Glycoprotein C of herpes simplex virus type 1 is essential for the virus to evade antibody-independent complement-mediated virus inactivation and lysis of virus-infected cells

Yasufumi Hidaka,* Yuichiro Sakai, Yasushi Toh and Ryoichi Mori

Department of Virology, School of Medicine, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812, Japan

Glycoprotein C (gC) of herpes simplex virus type 1 (HSV-1) is a receptor for the complement component C3b. We have previously isolated HSV-1 gC− strains (TN1, TN2 and TN3) from a patient with recurrent keratitis at three different times. These are very rare isolates because gC was thought to be essential for the virus in vivo. To determine whether gC modifies the interaction of complement with cell-free virus or virus-infected cells, we constructed gC+ recombinant viruses in which the intact gC gene of strain KOS was inserted into the TN1 virus genome. TN1 virus was inactivated by complement and TN1 virus-infected cells were lysed by complement; however, gC+ recombinant viruses became resistant to these effects of complement. These results suggest a role for gC in protection of both the virion envelope and the infected cell surface against damage by complement. TN1 virus was inactivated by complement from rats (Wistar, WKA, F344 and SD), guinea-pigs (Hartley) and humans, but not by complement from mice (C3H, DDD and BALB/c), which indicates that mice seem to be inappropriate as an experimental model for the study of HSV infection in which complement factors need to be considered.

Introduction

The envelope of herpes simplex virus type 1 (HSV-1) is composed of a cell-derived lipid bilayer and a number of virus-specified glycoproteins; at least seven HSV-1 glycoprotein genes, gB, gC, gD, gE, gG, gH and gI, have been mapped (Buckmaster et al., 1984; Spear, 1984; Longnecker et al., 1987). gB, gC and gD, which are present on the infected cell surface, act as the major antigenic determinants of HSV-1 (Glorioso et al., 1984) and the gC of HSV-1 (gC-1) has been reported to be recognized by HSV-1-specific cytotoxic T lymphocytes in a mouse model (Glorioso et al., 1985; Rosenthal et al., 1987). In infected cells, the gCs of HSV-1 and HSV-2 function as receptors for the complement component C3b (Cines et al., 1982; Friedman et al., 1984, 1986; Smiley et al., 1985; Seidel-Dugan et al., 1988; Huemer et al., 1989; Ghosh-Choudhury et al., 1990). Purified gC-1 modulates complement activation by accelerating the decay of C3bBb, the amplification convertase of the alternative complement pathway (Fries et al., 1986). Consequently, it is argued that gC confers on the virus a means of modifying the activity of certain human complement components, thereby evading immune recognition by this mechanism. However, there are only a few reports on the interaction between complement and gC on the viral envelopes or the infected cell surface, and very few studies have been performed in the absence of antibody.

We previously reported very rare gC− mutants (strains TN1, TN2 and TN3) isolated from a patient with recurrent keratitis (Hidaka et al., 1990). In the present study, we used the clinical gC− isolates to investigate the interaction between gC and complement in the absence of antibody. We constructed a recombinant gC+ TN1 virus, into which the intact gC gene of the KOS strain of HSV-1 had been transferred, and examined its variation in complement sensitivity. The transfer of gC made the gC− TN1 virus resistant to complement both as cell-free virus and in virus-infected cells, confirming the finding that laboratory gC− mutants but not gC+ strains are inactivated by complement in the absence of antibody (McNearney et al., 1987). Furthermore we examined the TN1 virus and the recombinant gC+ TN1 virus for species differences in virus inactivation by complement. Rat complement readily inactivated the TN1 virus, but mouse complement had little activity against the virus, supporting the finding that gC is not required for HSV infection in mice (Dix et al., 1983; Johnson et al., 1986).

Methods

Cells and virus strains. Vero cells grown in Eagle’s MEM supplemented with 5% calf serum were used for virus propagation, titration and transfection. The virus strains used were HSV-1 strain KOS, a
Fig. 1. Maps of the HSV-1 strain KOS genome and of the plasmid used in this study. The position of BamHI fragments I and Q are shown in the map of KOS. In pTK1.0-gC open bars indicate the regions derived from BamHI fragment Q, the closed bar indicates the region from BamHI fragment I and the line indicates that from pUC18. The position and the direction of transcription of the gC gene and the TK gene are shown by arrows. Restriction endonuclease sites: Ba, BamHI; S, SalI; E, EcoRI; X, XbaI; Bg, BglII. The lines above the abbreviations indicate blunt ends generated using the Klenow fragment of DNA polymerase I.

Laboratory strain of HSV-1, HSV-1 gC- mutant strain TN1, a clinical isolate from human keratitis (Hidaka al., 1989) but this was not thought to affect the results of the experiments. Vero cells were cotransfected with mixtures of the appropriate virus DNA and recombinant plasmids as described by Lee al. (1982) and DeLuca al. (1985). Calcium phosphate precipitates were prepared to contain 1 μg TN1 DNA, 1 μg BamHI-digested pTK1.0-gC DNA and 10 μg of Vero cell DNA per 0.5 ml. After a 4 h exposure to the precipitates, the cells were treated with 15% (v/v) glycerol, fresh medium was added and the cultures were incubated at 37 °C for 4 or 5 days. Selection of ACV-resistant mutants from the progeny of these transfections was done under medium containing 1% methicillulose and 10 μg ACV per ml. The ACV-resistant clones obtained by this selection procedure were purified by two cycles of plaque cloning. Five clones obtained from this experiment were designated C-1, C-2, C-3, C-4 and C-5.

Immunofluorescence test of recombinant viruses. Recombinant viruses were inoculated onto Vero cells, cells were harvested when the c.p.e. reached about 100% and an immunofluorescence test was conducted with anti-gC-1 monoclonal antibody (Syva HSV-1 reagent).

[^14C]Glucosamine-labelled viral glycoprotein analysis. Labelling of the viral glycoproteins was performed as previously described (Hidaka al., 1990).[^14C]Glucosamine (final concentration, 2 μCi/ml) was introduced into the virus-infected Vero cells 4 h post-infection (p.i.), the cells were harvested 20 h later and solubilized using cell lysis medium containing 0.5%, NP40. The samples were solubilized in a buffer containing 1% SDS and 1-3% 2-mercaptoethanol, incubated at 80 °C for 2 min and separated electrophoretically on 7-5% polyacrylamide gels cross-linked with N,N’-diallyltartardiamide. Autoradiographic exposure was carried out at — 80 °C for 3 weeks.

Restriction endonuclease analysis of recombinant viruses. Viral DNA was prepared from virus particles purified by glycerol gradient centrifugation as described by Denniston al. (1981), and digested with BamHI. After electrophoresis in a 0.7% agarose gel, the DNA fragments were stained with ethidium bromide and visualized with a u.v. transilluminator and photographed. Furthermore, to confirm that recombination had occurred, Southern blot analysis was performed as described by Sambrook al. (1989). After electrophoresis in an agarose gel, the DNA was denatured and transferred to nitrocellulose filters. The filters were baked at 80 °C for 2 h, prehybridized, hybridized with 32P-labelled BamHI fragment Q from strain KOS as a probe and washed twice with 2 x SSC (1 x SSC is 0.15 M-NaCl, 0.015 M-sodium citrate), 0 1% SDS at room temperature and twice with 1 x SSC, 0.1% SDS at 60 °C for 20 min. Autoradiographic exposure with an intensifying screen was carried out at — 80 °C for 6 h.

Complement-mediated virus inactivation assay. A complement-mediated virus inactivation assay was performed to determine the sensitivity of cell-free virus to complement. Virus was titrated on Vero cells and diluted to 5 x 10^5 to 1 x 10^1 p.f.u./ml with phosphate-buffered saline (PBS) containing 2% heat-inactivated calf serum and 50 μgidgetamicin per ml (virus diluent). Virus solution (0.9 ml) and either 0.1 ml virus diluent as a control or 0.1 ml serum as a source of complement were mixed well on ice. The control mixture was titrated directly and the test mixture was titrated after incubation at 37 °C for 1 h. The rate of reduction in virus titre was calculated as —log_{10} (titre of virus surviving/virus titre of control).

Inhibition of complement-mediated virus inactivation. A complement-mediated virus inactivation assay was also performed by using heat-inactivated (56 °C for 30 min) sera, or Wistar rat sera pretreated at a ratio of 1:1 with either anti-rat C3 goat serum diluted to 1/1, 1/5 or 1/50, or virus diluent. These mixtures were incubated at 37 °C for 1 h. Complement-mediated virus inactivation assays were performed with these pretreated sera. In the experiment on blocking by anti-rat C3 goat serum, the final concentration of rat serum used was half that of the usual assay.

One-step growth curve in the presence of complement. Vero cells (1 x 10^6 cells/well) were infected with TN1 virus or the C-3 recombinant at a multiplicity of 20 p.f.u./cell. After adsorption at 37 °C for 1 h, the inocula were aspirated gently and the monolayers were rinsed three times with PBS. After addition of 1 ml of medium per well, the infected cells were incubated at 37 °C and at 4 h, 10 h and 22 h p.i.,
0.1 ml of rat serum or 0.1 ml of virus diluent was added to each well, i.e. the culture that was harvested after 24 h had received complement three times. At 3 h, 6 h, 12 h and 24 h p.i. the cells and the supernatant fluid were harvested separately; the cells were suspended in 2 ml virus diluent, disrupted by sonication and centrifuged at 3000 r.p.m. for 10 min. The virus suspensions were titrated on Vero cells and the titre of virus recovered was expressed in p.f.u./cell.

**Statistical analysis.** Student's t-test was used for the analysis of complement-mediated virus inactivation assays.

---

**Results**

**Characterization of recombinant viruses**

The ACV-resistant viruses were examined for gC expression by an immunofluorescence test using anti-gC-1 antibody (Syva HSV-1 reagent). Vero cells infected with TN1 and C-4 were not stained by this anti-gC-1 monoclonal antibody but cells infected with the C-1, C-2, C-3 and C-5 recombinants were stained clearly, as was KOS. The progeny clones were examined for gC expression by glucosamine-labelled viral glycoprotein analysis. gC of the mature size was observed in KOS, C-1, C-2, C-3 and C-5 but not in TN1 (data not shown).

Restriction endonuclease analysis of viral DNA showed that recombination had occurred in the progeny clones. DNAs from TN1, C-1, C-2, C-3, C-4 and C-5 were digested with BamHI; the restriction fragment patterns are shown in Fig. 2. In C-1, C-3 and C-5, the BamHI fragment Q (3.6 kb) disappeared and a novel 7.0 kb band was seen. This 7.0 kb band was thought to be the BamHI fragment Q into which SalI-BamHI fragment R-I had been inserted. To confirm that recombination had occurred, Southern blot analysis was performed; the results are shown in Fig. 3. The 7.0 kb bands seen with C-1, C-3 and C-5 and the 3.6 kb bands seen with KOS and C-2 hybridized with the KOS BamHI fragment Q probe, which contained the TK gene. These results showed that recombination occurred in the region around the TK gene in C-1, C-3 and C-5 as expected, but not in C-2. The recombinant viruses were converted from gC- to gC+ in C-2, recombination was thought to have occurred between the inactive gC gene of TN1 and the SalI-BamHI fragment R-I of pTKI-0-gC. Therefore gC expression was seen in C-2 but the electrophoretic pattern of its DNA did not change (Fig. 3).

**Complement-mediated virus inactivation assay of recombinant viruses**

Complement-mediated virus inactivation assays were performed with fresh guinea-pig serum. The reduction rates in KOS and TN1 virus titres were 0.19 ± 0.05 and 1.05 ± 0.12, respectively, which showed that KOS was resistant and TN1 was sensitive to the action of complement. The rates of reduction in titres of C-1, C-2, C-3 and C-5 were 0.20 ± 0.06, 0.19 ± 0.01, 0.22 ± 0.06 and 0.15 ± 0.03, respectively (Fig. 4), significantly lower than that of TN1 (P < 0.01 for C-2 and C-5; P < 0.05 for C-1 and C-3). These results suggest that gC expression might play an important role in resistance to complement because only transfer of the intact gC gene could make the gC-, complement-sensitive TN1 complement-resistant to the same degree as the gC+ standard KOS.

**Species variation in complement-mediated virus inactivation**

Inactivation of TN1 and C-3 by complement was examined using sera from guinea-pig, three strains of mice, four strains of rats and humans. TN1 was inactivated by guinea-pig and human sera and strongly inactivated by rat sera but was not inactivated by the mouse sera (Fig. 5a).
Fig. 3. Southern blot analysis of recombinant viruses. BamHI fragment Q from strain KOS was used as a probe. Lanes 1 to 5; KOS, C-1, C-2, C-3 and C-5. The sizes (kb) of hybridized bands are shown.

Fig. 4. Inactivation of infectivity of HSV-1 gC⁺ or gC⁻ mediated by guinea-pig complement. The error bars show s.d. K, KOS; T, TN1; 1, 2, 3 and 5, C-1, C-2, C-3 and C-5, respectively.

Inactivation of the gC⁺ recombinant C-3 by complement was not observed using the guinea-pig, mouse and human sera. However, the rat sera inactivated C-3 despite the expression of gC, although the inactivation of C-3 was far less than that of TN1 (Fig. 5b).

Inhibition of complement-mediated virus inactivation

Fig. 5. Inactivation of strains TN1 (a) and C-3 (b) mediated by complement from various animals. The error bars show s.d. GP, guinea-pig complement; 1, mouse strain C3H; 2, mouse strain DDD; 3, mouse strain BALB/c; 1*, rat strain Wistar; 2*, rat strain WKA; 3*, rat strain F344; rat strain 4*, SD.

We examined whether inactivation of the virus by the various kinds of sera shown in Fig. 5 was directly mediated by complement. The guinea-pig and Wistar rat sera lost their ability to inactivate when they had been heated at 56 °C for 30 min (Fig. 6a). Wistar rat sera pretreated with anti-rat C3 goat serum were inactivated in a dose-dependent fashion and the activity was completely blocked by pretreating the rat serum with
The role of HSV-1 gC

Role of gC on infected cells

Fig. 7 shows one-step growth curves for TN1 and C-3 in the presence or absence of rat complement. Although the growth of TN1 was decreased by rat complement, that of C-3 was not. It was observed microscopically that TN1 virus-infected cells were disrupted and detached from the bottom of the well after the addition of complement 22 h p.i. This seemed to be the reason why the titre of TN1 in cells in the presence of complement 24 h p.i. was less than that in cells at 12 h p.i. Although many TN1 viruses must have been released into the supernatant, the virus titre of the supernatant at 24 h was only slightly increased and thus the released viruses are thought to have been inactivated by complement. These results suggest that gC has an important role in protecting the virus from damage by complement not only on the virus envelope but also on the infected cell surface.

Discussion

In this study, we have shown that a gC- clinical isolate of HSV-1 was susceptible to the action of complement, whereas gC+ virus strains were not. We have confirmed that gC was the main reason for resistance to complement by transferring the gC gene into the gC- virus. Furthermore, we have shown a species difference in complement-mediated virus inactivation and that gC has a role on both the virus envelope and the infected cell surface.

Several enveloped viruses activate the complement cascade, which results in viral lysis in the absence of antibody. They include Epstein–Barr virus (McConnell et al., 1978), respiratory syncytial virus (Smith et al., 1981; Kaul et al., 1984; Edwards, K. M. et al., 1986), mumps virus (Hirsch et al., 1986), measles virus (Sissons et al., 1980), Sendai virus (Okada & Okada, 1981), influenza virus (Lambre et al., 1982), oncornaviruses (Bartholomew et al., 1978), feline leukaemia virus (Kobilinsky et al., 1980), and vesicular stomatitis virus and infectious bovine rhinotracheitis virus (Grewal & Babiuk, 1980). However such a phenomenon is not observed with HSV. Van Strijp et al. (1988) have shown that antibody is not required for the activation of complement in HSV-1-infected cells and Harris et al. (1990) have shown that gC prevents HSV-1-infected cell lysis and complement-mediated neutralization in the presence of antibody. Our findings show that gC has a marked protective effect against antibody-independent complement inactivation of free virus. gC also blocks the effect of complement on infected cells and allows the virus to continue growth. These results suggest that
protection by gC is most important for HSV both in the early stage of infection, before antibodies and cytotoxic T lymphocytes appear, and in severe cases in which generalized spread of HSV occurs by viraemia. gC seems to offer a survival advantage to HSV by interfering with complement activation in vivo and therefore HSV gC- strains are rarely isolated from human material, although spontaneous gC- mutants of HSV have frequently emerged in cell cultures (Hoggan & Roizman, 1959).

We also found a species difference in complement-mediated virus inactivation. Rat, guinea-pig and human complement inactivated the HSV-1 gC- strain, but complement from various strains of mice did not. Dix et al. (1983) and Johnson et al. (1986) have shown that gC- HSV can kill mice and hence gC is not essential for pathogenicity in mice. Because their sera did not inactivate gC- HSV, it may be inappropriate to use mice as a model of HSV infection in which complement factors need to be considered because the effect of complement was different from that in humans.

There are two possible reasons why the complement activity differs between species. One is the difference in affinity between the gC of HSV and the complement component in each animal. This difference in affinity was thought to be due to differences in either complement or glycoprotein structure, e.g. a difference in glycosylation level. In this study only viruses propagated in Vero cells were used and therefore it may be useful to examine viruses propagated in other cell types. Another possibility is that an unknown regulatory factor of complement is brought from Vero cells to the virus envelope; this factor could affect complement activity in some species, but not the rat.

The gC of HSV is a C3b receptor and protects the virus by interfering with the activation of complement, acting as a regulatory protein. Among viruses, HSV-1, HSV-2 and equine herpesvirus bind C3b (McNearney et al., 1987; Bielefeldt Ohmann & Babiuk, 1988), vaccinia virus has a protein with structural homology to C4-binding proteins (Kotwal & Moss, 1988) and Epstein–Barr virus accelerates decay of C3 convertase in the alternative pathway (Mold et al., 1988). Among microorganisms, Candida species bind degradation products of C3 (Edwards, J. E., et al., 1986) and Schistosoma species bind C3 (Tarleton & Kemp, 1981). Although the function of HSV-1 gC has been examined, the role of the other complement-binding proteins remains obscure. It would be intriguing to investigate whether other microorganisms have protective functions against complement, which is one of the earliest stages of host defence against microorganisms.

The authors are grateful to Dr Mary Louise Robbins for reviewing the manuscript. This work was supported by a grant from Yakult Honsha Co., Ltd. (Tokyo, Japan).

References


Lambre, C. R., Kazatchkine, M. D., Maillet, F. & Thibon, M. (1982). Guinea-pig erythrocytes, after their contact with influenza virus, acquire the ability to activate the alternative complement pathway through virus-induced desialation of the cells. *Journal of Immunology* 128, 629–634.


(Received 25 October 1990; Accepted 14 January 1991)