Utilization of Sialic Acid as a Coreceptor Enhances Reovirus Attachment by Multistep Adhesion Strengthening*

Many serotype 3 reoviruses bind to two different host cell molecules, sialic acid and an unidentified protein, using discrete receptor-binding domains in viral attachment protein, α1. To determine mechanisms by which these receptor-binding events cooperate to mediate cell attachment, we generated isogenic reovirus strains that differ in the capacity to bind sialic acid. Strain SA+, but not SA−, bound specifically to sialic acid on a biosensor chip with nanomolar avidity. SA+ displayed 5-fold higher avidity for HeLa cells when compared with SA−, although both strains recognized the same proteinaceous receptor. Increased avidity of SA+ binding was mediated by increased \( k_{\text{on}} \). Neuraminidase treatment to remove cell-surface sialic acid decreased the \( k_{\text{on}} \) of SA+ to that of SA−. Increased \( k_{\text{on}} \) of SA+ enhanced an infectious attachment process, since SA+ was 50–100-fold more efficient than SA− at infecting HeLa cells in a kinetic fluorescent focus assay. Sialic acid binding was operant early during SA+ attachment, since the capacity of soluble sialyllactose to inhibit infection decreased rapidly during the first 20 min of adsorption. These results indicate that reovirus binding to sialic acid enhances virus infection through adhesion of virus to the cell surface where access to a proteinaceous receptor is thermodynamically favored.

The ligation of a virus particle to specific cell-surface molecules is the primary interaction between the virus and its host, and as such it is a critical determinant of viral disease outcome and a potential target for antiviral therapy. Studies with enveloped mammalian viruses, including members of the herpesvirus (1–3) and retrovirus (4–6) families, have demonstrated that viral attachment is more complex than a bimolecular interaction between a viral attachment protein and a cellular receptor. Instead, attachment strategies employed by these enveloped viruses involve multiple interactions between several viral and cellular molecules, and these interactions are often accompanied by dramatic conformational rearrangements of viral proteins. In addition, many enveloped viruses employ an adhesion-strengthening attachment strategy in which primary virus-cell interactions require low affinity adhesion of the virus to common cell surface molecules that are often carbohydrate in nature (7–9). This initial phase of attachment is then followed by higher affinity interactions between the virus and a secondary receptor on permissive cells, an event that often triggers virus entry through direct membrane fusion or receptor-mediated endocytosis (10–13).

In contrast to our developing understanding of mechanisms of enveloped virus attachment, less is known about attachment strategies of nonenveloped viruses, a group that includes several important human pathogens. The mammalian reoviruses offer a useful experimental system to dissect the contributions of discrete steps in the viral replication cycle to pathogenesis in the infected host (14). Following oral inoculation into newborn mice, serotype 1 (T1) reovirus strains spread hematogenously to the central nervous system and replicate within ependymal cells, resulting in hydrocephalus. In contrast, serotype 3 (T3) reovirus strains utilize neural spread pathways to gain access to the central nervous system, where they replicate in a wide variety of neurons and cause encephalitis (15, 16). Since reovirus contains a segmented genome, it is possible to link pathogenic phenotypes to individual viral genes using reassortant viruses. Using this approach, it was determined that the mode of spread in the host (17) and cell tropism in the central nervous system (18, 19) segregates with the viral S1 gene segment, which encodes the viral attachment protein, α1 (20, 21). These findings suggest that reovirus tropism and disease outcome are determined by interactions between cell-surface receptors and the α1 protein.

The α1 oligomer has two structural domains, an aminoterminal fibrous tail, which anchors α1 at each of the icosahedral vertices of the virion, and a compact globular head located at the carboxyl terminus (22, 23). T3 α1 recognizes at least two cellular molecules using discrete, independently functional receptor-binding domains (RBDs). Sequences in the α1 head domain of all T3 strains bind to an unknown cellular molecule that is probably protein in nature. Recognition of the α1 head receptor is critical for virus binding and infection in vitro (24–30), and this interaction modulates tropism and disease outcome in infected mice (31–33). Sequences in the α1 tail domain

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1 The abbreviations used are: T1 and T3, serotype 1 and 3, respectively; FFU, fluorescent focus units; RBD, receptor-binding domain; MEL, murine erythroleukemia; mAb, monoclonal antibody; UTR, untranslated region; SPR, surface plasmon resonance; PBS, phosphate-buffered saline; D-PBS, Dulbecco’s phosphate-buffered saline; SLL, α-sialyllactose; pfu, plaque-forming unit(s); MOI, multiplicity of infection.
of some T3 strains confer the capacity to bind terminal α-linked sialic acid residues on glycosylated cell surface structures (30, 34–39). Recognition of sialic acid by the σ1 tail RBD can serve as a tropism determinant for some cell types in culture (38, 40); however, it is not clear whether binding to sialic acid is sufficient to permit virus entry or merely facilitates σ1-head receptor interactions. The mechanisms by which sialic acid binding and σ1 head receptor binding cooperate to achieve stable, cell-specific reovirus attachment and entry in cultured cells and in the infected host are unknown.

The presence of discrete RBDS in σ1 suggests that reoviruses employ multi-step binding processes analogous to those described for some enveloped viruses. However, attachment mechanisms used by reovirus may be unique, since reoviruses are nonenveloped, and both RBDS exist at a distance of a few hundred angstroms within the same viral protein. Studies to determine mechanisms by which the tail and head RBDS of σ1 cooperate to achieve stable virus-cell association, specific host cell tropism, and modulation of disease in the infected animal have not been possible to date, since isogenic virus strains differing only in the capacity to utilize a distinct receptor have not been available. To address these questions, we selected reovirus mutants that differ in sialic acid-binding capacity, and we used reassortant genetics to place the σ1-encoding S1 genes of these strains into identical genetic backgrounds. Using these strains, we demonstrate that reovirus attachment is a multi-step process, with primary sialic acid binding serving as a low affinity adhesion event that accelerates virus attachment but must be followed by σ1-head receptor interactions to permit efficient virus entry and infection.

MATERIALS AND METHODS

Cells, Viruses, and Antibodies—Spun-adapted murine L929 (L) cells were grown in either suspension or monolayer cultures in Joklik's modified Eagle's minimal essential medium (Irvine Scientific, Santa Ana, CA) supplemented with 5% fetal bovine serum (Intergen, Purchase, NY), 2 mM L-glutamine, 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 0.25 μg/ml of amphotericin (Irvine Scientific). Suspension cultures of murine erythroleukemia (MEL) cells were grown in F-12 medium (Irvine Scientific) supplemented to contain 10% fetal bovine serum, L-glutamine, and antibiotics as for L cells. HeLa cells were maintained in monolayer culture in Dulbecco's minimal essential medium (Life Technologies, Inc.) supplemented to contain 10% fetal bovine serum, L-glutamine, and antibiotics as for L cells. Reovirus strains T1L, T3C44 (41, 42), and T3C44-MA (38) are laboratory stocks. Viral titer (expressed as plaque-forming units (pfu)/ml) was determined by plaque assay on L-cell monolayers (43). Purified virions were prepared by using third-passage cell lysate stocks of plaque-purified reovirus as described previously (22). Viral particle concentrations were determined by measurement of A260 using a conversion factor of 2.1 × 1012 viral particles/mg A260 (44). Particle/pfu ratios of stocks used for viral infectivity assays were ~250:1. Murine monoclonal antibodies (mAbs) 9BG5 (G5) (45) and 5C6 (46) were prepared from hybridoma culture supernatants (Cell Culture Center, Minneapolis, MN), and Fab fragments (Fabs) of each were prepared using the Immunopure Fab Purification system (Pierce) according to the manufacturer's instructions.

Generation of Reovirus Mutants Differing in the Capacity to Bind Sialic Acid—Reassortant viruses were isolated by coinfecting monolayers of L cells with T1L and either T3C44 or T3C44-MA. Coinfections, plaque-purification, amplification of progeny viruses, and genotyping of reassortant viruses were performed as described previously (47). Two sequential rounds of reassortment were performed to obtain monoresortant viruses that retained the S1 gene segment from the T3 parental virus with all other gene segments derived from T1L. These monoresortant strains were designated T3C44-S1 and T3C44-MA-S1, abbreviated SA and SA+, respectively, throughout this paper. Electrophoresis of monoresortant gene segments was verified by purifying genomic dsRNA from CsCl-band purified virions (5 × 1011 particles) using TriReagent (Molecular Research Center, Cincinnati, OH). Purified gene segments were resolved in 7% SDS-polyacrylamide gels for 550 (small class gene segments), 950 (medium class gene segments), or 1380 (large class gene segments) mA/h at constant current, stained with EtBr, and visualized by UV transillumination. Following reassortment, the S1 gene segments of both strains were amplified by reverse transcription-polymerase chain reaction using primers complementary to the 5′- and 3′-untranslated regions (UTRs) (48), cloned into the pCR 2.1 vector, and sequenced using T4 DNA polymerase (Sequenase 2.0, U.S. Biochemical Corp.). In addition, the S1 gene UTR region of each strain was sequenced by direct sequencing of purified viral genomic RNA as described previously (37).

Assessment of Virus-Sialic Acid Interactions Using Surface Plasmon Resonance (SPR)—Glycoporin and asialglycoporin (1 mg/ml in PBS (Sigma) were biotinylated by incubation with a 10–100-fold molar excess of sulfo-NHS-biotin (Pierce) at room temperature for 2 h. Biotinylated glycoporin or asialglycoporin was injected at a concentration of 5–50 μg/ml in PBS across duplicate flow cells of a BIAcore streptavidin chip at a flow rate of 5 μl/min using a BIAcore 2000 instrument (Amersham Pharmacia Biotech). Sensor chip flow cells were coated with 1000–2000 resonance units of each cell, followed by rinsing in running buffer (Dulbecco’s PBS (D-PBS)) to remove unbound protein. Purified virions of strains SA− and SA+ were injected across the conjugated chip surfaces at various concentrations at a flow rate of 25 μl/min. Following virus binding, chip surfaces were regenerated with a 1-min pulse of 1 M NaCl, 20 mM NaOH. Surfaces treated in this manner showed no significant decrease in virus-binding capacity for up to 30 cycles of regeneration. In some experiments, virions were preincubated with α-sialyllectose (SLL) or lactose (Sigma) at room temperature for 1 min prior to injection. Since the refractive index of concentrated virus and saccharide solutions caused significant bulk shift effects during injection, specific binding of virions to sialic acid was calculated by subtracting resonance units measured on asialglycoporin-coated reference surface from binding to the glycoporin-coated surface prior to data analysis. No time-dependent increase in virus binding to asialglycoporin was detected under any conditions. Affinity constants of strain SA+ binding to sialic acid were determined using simultaneous k_d/k_a nonlinear regression with BIAevaluation 3.0 software (Amersham Pharmacia Biotech), assuming either a 1:1 Langmuir binding model or a bivalent analyte binding model (49–51).

Neuraminidase Treatment of Cells to Remove Sialic Acid—Terminal sialic acid residues were removed from cell-surface carbohydrates by incubating 5 × 106 cells/ml at 37 °C for 1 h in 1.0 ml of D-PBS containing 40 milliunits/ml Arthrobacter ureafaciens neuraminidase (Sigma) (38). Cells were washed in D-PBS to remove neuraminidase, resuspended in D-PBS, and processed for binding assays.

Virus Radioligand Binding Assays—Purified virus particles (2–4 × 1012/ml in D-PBS) were iodinated using the IODO-GEN two-step method (Pierce). Na125I (2.5 μCi) was oxidized for 6 min in IODO-GEN buffer, and iodinated virions were separated by centrifugation at 1.5–1.6 ml of purified virus at room temperature for 6 min. Viruses was separated from unincorporated 125I on 10-ml dextran D-Salt columns (Pierce) followed by dialysis against 4 liters of D-PBS. Labeled virus was stored at 4 °C in the presence of 10 mM NaN3, 5 mM 2-deoxyglucose, and 2 mM NaF (Sigma). Specific activity of iodinated virus was 0.5 to 1 × 106 cpm/particle. Greater than 85% of cpm was precipitable from iodinated virus preparations using 10% trichloroacetic acid precipitation or immunoprecipitation with anti-reovirus mAbs. Iodinated virion preparations were used for up to 4 weeks after iodination. Iodination of virions did not affect σ1 function as assessed by hemagglutination assay or interaction with conformationally sensitive anti-σ1 mAb G5, and no iodination of σ1 was detected following SDS-polyacrylamide gel electrophoresis analysis of labeled reovirus virions.

Radioligand binding assays were designed, and data were analyzed using general guidelines and equations as described (52). HeLa cells were detached from flasks by incubation in PBS plus 3 mM EDTA at 37 °C for 30 min followed by gentle pipetting. L cells were obtained from suspension cultures. Cells were pelleted at 250 × g for 5–6 min, resuspended at 2–4 × 107/ml in D-PBS supplemented with metabolic inhibitors (10 mM NaN3, 5 mM 2-deoxyglucose, and 2 mM NaF), and incubated at 37 °C for 15–20 min to deplete cellular ATP. This treatment abolishes receptor-mediated endocytosis in HeLa cells (53) and in L cells. For experiments in which neuraminidase-treated cells were used, 40 milliunits/ml neuraminidase were included with metabolic inhibitors, and incubations were extended to 1 h. Iodinated virus was diluted in 1:5 or 1:100 mixing media containing metabolic inhibitors and Complete EDTA-free protease inhibitor mixture (Roche Molecular Biochemicals; final concentration 0.5× in all binding assays). Virus and cells were incubated in 1.5-ml Eppendorf tubes at room temperature for various intervals with continuous rotation. The viability of cells incubated in this manner was...
not diminished for up to 8 h as determined by trypan blue exclusion. At the lowest concentrations of virus used in these experiments, equilibrium was reached within 6 h, and cell-bound virus was stable for up to 8 h. Cell-bound virus was captured by vacuum filtration onto Membralyn MF MB filters (5-μm pore size) (Whatman), followed by rinsing for 4 s with 50 ml of cold PBS. Filters were air-dried, and bound virus was quantitated by liquid scintillation counting in 4 ml of BIOSAFE II fluid (Research Products International, Mt. Prospect, IL) using a Beckman LS6500 counter (Beckman Instruments). Nonspecific binding was determined at several concentrations of labeled virus by incubating duplicate samples in the presence of 0.8–1 × 10^5 unlabeled virions, the maximum concentration of virus shown to compete virus without isotope dilution. Nonspecific binding was found to be a linear function (ranging from 0.5 to 1%) of input cpm under all conditions used and did not increase with time. For competition experiments, iodinated and unlabeled virions were added simultaneously to cells. For experiments assessing the effect of SLL or Fab on virus attachment, iodinated virions were preincubated with the indicated concentration of each reagent at 37 °C for 30 min.

For kinetic assays, virus association (k_{on}) and dissociation (k_{off}) binding constants were derived from the observed rate of virus binding (k_{obs}) over time using the formula,

\[ k_{obs} = k_{on}[\text{virus}] + k_{off} \]  
(Eq. 1)

and by comparing k_{obs} for each strain at multiple virus concentrations to exclude the contribution of k_{off}. This method permits the simultaneous determination of k_{on} and indirect inference of k_{off} (52).

Assessment of Virus Growth—Purified virions were diluted in gelatin saline or incubated at 37 °C for 30 min in PBS alone, 50 μg/ml G5 or 5C6 Fab, or 10 mM SLL or lactose. Tissue culture medium was aspirated, and 1 ml of complete culture medium was added. Cells were incubated at 37 °C for various intervals, followed by two cycles of freezing and thawing to release progeny virions. Titers of infectious virions were quantitated by plaque assay on L cells (43).

Fluorescent Focus Assays of Viral Infectivity—Unlabeled, purified virions were preincubated in PBS alone, 50 μg/ml G5 or 5C6 Fab, or 10 mM SLL or lactose at 37 °C for various intervals. To directly measure infectivity of treated virions, virus inocula were adsorbed to confluent HeLa cell monolayers (2 × 10^5 cells/well) as for growth experiments. Following incubation at 37 °C for 18 h to permit completion of a single round of viral replication, cell monolayers were fixed with 1 ml of methanol at −20 °C for a minimum of 30 min. Fixed monolayers were washed twice in PBS, blocked with 5% immunoglobulin-free BSA (Sigma-Aldrich) in PBS, and incubated at 37 °C for 30 min with protein A-affinity purified rabbit anti-reovirus serum (54) at a 1:800 dilution in PBS plus 0.5% Triton X-100. Monolayers were washed twice in PBS plus 0.5% Triton X-100 and incubated at 37 °C for 30 min with a 1:1000 dilution of anti-rabbit goat Ig conjugated with Alexa488 (Molecular Probes, Inc., Eugene, OR). Monolayers were washed twice, and infected cells were visualized by indirect immunofluorescence. Infected cells were identified by the presence of intense cytoplasmic fluorescence that was excluded from the nucleus. No background stain was noted on uninfected control monolayers. Reovirus antigen-positive cells were quantitated by counting fluorescent cells in three random fields of view per well at 100–400 × magnification.

RESULTS

Generation of Isogenic Reovirus Mutant Strains Differing in the Capacity to Bind Sialic Acid—To dissect the contributions of discrete reovirus-receptor interactions to virus attachment and host cell infection, we used reassortant genetics to construct isogenic reovirus strains that differ only in the capacity to bind sialic acid. T3C44 is a T3 reovirus field isolate that does not bind sialic acid (34, 38). Following two successive rounds of coinfection and genetic screening, two viruses were isolated that contained the S1 gene of either T3C44 (strain SA−) or T3C44-MA (strain SA+), and all other gene segments from T1L (Fig. 1). Following reassortment, the S1 genes of both monoreassortant strains were sequenced and were found to contain no additional mutations (data not shown).

Quantitation of α1-Sialic Acid Interactions Using an SPR Biosensor—To obtain a quantitative assessment of the avidity of SA− and SA+ for sialic acid, we utilized SPR to measure interactions between virions and sialic acid residues in real time in the absence of molecular labeling. The erythrocyte glycoprotein bound by T3 strains in hemagglutination assays is glycophorin A (55), and treatment of glycoporphin with neuraminidase to remove terminal sialic acid residues abrogates virus binding to this protein (37, 39). We therefore used biotin/streptavidin chemistry to attach glycophorin and asialoglycoporphin to an SPR detector chip and assessed the capacity of SA− and SA+ to bind to these surfaces. The asialoglycoporphin-coated chip served as a reference surface to measure nonsialic acid-dependent interactions between virus and either glycophorin or the chip surface (49, 50). We found that SA− was incapable of binding to either glycophorin or the asialoglycoporphin surface, while SA+ bound saturably and specifically to glycophorin (Fig. 2A) but not to asialoglycophorin (data not shown). To confirm that binding of SA+ to glycophorin was sialic acid-mediated, we tested the capacity of SLL, a soluble trisaccharide that mimics the α-linked sialic acid residues of cellular oligosaccharide chains, to inhibit virus binding to the glycophorin-coated biosensor surface (Fig. 2B). SLL inhibited binding of SA+ to glycophorin in a dose-dependent manner, displaying an IC_{50} of ~500 μM and achieving complete inhibition at 4 mM. Preincubation of virions with 4 mM lactose had only a modest inhibitory effect on SA+ binding to glycophorin, indicating that the effect of SLL was mediated specifically by the sialic acid residue (Fig. 2B).

Since SPR measurements of virus-sialic acid interactions in real time, it was possible to directly quantitate the apparent avidity, on-rate, and off-rate of reovirus for sialic acid using nonlinear regression analysis (Table I). When SA+ binding to
glycophorin was analyzed in this manner, assuming a reversible, 1:1 binding model (49, 51), the avidity of virus for the glycophorin surface was 4.8 (± 1.9) × 10^{-9} M. In addition, analysis of SA+ binding to sialic acid suggests that this interaction is likely to be multivalent, since nonlinear regression analysis of SA1 binding to sialic acid suggests that this interaction is likely to be multivalent, since nonlinear regression analysis of SA1 binding to sialic acid suggests that this interaction is likely to be multivalent, since nonlinear regression analysis of SA1 binding to sialic acid suggests that this interaction is likely to be multivalent, since nonlinear regression analysis of SA1 binding to sialic acid suggests that this interaction is likely to be multivalent, since nonlinear regression analysis of SA1 binding to sialic acid suggests that this interaction is likely to be multivalent, since nonlinear regression analysis of SA1 binding to sialic acid suggests that this interaction is likely to be multivalent, since nonlinear regression analysis of SA1 binding to sialic acid suggests that this interaction is likely to be multivalent, since 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Viral yields were assessed in triplicate experiments. Error bars expressed as viral yield, is equal to the log<sub>10</sub> of pfu/ml at 24 h divided by that obtained under equilibrium conditions (Table I). These results suggest that SA<sub>1</sub> bound more rapidly to the HeLa cell surface than SA<sub>2</sub>. Transformation of these data to eliminate the contribution of virus infection in some types of cells.

**Kinetic Binding of SA<sub>1</sub>- and SA<sub>2</sub> to HeLa Cells**—To determine whether sialic acid binding affects the association (k<sub>on</sub>) or dissociation (k<sub>off</sub>) rate of reovirus binding, we performed kinetic binding assays with SA<sub>1</sub>- and SA<sub>2</sub>-on HeLa cells (Fig. 6). During the association phase, SA<sub>1</sub> displayed more rapid binding and a higher final equilibrium level than SA<sub>2</sub>—(Fig. 6A). When observed association rates (k<sub>on</sub>) were assessed at various virus concentrations (Fig. 6, B and C) during the first 60 min of incubation, we found that the binding rate of each strain was proportional to the input concentration of virus, but that SA<sub>1</sub> bound more rapidly to the HeLa cell surface than SA<sub>2</sub>. Transformation of these data to eliminate the contribution of k<sub>off</sub> demonstrated that SA<sub>1</sub> displayed a k<sub>on</sub> of 6 × 10<sup>6</sup> M<sup>-1</sup> s<sup>-1</sup> while the k<sub>on</sub> of SA<sub>2</sub> was approximately 16-fold more rapid, at 1 × 10<sup>8</sup> M<sup>-1</sup> s<sup>-1</sup> (Fig. 6C, Table I). Thus, sialic acid binding increases the avidity of reovirus for HeLa cells by accelerating the on-rate of virus-cell interaction.

The derivation of k<sub>on</sub> by comparison of k<sub>off</sub> at various virus concentrations allows the indirect inference of the k<sub>off</sub> of virus binding, since k<sub>off</sub> is independent of the free virus concentration (52). Interestingly, although SA<sub>1</sub> displayed a higher avidity for HeLa cells than SA<sub>2</sub> under equilibrium conditions, the initial k<sub>off</sub> for SA<sub>1</sub> was 15-fold more rapid than that of SA<sub>2</sub>. This k<sub>off</sub> would result in an initial K<sub>eq</sub> of SA<sub>1</sub>-for HeLa cells of 6.8 × 10<sup>-10</sup> M<sup>-1</sup>, 14-fold less avid than that measured at equilibrium. In contrast, the kinetic K<sub>eq</sub> of SA<sub>2</sub> was 7.5 × 10<sup>-10</sup> M, similar to that obtained under equilibrium conditions (Table I). These results suggest that SA<sub>1</sub>, but not SA<sub>2</sub>, undergoes a time-dependent increase in its avidity for HeLa cells, likely mediated by a decrease in k<sub>off</sub> as binding approaches equilibrium.

**Effect of Cell-Surface Sialic Acid on Cell-Attachment Kinetics of SA<sub>1</sub>- and SA<sub>2</sub>**—To confirm that the enhanced attachment kinetics of SA<sub>1</sub> are dependent on cell-surface sialic acid, we determined the effect of neuraminidase treatment of cells on attachment rates for each virus strain (Fig. 7). Preincubation of HeLa cells with neuraminidase did not significantly alter the avidity of SA<sub>2</sub> for these cells (Fig. 7A, Table I). The binding avidity of SA<sub>1</sub>-on neuraminidase-treated HeLa cells was reduced to precisely that of SA<sub>2</sub>—(Fig. 7A, Table I). The decrease in affinity of SA<sub>1</sub> for neuraminidase-treated HeLa cells was due to a decreased rate of association of this strain at lower virus concentrations than SA<sub>2</sub>—on both cell types (Fig. 5, A and C). However, the difference between maximal binding levels of SA<sub>1</sub>- and SA<sub>2</sub>-was greater on HeLa cells than L cells. When steady state binding results were analyzed using a Scatchard transformation (57), both strains demonstrated linear plots on both cell types (Fig. 5, B and D). Therefore, at concentrations of virus used in these experiments, these viruses recognize thermodynamically uniform receptor populations at equilibrium. The avidity (expressed as apparent K<sub>eq</sub>) for L cells was 3.3 × 10<sup>-11</sup> M, (Table I), whereas the avidity of SA<sub>1</sub>-to L cells was only slightly higher, with a K<sub>eq</sub> of 2.5 × 10<sup>-11</sup> M. The nearly equivalent avidities of SA<sub>2</sub>- and SA<sub>1</sub>-for L cells are consistent with the capacity of these strains to replicate with equal efficiency in this cell type (Fig. 3). In contrast, the avidity of SA<sub>1</sub>-for HeLa cells was 2.5 × 10<sup>-10</sup> M, approximately 8-fold lower than that observed on L cells. SA<sub>2</sub>-avidity for HeLa cells was significantly higher than SA<sub>1</sub>-with a K<sub>eq</sub> of 5 × 10<sup>-11</sup> M. Thus, the capacity to bind sialic acid increases the avidity of reovirus for HeLa cells by 5-fold but has little effect on the avidity of either strain for L cells. The enhanced avidity of SA<sub>1</sub>-for HeLa cells correlates with the capacity of this strain to replicate more efficiently than SA<sub>2</sub>—in this cell type and suggests that the thermodynamics of virus-cell interaction can be modulated by sialic acid binding in a manner that enhances virus infection in some types of cells.

**Steady State Binding of SA<sub>1</sub>- and SA<sub>2</sub> to L Cells and HeLa Cells**—To determine the mechanisms by which sialic acid binding enhances reovirus infection of HeLa cells, we performed radioligand binding studies using <sup>125</sup>I-labeled SA<sub>1</sub>- and SA<sub>2</sub>-virions incubated with intact L cells and HeLa cells. Cells were ATP-depleted to inhibit endocytic processes (53) and incubated with increasing concentrations of iodinated virions until equilibrium was reached. Binding of SA<sub>1</sub>- and SA<sub>2</sub>-to both L cells and HeLa cells was saturable and specific, with SA<sub>1</sub>-capable of achieving higher maximal binding and approaching saturation
Consistent with the hypothesis that accelerated adsorption of SA\(_1\) mediates enhanced infectivity on HeLa cells, we also find that neuraminidase treatment of HeLa cells reduces viral yield of SA\(_1\) to that of SA\(_2\) (data not shown). These data indicate that cell-surface sialic acid is required to enhance the attachment rate of SA\(_1\), but that the head receptor bound by these strains does not require sialylation for its function in virus binding.

**Capacity of SA\(_2\) and SA\(_1\) to Compete for Cell-Surface Receptors**—To determine whether the mutation in the \(s1\) tail that confers sialic acid binding to strain SA\(_1\) also alters the nature of the interaction between the \(s1\) head and its receptor, we tested the capacity of each strain to compete the binding of the heterologous strain to HeLa cells (Fig. 8). Strain SA\(_2\) and SA\(_1\) competed their own capacity to bind HeLa cells in a dose-dependent manner, with a 50-fold excess of unlabeled virus being sufficient to reduce binding of labeled virus to the level of background (Fig. 8, A and B). Unlabeled SA\(_1\) efficiently competed binding of strain SA\(_2\), with dramatic inhibition of SA\(_2\) attachment observed in the presence of a 5-fold molar excess of SA\(_1\) (Fig. 8A). The enhanced capacity of SA\(_1\) to compete binding of SA\(_2\) is likely due to the accelerated \(k_{on}\) of SA\(_1\) (Fig. 6, Table I), which would enable it to saturate cell-surface receptors more rapidly than SA\(_2\). In contrast, even a 100-fold excess SA\(_2\) had minimal effect on the binding of SA\(_1\), which suggests that strain SA\(_1\) has acquired the capacity to bind an additional receptor molecule, presumably sialic acid (Fig. 8B).

To test this hypothesis, HeLa cells were treated with neuraminidase to remove sialic acid, and competition profiles of each strain were repeated (Fig. 8, C and D). In the absence of cell-surface sialic acid, both strains bound equivalently to HeLa cells and were capable of competing the attachment of the heterologous strains with identical efficiency. These results indicate that the head receptor bound by strains SA\(_2\) and SA\(_1\) is identical and suggest that the capacity to bind sialic acid confers a competitive advantage in the capacity of strain SA\(_1\) to bind to this receptor. Furthermore, these data suggest that SA\(_1\) can associate with the HeLa cell surface in a stable manner via binding to sialic acid in the absence of \(s1\)-head receptor ligation.

**Defining the Role of Sialic Acid Binding in Attachment of Infectious Reovirus Virions to Biologically Relevant Receptors**—Since the vast majority of reovirus particles (>99%) are not capable of productive infection in a plaque assay, it remained a
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Purified, radiolabeled virions of each strain were incubated with 1 × 10^6 cells for the times shown. Binding reactions, separation of bound from free virus, and quantitation of specific binding were performed using conditions identical to those used for the equilibrium binding studies shown in Fig. 5. A, association of strains SA- and SA+ with HeLa cells. Radiolabeled virions (3 × 10^11 particles) of each strain were incubated with cells, and bound virus was quantitated at the times indicated following vacuum filtration. Nonspecific binding was calculated following vacuum filtration. Nonspecific binding was calculated by subtracting the data from parallel incubations in the presence of excess unlabeled virus. Non-specific binding did not increase with time and is subtracted from the data shown.

FIG. 6. Kinetics of attachment of SA- and SA+ to HeLa cells. Purified, radiolabeled virions of each strain were incubated with 1 × 10^6 cells for the times shown. Binding reactions, separation of bound from free virus, and quantitation of specific binding were performed using conditions identical to those used for the equilibrium binding studies shown in Fig. 5. A, association of strains SA- and SA+ with HeLa cells. Radiolabeled virions (3 × 10^11 particles) of each strain were incubated with cells, and bound virus was quantitated at the times indicated following vacuum filtration. Nonspecific binding was calculated following vacuum filtration. Nonspecific binding did not increase with time and is subtracted from the data shown. B and C, data used for determination of association (k_on) and dissociation (k_off) rate constants of strains SA- and SA+ on HeLa cells. B, cell-associated virus was quantitated following incubation of virus at the indicated concentrations for the times shown, and untransformed data were plotted as a function of time. C, to determine k_on for each concentration of virus used, data from B were replotted as ln([virus bound at equilibrium]/[virus bound at equilibrium] − [virus bound at time point]) (52). Linear regression analysis was used to determine k_on, equal to the slope of each binding curve. The k_off values from C were plotted as a function of virus concentration to calculate k_off and k_on in Table I. Experiments were performed in duplicate, and average specific binding is shown. Error bars represent the range of data obtained.

DISCUSSION

To dissect mechanisms by which utilization of sialic acid as a coreceptor contributes to virus attachment and tropism, we isolated reovirus strains that differ solely in the capacity to bind sialic acid. Although we cannot formally exclude the existence of mutations in these strains in gene segments other than S1, four lines of evidence argue against this possibility. First, since T1L has been passaged in L cells since its isolation (58) and reovirus strains are exceptionally genetically stable in this cell line (47, 48), it is unlikely that additional mutations were selected in other gene segments during the few cycles of replication required to generate the monoreassortant strains.

formal possibility that biochemical assessments of the role of sialic acid binding in reovirus attachment might be applicable only to inactive particles, while infectious particles might be have differently. In addition, it seemed possible that binding to sialic acid might represent a nonproductive attachment route that is biochemically detectable but biologically irrelevant. To exclude these possibilities, we assessed the binding rates of strains SA- and SA+ using a fluorescent focus assay for attachment of infectious particles to individual cells in the presence of specific inhibitors of each σ1 RBD (Fig. 9). Purified virions of SA- and SA+ were titrated on L cells, and equal numbers of pfu were preincubated with either SLL, lactose, G5 Fabs, or 5C6 Fabs for 30 min. Following incubation with these RBD inhibitors, virus was adsorbed to HeLa cell monolayers for increasing periods of time, unbound virus was removed by thorough washing, and cells were incubated at 37 °C for 18 h to permit viral entry and a single round of replication. Infected cells were visualized by immunofluorescence. Both virus strains displayed a linear, time-dependent increase in numbers of infected cells (Fig. 9, A and B). However, infection rates of SA+ were dramatically accelerated in comparison with SA-, with SA+ displaying 50–100-fold more fluorescent focus units (FFU) than SA- at each time point (Fig. 9, A and B). Differences in the capacity of SA- and SA+ to generate FFU were not inherent to the assay, since these strains produce FFU with equivalent kinetics on L cells (data not shown). Preincubation with SLL significantly decreased the attachment rate of SA+ but not SA-, indicating that the enhanced rate of infection displayed by SA+ was dependent on virus-sialic acid interactions (Fig. 9B). Neutralizing mAb G5 potently and specifically inhibited infection with either SA- or SA+ (Fig. 9, A and B). These data indicate that the attachment kinetics demonstrated using purified viral particles parallel those of infectious particles and suggest that sialic acid binding enhances a productive binding pathway for reovirus virions.

Temporal Coordination of Reovirus-Sialic Acid and Reovirus-Head Receptor Interactions—Since the capacity to bind sialic acid enhances the association rate of strain SA+ binding to HeLa cells, we hypothesized that sialic acid binding might serve as the initial interaction between the virion and the cell surface. To determine whether adhesion of reovirus to cell-surface sialic acid temporally precedes interaction of the σ1 head with its receptor, we compared the capacity of SLL and G5 to inhibit infection of SA+ when added at various times during the adsorption phase (Fig. 10). SLL substantially (−90%) inhibited viral infection by SA+ when added at either 30 or 0 min prior to adsorption. However, SLL exhibited a time-dependent decrease in its capacity to inhibit attachment of SA+ between 0 and 20 min of adsorption, with almost all inhibitory effect lost by 30 min of adsorption. In contrast, G5 Fabs inhibited viral infection by >75% when added at any time prior to or during adsorption (up to 45 min). These findings suggest that the enhancement of infectivity conveyed by the capacity to bind sialic acid is mediated by adhesion of virus to cell-surface sialic acid as a primary step, but that this interaction is followed by transition to a sialic acid-independent interaction mediated by the σ1 head.
Second, SA\textsuperscript{−} and SA\textsuperscript{+} demonstrate cell-specific tropism previously attributed to the S1 gene segments of their respective T3 parent strains (38). Third, the same cellular molecule is bound by the σ1 head of SA\textsuperscript{−} and SA\textsuperscript{+}, since SA\textsuperscript{+} fully competes binding of SA\textsuperscript{−} on HeLa cells, and both viruses cross-compete on neuraminidase-treated cells. Finally, differences in binding, virus-induced signaling and apoptosis, and replication between SA\textsuperscript{−} and SA\textsuperscript{+} in HeLa cells are mediated solely by binding to sialic acid (this report).\textsuperscript{3} Thus, SA\textsuperscript{−} and SA\textsuperscript{+} represent a site-directed mutant pair that differs genetically with respect to a single amino acid, Leu\textsuperscript{204} (SA\textsuperscript{−}) → Pro\textsuperscript{204} (SA\textsuperscript{+}), in the σ1 tail domain and phenotypically in the capacity to bind sialic acid as a viral coreceptor. These strains represent the first reovirus mutants to be isolated that differ solely in the capacity to bind a known reovirus receptor.

We found that the capacity to bind sialic acid has distinct, cell type-dependent effects on reovirus growth that correlated with virus binding avidity and kinetics. MEL cells display an absolute requirement for binding to sialic acid, since SA\textsuperscript{−} did not replicate in these cells. In contrast, L cells display no requirement for sialic acid binding, since both SA\textsuperscript{−} and SA\textsuperscript{+} grew efficiently in these cells. Consistent with this result, we found that the steady-state avidities of these strains for L cells were equivalent. An intermediate requirement for binding to sialic acid was found for HeLa cells. Although both strains were capable of infecting HeLa cells, SA\textsuperscript{+} produced greater yields than SA\textsuperscript{−}. At equilibrium, the avidity of SA\textsuperscript{+} for HeLa cells was 5-fold higher than that of SA\textsuperscript{−}, and this increased avidity was attributable to an accelerated $k_{on}$ of SA\textsuperscript{+}. Enhanced steady-state and kinetic binding of SA\textsuperscript{+} to HeLa cells required cell surface sialic acid, since the avidity and association rate of SA\textsuperscript{−} decreased to that of SA\textsuperscript{−} when assessed using neuraminidase-treated cells. In contrast, although the $k_{on}$ of SA\textsuperscript{+} binding to L cells was modestly enhanced relative to SA\textsuperscript{−}, the $k_{on}$ of SA\textsuperscript{−} binding to L cells was as rapid as observed for SA\textsuperscript{+} binding to HeLa cells (data not shown), suggesting that this $k_{on}$ represents a kinetic threshold necessary for efficient viral adsorption to cultured cells. It is important to note that given the complex nature of the interactions between a multivalent viral ligand and the cell surface, binding constants derived in this study for SA\textsuperscript{−} and SA\textsuperscript{+} do not represent absolute biophysical properties of any single ligand-receptor interaction occurring during the process of virus adsorption. Instead, analysis of viral binding using the assumptions of mass action enables the comparison of the relative avidity and kinetics of SA\textsuperscript{−} and SA\textsuperscript{+} binding to cells.

To exclude the possibility that the accelerated binding observed for SA\textsuperscript{+} was applicable only to noninfectious particles (59), we compared the capacity of infectious particles of SA\textsuperscript{−} and SA\textsuperscript{+} to bind to HeLa cells using a fluorescent focus assay that directly detects infected cells. We found that SA\textsuperscript{+} was 50–100-fold more efficient than SA\textsuperscript{−} at establishing a biologically productive binding event. Enhanced kinetics of SA\textsuperscript{+} infectivity were mediated at the attachment step and were dependent on virus binding to cell-surface sialic acid, since preincubation of virus with SLL dramatically reduced the efficiency of SA\textsuperscript{+} infection. The capacity of SLL to inhibit infectious binding of SA\textsuperscript{+} was most potent when virus was preincubated with this compound, and inhibition decreased rapidly over the first 30 min of adsorption to the cell surface. In contrast, the capacity of a neutralizing antibody to the σ1 head to inhibit infectious attachment remained high throughout the course of the adsorption phase.

A number of conclusions can be drawn from these results. First, the enhanced attachment of SA\textsuperscript{+} measured in radioligand binding experiments can be correlated with an enhanced rate of productive host cell infection. Second, enhanced attachment mediated by sialic acid binding is operant early in adsorption and may represent the initial attachment event between virus and cell. Third, virus binding to sialic acid alone is not likely to mediate efficient virus infection of HeLa cells, since interaction between σ1 and the head receptor continues to play a critical function after the sialic acid-mediated adsorption phase is complete. Finally, reovirus attachment is likely to be a temporally regulated process, since an early, sialic acid-dependent phase can be separated from a later, sialic acid-independent phase. This multiphasic nature of reovirus ad-

\textsuperscript{3} J. L. Connolly, E. S. Barton, and T. S. Dermody, submitted for publication.
sorption is consistent with binding constants for SA\textsubscript{1} on HeLa cells, in which the initial \( K_D \) of SA\textsubscript{1} as determined kinetically was characterized by a rapid \( k_{off} \) and was 14-fold weaker than the saturation avidity but transitioned at equilibrium to a higher avidity binding state.

Taken together, these findings support a \( \sigma_1 \) head receptor-dominant adhesion-strengthening model of reovirus attachment (Fig. 11). For sialic acid-binding reovirus strains, the initial interaction between the viral particle and the host cell is likely to be mediated by cell-surface sialic acid. This interaction may involve multivalent virion-sialic acid interactions and has an apparent avidity of \( \sim 10^{-9} \) M as measured on a biosensor surface. By virtue of its rapid association rate, this interaction adheres the virion to the cell surface, enabling it to diffuse laterally until it interacts with the \( \sigma_1 \) head receptor molecule. This secondary interaction is the only binding event available to strains that do not bind sialic acid and may be necessary and sufficient for virus endocytosis. This attachment strategy is similar to that proposed for the \( \alpha \)-herpesviruses, where primary virus-cell interactions occur between viral glycoproteins and heparin sulfate, but virus penetration is facilitated by binding to one of a family of herpesvirus entry mediators (1, 2, 13, 60–62).

Multiple mechanisms may explain the enhanced kinetics of \( \sigma_1 \)-head receptor interaction that are observed in the presence of sialic acid binding. The accelerated attachment rate may simply be due to an increase in relative concentrations of virus and head receptor that occurs when the virus adheres to the cell surface. Alternatively, the virus binding site on the \( \sigma_1 \) head receptor may be proximal to the cell surface or otherwise sterically inaccessible to soluble virus. In this scenario, virus binding to sialic acid may serve to insert the \( \sigma_1 \) head into the glycocalyx to permit interaction between this receptor and \( \sigma_1 \) (63, 64). Finally, \( \sigma_1 \)-sialic acid interactions may induce a conformational change in \( \sigma_1 \) that places the head RBD in a state that displays higher affinity for its receptor, in a mechanism analogous to CD4-induced conformational changes that expose chemokine receptor-binding residues on HIV gp120 (4–6), although a \( \sigma_1 \) conformational change alone is not sufficient to enhance attachment, since incubation with SLL inhibits rather
than enhances SA+ binding. Discriminating between these models will require identification and characterization of the cellular molecule bound by the \( \sigma 1 \) head.

In addition to accelerating virus attachment through adhesion to the cell surface, the capacity to bind sialic acid may alter the biological outcome of reovirus infection. For example, sialic acid-binding reovirus strains may function as lectins, cross-linking sialylated cellular molecules and perturbing intracellular signaling pathways (65, 66). Alternatively, sialic acid binding may enhance the capacity of reovirus particles to induce cross-linking or activation of the \( \sigma 1 \) head receptor, an event that may have downstream effects, depending on the nature of this molecule. Strain SA+ induces NF-\( \kappa B \) activation in infected cells, thereby triggering apoptotic cell death (67, 68). Strain SA− neither activates NF-\( \kappa B \) nor causes apoptosis, even at doses that result in infection of all cells in a culture. These results support the hypothesis that binding to sialic acid may dramatically alter steps in virus-cell interaction following attachment and entry. Accordingly, although we favor a model whereby sialic acid binding enhances reovirus infection by accelerating adsorption (Fig. 11), our data are also consistent with sialic acid-mediated signaling events enhancing yield of SA+ in HeLa cells at a post-attachment step by altering the metabolic state of the host cell.

One implication of the adhesion-strengthening model for reovirus attachment proposed here is that the relative expression levels of sialic acid and head receptor on a given cell type may determine the types of virus strains that can infect these cells. On cells that express high levels of head receptor, sialic acid binding may be an absolute requirement for infectivity, as is the case for MEL cells. At intermediate levels of head receptor, sialylated cell-surface molecules may significantly enhance the efficiency of viral attachment, like the situation observed for HeLa cells. Conversely, on cells that express low levels of head receptor, sialic acid binding may be an absolute requirement for infectivity, as is the case for MEL cells. At intermediate levels of head receptor, sialylated cell-surface molecules may significantly enhance the efficiency of viral attachment, like the situation observed for HeLa cells. Finally, on cells that do not express the head receptor, binding to sialic acid may represent a “dead end” binding event that does not support viral infection. These attachment scenarios may have varying degrees of importance during discrete phases of replication in the infected animal.

Despite the fact that neuroinvasion and neurovirulence phenotypes of T3 reovirus strains have been genetically mapped to the \( \sigma 1 \)-encoding S1 gene, there exists little understanding of which \( \sigma 1 \)-receptor interactions dictate the exquisite neural tropism of these viruses. Since all detailed studies of T3 pathogenesis have used sialic acid-binding strains, the role of this receptor in neural tropism and disease is unknown. Interestingly, a sialic acid-binding T3 strain has been shown to cause apoptosis in the neonatal central nervous system (69) and heart (70), suggesting that binding to this carbohydrate may induce signaling alterations in the host as it does in cultured cells. The enhanced \( k_{on} \) of SA+ may be particularly important in permitting spread through rapidly moving body fluid such as
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blood or lymph (71), where adhesion to endothelial cells might permit virus to concentrate at the cell surface in a manner analogous to selectin-mediated lymphocyte rolling (56). In addition, results from the current study suggest that sialic acid binding is critical for infectivity of cell types that express the α1 head receptor at low levels, which predicts that SA+ would display expanded tissue tropism in the host relative to SA−. Future studies using these reovirus strains that differ solely in the primary interaction between a reovirus and endothelial cells (57, 58) should explore the mechanisms by which interaction of a virus with a common cell-surface carbohydrate contributes to virus-induced cell death and disease in the infected host.

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REFERENCES

1. Rajcani, J., and Vojvodova, A. (1998) Acta Virol. 42, 103–118
2. Montgomery, R. I., Warner, M. S., Lum, B. J., and Spear, P. G. (1996) Cell 87, 427–436
3. Spear, P. G., Shieh, M. T., Herold, B. C., WuDunn, D., and Koshy, T. I. (1992) Adv. Exp. Med. Biol. 313, 341–353
4. Dragic, T., Litwin, V., Allaway, G. P., Martin, S. R., Huang, Y., Nagashima, K. A., Cayanan, C., Maddon, P. J., Koup, R. A., Moore, J. P., and Paxton, W. T. (1996) Nature 384, 144–149
5. Ugolini, S., Mondor, I., and Sattentau, Q. J. (1999) Trends Microbiol. 7, 103–118
6. Berger, A. E., Murphy, P. M., and Farber, J. M. (1999) J. Virol. 73, 657–673
7. Leung, L., Gerard, N. P., Wyatt, R., Choe, H., Parolin, C., Ruffing, N., Borsetti, A., Cardosa, A. A., Denjardin, E., Newman, W., Gerard, C., and Sodroski, J. (1996) Nature 384, 179–183
8. Domu, R., and Peipert, S. C. (1997) J. Virol. 71, 1375–1380
9. Trkola, A., Dragic, T., Arthos, J., Bulley, J. M., Olson, W. C., Allaway, G. P., Cheng-Mayer, C., Robinson, J., Maddon, P. J., and Moore, J. F. (1996) Nature 384, 184–187
10. Wu, L., Gerard, N. P., Wyatt, R., Choe, H., Parolin, C., Ruffing, N., Borsetti, A., Cardosa, A. A., Denjardin, E., Newman, W., Gerard, C., and Sodroski, J. (1996) Nature 384, 179–183
11. Domu, R., and Peipert, S. C. (1997) J. Virol. 71, 1375–1380
12. White, J. J., Engler, J., and Lee, P. W. K. (1998) J. Gen. Virol. 79, 1625–1632, Lippincott-Raven, Philadelphia
13. Whitbeck, J. C., Pang, C., Lou, X., Rus, W. S., PoniDeLeon, M., Peng, T., Nicola, A. V., Montgomery, R. I., Warner, M. S., Soulika, A., Spruce, L. A., Moore, W. T., Langrim, J. D., Spear, P. G., Cohen, G. H., and Eisenberg, R. J. (1997) J. Virol. 71, 6863–6869
14. Tyler, K. L., and Fields, B. N. (1996) in Fields Virology (Fields, B. N., Knipe, D. M., and Howley, P. M., eds) 3rd Ed., pp. 356–364, Lippincott-Raven, Philadelphia
15. Fields, B. N., Knipe, D. M., and Howley, P. M., eds) 3rd Ed., pp. 1557–1596, Lippincott-Raven, Philadelphia
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Baribaud, I., Hou, W., Zou, C., Geraghty, R. J., Spear, P. G., Eisenberg, R. J., and Cohen, G. H. (1999) J. Virol. 73, 8127–8137
61. Sarrias, M. R., Whitbeck, J. C., Rooney, I., Spruce, L., Kay, B. K., Montgomery, R. I., Spear, P. G., Ware, C. F., Eisenberg, R. J., Cohen, G. H., and Lambris, J. D. (1999) J. Virol. 73, 5681–5687
62. Shukla, D., Rowe, C. L., Dong, Y., Racaniello, V. R., and Spear, P. G. (1999) J. Virol. 73, 4493–4497
63. Pickles, R. J., Fahrner, J. A., Petrella, J. M., Boucher, R. C., and Bergelson, J. M. (2000) J. Virol. 74, 6050–6057
64. Yeung, S., Bockhold, K., and Tufare, F. (1999) Gene Ther. 6, 1536–1544
65. Perillo, N. L., Marcus, M. E., and Baum, L. G. (1998) J. Mol. Med. 76, 402–412
66. Rott, O., Charriere, J., and Cash, E. (1996) Med. Microbiol. Immunol. 184, 185–193
67. Connolly, J. L., Rodgers, S. E., Clarke, P., Ballard, D. W., Kerr, L. D., Tyler, K. L., and Dermody, T. S. (2000) J. Virol. 74, 2981–2989
68. Rodgers, S. E., Barton, E. S., Oberhaus, S. M., Pike, B., Gibson, C. A., Tyler, K. L., and Dermody, T. S. (1997) J. Virol. 71, 2540–2546
69. Oberhaus, S. M., Smith, R. L., Clayton, G. H., Dermody, T. S., and Tyler, K. L. (1997) J. Virol. 71, 2100–2106
70. DeBiasi, R., Sherry, B., and Tyler, K. (1999) in American Society for Virology 18th Annual Meeting, July, 10–14, Amherst, MA
71. Williams, A. F. (1991) Nature 352, 473–474