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Pathogenicity of glycoprotein C negative mutants of herpes simplex virus type 1 for the mouse central nervous system

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Summary

A previous study from our laboratory showed that a mutant of herpes simplex virus type 1 (HSV-1), strain KOS-321, carrying a deletion in the structural gene for glycoprotein C (gC) had reduced pathogenicity for the mouse central nervous system when compared to the wild-type virus (Kümel et al., 1985). In this study, eight additional gC negative (gC^-) mutants derived from KOS-321 were shown to vary widely in their ability to induce lethal encephalitis in female DBA/2 mice following intracerebral inoculation. This variation in virulence showed no correlation with thymidine kinase activity. One less virulent gC^- strain, gC^-39, was further studied to determine whether the neurovirulent phenotype could be restored by rescue of the gC gene using standard marker rescue cotransfection procedures. The resulting progeny contained 2% gC^+ recombinant virions and was tested for its ability to cause encephalitis. Although this progeny had increased virulence, it was not attributable to the acquisition of the gC gene since passive immunization of mice with a pool of anti-gC monoclonal antibodies had no effect on the development of encephalitis and only gC^- viruses were isolated from diseased brain tissues. In agreement with these findings, individual plaque-purified gC positive (gC^+) virus recombinants were shown not to have been restored to the wild-type virus level of neurovirulence. It is concluded that gC is not a virulence determinant in this mouse

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model of HSV-induced encephalitis and that cotransfection procedures can induce additional mutations that affect viral pathogenesis.

Herpes simplex virus; Glycoprotein C; Virulence

Introduction

Herpes simplex virus type 1 (HSV-1) is a major cause of encephalitis in human adults, accounting for over 1000 cases annually in the United States (Centers for Disease Control, 1983). The clinical importance of HSV-1 is rooted in its ability to establish latency in neurons of sensory ganglia, which has made HSV infections difficult to eliminate from the human population. Once infected, the host's nervous system serves as a life-long reservoir for recrudescence and viral spread to other individuals.

Few HSV-1 neurovirulence factors have been identified. Several investigators have observed reduced ability of the virus to produce encephalitis or establish ganglionic latency in association with decreased levels of HSV thymidine kinase enzyme activity (Field and Darby, 1980; Sibrack et al., 1982; Stanberry et al., 1985; Tenser et al., 1985). HSV intertypic recombinant viruses and different natural strains of HSV-1 with decreased neurovirulence have been partially restored to the pathogenic phenotype by marker rescue with specific DNA restriction fragments derived from virulent HSV-1 viruses (Rosen et al., 1985; Thompson et al., 1983, 1985, 1986; Larder et al., 1986; Javier et al., 1987). However, the specific protein product(s) which contribute to virulence have not been identified. In addition, specific genes have been inactivated by deletion mutagenesis using the viral TK gene in mutant selection (Post and Roizman, 1981) and the mutant viruses tested for alterations in neurovirulence. This approach has shown that an immediate early gene, ICP22, is required for viral pathogenesis for the mouse central nervous system (Sears et al., 1985).

Glycoproteins have been identified as virulence factors in studies of other viruses such as rabies (Dietzschold et al., 1983) and murine coronavirus (Fleming et al., 1986). Among herpesviruses, glycoprotein gI, a pseudorabiesvirus structural component that plays a role in virus release, has been reported to be important to viral neurovirulence (Mettenleiter et al., 1987). Although HSV encodes at least eight glycoproteins (Spear, 1985), their role in neuropathogenesis is largely unexplored. Only three of these, gB, gD, and gH, are known to be required for virus production (Sarmiento et al., 1979; Gompels and Minson, 1986) and thus far only gB has been implicated in viral pathogenesis (Kümel et al., 1985; Weise et al., 1987). The consistent presence in clinical isolates of the remaining glycoproteins that are nonessential for virus production in cell culture indicates that they must play a role in the virus life cycle in vivo and may contribute to viral pathogenesis. In support of this view, Weber et al. (1987) have shown that a HSV-1 mutant carrying a chain terminating Tn5 insertion in the gene encoding glycoprotein G has reduced neuro-

virulence in mice. HSV-1 gE (Para et al., 1982) and gI (Johnson and Feenstra, 1987) have been associated with virus-specific binding activity for the Fc component of IgG. Evidence that binding of nonimmune IgG to infected cell membranes can protect these cells from cytolytic immune mechanisms (Adler et al., 1978) suggests that this activity may alter immune recognition of the virus in vivo. Glycoprotein C plays a central role in the induction of humoral and cell-mediated immune responses during infection (Oakes et al., 1980; Glorioso et al., 1985; Marlin et al., 1985; Kümel et al., 1985; Nash et al., 1985; Rosenthal et al., 1987) and has the unique property of functioning as a receptor for the C_3b component of complement (Friedman et al., 1984, 1986). This activity has been shown to interfere with activation of the alternative complement pathway (Fries et al., 1986), suggesting that gC may act as an important virulence determinant by protecting virus from complement-mediated anti-viral host defense mechanisms. In agreement with these predictions, the presence of gC in virion envelopes of infected cell membranes can interfere with complement-dependent virus neutralizing cytolytic antibodies (McNearny et al., 1987).

A previous study in our laboratory demonstrated that a mutant of HSV-1 strain KOS-321, gC^-39, with a deletion of the coding sequence of the gC gene, was less pathogenic than wild-type virus when inoculated intracerebrally into mice (Kümel et al., 1985). Four additional monoclonal antibody resistant (*mar*) mutants carrying antigenically altered gC molecules were also less pathogenic (Kümel et al., 1985). These findings prompted us to further explore the role of HSV-1 gC as a virulence factor.

In this study, we showed that the ability of eight additional gC^- mutant strains of HSV-1 to induce encephalitis in mice was highly variable. Studies of one gC^- strain with reduced neurovirulence by marker rescue to the gC^+ phenotype, in combination with passive immunization with monoclonal antibodies against gC, demonstrated that the gC^+ recombinant progeny did not show increased neurovirulence. Rather, it was the gC^- virions within the cotransfection progeny that had acquired the more pathogenic phenotype. These results were confirmed by an experiment showing virulence had not been restored to the gC^+ recombinants. These findings argue that gC is not an important virulence factor and indicate that the use of transfection procedures to marker-rescue a virulence determinant must be viewed with caution and should be carefully controlled.

Materials and Methods

Cells and viruses

Vero cells, BSC-1 cells, and human embryonic lung cells were grown in modified Eagle's minimum essential medium (MEM, GIBCO laboratories, Grand Island, NY) supplemented with 10% fetal calf serum (FCS, GIBCO) as described previously (Glorioso et al., 1980). Cell lines and virus stocks were mycoplasma free.

A plaque-purified isolate of wild-type HSV-1 strain KOS, designated KOS-321, was used as the parental gC^+ virus. The gC^- mutants were derived from KOS-321 following selection for replication in the presence of neutralizing monoclonal antibodies against gC (Holland et al., 1983b; Homa et al., 1986). Three of the nine mutants, gC^-5, gC^-13, gC^-39, have been shown to have an identical 1.7 kilobase deletion of the structural gene of gC. A fourth mutant, gC^-3, has a single nucleotide deletion resulting in premature termination of protein elongation, and lacks the carboxy-terminal 19 amino acids containing the transmembrane anchor sequence (Homa et al., 1986). As a result, gC is not detected on gC^-3 infected cell membranes, but is secreted as a truncated molecule into the medium. The molecular basis of the gC^- phenotype of gC^-17, gC^-20, gC^-32, gC^-40, and gC^-43 has not been determined. Virus stocks were produced by infection of Vero cells at low multiplicity at 37 °C and titers were determined by plaque assay.

Marker rescue at gC^-39

Mutant gC^-39 DNA was extracted from extracellular virions and infected cell lysates as described previously (Sandri-Goldin et al., 1981). The viral DNA released from virions was further purified by cesium chloride density gradient ultracentrifugation. BSC-1 monolayers were cotransfected with infectious gC^-39 DNA and a chimeric plasmid containing an HSV-1 KOS-321 restriction fragment spanning the gC gene by the calcium-phosphate precipitation method described by Graham and Van der Eb (1973) and modified for use in our laboratory (Sandri-Goldin et al., 1981). The construction of plasmids pFH60 and pFH60-ESt65 used in the cotransfections has been reported (Holland et al., 1984). Plasmids were digested with the appropriate restriction enzymes to excise the viral insert before use in cotransfections. Following incubation of cells with the cotransfecting DNA for 4 h, the cells were shocked with 25% DMSO to increase membrane permeability. Virus progeny plaques from cotransfections were examined for the presence of gC^+ virions using the immunoperoxidase staining technique, the "black plaque" assay (Holland et al., 1983a). Anti-gC antibody in the assay was a pool of monoclonal antibodies (Holland et al., 1984). Rescue frequency was determined by comparing the proportion of black plaques (gC^+) to white plaques (gC^-) on cell monolayers counterstained with crystal violet. Approximately 2000 plaques were counted to determine the percent rescue. Viable gC^+ virus was isolated from black plaques stained in the absence of glutaraldehyde fixation of infected cell monolayers (Johnson et al., 1986). Each isolate was plaque-purified three times and the black plaque assay repeated on each stock to confirm the gC^+ phenotype.

Immunoprecipitation

The rescued gC^+ isolates from the cotransfection of gC^-39 and the wild-type fragment were used to infect human embryonic lung cell monolayers at a multiplicity of 10. At 5 h post-infection, 20 μCi of [^{35}S]methionine in methionine-free MEM were added and incubated for 12 h. Infected cell lysates were precipitated with a

pool of gC-specific monoclonal antibodies, and the immune precipitates subjected to SDS–polyacrylamide slab gel electrophoresis as previously described (Holland et al., 1983b). Fluorographs of dried gels were made on Kodak XR-5 film exposed at −70 °C.

Thymidine kinase assay

Viruses were assayed for thymidine kinase activity in Vero cells using the Ara-T yield reduction method described previously (Tenser et al., 1979). A thymidine-kinase negative strain of HSV-1, KOS-018 (R. Tenser, Pennsylvania State Univ., Hershey, PA) was used as a negative control.

Mouse inoculation

Six-week-old female DBA/2 mice (Jackson Laboratories, Bar Harbor, ME) were conditioned for one week. Mice were anesthetized with methoxyflurane inhalation and 10 μl of virus diluted in MEM were injected with a 10 μl syringe fitted with a 26-gauge needle (Hamilton Co., Reno, Nevada) inserted to a depth of 3 mm into the right cerebral hemisphere. Serial half-log dilutions of KOS-321 or gC^-39 were each inoculated into groups of five mice. Mortality was recorded for 14 days post-inoculation and the 50% lethal dose (LD_{50}) calculated by the method of Reed and Muench (1938).

Virulence testing of marker rescued gC^-39

Each of fifty-six mice were inoculated with 10^4 PFU of the cotransfection progeny derived from the marker rescue experiment described above. Another group of 56 mice were inoculated with 10^4 PFU/mouse of a mixture of 2% KOS and 98% gC^-39, the same proportions of gC^+ and gC^- virions found in the cotransfection progeny. Twenty of the mice were observed separately for mortality over a period of 14 days. Of the remaining 36 mice, three survivors were sacrificed on each of days 1–6 post-inoculation. Their brains were removed, weighed, pooled, homogenized and suspended 1 : 10 in MEM. Virus concentrations in brain tissue (PFU/gram) were determined by plating supernatants on confluent monolayers of Vero cells. In addition, the numbers of gC^+ and gC^- viral plaques from brain tissue supernatants were determined using the immunoperoxidase black plaque assay. Additional control groups of ten mice each were inoculated with 10^4 PFU of gC^-39, or 10^3 PFU of KOS-321 and observed for mortality.

Passive immunization with monoclonal antibodies

Mice were passively immunized by intraperitoneal injection of a pool of three monoclonal antibodies directed against gC (C3, C4, C11) and then inoculated with either the cotransfection progeny or a mixture of 2% KOS and 98% gC^-39. Each of these monoclonal antibodies has been shown previously to protect against en-

cephalitis when given intraperitoneally in passive immunization experiments (Kümel et al., 1985). The 50% endpoint virus neutralization titers of monoclonal antibodies C3, C4, and C11 were 5120, 5120 and 40 960, respectively. One-tenth of a milligram of each of the three monoclonal antibodies was solubilized together in 0.5 ml PBS and administered 24 h prior to intracerebral inoculation of virus. Control mice were sham-injected with PBS. Forty-six mice were challenged with 10^4 PFU/mouse of the cotransfection progeny, or the 2% KOS and 98% gC$^-$39 virus mixture, and mortality rates (10 mice) and brain tissue titers of gC$^+$ and gC$^-$ virus (36 mice) determined as described above.

Statistical analysis

Statistical comparisons of mortality among groups of mice were performed by Chi-square analysis or Fisher's exact test, with significance defined as $P < 0.05$. In order to determine if there were correlations between thymidine kinase activities and mortality rates, correlation coefficients were determined by the method of least squares (Bourke et al., 1985).

Results

Neurovirulence of gC$^-$ mutants of HSV-1 KOS-321

The mortality rates for nine gC$^-$ mutants and the KOS-321 wild-type virus were compared as shown in Table 1, A. The mortality rate for the wild-type virus population was 100% for 50 female DBA/2 mice inoculated intracerebrally with 10^3 PFU/mouse. In a separate experiment, the LD_{50} for the wild-type virus was determined to be 10^2 PFU/mouse, in agreement with earlier findings (Kümel et al., 1985). The mortality rates for the nine gC$^-$ mutants at a dose of 10^3 PFU/mouse ranged from 0–80% (Table 1, A). Even the mortality rates for mutants gC$^-$5, gC$^-$13, and gC$^-$39, which carry the identical deletion of the gC gene (Homa et al., 1986), were variable; gC$^-$5 and gC$^-$39 caused no deaths while gC$^-$13 killed 4 out of 5 animals.

The variation in mortality observed among the gC$^-$ mutants suggested that there might be an inherent variability in neurovirulence among individual virions within the parent KOS population. Accordingly, virus from 10 plaque-purified isolates from KOS-321 were inoculated into mice. Mortality rates were found to be high and very similar among isolates (Table 1, B). Therefore, the variability among the gC$^-$ mutants could not be attributed to differences in virulence within the parent virus stock.

Since diminished virulence of HSV-1 had been previously associated with low or absent thymidine kinase activity (Field and Darby 1980; Sibrack et al., 1982; Stanberry et al., 1985; Tenser et al., 1985), the activity of that enzyme for each gC$^-$ mutant was determined using the Ara-T yield reduction assay (Tenser et al., 1979). Although some variation of tk activity among the gC$^-$ mutants was observed, there

TABLE 1

MORTALITY IN SIX-WEEK-OLD DBA/2 FEMALE MICE INOCULATED INTRACEREBRALLY WITH HSV-1 STRAIN KOS-321 AND GLYCOPROTEIN C NEGATIVE (gC^-) MUTANTS OF KOS-321.

Virus strain [b]	Mortality [c]	Thymidine kinase activity [d]
A. gC negative mutants of KOS-321 [a]		
KOS-321 stock	50/50	2.4
gC^-39 [e]	0/5	3.2
gC^-3	0/5	2.3
gC^-4 [e]	0/5	2.2
gC^-20	1/5	2.0
gC^-43	1/5	2.1
gC^-40	2/5	2.5
gC^-13 [e]	4/5	2.6
gC^-17	4/5	3.3
gC^-32	4/5	2.0
B. Individual isolates of KOS [f]		
No. 1	5/5	–
No. 2	4/5	–
No. 3	5/5	–
No. 4	5/5	–
No. 5	5/5	–
No. 6	4/5	–
No. 7	5/5	–
No. 8	5/5	–
No. 9	5/5	–
No. 10	4/5	–

[a] All gC^- mutants were derived from KOS-321 by escape from neutralization with a pool of gC-specific monoclonal antibodies and rabbit complement (Holland et al., 1983b).
[b] Inoculum is 10^3 PFU/mouse in 10 μl PBS.
[c] Number of deaths/number inoculated.
[d] Log_{10} PFU reduction of viral titer in presence of 50 μM Ara-T.
[e] Mutants with identical 1.7 kb deletions of the structural gC gene.
[f] Thymidine kinase activity not measured.

was no correlation between the level of tk activity and mortality ($r = 0.17$; Table 1, A). All HSV-1 gC^- mutants demonstrated at least 2 logs reduction in virus titer, indicating the presence of significant enzyme activity.

Neurovirulence of marker rescued gC^-39

The observation that there was variation in mortality rates among mice inoculated with nine gC^- mutants, whereas there was little variation in mortality rates among mice inoculated with ten wild-type virus isolates, suggested that gC may not play a role in neuropathogenesis. It is possible that the gC^- strains are not genetically the same in other loci, which in combination with the absence of gC,

might result in reduced neurovirulence. That is, the absence of gC would only affect neurovirulence in the context of particular alleles in other viral genes. Alternatively, the more virulent gC^- mutants might possess other virulence factors that compensate for their lack of gC. To explore these possibilities, the gC gene in a strain with reduced neurovirulence, gC^-39, was restored by marker rescue using a cloned restriction fragment carrying the wild-type gC gene. If restoration to the gC^+ phenotype also restored virulence to the wild-type level, then it might be concluded that gC contributes to neurovirulence, but only on a particular genetic background.

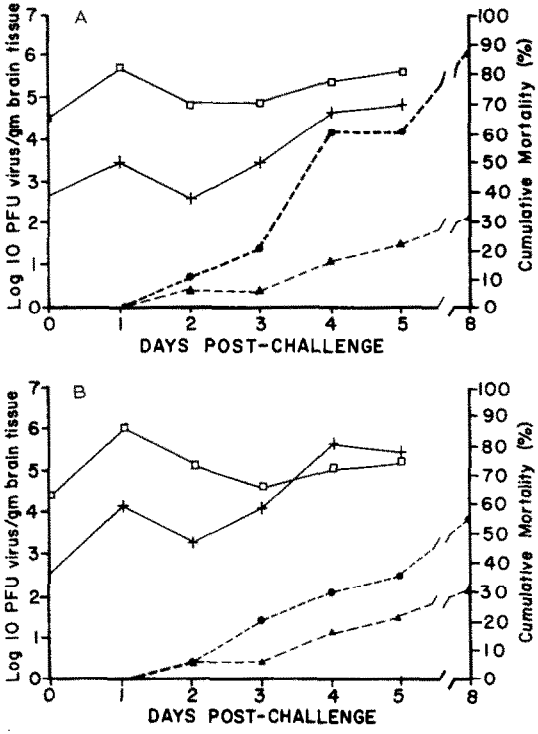

Fig. 1A. Viral titers of gC^+ (+———+) and gC^- (□———□) virus from pooled brain homogenates from three surviving mice/day. Cumulative mortality rate for twenty 6-week-old female DBA/2 mice inoculated intracerebrally with 10^4 PFU/mouse of cotransfection progeny derived from cotransfection of gC^-39 with gC^-39 DNA and plasmid pFH60 containing the wild-type KOS gC gene (●------●). Cumulative mortality rate for 10 mice injected with 10^4 PFU/mouse of the gC^- deletion mutant, gC^-39 (▲------▲).

Fig. 1B. Viral titers of gC^+ (+———+) and gC^- (□———□) virus from pooled brain homogenates from three surviving mice/day. Cumulative mortality rate for twenty 6-week-old female DBA/2 mice inoculated intracerebrally with 10^4 PFU/mouse of a mixture of 2% gC^+ KOS-321 and 98% gC^-39 containing the same percentages of gC^+ and gC^- virions as the marker rescue progeny in Fig. 1A (●------●). Cumulative mortality rate for 10 mice injected with 10^4 PFU/mouse of the gC^- deletion mutants, gC^-39 (▲------▲).

The mutant gC$^-$39 had a 200-fold reduction in virulence ($LD_{50} = 10^{4.5}$ PFU/mouse, data not shown) in comparison to KOS-321 and sustains a 1.7 kb deletion of the gC structural gene (Homa et al., 1986), ensuring that spontaneous reversion to the gC$^+$ phenotype would not occur.

Cotransfection of infectious gC$^-$39 DNA with pFH60, a clone containing the KOS gC gene and flanking sequences was performed and a rescue frequency of 2% gC$^+$ virus plaques was observed among the progeny. As expected, control cotransfections with gC$^-$39 DNA and pFH60-Est65, containing a KOS fragment that does not overlap the deletion in gC$^-$39, did not result in any gC$^+$ virus recombinants.

Previous work by Thompson et al. (1983a, b) demonstrated that increased mortality following direct brain inoculation of viral progeny derived from marker rescue experiments could be used to identify viral sequences that contribute to the virulence of HSV-1. Following this approach, the progeny obtained from cotransfection of gC$^-$39 DNA with pFH60 was inoculated into mice at a concentration of 10^4 PFU/mouse (Fig. 1A). Cumulative mortality among mice infected with the marker rescue progeny (18/20) was significantly greater than that in mice inoculated with gC$^-$39 (6/20) ($P = 0.0001$, Fisher's exact test). The proportion of gC$^+$ virions from brain tissue increased from the input inoculum of 2% to 19% by day 5 (Fig. 1A). These results are compatible with the interpretation that the gC$^+$ virions within the cotransfection progeny were responsible for the increased neurovirulence.

A reconstruction mixture of 2% KOS-321 and 98% gC$^-$39 was inoculated into a separate group of animals in order to mimic the same proportions of gC$^+$ and gC$^-$ virions as found in the cotransfection progeny (Fig. 1B). While cumulative mortality induced by the reconstruction mixture (11/20) was increased in comparison to that observed following inoculation with gC$^-$39 (6/20), this difference was not statistically significant ($P > 0.1$, Chi-square). In addition, the cumulative mortality rate for mice inoculated with the reconstruction mixture (Fig. 1B) was significantly less than that observed for animals inoculated with the cotransfection progeny ($P = 0.02$, Fisher's exact test; Fig. 1A). Because of this lower mortality, it might be expected that the proportion of gC$^+$ virions in brains of mice inoculated with the reconstruction mixture would be less than that for mice inoculated with the cotransfection progeny. Paradoxically, the proportion of gC$^+$ virus on day 5 in the brains of animals infected with the more virulent cotransfection progeny (19%, Fig. 1A) was lower than that observed among the animals receiving the reconstruction mixture (78%, Fig. 1B). These findings suggested that the gC$^-$ virions within the cotransfection progeny, rather than the gC$^+$ recombinants, were responsible for the increased virulence of the cotransfection progeny.

Neurovirulence of individual gC$^+$ isolates from the cotransfection progeny

In order to confirm that gC$^+$ recombinants were not responsible for the increased virulence of the marker rescue progeny, the mortality rate induced by each of 8 individual plaque-purified gC$^+$ recombinants was determined. As seen in Table 2, the mortality rates for individual recombinants were always much lower than those seen after inoculation of individual plaque isolates for KOS-321 (Table 1, B) and

TABLE 2

MORTALITY IN SIX-WEEK-OLD FEMALE DBA/2 MICE INOCULATED INTRACEREBRALLY WITH 10^3 PFU OF INDIVIDUAL gC^+ ISOLATES FROM COTRANSFECTION PROGENY. [a]

Virus strain [b]	Mortality [c]	Thymidine kinase activity [d]
KOS-321	5/5	2.4
gC^+ No. 1	1/5	2.2
gC^+ No. 2	0/5	1.4
gC^+ No. 3	0/5	1.8
gC^+ No. 4	1/5	3.1
gC^+ No. 5	0/5	2.0
gC^+ No. 6	0/5	4.4
gC^+ No. 7	0/5	4.2
gC^+ No. 8	2/4	3.1

[a] Cotransfection progeny from gC^-39 cotransfected with gC^-39 DNA and plasmid pFH60 from KOS-321.

[b] Nos. 1–8 are individual plaque-purified gC^+ isolates from the cotransfection progeny.

[c] Number of deaths/number inoculated.

[d] Log_{10} PFU reduction in viral titer in presence of 50 μM Ara-T.

were similar to that shown for the gC^-39 parent (Table 1, A). Likewise, the summed mortality for the eight gC^+ recombinants (4/40; Table 2) was significantly less than that found for wild-type KOS-321 virus (37/40; Table 1, B; $P < 0.001$, Fisher's exact test). Each of the gC^+ recombinants was shown to express the fully processed wild-type gC gene product, as determined by SDS–PAGE analysis of radioimmunoprecipitates of infected cell extracts (data not shown).

Passive immunization of mice with anti-gC monoclonal antibodies

The previous experiment suggested that the gC^+ recombinants in the cotransfection progeny were not responsible for the increased virulence, but rather that deaths were caused by gC^- virus. To confirm this hypothesis, mice were passively immunized with monoclonal antibodies against gC and inoculated 24 h later with the cotransfection progeny. This procedure would inhibit the replication and spread of gC^+ virus through the brain and could distinguish between neurovirulent gC^- and gC^+ virus in the cotransfection progeny. The cumulative mortality rate was 80% (8/10) for immunized mice receiving the cotransfection progeny (Fig. 2A) and was not significantly different from the 90% cumulative mortality rate (18/20) in unimmunized animals (Fig. 1A). However, only gC^- virions were found in brains of immunized mice inoculated with the cotransfection progeny by day 2 post-inoculation (Fig. 2A). Titers of gC^- virus increased rapidly and were similar to those from nonimmunized animals (Fig. 1A) throughout the 5 day post-inoculation period. As expected, the cumulative mortality rate in immunized mice inoculated with gC^-39 (40%, 4/10; Fig. 2A) was essentially the same as seen in nonimmunized mice (30%, 3/10; Fig. 1A). These results clearly showed that the gC^- virions were responsible

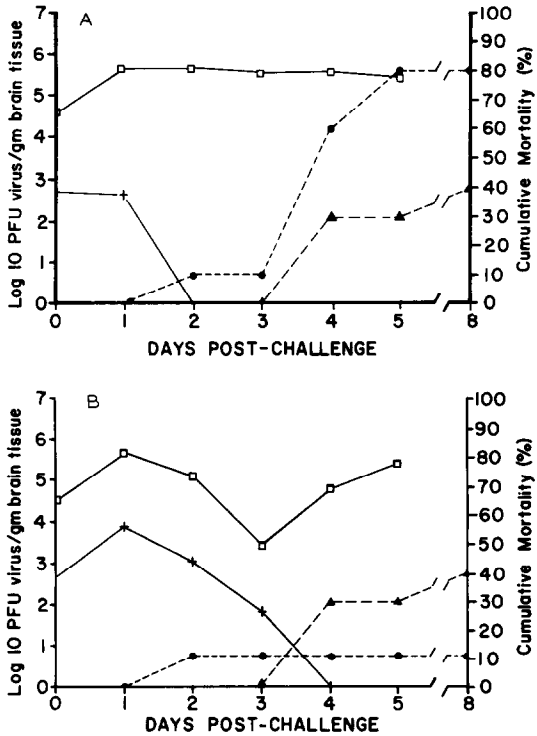

Fig. 2A. Viral titers of gC^+ (+ ——— +) and gC^- (□———□) virus from pooled brain homogenates from three surviving mice/day. Cumulative mortality rate in ten 6-week-old female DBA/2 mice inoculated intracerebrally with 10^4 PFU/mouse of cotransfection progeny derived from cotransfection of gC^-39 with gC^-39 DNA and plasmid pFH60 containing the wild-type KOS gC gene (●------●). The mice were passively immunized with anti-gC monoclonal antibodies 24 h prior to virus inoculation. Cumulative mortality rate in 10 passively immunized mice injected with 10^4 PFU/mouse of the gC^- deletion mutant gC^-39 (▲------▲).

Fig. 2B. Viral titers of gC^+ (+ ——— +) and gC^- (□———□) virus from pooled brain homogenates from three surviving mice/day. Cumulative mortality rate in ten 6-week-old female DBA/2 mice. The mice were passively immunized with anti-gC monoclonal antibodies, and inoculated intracerebrally with 10^4 PFU/mouse of a mixture of 2% gC^+ KOS-321 and 98% gC^-39 (●------●). Cumulative mortality rate in 10 passively immunized mice injected with 10^4 PFU/mouse of the gC^- deletion mutant gC^-39 (▲------▲).

for the increased virulence of the cotransfection progeny. In contrast, the cumulative mortality rate in passively immunized mice inoculated with the mixture of 2% KOS and 98% gC^-39 was only 10% (1/10; Fig. 2B), compared to 55% (11/20) in nonimmunized animals (Fig. 1B). This indicated that the gC^+ KOS-321 virions in the reconstruction mixture, although only a small proportion of the total population, contributed significantly to the deaths of the nonimmunized mice. Therefore, the gC^-39 virions were not virulent unless gC^-39 DNA has been subjected to the transfection procedure.

Discussion

In a previous paper, we reported that a gC^- strain of HSV-1 (KOS-321) had reduced virulence for the central nervous system of mice (Kümel et al., 1985). Here, further studies were carried out in an attempt to confirm that glycoprotein C of HSV-1 is a virulence determinant using a lethal mouse model of encephalitis. The wide variability in virulence among nine gC^- mutants derived from KOS-321 was unexpected because they were all selected for resistance to monoclonal antibodies against gC from the same parental virus stock. Thus, there was no known selection pressure on any other virus genes. Even the three gC^- mutants with identical deletions of the gC gene showed variation in virulence. The lack of a consistent correlation between mortality rates and the absence of gC implied that HSV-1 gC is not an essential virulence factor. In agreement with this conjecture, marker rescue of a gC^- deletion mutant was not successful in restoring virulence to the gC^+ recombinants. Similarly, we previously had been unable to show any difference in virulence between an HSV-2 isolate and its gC^- mutant (Johnson et al., 1986). Dix et al. (1983) also found no differences in virulence between a gC^+ strain (mP) of HSV-1 and a single gC^- mutant (MP) in BALB/c mice infected by either footpad or intracerebral routes of inoculation.

The genomic location of the HSV-1 gC gene is within map units 0.628–0.646 (Holland et al., 1984), a region which differs from the genomic locations that other groups have associated with virulence. Intertypic transfection experiments with HSV-1, strain KOS (M), have identified a locus within map units 0.25–0.53 conferring enhanced neurovirulence after footpad inoculation (Thompson et al., 1986). Within the same map region, Weise et al. (1987) showed that transfection of the *Bam*HI G fragment of HSV-1 from a virulent to a less virulent strain enhanced the virulence of the recombinants. They speculated that glycoprotein B, encoded within this region, might be a virulence determinant. Centifanto-Fitzgerald et al. (1982) found differences in rabbit corneal morphology following transfection of HSV-1 strain F DNA with the *Bgl*II F fragment (map units 0.70–0.83) from HSV-1, strain MP. Within a similar genomic region, map units 0.7–0.8, Rosen et al. (1985a, b) and Thompson et al. (1983a, b, 1985, 1986) identified HSV-1 fragments coding for factor(s) enhancing HSV-1 neurovirulence in tree shrews and mice, respectively. In addition, the viral thymidine kinase gene is located at map units 0.305–0.315 (McKnight, 1980), and thus would not be directly affected by our cotransfection experiments. The lack of correlation between virulence and thymidine kinase activity among gC^- mutants and gC^+ recombinants indicated that the variation in virulence seen in our experiments was probably not due to mutations of the thymidine kinase gene.

The increased virulence of gC^- virions from the cotransfection progeny presumably resulted from transfection-induced mutations in genes coding for unknown virulence factors. Other investigators have also reported increases in virulence of HSV following transfection of viral DNA. Thompson et al. (1986) studied an isolate related to HSV-1 KOS-321, designated KOS (M), which is virulent only by the intracerebral route and not via footpad inoculation. They reported that transfection

of KOS (M) DNA without a cotransfecting fragment resulted in increased neurovirulence in 10–20% of transfection progeny when tested by footpad inoculation. This presumably occurred by inducing mutations at unknown loci (Thompson et al., 1986). Schröder and Kümel (1986) similarly reported increased virulence among 10% of HSV-1 DNA transfections. In other experimental genetic systems, transfection has been shown to produce multiple mutations in shuttle plasmid vectors (Calos et al., 1983; Razzaque et al., 1983). Our results indicating increased virulence of gC^- HSV following transfection suggest that results of virulence studies using this technique should be carefully controlled and interpreted with caution.

Our approach to the identification of viral recombinants differed from that of other investigators. For example, Thompson et al. (1983a, 1983b) screened cotransfection progeny for neurovirulence in mice, plaque-purified the virus recovered from brain tissues, and then reinoculated mice for LD_{50} determinations. Virulent cotransfection progeny virions were examined for evidence of recombination with the cotransfected fragment from the virulent HSV-1 parent only after passage through animals. In contrast, our experiments avoided prior in vivo passage of the transfection progeny by utilizing monoclonal antibodies in the immunoperoxidase plaque assay to identify and recover individual gC^+ recombinants. Monoclonal antibodies also allowed us to measure the proportions of gC^+ and gC^- virions in brain tissue post-inoculation and to perform experiments with a reconstructed gC^+/gC^- virion mixture. In addition, passive immunization experiments with monoclonal antibodies selectively eliminated recombinant gC^+ virions from the central nervous system and allowed us to conclusively demonstrate that the gC^- virions were responsible for the increased virulence of the cotransfection progeny. Passive immunization with monoclonal antibodies should be a useful technique for studying the role of other HSV encoded proteins expressed on infected cell membranes as virulence factors.

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