Binding of Complement Component C3b to Glycoprotein C Is Modulated by Sialic Acid On Herpes Simplex Virus Type 1-Infected Cells

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Neuraminidase treatment of cells infected with herpes simplex virus type 1 (HSV-1) markedly enhanced the binding of complement component C3b to HSV 1 glycoprotein C (gC). When HSV-1 was grown in BHK RicR14 cells in which glycoproteins had reduced amounts of N-linked complex oligosaccharides, including sialic acid, the binding of C3b to gC was markedly enhanced. We used neuraminidase treatment to demonstrate that cloning the gC gene from the HSV-1 F strain into an HSV-1 mutant which fails to express gC converted the mutant virus from C3b receptor negative to receptor positive. These results further support a role for gC as a C3b receptor and indicate that sialic acid modifies receptor activity.

Herpes simplex virus type 1 (HSV-1) induces a receptor for the third component of complement on the plasma membrane of infected cells (2). The binding of complement component of C3b to this receptor is blocked by monoclonal antibody to HSV-1 glycoprotein C (gC) but not by monoclonal antibodies to HSV-1 gB, gD, or gE, (8). No C3b receptors are expressed after infection with the HSV-1 MP virus (8), a mutant which fails to express gC on infected cells (10, 18).

Posttranslational processing of gC involves the addition of sialic acid to the precursor form of the glycoprotein (3). Sialic acid is important in the regulation of complement activation (11, 14, 22) and is known to modify the binding of immunoglobulin G (IgG) to HSV-1-induced Fc receptors (4, 19) and to Fc receptors present on T lymphocytes (24). In this study, we evaluate the role of sialic acid on gC in modifying the binding of C3b to the viral receptor.

Human endothelial cells were harvested from umbilical veins and cultured on fibronectin-coated wells as previously described (5, 8, 16, 17). C3b receptors were detected 20 h after HSV-1 infection (NS strain) (9) by erythrocyte rosetting and by binding assays with ⁵¹Cr-labeled erythrocytes (8). Complement-coated erythrocytes were incubated with the monolayer for 2 h at 37°C, unbound erythrocytes were removed by washing, and monolayers were observed for rosettes. To determine the percentage of ⁵¹Cr binding, adherent erythrocytes were lysed with distilled water, and the release of ⁵¹Cr was measured. Blocking experiments were performed by incubating the monolayers with monoclonal antibody to HSV-1 gC for 30 min before the addition of erythrocytes (8). For neuraminidase experiments, monolayers were incubated for 30 min at 37°C with 0.05 U of neuraminidase (type x, from *Clostridium perfringens* [Sigma Chemical Co., St. Louis, Mo.]; 160 U/mg of protein). The enzyme was removed by washing, and complement-coated erythrocytes were added.

As in our previous studies (2, 8), EAC1423 cells (C3b-coated erythrocytes) bound to HSV-1-infected endothelial

cells but not to uninfected cells (by the ⁵¹Cr assay shown in Fig. 1, $t = 5.10$, $P < 0.01$); monoclonal antibody to gC specifically blocked the binding of EAC1423 cells, and in the absence of C3, binding of erythrocyte indicator cells (EAC142) did not occur. When endothelial cell monolayers were treated with 0.05 U of *C. perfringens* neuraminidase before the erythrocyte indicator cells were added, there was a marked increase in the rosetting of EAC1423 cells. Without neuraminidase, 50 to 75% of infected cells formed EAC1423 rosettes which were moderately dense (10 to 20 erythrocytes bound per cell). After neuraminidase treatment, 100% of the cells showed a very dense pattern of rosetting (>20 erythrocytes per cell). By the ⁵¹Cr binding assay, attachment of EAC1423 cells was greater to the enzyme-treated cells, $32.0 \pm 5.7\%$ (standard deviation), than to sham-treated cells, $8.5 \pm 1.5\%$ (Fig. 1, $t = 4.22$, $P < 0.01$). This additional binding of EAC1423 cells was totally blocked by monoclonal antibody to gC. Monoclonal antibody to gE had no blocking effect on the binding of EAC1423 cells. Neuraminidase did not increase the binding of EAC1423 cells to uninfected monolayers, nor did it increase the binding of EAC142 cells to HSV-1-infected endothelium (Fig. 1).

As a control for impurities in the neuraminidase preparation, a second source of neuraminidase was evaluated. Identical results were obtained with similar concentrations of *Vibrio cholerae* neuraminidase. In addition, for some experiments, 200 mM γ -D-galactolactone was added with the neuraminidase to the monolayer. This control was included to prevent impurities, if present, in the neuraminidase preparation from cleaving galactose residues from the glycoproteins. Results obtained were similar to those described above; i.e., a marked increase in the binding of EAC1423 cells was observed. As an additional control, neuraminidase was added to the EAC1423 cells rather than to the infected monolayer. This did not significantly alter the binding of the erythrocyte indicator cells to HSV-1-infected monolayers.

To determine the kinetics of the neuraminidase effect on C3b receptors, HSV-1-infected monolayers were incubated with neuraminidase for 1, 10, 20, 30, 45, or 120 min at 37°C. Neuraminidase was removed by washing, and erythrocyte indicator cells were added for 2 h at 37°C. After 1 min of incubation of the enzyme with the monolayer, rosetting of

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FIG. 1. Binding of ⁵¹Cr-labeled EAC1423 cells to sham- (routine) or neuraminidase-treated HSV-1-infected human umbilical vein endothelial cells. EAC1423 binding represents the mean of seven experiments. Blocking studies with anti-gC monoclonal antibody is the mean of two experiments.

EAC1423 cells increased from 25 to 75%. After 20 min, very dense rosetting was detected around 100% of infected cells. By the ⁵¹Cr binding assay, near-maximal binding was detected after 30 min of incubation with neuraminidase.

To determine the optimal concentration of neuraminidase, various amounts of enzyme (0.02, 0.05, 0.10, and 0.25 U) were added to the monolayer in 0.2-ml volumes for 30 min. Even at the lowest concentrations examined (0.02 U), a marked increase in the binding of ⁵¹Cr-labeled EAC1423 cells was detected. Higher concentrations increased the binding of EAC1423 cells only slightly.

We examined the expression of C3b receptors on BHK and BHK RicR14 cells (kindly provided by R. C. Hughes, London, England). The latter were evaluated to further support a role for sialic acid in modulating receptor function. BHK RicR14 cells are mutant BHK cells deficient in *N*-acetyl glucosaminyl transferase 1, an essential enzyme in the assembly of complex *N*-linked oligosaccharides (20, 21, 27). In HSV-1-infected BHK RicR14 cells, truncated forms of viral *N*-linked glycoproteins with reduced amounts of *N*-acetyl glucosamine, galactose, and sialic acid accumulate on the infected cell surface. In spite of the differences in glycosylation, HSV-1 replicates in BHK RicR14 cells to yields similar to those in parental BHK cells (1).

C3b receptors were detected on HSV-1-infected BHK and BHK RicR14 cells. As on human umbilical vein endothelial cells, C3b receptors were not present on uninfected cells. In the absence of C3, erythrocyte indicator cells did not form rosettes on HSV-1-infected cells, although the background binding as determined by the ⁵¹Cr assay was considerably higher than that measured on endothelial cells ($10.5 \pm 5.5\%$ on BHK cells and $7.7 \pm 0.5\%$ on BHK RicR14 cells). The results of neuraminidase treatment of BHK cells demonstrated a marked increase in the binding of ⁵¹Cr-labeled EAC1423 cells to HSV-1-infected cells (Fig. 2). When HSV-1 was grown in BHK RicR14 cells, expression of C3b receptors was enhanced compared with wild-type BHK cells ($59.1 \pm 2.4\%$ versus $21.5 \pm 5.9\%$, $t = 5.90$, $P < 0.05$, Fig. 2).

This extensive binding of EAC1423 cells to the mutant BHK cells supports the results of the neuraminidase experiments.

The amount of gC on the cell surface may influence the extent of C3b binding; therefore, we compared the amount of gC on the surface of BHK cells with that on BHK RicR14 cells. At 24 h postinfection, saturating concentrations of anti-gC monoclonal antibodies were added to infected BHK and BHK RicR14 cells. Antibody binding to infected cells was measured by ¹²⁵I protein A (Amersham Corp., Arlington Heights, Ill.). By this assay, equivalent concentrations of gC were present on the surface of the two cell types at a time when detection of C3b receptors was markedly enhanced on BHK RicR14 cells.

As expected, neuraminidase treatment of BHK RicR14 cells had little effect on the binding of EAC1423 to C3b receptors (Fig. 2), presumably because on these cells gC already contained reduced amounts of sialic acid. On both BHK and BHK RicR14 cells, monoclonal antibody to gC blocked binding of EAC1423 cells to background levels, indicating the specificity of the binding (Fig. 2).

To demonstrate that gC is altered by neuraminidase treatment and by growth in BHK RicR14 cells, cell extracts were prepared 20 h postinfection, and immunoblots were performed as described in the legend to Fig. 3. Neuraminidase treatment of extracts from HSV-1-infected endothelial cells changed the migration position of fully glycosylated gC (lane 2) and gD (lane 4). The latter was examined to provide additional support for the alterations of the viral glycoproteins by neuraminidase treatment. In BHK RicR14 cells, gC migrated at a position similar to that of the precursor form of gC (lane 6).

We used neuraminidase treatment to evaluate the expression of C3b receptors on human umbilical vein endothelial cells after infection with mutants of HSV-1. The strains examined included MP (12), which does not express gC (10, 18); MP 6-9A, which has a wild-type allele of gC from the HSV-1 F strain inserted into the thymidine kinase gene of MP (kindly provided by P. Spear, Chicago, Ill.) (15); and the

FIG. 2. Binding of ⁵¹Cr-labeled EAC1423 cells to HSV-1-infected BHK and BHK RicR14 mutant cells following sham (routine) or neuraminidase treatment of infected monolayers. Values represent the mean of two experiments.

FIG. 3. Infected cell extracts from HSV-1-infected BHK cells were incubated with neuraminidase (0.05 U) for 1 h at 37°C and run by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with 10% polyacrylamide. The proteins resolved by electrophoresis were transferred to nitrocellulose paper, and immunoblotting was performed (26) with rabbit polyclonal immunoglobulin G anti-gC (lanes 1 and 2) or anti-gD (lanes 3 and 4). Lanes 1, gC without exposure to neuraminidase; 2, gC after neuraminidase treatment; 3, gD without exposure to neuraminidase; 4, gD after neuraminidase treatment. Both gC and gD are altered by neuraminidase treatment. In separate experiments, infected cell extracts were prepared from BHK cells or BHK RicR14 cells, and immunoblots were performed with anti-gC serum. Lane 5, gC in BHK cells. Lane 6, gC in BHK RicR14 cells. A truncated form of gC is detected in BHK RicR14 cells.

HSV-1 F strain. The parental source of gC, the HSV-1 F strain, expressed C3b receptors which were enhanced by neuraminidase treatment and blocked by monoclonal antibody to gC (Fig. 4a). Human endothelial cells infected with MP did not express C3b receptors either with or without neuraminidase treatment (Fig. 4b). After infection by MP 6-9A, rosetting of EAC1423 cells was detected around 5 to 10% of endothelial cells. Anti-gC monoclonal antibody reduced rosetting to <1%, indicating specific binding. By the ^{51}Cr assay, binding of EAC1423 cells to MP 6-9A-infected cells was $5.0 \pm 2.9\%$ (Fig. 4c) which, however, was not significantly different from binding to MP-infected cells ($1.2 \pm 0.1\%$; $t = 2.02$, $P = 0.1$, comparing the means of three experiments). When MP6-9A-infected cells were treated with neuraminidase, rosettes were detected around 50% of the cells, and binding of ^{51}Cr -labeled EAC1423 cells was $31.7 \pm 4.4\%$ (Fig. 4c, $t = 5.01$, $P < 0.001$, compared with binding to neuraminidase-treated, MP-infected cells). Rosetting and binding of ^{51}Cr -labeled EAC1423 cells were partially blocked by anti-gC monoclonal antibody and were totally blocked by anti-gC polyclonal serum (Fig. 4c). These results indicate that cloning the DNA which codes for gC of F strain into MP

results in expression of a C3b receptor on infected cells. The removal of sialic acid with neuraminidase enhances recognition of the receptor.

Five laboratory-adapted strains (McIntyre, Patton, HF, F, and KOS) were examined for C3b receptor activity on human umbilical vein endothelial cells. EAC1423 cells formed rosettes around 50 to 75% of infected cells with each strain tested, except for KOS. With KOS only a rare infected cell (1 to 2%) developed a rosette. However, when KOS-infected cells were treated with neuraminidase, C3b receptors were readily detected, both by rosetting and by ^{51}Cr binding assays. By the latter assay, EAC1423 binding without neuraminidase treatment was at background levels, which increased to $16.0 \pm 2.6\%$ after treatment with neuraminidase ($t = 10.88$, $P < 0.01$). Neuraminidase had no effect on the binding of control EAC142 cells to the infected monolayer. Monoclonal antibody to gC completely blocked the binding of EAC1423 cells to the neuraminidase-treated KOS-infected cells.

HSV-1 infection induces C3b receptors on a variety of cell types (25). We examined whether neuraminidase treatment modifies the binding of EAC1423 cells to gC on these other cells. With neuraminidase treatment (0.05 U), a marked increase in rosetting of EAC1423 cells to HSV-1-infected human embryonic lung fibroblast cells (MRC-5 strain) was noted. Without neuraminidase treatment, 25% of MRC-5 cells had rosettes which were sparsely distributed over the cell surface. After treatment with neuraminidase, 75 to 100% of the cells had rosettes which were densely packed around the infected cells. By the ^{51}Cr assay, neuraminidase treatment increased binding of EAC1423 cells from $5.0 \pm 0.4\%$ to $36.6 \pm 2.8\%$ ($t = 14.90$, $P < 0.001$). A similar increase in binding was demonstrated on HSV-1-infected human embryonic kidney cells. Neuraminidase treatment changed the percentage of cells showing rosettes from 50 to 100%. The binding of ^{51}Cr -labeled EAC1423 cells also increased from $9.4 \pm 3.3\%$ to $31.7 \pm 5.4\%$ ($t = 3.76$, $P < 0.02$).

We previously reported that cells infected with HSV-2, cytomegalovirus, varicella-zoster virus, adenovirus 7, or mumps or measles viruses do not express C3b receptors (25). We examined whether neuraminidase treatment of cells infected with these viruses unmasks C3b receptors. No C3b receptors were detected with either sham or neuraminidase treatment. Neuraminidase did, however, enhance detection of HSV-1- and HSV-2-induced Fc receptors. By the ^{51}Cr assay, binding of IgG-coated erythrocytes to HSV-1-infected cells was $9.2 \pm 2.0\%$ without neuraminidase, compared with $20.3 \pm 2.8\%$ after neuraminidase treatment ($t = 5.57$, $P < 0.01$).

Several functions have been assigned to C3b receptors (CR 1) present on blood cells including human erythrocytes, polymorphonuclear leukocytes, monocytes, and lymphocytes. Functions include binding and receptor-mediated internalization of C3b-coated antigen-antibody complexes, inhibition of classical and alternative pathway C3 convertase activity, and blood factor H-like activity converting C3b to the inactive product iC3b (6, 7, 13). If the C3b receptor on HSV-1-infected cells functions in a similar manner, it may protect the virus-infected cell from complement-mediated injury. In this report we demonstrate that sialic acid modulates C3b receptor expression on HSV-1-infected cells. Whether this increased binding occurs because of reduced negative charge, conformational changes in gC, or unmasking of the C3b binding site on gC remains to be determined. We also show variability among HSV-1 strains in C3b receptor expression. Host cell differences in glycosylation of

FIG. 4. (a) C3b receptor expression after infection of human umbilical vein endothelial cells with the HSV-1 F strain. Neuraminidase treatment of infected cells increases the binding of EAC1423. Receptor detection is blocked by anti-gC monoclonal antibody. (b) MP mutant fails to express C3b receptors, even after neuraminidase treatment. (c) Reappearance of C3b receptors in the MP 6-9A mutant which contains cloned DNA from the gC gene of the HSV-1 F strain (15). Receptor detection is partially blocked by anti-gC monoclonal antibody and totally blocked by anti-gC polyclonal serum.

HSV-proteins have been reported (23); therefore, even the same strain of HSV-1 may vary in the extent to which it expresses C3b receptors, depending on which organ or cell type is infected. These differences may be important causes of variations in strain virulence or in understanding the propensity for HSV-1 to cause disease in particular body organs.

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