HERPES SIMPLEX VIRUS GLYCOPROTEINS gC-1 AND gC-2 BIND TO THE THIRD COMPONENT OF COMPLEMENT AND PROVIDE PROTECTION AGAINST COMPLEMENT-MEDIATED NEUTRALIZATION OF VIRAL INFECTIVITY

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Previous studies have demonstrated that C3b-coated erythrocytes rosette with herpes simplex virus I (HSV-I)-infected endothelial, epithelial, and fibroblastic cells (1–3). Glycoprotein C (gC-1) of HSV-1 was postulated to mediate this binding because antibodies to gC-1 inhibited this reaction (2, 4). Also, cells infected with HSV-1(MP) (a gC-1$^-$ mutant) did not exhibit C3b-dependent rosette formation, whereas cells infected with HSV-1(MP)6-9A [a derivative of HSV-1(MP) with a functional gC-1 gene insertion] did form rosettes (4, 5).

The discovery of a viral protein exhibiting C3b binding activity was an unanticipated finding. Its biologic significance has not been entirely elucidated. However, with the realization that many C3b- and C4b-binding proteins possess regulatory roles in the complement cascade and inhibit activation on autologous tissue (6), it became apparent that such a protein on the virus envelope or on a virally infected cell could inhibit complement activation. Purified gC-1 has recently been shown to possess decay-accelerating activity for the alternative pathway C3 convertase and, by this mechanism, could block the effects of complement (7). This same functional activity is possessed by two human complement-regulatory proteins, decay-accelerating factor and the C3b/C4b receptor (CR1) (6).

In previous studies, cells infected with HSV-2 as well as cytomegalovirus and varicella zoster virus failed to form rosettes with C3b-coated erythrocytes, suggesting that these viruses might not encode a C3-binding protein (2, 4). In this report we confirm by affinity chromatography prior results demonstrating that gC-1 expressed by HSV-1-infected cells is a C3 binding protein. By this same methodology we present evidence that gC-2 expressed by HSV-2-infected cells also binds to human C3. In addition, we show that the presence of gC-1 or gC-2

Studies performed in the laboratory of J. P. Atkinson were supported by National Institutes of Health Grant 5 R01-AI19642, the Monsanto Corporation, and the Lottie Caroline Hardy Trust Fund. Work done in the laboratory of P. G. Spear was supported by grants from the National Cancer Institute (CA-21776 and CA-19264) and a Marietta Klinman Memorial Grant from the American Cancer Society. Christine Odell was a trainee on NIH grant 5T32AI07182.

in virions provides protection against complement-mediated neutralization of viral infectivity.

Materials and Methods

Cells and Virus Strains. HEp-2 and Vero cells, obtained from the American Type Culture Collection (Rockville, MD), were grown in DMEM supplemented with 10% FCS and in medium 199 supplemented with 5% FCS, respectively. The virus strains used were HSV-1(HFEM), a laboratory strain of HSV-1; HSV-1(MP), a gC^- mutant (8); HSV-1(MP-6-9A, a derivative of HSV-1(MP) containing a functional gC-1 gene [from strain HSV-1(F)] inserted in its thymidine kinase gene (9); HSV-1(MP)801-1, a derivative of HSV-1(MP) containing a functional gC-2 gene [from strain HSV-2(333)] inserted into its thymidine kinase gene (10); and HSV-2 (G), a low-passage clinical isolate of HSV-2.

Preparation of Labeled Extracts from Infected Cells. HEp-2 cells ($\sim 1.5 \times 10^7$ cells/monolayer culture) were inoculated with virus at multiplicity of infections of 10–20 PFU/cell in PBS (10 mM Na_2HPO_4, 1.5 mM K_2HPO_4, 140 mM NaCl, 3 mM KCl, 0.5 mM $MgCl_2$, and 1 mM $CaCl_2$, pH 7.4) containing 0.1% glucose and 1% BSA (PBS/G/BSA)[1]. After allowing adsorption of virus for 2 h at 37°C, the inoculum was removed and replaced with medium 199 containing a radioactive precursor, either [^{35}S] methionine at 50 μCi/ml (the medium 199 was altered to contain 10% the usual concentration of methionine and supplemented with 10 mM Hepes) or [^{14}C]glucosamine at 0.5 μCi/ml. At 18–24 h after addition of virus, extracts of the infected cells were prepared. The cell monolayers were washed with cold PBS and the cells scraped into PBS. The cell pellets ($\sim 1.5 \times 10^7$ cells per pellet) were suspended in 2 ml of solubilization buffer (PBS containing 1% NP-40, aprotinin at 100 U/ml and, in some cases, 1% sodium deoxycholate or 1 mM PMSF). Nuclei were removed from the cell lysates by low-speed centrifugation, and the cytoplasmic fractions were frozen at −80°C until use.

Before the affinity chromatography or immunoprecipitation, each solubilized preparation was thawed, dialyzed at 4°C against PBS (150 mM NaCl, 10 mM K_2HPO_4, 1.5 mM NaH_2PO_4, pH 7.4) containing 1% NP-40, 2 mM PMSF, 1 μM pepstatin, 3 mM EDTA, and 2 mM iodoacetamide, and then centrifuged at 80,000 *g* in a Beckman L8-80M ultracentrifuge for 1 h at 4°C.

Affinity Chromatography. Human iC3 and iC4 were coupled to Sepharose 4B via CNBr activation at 2 mg of iC3 or iC4/ml Sepharose (11). BSA (Sigma Chemical Co., St. Louis, MO) and human IgG (fraction II; Miles Scientific, Naperville, IL) were coupled to Sepharose 4B at 10 mg/ml. Coupling efficiency was >90%. Human C3b was coupled to thiol-Sepharose as previously reported (12, 13). The protein-Sepharose conjugates were stored in borate-buffered saline (0.15 M NaCl, 0.2 M H_2BO_3, pH 8.0), 1% NP-40, 0.02% NaN_3. Before use, the Sepharose beads were washed with PBS, 1% NP-40.

Solubilized preparations of cells were preincubated with BSA- or IgG-Sepharose on a rotator for 1 h at room temperature. The supernatant was then incubated for 1 h with control (BSA or IgG) Sepharose or specific (iC3, iC4, C3b) Sepharose. The supernatant was removed and beads transferred to a polypropylene column (11 ml; Bio-Rad Laboratories, Richmond, CA) with 3 ml of a 1:3 dilution of PBS + 1% NP-40, 2 mM PMSF. The conjugated Sepharose was washed three times with 4 ml of a 1:3 dilution of PBS, 1% NP-40, and then eluted with 400 mM NaCl, 1% NP-40. The eluted material was dialyzed overnight at 4°C against water. After lyophilization and acetone precipitation, the pellet was resuspended in a reducing sample buffer for gel electrophoresis (0.25 M Tris-base, 2% SDS (wt/vol), 10% glycerine, 10^{-3}% bromphenol blue (wt/wt), 2-ME (5% vol/vol), pH 6.8) and heated at 80°C for 5 min.

Immunoprecipitation and SDS-PAGE Analysis. The antibodies used were as follows: ascitic fluid containing mAb to gC-1 (II73-1, II474-1, II475-1, II512-3, II529-1) or to gC-2 (III-188-4, III-211-1, III-596-1) were lyophilized and resuspended to original volume in PBS, pooling those antibodies specific for the same glycoprotein (14). Ascitic fluid containing mAb MOPC-21, of unknown specificity, was treated as above and used as

[1] *Abbreviation used in this paper:* PBS/G/BSA, PBS with glucose and BSA.

control. The solubilized preparations of infected cells were first precleared with *Staphylococcus aureus* protein A (Cowan I Strain; Calbiochem-Behring, La Jolla, CA). This suspension was centrifuged and the supernatant was removed and incubated with either specific or nonspecific mAb for 1 h at 4°C. The preparations were then incubated with protein A for 15 min, washed three times with 1 ml of PBS, 1% NP-40, and the bound proteins were eluted with sample buffer for electrophoresis (described above). The samples were heated at 80°C for 5 min, centrifuged, and the supernatant was analyzed by SDS-PAGE and autoradiography (15).

Neutralization of Viral Infectivity. In a volume of 2.5 ml, the reaction mixtures contained virus at 500–1,000 PFU/ml, human serum at a final dilution of 1:15 and, in some instances, a heat-inactivated rabbit antiserum containing neutralizing antibodies specific for HSV gD at a final dilution of 1:125. The diluent for all components of the reaction mixture was PBS/G/BSA. The human serum was either heated to 56°C for 30 min to inactivate complement components, or was unheated, or was a mixture of heated and unheated samples. The human serum chosen for use was shown to be free of anti-HSV antibodies by Western blot analysis and by absence of neutralizing activity in heat-inactivated samples. The reaction mixtures were prepared on ice and then incubated at 37°C for 1 h. 1-ml samples were then plated on monolayers of Vero cells in 25-cm^2 flasks to permit plaque development in a standard plaque assay.

Results

Identification of C3-binding Proteins from Extracts of HSV-1- and HSV-2-Infected Cells. Although published evidence clearly demonstrates that gC-1 is required for the C3b binding activity of HSV-1-infected cells, the binding of solubilized gC-1 to C3b or iC3 has not yet been reported. Our first series of experiments was designed to determine whether gC-1 could bind to iC3-Sepharose columns. Fig. 1 shows that two polypeptides solubilized from HSV-1(HFEM)-infected cells bound to the column. These polypeptides could be labeled with either [^{35}S]-methionine or [^{14}C]glucosamine, and their M_r (110,000–130,000 and 90,000–95,000) are similar to those of the mature and precursor forms, respectively, of gC-1 (16, 17). Two lines of evidence demonstrate that the polypeptides bound to iC3-Sepharose are products of the gC-1 gene. First, the gC$^-$ mutant HSV-1(MP) failed to produce either polypeptide, whereas HSV-1(MP)6-9A produced both (Fig. 1). The latter virus strain is a derivative of HSV-1(MP) containing an insertion of a functional gC-1 gene (9). Moreover, in experiments in which the extract was first exposed to iC3-Sepharose and then immunoprecipitated with antibodies to gC-1, or vice versa, the quantity of the peptides specifically recovered in the second step was markedly depleted (Fig. 1). The mature forms of gC-1 are heterogeneous in M_r, due in part to heterogeneity in sialation (17, 18). It is of interest that, in comparison with the forms of gC-1 in immunoprecipitates, the iC3-binding material is relatively enriched for the precursor form and the faster-migrating, least-sialated mature forms of gC-1.

Similar experiments were done with extracts from HSV-2(G)-infected cells. As shown in Figs. 2 and 3, a glycopeptide of M_r 75,000–85,000 bound to iC3-Sepharose but not to IgG- or iC4-Sepharose. No iC3-binding material was isolated from mock-infected cells. The HSV-2 glycoprotein that is antigenically and evolutionarily related to gC-1 is designated gC-2 and has an M_r similar to that of the iC3-binding glycopeptide (19). Moreover, immunoprecipitation of HSV-2-infected cell extracts with anti-gC-2, but not anti-gC-1 or MOPC, removed the iC3-binding glycopeptide (Figs. 3 and 4). Finally, the insertion mutant HSV-

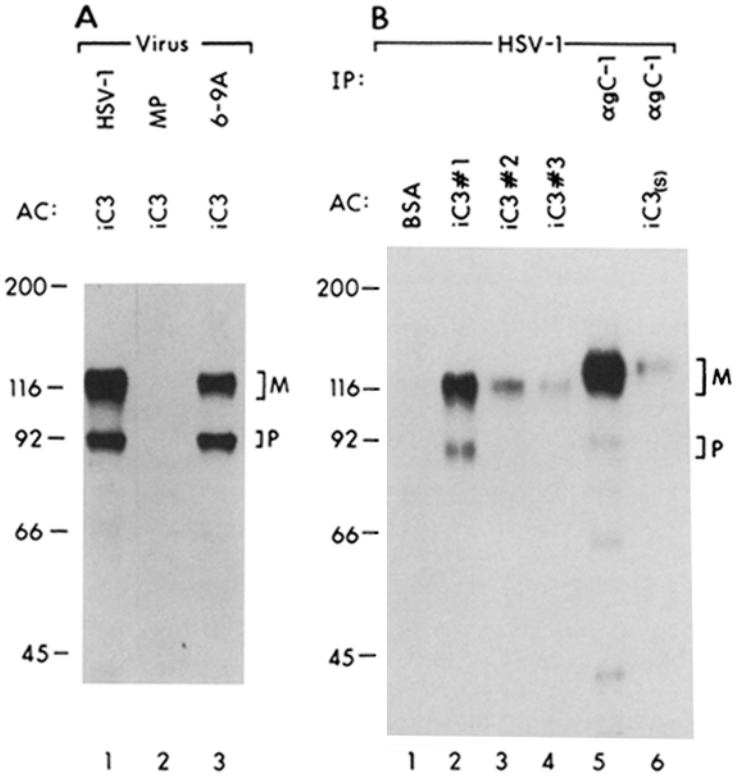

FIGURE 1. Autoradiographs of proteins isolated by iC3-affinity chromatography or immunoprecipitation from extracts of HSV-1-infected HEp-2 cells. *A*. Comparison of three HSV-1 strains [HSV-1(HFEM), HSV-1(MP) (gC-1$^-$ mutant) or HSV-1(MP)6-9A (gC-1 recombinant)]. *B*. Comparison of proteins isolated by affinity chromatography or immunoprecipitation. Lane *5* represents the proteins isolated by immunoprecipitation with antibodies to gC-1. In lanes *2–4*, the preparation was subjected to sequential affinity chromatography. The last supernatant was then immunoprecipitated with antibodies to gC-1 [^{35}S]methionine (lane *6*). *AC*, affinity chromatography; *IP*, immunoprecipitation; *M*, mature form of gC-1; *P*, precursor form of gC-1; *αgC-1*, mAb to gC-1.

1(MP)801-1 produces iC3-binding material of the appropriate M_r. This mutant is a derivative of HSV-1(MP) containing an insertion of a functional gC-2 gene.

For viral extracts containing gC-1 or gC-2, affinity chromatography with C3b-thiol-Sepharose produced identical results to that with iC3 (not shown).

gC Expression and Complement-mediated Neutralization. To determine whether presence of gC-1 or gC-2 in the virus might influence complement-mediated neutralization of infectivity, neutralization studies were done with the gC$^-$ mutant HSV-1(MP) and the recombinants expressing gC-1 or gC-2 [HSV-1(MP)6-9A and HSV-1(MP)801-1]. This particular set of viruses was chosen because all virion components except gC are identical for the three viruses, and because the two recombinants produce comparable quantities of gC-1 and gC-2. These isogenic viruses, differing only in their expression of gC, were incubated with human serum from a seronegative individual in the presence or absence of rabbit anti-HSV antibodies. Various proportions of heated and unheated human serum were present in each reaction mixture such that concentrations of active comple-

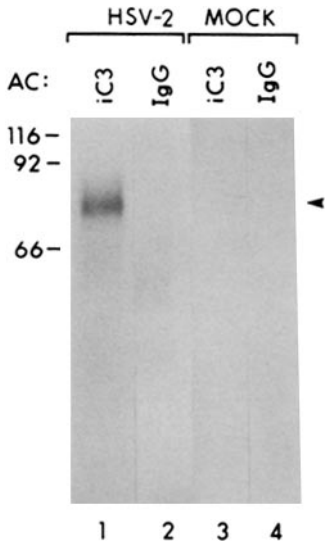

FIGURE 2. Autoradiograph of proteins isolated by affinity chromatography from extracts HSV-2- or mock-infected HEp-2 cells incubated with iC3- or IgG-Sepharose, labeled with [^{14}C]-glucosamine. *Arrowhead,* gC-2.

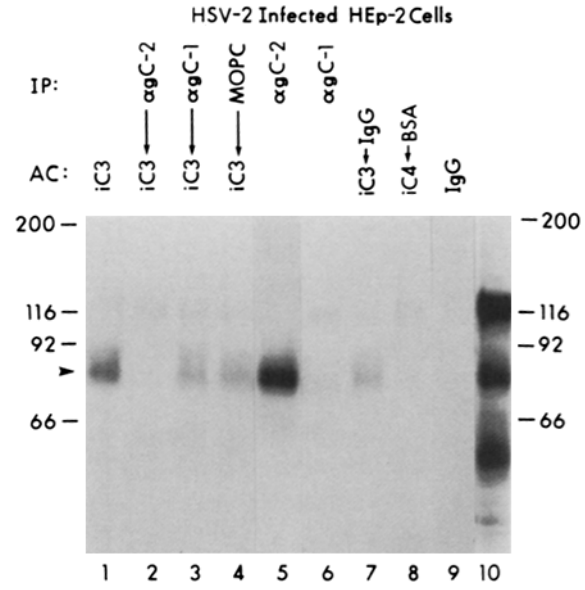

FIGURE 3. Autoradiograph of proteins isolated by affinity chromatography or immunoprecipitation from extracts of HSV-2-infected HEp-2 cells. The samples in lanes *2–4* were first subjected to immunoprecipitation before being incubated with iC3-Sepharose. In lanes *7* and *8*, the solubilized preparation was first preincubated with IgG- or BSA-Sepharose before being incubated with iC3- or iC4-Sepharose. In lane *10* the solubilized preparation was heated in sample buffer and applied to the gel to demonstrate the major radiolabeled bands, labeled with [^{14}C]glucosamine. *αgC-2, αgC-1,* and *MOPC,* mAb to gC-1, gC-2, and of unknown specificity, respectively. *Arrowhead,* gC-2. Note that antibodies to gC-2 specifically remove the iC3-binding protein.

ment components differed while concentrations of total serum protein remained constant. Fig. 5*A* shows that human complement enhanced the neutralization mediated by anti-HSV antibodies in a dose-dependent fashion, but only for the gC$^-$ mutant HSV-1(MP). The recombinant viruses expressing gC-1 or gC-2 appeared to be protected from the neutralizing effects of complement. Similar results were obtained when the three virus strains were incubated with human serum in the absence of anti-HSV antibodies (Fig. 5*B*), suggesting that there is also antibody-independent neutralization by complement.

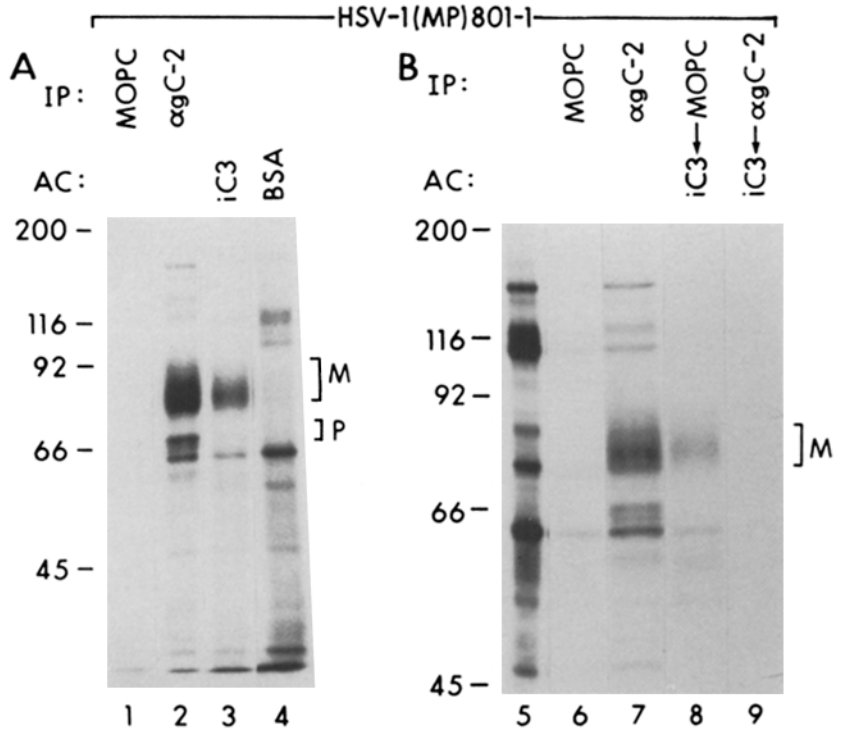

FIGURE 4. Autoradiograph of proteins isolated by affinity chromatography or immunoprecipitation from extracts of HSV-1(MP)801-1-infected HEp-2 cells. *A*. Comparison of the proteins isolated by the two procedures. *B*. Samples in lanes *8* and *9* were first subjected to immunoprecipitation, and then the supernatants were incubated with iC3-Sepharose. In lane *5*, the solubilized preparation was heated in sample buffer to demonstrate the major radiolabeled bands. Definitions are identical to those of Figs. 1 and 3. Note that the bands isolated by either procedure align. Label is [^{35}S]methionine.

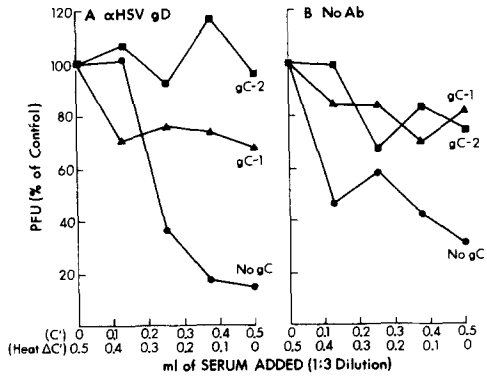

FIGURE 5. Effects of human complement on HSV infectivity. Unheated and heated samples of human serum (diluted 1:3) were mixed in the proportions indicated for addition to the neutralization reaction mixtures as described in the text. Anti-HSV neutralizing antibodies were present(*A*) or absent (*B*). The values presented are the average of duplicate determinations (the values taken as 100% range from 52 to 533). The concentration of anti-HSV serum used in *A* was chosen so that the number of PFUs obtained in the presence of anti-HSV serum plus heated human serum was 20–50% of the number obtained in the presence of heated human serum alone. The virus strains used were the gC$^-$ mutant HSV-1(MP) or recombinants derived from HSV-1(MP) by insertion of a functional gC-1 or gC-2 gene. This study is representative of six similar experiments.

Discussion

Several prior reports have established that C3b-coated red blood cells form rosettes with HSV-1-infected cells (1–5). In those reports, multiple cell lines were used, and C3b-dependent rosette formation correlated with the expression of gC-1. Pretreatment of these cells with mAb to gC-1 inhibited this reaction. Moreover, purified gC-1 exhibited decay-accelerating activity for the C3 convertase of the alternative pathway (7).

The use of iC3 affinity chromatography has contributed some additional insights into the nature of this interaction between activation fragments of human C3 and gC-1. First, this work provides further evidence that gC of HSV-1 is a C3-binding protein. iC3 and C3b are functionally equivalent activation fragments of C3 (20, 21), and we have obtained binding to both. In addition, both the mature and precursor forms of gC-1 bind to iC3-Sepharose. In fact, the precursor form and less sialylated mature forms may bind preferentially. The gC-1 precursor has *N*-linked sugars of a high mannose type, and in the mature form these are converted to complex carbohydrate moieties in the Golgi (18, 22). Also, *O*-linked sugars are attached to gC-1 as the protein moves through the Golgi (18, 23, 24). These data suggest that the attachment of the *O*-linked sugars or conversion of high mannose to complex oligosaccharides alters the binding of gC-1 to iC3. Of interest in this regard is that pretreatment of HSV-1-infected cells with neuraminidase increases their ability to form rosettes (3, 5). These data are also of note relative to human CR1, a C3b/C4b complement receptor and regulatory protein that may be antigenically (3) as well as functionally related to gC-1. Carbohydrate has been shown to be important to the stability, transport, and binding efficiency of human CR1 (25).

A new finding in this report is that HSV-2, as well as HSV-1, expresses a C3b binding glycoprotein, despite previous indications that HSV-2 might not exhibit this activity (4, 5). In the studies herein, gC-2 from two viral strains, a low-passage clinical isolate [HSV-2(G)] and gC-2, derived from a laboratory strain [HSV-2(333)] both bound to iC3-Sepharose. The genes for gC-1 and gC-2 occupy colinear positions on their respective viral genomes (10) and are homologous in sequence (26, 27). The glycoproteins specified by these genes are quite different in size, however, in part because gC-1 contains a stretch of 27 amino acids near its NH_2-terminus that are not present in gC-2 (26, 27). Moreover, the number of antigenic determinants shared by the two glycoproteins seems to be limited in comparison with the unique antigenic determinants (14, 19, 28, 29). Although these two homologous glycoproteins have diverged with time more than some of the other homologous HSV-1 and HSV-2 glycoproteins, it appears that they retain a common affinity for C3b and very likely also retain common activities associated with this binding activity. Comparisons of the deduced amino acid sequences for gC-1 and gC-2 reveal the presence of highly conserved regions. It remains to be determined whether the C3b-binding domain can be mapped to any of these conserved domains. To further investigate this issue, the deduced protein sequences of gC-1 and gC-2 have been compared with the sequences of members of the human C3b/C4b binding protein family (6). The comparisons indicate the presence of numerous homologous regions between gC-1, gC-2, and the human proteins (V. M. Holers, unpublished data). These results suggest an

evolutionary relationship between viral glycoprotein C and this family. Experiments are currently underway to determine whether these specific peptide sequences are functionally relevant to the binding of gC-1 or gC-2 to C3.

It is unclear why cells infected with HSV-1 but not with HSV-2 express C3b-binding activity on their surfaces as detected by the rosette assay. Considering their amino acid homology, and in view of this study that gC-2 can bind to C3, one would expect HSV-2-infected cells to rosette with C3-coated erythrocytes. Several factors that affect rosette formation, such as surface density of receptors, membrane mobility, and cytoskeletal interactions may not be optimal for HSV-2-infected cells. For example, HSV-2 produces less gC-2 in cultures of cells in comparison with amounts produced by the recombinant HSV-1(MP)801-1 (10) or amounts of gC-1 produced by HSV-1 (P. Spear, unpublished data). One consequence may be reduced surface density of receptors in cultured cells infected with HSV-2. It is also possible that differences in glycosylation between gC-1 and gC-2 may alter tertiary structure and may in part be responsible for the failure of HSV-2-infected cells to rosette with C3b-coated erythrocytes. Pretreatment with neuraminidase did not enhance rosetting by HSV-2-infected cells (5), but possibly further manipulation of the carbohydrate moieties would result in successful rosetting studies. This study demonstrates that the apparent absence of C3b-binding activity on the surfaces of HSV-2-infected cells cannot be explained by the failure of HSV-2 to express a C3b-binding glycoprotein.

A second observation of interest in our studies concerns the protection against complement-mediated viral neutralization by gC-1 and gC-2. In these neutralization assays, mutant [HSV-1(MP)] and recombinant strains [HSV-1(MP)6-9A and HSV-1(MP)801-1] were compared with respect to sensitivity to neutralization by complement in the presence or absence of anti-HSV antibody. The use of these recombinant strains allowed direct assessment of the effects of gC-1 or gC-2 expression against the common genetic background of the gC^- viral mutant. In this study, as outlined in Fig. 5, the polyclonal anti-gD serum caused some neutralization in the absence of a complement source, and was used at a concentration that neutralized ~50% of input virus. These studies suggest that gC-1 and gC-2 provide protection against complement-mediated viral neutralization in the presence of antibody. Also, in the absence of antiviral antibody, fresh but not heated serum enhanced neutralization to a greater extent for the gC^- mutant compared with the $gC\text{-}1^+$ and $gC\text{-}2^+$ strains.

In the experimental system without antibody, the alternative complement pathway would be operative, and is likely to be responsible for viral neutralization. The protection provided by gC-1 and gC-2 could be accounted for by the observation that they promote decay acceleration of the alternative pathway convertase, as has been directly shown for gC-1 (7). The alternative pathway convertase is a bimolecular enzyme complex that cleaves C3 to C3b, and C3b is the major opsonic fragment of the complement system. Cleavage of C3 is also necessary to initiate the lytic complex. By preventing the formation of and accelerating the decay of already-formed complexes, gC-1, and by analogy gC-2, could inhibit neutralization of the virus by the alternative complement pathway. The alternative complement pathway is one of the host's primary means of controlling infections before and during the development of immunity (30), and

the data suggest that gC-1 and gC-2 impede immunosurveillance of this system by interfering with the complement cascade at the critical convertase step of the alternative pathway. Complement enhancement of antibody-mediated neutralization was also less apparent for the gC-1^+ and gC-2^+ strains than for the gC^- strain. In this system both the classical and alternative pathway could be important in viral neutralization. It is not clear whether the decay acceleration of the alternative pathway C3 convertase is entirely responsible for this effect or whether other effects also result from the C3-binding activity of gC-1 and gC-2.

In summary, the viral synthesis of C3b-binding glycoproteins such as gC-1 and gC-2 represents one of several mechanisms by which the herpes simplex viruses may evade the immune system. Both the viruses and virally infected cells are coated with these proteins, which are analogous in function and possibly structure to complement-regulatory proteins that protect host tissue from complement-mediated damage (6). Manipulation of the host's immune system should result in a survival advantage for the virus. Similar means to abrogate the activation of complement in the host may have evolved in other microorganisms as an adaptation to enhance infectivity.

Summary

Cells infected with herpes simplex virus type 1 (HSV-1) form rosettes with C3b-coated erythrocytes, whereas cells infected with herpes simplex virus type 2 (HSV-2) or other herpes viruses do not. It was reported that glycoprotein C of HSV-1 (gC-1) mediates the binding of C3b-coated erythrocytes to infected cells and has regulatory (decay-accelerating) activity for the alternative pathway C3 convertase of human complement. We show here that solubilized gC-1 binds to iC3-Sepharose affinity columns. We also report that solubilized gC-2, the genetically related glycoprotein specified by HSV-2, binds to iC3-Sepharose. mAb specific for gC-1 or gC-2 and mutant viral strains were used to identify the C3-binding glycoproteins. In other experiments, HSV-1 mutant strains and recombinants, differing only in their expression of gC, were tested for sensitivity to neutralization by human complement in the presence or absence of antibodies specific for HSV gD. In either case the gC^- strain was most sensitive. Expression of gC-1 or gC-2 by isogenic insertion mutants provided protection against complement-mediated neutralization. These results indicate that the genetically and structurally related gC-1 and gC-2 share the functional activity of binding to human C3 and enhance viral infectivity.

We thank Ms. Pat Stewart and Mrs. Lorraine Whiteley for their excellent secretarial efforts.

Received for publication 11 June 1987 and in revised form 4 August 1987.

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