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Immune Evasion Properties of Herpes Simplex Virus Type 1 Glycoprotein gC

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Herpes simplex virus type 1 (HSV-1) glycoprotein gC binds complement component C3b, and purified gC inhibits complement activation. Two HSV strains carrying mutations in the gC gene which rendered them unable to bind C3b were compared with wild-type and marker-rescued viruses to evaluate the role of gC on the virion in protecting HSV-1 from complement-mediated neutralization. The gC mutant viruses were markedly susceptible to neutralization by nonimmune human serum, showing up to a 5,000-fold decline in titer after 1 h of incubation with serum. In contrast, wild-type or marker-rescued viruses showed a twofold reduction in titer. Studies with hypogammaglobulinemic and immunoglobulin G-depleted serum supported the observation that neutralization occurred in the absence of antibody. Neutralization of gC mutant strains by nonimmune serum was rapid; their half-life was 2 to 2.5 min, compared with 1 h for wild-type virus. Ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA)-treated human serum or C4-deficient guinea pig serum failed to neutralize gC mutant strains, indicating a role for components of the classical complement pathway. gC had little additional effect on neutralization by the combination of antibody plus complement compared with complement alone. The results indicate that the magnitude of the protection offered by gC-1 is larger than previously recognized; that in the absence of gC-1, complement neutralization is rapid and is mediated by components of the classical complement pathway; and that gC mainly protects against antibody-independent complement neutralization, suggesting a probable role for gC early in infection, before antibodies develop.

Viruses use a variety of strategies to evade immune attack. Certain RNA viruses, including influenza virus, lymphocytic choriomeningitis virus (LCMV), and human immunodeficiency virus, escape humoral or cellular immunity by mutating antigens that are immune targets (18, 43, 44). A strategy particularly common among DNA viruses, which are less capable of frequent and rapid mutations, is to synthesize proteins that modify host immune attack. Adenovirus proteins encoded by the E3 and E1B regions protect infected cells from cytolysis by tumor necrosis factor, a macrophage cytokine (20). The E3 region of adenovirus encodes a 19-kDa protein that binds class I major histocompatibility complex (MHC) antigens, thereby preventing MHC-I expression at the cell surface and subsequent lysis by cytotoxic T cells (20). Vaccinia virus secretes a 35-kDa protein, VCP, that binds complement component C4b and inhibits classical complement pathway activation (34). Deletion of the VCP gene decreases vaccinia virus virulence in rabbits (31). Vaccinia virus also secretes a protein encoded by the B15R open reading frame that binds interleukin-1β (IL-1β), blocking binding to its receptors on T cells (2, 50). Herpesviruses encode proteins that interact with immune mediators. Epstein-Barr virus (EBV) encodes the BCRF1 gene, the product of which has sequence homology with IL-10 and mimics IL-10 activity by inhibiting synthesis of gamma interferon by T helper cells, an effect that hinders immune clearance of virus (27, 40). Herpesvirus saimiri encodes a complement control protein homolog that has sequence similarity to complement-regulatory proteins, particularly decay-accelerating factor (CD55) and membrane cofactor protein (CD46) (1). In addition, herpesvirus saimiri encodes a CD59-like protein that inhibits complement lysis and is postulated to function by a mechanism similar to that of CD59, which regulates activation of C9 (46). Herpes simplex virus (HSV) immediate-early protein ICP47 blocks the TAP (transporter associated with antigen processing) system, preventing association of HSV peptides with MHC class I molecules and inhibiting lysis of infected cells by cytotoxic $CD8^+$ T cells (16, 26, 55). HSV-1, HSV-2, and varicella-zoster virus (VZV) encode glycoproteins that serve as receptors for the Fc domain of immunoglobulin G (IgG) (3, 9, 32, 33, 36, 42). The HSV-1 Fc receptor is formed by gE and gI; the former has sequence homology with mammalian Fc receptors (7). This receptor binds nonimmune and immune IgG, the latter by bipolar bridging, i.e., antibody binding by its Fab end to HSV antigen and by its Fc end to the viral Fc receptor (13). Bipolar bridging of antibody modifies effector functions mediated by the Fc region, including antibody-dependent cellular cytotoxicity, binding of C1q, and antibody-mediated complement-enhanced virus neutralization (8, 9, 13). HSV-1 and -2, pseudorabies virus, bovine herpesvirus 1, and equine herpesvirus 1 bind complement component C3b, a function mapped to gC of HSV-1 and -2 or to gC homologs of pseudorabies virus and bovine herpesvirus 1 (4, 12, 14, 28, 35). gC-1 protects HSV-1-infected cells from lysis by antibody plus complement; however, the most striking protection is against lysis mediated by complement alone (22).

Considerable information has been reported on the role of HSV gC-1 in protecting cell-free virus from complement-mediated neutralization, yet important questions remain (17, 22, 25, 39). These include the magnitude of the protection pro-

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vided by gC-1, the kinetics of complement-mediated neutralization, the complement pathway involved in neutralization, and the role of complement alone versus antibody plus complement in neutralization. To address these issues, we performed neutralization assays using complement alone or antibody plus complement and compared wild-type virus with HSV strains carrying mutations in the gC gene as well as marker-rescued viruses. The gC mutant strains were unable to bind C3b and were noted to be ≥5,000-fold more susceptible to complement neutralization than wild-type virus, which represents several orders of magnitude greater protection by gC-1 against complement neutralization than previously reported (25, 39). Neutralization of gC mutant strains by nonimmune human serum was rapid, the half-life, or time to 50% neutralization ($t_{1/2}$), was 2 to 2.5 min, compared with 1 h for wild-type virus, and neutralization required components of the classical complement pathway. The protection provided by gC-1 against antibody plus complement was similar to that against complement alone, indicating that gC-1 is likely to be important early in infection, before antibodies develop.

MATERIALS AND METHODS

Cells and virus pools. HSV pools were prepared in Vero or HEp-2 cells grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cells were infected at a multiplicity of infection of 5, and supernatant fluids were harvested 20 to 24 h postinfection. Supernatants were clarified by centrifugation at 10,000 × *g* for 20 min, and then virus was pelleted at 100,000 × *g* for 90 min, resuspended in 0.5 ml of phosphate-buffered saline (PBS), placed on a 5-ml 5 to 70% sucrose gradient, and centrifuged at 150,000 × *g* for 140 min. Fractions of 0.5 ml were collected, and those containing peak titers were pooled, dialyzed against PBS at 4°C, aliquoted, and stored at −70°C.

Neutralization assay. Antibody-independent complement neutralization was performed by incubating equal volumes of purified virus with nonimmune human serum as source of complement for 1 h at 37°C. For some experiments, additional complement was added to the virus-serum mix at 20 min. The neutralizing effects of complement were determined by preparing serial 10-fold dilutions of the virus-serum mix, adding these to duplicate wells of Vero cells for 1 h at 37°C, washing the cells to remove unbound virus, and overlaying them with 0.5% agarose. After 72 h, 0.06% neutral red was added, and plaques were counted the following day (22). Results are expressed as geometric mean titers ($\log_{10}$) ± standard error (SE). Antibody-plus-complement neutralization was performed by incubating virus with various dilutions of pooled human IgG (Sigma Chemical Co., St. Louis, Mo.) and nonimmune human serum as a source of complement. After 1 h at 37°C, cells were infected and virus titers were determined as described above (22).

Complement reagents. To inactivate classical and alternative complement pathways, serum was treated with 10 mM EDTA; to inactivate the classical complement pathway only, serum was treated with 8 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) and 2 mM Mg^{2+} (22). Herpesvirus-nonimmune sera were obtained from four healthy adult volunteers. It was critical for our studies to use sera that did not contain antibodies to herpesvirus; therefore, the nonimmune status was determined by several assays: (i) neutralizing antibody assays that demonstrated no loss of titer when virus was incubated with EDTA-treated serum compared with PBS; (ii) HSV enzyme-linked immunosorbent assay (ELISA); and (iii) HSV-1 and -2 Western blot (immunoblot) analysis. These studies were performed in the Clinical Virology Laboratory at Children's Hospital of Philadelphia. One additional nonimmune serum was obtained from a 31-month-old child who was a candidate for bone marrow transplantation at Children's Hospital of Philadelphia. The serum was negative for antibodies to HSV-1 and -2, cytomegalovirus (CMV), and VZV by ELISA and to EBV by immunofluorescence. The following titers for EBV were obtained: viral capsid antigen IgG, <1:40; early antigen, diffuse, <1:10; early antigen, restricted, <1:10; and EBV nuclear antigen, <1:2. Hypogammaglobulinemic human serum was obtained from a 27-year-old woman who was free of infection and not receiving immunoglobulin therapy. Serum had the following immunoglobulin concentrations: IgG, 46 mg/dl (normal, 800 to 1,500 mg/dl); IgA, 4 mg/dl (normal, 90 to 325 ml/dl); and IgM, 33 mg/dl (normal, 45 to 150 mg/dl). IgG-depleted human serum was prepared by passing EDTA-treated nonimmune serum over a protein G column as instructed by the manufacturer (Pharmacia Biotech, Uppsala, Sweden). The IgG purification was repeated twice, resulting in removal of >95% IgG. Prior to use in neutralization assays, the IgG was dialyzed against PBS to remove EDTA. C4-deficient guinea pig serum was purchased from Sigma, as was purified human C4, which was used to reconstitute the C4-depleted serum. The total hemolytic complement activity (50% hemolytic complement units) of sera used for neutralization assays was measured by incubating serial twofold dilutions of serum with antibody-coated sheep erythrocytes as instructed by the manufacturer (Sigma). This assay was used to adjust the dilution of hypogammaglobulinemic, IgG-depleted, or nonimmune human serum used in the complement neutralization assay so that similar levels of total hemolytic complement activity were being compared.

Purified IgG. Nonimmune IgG was purified from human serum by DEAE-Affi-Gel blue chromatography as instructed by the manufacturer (Bio-Rad Laboratories, Hercules, Calif.). Peak fractions at an optical density at 280 nm were combined, dialyzed against PBS at 4°C, concentrated to 20 mg/ml, and stored in aliquots at −20°C.

Preparation and characterization of recombinant viruses. Mutants and rescued viruses were derived from HSV-1 strain NS, a low-passage-number clinical isolate (14). NS-gC_{null} has the entire gC-1 protein-coding sequences replaced by the *lacZ* gene under the control of ICP6 regulatory signals. The ICP6::*lacZ* cassette was obtained from pD6p (19). To recombine the cassette back into virus, a 5′ and 3′ gC-1 flanking sequence vector was prepared. *Sal*I-*Bam*HI was excised from the *Bam*HI I fragment of NS DNA (sequence numbers 94853 to 98250; GenBank accession numbers X14112, D00317, D00374, and S40493) and cloned into the *Sal*I and *Bam*HI sites of pSP64 (15). *Nru*I and *Sau*I were used to excise sequences extending from 20 bp 5′ of the gC-1 protein start codon to 100 bp 3′ of the stop codon (sequence numbers 96288 to 97943). The flanking sequence vector was blunt ended, and the ICP6::*lacZ* cassette was cloned between the 5′ and 3′ gC-1 sequences. This created a gC-1 flanking sequence plasmid containing ICP6::*lacZ* flanked by 1,432 bp of NS DNA on the 5′ end and 308 bp on the 3′ end. Recombinant NS-gC_{null} virus was prepared by cotransfecting Vero cells with NS DNA and the *lacZ*-containing flanking sequence plasmid. Blue plaques were selected and twice purified, and then virus pools were prepared (19).

Strain ns-1 is a monoclonal antibody-resistant (*mar*) mutant that has been previously described (22). It is a gC-1 mutant of NS that escaped neutralization when grown in the presence of anti-gC-1 monoclonal antibody 1C8 and rabbit serum as a source of complement. Our previous studies demonstrated that ns-1 gC protein is smaller than the wild type, with precursor and processed forms of 50 to 60 kDa, compared with 100 to 120 kDa for wild-type gC-1. ns-1 gC protein is detected within cells and is not expressed at the cell surface (22). ns-1 gC DNA sequence analysis was performed by using PCR to amplify sequences extending from 38 bp 5′ of the start codon to 20 bp 5′ of the stop codon. The sequences of the 5′ and 3′ PCR primers were 5′-AACGCTAGCCGATCCCTC-3′ and 5′-ACTGTGATGTGCGGACGA-3′, respectively. The amplified PCR fragment was cloned into the TA cloning vector (Invitrogen Corp., San Diego, Calif.), and the entire fragment was sequenced by using a panel of primers located at various positions along the gene.

Rescued viruses rns-1 and rNS-gC_{null} were constructed by using the entire *Bam*HI I fragment of NS to rescue the gC-1 defects in ns-1 and NS-gC_{null}. Marker-rescued viruses were selected by using rabbit polyclonal anti-gC-1 antibody (rabbit 47) in an immunoperoxidase assay (29). Virus was twice purified by limiting dilutions, and pools were prepared as described above.

Western and Southern blots. For gC-1 Western blots, 10^6 PFU of sucrose gradient-purified virus was loaded per lane, electrophoresed on sodium dodecyl sulfate (SDS)–10% polyacrylamide gels, transferred to Immobilon polyvinylidene difluoride membranes (Millipore Corp., Bedford, Mass.), and probed with anti-gC-1 antibody Bac-15, which was prepared by immunizing rabbits with baculovirus-expressed truncated gC-1 protein (47, 54). Southern blotting was performed on *Bam*HI digests of viral DNA that were probed with biotin-labeled gC-1 DNA sequences obtained as an *Hin*dIII digest of pSH140 or with biotin-labeled *lacZ* sequences obtained by *Bam*HI digestion of pBAG (30, 45). Chemiluminescence was used for detection.

C3b rosettes. Vero cells were infected at a multiplicity of infection of 1 with wild-type, gC mutant, or marker-rescued virus. After 20 h, cells were removed with cell dissociation buffer (Gibco BRL, Gaithersburg, Md.), treated with 0.08 U of type X *Clostridium perfringens* neuraminidase (Sigma) per ml for 30 min at 37°C, incubated with C3b-coated sheep erythrocytes for 2 h at 37°C, and observed for rosetting, defined as ≥4 erythrocytes attached per cell (14, 51).

RESULTS

Phenotypic and genotypic characterization of mutants and rescued viruses derived from HSV-1 strain NS. Each of the viruses used in this study was characterized as follows.

(i) Western blotting using purified virus to detect gC-1. HSV-1 strains were purified by sucrose gradient centrifugation. Equal titers (10^6 PFU per lane) were analyzed by Western blotting for the presence of gC-1. Wild-type NS and rescued virus strains rns-1 and rNS-gC_{null} contained full-length gC-1, while ns-1 and NS-gC_{null} did not contain gC-1 (Fig. 1; Table 1).

(ii) Southern blotting to detect HSV-1 gC DNA. HSV-1 DNA was purified from infected cells, digested with *Bam*HI, and evaluated for gC-1, using a 1.6-kb probe that spans the entire gC-1 protein-coding sequence. gC-1 DNA was detected in NS, ns-1, and rescued strains rns-1 and rNS-gC_{null} but not

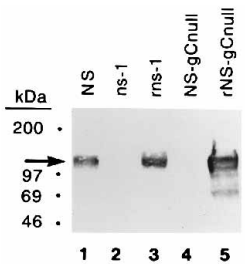

FIG. 1. Western blot to detect gC-1. Approximately 10^6 PFU of sucrose gradient-purified HSV NS (lane 1), ns-1 (lane 2), rns-1 (lane 3), NS-gC_{null} (lane 4), and rNS-gC_{null} (lane 5) was electrophoresed on an SDS–10% gel, transferred to a nylon membrane, and probed with anti-gC-1 antibody Bac-15. gC-1 protein of the expected size was detected on the virions of NS, rns-1, and rNS-gC_{null}, while gC-1 was not detected on ns-1 or NS-gC_{null}. Lane 5 was overloaded, which explains the additional bands. Molecular masses are indicated on the left, and an arrow indicates the position of gC-1.

in NS-gC_{null}. When NS-gC_{null} was probed to detect *lacZ* sequences, we detected the expected 4.6-kb band, which was absent in the rescued strain rNS-gC_{null} (not shown).

(iii) DNA sequence analysis of gC-1 mutant ns-1. The results presented above indicate that ns-1 contains full-length gC-1 DNA but does not express gC-1 on the virion. To determine the DNA sequence, ns-1 gC DNA was amplified by PCR. DNA sequencing revealed the insertion of a single cytosine after codon 165, resulting in a frameshift mutation. A new stop codon appeared after codon 289. This results in a smaller protein than NS gC, which by comparison ends at amino acid 511. In addition, the mutant form contains 124 amino acids at the C terminus which are not found in wild-type gC-1. Thus ns-1 encodes an altered intracellular protein that is not expressed on the virion envelope or at the infected cell surface but, as previously reported, is detected in the cytoplasm of infected cells (Table 1) (22).

(iv) C3b rosetting assays. Vero cells were infected with the various HSV-1 strains, and C3b binding was measured by a rosetting assay. Cells infected with NS, rns-1, and rNS-gC_{null} formed rosettes, while cells infected with ns-1 and NS-gC_{null} did not (Table 1), indicating the inability of the gC mutants to bind C3b, while wild-type and marker-rescued strains were intact for this function.

gC-1 protects HSV from complement neutralization by nonimmune human serum. Previously we showed that gC-1 protects HSV-1 from antibody-plus-complement neutralization. To evaluate the role of gC-1 in protecting virus from antibody-independent complement neutralization, purified NS, gC mutant, or marker-rescued virus was incubated for 1 h at 37°C with nonimmune human serum as the source of complement. As controls, viruses were incubated with PBS or with the same serum treated with 10 mM EDTA to inactivate classical and alternative complement pathways. When NS was incubated with active serum (untreated), there was a twofold (0.3 $\log_{10}$) reduction in titer (Fig. 2). In contrast, when gC mutant strains NS-gC_{null} and ns-1 were incubated with active serum, there was an approximately 100-fold (2 $\log_{10}$) reduction in titer (Fig. 2). Thus, in the absence of gC, complement markedly reduced the titers of HSV-1. When viruses rns-1 and rNS-gC_{null}, which have the phenotype of wild-type gC-1, were incubated with nonimmune human serum, they resisted complement neutralization, indicating that the protection is mediated by gC-1 (Fig. 2).

To ensure that antibody to HSV was not influencing these results, three additional HSV-1 and -2 antibody-negative donors were evaluated. Each serum caused either a small or no reduction in titer when incubated with NS. In contrast, each serum reduced the titer of ns-1 approximately 100-fold (Fig. 3A). Since virus was grown in African green monkey (Vero) cells, while human serum was used as the source of complement, the possibility exists that cross-species antibodies were responsible for complement activation (41, 53, 57). Therefore, neutralization experiments were repeated with virus grown in human cells (HEp-2) or with serum adsorbed against uninfected Vero cells to remove possible cross-reactive antibodies. Complement neutralization results were unchanged from those reported above (results not shown). Several additional lines of experiments indicated that immunoglobulins were not required for complement-mediated neutralization. First, we evaluated serum from a child which lacked antibodies to CMV, EBV, and VZV in addition to HSV-1 and -2. This serum had been stored at 4°C for several weeks and had a total hemolytic complement titer that was eightfold lower than that of the reference nonimmune serum used for Fig. 2. The neutralizing activity of the child's serum was comparable to the reference serum when the latter was diluted to contain the same titer of complement (result not shown). Second, hypogammaglobulinemic human serum neutralized gC mutant ns-1 (Fig. 3B). Third, nonimmune human serum depleted of >95% IgG neutralized ns-1 (Fig. 3C). These results suggest that complement neutralization of gC mutant strains does not require antibody.

Input virus titer, serum concentration, and replenishing the complement supply affect complement neutralization. To further assess the magnitude of complement neutralization, the conditions of the assay were modified. First, the effects of input virus titer were evaluated (Table 2). At input titers of up to 2.3 $\log_{10}$, nonimmune serum reduced titers to undetectable levels (≤200-fold reduction). At progressively higher input titers, nonimmune serum resulted in a 16- to 200-fold (1.2 $\log_{10}$ to 2.3 $\log_{10}$) reduction in titer. At a virus titer of 6.9 $\log_{10}$, serum decreased the titer to 5.7 $\log_{10}$ (16-fold reduction), which represents neutralization of >7 million PFU. The results indicate that as the virus titer increases, complement is not able to completely neutralize gC mutant virus, presumably because of consumption of complement components or the presence of

TABLE 1. Characterization of NS, gC mutants, and marker-rescued viruses[a]

Virus	Description	C3b rosettes	Result of Western blotting	Genotype
NS	Low-passage-no. clinical isolate	Pos	wt	wt
NS-gC_{null}	ICP6::*lacZ* replaces gC-1 DNA	Neg	Neg	gC null
ns-1	*mar* mutant	Neg	Truncated intracellular protein, no gC-1 on the virion envelope	Frameshift mutation after codon 165, new stop codon after 289
rNS-gC_{null}	Rescue of NS-gC_{null} with NS gC-1	Pos	wt	wt
rns-1	Rescue of ns-1 with NS gC-1	Pos	wt	wt

[a] Pos, positive; wt, wild type; Neg, negative.

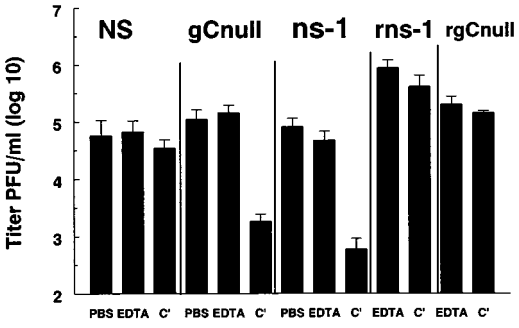

FIG. 2. gC protects HSV-1 from complement neutralization. Viruses were mixed with either PBS, nonimmune human serum treated with EDTA to inactivate complement (EDTA), or active serum (C′) for 1 h at 37°C, and then the titer was determined by plaque assay. Results shown represent the geometric mean titers ± SE. The numbers of experiments performed were 10 for NS, 4 for NS-gC_{null} (gCnull), 12 for ns-1, 2 for rns-1, and 4 for rNS-gC_{null} (rgCnull). In comparison of EDTA-serum with active serum for NS-gC_{null} or for ns-1, was $P < 0.001$. In contrast, the P values were not significant in comparison of EDTA-serum with active serum for NS, rns-1, and rNS-gC_{null}.

inhibitors. Next, fresh serum was added to the virus-complement mix after 20 min, which further reduced the titers and produced a 5,000-fold (3.7 $\log_{10}$) reduction of gC mutant ns-1 (Table 2). This result indicates that the availability of fresh complement affects the extent of neutralization. To further evaluate the effects of complement concentration, serial two-fold dilutions of serum were incubated with 100 PFU (2 $\log_{10}$) of NS or gC mutant strain for 1 h at 37°C. Neutralization of gC mutants was complete at a 1:5 dilution, >80% at a 1:20 dilution, and >50% at a 1:40 dilution of serum (Fig. 4). In contrast, only a small reduction (19%) in titer of NS was detected at a 1:5 dilution, and no reduction was detected at a ≥1:10 dilution. The results indicate that complement concentrations and virus titer affected neutralization and that the maximum reduction achieved was ≥5,000-fold.

Complement neutralization of gC mutant strains is very rapid. To determine the kinetics of neutralization, 4 to 5 $\log_{10}$ of NS or gC mutant strains ns-1 and NS-gC_{null} was incubated with nonimmune human serum. After 1, 5, 10, 20, 40, or 60 min, EDTA was added to stop complement activation, and the virus titer was determined by plaque assay. At 60 min, neutralization of NS was 2-fold, while titers of gC mutants fell approximately 100-fold (Fig. 5A). The steepest slope in the neutralization curve for gC mutants was over the first 10 min. The $t_{1/2}$s in serum of ns-1 and NS-gC_{null} were calculated from these results. The $t_{1/2}$ for ns-1 was 2 min, and that for NS-gC_{null} was 2.5 min (Fig. 5B). To calculate the $t_{1/2}$ for NS, virus was incubated with serum and titers were determined at 30-min intervals for up to 3 h (Fig. 5C). The $t_{1/2}$ for NS was 60 min. These results highlight the impressive speed of complement neutralization of gC mutant strains and the degree of protection provided by gC-1.

TABLE 2. Effects of virus titer and replenishing the complement supply on neutralization of gC mutant ns-1

Complement added to virus:	Initial titer	Titer after 60 min of incubation with complement	Reduction in titer ($\log_{10}$)
Once, at $t = 0$	$10^{1.9}$	0	1.9 (total neutralization)
	$10^{2.3}$	0	2.3 (total neutralization)
	$10^{3.7}$	$10^{1.4}$	2.3
	$10^{4.5}$	$10^{2.7}$	1.8
	$10^{6.9}$	$10^{5.7}$	1.2
Twice, at $t = 0$ and 20 min	$10^{3.7}$	0	3.7 (total neutralization)

Neutralization requires components of the classical complement pathway. Serum was treated with 8 mM EGTA–2 mM Mg^{2+}, which inactivates the classical complement pathway and leaves the alternative complement pathway intact (Fig. 6). Mutant ns-1 was not neutralized by EGTA-treated serum, supporting a role for components of the classical complement pathway. As further evidence, C4-deficient guinea pig serum was used as a source of complement, since C4 is required for activation of the classical pathway (Fig. 6). Intact guinea pig serum neutralized gC mutant ns-1; however, C4-deficient guinea pig serum failed to neutralize ns-1. C4-deficient serum was reconstituted with physiologic concentrations of purified human C4 (400 μg/ml), which restored levels of hemolytic complement activity back to those of intact serum and resulted in neutralization of ns-1 (Fig. 6). These results support a role

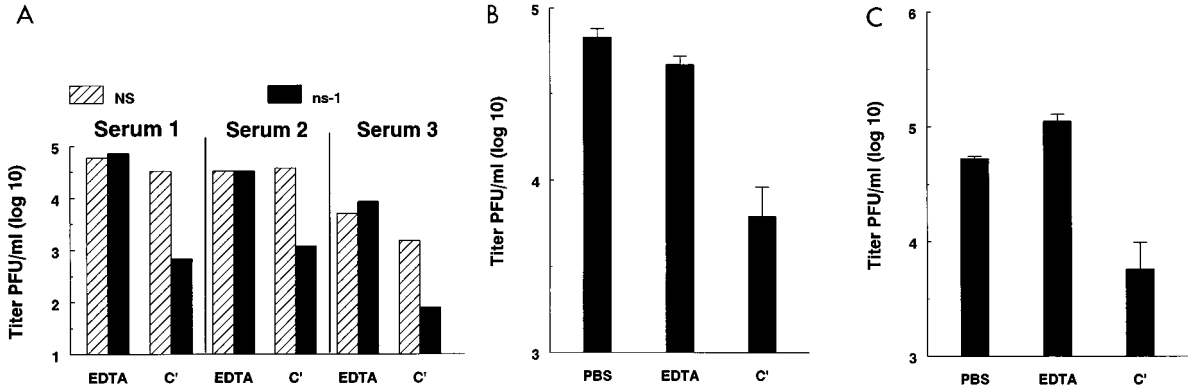

FIG. 3. (A) Neutralization of NS and ns-1 by three different nonimmune human sera yields results comparable to those shown in Fig. 2. Virus was incubated with EDTA-treated (EDTA) or active (C′) serum, and the titer was determined. Each serum was tested once. (B) Hypogammaglobulinemic human serum neutralizes gC mutant ns-1. Strain ns-1 was incubated with either PBS, hypogammaglobulinemic serum treated with EDTA (EDTA), or active serum (C′) for 1 h at 37°C, and then the titer was determined by plaque assay. Results shown are means ± SE of two separate experiments. (C) Nonimmune human serum depleted of IgG neutralizes gC mutant ns-1. Strain ns-1 was incubated with either PBS, IgG-depleted serum treated with EDTA (EDTA), or active serum (C′) for 1 h at 37°C, and then the titer was determined. Results shown are the means ± SE of three separate experiments.

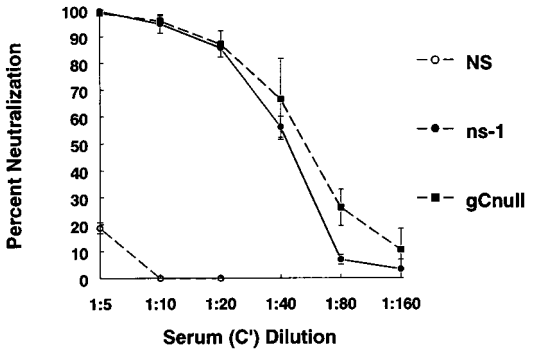

FIG. 4. Complement neutralization in assays using various dilutions of nonimmune human serum demonstrates activity against gC mutant strains at titers of ≤1:40. NS or gC mutant strains were incubated with complement (C′) for 1 h at 37°C, and then the titer was determined by plaque assay. The percent neutralization was calculated by comparing virus titers in active serum and EDTA-serum. Results are the means ± SE of two separate experiments.

for the classical complement pathway in neutralization of gC mutant strains.

The role of gC in antibody-plus-complement neutralization. To determine the role of gC in protecting HSV-1 against neutralization by a combination of antibody plus complement, pooled human IgG was used as source of antibody and a 1:5 dilution of nonimmune human serum was used as the source of complement (Fig. 7). The concentration of pooled human IgG (50 or 100 μg/ml) was selected to neutralize the infectivity of 50% (0.3 $\log_{10}$) of ns-1 or NS, respectively. As noted previously, complement alone had little effect on the titer of NS but had a marked effect on the titer of ns-1. When antibody and complement were used together, enhanced neutralization of NS and ns-1 occurred; however, the effect was not dramatic, particularly compared with the effect of complement alone on ns-1. We performed additional experiments in which the antibody was incubated at various concentration with nonimmune human serum at 1%, a concentration that has little neutralizing activity against gC mutant strains. For NS, ns-1, and NS-gC_{null}, the concentration of antibody required to neutralize 50% of virus was slightly lower (less than twofold) when complement was present; however, gC offered minimal or no advantage to the virus, since the effects of complement were similar for the wild type and the gC mutants (result not shown). Therefore, the effects of gC-1 are most impressive in protecting HSV against antibody-independent complement neutralization.

DISCUSSION

The main observations of this study are the marked reduction in titer of HSV-1 gC mutants in comparison with wild-type virus when the strains are exposed to complement, the rapid kinetics of neutralization of gC mutants, the fact that the classical complement pathway mediates neutralization by nonimmune serum, and the demonstration that gC contributes mainly to protection against antibody-independent complement neutralization. Several other studies, including one from our group, have reported on complement neutralization of gC mutant viruses (17, 22, 25, 39). An unexpected finding of this study was the extensive neutralization by nonimmune serum. Our previous report did not examine the effect of complement alone on HSV infectivity; rather, we examined the role of gC-1 in preventing neutralization by a combination of antibody plus complement (22). Differences in antibody-plus-complement neutralization between NS and gC mutant ns-1 were approximately 100-fold. From our current results, we now conclude that the protection offered by gC-1 was mainly directed against antibody-independent complement neutralization. A report by Hidaka et al. examined a naturally occurring gC-1 mutant, TN1, isolated from a patient with recurrent HSV keratitis (25). The authors reported 1,000-fold reductions in titer of TN1, using rat serum as the source of complement, but did not examine cross-species antibodies. In contrast to our results, they detected only a fourfold (0.6 $\log_{10}$) reduction in titer, using nonimmune human serum. In their study, virus was used at a titer of 5×10^5 to 5×10^6 PFU/ml and complement was used at a 1:10 dilution. As apparent from Fig. 4 and Table 2, the input titer and concentration of complement markedly affect the extent of neutralization detected, which may explain

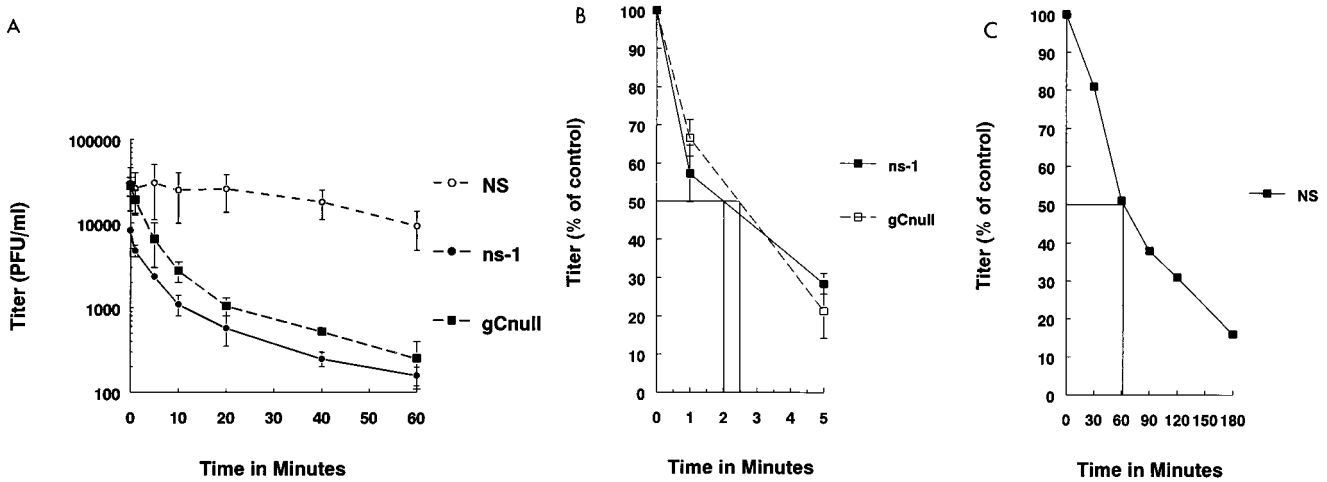

FIG. 5. (A) Complement neutralization of gC mutant strains is rapid. At various times after mixing of virus with nonimmune human serum, complement was inactivated by adding EDTA, and the virus was titered by plaque assay. Results are the means ± SE of two separate experiments. (B) The $t_{½}$ in nonimmune human serum of gC mutant strain ns-1 or NS-gC_{null} is 2 or 2.5 min, respectively. The $t_{½}$ was calculated from the results shown in panel A. The following formula was used to calculate the titer as percentage of the control value $100 \times (1 -$ titer of virus in active serum/titer of virus in EDTA-serum). Results are the means ± SE of two separate experiments. (C) The $t_{½}$ in nonimmune human serum of wild-type NS is 1 h. The $t_{½}$ was determined by incubating NS with serum for the times shown and determining the titer by plaque assay. The formula specified above was used to calculate the titer as percentage of the control value.

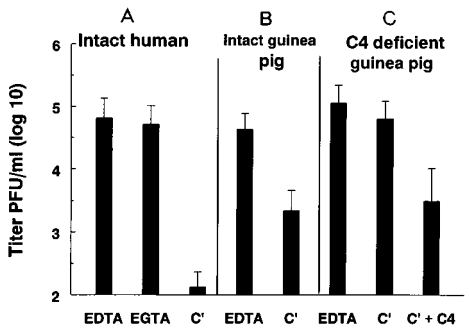

FIG. 6. Complement neutralization of gC mutant ns-1 requires components of the classical complement pathway. Nonimmune human serum was treated with EDTA to inactivate the classical and alternative complement pathways (EDTA), treated with EGTA to inactivate the classical pathway (EGTA), or left untreated (C′ in panel A) and incubated with gC mutant ns-1 for 1 h at 37°C; the titer was determined by plaque assay. For some experiments, the source of complement was intact guinea pig serum (C′ in panel B), C4-deficient guinea pig serum (C′ in panel C), or C4-deficient serum reconstituted with physiologic concentrations (400 μg/ml) of purified human C4 (C′ + C4). Results are means ± SE of three separate experiments for intact human and C4-deficient guinea pig sera and of six experiments for intact guinea pig serum.

the apparent discrepancies between our study and that of Hidaka et al. A study by McNearney et al. used a laboratory adapted HSV-1 mutant, MP, as the gC-negative strain and compared it with (MP)6-9A, a gC-1 recombinant derived from MP that has gC-1 protein expressed at the thymidine kinase locus and under the control of the thymidine kinase promoter rather than at its natural site (39). At a 1:3 dilution of serum, the authors noted approximately 80% neutralization (<1 $\log_{10}$) of the gC mutant, compared with 35% for the gC-1 marker-rescued strain. The results indicate that MP and NS differ in susceptibility to complement neutralization. Strain differences may be attributable to the ability of virus to activate complement or to the effectiveness of gC in modifying complement activation once it occurs. The fact that gC-null virus is neutralized by serum indicates that gC is not required for complement activation and suggests that gC may not be the only viral protein influencing complement-mediated neutralization. A recent study by Gerber et al. evaluated the role of HSV-2 gC in protection against complement neutralization by nonimmune human serum (17). For comparison purposes, the authors studied a gC-1 deletion mutant constructed in HSV-1 KOS. With undiluted human serum, neutralization was >95% (>1 $\log_{10}$), which is consistent with our results. An important observation of our study is that the extent of complement neutralization varies with input titer, complement dilution, and replenishment of the complement supply. Under conditions in which complement was replenished after 20 min, we showed a 5,000-fold (3.7 $\log_{10}$) reduction in titer of mutant virus, while at an input titer of 6.9 $\log_{10}$, we demonstrated a 16-fold reduction (1.2 $\log_{10}$) during 1 h of exposure to complement, which reflects the neutralization of approximately 7 million PFU (Table 2).

Complement neutralization was rapid for gC mutant viruses, with a $t_{1/2}$ of 2 to 2.5 min, compared with 1 h for wild-type virus. A study by Daniels et al. examined the kinetics of HSV-1 neutralization by antibody and complement and in particular evaluated the role of C1 and C4 in antibody-dependent neutralization of HSV-1 (6). As in the present study, neutralization occurred within minutes; however, our study differs in that we examined complement neutralization in the absence of antibody and focused on the role of gC in preventing complement neutralization. Other human viruses, including EBV (41), LCMV (57), and measles virus (49), have been shown to be neutralized by nonimmune human serum. EBV and LCMV activate the classical complement pathway, while measles activates the alternative complement pathway. Although EBV and LCMV are neutralized by nonimmune serum via the classical complement pathway, cross-reacting antibodies are required to activate complement; for EBV, the antibodies appear to be directed against HSV-1 (41), while for LCMV, natural antibodies are detected against host cells (L-929) used to grow the virus (57). Of the human viral pathogens, only human immunodeficiency virus has been shown to activate the classical complement pathway in the absence of antibody (10). Host cell complement-regulatory proteins, including decay-accelerating factor (CD55) and homologous restriction factor (CD59), are incorporated into the virion envelope and prevent human immunodeficiency virus neutralization by nonimmune serum (38). This is somewhat analogous to the situation with HSV-1 except that complement neutralization of HSV-1 is prevented by incorporation of a virus-encoded glycoprotein, gC, into the virion envelope.

The evidence that cross-reactive antibodies are not needed for neutralization of HSV-1 gC mutants includes the following findings: (i) five different nonimmune sera, including one obtained from a child who was negative for antibodies to HSV-1 and -2, CMV, EBV, and VZV, mediated neutralization; (ii) complement neutralization was mediated by hypogammaglobulinemic serum; (iii) IgG-depleted serum was effective in complement neutralization; and (iv) complement neutralization occurred when gC mutant virus was purified in human cells (HEp-2) or when serum was absorbed against Vero cells to remove potential cross-reacting antibodies. These findings make it unlikely that low levels of cross-reactive antibodies activate complement. Recent evidence suggests that cross-reacting antibodies may be difficult to detect (53). Cross-reacting antibodies to Gal(α1-3)Gal, a terminal carbohydrate expressed by mammals but absent in humans, were found to mediate antibody and complement neutralization of animal retroviruses, using nonimmune human serum as the source of complement. Our results suggest that cross-reacting antibodies are not required for complement neutralization of HSV-1 gC mu-

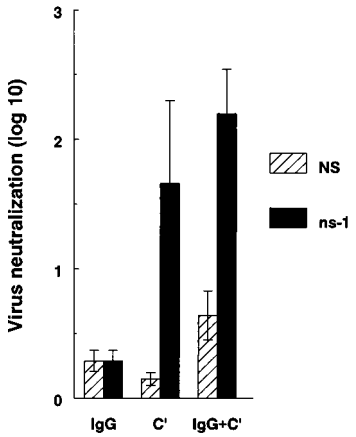

FIG. 7. Antibody, complement, or antibody-plus-complement neutralization of NS and ns-1 indicates that the protection provided by gC-1 is mainly because of its effect on complement alone. Approximately 10^5 PFU of ns-1 or NS was incubated with anti-HSV IgG at 50 or 100 μg/ml, respectively, which resulted in 50% neutralization, with complement (C′) alone at a 1:5 dilution of nonimmune human serum, or with IgG plus complement. After 1 h at 37°C, titers were determined by plaque assay. Results are the means ± SE of two separate experiments.

tants. Although it remains possible that low levels of cross-reactive antibodies were undetected, our results clearly indicate that human serum from HSV-naive individuals readily neutralizes gC mutant strains.

Our previous report demonstrated that gC-1 expressed at the cell surface protects infected cells from lysis by nonimmune serum mediated by the alternative complement pathway (22). In the current study, we demonstrate that gC-1 protects cell-free virus against neutralization mediated by the classical complement pathway. gC-1 binds C3b, which is central to both complement pathways, providing an explanation for the role of gC-1 in modifying both the classical and alternative pathways. Complement was active in neutralizing gC mutants at serum dilutions of $\leq$1:40. Concentrations of complement are highest in serum, but complement proteins are found in many body fluids, including oral and genital secretions, which supports a role for gC in protecting virus or infected cells against complement-mediated mucosal immunity (23, 58). The mechanism by which complement neutralizes gC mutants remains to be defined. Mechanisms described for other viruses include aggregation or clumping of viruses to reduce infectivity, coating of virus to block attachment or entry, and viral lysis (reviewed in reference 5). Studies are in progress to evaluate the possible mechanisms.

In addition to its complement-modulating properties, gC mediates binding of virus to heparan sulfate on cells (24, 52, 59). Are these two functions of gC linked? Structural studies suggest that the amino-terminal domain of gC-1 is not involved in C3b binding but does participate in binding to heparan sulfate, suggesting that these two functions are carried out by different regions of the glycoprotein (30, 54). Nevertheless, observations supporting a possible link between these two functions include (i) the interesting observation that heparin is an inhibitor of complement activation, and therefore gC binding to heparan sulfate may augment the inhibitory activity mediated by gC (11, 56); and (ii) the finding that mammalian C3 receptors are widely distributed on cells, and therefore activated C3 fragments on HSV may enhance infection by promoting virus attachment to complement receptors on cells (48). *Candida albicans* expresses a protein that mediates adhesion of the organism to cells; the protein is antigenically related to complement receptor type 3, a member of the integrin family of adhesin molecules, and the protein binds complement component iC3b. Therefore, this protein is similar to gC in that it binds complement and mediates attachment to cells (21). Future experiments should enable us to understand the relative importance of gC in complement binding and in cell attachment. The impressive protection offered by gC against complement neutralization suggests that this function is likely to be important in vivo.

The in vivo biologic relevance of complement neutralization is under investigation by our group (37). Given the ability of guinea pig serum to neutralize gC mutants (Fig. 6), guinea pigs appear to be suitable laboratory animals for pathogenesis studies. Our results of studies using a vaginal model of infection indicate that gC mutant strains grow to titers approximately 100-fold lower than those of wild-type or marker-rescued virus and that the gC mutant strains cause less severe vaginitis. Of interest, when gC mutant virus was inoculated into C3-deficient guinea pigs, vaginitis was more severe than in complement-intact animals and vaginal titers were 100-fold higher. In C3-deficient guinea pigs, no differences between gC mutant and wild-type viruses were detected. These preliminary studies support a role for the interaction between gC and complement in pathogenesis and suggest that our in vitro results correlate with in vivo virulence.

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