was not observed with [^{35}S]methionine labeling in the absence of the analog, presumably because the precursor was glycosylated very rapidly to the heavily labeled terminal form. In the *ts*-606-infected cells, in the presence of 2-deoxy-D-glucose, label accumulated at 34°C

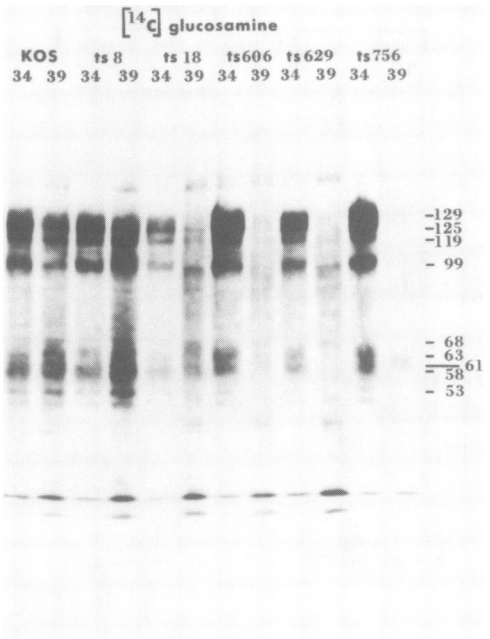

FIG. 5. *Comparison of HSV-1 glycoproteins synthesized by wild-type and ts mutant viruses during infections at 34 and 39°C. Infected cell glycoproteins were intrinsically radiolabeled with [^{14}C]glucosamine from 4 to 24 h after infection. Whole cell extracts were prepared in ESS and analyzed by sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis. Radiolabeled proteins were visualized by fluorography. Molecular weights (×10^3) are shown on the right.*

in the 92-kdal precursor, but the amount of this protein was greatly reduced at 39°C. *ts*-606-infected cells showed no precursor accumulation at 34 or 39°C in the absence of the analog. The finding that little or no 92-kdal protein accumulated at 39°C in *ts*-606-infected cells in the presence of 2-deoxy-D-glucose suggests that the complementation group E mutant lesion is not in the glycosylation of viral polypeptide precursors, but rather a failure to synthesize normal amounts of the precursors themselves at the restrictive temperature. Another interpretation of the results is that the glycoprotein precursors are indeed synthesized at 39°C but are quickly degraded and, therefore, not available for glycosylation.

Virus-specific polypeptides in wild-type- and mutant-infected cells. *ts*-8, *ts*-18, and group E mutants all have DNA-negative phenotypes on infection at 39°C (unpublished data). Previous reports have shown that herpesvirus *ts* mutants with DNA-negative phenotypes exhibit marked differences in polypeptide profiles compared with wild type during infections at a nonpermissive temperature (6, 7, 21, 24). Intrinsic labeling experiments with [^{35}S]methionine were undertaken to characterize the viral polypeptides synthesized under nonpermissive conditions by group E, *ts*-8, and *ts*-18 mutant infections. The polypeptide profiles are shown in Fig. 7. At 34°C, wild-type and all mutant infections had essentially the same protein profiles. At 39°C, *ts*-8 infections showed no appreciable differences in banding patterns compared with wild-type infections. *ts*-18 showed reduced amounts of ICP 154, ICP 134, and ICP 125. ICP 104 was not detectable, whereas the other proteins remained at wild-type levels. The group E mutant infections differed dramatically from wild-type infections in the ability to produce viral peptides at 39°C. Two viral peptides not observed in the wild-type profile were produced by group E infections. These were ICP 136 and ICP 175, and were demonstrated most strikingly in the *ts*-756 infection. Another striking feature was the overproduction of ICP 148 and ICP 134.

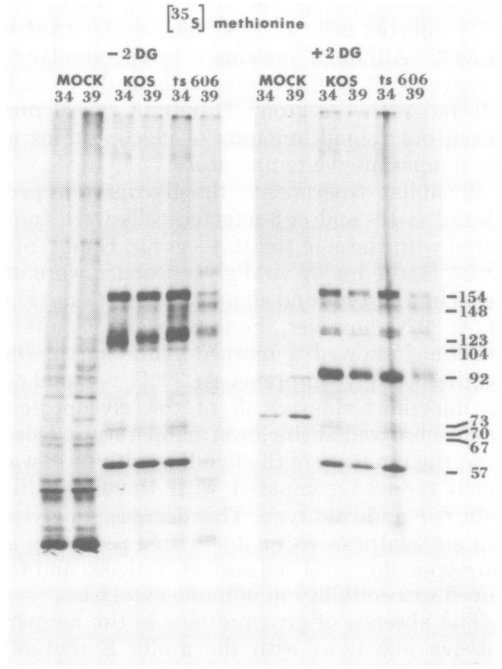

FIG. 6. *Comparison of the effect of 2-deoxy-D-glucose (2DG) at 34 and 39°C on wild type and ts-606 (group E) glycoprotein syntheses. Glycosylation of infected cell glycoprotein precursors was inhibited with 10 mM 2-deoxy-D-glucose added at 5 h after infection. Infected cell proteins were radiolabeled with [^{35}S]methionine from 4 to 24 h. Whole cell extracts were prepared in ESS and analyzed by sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis, and the proteins were visualized by fluorography. Molecular weights (×10^3) are shown on the right.*

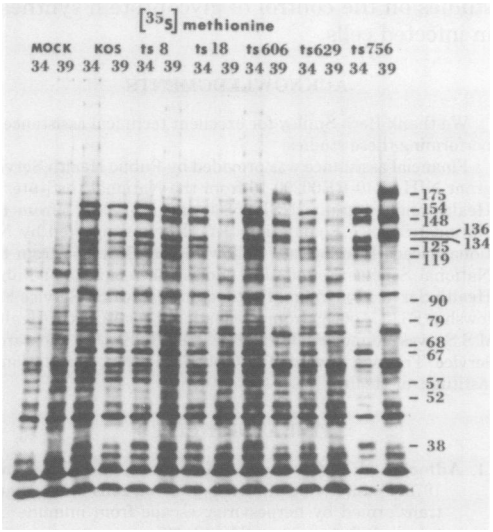

FIG. 7. *Comparison of wild-type- and ts mutant-infected cell proteins radiolabeled with [35S]methionine at 34 and 39°C. Infected cell proteins were intrinsically radiolabeled with [35S]methionine from 4 to 24 h after infection. Whole cell extracts were prepared in ESS and subjected to sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis. The molecular weights (×10³) of the proteins bands visualized by fluorography are shown on the right.*

The significance of these changes is not known, although ICP 175 overproduction has been reported in other DNA-negative *ts* mutants of HSV-1 (6, 7, 21). Previously, all of these proteins have been reported to be nonstructural proteins (6, 7, 21). The group E mutants underproduce or fail to produce all other viral peptides. These results suggest that group E mutants contain a *ts* lesion which prevents efficient production of late viral proteins.

DISCUSSION

These studies were initiated with the aim of identifying *ts* mutants of HSV-1 strain KOS that fail to induce the synthesis of viral cell surface antigens involved in complement-mediated immune cytolysis. This was accomplished by testing cells infected with representative *ts* mutants from 16 complementation groups which were available in our laboratory for susceptibility to immune lysis. It was our hope that this screening would identify mutants that either fail to produce any viral cell surface antigens under nonpermissive conditions or fail to produce one or several essential target antigens required for combining with cytolytic antibody. Although variations in immune lysis were observed, the members of only one complementation group, group E mutants, were highly resistant to immune lysis in a *ts* fashion. The characterization of viral glycoprotein synthesis and viral cell surface antigen expression revealed that group E mutants belonged to the category of immune lysis-resistant mutants that are defective for the synthesis of late viral proteins, including the cell surface glycoproteins.

Three independently arising group E mutants, *ts*-606, *ts*-629, and *ts*-756, were examined in these studies, and all behaved similarly, suggesting that the phenotype of these mutants is characteristic of the mutant gene. Cells infected with the three mutants are completely resistant to complement-mediated immune cytolysis at 39°C. The resistance is not due to the absence of a specific antigen, but rather to the absence of a whole set of antigens which are normally introduced into cell surface membranes during infection. These viral antigens cannot be detected on nonpermissively infected cell surfaces by cell surface labeling procedures followed by immunoprecipitation. The failure to synthesize cell surface antigens in group E mutant-infected cells is probably not directly related to the DNA-negative phenotype of the mutants. *ts*-8 and *ts*-18 also show DNA-negative phenotypes (unpublished data), but cells infected with these mutants synthesize normal or near normal amounts of virus proteins and show normal or near normal levels of complement-mediated cytolysis.

The precise function governed by the group E locus is not known. The defect appears not to be in the glycosylation process itself, but rather in the synthesis of the peptide precursors. Courtney (5) showed that in the presence of the mannose analog 2-deoxy-D-glucose, the major envelope glycoprotein, VP 123, disappeared, with the concomitant appearance of a 92-kdal polypeptide, the presumed nonglycosylated precursor of VP 123. During infection at 34°C with the group E *ts*-606 mutant in the presence of the analog, a 92-kdal polypeptide accumulates, whereas VP 123 is not detected. At 39°C the amount of the 92-kdal polypeptide is greatly reduced. The data suggest a defect in the synthesis of the polypeptide precursor of the VP 123 glycoprotein. Similar data on precursor synthesis for the other glycoproteins are not available in our analysis. We have not been able to label any polypeptides with [14C]glucosamine at 39°C with the group E mutants.

The group E locus has many of the characteristics expected of a regulatory gene controlling the synthesis of late proteins. At least two immediate early proteins, VP 175 and VP 136, have been observed in nonpermissive infections (6, 7, 20, 21). Indeed, both are overproduced under these conditions. Courtney and Benyesh-Mel-

nick (6) have reported overproduction of VP 175 and VP 134 in nonpermissive infections with HSV-1 KOS mutants *ts*-B2 and *ts*-B21 (22). Preston (21) has suggested that VP 175 functions at the transcriptional level for the synthesis of many of the later viral messengers.

The group E mutants described here are probably identical to mutants of HSV-1 isolated in other laboratories. The strain KOS mutant *ts*-B2 of complementation group B of Schaffer et al. (22) fails to complement the group E mutant *ts*-901 (23). Mutant *ts*-B2 also fails to complement (23) the D mutants of strain 17 (4) and the *ts*-LB2 mutant of strain HFEM (11). Finally, the latter two mutants fail to complement each other in mixed infections (15). In keeping with the new HSV-1 nomenclature proposed by Schaffer et al. (23), these mutants should be considered members of HSV-1 complementation group 1.2. Nonpermissive infections with these mutants are DNA negative, and a large number of late proteins are not observed (4, 6, 7, 11). Dixon and Schaffer (unpublished data) have confirmed our finding with their group B mutants, namely, that mutant-infected cells fail to produce viral glycoproteins. Marker rescue and intertypic recombination experiments physically map these mutations in the S region of the viral genome (15, 29, 30; D. Parris, R. Dixon, and P. Schaffer, unpublished data; R. Sandri, M. Levine, and J. C. Glorioso, unpublished data). A recent report (21) describes the characteristics of the *ts* K mutant of HSV-1 strain 17. This mutant has a phenotype similar to that of the group 1.2 mutants. Immediate early proteins are made under nonpermissive conditions, but late proteins are not. The mutation also maps in the S segment of the virus genome (29, 30).

Results from this study demonstrate that the resistance of group E mutant infections to complement-mediated immune lysis is correlated with the absence of viral cell surface antigens. To date, there has been no evidence to suggest that the presence of any one particular surface antigen is essential for complement-mediated immune lysis. It is possible that many of these antigens can participate in immune lysis reactions, provided that specific cytolytic antibodies are present (9, 10, 19). Specific lesions in viral cell surface antigen expression have been found in analyses of other *ts* mutants of strain KOS (J. C. Glorioso, T. C. Holland, and M. Levine, unpublished data). The role of these specific peptides in the immune response to HSV-1 infections is currently under investigation. The group E mutants continue to be useful in these studies as mutants representing one end of the spectrum of variants in viral cell surface antigen expression. The group E mutants will also be useful in studies on the control of glycoprotein synthesis in infected cells.

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