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<td>GRADE / DEGREE:</td>
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<td>ANNÉE D'OBTENTION / YEAR GRANTED:</td>
<td>2003</td>
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<td>TITRE DE LA THÈSE / TITLE OF THESIS:</td>
<td>Virus Receptor Interactions of Enterovirus 70: Roles of Sialic Acid and Decay-Accelerating Factor</td>
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Biochemistry, Microbiology and Immunology
FACULTÉ, ÉCOLE, DÉPARTEMENT - FACULTY, SCHOOL, DEPARTMENT

TITRE DE LA THÈSE - TITLE OF THE THESIS

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Roles of Sialic Acid and Decay-Accelerating Factor

A Thesis Submitted to the Faculty of Graduate and Postdoctoral Studies

University of Ottawa

In Partial Fulfillment of the Requirements for the Degree of
Doctor of Philosophy

Department of Biochemistry, Microbiology and Immunology
Faculty of Medicine

By

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0-612-79283-8
ABSTRACT

The interaction of viruses with host cell receptors is the initial step in viral infection and is an important determinant of virus host range, tissue tropism and pathogenesis. The complement regulatory protein decay-accelerating factor (DAF/CD55) is an attachment receptor for Enterovirus 70 (EV70), a member of the family Picornaviridae commonly associated with an eye infection in humans known as acute hemorrhagic conjunctivitis. In earlier work, the EV70 receptor on erythrocytes responsible for viral hemagglutinating activity was shown to be sensitive to neuraminidase, implying an essential role for sialic acid in virus attachment. Here, these findings are extended to show that cell surface sialic acid is required for EV70 binding to nucleated cells susceptible to virus infection, and that sialic acid binding is important in productive infection. Through the use of site-directed mutagenesis to eliminate the single N-linked glycosylation site of DAF and of a chimeric receptor protein in which the O-glycosylated domain of DAF was replaced by a region of the HLA-B44 molecule, a role in EV70 binding for the sialic acid residues of DAF was excluded, suggesting the existence of at least one additional, sialylated EV70-binding factor at the cell surface. Treatment of cells with metabolic inhibitors of glycosylation excluded a role for the N-linked oligosaccharides of glycoproteins, but suggested that O-linked glycosylation is important for EV70 binding. Finally, the virus-receptor interactions of EV70 in physiologically relevant human corneal cell lines and tissue were investigated. Sialic acid, but not DAF, was found to be an essential component of the EV70 receptor on ocular cells. These results provide proof of principle for the novel application of human corneal equivalents as a model system for infections of the eye.
ACKNOWLEDGEMENTS

First and foremost, thanks are due to Dr. Ken Dimock, a man for whom I have a great deal of respect, and about whom many accolades could be written. In addition to his guidance and mentoring in the practice of science, I am grateful for the care, concern and respect with which he treats those who work for and with him, and for his example of balancing the demands of work with those of home and family.

The contributions of the members of my thesis advisory committee are acknowledged with gratitude: Dr. Lionel Filion, Dr. Kathie Wright, and Dr. Robert Korneluk. I also appreciate the assistance of the various members, past and present, of the Dimock laboratory, of our neighbours in the Brown, Wright, Webb, Sattar and Dillon labs, and those of others, more far-flung, in the department.

Thanks are due to P. Urvil and B. Nowicki for kindly supplying the CHO cells expressing the DAF/HLA-B44 chimera and controls, to O. B. Spiller for providing the NIH/3T3 cells expressing human DAF, and to E. Suuronen and M. Griffith for supplying immortalized human corneal cell lines and tissues.

Financial support from the Natural Sciences and Engineering Research Council of Canada (NSERC), an Ontario Graduate Scholarship in Science and Technology, and the University of Ottawa made this work possible.

Finally, I am thankful for the support of many family members and friends, especially of Ann, my wife, and Molly, our daughter.

This thesis is dedicated to the glory of God, "maker of all things visible and invisible".
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INTRODUCTION

I. Acute Hemorrhagic Conjunctivitis

In the summer of 1969, a previously unknown infectious disease of humans appeared in western Africa and began to spread with alarming rapidity. Because its emergence coincided with the first American lunar landing, the disease, an unusual form of conjunctivitis caused by a previously undescribed picornavirus, became popularly known as Apollo 11 Disease. The more technical name Acute Hemorrhagic Conjunctivitis (AHC) eventually gained universal acceptance.

1. Clinical Picture

Signs and symptoms most commonly associated with AHC include the rapid onset (usually within 24 hours of exposure) of swelling of the conjunctival epithelia of both the globe of the eye (bulbar conjunctiva) and of the lids (palpebral conjunctiva), excessive tearing and serous discharge, pain, foreign-body sensation, and sensitivity to light (Wolken, 1974; Dawson et al., 1974; Higgins, 1982; Editorial, 1982a; Yin-Murphy, 1984; Wright et al., 1992). The inflammation is often accompanied by subconjunctival hemorrhages, which vary in extent from pinpoint spots (petechiae) to large areas of frank hemorrhage; the name of the disease is derived in part from this dramatic feature, which is often a cause of concern for patients. Corneal involvement, usually in the form of a punctate epithelial keratitis, has been observed to varying extents in most outbreaks, although vision typically remains unimpaired (Jones, 1972; Nabli, 1973; Quarcoo, 1973; Yang et al., 1975; Likar et al., 1975; Whitcher et al., 1976; Sklar et al., 1983; Asbell et
al., 1985; Babalola et al., 1990). Pre-auricular lymph gland enlargement and tenderness occur less frequently. With the exception of those rare cases of AHC accompanied by neurological disease (see below), the majority of cases are benign and self-limiting, and a complete recovery is usually made within one or two weeks.

Neurological complications associated with AHC were first noted in India (Bharucha and Mondkar, 1972; Wadia et al., 1973; Kono et al., 1974; Wadia et al., 1981), where patients developed fever and malaise, soon followed by acute flaccid motor paresis or paralysis primarily in the lower limbs, within days or weeks of the onset of AHC. Recovery was slow, and in approximately half of the cases a permanent incapacitation resulted. Additional cases of neurological disease accompanying AHC were later reported from Senegal (Hung and Kono, 1979), Thailand (Phuapradit et al., 1976; Kono et al., 1977), Taiwan (Yang et al., 1975; Hung et al., 1976; Hung, 1981), India (Chaturvedi et al., 1975; John et al., 1981; Wadia et al., 1981; Thakur, 1981; Mondkar, 1981; Katiyar et al., 1981; Kono et al., 1981a; Singhal et al., 1982; Katiyar et al., 1983; Wadia et al., 1983a; Wadia et al., 1983b; Chopra et al., 1986), Japan (Hung and Kono, 1979), the United States (Centers for Disease Control, 1981b; World Health Organization, 1981b; Sklar et al., 1983), Panama (Reeves et al., 1986), and Puerto Rico (Waterman et al., 1984). The incidence has been estimated to be in the range of 1:10,000 to 1:17,000 among cases of AHC (Hung and Kono, 1979). Most commonly the neurological disease resembled acute poliomyelitis, as in the original Indian cases described above. Clinical observations and electrophysiological studies of these cases suggested the involvement of the anterior horn cells of the spinal cord, a conclusion that was supported by the post mortem analysis of an animal model of the disease generated in cynomolgus monkeys.
(Kono et al., 1973). In certain other cases, the neurological complications of AHC manifested as various cranial nerve palsies, and in a few rare cases as encephalitis (World Health Organization, 1982b). One patient died of respiratory failure as a result of acute ascending transverse myelitis two weeks after developing AHC (Wadia et al., 1981). The sudden emergence of a new infectious disease of humans, with a demonstrated capacity for pandemic spread matched only by that of influenza (see below), and with the potential to cause serious and permanent neurological illness, warrants serious attention.

2. Pandemic Spread

Chatterjee et al. (1970) were the first to describe the unusual new form of conjunctivitis that emerged and spread rapidly through Ghana in western Africa between June and October of 1969. From this initial focus (Kono, 1985), the disease spread east and south along the coast of western Africa during 1969 and 1970 (World Health Organization, 1971), with outbreaks being sequentially reported from Nigeria (Parrott, 1971) to Gambia (Editorial, 1973). In 1971, AHC spread through northern and eastern Africa, including Morocco (Chomel et al., 1973; Nejmi et al., 1974), Tunisia (Nabli, 1973; Whitcher et al., 1976) and Kenya (Metselaar et al., 1976). The disease also appeared in Java, Indonesia in 1970 (Kono, 1975) and quickly spread to Singapore (Yin-Murphy and Lim, 1972). In 1971 AHC affected most of southeast Asia (Yang et al., 1975; Kono, 1975) and the Indian subcontinent (Pramanik, 1971; Roy et al., 1972; Chaturvedi et al., 1975), extending northwards as far as Japan (Kono et al., 1972). Isolated outbreaks also were reported from several locations in Europe, including London (Jones, 1972), Moscow (Hierholzer et al., 1975), Lyon (Mirkovic et al., 1973) and northern Yugoslavia (Likar et al., 1975).
During the remainder of the 1970s, smaller outbreaks of AHC occurred, as the disease began to circulate endemically in those regions affected in the initial pandemic (Phuapradit et al., 1976; Mathur et al., 1977; Kono et al., 1977; Arnow et al., 1977). In 1980, the beginnings of a second pandemic were signalled by outbreaks of AHC in Africa (Nairobi, Kenya; World Health Organization, 1980) and Southeast Asia (Singapore and Malaysia; Yin-Murphy, 1984). In the same year, a localized outbreak among southeast Asian refugees arriving in California was also reported (Centers for Disease Control, 1980a; Centers for Disease Control, 1980b; Bernard et al., 1982). In 1981, widespread AHC epidemics were reported throughout Africa and Asia, including Nigeria (World Health Organization, 1981a; McMoli et al., 1984), the Democratic Republic of Congo (formerly Zaire) (Desmyter et al., 1981; World Health Organization, 1981b), Pakistan (World Health Organization, 1981c; World Health Organization, 1981f), India (World Health Organization, 1981a; World Health Organization, 1982b; Pal et al., 1983b), Bangladesh (Hossain et al., 1983), the United Arab Emirates (World Health Organization, 1981c), and Yemen (World Health Organization, 1982a). The first reports of AHC transmission in the western hemisphere emerged from Surinam in South America (World Health Organization, 1982e), making AHC a truly global pandemic. The disease spread rapidly throughout South and Central America and the Caribbean (World Health Organization, 1981a; World Health Organization, 1981b; World Health Organization, 1981c; Centers for Disease Control, 1981d; World Health Organization, 1981d; World Health Organization, 1981e; Centers for Disease Control, 1981e; Waterman et al., 1984; Asbell et al., 1985; Reeves et al., 1986), affecting Brazil, Colombia, Guyana, Panama, Honduras, Belize, Costa Rica, Guatemala, El Salvador, Nicaragua, Mexico,
Cuba, Haiti, Antigua & Barbuda, Bahamas, Dominican Republic, Puerto Rico and Trinidad & Tobago. Outbreaks in the United States (Key West and Miami, Florida and in North Carolina) in September and October of 1981 attracted considerable attention (Centers for Disease Control, 1981a; Centers for Disease Control, 1981b; World Health Organization, 1981b; Centers for Disease Control, 1981c; World Health Organization, 1981c; World Health Organization, 1981e; Centers for Disease Control, 1981f; World Health Organization, 1982e; Sklar et al., 1983; Patriarca et al., 1983; Malison et al., 1984). Later in 1981 and well into 1982, AHC spread through the islands of the western and South Pacific (Centers for Disease Control, 1982; World Health Organization, 1982c; World Health Organization, 1982e; World Health Organization, 1982f; Onorato et al., 1985), eventually reaching New Zealand (World Health Organization, 1982d).

Since 1982, the global incidence of AHC has returned to a pattern similar to that of the inter-pandemic period, with periodic outbreaks in previously affected areas (Wulff et al., 1987; Goh et al., 1990; Babalola et al., 1990; Ramia and Arif, 1990; Bern et al., 1992; Orillac and Langford, 1993; Bhide et al., 1994; Yamazaki et al., 1995; Uchio et al., 1996). Such occurrences continue up to the present, with recent reports emerging from India (Maitreyi et al., 1999; Wairagkar et al., 1999), Japan (Uchio et al., 1999) and Israel (Shulman et al., 1997).

Because records are incomplete for many of the outbreaks of AHC, it is difficult to estimate the total number of persons affected by the disease. Towards the end of the second pandemic the global incidence was estimated to be 80 million cases (Wadia et al., 1983a; Wadia et al., 1983b); after twenty years of additional sporadic outbreaks, the true number is undoubtedly higher still.
3. Epidemiology

It was established early on that the primary mode of AHC transmission is from eye to eye, via hands or other contaminated articles (fomites), such as bedding material (Editorial, 1973). Infected individuals are capable of transmitting the disease for at least eight days after onset, even after clinical signs and symptoms have completely resolved (Kuritsky et al., 1983); one study found that subclinical infection persisted for more than 60 days post-exposure (Pal et al., 1983b). Secondary attack rates in affected homes are consistently very high, often 100% (Wolken, 1974; Asbell et al., 1985), and community attack rates can reach levels of over 60% during an epidemic (Hossain et al., 1983; Reeves et al., 1986). The majority of cases worldwide have occurred in coastal tropical and semi-tropical zones, and the relative humidity of the air has been found to have a major impact on the survival of the virus in the environment, which may help to explain this geographic distribution (Sattar et al., 1988). Overcrowding and poor hygienic conditions contribute to the spread of the disease (Waterman et al., 1984), a fact that is strikingly illustrated by the discrepancies in attack rates between upper and lower class groups during various outbreaks (Parrott, 1971; Arnow et al., 1977; Patriarca et al., 1983; Onorato et al., 1985; Reeves et al., 1986). Males and females are generally affected equally, and although certain early studies suggested a lower incidence of AHC among young children (Kono, 1975), this observation was not corroborated by data collected during the second pandemic (Patriarca et al., 1983; Malison et al., 1984). In contrast, the neurological complications of AHC appear to affect primarily adult males (Hung and Kono, 1979; Editorial, 1982b; Wadia et al., 1983b; Chopra et al., 1986), perhaps the most significant clinical/epidemiological difference between the AHC-associated neurological
disease and acute poliomyelitis, the latter of which affects predominantly children and adolescents.

II. Enterovirus Type 70

1. Causative Agent of Acute Hemorrhagic Conjunctivitis

From the outset, a viral etiology was suspected for AHC, although initial virus isolation attempts were unsuccessful (Chatterjee et al., 1970; Parrott, 1971; World Health Organization, 1971; Roy et al., 1972). Prior to the first pandemic of AHC, various adenovirus serotypes were most commonly associated with epidemic conjunctivitis (Yin-Murphy, 1984), and it was suspected that a particularly virulent adenovirus strain was responsible for the new illness. Surprisingly, a picornavirus having the attributes of an enterovirus, yet not neutralized by antisera recognizing any of the previously known enteroviruses, was isolated in Japan during the epidemic in that country in 1971 (Kono et al., 1972); this agent was soon officially recognized as a new serotype, Enterovirus type 70 (EV70) (Mirkovic et al., 1973; Melnick et al., 1974). The isolation of identical or very similar (antigenically cross-reacting) enteroviruses from cases of AHC was later reported from various locations in Africa, Asia and Europe (Jones, 1972; Yin-Murphy and Lim, 1972; Nabli, 1973; Higgins and Scott, 1973; Higgins et al., 1974; Nejmi et al., 1974; Chaturvedi et al., 1975; Yang et al., 1975; Likar et al., 1975; Metselaar et al., 1976; Whitcher et al., 1976). The majority of paired acute and convalescent phase sera from AHC patients in various geographical locations showed a four-fold or greater rise in neutralizing titre to EV70, providing additional support for the role of this agent in the disease (Kono et al., 1975). A final confirmation of the causative role of EV70 in AHC
was provided by accidental laboratory-acquired infections (Sasagawa et al., 1976). Although several successful isolations of EV70 during the second pandemic in 1980-1982 were reported (World Health Organization, 1980; Centers for Disease Control, 1980a; Hatch et al., 1981; World Health Organization, 1981f; Centers for Disease Control, 1981f; World Health Organization, 1982b; Hossain et al., 1983; Malison et al., 1984; Waterman et al., 1984; Asbell et al., 1985), virus recovery rates were significantly lower than in previous years (Desmyter et al., 1981; World Health Organization, 1981g).

In the years since the end of the second pandemic, EV70 has been even more resistant to isolation attempts; consequently, confirmation of its role in outbreaks of AHC has more often been achieved by serological studies (Yin-Murphy, 1976; Kuritsky et al., 1983; Hierholzer et al., 1984), immunofluorescence (Kurata et al., 1983; Pal et al., 1983a; Pal et al., 1983b; Anderson et al., 1984; Bhide et al., 1994; Maitreyi et al., 1999), analysis of tear specimens for virus particles and early-appearing neutralizing antibody (Yin-Murphy et al., 1985; Shulman et al., 1997), enzyme-linked immunosorbent assay (ELISA) (Anderson et al., 1984; Wulff et al., 1987) and RT-PCR (Uchio et al., 1996; Shulman et al., 1997; Maitreyi et al., 1999; Uchio et al., 1999).

Conclusive evidence for the role of EV70 in AHC-associated neurological disease has been more difficult to obtain. As described previously, certain signs and symptoms of the illness closely resemble those of acute poliomyelitis, and many (but not all) of the cases of neurological complications accompanying AHC occurred in areas of the world where polioviruses were still circulating. Initially, the neurovirulence of EV70 was suggested by epidemiological and serological evidence, by virtue of the temporal association of the neurological disease with AHC, and of the presence of high and/or rising neutralizing
titres against the virus in the sera of patients (Bharucha and Mondkar, 1972; Wadia et al., 1972; Wadia et al., 1973; Kono et al., 1974; Chaturvedi et al., 1975; Green et al., 1975; Hung et al., 1976; Phuapradit et al., 1976). However, elevated titres to all three serotypes of poliovirus were also often detected. Kono et al. proceeded to analyze neutralizing antibodies against poliovirus and EV70 by sucrose density gradient centrifugation, and found that anti-EV70 activity was found in both the 19S (corresponding to the IgM isotype) and 7S (IgG) fractions, whereas neutralizing antibody to all three poliovirus serotypes was restricted to the 7S fraction; the presence of IgM antibodies to EV70 was interpreted as indicating a relatively recent infection, whereas the anti-poliovirus IgG was suggested to be maintenance antibody (Kono et al., 1977). Attempts to isolate EV70 from the central nervous system (CNS) have been uniformly unsuccessful. However, strong evidence for the presence of EV70 in the CNS was obtained during the second pandemic: analysis of the cerebrospinal fluid (CSF) of patients afflicted with AHC-associated neurological disease revealed high and rising titres of neutralizing antibody to EV70 (John et al., 1981; Kono et al., 1981a; Katiyar et al., 1983; Wadia et al., 1983a; Chopra et al., 1986), whereas Pal et al. used immunofluorescence to demonstrate the presence of EV70 antigen in isolated spinal cord cells (Pal et al., 1986). Finally, the neurovirulence of EV70 in experimental animals was demonstrated with the reproduction of the polio-like disease in cynomolgus monkeys after intraspinal and intrathalamic inoculation (Kono et al., 1973); expanded studies involving additional virus isolates in more sensitive monkey species have been called for but not completed (Arya, 1984). The route by which the virus accesses the CNS remains unknown. By analogy with poliovirus (Ohka and Nomoto, 2001), possibilities include
the spread to secondary sites of infection during viremia, or direct viral entry to the peripheral nervous system at the primary site of replication (i.e., the conjunctivae), followed by migration to the CNS (Hung and Kono, 1979).

A second, antigenically distinct enterovirus was isolated from some cases of AHC in Singapore in 1970 and Hong Kong in 1971 (Yin-Murphy and Lim, 1972; Yin-Murphy, 1973; Higgins et al., 1974), and was later found to be an antigenic variant of coxsackievirus A24 (CAV24v) (Mirkovic et al., 1974). This virus possesses a very low level of sequence similarity with EV70 (Natori et al., 1984), and is believed to have emerged independently at approximately the same time (Miyamura et al., 1990; Ishiko et al., 1992). AHC due to CAV24v has not been associated with neurological complications.

2. Physical Properties

Initially, it was the physicochemical properties of AHC virus that led investigators to classify the etiological agent as an enterovirus within the family Picornaviridae (Kono et al., 1972; Jones, 1972; Yin-Murphy and Lim, 1972; Mirkovic et al., 1973; Nabli, 1973; Chomel et al., 1973; Yin-Murphy, 1973; Higgins et al., 1974; Nejmi et al., 1974; Likar et al., 1975). Specifically, electron microscopy revealed an unenveloped capsid of 25-30 nm in diameter, possessing apparently cubic symmetry. Infectivity of the virus was not significantly reduced by exposure to acid (pH 3.0), chloroform, ether, detergent (sodium deoxycholate), or elevated temperature (50°C, in the presence of 1M MgCl₂). The failure of 5-iododeoxyuridine to inhibit viral replication and the staining of virions and infected cells with acridine orange suggested an RNA genome. Identification
of the virus as an enterovirus was further supported by the characteristic cytopathic effect produced in infected cell cultures in vitro.

More detailed physical characterization of the virus further supported this early classification. The buoyant density of the virus was determined to be 1.34 g/mL in cesium chloride (Yamazaki et al., 1974; Higgins et al., 1974). RNA extracted from virus particles was shown to be single-stranded owing to its sensitivity to RNase A, and to have a sedimentation coefficient of 34S and a relative molecular weight of 2.5 x 10^6 (Yamazaki et al., 1974). Analytical studies of the virion structural proteins revealed the characteristic four picornaviral polypeptides: VP1 (35 kDa), VP2 (28 kDa), VP3 (27 kDa) and VP4 (9 kDa) (Esposito and Obljeski, 1976).

Picornavirus capsids consist of 60 protomers, each containing one copy of each of the four viral structural proteins, VP1 through VP4. VP1, VP2 and VP3 together make up the icosahedral viral shell, of approximately 30 nm in diameter, containing the single stranded, positive sense, 7-8.5 kb RNA genome; VP4 resides at the RNA-capsid interface (Smith and Baker, 1999). High-resolution three-dimensional structures have been obtained for a number of picornaviruses, revealing the presence of a canyon, 15-20 Å deep, surrounding each of the five-fold vertices of enterovirus and rhinovirus capsids (Smith and Baker, 1999; Rossmann et al., 2000; Rossmann et al., 2002; Hogle, 2002). A cavity within VP1 at the base of the canyon was found to contain a hydrophobic "pocket factor", postulated to be important in conferring structural stability to the virion (Rossmann et al., 2000; Hogle, 2002). Structural information for EV70 itself has not yet been obtained.
3. Origins and Phylogeny

The sudden appearance of a previously undescribed enterovirus prompted speculation as to its origin. Early hypotheses included a previously circulating human enterovirus that acquired new pathogenic properties, or an animal or insect virus that crossed the species barrier (Kono, 1975). Subsequent serological studies of healthy human populations in endemic areas prior to the appearance of AHC revealed a very low level of circulating virus neutralizing antibodies, suggesting that EV70 was not prevalent in humans prior to the first pandemic (Kono et al., 1975; Hierholzer et al., 1975; Minami et al., 1981; Kono et al., 1981b). The possibility of a zoonotic origin of EV70, on the other hand, was supported by the detection of neutralizing antibody to EV70 in the sera of various domestic animals (Kono et al., 1975; Kono et al., 1981c), and by the observation that EV70 has the ability, rare among human enteroviruses, to replicate in vitro in cells derived from a variety of non-primate sources (Yoshii et al., 1977). Oligonucleotide analysis of the genomes of EV70 strains isolated in various geographic areas at various times indicated that all isolates were closely related (Kew et al., 1983) and derived from a common ancestor that emerged in Western Africa in 1966/67, several years before the onset of the first pandemic (Takeda et al., 1984; Miyamura et al., 1986); subsequent sequence and phylogenetic analysis supported this conclusion (Takeda et al., 1994).

The *Picornaviridae* comprise a large family of important human and animal pathogens including such well-known members as the polioviruses, foot-and-mouth disease virus and hepatitis A virus. The family is currently divided into six genera: Enterovirus (including coxsackieviruses and echoviruses), Rhinovirus, Cardiovirus, Aphthovirus, Hepatovirus, and Parechovirus; the addition of several additional genera (including
Erbovirus, Teschovirus and Kobuvirus) containing newly described picornaviruses has been proposed (King et al., 2000). The publication of the complete genome sequence of EV70 (Takeda, 1989; Ryan et al., 1990) confirmed its earlier classification, based on physicochemical and biological properties, as an enterovirus. Phylogenetic analysis of the 5'-untranslated region (5'-UTR) led to the clustering of EV70 with CAV21, CAV24, and the polioviruses, whereas analyses of the coding region and 3'-UTR resulted in EV70 grouping separately from all other enteroviruses analyzed (Auvinen et al., 1989; Poyry et al., 1996). In keeping with this latter finding, EV70 is officially classified as the prototype member of the *Human enterovirus D* species, of which enterovirus 68 is the only other member (King et al., 2000). Recent work has found that human rhinovirus 87 clusters separately from the rest of the rhinovirus serotypes and close to EV70 (Savolainen et al., 2002).

### 4. Unusual Attributes

In addition to its intriguing identity as a newly emerged human pathogen, EV70 has also attracted interest due to several unusual attributes. The virus is noteworthy as a naturally occurring temperature-sensitive “mutant”, with optimal replication occurring at approximately 33°C, the normal temperature of the conjunctivae, with somewhat elevated temperatures (39°C) being nonpermissive for the growth of most isolates (Miyamura et al., 1974; Miyamura et al., 1976; Stanton et al., 1977). Subsequent studies attempting to discover the molecular basis for this temperature sensitivity indicated that RNA replication (Miyamura et al., 1978; Takeda et al., 1982), and, in particular, a lack of uridylylation of the genome-linked viral protein VPg (an essential step in the initiation of
RNA synthesis) is impaired at the higher temperature (Miyamura et al., 1984; Takeda et al., 1989).

Less well understood is the unusual tissue tropism of EV70 for the eye and/or CNS; only very rarely has the virus been isolated from the gut, and its rapid spread during outbreaks of AHC suggests that even if occasional replication in the enteric tract does occur, it is not likely to be important in the life cycle of the virus in the infected host. Although not completely unique in this respect (e.g., CAV21 appears to replicate almost exclusively in the upper respiratory tract; Higgins, 1982), a prominent role for intestinal infection is typically seen in the pathogenesis of members of the genus Enterovirus, as the name suggests.

In addition, as alluded to above, the ability of EV70 to replicate in vitro in cells derived from a wide range of non-primate species, possibly reflecting an animal origin, is also uncommon among human enteroviruses (Yoshii et al., 1977).

A long-term objective of our laboratory is to determine the molecular basis underlying the unusual tissue tropism and broad in vitro host range of EV70. Because both of these properties of the virus involve interaction with the host cell, a brief consideration of the viral life cycle and of the major points of virus-cell interaction follows.

5. Virus-Cell Interactions

The initial step in the enterovirus infectious cycle is attachment of the virus to one or more specific host cell receptors. [For many virus families, including Picornaviruses, Retroviruses and Herpesviruses, important distinctions have been made between cell surface molecules involved strictly in the attachment of virions to the cell surface, and those mediating additional steps in virus internalization (Evans and Almond,
In this thesis, the term ‘receptor’ is used for any molecule enabling binding. Receptor binding may permit virus entry in certain cases, or additional factors may be required.] The details and subcellular location (i.e., at the cell surface or within endosomes) of cell entry by enteroviruses remain poorly understood; however, the viral genome is ultimately introduced into the cytoplasm of the target cell, a step referred to as “uncoating”; for a review of enterovirus penetration and uncoating, see (Hogle, 2002). The positive-sense viral RNA then functions as messenger RNA (mRNA) to direct the synthesis of viral gene products by the host cell translation machinery. The viral mRNA encodes a single polyprotein that is autolytically cleaved in a controlled, stepwise fashion, eventually yielding individual viral proteins, including the capsid proteins (VP1 through VP4) and the RNA-dependent RNA polymerase. A highly efficient shutdown of host cell macromolecular synthesis soon follows viral protein synthesis. Using the viral genomic RNA as a template, the RNA-dependent RNA polymerase transcribes negative-sense, complementary RNA as a replicative intermediate; RNA replication is physically associated with intracellular membranes. Additional positive sense transcript is then amplified and employed in ongoing viral protein synthesis, RNA replication, and, eventually, as genomic RNA to be packaged within newly assembled virions. Release of progeny virus is often achieved by lysis of the infected cell, although productive infection in the absence of any observable cytopathic effect also occurs with certain viruses (including EV70) in certain cell types. For a detailed overview of the picornavirus replication cycle, see (Racaniello, 2001).

The dependence of a virus on a specific receptor molecule for cell entry has obvious implications for tissue tropism and species host range: only those cells and tissues in a
mixed culture or in the infected host that express a functional receptor will be susceptible to infection, thus providing an important restriction on viral spread and pathogenesis. The links between receptor usage, tropism, host range and pathogenesis have been established for a number of viruses (Schneider-Schaulies, 2000), and virus-receptor interactions among the Picornaviridae are among the most extensively characterized (Evans and Almond, 1998; Rossmann et al., 2000; Rossmann et al., 2002; Hogle, 2002).

As a group, picornaviruses recognize a wide variety of cell surface receptors, and in many cases, clustering of viruses according to their associated disease parallels clustering by receptor usage. Thus, all three poliovirus serotypes recognize a single receptor (CD155), all but one of the more than one hundred rhinovirus serotypes bind to intercellular adhesion molecule 1 (ICAM-1) or to the low-density lipoprotein receptor, and all six coxsackie B viruses use the coxsackievirus-adenovirus receptor (CAR) protein for cell attachment and entry (Rossmann et al., 2000).

However, the ability of a virus to gain entry to the cytoplasm is not sufficient to guarantee productive infection. Possessing minimalist genomes, picornaviruses depend on various host factors for the execution of most of their essential functions, including translation of viral proteins and replication of viral RNA. Although many of the required cellular factors are also fundamental to basic cellular processes and, as such, are widely expressed, it is clear that intracellular determinants must play a role in restricting the tropism of picornaviruses in vivo and in vitro (Andino et al., 1999); for example, in transgenic mice engineered to express the human poliovirus receptor, only a restricted subset of tissues expressing the virus receptor were found to be susceptible to infection (Koike et al., 1991; Ren and Racaniello, 1992). Similarly, although the cellular factors
involved have not yet been identified, it is clear that nucleotides in the 5’UTR of poliovirus and coxsackievirus B3 (CBV3) contribute to the neurovirulence phenotypes of those viruses (Gromeier et al., 1999; Harvala et al., 2002). For poliovirus, those nucleotides required for neurovirulence have been localized to stem-loop structures in the internal ribosome entry site (IRES) element, which is involved in the initiation of viral protein synthesis (Gromeier et al., 1999).

III. Hypothesis

While recognizing that intracellular determinants of replication may also be involved, the work described in this thesis has been directed towards testing the hypothesis that virus-receptor interactions constitute an important molecular basis for the tissue tropism, pathogenesis, and species host range of EV70, which are atypical for a human enterovirus.

IV. Enterovirus 70 Receptors

At the outset of the work described in this thesis, two molecules had been implicated in the attachment of EV70 to human cells: sialic acid and decay-accelerating factor.

1. Sialic Acid

Early work on the cellular receptor for EV70 made use of viral hemagglutination studies to demonstrate that sialic acid, a negatively charged terminal sugar residue commonly found in the oligosaccharides of cell surface glycoproteins and glycolipids, was involved in the binding of the virus to human erythrocytes (Utagawa et al., 1982). Although
providing important preliminary information, the physiological relevance of this interaction to the life cycle of the virus remained to be established.

2. Decay-Accelerating Factor

In an effort to identify EV70 attachment receptors, monoclonal antibodies (mAbs) were raised against HeLa cell plasma membranes, and screened for their ability to protect the cells from virus infection (Karnauchow et al., 1996). A single protective mAb was obtained, which was later found to react with decay-accelerating factor (DAF; CD55).

DAF is a widely expressed complement regulatory protein involved in protecting host cells from damage by the autologous complement system (Lublin and Atkinson, 1989; Nicholson-Weller and Wang, 1994). It is a member of the ‘regulators of complement activation’ (RCA) family of proteins, members of which possess variable numbers of ~60 amino acid sequences termed complement control protein (CCP) domains (also known as short consensus repeats, or SCRs). DAF possesses four such domains, of which the first, CCP1, and to a lesser extent the second, CCP2, have been implicated in the interaction with EV70, as determined by testing virus binding to chimeric molecules composed of different combinations of the CCPs of DAF and the closely related molecule CD46; sequences in CCP1 of DAF were found to be necessary for EV70 binding, whereas optimal binding was also dependent on the presence of CCP2 (Karnauchow et al., 1998). The CCP modules are mounted upon a heavily O-glycosylated, serine- and threonine-rich, mucin-like stalk domain, and a single complex-type N-linked glycosylation site is located at the interface of CCP1 and CCP2 (Lublin et al., 1986) (see panel A of Figure 5). The predominant form (~90%) of the molecule is inserted into the outer leaflet of the plasma membrane by a glycosyl-phosphatidylinositol
A covalently cross-linked homodimer of DAF has also been described (Nickells et al., 1994). The relative molecular weight of the monomeric, membrane-bound form of DAF ranges from 70,000 to 80,000, presumably due to variations in the nature and extent of glycosylation (Lublin et al., 1986).

DAF protects host cells by promoting the disassociation of (i.e., accelerating the decay of) the two subunits comprising the C3/C5 convertase heterodimers of both the classical and alternative complement pathways, thereby interrupting the activation cascade of both pathways at the point at which they converge into the effector branch of the system (Lublin and Atkinson, 1989; Nicholson-Weller and Wang, 1994). Residues involved in the modulation of complement components have been localized to CCP domains 2, 3, and 4 (Coyne et al., 1992; Brodbeck et al., 1996; Brodbeck et al., 2000; Kuttner-Kondo et al., 2001), although a certain level of cryptic decay-accelerating activity has also been detected in CCP1 (Christiansen et al., 2000). Crystal structures have been presented for other members of the RCA family [e.g., membrane co-factor protein, CD46 (Casasnovas et al., 1999), and vaccinia virus complement control protein (Murthy et al., 2001); for a review, see Kirkitadze and Barlow, 2001], but no high resolution structural information is yet available for DAF itself. By using structural data from related proteins, a model of the three-dimensional structure of DAF has been proposed, in which the CCP domains of the molecule assume a helical conformation (Kuttner-Kondo et al., 1996).

In addition to its interactions with the C3/C5 convertases of the complement system, DAF has been identified as a ligand (via CCP1) for CD97, an activation-induced
leukocyte antigen (Hamann et al., 1996), and as part of a lipopolysaccharide (LPS)-
binding complex, along with CD14 and the soluble LPS-binding protein (el-Samalouti et 
al., 1999; Heine et al., 2001). DAF has also been implicated as a cellular receptor for a
number of other pathogens, including various enteroviruses in addition to EV70 (Ward et 
al., 1994; Bergelson et al., 1994; Shafren et al., 1995; Bergelson et al., 1995; Shafren et al.,
1997a; for a more detailed treatment of the use of DAF by other enteroviruses, see 
Discussion), and several strains of Escherichia coli that express Dr or related adhesins 
(Nowicki et al., 1993; Pham et al., 1995). It has been demonstrated by several groups that 
the binding of various ligands to DAF results in intracellular changes triggered by signal 
transduction through DAF (Davis et al., 1988; Shenoy-Scaria et al., 1992; Shibuya et al.,
1992; Peiffer et al., 1998; Heine et al., 2001; Tieng et al., 2002).

V. Objectives

As this work began, the relation between DAF binding and the tissue tropism of EV70 
remained uncertain; it was clear, however, that the use of DAF as a receptor could not 
onely account for the tropism of the virus. Although DAF is expressed at the cell surface 
of conjunctival and corneal epithelia (Medof et al., 1987b; Lass et al., 1990; Bora et al.,
1993), and DAF antigen has been detected in the CSF (Medof et al., 1987b), expression 
of the molecule is much wider than those tissues infected by EV70, and in fact includes 
most cell types exposed to serum (Nicholson-Weller and Wang, 1994). Further, the 
recognition of DAF by other enteroviruses that cause clinical syndromes quite different 
from AHC argues against DAF binding being the sole, or perhaps even an important, 
determinant of tropism and pathogenesis for EV70.
Similarly, the role of sialic acid in EV70 attachment and entry, and the relationship, if any, between the observed requirements for sialic acid and for DAF remained unclear. Might the virus specifically require the sialic acid residues of DAF for attachment? Intriguingly, the N-linked oligosaccharide of DAF resides at the interface of CCP domains 1 and 2 (Coyne et al., 1992), the same region of the molecule that was implicated in EV70 binding (Karnauchow et al., 1998). In addition, although further removed from the identified site of EV70 interaction, the O-linked oligosaccharides of DAF in the stalk region have been shown to be heavily sialylated (Lublin et al., 1986).

The initial objective of this work, then, was to confirm the role played by sialic acid in EV70 binding to nucleated cells susceptible to virus infection. Subsequently, a possible requirement for the sialic acid residues of DAF in EV70 binding was tested, and a preliminary characterization of the nature of the sialoconjugate involved in virus attachment was undertaken. Finally, the roles of DAF and sialic acid in EV70 interactions with immortalized human corneal cells and tissues, a novel, physiologically relevant system, were explored.
MATERIALS AND METHODS

I. Cells, cell culture and viruses

HeLa cells were obtained from the National Institute of Allergy and Infectious Diseases AIDS Research and Reference Reagent Program, Bethesda, MD; LLC-MK2 cells (rhesus monkey (*Macaca mulatta*) kidney) from Flow Laboratories, Rockville, MD; and CV-1 cells (African green monkey (*Cercopithecus aethiops*) kidney) from the American Type Culture Collection (ATCC), Rockville, MD. U-937 cells (human histiocytic lymphoma) were from L. Filion, and NIH/3T3 (murine fibroblast) cells were provided by E. G. Brown, both of the Department of Biochemistry, Microbiology and Immunology at the University of Ottawa, Ottawa, Canada. NIH/3T3 cells constitutively expressing human DAF have been described previously (Spiller et al., 2000) and were from O. B. Spiller in the Department of Medicinal Biochemistry, University of Wales College of Medicine, Cardiff, U.K. Chinese hamster ovary (CHO) cells constitutively expressing human DAF or a DAF/HLA-B44 chimera (amino acid residues 1 through 257 of DAF fused to the carboxyl terminus of HLA-B44 from amino acid 66 through the stop codon) were obtained from B. Nowicki, Dept. of Microbiology and Immunology, University of Texas Medical Branch, Galveston, TX and have also been previously described (Coyne et al., 1992). HeLa cells, NIH/3T3 cells and derivatives, and CHO cells and derivatives were maintained in Eagle’s minimal essential medium containing Earle’s salts (MEM), as previously described (Karnauchow et al., 1996); U-937 cells were grown in RPMI 1640 medium containing 10% fetal bovine serum (FBS). All of the above media and supplements were from Invitrogen Life Technologies.
Human corneal equivalents and their constituent cell lines have been described elsewhere in detail (Griffith et al., 1999). Briefly, three major cell layers comprise the complete cornea; moving from anterior to posterior, these are the stratified squamous epithelium, followed by a stromal layer consisting of keratocytes dispersed in a collagenous matrix, and finally the endothelial monolayer that lines the intraocular surface. Cells of human origin were isolated from each cell layer and immortalized by induced expression of SV40 large T antigen (epithelial cells) or human papillomavirus genes E6 and E7 (stromal and endothelial cells). Epithelial cells were cultivated in keratinocyte serum-free medium (Invitrogen Life Technologies), and stromal and endothelial cells were grown in medium 199 (Sigma), supplemented with 10% FBS and 1% insulin-transferrin-selenium (Sigma). Corneal equivalent tissues were constructed and maintained in the lab of M. Griffith, University of Ottawa Eye Institute, Ottawa, Canada, by E. Suuronen, until the epithelium was estimated to be approximately four to six cell layers deep (approximately ten days), at which time experiments were conducted.

Enterovirus type 70, prototype strain J 670/71, was obtained from M. Hatch and M. Pallansch, Centers for Disease Control and Prevention, Atlanta GA, and was passaged in LLC-MK2 as described (Karnauchow et al., 1996). Echovirus 11 (E11), strain Gregory, was from S. Lee at the National Centre for Enteroviruses, Halifax, NS, Canada and was also passaged in LLC-MK2 cells. Vaccinia virus strain vTF7-3 was purchased from ATCC, and was propagated in CV-1 cells.

II. Virus binding assay

Adherent cells were detached by incubation in 15 mM EDTA in Tris-buffered saline (TBS). For each replicate, 5 x 10^5 cells were washed once in TBS, and a non-saturating
amount (1,000-10,000 cpm) of $^{35}\text{S}$-radiolabelled EV70 ($^{35}\text{S}$-EV70; prepared as in Karnauchow et al., 1996) or $^{35}\text{S}$-E11 in serum-free medium (final volume 50 µL) was added to the cells. After incubation at 33°C (EV70) or 37°C (E11) for 1 h, the cells were pelleted and the supernatant was removed. The cells were washed once with serum-free medium and pelleted again, and the resulting supernatant was pooled with that obtained previously; this pooled supernatant represented the unbound fraction of input virus. The cells, with associated virus particles, were resuspended, representing the bound fraction of input virus, and samples were analyzed by liquid scintillation counting. A representative binding assay, showing binding of $^{35}\text{S}$-EV70 to HeLa and U-937 cells, is shown in Figure 1.

**III. Inhibition of virus binding**

Cells were processed as in the virus binding assay described above. Prior to the combining of cells and $^{35}\text{S}$-labelled virus, cells were incubated (as indicated) in protease-free *Vibrio cholerae* neuraminidase (Roche) in 150 µL of serum-free medium for 30 min at 37°C, or in the presence of 22 µg/mL anti-DAF monoclonal antibody EVR1 for 60 min at 37°C, and then washed twice with TBS. EVR1 recognizes complement control protein domain 1 (CCP1) of human DAF and has been shown to block EV70 binding to and infection of HeLa cells (Karnauchow et al., 1996). Alternatively, $^{35}\text{S}$-labelled EV70 was incubated at 33°C for 60 min in a final volume of 50 µL of serum-free medium with the indicated concentrations of sialic acid (N-acetyl neuraminic acid; Sigma) prior to assaying virus binding.
Figure 1 Representative binding of $^{35}$S-labelled EV70 to HeLa and U-937 cells.

Cells were incubated for 1 h at 33°C in a final volume of 50 μL of serum-free cell culture medium containing the indicated volumes of $^{35}$S-labelled EV70 (ranging from approximately 1,000–100,000 cpm). Based on these and similar results obtained with other preparations of $^{35}$S-labelled EV70, a volume of approximately 1 μL of virus stock was generally used in virus binding assays.
IV. Infection of cells and tissues with EV70

For adherent cells, multi-well dishes were infected at 80-100% confluency, 48-72 h after seeding. The cells in a representative well were detached with trypsin and counted to estimate the multiplicity of infection (MOI). Suspension cells were infected in the log phase of growth and were also counted prior to infection. For both adherent and suspension cultures, cells were inoculated with EV70 at an MOI of 5 in a minimal volume (250-400 µL) and incubated for 1 h at 33°C. Cultures were then washed twice with TBS, culture medium was replaced, and cells were incubated at 33°C; the beginning of this incubation was considered as time zero. Infected monolayers or aliquots of infected suspension cells were removed at various times, and subjected to three cycles of freezing and thawing. The released virus was quantified by plaque assay on LLC-MK2 monolayers according to standard protocols.

For infection of human corneal equivalents with EV70, the number of epithelial cells exposed at the outermost layer was estimated based on epithelial cell density in monoculture, and an estimated MOI of 0.2, in a volume of 100 µL per construct, was used as inoculum. Constructs were incubated at 33°C for 1 h, washed twice with TBS, and then incubated at 33°C. Individual constructs were transferred by sterilized forceps to 12 x 75 mm tubes containing 1 mL of serum-free medium and stored at -70°C at various times. Tissues were disrupted by sonication on ice with a probe sonicator (6 W, 30 s), and the titre of infectious virus in the sonicated mixture was determined by plaque
assay on LLC-MK₂ cells. Controls were included for the effect of sonication on the virus, and for possible trapping of virus particles by the corneal tissue.

V. Elimination of the N-linked glycosylation site of DAF

A mutagenic positive-sense primer was synthesized, 5’-ATCTGCCCTTAAGGGCAGTCAATGGTCAGATATTGAAGAGTTCTGCAGTCGTAGCTGCGAGGTG-3’, spanning nucleotides 291 to 353 of the DAF cDNA (numbering as in Medof et al., 1987a) and designed to introduce an A-to-G mutation at position 337 (bold), altering the derived amino acid sequence from Asn to Ser at position 61 of the mature polypeptide (N61S). This primer also incorporated the unique Afl2 site (underlined) in the DAF cDNA. The negative sense primer, 5’-GTGGTGACCTTGGAA-3’, incorporated a unique PpuM1 restriction site (underlined) and spanned residues 919 to 937 of the DAF cDNA. The resulting PCR amplicon was inserted between the Afl2 and PpuM1 sites in the wild-type DAF sequence following Afl2 and PpuM1 digestion.

VI. Transient expression of DAF and DAF N61S

DAF and DAF N61S cDNAs were inserted between the Neo1 and EcoR1 sites of the pCITE-2a(+) vector (Novagen). Twelve-well cell culture dishes were seeded with NIH/3T3 cells and transfected with the different constructs with LipofectAmine™ reagent (Invitrogen Life Technologies) according to the manufacturer’s instructions. After 24 h, cells were infected with vaccinia virus strain vTF7-3, encoding the T7 RNA polymerase (Fuerst et al., 1986), at a multiplicity of infection of 15 PFU/cell. After an additional 24 h, cells were detached from the culture dishes. A fraction of the cells was processed for
$^{35}$S-labelled virus binding, as described above, while the remaining fraction was used to measure transgene expression by flow cytometry, as described below. Virus binding data were normalized for DAF construct expression, using the average of the mean fluorescence intensities for the four monoclonal antibodies used; expression levels of wild-type DAF and DAF N61S varied by two-fold or less.

**VII. Flow cytometry**

Flow cytometric analysis of DAF expression was described previously (Karnauchow et al., 1998). Briefly, analyses were performed using monoclonal antibodies EVR1, 11D7 (CCP1-specific) (Coyne et al., 1992), IF7 (CCP2-specific) (Bergelson et al., 1994) and 8D11 (CCP4-specific) (Coyne et al., 1992) as primary antibodies, as indicated, at final concentrations of 0.2 μg/mL. 11D7 and 8D11 were from W. Rosse, Duke University Medical Center, Durham, NC, and IF7 was from R. Finberg, Dana-Farber Cancer Institute, Boston, MA. Goat anti-[mouse IgG(H+L)] conjugated to fluorescein isothiocyanate (FITC) (Roche) was used as the secondary antibody (diluted 1:1000 from the supplied stock).

**VIII. Metabolic inhibitors of glycosylation**

To inhibit N-linked glycosylation, cells were incubated in culture medium containing 0.2 μg/mL tunicamycin (Sigma) for 24 h prior to assaying virus binding (concentration selected as a result of titration of tunicamycin levels for activity and toxicity). Benzyl N-acetyl-α-D-galactosaminide (Benzyl GalNAc; Sigma), included in culture medium at a final concentration of 3 mM for 48 h prior to $^{35}$S-labelled virus binding, was used to inhibit O-linked glycosylation, as in (Nepomuceno et al., 1999; Oshima et al., 1999).
IX. Immunoblot analysis

Adherent cells were detached by incubation in 15 mM EDTA in TBS and washed twice. To release DAF from the cell surface, cells were resuspended in phosphatidylinositol-specific phospholipase C (PL-PLC) buffer (RPMI 1640 medium, 0.2% bovine serum albumin, 50 μM 2-mercaptoethanol, 10 mM HEPES, 0.1% sodium azide) containing 6 U/mL PL-PLC from Bacillus cereus (Sigma) and 1% (v/v) broad specificity protease inhibitor cocktail (Sigma). Following incubation at 37°C for 4 h, cells were pelleted and samples of the supernatant (containing equal amounts of total protein; Bio-Rad) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting onto polyvinylidene fluoride (PVDF) membranes (Millipore). DAF was visualized by incubating membranes in TBS containing rabbit anti-human DAF polyclonal immunoglobulin G (1:500 dilution; Research Diagnostics Inc.), followed by protein A-alkaline phosphatase conjugate (1:1250 dilution; Sigma) and colorimetric substrate (5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium; Promega).
RESULTS

1. Sialic acid is involved in EV70 binding to and infection of susceptible cell lines

Previous results (Utagawa et al., 1982) implicated sialic acid as an essential component of the EV70 erythrocyte receptor, as evaluated by viral hemagglutination inhibition. Here, these findings were extended by testing whether sialic acid is involved in EV70 attachment to nucleated cells that are susceptible to EV70 infection. Binding to all cell lines examined was found to be neuraminidase sensitive, although to varying extents. As shown in Figure 2A, EV70 binding to HeLa cells decreased in a dose-dependent manner as a result of pre-incubation of the cells with the broad specificity, protease-free Vibrio cholerae neuraminidase. E11 binding to HeLa cells was measured under the same conditions as a control, as the results of Utagawa et al. (1982) indicated that E11-induced hemagglutination was not dependent on sialic acid, and it has been repeatedly shown that, like EV70, E11 recognizes DAF as a cellular receptor (Bergelson et al., 1994; Powell et al., 1998; Lea et al., 1998; Powell et al., 1999). As expected, E11 binding to HeLa cells was not affected by neuraminidase treatment (Fig. 2A), indicating that the observed inhibition of EV70 binding is specific, and not due to a general decrease in the ability of the cells to bind virus. EV70 binding to HeLa cells was also found to be inhibited, to a lesser extent, by the preincubation of the virus with free sialic acid in the range of 5 to 20 mg/mL (Fig. 3), further indicating a specific role for sialic acid in virus binding and arguing against the presence of a contaminating, receptor-destroying enzymatic activity in the neuraminidase preparation. EV70 binding to U-937 cells was particularly sensitive to neuraminidase pretreatment (Fig. 2B), whereas E11 binding was not adversely
Figure 2  Neuraminidase pretreatment inhibits EV70 binding to several susceptible cell lines.

Cells were incubated in the presence of the indicated concentration of neuraminidase for 30 min at 37°C. After washing, cells were incubated for 1 h with $^{35}$S-labelled EV70 (33°C) or E11 (37°C) (2,000–10,000 cpm). Virus binding data are presented as the mean ± SD of at least three experiments, relative to binding to untreated cells (i.e., 0 mU/mL neuraminidase). A., EV70 and E11 binding to HeLa cells; untreated cells bound 33% and 67% of input EV70 and E11, respectively. B., EV70 and E11 binding to U-937 cells; untreated cells bound 51% and 16% of input EV70 and E11, respectively. C., EV70 binding to NIH/3T3 cells constitutively expressing human DAF; untreated cells bound 30% of input EV70. ND, not determined.
**Figure 3** EV70 binding to HeLa cells is inhibited by the presence of sialic acid.

35S-labelled EV70 was incubated at 33°C for 60 min in a final volume of 50 µL with the indicated concentrations of sialic acid (N-acetyl neuraminic acid) prior to assaying virus binding. Virus binding data are presented as the mean ± SD of at least three experiments. Variable results were obtained, with a maximum inhibition of virus binding (at 20 mg/mL sialic acid) ranging from approximately 30% to approximately 70% of binding by untreated virus; the data presented here represent an intermediate result.
affected; in fact, binding appeared to be enhanced by the removal of cell surface sialic acid, possibly by rendering E11 virus binding sites on DAF more accessible. Expression of human DAF confers EV70 binding activity to the receptor-negative murine cell line NIH/3T3 and renders the cells susceptible to EV70 infection (Karnauchow et al., 1996; Karnauchow et al., 1998). As shown in Fig. 2C, this DAF-dependent EV70 binding activity was also inhibited by pretreatment of the cells with neuraminidase. (A similar pattern was observed for CHO cells constitutively expressing human DAF; see Fig. 16 in Appendix).

In order to rule out the possibility that neuraminidase treatment inhibited a critical post-binding event required for virus retention at the cell surface, virus binding experiments following neuraminidase treatment were also carried out at 4°C by A. Khan in our laboratory; similar results were obtained (data not shown). In addition to those cell lines shown in Figure 2, experiments in our lab have found that EV70 binding to a wide range of leukocyte cell lines, most of which support EV70 replication, is highly susceptible to neuraminidase pretreatment (Haddad et al., unpublished data).

In an effort to determine if the EV70 binding mediated by cell surface sialic acid leads to the productive infection of cells, U-937 cells were incubated with neuraminidase prior to EV70 infection; U-937 cells were used due to the strong sensitivity of the EV70 binding activity of this cell line to neuraminidase pretreatment (see Fig. 2B). As shown in Figure 4, the titre of EV70 released by neuraminidase-treated cells was an order of magnitude lower than that of untreated cells at all time points, demonstrating that binding to cell surface sialic acid by EV70 is an important step in cell entry. Infection of HeLa cells was also inhibited by neuraminidase pretreatment, although to a lesser extent.
Figure 4 Neuraminidase pretreatment inhibits EV70 infection of U-937 cells.

Cells were incubated in 50 mU/mL neuraminidase for 30 min at 37°C. After washing twice with TBS, cells were infected with EV70 at an MOI of 5 and incubated at 33°C. Aliquots were removed at the indicated times and EV70 titer ± SD for duplicate experiments was determined by plaque assay on LLC-MK₂ cells.
II. Sialic acid residues of DAF are not involved in EV70 binding

DAF has been identified as a HeLa cell receptor for EV70 (Karnauchow et al., 1996), and is known to possess both a single complex-type N–linked glycosylation site and a heavily sialylated, serine and threonine-rich O–glycosylated domain (Lublin et al., 1986; Coyne et al., 1992). The expression of DAF on receptor-negative NIH/3T3 cells resulted in an EV70 binding activity that was sensitive to neuraminidase (see Fig. 2C). Taken together, the above observations suggested that sialic acid residues involved in EV70 binding to cells might be localized on the DAF molecule.

DAF consists of four CCP domains mounted on a rod-like, serine and threonine-rich O-glycosylated region, bound to the outer leaflet of the plasma membrane by a glycosylphosphatidylinositol anchor (see (Lublin and Atkinson, 1989) and Fig. 5A). The site of EV70 binding on DAF has been localized to the N-terminal CCP domain, CCP1, with evidence also suggesting a role for CCP2 (Karnauchow et al., 1998); the single N–linked glycosylation site of DAF is located in the same region, at the interface of CCP domains 1 and 2 (Coyne et al., 1992). Therefore, it was hypothesized that sialic acid residues in the complex-type N-linked oligosaccharide may contribute to EV70 binding to DAF. To test this hypothesis, a derivative of DAF was prepared by site-directed mutagenesis, altering the asparagine residue to serine in the N–linked glycosylation consensus sequence (Asn-X-Ser), resulting in Ser-Arg-Ser. When transiently expressed in the receptor-negative cell line NIH/3T3, this mutant form of DAF (DAF N61S), was observed to bind EV70 as efficiently as the wild-type control (Fig 6A). Additional
Figure 5 Simplified schematic representation of DAF and DAF/HLA-B44.

The four complement control protein (CCP) domains are numbered. DAF is anchored in the plasma membrane by a glycosyl-phosphatidylinositol (GPI) linkage, whereas DAF/HLA-B44 consists of the four CCP domains of DAF mounted on the membrane proximal, transmembrane and cytoplasmic domains of the HLA-B44 molecule. N-CHO, unique N-glycosylation site; O-CHO, O-glycosylation rich domain. A., DAF. B., DAF/HLA-B44.
Figure 6 Elimination of the N-linked carbohydrate of DAF has no effect on EV70 binding.

A., An A-to-G mutation was introduced into DAF cDNA, resulting in an Asn-to-Ser substitution (N61S) in the DAF polypeptide. Vector-only control (Vector), wild-type (DAF) and mutant (DAF N61S) constructs were transfected into the EV70 receptor-negative cell line NIH/3T3. $^{35}$S-EV70 binding was determined after 48 h, and normalized for DAF construct expression as determined by flow cytometry (see Materials and Methods). B., EV70 binding to both wild-type and mutant DAF is sensitive to neuraminidase. Transfection and virus binding were performed as in A, except that cells were incubated in serum-free medium (Control) or in serum-free medium containing 50 mU/mL neuraminidase for 30 min at 37°C before $^{35}$S-EV70 binding. For both panels, data are presented as mean ± SD for at least three experiments.
experiments showed that the EV70 binding activity of cells expressing DAF N61S was sensitive to neuraminidase, similar to that of the wild-type (Fig 6B), further establishing that the sialic acid residues critical for EV70 binding are not a component of the N-linked glycan of DAF.

As mentioned above, the DAF molecule also contains a heavily sialylated O-glycosylated region which projects the four CCP domains above the plasma membrane. This region has been demonstrated to serve an essential but non-specific spacer function in the complement regulatory function of DAF (Coyne et al., 1992), as well as in the ability of DAF to function as a receptor for Echovirus 7 (Clarkson et al., 1995) and for certain strains of pathogenic *Escherichia coli* bearing Dr or related adhesins (Nowicki et al., 1993). Although it seemed unlikely that sialic acid residues in this domain, located at a distance from the identified EV70 binding site at the amino terminus of the molecule, would play a direct role in the binding of the virus, it was imperative to test this possibility. Therefore, the EV70 binding activity of a chimeric construct (DAF/HLA-B44; described in Coyne et al., 1992) consisting of the four CCP domains of DAF mounted on the membrane proximal, transmembrane and cytoplasmic domains of the HLA-B44 molecule (Fig. 5B) was examined. Figure 7 shows that the replacement of the O-glycosylated domain of DAF had no significant effect on the EV70 binding activity of the molecule, indicating that this region, and its associated sialic acid residues, play no direct and necessary role in virus binding. Expression of a second, similar chimeric construct, DAF/HLA-A26, could not be detected at the cell surface (see Fig. 15 in Appendix). A complementary approach employed to test the role of the O-linked glycosylation of DAF made use of a glycosylation mutant cell line reported to synthesize
Figure 7 The O-glycosylated domain of DAF is not directly involved in EV70 binding.

CHO cells constitutively expressing wild-type DAF or a chimeric construct consisting of the four CCP domains of DAF fused to the majority of the HLA-B44 molecule (DAF/HLA-B44), and control cells transfected with vector alone, were assayed for EV70 binding. Virus binding results were normalized for DAF construct expression level. Data are presented as mean ± SD for triplicate experiments.
glycoproteins and glycolipids with truncated or absent oligosaccharide chains; however, a stable transfectant of this cell line constitutively expressing human DAF did not display the expected phenotype (see Fig. 16 in Appendix).

III. **O-Linked, but not N-linked, oligosaccharides are involved in EV70 binding**

Having excluded the possibility that sialic acid residues on the DAF molecule are critical for EV70 binding to cells, it was decided to evaluate more generally the role of N-linked and O-linked carbohydrate moieties of cell surface glycoproteins in EV70 binding. The effect of specific inhibitors of glycosylation on the ability of cells to bind EV70 was therefore assessed.

Tunicamycin is commonly used to test the role of N-linked glycoproteins in cellular processes; it specifically inhibits the transfer of N-acetylglucosamine to dolichol monophosphate, a very early step in the synthesis of the high-mannose core oligosaccharide (Duksin and Mahoney, 1982). HeLa cells and U-937 cells were cultivated in the presence of 0.2 µg/mL tunicamycin for 24 h, and then processed for EV70 binding. Tunicamycin-treated cells were indistinguishable from untreated cells when examined microscopically, and although the number of viable cells (as indicated by the exclusion of 0.05% trypan blue) was consistently lower for tunicamycin-treated than for untreated cells, cell density varied by less than a factor of two between the two populations. As shown in Fig. 8A, tunicamycin treatment had no effect on EV70 binding to either cell line. To verify that tunicamycin was effective at inhibiting N-linked glycosylation under these conditions, an immunoblot of DAF cleaved from the surface of intact cells by the action of PI-PLC was prepared. DAF released from HeLa cells
Figure 8 N-linked glycans are not involved in EV70 attachment.

A., HeLa or U-937 cells, as indicated, were incubated in cell culture medium alone (Control) or in cell culture medium containing 0.2 µg/mL tunicamycin (Tunic) for 24 h at 37°C prior to 35S-EV70 binding. Data are presented as mean ± SD for three experiments. B., Immunoblot analysis of DAF released from tunicamycin-treated and untreated HeLa cells by digestion with PI-PLC. The apparent molecular weights of prestained protein markers are shown at left.
cultivated in the presence of tunicamycin showed a decrease in relative molecular weight compared to DAF released from untreated control cells that was consistent with the loss of its single N-linked carbohydrate moiety, as reported previously (Lublin et al., 1986) (Fig. 8B).

To investigate the role of the O-linked carbohydrate of cell surface glycoproteins on EV70 binding, cells were cultivated in the presence of Benzyl GalNAc. Benzyl GalNAc inhibits the N-acetyl-α-D-galactosaminyl transferase, which catalyzes the first step in the biosynthesis of O-linked glycans (Kuan et al., 1989). HeLa cells treated with Benzyl GalNAc showed an approximately 75% decrease in EV70 binding compared to untreated control cells (Fig. 9A), and U-937 cells showed a greater than 50% decrease in EV70 binding relative to untreated controls (Fig. 9B). No significant difference in either cell density or in appearance when examined microscopically was detected between Benzyl GalNAc-treated and control cells. To verify that the effect observed on EV70 binding was specific, and not due to toxicity of the inhibitor, E11 binding was once again measured as a control. No significant change in E11 binding to HeLa cells treated with Benzyl GalNAc was observed (Fig. 9A), and E11 binding to U-937 cells was somewhat enhanced by the treatment (Fig. 9B). This latter observation parallels the increase in E11 binding to U-937 cells treated with neuraminidase (see Fig 2B), and is consistent with a role for sialic acid, or oligosaccharides more generally, in masking E11 virus binding sites. Immunoblot analysis of DAF released from Benzyl GalNAc-treated and untreated cells revealed similar expression levels at the cell surface (Fig. 9C); this analysis also confirmed that the size of DAF obtained from Benzyl GalNAc-treated cells was consistent with the inhibition of O-linked glycosylation.
Figure 9 Disruption of O-linked glycosylation inhibits EV70 binding.

A., HeLa cells were incubated in the presence or absence of 3 mM Benzyl GalNAc for 48 h at 37°C prior to $^{35}$S-EV70 or $^{35}$S-E11 binding. B., As in A., with U-937 cells in place of HeLa cells. For both A and B, data are presented as mean ± SD for three experiments. C., Immunoblot analysis of DAF released from Benzyl GalNAc-treated and untreated HeLa cells by digestion with PI-PLC. The relative molecular weights of prestained protein markers are shown at left.
IV. Sialic acid, but not DAF, is required for EV70 binding to human corneal cell lines

Up to this point, work on EV70 receptors has been conducted with commonly available cell culture systems, including HeLa, LLC-MK₂, NIH/3T3, CHO, and human leukocyte cell lines, rather than in more physiologically relevant ocular cell lines. In part, this has been due to the widespread availability of the former, to the fact that EV70 grows to relatively high titers in these cell lines or their derivatives, and to the historic use of certain of these lines in virus-receptor interaction studies. In contrast, verified cell lines of ocular origin have not been generally available until quite recently. The Chang cell line, an older line once believed to have been derived from human conjunctiva, has recently been shown to be contaminated by HeLa cells (ATCC; http://www.atcc.org/). It was not until the mid-1990s that verified, continuous cell lines of ocular origin, immortalized by the induced expression of viral oncogenes, were reported (Kahn et al., 1993; Araki-Sasaki et al., 1995; Offord et al., 1999). A further significant advance was made in 1999, when in vitro tissue engineering techniques were successfully employed in the construction of human corneal equivalents from immortalized cell lines (Griffith et al., 1999).

Here, the preliminary characterization of the virus-receptor interactions of EV70 with cells derived from each of the three primary cell layers of the cornea (epithelium, stroma and endothelium; see Materials and Methods for a more complete description) and with assembled human corneal equivalents is described.
Moderate to high expression of DAF in human corneal and conjunctival epithelia has been previously detected by immunohistochemistry and flow cytometry (Medof et al., 1987b; Lass et al., 1990; Bora et al., 1993). Here, in contrast, expression of DAF by the immortalized corneal epithelial cells was found by flow cytometry to be quite low (Figure 10A). This difference may be due to a change in DAF expression upon immortalization, to variation in levels of DAF expression between cells or individuals, or to technical differences in the methods used to detect DAF antigen (e.g., different antibodies). Slightly higher levels of DAF expression were detected on the stromal cells (Fig. 10B), and higher again on endothelial cells (Fig. 10C).

Virus binding and binding inhibition studies were undertaken to determine the roles of DAF and sialic acid in EV70 attachment to the different corneal cell lines. Strikingly, monoclonal antibody blockade of DAF did not affect EV70 binding to any of the corneal cell lines (Fig. 11). This was in contrast to virus binding to HeLa cells, which was reduced by approximately 90% by pre-incubation of the cells with the anti-DAF monoclonal antibody (Fig. 11), consistent with previous reports (Karnauchow et al., 1996). The lack of a role for DAF in EV70 binding to corneal cells was further supported by the observation that, for epithelial cells, despite having the lowest levels of DAF expression (see Fig. 10), EV70 bound most strongly to this cell line, to a level equivalent to binding to HeLa cells. Virus binding to stromal cells was somewhat lower, whereas binding to endothelial cells was the weakest, only 20-25% of the level of HeLa and epithelial cells. In contrast to the results concerning DAF, and as observed for all cell lines tested to date, sialic acid was found to be a critical component of the EV70 receptor on all three corneal cell types (Fig. 11).
Figure 10 DAF expression by immortalized human corneal cells.

Flow cytometric analysis of immortalized human corneal epithelial (A), stromal (B) and endothelial (C) cells for expression of DAF was performed with monoclonal antibody EVRI and FITC-conjugated anti-mouse IgG secondary antibody. Cellular autofluorescence and a control population incubated with secondary antibody alone are also shown for each cell line. The ratios of mean fluorescence intensity for the test sample (containing both primary and secondary antibodies) to that of control containing secondary antibody alone were 1.4, 1.8 and 3.3 for epithelial, stromal and endothelial cells, respectively.
Figure 11 Sialic acid, but not DAF, is required for EV70 binding to corneal cell lines.

Prior to virus binding, corneal and HeLa cells were incubated in the presence of 50 mU/mL neuraminidase for 30 min or 22 μg/mL EVR1 for 60 min at 37°C. Results are presented as the mean ± SD of at least three experiments, relative to binding to untreated control cells. In these experiments, untreated HeLa and corneal epithelial cells bound approximately 15% of input EV70, stromal cells bound on average approximately 11%, and endothelial cells bound 3-4% of input virus.
Unfortunately, virus binding studies on corneal tissue equivalents were not possible due to high, non-specific background binding to the constructs; the non-specific nature of the interaction was confirmed by the inability of unlabelled homologous competitor virus to displace radiolabelled EV70.

V. **Productive EV70 infection of human corneal equivalents**

In addition to investigations of virus binding, corneal cells and tissues may prove useful in validating and extending, in a physiologically relevant setting, earlier findings pertaining to EV70 entry and replication. Thus, the ability of EV70 to infect each of the three corneal cell lines in monoculture, as well as the assembled corneal constructs, was assessed. Figure 12 shows that all three corneal cell lines support productive EV70 infection, with similarly shaped growth curves obtained for each, and Figure 13 shows the productive infection of the assembled corneal constructs. Only epithelial cells showed cytopathic effects upon EV70 infection in monoculture, and no cytopathic effects were detectable for any of the three cell types during infection of the assembled corneal constructs.
Figure 12 All three corneal cell lines support productive EV70 infection.
Epithelial (◆), stromal (■) and endothelial (▲) cells were infected at an MOI of 5 and incubated at 32-33°C. Aliquots were removed at the indicated times and EV70 titer ± SD for duplicate experiments was determined by plaque assay on LLC-MK₂ cells.
Figure 13 Productive infection of human corneal equivalents by EV70.

$3 \times 10^4$ plaque-forming units of EV70 were added to each cornea, an estimated MOI of 0.2 with respect to the outermost layer of epithelial cells. The infected constructs were incubated at 32°C, and individual corneal equivalents were removed at the indicated times and frozen at -70°C. Tissues were thawed and disrupted by sonication, and the titer of released virus (± SD for duplicate experiments) was determined by plaque assay on LLC-MK2 cells.
DISCUSSION

Sialic acid binding has been observed for a wide variety of viruses, including both DNA and RNA viruses, and for both enveloped and non-enveloped virions. Included among these are orthomyxoviruses (Suzuki et al., 2000), respiroviruses (within the family Paramyxoviridae) (Suzuki et al., 2001), certain strains of animal rotaviruses (Mendez et al., 1999), polyomavirus (Bauer et al., 1999), transmissible gastroenteritis coronavirus (TGEV) (Krempf et al., 2000), certain strains of adenoviruses (Arnberg et al., 2000b) (including adenovirus type 37, a causative agent of epidemic keratoconjunctivitis and thus sharing ocular tropism with EV70; Arnberg et al., 2000a; Arnberg et al., 2002), reoviruses (Barton et al., 2001a) and several members of the Paroviridae (Kaludov et al., 2001) [see references and works cited therein]. The use of sialic acid as a receptor has also been described amongst the Picornaviridae, including encephalomyocarditis virus (EMCV) (Jin et al., 1994), Theiler's murine encephalomyelitis virus (TMEV) (Zhou et al., 2000), bovine enterovirus 261 (Stoner et al., 1973), and human rhinovirus 87 (HRV87) (Uncapher et al., 1991). (Interestingly, a recent study of the genetic relationships among HRV prototype strains has shown that HRV87 clusters separately from the remaining HRVs and close to EV70 (Savolainen et al., 2002)). Binding to sialic acid may also have some parallels with the widespread use of another class of ubiquitous anionic cell surface carbohydrates, glycosaminoglycans, and in particular heparan sulfates, in cell attachment by a variety of viruses and other pathogens (Rostand and Esko, 1997; Spillmann, 2001).

Recent work in several laboratories has advanced our understanding of the role of sialic acid binding in host cell infection and pathogenesis. Barton et al. (2001a) recently
demonstrated that reovirus binding to sialic acid increases the avidity of cell attachment and enhances productive infection, possibly by providing a locus for the initial virus-cell interaction in a multi-step adhesion strengthening strategy. Sialic acid binding is then followed by a higher affinity binding to a secondary receptor critical for virus entry, junction adhesion molecule (Barton et al., 2001b). Within such a model, the capacity to bind cell surface sialic acid may be an important determinant of cell and tissue tropism in situations where the secondary entry receptor is expressed at low levels, or where infection of cells proceeds in an environment of rapidly moving body fluid, such as blood, lymph, or tears. Virus binding to sialic acid may also activate cellular signaling pathways, and thus may have important effects downstream of virus attachment on cellular metabolism and the outcome of infection. For example, reovirus binding to cell surface sialic acid is an important mediator in the activation of nuclear factor κB (NF-κB) and in the induction of apoptosis (Connolly et al., 2001).

In the case of TMEV, the capacity to bind sialic acid has a pronounced effect on viral pathogenesis. Infection with certain strains of TMEV results in viral persistence in the CNS, accompanied by demyelination, whereas other strains cause an acute encephalitis. Differential binding to sialic acid residues on the cell surface may contribute to this striking difference in the in vivo outcomes, with sialic acid binding being a unique property of the demyelinating, persistent group (Jnaoui et al., 2002); recent work has further shown that the persistent strains of Theiler’s virus specifically recognize sialic acid moieties on N-linked glycoproteins (Shah and Lipton, 2002).

Similarly, the sialic acid binding activity of TGEV contributes to the pathogenicity of this virus; in addition to a possible role of sialic acid binding in enhancing cell attachment and
entry (Schwegmann-Wessels et al., 2002), it has been speculated that the binding of sialylated macromolecules to the virus surface may increase the stability of the virus against detergent-like bile salts encountered in the gastrointestinal tract (Krempl et al., 2000).

In each of the above examples, the ability to elucidate the role of the sialic acid binding activity in infection and pathogenesis was made possible by the availability of virus strains differing in their capacity to bind sialic acid. What role, if any, the sialic acid binding activity of EV70 plays in vivo is unknown. In the present study, the prototype J 670/71 strain of EV70 was employed; the sialic acid binding activity of other EV70 strains has not been evaluated.

EV70 is the only human enterovirus to date observed to require cell surface sialic acid for attachment; sialic acid binding was also found to be important for infection of susceptible cells, as was shown previously for bovine enterovirus 261 (Stoner et al., 1973). The data presented here also indicate that the absence of the sialic acid residues contained in the single, complex-type N-linked oligosaccharide or in the heavily sialylated O-linked oligosaccharides of DAF did not significantly reduce virus binding to transfected cells expressing the receptor variants. Taken together, these results suggest that at least one additional sialylated receptor molecule, apart from DAF, is involved in EV70 attachment and infection.

The requirement for an additional receptor is consistent with previously reported findings for some other DAF-binding human enteroviruses, which include certain variants or isolates of CBV serotypes 1, 3 and 5 (Shafren et al., 1995; Bergelson et al., 1997; Martino et al., 1998; Schmidtke et al., 2000), CAV21 (Shafren et al., 1997a), and various
echovirus serotypes (Ward et al., 1994; Bergelson et al., 1994). Many of these viruses have been shown to require additional factors for cell entry and infection; for example, CBV3 and CAV21 require the expression of coxsackievirus-adenovirus receptor (CAR) (Shafren et al., 1997b) and intercellular adhesion molecule 1 (ICAM-1) (Shafren et al., 1997a), respectively (although it has also been shown that DAF that has been cross-linked with antibody can support CAV21 entry in the absence of ICAM-1 (Shafren et al., 1998; Shafren, 1998)). Several of the DAF-binding echoviruses may also require accessory molecules in addition to DAF for cell attachment and infection (Powell et al., 1998); candidates that have been proposed include β2-microglobulin (Ward et al., 1998) and CD59 (Goodfellow et al., 2000). In the above cases, it has been proposed that DAF serves as an initial, reversible, low affinity virus attachment site, which facilitates or enhances binding to a higher affinity entry factor, the latter of which may also support virus infection in the absence of DAF. This model is consistent with the inability of DAF to induce A particle formation in echovirus 7 (Powell et al., 1997) (the A particle is an altered picornavirus capsid conformation believed to be an intermediate in the viral entry process; see Huang et al., 2000), and with the low affinity and rapid dissociation of E11-DAF complexes (Lea et al., 1998). Recent picornavirus-receptor structure determinations lend additional support to this model. The majority of known rhinovirus and enterovirus entry receptors belong to the immunoglobulin superfamily (IGSF); the amino-terminal domains of these molecules bind to residues within the canyon surrounding the viral capsid five-fold axis of symmetry (Kolatkar et al., 1999; Xing et al., 2000; He et al., 2000; Belnap et al., 2000; Xiao et al., 2001; He et al., 2001). This interaction is believed to destabilize the virus and induce uncoating, possibly by displacing the hydrophobic pocket
factor bound below the base of the canyon (Smith and Baker, 1999; Rossmann et al., 2000; Rossmann et al., 2002; Hogle, 2002). In contrast, structures of several picornaviruses bound to non-IGSF receptor molecules, including DAF and the low-density lipoprotein receptor (the latter of which is also unable to induce A particle formation in its viral ligands, the minor receptor group rhinoviruses (Rossmann et al., 2002)), reveal that these molecules do not interact with residues within the canyon, but rather bind at the top of the five-fold axis of symmetry, or close to the two-fold axis of symmetry (Hewat et al., 2000; He et al., 2002; Stuart et al., 2002), suggesting that an additional molecule(s) may be involved in virus entry.

A version of this model may also be applicable to the binding of EV70 to susceptible cells, in which the interaction of the virus with DAF may promote initial cell adhesion, followed by higher affinity binding to the putative secondary factor in a sialic acid-dependent manner. This would constitute a reversal of the common paradigm for sialic acid binding among various other virus families, namely that of sialic acid constituting the initial, low affinity interaction step that facilitates virus binding to the primary receptor (e.g., reovirus; see above). However, certain of the observations reported here are inconsistent with this latter model for EV70-receptor interactions. For example, NIH/3T3 cells do not bind the virus in the absence of DAF (see Fig. 6 and Karnauchow et al., 1998), but binding to transfected cells expressing DAF is dependent on sialic acid (see Figs. 2 and 6), suggesting that the sialic acid residues involved in EV70 binding to NIH/3T3 cells are not able to mediate virus binding directly or to facilitate virus binding to an additional high-affinity receptor in the absence of DAF. The same pattern is observed with CHO cells (see Fig. 16 in Appendix). Similarly, when DAF is blocked or
enzymatically removed from HeLa cells, EV70 binding is prevented (see Fig. 11 and Karnauchow et al., 1996), although binding to this cell line is also sialic acid-dependent (see Fig. 2).

On the other hand, results with the three immortalized human corneal cell lines (see Fig. 11) and with various human leukocyte cell lines in our laboratory (Haddad et al., unpublished), suggest that, at least under certain conditions, DAF binding is not required for EV70 attachment to cells, though binding remains dependent upon the presence of sialic acid. With these cell lines, the virus may employ an alternative low-affinity attachment receptor, functioning in an analogous manner to DAF; this alternative receptor may not be expressed or may be present at such low levels on NIH/3T3 and HeLa cells that access to DAF becomes obligatory. A schematic diagram illustrating this model is presented in Figure 14. Alternatively, EV70 may access a sialylated attachment molecule directly on ocular and leukocyte cell lines, with no requirement for DAF or for an alternative low-affinity sequestration receptor. Measuring the relative affinities of the interactions of EV70 with DAF and sialic acid (e.g., by surface plasmon resonance) may help to resolve some of these uncertainties.

The data presented here also rule out a direct or an indirect role in EV70 attachment for the oligosaccharides underlying the sialic acid residues of DAF. The interaction of DAF with other human enteroviruses has also generally been found to be independent of the N-linked and O-linked glycosylations (Clarkson et al., 1995;Powell et al., 1997;Powell et al., 1999). Only for CAV21 has a role for a nonpeptide modification of DAF been suggested; CAV21 hemagglutination inhibition by soluble DAF variants lacking the N-linked glycan was ineffective, and hemagglutination inhibition by a partially N-
Figure 14  Model for EV70-receptor interactions.

The virus is represented as the beige polygon (drawn smaller than to scale), DAF is shown in burgundy, sialic acid residues in teal, and proposed and/or unknown molecules in gray. See text for details.
Low affinity, reversible attachment to DAF or alternate receptor

Higher affinity, sialic acid-dependent binding
glycosylated construct containing all four CCPs of DAF was significantly less effective for CAV21 than it was for all other viruses tested (Powell et al., 1999). Interestingly, CAV21 is the only DAF-binding enterovirus apart from EV70 that has been found to interact with the N-terminal CCPI domain of DAF (Shafren et al., 1997a); however, the interaction of the two viruses with DAF appears to differ with respect to the requirement for the N-linked oligosaccharide. It has been suggested that the ability to bind DAF has evolved on multiple occasions among the enteroviruses, based on the observation that three of the four enterovirus species possess at least one representative demonstrating DAF-binding activity, and that the different species have been found to interact with different domains of the molecule (Powell et al., 1999). Since EV70 and CAV21 are representatives of separate species, it is perhaps not surprising that the two viruses appear to interact with DAF in distinct ways with respect to the requirement for N-glycosylation. The apparently convergent evolution and widespread use of DAF as an attachment receptor among the enteroviruses has prompted some speculation as to what selective advantage the use of this molecule offers this class of viruses (Lindahl et al., 2000). The answer may be as simple as the widespread expression of the molecule, permitting its use as a sequestration receptor by viruses with a broad range of tropisms, or its accessibility, projected relatively far above the plasma membrane by its mucin-like stalk domain (see below). Alternatively, the use of DAF may permit the exploitation of a signaling pathway activated by virus binding that may enhance virus uptake, replication, or spread, or inhibit an antiviral defense mechanism (Ward et al., 1998). The clustering of GPI-linked proteins, including DAF, in lipid microdomains may enhance infectivity by
increasing local receptor concentration, as well as that of potential co-receptors and signaling molecules (Fivaz et al., 1999).

The results obtained with the metabolic inhibitors of protein glycosylation, tunicamycin and Benzyl GalNAc, provide preliminary information about the nature of the sialylated molecular species involved in EV70 binding. The absence of an effect with tunicamycin treatment suggests that the N-linked oligosaccharides of glycoproteins, along with any associated sialic acid residues, are dispensable for EV70 attachment. In contrast, disruption of O-linked glycosylation inhibited EV70 binding. O-linked glycans of cell surface glycoproteins are often found clustered together in mucin-like domains (as is the case with DAF), where they confer an extended, rod-like structure to the polypeptide chain (Jentoft, 1990). Thus, the inhibition of virus binding by Benzyl GalNAc treatment may be due directly to the loss of sialic acid associated with O-linked oligosaccharides on the cell surface, although it is also possible that interfering with O-glycosylation results in structural or conformational perturbation of a virus-binding factor in such a way as to indirectly prevent EV70 interaction. In addition, whereas it is clear that Benzyl GalNAc inhibits O-linked glycosylation of proteins (Kuan et al., 1989), its effects on glycolipid glycosylation have not been reported, and a potential role for sialylated glycolipids in EV70 binding and entry is currently under study.

For many of the viruses that bind sialic acid, additional details relating to the specificity of the interaction have been elucidated, including the type of linkage between the sialic acid and the underlying oligosaccharide, the nature of the underlying oligosaccharide, and the size and location of the acyl chain. For example, human polyomavirus recognizes N-linked oligosaccharides with \( \alpha-2,6 \) and \( \alpha-2,3 \) linked sialic acid (Chen and
Benjamin, 1997; Liu et al., 1998), and influenza A virus and murine polyomavirus are sensitive to even slight alterations in the length of the N-acyl chain of receptor sialic acid (Herrmann et al., 1997; Keppler et al., 1998). Such information can be obtained through the use of neuraminidases, sialyl transferases and/or lectins with specificity for particular neuraminic acids or linkages, or by testing virus binding to mutant cell lines. The V. cholerae neuraminidase used in the present work has a broad substrate specificity, and as such was suitable for an initial evaluation of the role of sialic acid in EV70 binding; future work will characterize more precisely the specificity of the sialic acid species and linkage recognized by EV70.

It appears likely that EV70 possesses the potential to bind to a spectrum of molecules at the cell surface bearing sialic acid in the correct linkage and context, rather than to a unique sialoconjugate. However, on a given cell type the suitable candidate receptors may be restricted to a relatively small subset, especially when considering only those molecules expressed at significant levels. This situation may result in a certain molecule becoming, in effect, a unique sialylated receptor for the virus, as described for influenza C virus in the Madin-Darby canine kidney cell line; among those sialoglycoproteins expressing the required sialic acid, N-acetyl-9-O-acetylneuraminic acid, only a particular cellular sialoglycoprotein (termed gp40) was found to be present at the cell surface to a significant extent, and was thereby found to possess the characteristics of a unique receptor (Zimmer et al., 1995).

The novel use of immortalized human corneal cells and in vitro-assembled corneal equivalents for experiments with EV70 permits validation and extension of earlier results in a physiologically relevant system. The results obtained here indicate that DAF is not
required for virus binding to ocular cells; this was also recently shown to be the case for several leukocyte cell lines (Haddad et al., unpublished). In contrast, sialic acid has been found to be a critical component of the attachment receptor on all cell lines examined to date, including the three corneal cell lines.

Corneal cell lines and tissues represent ideal culture systems in which to pursue further investigations of EV70-receptor interactions, as well as studies of virus entry pathways. Recently, considerable attention has been devoted to identifying cellular endocytic pathways that may be exploited by various viruses to gain access to the interior of the host cell (Sieczkarski and Whittaker, 2002); among the Picornaviridae, clathrin-dependent endocytosis has been recently implicated in rhinovirus type 2 entry (Bayer et al., 2001), and caveolae were shown to be involved in the entry of echovirus type 1 (Marjomaki et al., 2002). The possible role for endocytic pathways in EV70 entry has not yet been evaluated. Corneal cultures may also prove useful in exploring the efficacy of potential antiviral agents, the possible roles of different signaling pathways in EV70 entry, and cellular responses to infection in a physiologically relevant setting. In similar work, the release of immunomodulatory factors from immortalized corneal epithelial cells upon infection with adenovirus type 37 was recently described (Chang et al., 2002). Early results with EV70 and CAV24v demonstrated the release of interferons from cells upon infection, as well as the potential therapeutic value of interferons or inducers of interferon as prophylactics to inhibit viral replication (Stanton et al., 1977; Langford et al., 1980; Langford et al., 1983; Stansfield et al., 1984; Langford et al., 1988). Other agents, including arildone (Langford et al., 1985), benzimidazoles (Langford et al., 1995) and the
human rhinovirus 3C protease inhibitor AG7088 (Patick et al., 1999) have also been shown to have antiviral activity against EV70.

Corneal epithelial cells are arguably the most logical cell line in which to initially conduct the above investigations, being contiguous with conjunctival epithelium and thus representative of the cell type first encountered by the virus in the eye, and given that corneal involvement during AHC is commonly reported. The use of the assembled human corneal equivalents as a model tissue in which to confirm the results obtained with epithelial cells in monolayer is nevertheless an important step. Even within the epithelium, stratification during assembly is accompanied by differentiation into at least three cell layers: the outermost (‘apical’ cells; squamous epithelium), the central (‘wing’ cells; polygonal epithelium) and the innermost (‘basal’ cells; columnar cuboidal epithelium) (Griffith et al., 2002); cellular changes accompanying this differentiation may affect interactions of the cells with virus and/or antiviral agents.

The corneal cells and tissues used in the work described here do not represent the first attempt to employ ocular cells in studies of EV70. Earlier work showed that the virus grew readily in primary epithelial cells derived from mouse, rabbit and monkey conjunctival and corneal tissue (Langford and Stanton, 1980); however, the immortalized cells used here offer the obvious advantages of ease of use and continuous culture, as well as being of human origin. A rabbit model of AHC due to EV70 has also been described (Langford et al., 1986), which could provide complementary benefits to the use of the human corneal culture (although another group has reported the inability to reproduce this infection in the eyes of rabbits (Yamazaki and Miyamura, 1989)). In vitro corneal cultures are less expensive and cumbersome than the rabbit model, but the latter
(or an alternative animal model) would provide the opportunity to address additional questions such as host defense responses or the efficacy of potential vaccine candidates. The productive infection of immortalized human corneal cell lines and corneal equivalent tissues with EV70 described here also provides an important proof of principle for the use of these culture systems as in vitro models for other ocular pathogens, including viral, bacterial, parasitic and fungal agents.
APPENDIX
Figure 15 The failure of a DAF/HLA-A26 construct to bind EV70 is due to the absence of detectable expression of the molecule at the cell surface.

A. A DAF/HLA-A26 chimera (amino acid residues 1 through 257 of DAF fused to the carboxyl terminus of HLA-A26, from amino acid 120 through the stop codon) was prepared as follows. A positive-sense primer was synthesized, 5'-CACACACCGTCCGTGGTGGTGGTTAGATAATGTATGGCTGCAGA-3', spanning nucleotides 364 to 382 of the HLA-A26 cDNA and incorporating the unique Rsr2 site (underlined), and a tetraglycine “hinge” region (bold, italicized). The negative sense primer, 5'-ACACACTCTAGATCACACTTTCACAAGCTGTGAGAGAC-3', incorporated a unique XbaI restriction site (underlined) and spanned residues 1079 to 1103 of the HLA-A26 cDNA. A commercially obtained HLA-A26 cDNA clone (Invitrogen) was used as template. The resulting PCR amplicon was digested with Rsr2 and XbaI and inserted between the PpuM1 and XbaI sites of the pcITE-2a(+) vector containing the wild-type DAF sequence, after digestion of the vector with PpuM1 and XbaI. (PpuM1 cleaves within the coding region of the HLA-A26 cDNA; therefore Rsr2, which generates an overhang compatible with that of PpuM1 but does not cleave within HLA-A26 cDNA, was used in this construction.) Vector-only control (Vector), wild-type (DAF) and receptor chimera (DAF/HLA-B44) constructs were transfected into the EV70 receptor-negative cell line NIH/3T3 as described in Materials and Methods, and °S-EV70 binding was determined after 48 h. Virus binding results are presented as the mean ± SD of at least three experiments. These data appear to indicate that the DAF/HLA-A26 chimera was unable to bind EV70, suggesting that the O-glycosylated domain of DAF may be required for virus binding.

B. Flow cytometric analysis of DAF expression was performed with monoclonal antibody EVR1 and FITC-conjugated anti-mouse IgG secondary antibody, as described in Materials and Methods. The ratios of mean fluorescence intensity for the test sample (containing both primary and secondary antibodies) to that of control containing secondary antibody alone are shown for each transfected cell population. These data reveal that the DAF/HLA-A26 receptor chimera was not expressed at detectable levels at the cell surface.

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Figure 16 A cell line reported to be a glycosylation mutant did not display the expected phenotype.

A. The CHO derivative cell line ldID has been reported to lack the enzyme UDP-Gal/UDP-GalNAc 4-epimerase, and thus to be unable to synthesize UDP-galactose and UDP-GalNAc; in the absence of an exogenous source of monosaccharides, this defect results in the synthesis of glycoproteins and glycolipids with truncated or absent oligosaccharide chains (Kingsley et al., 1986). A stable transfectant constitutively expressing human DAF (ldID-DAF) has also been described (Reddy et al., 1989). CHO, CHO-DAF, ldID, and ldID-DAF cells were obtained from M. Krieger (Massachusetts Institute of Technology). The cells were cultivated under conditions reported to result in the synthesis of truncated oligosaccharides in ldID and ldID-DAF cells (0.5% defined and supplemented calf serum for 48 h) and were processed for EV70 binding. Where indicated, cells were incubated in the presence of 50 mU/mL neuraminidase for 30 min at 37°C prior to virus binding. Results are presented as the mean ± SD of at least three experiments. These data appear to demonstrate that EV70 binding is DAF-dependent (compare CHO to CHO-DAF and ldID to ldID-DAF binding), sialic acid dependent (compare neuraminidase-treated CHO-DAF and ldID-DAF binding to that of untreated cells) and also that EV70 binding to DAF is unaffected by the absence of both N- and O-linked glycosylation (compare CHO-DAF to ldID-DAF binding).

B. DAF released from CHO-DAF and ldID-DAF cells by digestion with PI-PLC was analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting, as described in Materials and Methods. The apparent molecular weight of one of the prestained protein markers is shown at left. These data reveal that the ldID-DAF cells do not display the expected phenotype; DAF released from the surface of CHO-DAF and ldID-DAF cells migrates to exactly the same position, indicating that it is glycosylated to the same extent in both cell lines.
REFERENCES


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