

Sialic Acid Is a Cellular Receptor for Coxsackievirus A24 Variant, an Emerging Virus with Pandemic Potential[∇]

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Received 16 November 2007/Accepted 26 December 2007

Binding to target cell receptors is a critical step in the virus life cycle. Coxsackievirus A24 variant (CVA24v) has pandemic potential and is a major cause of acute hemorrhagic conjunctivitis, but its cellular receptor has hitherto been unknown. Here we show that CVA24v fails to bind to and infect CHO cells defective in sialic acid expression. Binding of CVA24v to and infection of corneal epithelial cells are efficiently inhibited by treating cells with a sialic acid-cleaving enzyme or sialic acid-binding lectins and by treatment of the virus with soluble, multivalent sialic acid. Protease treatment of cells efficiently inhibited virus binding, suggesting that the receptor is a sialylated glycoprotein. Like enterovirus type 70 and influenza A virus, CVA24v can cause pandemics. Remarkably, all three viruses use the same receptor. Since several unrelated viruses with tropism for the eye use this receptor, sialic acid-based antiviral drugs that prevent virus entry may be useful for topical treatment of such infections.

Two members of the family *Picornaviridae*, coxsackievirus A24 variant (CVA24v) and enterovirus type 70 (EV70), are responsible for the majority of cases of acute hemorrhagic conjunctivitis (AHC). AHC is characterized by conjunctivitis, keratitis, foreign body sensation, and pain, but respiratory symptoms and severe neurological symptoms similar to those caused by poliovirus (acute flaccid paralysis) have also been reported (7, 44, 64, 67). AHC was first reported in Ghana in 1969 (10), and since then numerous epidemics and three pandemics have occurred: in 1969 to 1971 (36), 1980 to 1981 (18, 27), and in 2002 to 2004. It has been estimated that there have been about 100 million cases of AHC since identification of the causative agents (1). With a few exceptions, most outbreaks during the past 15 to 20 years, including the 2002 to 2004 pandemic, have been caused by CVA24v. The last pandemic started in the eastern hemisphere in 2002, probably in South Korea, where more than one million people were reported to be affected over a three-month period (41). It continued with an outbreak in Malaysia the same year (18). In 2003, outbreaks of AHC were reported from South Korea (46), India (19), Nepal (25), Tunisia (60), and Congo (29), and in the western hemisphere from Nicaragua, Honduras, Guatemala, El Salvador, and Caribbean countries (45), French Guiana and the West Indies (15), Puerto Rico (2), and Brazil (38). In 2004, more outbreaks hit both the western (58) and eastern (29) hemispheres.

To date, cellular receptors used by coxsackie A viruses have been described only for CVA9 and CVA21, but neither of these viruses is associated with AHC. CVA9 uses $\alpha_v\beta_3$ inte-

grins, $\alpha_v\beta_6$ integrins, and/or glucose-regulated protein 78 as cellular receptors (48, 59, 62), whereas CVA13, -15, -18, and -20 use intercellular adhesion molecule 1 (ICAM-1) (39) and CVA21 uses CD55 and/or ICAM-1 (53).

EV70, the other member of the *Picornaviridae* family that causes AHC has been found to use either CD55 (26) or sialic acid (1) as a cellular receptor, and the choice of receptor may depend on the cell line under investigation. Early on members of the *Picornaviridae* family were shown to compete with members of the *Adenoviridae* family for receptors (32). The coxsackie-adenovirus receptor (CAR) was the first cellular receptor shown to be used by both virus families (9). Subsequently, sialic acid has been shown to be used by both EV70 and specific adenoviruses that cause epidemic keratoconjunctivitis (5, 6). Other receptors shared by these two unrelated virus families are heparan sulfate, integrins, and members of the regulators of complement activation protein family (49, 69). In this study we set out to investigate whether CVA24v can make use of any of the receptors used by members of the *Picornaviridae* or *Adenoviridae*. We identified sialic acid as a cellular receptor for CVA24v on human ocular cells, and we conclude that usage of sialic acid is a common feature of at least three different viruses with pandemic potential: CVA24v, EV70, and influenza A viruses. Another common feature of these viruses is their primary sites of replication in humans: the eyes and airways. Usage of sialic acid has previously been suggested to explain, at least in part, the tropism of these viruses (42). Here we provide results that support this suggestion.

MATERIALS AND METHODS

Cells and viruses. CHO-MOCK, CHO-CAR, CHO-CD55, CHO-CD46, CHO-ICAM-1, Lec2, Pro-5, psgB-618, HCE (human corneal epithelial), NHC (normal human conjunctiva), A549 (alveolar), Hep2 (larynx), HeLa (cervix), and GMK (green monkey kidney) cells were grown as described in the references listed in Table 1, except for GMK cells, which were grown in Dulbecco's modified essential medium (DMEM; Sigma-Aldrich, St. Louis, MO) supplemented with

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[∇] Published ahead of print on 9 January 2008.

TABLE 1. Cell lines used in this study and expression of candidate receptors

Cell line	Candidate receptor expression ^a						Reference
	SA	GAG	hCAR	hCD55	hCD46	hICAM-1	
Pro-5	+	+	—	—	—	—	55
CHO-CAR	+	+	+	—	—	—	9
CHO-MOCK	+	+	—	—	—	—	9
CHO-CD55	+	+	—	+	—	—	33
CHO-CD46	+	+	—	—	+	—	31
CHO-ICAM-1	+	+	—	—	—	+	20
pgsB-618	+	—	—	—	—	—	16
Lec2	—	+	—	—	—	—	13
HCE	+	+	+	+	+	+	4
NHC	+	ND	+	+	+	+	14
A549	+	ND	+	+	+	+/-	30
Hep2	+	ND	+	+	+	—	37
HeLa	+	ND	+	+	+	+/-	51
GMK	+	ND	+	+	+	+/-	21

^a Abbreviations: SA, sialic acid; GAG, glycosaminoglycan; hCAR, human coxsackie-adenovirus receptor; hICAM-1, human intracellular adhesion molecule 1; and ND, not determined.

10% fetal calf serum (FCS; Sigma-Aldrich), HEPES at pH 7.4 (EuroClone, Milan, Italy), and penicillin-streptomycin (PEST; Gibco, Carlsbad, CA). The CVA24v strain used in this work (110390) originates from the outbreak of AHC in Malaysia in 2002 to 2003 and was isolated in Hep2 cells (18). New stocks were propagated in NHC cells. ³⁵S-labeled CVA24v virions were propagated as follows. NHC cells were incubated with freshly prepared CVA24v stocks in serum-free medium containing DMEM, HEPES, and PEST. After one hour, unadsorbed virions were removed by washing with phosphate-buffered saline (PBS; Medicago AB, Uppsala, Sweden), and cells were starved of methionine and cysteine in Met/Cys-free medium (Sigma-Aldrich) with 1% FCS (Sigma-Aldrich) for three hours and then incubated with Met/Cys-free medium supplemented with ³⁵S-Met/Cys mixture (NEG-772 Easytag express protein-labeling mix; Perkin-Elmer, Wellesley, MA). Thirty hours after infection, Triton X-100 (Sigma-Aldrich) was added to a final concentration of 0.5%. After 15 min of centrifugation at 3,000 × g, sodium dodecyl sulfate (VWR, Leicestershire, United Kingdom) was mixed with the supernatant to a final concentration of 0.5%. The mixture was laid onto a 30% sucrose solution and centrifuged for 3 h at 113,000 × g at 4°C. The pellets were dissolved in 4 ml of 10 mM Tris-HCl, pH 7.5, and sonicated for 20 s. The mixture was loaded onto a discontinuous gradient of 1.2 and 1.4 g/ml CsCl and centrifuged at 107,000 × g for 17 h at 4°C. The virion band was harvested and desalted on a NAP-10 column (Amersham Biosciences, Uppsala, Sweden) and stored in Tris-buffered saline with 10% glycerol at -80°C until use. Unlabeled virions were propagated essentially as described above except for the ³⁵S-Met/Cys-labeling step, which was omitted.

Binding and binding inhibition assays. The binding assay was performed essentially as described previously (5). Briefly, adherent cells were detached with PBS containing 0.05% EDTA (PBS-EDTA; Merck, Darmstadt, Germany) and recovered in growth medium for one hour at 37°C. After washing, 2 × 10⁵ cells/sample were incubated with 5,000 ³⁵S-labeled CVA24v virions (physical particles) per cell in 100 μl binding buffer (BB) consisting of DMEM, PEST, HEPES (pH 7.4), and 1% bovine serum albumin (Roche AB, Stockholm, Sweden) at 4°C with gentle agitation. One hour later, unbound virions were removed by washing, and the cell-associated radioactivity was measured with a Wallac 1409 scintillation counter (Perkin-Elmer). The experiments were varied in that (i) cells were pretreated with *Vibrio cholerae* neuraminidase (10 mU/100 μl BB; Sigma Aldrich) and washed, prior to incubation with virions, (ii) cells were preincubated with *Sambucus nigra* lectin (SNA), *Maackia amurensis* II lectin (MAA II), or wheat germ agglutinin lectin (WGA) (all from Vector Laboratories, Burlingame, CA) at 20 μg/100 μl in BB prior to incubation with virions, or (iii) virions were preincubated with 13-valent sialic acid-human serum albumin (SA-HSA) (24) in BB prior to incubation with cells. Each experiment was performed at least twice with duplicate samples in each experiment.

Infection and infection inhibition assays. The infection assay was performed essentially as described previously (5). Briefly, 2 × 10⁵ adherent cells were incubated with various numbers of unlabeled CVA24v virions (physical particles) at 4°C, which allows binding to cells but not internalization. One hour later, unbound virions were removed by washing, and the cells were incubated at 37°C. Sixteen to eighteen hours later, the cells were fixed in 99.5% methanol and stained through incubation with (i) mouse monoclonal antibodies against en-

terovirus VP1 (DakoCytomation, Glostrup, Denmark) diluted 1:200 in PBS, for one hour at room temperature, and (ii) fluorescein isothiocyanate (FITC)-labeled rabbit anti-mouse immunoglobulin G (DakoCytomation) diluted 1:100 in PBS, for one hour at room temperature. After each incubation, the cells were washed to remove unbound antibodies. Infected cells (i.e., antigen-positive cells) were then quantified with a fluorescence microscope (Axioskop2; Zeiss, Jena, Germany) at ×20 magnification; this was linked to a digital camera (AxioCam MRm; Zeiss) and Axiovision AC software (Zeiss). Counting of infected cells was performed using ImageJ software (downloaded from <http://rsb.info.nih.gov/ij/>). The experiments were varied in that cells were (i) pretreated with 10 mU *V. cholerae* neuraminidase in 300 μl PBS and washed, prior to incubation with virions, or (ii) pretreated with 20 μg WGA in 300 μl PBS, prior to incubation with virions. Each experiment was performed at least twice with duplicate samples in each experiment.

Flow cytometry. NHC, HCE, A549, HeLa, and Hep2 cells were rinsed and harvested with 0.05% EDTA in PBS, counted, and recovered in DMEM containing HEPES (pH 7.4), PEST, and 2% bovine serum albumin, for one hour at 37°C. Cells were then resuspended in PFN buffer (PBS containing 1% FCS and 0.05% Na₃N) (from J. T. Baker, Tamro MedLab AB, Mölndal, Sweden) and transferred to 96-well plates with 5 × 10⁵ cells per well, in a volume of 100 μl. The cells were then incubated with PFN containing biotinylated MAA II, SNA, or WGA (1 μg/100 μl) or monoclonal antibodies to CD55 (IF7; diluted 1:200), CD46 (FITC conjugated) (E4.3; 0.5 μg/100 μl; Ancell, Bayport, NM), ICAM-1 (FITC conjugated) (6.5B5; 0.1 μg/100 μl; DakoCytomation), or CAR (RmcB; 1 μg/100 μl; Millipore, Charlottesville, VA) and incubated for one hour on ice. Thereafter, the cells were washed with PFN and (i) in the case of the lectins, incubated for one more hour in the dark with FITC-labeled streptavidin in PFN (0.5 μg/100 μl; DakoCytomation) or (ii) in the case of anti-CD55 and anti-CAR, incubated with FITC-labeled rabbit anti-mouse antibody in PFN (0.1 μg/100 μl; DakoCytomation). The cells were washed with PFN and resuspended in 200 μl PBS per sample, transferred to fluorescence-activated cell sorter tubes (Becton Dickinson, Franklin Lakes, NJ) and then analyzed using a FACScan flow cytometer (Becton Dickinson).

RESULTS

CVA24v requires sialic acid for efficient binding to CHO cells. In order to investigate whether any of the previously identified picornavirus and adenovirus receptors can also be used by CVA24v, we first investigated binding of metabolically ³⁵S-labeled CVA24v virions to CHO cell lines that expressed specific receptors in *trans* or were deficient in endogenous receptor expression, and then we compared these results with binding of CVA24v to ordinary CHO cells. CVA24v virions bound with similar efficiency to the reference CHO cell line Pro-5, which expresses both sialic acid and heparan sulfate,

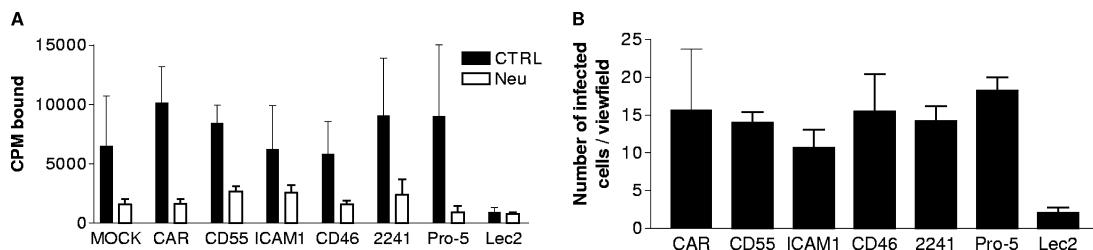


FIG. 1. CVA24v requires cell surface sialic acid for efficient binding to and infection of CHO cells. (A) CHO cells in suspension were first pretreated at 37°C for one hour with or without *V. cholerae* neuraminidase, incubated with 5,000 ³⁵S-labeled CVA24v virions per cell at 4°C for one hour, washed to remove unbound virions, and then analyzed in a scintillation counter for cell-associated radioactivity. CPM, counts per minute. (B) Adherent CHO cells were infected with 20,000 CVA24v virions per cell, fixed in methanol, and stained as described in Materials and Methods. CVA24v antigen-positive cells were then quantified in a fluorescence microscope. The results are presented as means ± standard deviations of duplicate samples from at least three independent experiments (MOCK, CHO-MOCK [mock with respect to CAR]; CAR, CHO-CAR; CD55, CHO-CD55; ICAM1, CHO-ICAM-1; CD46, CHO-CD46; 2241, CHO-2241; Lec2; and Pro-5 [parental cell line to Lec2]).

and to CHO cell lines transfected with cDNAs encoding human CAR (CHO-CAR), CD55 (CHO-CD55), CD46 (CHO-CD46), ICAM-1 (CHO-ICAM-1), and also to a CHO cell line that is deficient in synthesis of glycosaminoglycans (pgsB-618) (Fig. 1A; Table 1). However, CVA24v bound much more weakly to Lec2, a CHO cell line that is deficient in sialic acid expression. When sialic acid was removed from cell surfaces by treatment with neuraminidase, the binding of CVA24v virions to all CHO cell lines was reduced by 65 to 85%, except for Lec2 cells, for which the originally low binding was reduced further by 10% only.

To test whether any of these molecules is also important for subsequent entry and production of viral proteins, we allowed CVA24v virions to infect the same range of CHO cells and then quantified the numbers of cells positive for CVA24v antigens (Fig. 1B). Similar to the binding experiment, all cells were equally infected (range: 11 to 18 infected cells per viewfield), except for Lec2, where only two infected cells per viewfield were detected. These data suggested that sialic acid allows not only virus binding but also the subsequent steps in infection.

CVA24v efficiently binds to and infects ocular cells. To identify human cell lines that would be suitable for further characterization of CVA24v receptors, we first compared the ability of CVA24v to bind to and infect various epithelial cell lines that have been used previously for successful isolation of CVA24v (HeLa, A549, Hep2, and GMK) and also two additional epithelial cell lines that reflect the pronounced tropism

of CVA24v for the eye, NHC cells and HCE cells. Production of CVA24v antigens was most pronounced in NHC cells, followed by HCE and Hep2 cells, suggesting efficient infection of these cell lines (Fig. 2A). Fewer antigens were found in A549, HeLa, and GMK cells, suggesting less-efficient infection of these cells. The relative number of antigen-positive cells correlated reasonably well with the ability of CVA24v to bind to these cells (Fig. 2B). CVA24v bound most efficiently to NHC and Hep2 cells, with intermediate efficiency to A549, HCE, and HeLa cells, and with the least efficiency to GMK cells. In agreement with the ocular and upper respiratory tropism of CVA24v, epithelial cells derived from the conjunctiva (NHC), the cornea (HCE), and the larynx (Hep2) appeared to be most suitable for further studies.

NHC cells express high amounts of previously recognized virus receptors. Next, we set out to determine the relative levels of expression of sialic acid, CAR, CD55, CD46, and ICAM-1 on epithelial cell surfaces using flow cytometry (Fig. 3). Sialic acid is a cellular receptor for EV70 on ocular cells (1, 40) and for adenoviruses that cause epidemic keratoconjunctivitis (5, 6). NHC and HCE cells were found to express approximately three to four times as much sialic acid as A549, HeLa, Hep2, and GMK cells, as determined by WGA staining (Fig. 3A). However, since WGA also has affinity for *N*-acetylglucosamine, it could not be excluded that some of the WGA binding was due to *N*-acetylglucosamine on cell surfaces. CAR has been suggested to be a cellular receptor for all six coxsackie B viruses (34) and for many, but not all, adenoviruses (47).

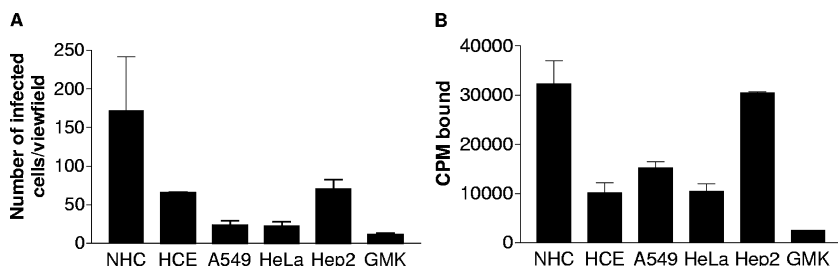


FIG. 2. CVA24v virions efficiently infect and bind to human ocular epithelial cells. (A) Adherent cells (2×10^5) were incubated with 5,000 CVA24v virions per cell at 4°C for one hour, washed to remove unbound virions, and incubated for 16 to 18 h at 37°C. The cells were fixed with methanol, stained as described in Materials and Methods, and quantified in a fluorescence microscope. (B) Binding experiments were carried out exactly as described for Fig. 1A, but without neuraminidase and with different cells. The results are presented as means ± standard deviations of duplicate samples from at least three independent experiments. CPM, counts per minute.

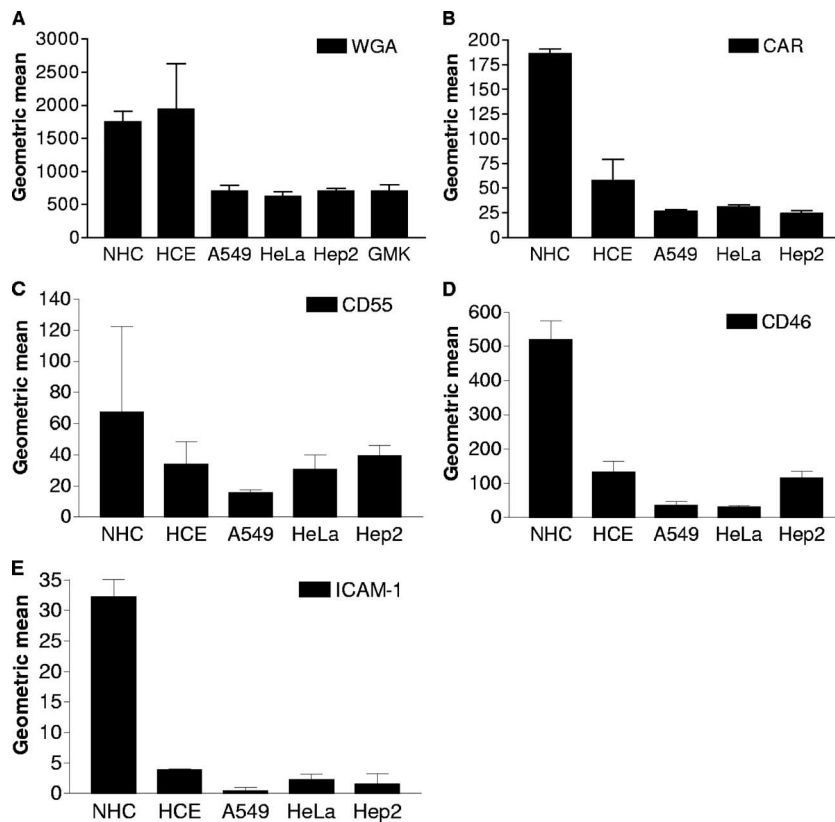


FIG. 3. Flow cytometry analysis of candidate receptors on epithelial cells. Epithelial cells (5×10^5) in suspension were incubated with biotinylated WGA to detect sialic acid, followed by incubation with streptavidin-FITC (A) or with monoclonal antibodies to human CAR (B), human CD55 (C), human CD46 (FITC conjugated) (D), or human ICAM-1 (FITC conjugated) (E), followed by FITC-conjugated anti-antibodies (against CAR or CD55), and analyzed by flow cytometry as described in Materials and Methods. Data represent the geometric means \pm standard deviations of the fluorescence measured in three experiments in duplicate.

CAR was expressed in relatively high amounts on NHC cells, in intermediate amounts on HCE cells, and in relatively low amounts on A549, HeLa, and Hep2 cells (Fig. 3B). CD55 and CD46 both belong to the protein family termed regulators of complement activation. CD55 is a receptor for echoviruses, coxsackieviruses, and EV70 on HeLa cells (8, 26, 52, 53), whereas CD46 is a receptor for seven out of nine species B adenoviruses (35). CD55 was found in relatively high amounts on NHC cells, in intermediate amounts on HCE, HeLa, and Hep2 cells, and in the lowest amounts on A549 cells (Fig. 3C), whereas CD46 was expressed in relatively high amounts on NHC cells, in intermediate amounts on HCE and Hep2 cells, and in relatively low amounts on A549 and HeLa cells (Fig. 3D). The only receptor known to be used by other coxsackie A viruses that was included in this study, ICAM-1, was found to be expressed in relatively high amounts on NHC cells but was hardly detectable on the other cell lines (Fig. 3E). Since species-specific monoclonal antibodies were used to detect expression of human CAR, CD55, CD46, and ICAM-1 proteins, the presence of these proteins was not investigated on GMK cells. The candidate receptor sialic acid was expressed in relatively high amounts on NHC and HCE cells. Therefore, these cell lines were included in all subsequent experiments.

CVA24v uses sialic acid as a receptor on corneal cells. To test whether sialic acid is a receptor for attachment of CVA24v

on human epithelial cells, we first treated NHC, HCE, and Hep2 cells with neuraminidase prior to binding of ^{35}S -labeled CVA24v virions. This resulted in a modest (28%) decrease in binding to NHC cells but a strong decrease in binding to HCE cells (85%) and to Hep2 cells (58%) (Fig. 4A). Next, we pre-incubated cells with WGA, a lectin that binds to both sialic acid and *N*-acetylglucosamine. Compared to control (without lectin), this resulted in inhibition of binding to NHC cells (46%), HCE cells (93%), and Hep2 cells (30%) (Fig. 4B). Since WGA also binds to *N*-acetylglucosamine, this molecule was considered to be a candidate receptor for CVA24v. However, up to 100 mM *N*-acetylglucosamine monosaccharides did not affect CVA24v binding to either NHC or HCE cells (data not shown), suggesting that *N*-acetylglucosamine is not a receptor for CVA24v and that the inhibitory effect of WGA is due to blocking of sialic acid on the cell surface. In a third type of binding experiment, sialic acid monosaccharides linked to human serum albumin (SA-HSA; 13-valent with respect to sialic acid) did not inhibit binding of CVA24v to NHC cells. However, increasing concentrations of SA-HSA efficiently inhibited binding to HCE cells (Fig. 4C). Similar results were obtained when the effect on CVA24v infection was investigated. Treatment of cells with neuraminidase or incubation of cells with WGA prior to infection with CVA24v resulted in a strong

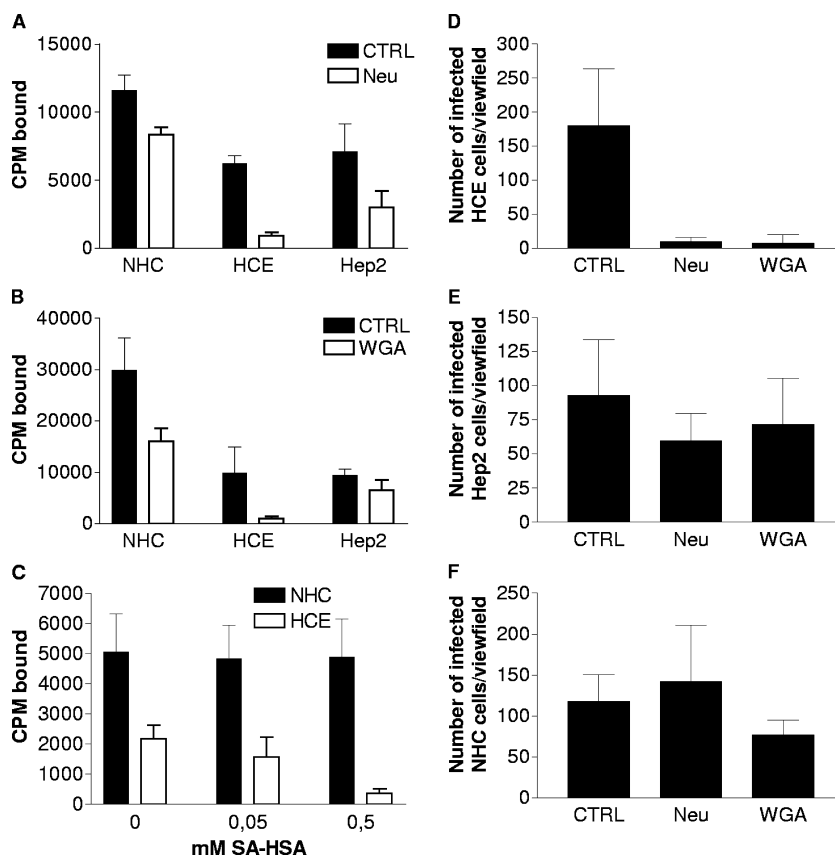


FIG. 4. Cell surface sialic acid is required for efficient binding of CVA24v to and infection of human ocular cells. Binding was carried out as described for Fig. 1. (A) Cells (2×10^5) in suspension were pretreated with or without neuraminidase from *V. cholerae* prior to incubation of the cells with 5,000 ^{35}S -labeled CVA24v virions per cell. (B) Cells (2×10^5) in suspension were preincubated with or without WGA prior to incubation with 5,000 ^{35}S -labeled CVA24v virions per cell. (C) ^{35}S -labeled CVA24v virions (5,000 virions/cell) were preincubated with or without 13-valent sialic acid linked to albumin (SA-HSA) prior to virion-cell incubation (2×10^5 cells/sample; concentrations of SA-HSA are in mM and with respect to sialic acid monosaccharides). (D to F) Adherent HCE (D), NHC (E), or Hep2 (F) epithelial human cell lines were pretreated with or without neuraminidase from *V. cholerae* or preincubated with or without WGA prior to incubation with virus (500 virions per cell) at 4°C . After one hour, the cells were washed to remove unbound virions and then incubated for 16 to 18 h at 37°C . They were fixed with methanol, stained as described in Materials and Methods, and quantified in a fluorescence microscope. The results are presented as means \pm standard deviations of duplicate samples from at least three independent experiments. CPM, counts per minute.

reduction in infection of HCE cells, whereas little or no effect was seen on infection of NHC and Hep2 cells (Fig. 4D to F).

Specific glycosidic bonds are not required for CVA24v binding to NHC and HCE cells. Four other viruses with pronounced ocular tropism, EV70 (40), Ad37 (5), avian influenza A virus H7N7 (23), and Newcastle disease virus (57), all bind more efficiently to sialic acid linked via $\alpha 2,3$ glycosidic bonds to galactose (SA $\alpha 2,3$ Gal) than to SA $\alpha 2,6$ Gal, suggesting that this could also be the case for CVA24v. To test this, we first set out to compare the relative expression of SA $\alpha 2,3$ Gal and SA $\alpha 2,6$ Gal on NHC and HCE cells. Using *Maackia amurensis* lectin (MAA II), which specifically binds SA $\alpha 2,3$ Gal (22), and SNA, which specifically binds SA $\alpha 2,6$ Gal (54), we found by flow cytometry that MAA II bound more efficiently than SNA to both NHC and HCE cells (Fig. 5), thus indicating that SA $\alpha 2,3$ Gal mainly, but also SA $\alpha 2,6$ Gal to some extent, is available to CVA24v on these cells. Preincubation of these cells with MAA II or SNA, or both together, prior to CVA24v binding showed that SNA is a slightly more-efficient inhibitor of CVA24v binding to NHC cells but also that neither of the

lectins alone efficiently inhibited CVA24v binding to HCE cells. However, when both lectins were incubated together, there was an efficient reduction in binding of CVA24v to both NHC cells (67%) and HCE cells (66%). Taken together, these experiments support the suggestion that sialic acid is a cellular receptor for CVA24v on ocular cells. However, we could not determine whether $\alpha 2,3$ or $\alpha 2,6$ glycosidic bonds between sialic acid and galactose are preferred for CVA24v binding to these cells.

The receptor used by CVA24v on NCH and HCE cells has a protein component. Since sialic acid can be linked to the cell surface through additional saccharides attached either to a protein or to a lipid, we wanted to determine whether the CVA24v receptor is a sialylated glycoprotein or a sialylated glycolipid (i.e., a ganglioside). To test this, we treated NHC and HCE cells with three different proteases. Bromelain and ficin are cysteine proteases; bromelain is relatively nonspecific, cleaving at several different amino acid sites, while ficin cleaves after aromatic amino acids. V8 protease, on the other hand, specifically cleaves polypeptides at Glu or Asp residues. All

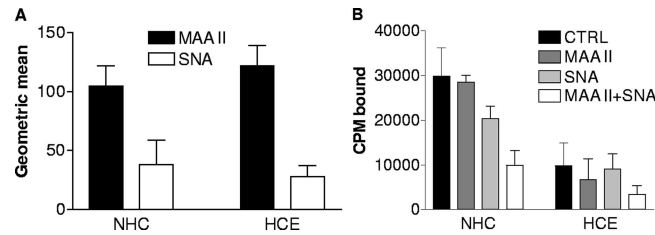


FIG. 5. Roles of SA α 2,3Gal and SA α 2,6Gal in binding of CVA24v to human ocular cells. (A) Flow cytometric analysis of expression of SA α 2,3Gal (MAA II binding) and SA α 2,6Gal (SNA binding) on NHC and HCE cells. Cells (5×10^5) in suspension were first incubated with biotinylated lectins, followed by streptavidin-FITC, prior to flow cytometric analysis. Data represent the geometric means \pm standard deviations of the fluorescence measured in three experiments in duplicate. (B) Effect of SA α 2,3Gal-binding MAA II and SA α 2,3Gal-binding SNA on CVA24v virion binding to NHC and HCE cells. Cells (2×10^5) in suspension were preincubated with lectins prior to incubation with 5,000 35 S-labeled CVA24v virions/cell. Cell-associated radioactivity (CPM [counts per minute] bound) was determined by using a scintillation counter. The results are presented as means \pm standard deviations of duplicate samples from at least three independent experiments.

three proteases efficiently reduced binding of CVA24v to both NHC and HCE cells, indicating that the cellular receptor used by CVA24v on these cells contains a protein component and that this component contains exposed and protease-sensitive cysteine, glutamic acid, and/or aspartic acid residues (Fig. 6). However, on NHC cells, ficin protease was the most efficient, since maximum inhibition was obtained at concentrations as low as ≤ 2 mU, whereas V8 protease was more efficient than bromelain and ficin on HCE cells, since maximum inhibition was already obtained at ≤ 2 mU. This suggests that there may be two nonidentical protein components that serve as receptors for CVA24v on corneal and conjunctival cells.

DISCUSSION

Members of the *Picornaviridae* use a broad range of cellular receptors, including CAR, CD55, ICAM-1, CD155 (poliovirus receptor), sialic acid, heparan sulfate, and various integrins (49). Several of these are also used by members of the *Adenoviridae*. In this study, we investigated whether any of these previously recognized receptors could also be used by CVA24v. Using cells unable to express sialic acid (Lec2), sialic acid-blocking lectins, soluble sialic acid-containing molecules, and enzymes (neuraminidase) that remove cell surface sialic acid, we have demonstrated that sialic acid is a major cellular receptor for CVA24v on CHO cells and on human epithelial

cells of corneal origin. On epithelial cells derived from human conjunctiva and larynx, sialic acid contributes to some extent to viral binding and infection, but alone, it is insufficient to mediate efficient binding and infection. Treatment of NHC and HCE cells with three different proteases resulted in a strong inhibition of the binding of CVA24v to ocular cells, which suggests that the cellular receptor(s) used by CVA24v on these cells contains at least one and probably two different protein components, thus excluding gangliosides as candidate receptors for CVA24v.

Replication of CVA24v takes place in palpebral and bulbar conjunctiva and also in corneal epithelial cells (63). Replication of a virus in the cornea leads to destruction of cells and is followed by punctate epithelial keratitis, which is thought to be the cause of foreign body sensation (64). This symptom is reported as being predominant in most outbreaks of AHC ($\sim 80\%$ of all cases) (12, 18, 25). Thus, replication in corneal cells contributes considerably to the clinical picture of AHC, so the use of sialic acid as a cellular receptor by CVA24v in the cornea is likely to affect the severity of AHC. On conjunctival cells, however, the role of sialic acid needs to be investigated further, and usage of additional receptors or coreceptors cannot be excluded.

In several cases, it has been demonstrated that viruses that bind to sialic acid show a preference for either SA α 2,3Gal or SA α 2,6Gal (42) and that this is largely associated with the

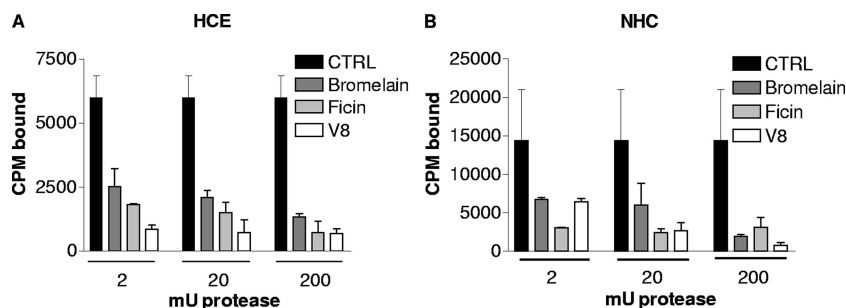


FIG. 6. Protease treatment of ocular cells inhibits binding of CVA24v virions to NHC and HCE cells. Cells (2×10^5) in suspension were pretreated with bromelain and ficin proteases (which cleave at cysteine residues) or V8 protease (which cleaves at glutamic acid or aspartic acid residues), prior to incubation with 5,000 35 S-labeled CVA24v virions/cell. Cell-associated radioactivity (CPM [counts per minute] bound) was determined by using a scintillation counter. Cell viability was assessed with the trypan blue method immediately after proteolytic digestion and no cytotoxic effects mediated by the protease treatment could be seen, compared to untreated control cells. The results are presented as means \pm standard deviations of duplicate samples from at least three independent experiments.

tropism of the virus. With few exceptions, the rule of thumb has been that respiratory viruses, such as human influenza A, bind to SA α 2,6Gal and ocular viruses, including EV70, bind to SA α 2,3Gal (40, 42). With this in mind, we first found it somewhat surprising that CVA24v was not inhibited more efficiently by SA α 2,3Ga-specific MAA II lectin. However, unlike EV70, which is rarely or never associated with respiratory disease, CVA24v is frequently associated with respiratory disease (28, 64, 66–68). Conjunctival secretions and transmission via direct contact are likely routes of transmission, but respiratory transmission may also occur (67, 68) and may even explain the rapid and extensive spread of AHC during outbreaks (67). Thus, the dual tropism of CVA24v for eyes and airways matches its capacity to bind to both SA α 2,3Gal and SA α 2,6Gal.

Immunity to AHC decreases considerably within as little as seven years (3). Accordingly, loss of herd immunity has been suggested to contribute to widespread transmission of AHC (2). The epidemic and pandemic potential of AHC-causing picornaviruses matches only one other viral disease: influenza (66). From the present work, we can conclude that one other feature that these viruses have in common is their usage of sialic acid as a cellular receptor. With few exceptions (50), avian influenza A viruses, including the highly pathogenic H5 and H7 subtypes, bind mainly to SA α 2,3Gal, whereas human influenza A viruses bind only to SA α 2,6Gal (56). Conjunctivitis seems to be a predominant symptom in zoonotic cases or outbreaks caused by H7 subtypes (17), and zoonotic cases caused by H5N1 strains have also been associated with conjunctivitis (11, 43, 61). When isolated from humans, however, H5N1 strains tend to have adapted to using both SA α 2,3Gal and SA α 2,6Gal (65). We conclude that usage of sialic acid as a receptor is a conserved feature among several oculo-respiratory viruses with pandemic potential. Further studies of the receptors used by these viruses may help us on the road to fully understanding the determinants of virus tropism and potentially also the development and establishment of pandemics caused by these viruses.

ACKNOWLEDGMENTS

We thank Jeffrey M. Bergelson for the generous gift of CHO-CAR and CHO-DAF cells and antibodies to DAF (IF7), John P. Atkinson for the generous gift of CHO-CD46 cells, and Magnus Evander for the generous gift of psgB-618 cells. We also thank our colleagues Ken Dimock and Mark Pallansch for critically reading the manuscript.

This work was supported by the Swedish Research Council (grants no. 2003-6008 and 2004-6174), the Kempe Foundation, the Swedish Society for Medical Research, the Swedish Society of Medicine, the Jeansson Foundation, the Sven and Dagmar Salén Foundation, and the Petrus and Augusta Hedlund Foundation.

The authors declare that there are no conflicts of interest in this study.

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