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DECAY-ACCELERATING FACTOR IS NOT THE ONLY RECEPTOR FOR ENTEROVIRUS 70

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ABSTRACT

Enterovirus 70 (EV70) belongs to the family Picornaviridae and is a human pathogen responsible for acute hemorrhagic conjunctivitis. Unlike most human enteroviruses, the host range of EV70 encompasses a wide variety of non-primate mammalian cells *in vitro*. We demonstrated previously that EV70 uses decay-accelerating factor (DAF/CD55), a 70 kDa glycosyl-phospatidylinositol (GPI)-anchored protein, as a receptor on HeLa cells. Others have shown that DAF also facilitates the attachment of other human enteroviruses, including a number of coxsackieviruses and echoviruses; however, for some of these viruses additional surface proteins are essential for virus entry and infection, such as intercellular adhesion molecule 1 (ICAM-1) for coxsackievirus A21, and the coxsackie-adenovirus receptor (CAR) for coxsackie B viruses. Binding of radiolabelled EV70 to HeLa cells is inhibited in the presence of DAF-specific monoclonal antibodies (mAbs) or by treating cells with phosphatidylinositol-specific phospholipase C (PI-PLC). One objective of the research described in this thesis was to determine if human DAF also functioned as a receptor for EV70 on other human cell lines. As shown here, binding of EV70 to human leukocyte lines, including U-937 and Jurkat cells, was unaffected by DAF-specific mAbs or PI-PLC treatment, suggesting the presence of a different receptor. Another objective was to determine whether ICAM-1 or human CAR function as receptors for EV70. It was demonstrated that expression of human DAF, but not ICAM-1 or human CAR, in receptor-negative murine NIH/3T3 cells, resulted in EV70 binding and infection. A third objective was to elucidate the potential role of non-primate DAF homologues in EV70

binding and infection. The results reported here show that mouse, rat, or pig DAF homologues expressed in NIH/3T3 cells were unable to mimic the receptor function of human DAF. EV70, therefore, must utilize molecules other than DAF for infection of a variety of human and non-human cell lines, and these molecules do not appear to include either ICAM-1 or CAR.

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LIST OF ABBREVIATIONS

3T3/hCAR	NIH 3T3 cells constitutively expressing hCAR
3T3/ICAM	NIH 3T3 cells constitutively expressing ICAM-1
3T3/pEF6	NIH 3T3 cells stably transfected with the pEF6 plasmid
3T3/xDAF	NIH 3T3 cells constitutively expressing x DAF, where x represents the species of origin of DAF
AHC	acute hæmorrhagic conjunctivitis
CCP	complement control protein repeat
CNS	central nervous system
CVA	group A coxsackievirus
CVB	group B coxsackievirus
DAF	decay-accelerating factor
DAF-GPI	GPI-anchored DAF
DAF-TM	transmembrane-anchored DAF
EV70	enterovirus 70
FBS	fetal bovine serum
FC buffer	flow cytometry buffer
FITC	fluorescein isothiocyanate
GPI	glycosyl-phosphatidylinositol
hDAF	human DAF
hCAR	human coxsackie-adenovirus receptor
HRV14	human rhinovirus 14

ICAM-1	intercellular adhesion molecule-1
mAb	monoclonal antibody
mDAF	mouse DAF
MEM	*minimal essential medium*
MOI	multiplicity of infection
MPH	murine PVR homologue
pDAF	pig DAF
PI-PLC	phosphatidylinositol-specific phospholipase C
PFU	plaque forming unit
PVR	poliovirus receptor
rDAF	rat DAF
TM	transmembrane domain

INTRODUCTION

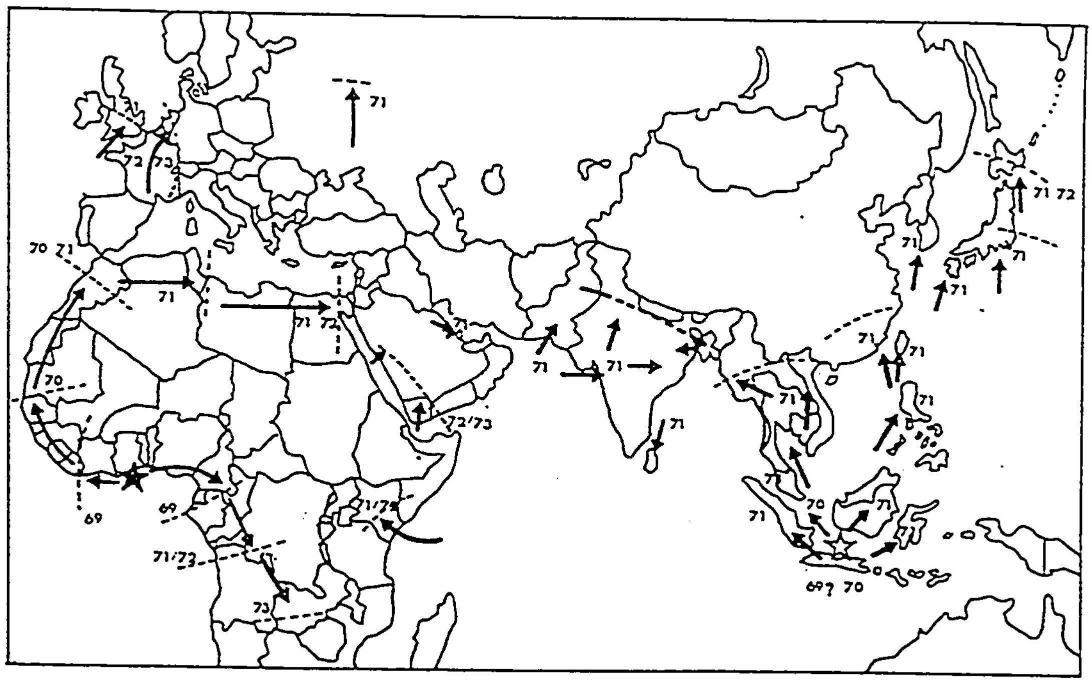
I. Enterovirus 70 (EV70) and Acute Hæmorrhagic Conjunctivitis (AHC)

a) AHC and the world

Although enteroviruses have been named after their ability to multiply in the enteric tract, infection with some (notably echovirus 7, coxsackievirus B2 and echovirus 11) has been accompanied by conjunctivitis (Melnick, 1996). Two other enteroviruses, Enterovirus 70 and a variant of coxsackievirus A24, have been responsible for a large number of outbreaks and epidemics of a distinct, highly contagious form of conjunctivitis termed Acute Hæmorrhagic Conjunctivitis or AHC.

AHC was first observed by Chatterjee *et al.* (1970) in June of 1969 in Ghana, West Africa (Ghendon, 1989). In October of that year, Lagos (Nigeria) experienced an outbreak of the disease (Parrott, 1971), which adopted the name 'Apollo disease' due to its association with the moon gazing brought about by the landing of Apollo 11 three months earlier. The disease soon affected other parts of Africa (figure 1), reaching Morocco (Nejmi *et al.*, 1974), Kenya (Metselaar *et al.*, 1976), Cameroon, Ivory Coast, Togo, Liberia, and Sierra Leone (Ghendon, 1989). By 1971, the disease had resulted in a pandemic affecting more than 10 million people, spreading to Eastern Asia with outbreaks in Indonesia, Malaysia, Cambodia, Thailand, Hong Kong, the Philippines, and Taiwan (Kono *et al.*, 1972), as well as Singapore (Yin-Murphy and Lim, 1972), India, Pakistan, Sri Lanka, mainland China, Korea, and Japan (Kono, 1975). According to Kono (1975), the outbreaks in Asia originated from a second focus in Java, Indonesia,

Figure 1. Trends of global spread of acute hemorrhagic conjunctivitis (1969-1973) indicating years of epidemic in different areas. Obtained from Kono (1975) with permission.



★ African focus ☆ Asian focus

and the speed of the spread was comparable to that of Asian influenza. The Middle East was spared although some cases were reported in Yemen and Saudi Arabia between 1972 and 1973 (Kono, 1975).

In the following years before the advent of the second pandemic, several outbreaks were reported in Thailand (Kono *et al.*, 1977), Kenya (Metselaar *et al.*, 1976), Burma and Taiwan (Kono and Uchida, 1977), as well as smaller ones in some European countries including the Netherlands, England, France, and Yugoslavia (Kono and Uchida, 1977).

The second pandemic occurred between 1980 and 1982, starting in Southern India (Ghendon, 1989) and spreading to most of the Asian and African countries affected in the first pandemic as well as to others, including Zaire, and the United Arab Emirates (Hoffman, 1982). In addition, the disease reached the western hemisphere this time, brought to Honduras in 1981 by sailors returning from India (Asbell, 1985), and spreading throughout Central and South America, including Panama (Reeves *et al.*, 1986), Puerto Rico (Waterman *et al.*, 1984), Costa Rica, Brazil, and the Carribean (Ghendon, 1989). Mexico and some American states were also affected (Sklar *el al.*, 1983, Malison *et al.*, 1984, Ghendon, 1989).

More localized outbreaks as well as sporadic cases have been reported since then, including in 1983 in India (Kaiwar *et al.*, 1983), in 1986 in Trinidad (Ghendon, 1989), in 1990 in Saudi Arabia (Ramia and Arif, 1990), and more recently in 1994 in Israel (Shulman *et al.*, 1997) and Japan (Uchio *et al.*, 1999), and in 1996 in India (Maitreyi *et al.*, 1999).

b) Discovery of the causative agent

In 1972, Kono *et al.* set out to search for the cause of the conjunctivitis experienced in Japan, whose clinical features were similar to the ones described for the disease that was sweeping through South Eastern Asia. They obtained several isolates from conjunctival scrapings and swabs of patients from three different outbreaks. The virus isolate was found to have the general characteristics of an enterovirus with a diameter of 29±1 nm. It could not be neutralized by antisera against known enteroviruses and it was suggested to be of a new serotype. Similar studies were conducted by Mirkovic *et al.* (1973) on representative strains from Morocco and Singapore, which altogether led to the classification of a new virus, Enterovirus type 70. Although it was later discovered that a variant of coxsackievirus A24 was also responsible for several AHC outbreaks, studies confirmed that EV70 was the cause of the two major pandemics (Kono *et al.*, 1975, Ghendon, 1989) and all of the outbreaks mentioned above.

c) Clinical presentation of AHC and treatment

This form of conjunctivitis is characterized by a short incubation period of 12 to 72 hours, averaging at 24 hours (Kono *et al.*, 1972, Hoffman, 1982, Uchida, 1989), usually affecting one eye at first but in most cases spreading to the other within one day (Kono and Uchida, 1977, Asbell *et al.*, 1985). Symptoms include itching, foreign-body sensation, ocular pain, discharge, burning sensation, photophobia, and subconjunctival hæmorrhage, the last being the most characteristic (Kono *et al.*, 1972, Kono and Uchida, 1977, Hoffman, 1982, Asbell *et al.*, 1985). Epithelial keratitis is often present (Kono and Uchida, 1977), and involvement of the preauricular lymph nodes is seen in a

majority of cases (Sasagawa *et al.*, 1982, Kono and Uchida, 1977). Occasional systemic involvement has been observed (Kono and Uchida, 1977, Hoffman, 1982). The disease is self-limiting and recovery is complete within 5 to 7 days (Kono *et al.*, 1972, Hoffman, 1982).

The highest proportion of symptomatic cases was seen in adults of the 20-30 or 30-40 age groups, depending on the region (Kono, 1975). The presence of antibody in a great majority of children suggested that infection in children was more common (Quarcoopome, 1979), even though it resulted in milder illness (Quarcoopome, 1979, Asbell *et al.*, 1985). The incidence of asymptomatic cases in adults is low, and reinfection has been observed (Wright *et al.*, 1992). In fact, Aoki and Sawada (1992) followed, for seven years, a group of patients who had been infected by EV70 in 1984, and found that the neutralizing antibody titre had constantly decreased every year, and was too low, by the end of the study, to confer resistance against reinfection.

There is no anti-viral treatment for AHC caused by EV70. Esposito and Obijeski (1976) found that the presence of zinc ions in tissue culture medium reduced virus titre and plaque size. According to Hoffman (1982), the use of zinc sulphate in methylcellulose was later suggested as temporary relief along with frequent irrigation with saline solution. Other compounds found to inhibit EV70 multiplication *in vitro* include guanidine-HCl (Miyamura *et al.*, 1974), arildone (Langford *et al.*, 1985), as well as the benzimidazoles enviroxime and enviradone (Langford *et al.*, 1995), which inhibited infection of conjunctival cells.

Rarely, EV70 infection results in neurological complications (Wadia *et al.*, 1973, Kono *et al.*, 1974, Stanton *et al.*, 1977, Kono *et al.*, 1977, Wadia *el al.*, 1983), although

symptoms develop weeks after the onset of ocular disease. Hung and Kono (1979) describe the neurovirulence of the J670/71 prototype strain as comparable to that of live attenuated poliovirus vaccine. Most patients experience symptoms of systemic illness prior to the initial neurologic symptoms of motor weakness and radicular pain in muscles and limbs (Kono and Uchida, 1977, Hung, 1989). This is most often followed by asymmetric flaccid motor paralysis or paresis, and in some cases by cranial nerve involvement (Kono and Uchida, 1977, Hung, 1989). The ability of EV70 to cause neurologic disease was proven by Kono *et al.* (1973) who reported that injection of the virus intraspinally or intrathalamically in monkeys resulted in varying degrees of neuromuscular dysfunction.

The incidence of involvement of the central nervous system (CNS) is estimated at 1 in 10,000 to 17,000 cases (Hung and Kono, 1979), and seems to be higher in males (Kono and Uchida, 1977, Hung, 1989). It has been suggested that this frequency is probably underestimated since CNS disease was not associated with AHC infection due to the rare number of cases (Kono *et al.*, 1974) and/or the two to three week gap between the onset of AHC and the appearance of neurologic disease (Kono and Uchida, 1977).

d) Characteristics of EV70

EV70 virions have a cubic symmetry, a diameter of 29±1 nm, and a buoyant density of 1.34 g/mL in CsCl (Kono *et al.*, 1972, Yamazaki *et al.*, 1974). The positive-stranded RNA genome has a sedimentation coefficient of 34S and a relative molecular weight of 2.5×10^6 (Yamazaki *et al.*, 1974). This RNA is 7390 nucleotides (nt) long and is composed of a 726 nt 5' nontranslated region (NTR) that is 85% similar to the

poliovirus 5' NTR, followed by an open reading frame of 6582 nt and a short 3' NTR of 82 nt (Ryan *et al.*, 1990).

Although EV70 resembles other members of its genus in many ways (Kono *et al.*, 1972), it does present some striking differences. Unlike most enteroviruses whose primary site of infection is the enteric tract, EV70 has an unusual *in vivo* tropism for the conjunctival epithelium (Yamazaki and Miyamura, 1989), and an optimal growth temperature of 32-34°C (Miyamura *et al.*, 1974), the temperature encountered in the conjunctiva. Replication of EV70 is almost completely inhibited at temperatures between 37°C and 39°C (Miyamura *et al.*, 1974, Stanton *et al.*, 1977), due to a lack of RNA transcription (Takeda *et al.*, 1982) probably caused by the lack of uridylylation of VPg at the restricted temperature (Takeda *et al.*, 1989). This temperature-sensitivity provides an explanation for the inability to isolate the virus from the enteric tract (Yamazaki and Miyamura, 1989), where the temperature is 37°C. It also implies that the virus is not transmitted by the oral-fecal route. In fact, EV70 is transmitted by direct touch or by fomites (Yamazaki and Miyamura, 1989). On the other hand, Stanton *et al.* (1977) have observed some adaptation to higher temperatures, thereby suggesting an explanation for how the virus may replicate in the CNS.

In contrast to its specific tropism *in vivo*, EV70 exhibits a wide host range *in vitro*. Unlike most human enteroviruses which multiply only in primate cell lines, EV70 was found to replicate in mouse L-cells (Stanton *et al.*, 1977), monkey, rabbit, and mouse conjunctival and corneal cells (Langford and Stanton, 1980), and a variety of murine, crecitine, leporine, porcine, and bovine kidney cell cultures (Yoshii *et al.*,

1977). Langford *et al.* (1986) reported developing a model of EV70 infection in rabbits but these observations have not been confirmed since.

e) Origin and evolution of EV70

Through the study of 18 EV70 isolates, Takeda *et al.* (1994) were able to construct a phylogenetic tree from which they estimated the virus' time of emergence to be August 1967 ± 15 months, evolving from a single focus. Based on EV70's wide host range and on the findings by Kono *et al.* (1975) that cattle and horse sera contained neutralizing substances to AHC virus (EV70), Yoshii *et al.* (1977) suggested that EV70 originated as an animal picornavirus. Virus neutralizing titres composed of IgM antibodies were also found in bovine, sheep, swine, goat, and chicken sera (Kono *et al.*, 1981, Sasagawa *et al.*, 1982). Interestingly, serum from monkeys was found to contain no or very low neutralizing titres. However, IgM cross-reactivity among human and animal picornaviruses has been described (Mertens *et al.*, 1983) and may explain the virus neutralizing titres found in animals.

Several studies have reported a constant and very high rate of nucleotide substitution in the EV70 genome (Takeda *et al.*, 1994, Yamazaki *et al.*, 1995, Yoshino *et al.*, 1998), although an extremely low percentage of these substitutions was found to be at the amino acid level. In fact, a non-synonymous substitution rate of as low as 0% was observed for some parts of the viral capsid proteins of the most recent isolate, Kumoi-2/93, when compared to the prototype strain J670/71 (Yamazaki *et al.*, 1995). In a recent study, Uchio *et al.* (1999) reported the highest number of cases in children aged 11 to 15, but milder clinical features than previously reported with a lower incidence of subconjunctival hæmorrhage and no neurological complications, during a 1994

epidemic in Okinawa, Japan. These observations as well as the difficulty of isolation of EV70 since the late 1980's seem intriguing considering that the genome coding sequence is virtually unchanged since the original appearance of the virus.

II. The family *Picornaviridae*

a) Taxonomy

Poliovirus, human hepatitis A and rhinoviruses are among the most important of the many human and agricultural viruses in the family *Picornaviridae* (Rueckert, 1996). Although the family has recently been expanded to encompass ten genera, the five major ones are the *Enteroviruses*, the *Rhinoviruses*, the *Aphthoviruses*, the *Cardioviruses* and the *Hepatoviruses*. So-called because of their usual tropism for the enteric tract, enteroviruses include the polioviruses, the coxsackieviruses, the echoviruses, the human enteroviruses 68-71 as well as several nonhuman enteric viruses. Rhinoviruses are the single most important etiological agents of the common cold with more than 100 serotypes infecting adults and children, as well as a few that infect cattle. Aphthoviruses infect cloven-footed animals and rarely humans, causing foot-and-mouth disease. The cardioviruses are picornaviruses of rodents and include Columbia SK virus, EMC virus, MM virus and mengovirus. The last genus includes human hepatitis virus A, which was previously known as human enterovirus 72. The more recent genera are the *Kobuviruses*, the *Teschoviruses*, the *Parechoviruses*, the *Aichiviruses*, and the *Erboviruses* (Pallansch and Roos, 2001).

b) Virus characteristics

The genomes of picornaviruses consist of single stranded positive sense ribonucleic acid (+RNA), and range from 7209 to 8450 bases in length. This RNA carries a covalently-attached small protein called VPg at its 5' terminus which is cleaved by a host protein prior to translation. It is suggested that VPg plays an important role in initiation of picornaviral RNA synthesis by acting as a primer (Racaniello, 2001). The 3' end is polyadenylated, and removal of this tail results in loss of infectivity (Racaniello, 2001).

Picornaviral genomic RNA encodes a large polyprotein, which is cleaved during translation by virus-coded proteinases into 11-12 end products, including viral coat proteins, several viral proteases and a viral RNA polymerase. This polypeptide coding sequence is flanked by a rather long 5' NTR that contains structures involved in translational initiation, and a relatively short NTR at the 3' end which contains a secondary structure that is involved in controlling the synthesis of viral RNA.

The RNA is tightly packed into a protein shell of icosahedral symmetry. This shell protects the RNA from nucleases and is involved in host range and tissue tropism due to its role in recognition of cellular receptors. It also carries directions for selection and packaging of the viral genome, provides a proteinase involved in virion maturation, and is involved in delivering the RNA genome into the cytoplasm of susceptible host cells (Rueckert, 1996).

c) Initiation of infection

Attachment of a virion to the cell surface is due to the specific binding of the antireceptor, a protein found throughout the surface of the virion, to receptors, most commonly glycoproteins, found on the cell plasma membrane (Roizman and Palese, 1996). Numerous studies in recent years have found that receptor complexes, rather than single cell surface proteins, are required for virus attachment and cell entry, leading to infection (Shafren *et al*, 1997a). For instance, CVA21 binds to the human DAF molecule but requires ICAM-1 to be coexpressed on cells for entry and infection (Shafren *et al*, 1997a). Viruses may have several types of antireceptors on their surface, or may have some that consist of multiple domains that recognize different receptors or coreceptors (Roizman and Palese, 1996).

The presence of ions in the environment also appears to be required for optimal attachment. For instance, group B coxsackieviruses as well as echovirus 6 exhibit a pH-dependant attachment profile (Rueckert, 1996). In contrast, the attachment of polioviruses is independent of pH within a certain range, although the process seems to be accelerated with the addition of monovalent cations. The divalent cations Ca^{2+} or Mg^{2+} seem to be required for the attachment of other members of the family *Picornaviridae,* such as rhinoviruses, FMDV and coxsackieviruses A9 and A13. This binding is inhibited by the presence of chelating agents such as EDTA. Although the effects of ions on attachment are clear, the mechanisms by which they function are not.

Attachment of viruses is normally a temperature and energy independent process, and in many cases leads to irreversible changes in the structure of the virion

(Roizman and Palese, 1996). This conformationally altered virion is called an A particle and is no longer infectious.

d) Viral uncoating and entry

The process of penetration immediately follows attachment and is energy-dependent (Roizman and Palese, 1996). For nonenveloped viruses, this may involve translocation of the entire virus across the plasma membrane, endocytosis of the virus particle leading to accumulation of virus particle inside vacuoles in the cytoplasm, or possibly delivery of the genome across the plasma membrane leaving the capsid in the extracellular environment.

The process of uncoating describes the events that follow penetration and lead to expression of the viral genome as well as to its replication (Roizman and Palese, 1996). The important feature is the exposing of the viral genome to the translational machinery inside the cell. In many cases, including picornaviruses, this is accomplished by stripping the RNA of its protective shell upon entry of the virus into the host cell.

The ability of a virus to infect a host cell by attaching, penetrating and uncoating renders the cell susceptible. This does not necessarily lead to virus multiplication, and the ability of a cell to support subsequent virus growth and replication is termed permissiveness. The opposite is also possible, whereby a cell is permissive but lacks the receptors that are required for the binding and entry of a certain virus. This can be tested by transfecting a non-susceptible cell with intact RNA from the virion, with RNA transcribed *in* vitro or even with cDNA cloned into expression vectors. This property of a cell can be exploited in the discovery of specific virus receptors.

e) Viral replication

After initial attachment of the virion to cellular receptors and release of the viral RNA into the cytoplasm, polyribosomes form on the genomic RNA, directing the synthesis of the polyprotein. As mentioned earlier, this polypeptide is cleaved by virus-coded proteinases during synthesis to form the different viral products, one of which is an RNA polymerase, required for the synthesis of the minus-stranded RNA complementary to the original viral genome. This is then used as a template to synthesize more (+)RNA for translation as well as for packaging into virions, once the relevant protein concentration is increased. Virus particles that are completed are eventually released by infection-mediated host cell lysis (Rueckert, 1996).

f) Picornaviral receptors

The attachment of a virus to its cell surface receptor is a key step in viral infection. Several studies have demonstrated that picornaviruses utilize a wide variety of molecules as receptors (Evans and Almond, 1998), including sialic acid, VCAM-1, $\alpha_2\beta_1$ integrin, and the LDL receptor. Some picornaviruses, including enteroviruses, may also use accessory factors, molecules which may enhance viral binding and infection. One such molecule is decay-accelerating factor (DAF) which has been identified as a receptor for several picornaviruses, including coxsackievirus A21, several of the echoviruses and group B coxsackieviruses, as well as enterovirus 70. DAF has been shown to function as a co-receptor with ICAM-1 and hCAR. These three receptors are further described in the next three sections.

Although receptors are a requirement for virus tropism, this is not always determined by the mere presence of these molecules. The poliovirus receptor (PVR),

for instance, is present on several organs that are not infected by the poliovirus. Similar observations have been made for the widely distributed glycoproteins ICAM-1 and DAF.

III. Decay-accelerating factor

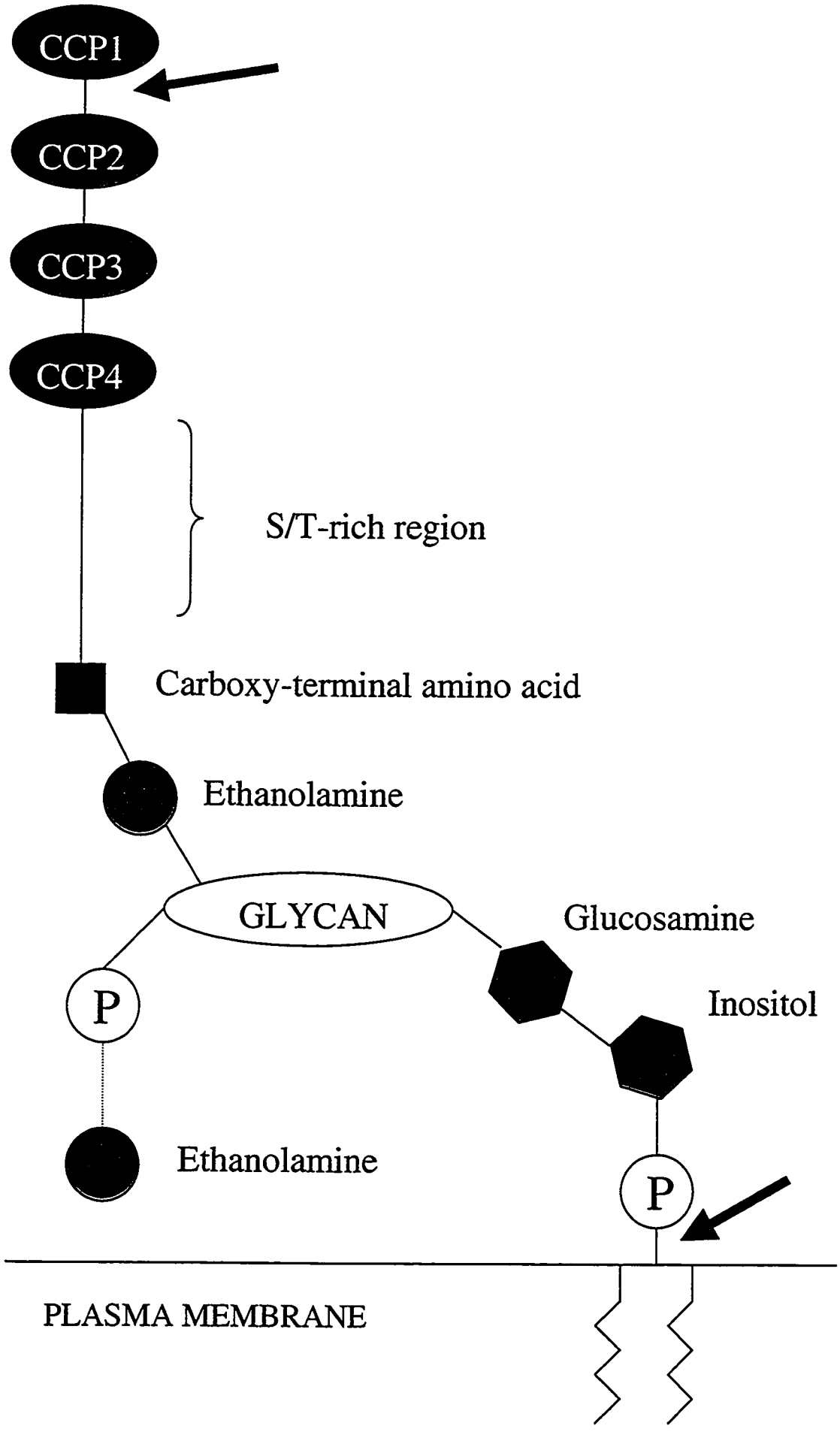
a) Human DAF (figure 2)

DAF is a 70 kDa member of the regulators of complement activation (RCA) family of proteins (Hourcade *et al.*, 1992), along with the plasma proteins C4-binding protein and factor H, the membrane proteins complement receptors type 1 (CR1) and type 2 (CR2), and membrane cofactor protein (MCP). These proteins protect tissues against autologous complement and play a role in the clearance of immune complexes.

The RCA cluster is located in the q32 region of chromosome 1. A common feature among the members of this family is the presence of repeated 60 amino acid-motifs called complement control protein repeats (CCP) or short consensus repeats (SCR) (Hourcade *et al.*, 1992). An amino acid sequence homology of 20 to 35 % is observed between the CCPs within a single protein and between repeats of different proteins.

RCA proteins involved in protection against autologous complement display either cofactor activity or decay accelerating activity, or both (Coyne *et al.*, 1992). The former activity involves the proteolytic cleavage and inactivation of the C3/C5 convertase, a function carried out by the plasma enzyme factor I in the presence of a required cofactor. As the name implies, DAF, which associates with C4b and C3b in the

Figure 2. Structure of decay-accelerating factor (DAF). **Black arrow** points to the site of PI-PLC cleavage. Green arrow indicates the site of N-glycosylation. Illustration is not to scale. Adapted from Nicholson-Weller and Wang (1994) and Udenfriend and Kodukula (1995).


CCP1
CCP2
CCP3
CCP4
S/T-rich region
Carboxy-terminal amino acid
Ethanolamine
GLYCAN
Glucosamine
P
Inositol
Ethanolamine
P
PLASMA MEMBRANE

membrane, is involved in the latter activity. It prevents the assembly and accelerates the decay of C3 convertases C4b2a and C3bBb of the classical and alternative pathways, respectively, by dissociating 2a and Bb from the binding sites (Fujita *et al.*, 1987).

DAF was first isolated by Nicholson-Weller *et al.* (1981) from guinea pig erythrocytes, and from human erythrocytes shortly afterwards (Nicholson-Weller *et al.*, 1982). Medof *et al.* (1984) observed that purified human DAF could incorporate into the membranes of rabbit erythrocytes thereby inhibiting their lysis by human complement. DAF incorporated into sheep red cell complement intermediates was shown to inhibit the assembly of C3/C5 convertases of both the classical and the alternative pathways. Treatment with specific anti-DAF antibodies reversed this effect (Medof *et al.*, 1984).

The DAF gene is composed of 11 exons spanning approximately 40 kb (Post *et al.*, 1990). Cloning of DAF cDNA revealed a 347-amino acid mature protein which contained four CCPs and a 70-amino acid serine/threonine (S/T)-rich region, with a single site for N-linked glycosylation at residue 61, between CCP 1 and CCP 2 (Caras *et al.*, 1987; Medof *et al.*, 1987b). Lublin *et al.* (1986) found that DAF is synthesized as a 43 kDa precursor species which is converted to a 46 kDa species prior to entering the Golgi. The modifications involve the cleavage of the signal peptide and the addition of a glycosyl-phosphatidylinositol (GPI) anchor, which was confirmed by the ability of phosphatidylinositol-specific phosopholipase C (PI-PLC) to release DAF from the cell surface (Davitz *et al.*, 1986; Medof *et al.*, 1986). DAF released by this treatment was slightly smaller than the membrane form and could not reincorporate into membranes. Signalling for the addition of the GPI moiety is by virtue of a hydrophobic peptide at the

C terminus of the protein (Udenfriend and Kodukula, 1995). Just like the signal peptide, this C-terminal peptide is also cleaved during processing (Udenfriend and Kodukula, 1995), followed by linkage of the GPI moiety before additional modifications occur in the Golgi (Lublin *et al.*, 1986; Medof *et al.*, 1986). O-glycosylation of the S/T-rich region occurs in the Golgi producing the 70 kDa species typically reported (Lublin *et al.*, 1986).

The domains required for DAF function were shown to be to CCPs 2, 3, and 4, and the S/T-rich region was found to be required only as a spacer projecting the CCPs away from the plasma membrane (Coyne *et al.*, 1992). The importance of the length of this spacer region was also observed in guinea pig DAF by Wang *et al.* (1998) who reported that isoforms with longer S/T regions were efficient at inhibiting complement. Brodbeck *et al.* (2000) found that the N-linked glycan of DAF is not involved in its regulatory function, but that the leucine at position 147 (in a hydrophobic area of CCP3) is essential for the regulation of the C3 convertase in both the classical and alternative pathways. A stretch of three lysines at positions 125-127 in the positively charged pocket between CCPs 2 and 3 was found to be necessary for the regulatory activity of DAF on the alternative pathway C3 convertase but played a lesser role in its activity on the classical pathway enzyme (Brodbeck *et al.*, 2000).

Although Davitz *et al.* (1986) claimed that DAF lacking the GPI anchor (due to release by PI-PLC) could not inhibit the C3 convertase, others have reported otherwise (Lublin and Coyne, 1991; Medof *et al.*, 1986; Moran *et al.*, 1992). In fact, transmembrane(TM)-DAF constructs, where the S/T-rich region has been replaced by the TM and cytoplasmic domains of either HLA-B44 or MCP, have been shown to

decrease C3 deposition with equal efficiency to DAF (Lublin and Coyne, 1991). This defies the suggestion that the increased lateral mobility displayed by GPI-anchored DAF (Thomas *et al.*, 1987) is important for its function.

DAF is a widely distributed molecule, found on the surface of platelets, polymorphonuclear leukocytes (PMN), monocytes, and B and T lymphocytes (Caudwell *et al.*, 1990; Kinoshita *et al.*, 1985; Nicholson-Weller *et al.*, 1985; Vuorinen *et al.*, 1999). DAF was detected on corneal and conjunctival epithelia (Lass *et al.*, 1990; Medof *et al.*, 1987a), corneal endothelium, retina, on lacrimal gland acinar cells, in tears, aqueous humor, and vitreous humor (Lass *et al.*, 1990). Medof *et al.* (1987a) also reported expression of DAF on epithelium in oral mucosa and salivary glands, esophagus, upper and lower GI tract, as well as in endocardium, synovial cells lining joint spaces, endometrial and epithelial cells lining uterus and cervix, saliva and urine (Medof *et al.*, 1987a). Complement-regulating DAF is also expressed on human spermatozoa (Cervoni *et al.*, 1993).

Several forms of DAF have been described. Firstly, cloning of DAF from HeLa cells revealed that the previously described GPI-anchored DAF arises from a splicing product with a coding frameshift near the C terminus (Caras *et al.*, 1987). The longer unspliced messenger RNA (mRNA) was a minor product but was translated. This form of DAF lacked the C-terminal hydrophobic region required for the attachment of the GPI anchor and could not be detected in transfected cells unless they were permeabilized. This led to the conclusion that this was a secreted form of DAF, later detected in several body fluids (Lass *et al.*, 1990). Secondly, a high molecular weight form of DAF, DAF-2, has been reported by Kinoshita *et al.* (1987). Like DAF, this 140

kDa protein displayed decay-accelerating activity, and reincorporated into red cell membranes. Treatment with PI-PLC and glycosidases demonstrated that DAF-2 possessed two GPI anchors and contained twice as much oligosaccharide as DAF (Nickells *et al.*, 1994). Along with other data, it was concluded that DAF-2 was a covalently cross-linked dimer of DAF. Thirdly, Medof *et al.* (1987a) and Lass *et al.* (1990) reported the detection of a 100 kDa form of DAF in tears. Alternative mRNA splicing and differential post-translational processing were suggested as possible mechanisms leading to the production of the larger DAF protein (Lass *et al.*, 1990). Lastly, Sayama *et al.* (1991) observed that DAF on human skin elastic fibers was resistant to PI-PLC treatment and suggested the existence of an anchoring mechanism different from that of a GPI link. No such mechanism has been described since, although the resistance observed could have been caused by palmitoylation of the inositol in the GPI anchor, as was observed on human erythrocyte acetylcholinesterase (Roberts *et al.*, 1988). Release of the palmitoyl group from GPI-anchored acetylcholinesterase abolished resistance to PI-PLC treatment. This resistance could also explain why Davitz *et al.* (1986) observed that PI-PLC treatment of erythrocytes released merely 10% of the DAF expressed as opposed to 70-80% and 60% on mononuclear cells and monocytes, respectively.

Besides its role in complement regulation, several other functions have been attributed to DAF. A role as a signal transmitter in T cell activation has been observed by Davis *et al.* (1988), with a requirement for the GPI anchor. The important role of the anchor was confirmed by Shenoy-Scaria *et al.* (1992) who observed transduction of late activation events in DAF-transfected cells but not in cells transfected with DAF-TM.

GPI-anchored DAF was found to be associated with the protein tyrosine kinases $p56^{lck}$ and $p59^{fyn\ 1}$, leading to the phosphorylation of several proteins. DAF was also reported to serve as a ligand for the transmembrane receptor CD97 (Hamann *et al.*, 1996), which was found to attach to CCP1 of DAF. This interaction has also been observed between murine homologues of DAF and CD97 (Qian *et al.*, 1999).

DAF is utilized as a receptor by several organisms. Gestational pyelonephritis-associated *Escherichia coli* (*E. coli*) has been shown to attach to SCRs 3 and 4 of DAF (Pham *et al.*, 1995). The Afa/Dr family of diffusely adhering *E. coli* also uses DAF as a receptor (Guignot et al., 2000). Several viruses utilize DAF for cell attachment and entry. Bergelson *et al.* (1994) demonstrated that echovirus 7 uses DAF as a receptor, and Clarkson *et al.* (1995) showed that this interaction required CCPs 2, 3, and 4, but not the GPI anchor nor the N-linked or O-linked glycosylations. As mentioned earlier for DAF's complement regulation function, the S/T-rich region was found to be useful only as a spacer (Clarkson *et al.*, 1995). Antibodies to DAF were also shown to protect HeLa cells from infection by echoviruses 6, 11, 12, 20, and 21 (Bergelson *et al.*, 1994), and coxsackievirus serotypes B1, B3, B5, and A21 were also shown to utilize DAF as a means of attaching to the surface of cells (Bergelson *et al.*, 1997b; Shafren *et al.*, 1995, 1997b, 1997c). The attachment of the coxsackie B viruses to DAF was shown to be dependent on the origin of the laboratory strain or the clinical isolate (Bergelson *et al.*, 1997b).

DAF was also identified as the receptor for EV70 in HeLa cells (Karnauchow *et al.*, 1996). Several monoclonal anti-DAF antibodies were shown to protect these cells from infection by EV70. DAF expressed transiently in murine NIH 3T3 cells allowed

these cells to bind radiolabelled virus. Construction of chimeras using MCP CCPs revealed that CCP 1 of DAF was required for the interaction with EV70, but that it was not sufficient, as binding of the virus was inhibited by 80% in the absence of CCP 2 (Karnauchow *et al.*, 1998).

b) Non-human DAF homologues

The expression of DAF has been demonstrated in several animals. In mice, a 60 kDa membrane protein was purified by Kameyoshi *et al.* (1989) and was found to protect erythrocytes against hemolysis by both mouse and human complement. Two different cDNAs were later cloned, and were unique in that they originated from two separate genes (Spicer *et al.*, 1995). Their sequences were found to be 83% identical at the nucleotide level, with most of the differences localizing to the 3' end of the cDNA. The sequences of the predicted proteins were 78% identical, contained four CCPs and a S/T-rich region, but differed in their attachment to the membrane: one form was GPI-anchored (mDAF-GPI) and the other transmembrane-anchored (mDAF-TM). The overall degree of identity of the amino acid sequences to that of human DAF (hDAF) was between 39 and 47%, although a larger similarity was observed in the CCP region, especially CCPs 2, 3, and 4 (56-59%), which are required for DAF function (Coyne *et al.*, 1992). By measuring mRNA levels in different tissues, Spicer *et al.* (1995) found that mDAF-GPI was expressed in brain, secretory epithelia, skeletal muscle, liver, testes, thymus, spleen, kidney, small intestine, and peripheral and mesenteric lymph nodes. mDAF-TM was expressed only in the lymph node, testes, and spleen (Harris *et al.*, 1999; Spicer *et al.*, 1995). Ohta *et al.* (1999) reported that both forms of mDAF, when transfected into CHO cells, could inhibit C3 deposition mediated by either the

classical or the alternative pathway. It was also observed that this complement regulatory activity was effective against rat complement but not human or guinea pig complement (Ohta *et al.*, 1999). The complement regulatory function of both isoforms was confirmed by Harris *et al.* (1999), who also reported several alternative splicing events at exon/intron junctions in the sequences encoding either mDAF-TM or mDAF-GPI, resulting in two more isoforms with a shorter or no S/T-rich region, one isoform deriving from the TM sequence but with a GPI anchor, and one other that derived from the GPI sequence and was predicted to be secreted.

Two forms of DAF, one GPI-linked and the other secreted, were identified in rats (rDAF, Hinchliffe *et al.*, 1998). RT-PCR analysis revealed that both forms, which are encoded by a single gene, were expressed in several tissues, especially the lungs and the testes. Although it is only 40% identical to hDAF, rDAF-GPI expressed on the surface of NIH 3T3 cells was as efficient as its human counterpart in reducing deposition of rat C3. Miwa *et al.* (2000) describe a TM form that is preferentially expressed in testes. Three isoforms were described in pigs, all unique in that they contained only three CCPs, with a mean amino-acid identity of 64% to the hDAF CCPs, as well as three potential sites for N-glycosylation (pDAF, Pérez de la Lastra *et al.*, 2000). Two of the isoforms were GPI-linked, one of which had a S/T-rich region that was shorter than the other's by seven amino acids, and the third isoform was a transmembrane form. Another unique feature encountered by Pérez de la Lastra *et al.* (2000) was the wider distribution of pDAF-TM, whose mRNA was detected in peripheral blood mononuclear cells (PMBC), kidney, lung, spleen, testes, and weakly in

granulocytes. Isolation of DAF from rabbit erythrocytes has been reported (Sugita *et al.*, 1987), and at least six isoforms were detected in guinea pigs (Wang *et al.*, 1998).

IV. Intercellular adhesion molecule 1

Intercellular adhesion molecule-1 (ICAM-1) is a 505 amino acid transmembrane protein belonging to the immunoglobulin superfamily (Van de Stolpe and Van der Saag, 1996). It consists of a charged cytoplasmic domain and five extracellular domains linked together by a hydrophobic transmembrane domain. ICAM-1 contains eight potential N-linked glycosylation sites, and is differentially glycosylated resulting in a molecular weight ranging between 80 and 114 kDa.

ICAM-1 serves as a ligand for several molecules, including the two integrins leukocyte function-associated antigen-1 (LFA-1) and Mac-1, soluble fibrinogen, and the extracellular matrix factor hyaluronan (Van de Stolpe and Van der Saag, 1996). ICAM-1 functions in cell to cell adhesion, and is involved in T-cell activation as well as recruitment of leukocytes to sites of inflammation, where ICAM-1 is often expressed in high levels. In fact, inflammatory responses are reduced in ICAM-1 knockout mice (Sligh *et al.*, 1993). Signalling through ICAM-1 has also been demonstrated, leading to cytokine regulation or activation of kinases. Although found on a wide variety of cell types including leukocytes, fibroblasts, and certain epithelial cells, ICAM-1 is normally expressed at low levels (Maio and Del Vecchio, 1991; Ohh and Takei, 1996).

ICAM-1 is also utilized as a receptor by microorganisms. Based on the observation that treatment of HeLa cells with ICAM-1 inducing factors correlated with

an increase in the binding of the major group human rhinoviruses (HRV) to these cells, Tomassini *et al.* (1989) were able to identify ICAM-1 as the receptor for these viruses. Marlin *et al.* (1990) confirmed this result by constructing and purifying a soluble form of ICAM-1 which was shown to efficiently inhibit rhinovirus infection. Shafren *et al.* (1997a) identified the same protein as the receptor for CVA21 and used monoclonal antibodies to trace the binding site to the N-terminal domain of the glycoprotein. Transfection of this molecule into baby hamster kidney cells rendered these cells permissive to both HRV14 and CVA21 (Grunert *et al.*, 1997). Malaria-infected erythrocytes were also reported to utilize ICAM-1 as a means of attachment (Berendt *et al.*, 1989).

V. Coxsackie-adenovirus receptor

By extracting the virus-receptor complex from infected HeLa cells, Mapoles *et al.* (1985) were first to isolate the 50 kDa CVB receptor which they named RP-a. An antibody was raised to this protein and shown to prevent all six serotypes of CVB from infecting HeLa cells and Buffalo green monkey kidney cells (Hsu *et al.*, 1988). This antibody was used by Bergelson *et al.* (1997a) to isolate the protein and clone the corresponding cDNA. Analysis of this sequence predicted a protein of 365 amino acids with a helical transmembrane domain, an extracellular portion consisting of two immunoglobulin-like domains, and a relatively long cytoplasmic tail of 107 amino acids. Not only did transfection of this cDNA into CHO cells render them permissive to CVB3 and CVB4, but it also allowed radiolabelled adenovirus 2 as well as isolated

adenovirus 2 fibers to bind the cells (Bergelson *et al.*, 1997a). Gene delivery of adenovirus 5 was shown to be greatly enhanced in the cells transfected with what was termed the human coxsackie-adenovirus receptor (hCAR) (Bergelson *et al.*, 1997a). Tomko *et al.* (1997) reported similar observations in murine NIH 3T3 cells. This receptor function was also observed for the murine homolog of this protein (Bergelson *et al.*, 1998; Tomko *et al.*, 1997), which shares 91% identity with its human counterpart (Bergelson *et al.*, 1997a).

VI. Rationale and objectives

Since the receptor for EV70 on HeLa cells has been identified as DAF (Karnauchow *et al.*, 1996), it is conceivable that the atypical wide host range of EV70 *in vitro* can be explained by DAF's wide distribution among non-primate mammals (Hinchliffe *et al.*, 1998; Kameyoshi *et al.*, 1989; Pérez de la Lastra *et al.*, 2000; Sugita *et al.*, 1987; Wang *et al.*, 1998). As mentioned earlier, the receptor function of murine CAR mirrors that of the human protein (Bergelson *et al.*, 1998; Tomko *et al.*, 1997). Conversely, it has been demonstrated that MPH, the murine ortholog of the poliovirus receptor (PVR) (Eberle et al., 1995). does not function as a receptor for poliovirus (Morrison and Racaniello, 1992). The first objective of this thesis was then set to elucidate whether DAF in non-primate mammalian cells was functioning as a receptor for enterovirus 70.

Another atypical characteristic of EV70 is its restricted tissue tropism for the conjunctival and corneal epithelium *in vivo*. Since involvement of the lymph nodes has

been reported (Sasagawa *et al.*, 1982, Kono and Uchida, 1977), it is possible that the spread of EV70 to the CNS occurs via the bloodstream. Several enteroviruses have displayed an ability to infect blood cells (Freistadt *et al.*, 1993, 1996; Vuorinen *et al.*, 1994, 1996, 1999), and preliminary experiments in our laboratory have demonstrated the same for EV70 (appendix A). It was therefore of interest to determine whether DAF was functioning as a receptor on human cells other than HeLa cells, and leukocyte cell lines expressing variable levels of hDAF (table 1) were of particular importance for the reasons mentioned above. This was set as the second objective of this thesis.

The involvement of more than one receptor in the attachment of specific enteroviruses has been suggested by several researchers. Powell *et al.* (1998) reported echoviruses binding to and infecting cells from which DAF has been removed or blocked. The use of two distinct receptors for CVBs was first suggested by Hsu *et al.* (1988), and later confirmed by Shafren *et al.* (1997c) who demonstrated that a strain of CVB3 required hCAR for infection even though it was capable of utilizing DAF as an attachment protein. Similar observations were made for CVA21 which bound DAF but required ICAM-1 for infection (Shafren *et al.*, 1997b). These two molecules were found to be closely associated, although no cytoplasmic interactions were required (Shafren *et al.*, 2000) for CVA21 infection. As for EV70, Yoshii *et al.* (1977) did observe that EV70 adsorbed to some cell lines without any sign of replication, thereby suggesting the involvement of factors other than the cellular receptor in the internalization of the virus.

Table 1. Expression of human DAF on the surface of leukocyte cells.[a]

Cell line	2Y[b]	1Y[c]	ratio[d]
HeLa T4	0.619	3.18	4.76
U-937	1.15	6.01	5.34
Jurkat	1.48	4.40	3.03
HL-60	1.66	1.67	1.00
THP-1	1.46	5.08	3.29
Daudi	1.17	5.49	5.14
Raji	4.75	6.89	1.44
A.201	0.69	1.21	1.76

[a] data expressed as mean fluorescence intensities (MFI)
[b] MFI with secondary antibody only
[c] MFI with both primary and secondary antibodies present
[d] ratio determined by dividing the MFI for 1Y by the MFI for 2Y

Based on these observations, the third objective of this thesis was to determine whether ICAM-1 or hCAR play a role in the attachment and/or internalization of EV70.

MATERIALS AND METHODS

I. Cell culture

a) Sources of cell lines

Non-adherent (suspension) cell lines. The T-cell lines Jurkat and A.201 were obtained from Dr. K. Wright (Department of Biochemistry, Immunology and Microbiology (BMI), University of Ottawa) and Dr. L. Poulin (Department of Microbiology, Laval University, Ste-Foy, QC), respectively. Daudi cells (B lymphoblasts) were a gift from Dr. M. Freedman (Department of Medicine, University of Ottawa), and Raji cells (B lymphocytes) from Dr. F. Diaz-Mitoma (Children's Hospital of Eastern Ontario, Ottawa, ON). THP-1 (monocytes) and HL-60 (promyeloblasts) cells were obtained from Dr. J. Angel (Department of Medicine, University of Ottawa), and the histiocytic cell line U-937 from Dr. L. Filion (Department of BMI, University of Ottawa).

Adherent cell lines. Rhesus monkey kidney cells, LLC-MK_2, were purchased from Flow Laboratories, Rockville, MD. Mouse embryo fibroblast cells, NIH 3T3, were a gift from Dr. S. Bennett (Department of BMI, University of Ottawa). Mouse fibroblast L-929 cells were provided by Dr. E. Brown (Department of BMI, University of Ottawa). HeLa and HeLa T4 cells were obtained from the National Institute of Allergy and Infectious Diseases (NIAID) AIDS Research and Reference Reagent Program, Rockville, MD. Human HEp-2 cells were purchased from the American Type Culture Collection (ATCC), Rockville, MD. NIH 3T3 cells expressing human, pig, mouse, and rat DAF

(3T3/hDAF, 3T3/pDAF, 3T3/mDAF, and 3T3/rDAF, respectively) were a gift from Dr. O. B. Spiller (Department of Medicinal Biochemistry, University of Wales College of Medicine, Cardiff, UK).

b) Culture

Media and supplements were purchased from GibcoBRL Life Technologies Canada (Burlington, ON) unless indicated otherwise. All of the non-adherent cell lines were grown as stationary suspension cultures in 25 cm^2 polystyrene flasks (Corning Glass Works, Corning, New York), or NUNC Life Science Products (Burlington, ON), at 37°C in a humidified 5% CO_2 atmosphere, and maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 50 μg/ml gentamycin sulphate (Roussel Canada, Montreal, PQ). Passaging of these cells required mixing and dispersing, followed by replacing 90-95% of the suspension with fresh medium, every 3-4 days.

All adherent cells were grown in medium consisting of Minimal Essential Medium containing Eagle's salts (MEM) supplemented with 0.2% (w/v) sodium bicarbonate, 2 mM L-glutamine, 50 μg/ml gentamycin sulphate and 10% FBS (NIH 3T3), 10% defined and supplemented bovine calf serum (d/sBCS, HyClone Laboratories Inc, Logan, UT) (HeLa, HeLa T4, and Hep-2) or 5% d/sBCS (LLC-MK_2). For stably transfected cells, growth medium was also supplemented with either 100 μg/ml Hygromycin B (Life Technologies) (3T3/h,p,m, and rDAF) or 10 μg/ml Blasticidin S HCl (Invitrogen Corp., Burlington, ON) (3T3/ICAM and 3T3/hCAR). Cells were grown in 100x20mm polystyrene culture dishes (Sarstedt Inc., Montreal, PQ, or NUNC Life Science Products) at 37°C in a humidified 5% CO_2 atmosphere.

Adherent cells were passaged as follows. Medium was aspirated and the cell monolayer was washed with warmed Tris-buffered saline (TBS; 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 5.6 mM glucose and 25 mM Tris-HCl pH 7.2). Trypsinization followed (0.05% trypsin and 0.5 mM EDTA) at 37°C for 3 to 5 minutes and the required proportion of cells was then transfered to fresh warmed medium by direct pipetting. Cells were passaged every 3-4 days and split at either a 1:12-15 ratio (NIH 3T3 and all stable transfectants) or a 1:5-10 ratio (HeLa, HeLaT4, Hep-2, and LLC-MK_2).

II. DNA Manipulations

a) DNA amplification

Double stranded DNA sequences were amplified using polymerase chain reactions in 1X PCR buffer (10 mM Tris-HCl, 50 mM KCl, and 0.08% Nonidet P40), 0.2 mM of each dNTP and 1.5 mM $MgCl_2$. Primers were added at a concentration of 0.5 μM each, and 1-10 μl of template DNA (<10 ng) was normally added. 2.5 units of *Taq* DNA polymerase were used and the final mix was diluted to 100 μl with sterile water. All reagents were supplied by MBI Fermentas, Flamborough, ON. A hot start protocol was used in which the reaction mixture was heated to 94°C, and denaturation occurred for 3 minutes prior to the addition of the Taq polymerase. Reactions were then allowed to proceed for 30 cycles as follows. Denaturation at 94°C for 45 seconds was followed by 1 minute of annealing at 50°C, and then 2.5 minutes at 72°C for extension. Samples were further incubated at 72°C for 30 minutes to allow for complete addition of single dA

nucleotides to both extremities of the amplification product. The primers and templates used are described in table 2.

Table 2. Templates and primers used in PCR.

GENE	TEMPLATE	PRIMERS
ICAM-1	PEFBOS-ICAM[a]	(+) CACACAGGATCCAACATGTCTCCCAGCAGCCCCCG
		(-) CACACAGGTACCGGGATAGGTTCAGGGAG
HCAR	pcDNA1-CAR[b]	(+) CACACAGGTACCCCACCATGGCGCTCCTG
		(-) CACACAGAATTCGACATATGGAGGCTCTATAC

[a] pEFBOS-ICAM was provided by Dr. D. Shafren (Department of Microbiology, University of Newcastle, Newcastle, Australia).
[b] pcDNA1-CAR was a gift of Tami Martino and Dr. P. Liu (Heart and Stroke/Richard Lewer Center of Excellence, University of Toronto, Toronto, ON).

b) DNA isolation and purification

Small scale isolation of plasmid DNA was carried out using a procedure modified from that described by Birnboim and Doly (1979). Bacterial cells were pelleted using a microcentrifuge and resuspended in 150 µL of ice-cold solution I (25 mM Tris-HCl pH 8.0, 10 mM EDTA, 50 mM glucose and 10 µg/ml DNAse-free RNAse A), to which 150 µL of freshly prepared solution II (1% SDS, 0.2 N NaOH) was added, for lysis, followed by gentle mixing. 150 µL of solution III (5 M KAcetate pH 4.8) was then added for neutralization, and samples were vigorously mixed and then centrifuged at 4°C for 5 minutes at 10,000xg in a microfuge. Proteins were then extracted with chloroform and DNA was precipitated with ethanol at 4°C for 10 minutes, pelleted (10,000xg) and resuspended in 50 µL of sterile water.

For certain applications (i.e. sequencing, transfections) isolation of plasmid DNA was carried out using the Wizard Plus Minipreps or Midipreps DNA Purification System (Promega, Madison, WI), as described by the supplier.

Isolation of DNA fragments, including PCR products, from low melting temperature agarose (SeaPlaque GTG, FMC Bioproducts, Rockland, ME) gels was performed by excising the fragment of interest from the gel and purifying it using the Wizard DNA Clean-Up System (Promega, Madison, WI). Gels made with GTG agarose were poured and used at 4°C.

c) DNA analysis

Gel electrophoresis was performed in 1% (w/v) agarose gels (Bio-Rad Laboratories Ltd., Mississauga, ON) in Tris-acetate buffer (TAE; 40 mM Tris-acetate, 2 mM EDTA), in a Horizon 58 submarine electrophoresis unit (GibcoBRL). Electrophoresis was carried out at 80-90 V for 30 minutes to 1 hour, and the gels were stained with ethidium bromide (0.5 μg/ml) for 10 minutes before visualization of the DNA using short wave ultraviolet light.

Sequencing of DNA was performed at the University of Ottawa Biotechnology Research Institute using a fluorescence-tag dideoxynucleotide chain termination method and a ABI prism 373 DNA sequencer (Applied Biosystems, Foster City, CA).

d) Cloning and transformations

PCR products were cloned into the pEF6/V5-His TOPO TA vector (Invitrogen Corp., Carlsbad, CA) and products of the TOPO reaction were used to transform chemically competent TOP10 One Shot *E. coli* (Invitrogen Corp) following the manufacturer's protocol. Briefly, 1 μL of the vector preparation and 1 μL of the salt

solution provided (1.2 M NaCl, 0.06 M $MgCl_2$) were added to 4 µL of purified PCR product and the mixture was incubated at room temperature for 15 minutes. 2 µL of this mixture were then added to 50 µL of TOP10 cells followed by brief gentle mixing and a 25-minute incubation on ice. The cells were then heat shocked at 42°C for 30 seconds and transferred immediately back on ice. 250 µL of prewarmed SOC (2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, and 10 mM $MgSO_4$ and 20 mM glucose) were then added to the cells and the mixture was incubated for 1 hour at 37°C with shaking. Subsequently, aliquots were plated on LB-ampicillin (2 % (w/v) LB base from GibcoBRL, 1.5% (w/v) agar and 100 µg/ml ampicillin) plates which were incubated at 37°C overnight. Randomly selected colonies were then grown in 3 ml LBA (2 % (w/v) LB base from GibcoBRL and 100 µg/ml ampicillin) medium overnight. Plasmid DNA was purified as described earlier, and correct orientation and nucleotide sequence of each of the cloned coding sequences were confirmed by restriction endonuclease and nucleotide sequence analysis.

III. Expression studies

a) Transfections

NIH 3T3 cells, grown to 50-60% confluency in 6-well dishes, were transfected using LipofectAMINE PLUS (Life Technologies), as follows. 1 µg of plasmid DNA was diluted in serum-free OPTIMEM (Life Technologies) medium in a total volume of 125 µL. 20 µL of the PLUS reagent was added and the mixture incubated at room temperature for 15 minutes. Diluted LipofectAMINE (5 µL reagent and 120 µL

OPTIMEM) was then gently mixed in, followed by another 15-minute incubation at room temperature. The medium on NIH 3T3 was replaced with 1 mL of prewarmed OPTIMEM and the transfection mixture was added. Cells were incubated with the transfection mixture at 37°C for 5 hours and then 3 mL of prewarmed MEM containing 10% FBS were added. Culture medium was replaced 48 hours after transfection with fresh MEM containing 10% FBS and 10 μg/mL blasticidin. Cultures were incubated for 10-14 days in the presence of blasticidin, monitored for levels of expression by flow cytometry, and sorted, as described below.

b) Flow cytometry and cell sorting

Adherent cells were disrupted with trypsin-EDTA for 3-5 minutes at 37°C. Cells ($3x10^5$) were then pelleted and washed once with FC buffer (phosphate-buffered saline [PBS, 137 mM NaCl, 2.7 KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4] containing 0.1% (w/v) sodium azide, and 2% (w/v) BSA). Cells were then resuspended in 100-150 μl of FC buffer containing the desired concentration of primary antibody (Table 3) and incubated at room temperature for at least 15 minutes. Cells were then washed with 4 mL of FC buffer, pelleted, and resuspended in 100-150 μl of FC buffer containing one of two secondary antibodies, FITC-conjugated goat anti-mouse (Roche, Indianapolis, IN) or anti-rat (PharMingen, San Diego, CL) immunoglobulin, diluted 1:1000 and 1:500,

Table 3. Primary antibody dilutions used for flow cytometry.[a]

Antibody	Mg/mL
11D7[b]	2.0
8D11[c]	2.0
Rmcb[d]	1.0
84H10[e]	1.0

[a] See section IIIc for antibody sources; [b] DAF-specific, CCP1; [c] DAF-specific, CCP4; [d] hCAR-specific; [e] ICAM-1-specific.

respectively. Following another incubation at room temperature for at least 15 minutes, the samples were diluted to 1 ml with FC buffer and analyzed using a Coulter EpicsXL-MCL flow cytometer. A minimum of 5000 events was counted for each sample in the established gate, and mean fluorescence intensities were determined from single parameter histograms generated with Epics XL 2.0 software. Controls for autofluorescence (no antibody) and nonspecific secondary antibody binding (secondary antibody only) were included in each analysis.

For sorting of 3T3 cells, OPTIMEM was used instead of FC buffer. 3-5 x 10^6 antibody-labelled cells were suspended in OPTIMEM and the most fluorescent 10-20% of the transfectants were sorted directly into culture medium containing 10% FBS and 10 μg/mL blasticidin using a Coulter EPICS Elite ESP cell sorter and EXPO V. 2.0 software (Applied Cytometry Systems). The selected cells were then pelleted and plated in a 6-well culture dish in the presence of 10 μg/mL blasticidin.

c) Sources of antibodies

The human DAF-specific monoclonal antibody (mAb), EVR1, has been described previously (Karnauchow et al, 1996). hDAF-specific mAbs 8D11 and 11D7 were a generous gift of Dr W Rosse (Department of Medicine, Duke University Medical center, Durham, NC). hDAF-specific mAb 1H4 was obtained from Dr. D. Lublin (Washington University School of Medicine, St. Louis, MO). The hCAR-specific monoclonal antibody, RmcB, was provided by Dr. J. Bergelson (Division of Immunological and Infectious Diseases, Children's Hospital of Philadelphia, Philadelphia, PA). The human CD54 (ICAM-1)-specific mAb, 84H10 (Serotec), was purchased from Cedarlane

Laboratories, Hornby, ON. The MBC1, 2C6, RDIII7, and MBC-PD1 mAbs, specific for hDAF, mDAF, rDAF, and pDAF, respectively, were kindly provided by Dr. B. Spiller.

IV) Virology

a) Sources of viruses

The strain of EV70 used was prototype strain J670/71 and was obtained from Dr. M. Hatch and Dr. M. Pallansch of the Centers for Disease Control, Atlanta, GA. Coxsackievirus B3 strain Cg was provided by Tami Martino and Dr P. Liu. Human rhinovirus 14 was a gift from Dr. K. Wright.

b) Production of stock virus

LLC-MK_2 cells were grown to confluency in 175 cm^2 culture flasks (Sarstedt Inc., Montreal, QC) and infected with either EV70 (33°C) or CVB3 (37°C, Cg strain) at a multiplicity of infection (MOI) of 0.2 for one hour in a total volume of 5 mL of medium per flask. The inoculum was then replaced with 20 mL of MEM and infection was allowed to proceed for 2-3 days. Cells were then frozen at -80°C, thawed, pelleted for 10 minutes at 4000xg and 4°C, and the virus-containing supernatant removed. Cells were then resuspended in 5 mL of the original supernatant and subjected to two more freeze-thaw cycles, using a liquid nitrogen bath and a 37°C bath, to ensure thorough lysis of the cells and maximum release of virus. Cell debris was then pelleted at 4000xg for 10 minutes at 4°C, and this supernatant was pooled with the former. Virus stock was stored at -80°C in aliquots of 1 mL. Virus titre was determined by plaque assay, as described below.

c) Radiolabeled virus

Labeling and extraction of virus. LLC-MK_2 cells were grown to confluency in 175 cm^2 culture flasks (Sarstedt Inc., Newton, NC) and infected with either EV70 (33°C) or CVB3 (37°C, Cg strain) at a MOI of 0.5 or 5, respectively, for one hour in a total volume of 6 mL of medium per flask. The inoculum was then removed and the cells were washed once with 6 mL of TBS per flask. 30 mL of methionine-free and cysteine-free medium (ICN Biomedicals, Montréal, QC) were then added to each flask and the cells were incubated at the appropriate temperature for three hours. Pro-Mix (^{35}S-methionine and ^{35}S-cysteine cell labelling mix, Amersham Pharmacia, Baie d'Urfé, QC) containing approximately 1.4 mCi was added to each flask, and the cells were further incubated (33°C for EV70 or 37°C for CVB3) until most of the cells displayed cytopathic effects (CPE), normally 30-50 hours after infection. Cells from different flasks were disrupted by freezing and thawing, pooled, transferred to centrifuge bottles and pelleted at 15,000xg for 15 minutes at 4°C. The supernatant was removed and kept on ice. The pellets were resuspended in serum-free medium (1.5 mL per flask) and lysed by three cycles of freezing and thawing (liquid nitrogen/37°C). Debris was pelleted at 15,000xg at 4°C for 15 minutes and this supernatant was pooled with the former.

Purification of labeled virus. Virus was pelleted by underlaying the pooled supernatants with 2 mL of 10% sucrose in R buffer (10 mM Tris-HCl, pH 7.5, 0.2 M NaCl, and 50 mM $MgCl_2$) and centrifuging for 4 hours and 30 minutes at 110,000xg at 4°C. Virus was resuspended in 125-250 µL of R buffer and subjected to velocity gradient centrifugation on a 10 mL linear 10-40% sucrose gradient for 2.5 hours at 154,000xg and 4°C. 400 µL fractions were collected, and the radioactivity in 2 µL samples was

determined in 4 mL of ScintiSafe™ Econo 1 cocktail (Fisher Scientific, Nepean, ON) using a RackBeta 'Excel' liquid scintillation counter (LKB Wallac, Turku, Finland). The three fractions containing the most radioactivity were pooled and stored at -80°C.

d) Infections

Adherent cells growing in 12-well culture dishes were counted and infected with virus (EV70, CVB3, or HRV14) at a MOI of 5 in a final volume of 300 µL of MEM for 1 hr at 33°C (EV70, HRV14) or 37°C (CVB3). Cells were washed twice with 2 mL of medium to remove unbound virus and then incubated at 33°C or 37°C in 0.5 mL of MEM supplemented with 10% FBS. Duplicate samples were frozen at -80°C, immediately following infection and washing, and at various times after infection.

For suspension cells, 10^6 cells were infected with EV70 for 1 hr at a MOI of 5 in a final volume of 100 µL of RPMI and then washed 3 times with 2 mL of RPMI. Cells were resuspended in 2 mL of RPMI supplemented with 10% FBS and duplicate 200 µL aliquots were withdrawn immediately, and at various times after infection, and frozen at -80°C.

The amount of infectious virus in each sample was determined by plaque assay on LLC-MK_2 cells (EV70 and CVB3) or HeLa cells (HRV14), as described below.

e) Plaque assays

Culture medium was removed from monolayers of LLC-MK_2 or HeLa cells grown to confluency in 12-well culture dishes and cells were infected with 0.3 ml of the various virus dilutions. Following incubation at 33°C (EV70 and HRV14) or 37°C (CVB3) for 1 hr, 1.5 mL of agarose overlay (1.5 % (w/v) sterile agarose (Bio-Rad) in freshly prepared antibiotic-free culture medium) was added. Cells were then further

incubated for 3-4 days at the appropriate temperature prior to staining. This involved fixing the monolayers with 2 ml of formol/saline (3.5-4.0% formaldehyde and 0.8% (w/v) NaCl) solution in each well, incubating at 37°C for at least half an hour, washing off the agarose with water and bathing the cells in 0.2 ml of 0.1% (w/v) crystal violet for 5 to 10 minutes at room temperature. The stain was washed off with water, plaques were counted and titers were calculated and expressed in plaque forming units per mL (PFU/mL).

f) Virus binding assays

Suspension cells ($1.5x10^6$) were pelleted at 800xg for 5-7 minutes, washed once with 3 mL of TBS, resuspended in 150 µL of OPTIMEM, and split into triplicate samples to a final concentration of 5×10^5 cells in 50 µL of OPTIMEM (Life Technologies) containing 3-6 X 10^3 cpm of radiolabelled EV70. Following a 1-hour incubation at 33°C, 250 µL of OPTIMEM were added to each sample, cells were pelleted, and the supernatant was transferred to scintillation vials. Cells were gently washed with 300 µL of OPTIMEM, pelleted again, and the second supernatant was pooled with the first to form the unbound fraction. The pellet (constituting the bound fraction) was resuspended in 600 µL of OPTIMEM and transferred to scintillation vials. 6 mL of scintillation cocktail (ScintiSafe Econo 1) was added to each vial and samples were counted as described earlier.

Adherent cells were grown to over 80% confluency in 12-well culture dishes, medium was removed, and each well was incubated in 300 µL OPTIMEM containing 3-6 X 10^3 cpm of radiolabelled virus for one hour at 33°C (EV70) or 37°C (CVB3). The inoculum was removed to a scintillation vial and replaced with 300 µL of OPTIMEM to

wash the cells and remove any remaining unbound virus, which was pooled with the previous fraction. The cell monolayers (which contain the bound fraction) were then disrupted with 300 µL of trypsin-EDTA for 3-5 minutes at 37° and removed to another vial. The wells were washed with another 300 µL of trypsin-EDTA which was pooled with the previous bound fraction. 6 mL of scintillation cocktail (ScintiSafe Econo 1) was added to each vial and samples were counted as described earlier.

When used as a control for the suspension cells, adherent HeLa T4 cells were disrupted with 500 µL of trypsin-EDTA for 3-5 minutes at 37°C and then treated like the suspension cells.

g) Virus binding inhibition assays

$3x10^6$ cells were pelleted and washed once with 4 mL of PBS. Half of the cells were then resuspended in 150 µL of OPTIMEM containing 11 or 110 µg/mL of the monoclonal antibody EVR1 or 6 µg/mL of the monoclonal antibody 1H4, and the other half in 150 µL of OPTIMEM only, as a control. Samples were then incubated for 1 hour at 37°C, washed with 4 mL of PBS and virus binding was assessed as described above.

IV. Enzymatic treatment

a) Phosphatidylinositol-specific phospholipase C (PI-PLC)

Suspension cells ($5x10^6$) were pelleted and washed 3 times with PBS. Cells were split into two samples and were resuspended in 50 µL of PI-PLC buffer (RPMI containing 0.2% (w/v) BSA, 50 µM 2-mercaptoethanol, 10 mM HEPES, and 0.1% (w/v) sodium azide). *Bacillus cereus* PI-PLC (Sigma, Oakville, ON) was added to one of the

samples to a final concentration of 6 units/mL. All cells were incubated at 37°C for 90 minutes and then washed twice with 4 mL of PBS. Approximately $8x10^5$ cells from each sample were removed and assayed by flow cytometry as described earlier. Virus binding was assessed on the remaining cells as described above.

Adherent cells were grown to confluency in 12-well culture dishes, washed twice with 1 mL of OPTIMEM and incubated in 300 µL of PI-PLC buffer containing *Bacillus cereus* PI-PLC in a final concentration of 0.3 units/mL, for 2 hours at 37°C. Control wells were treated similarly but without enzyme. All wells were then washed twice with 1 mL of OPTIMEM, and virus binding was assessed as described above.

b) Neuraminidase

Cells ($1.5x10^6$) were pelleted and resuspended in 150 µL OPTIMEM. *Vibrio cholerae* neuraminidase (Roche) was added to test samples to a final concentration of 25 mU/mL. All cells were incubated at 37°C for 30 minutes, washed twice with 4 mL of PBS, and virus binding was assessed as described above.

RESULTS

I. Non-primate DAF homologues are not receptors for EV70

The first objective of this thesis was to determine whether DAF from non-primate mammals was functioning as a receptor for enterovirus 70, thereby explaining its wide host range *in vitro*. Yoshii *et al.* (1977) reported that cell lines of mouse and pig origin were susceptible and permissive to infection by EV70. hDAF has been identified as a receptor for EV70 on HeLa cells (Karnauchow *et al.*, 1996), and homologues of DAF were shown to be expressed on several mouse, rat, and pig tissues (Hinchliffe *et al.*, 1998; Kameyoshi *et al.*, 1989; Pérez de la Lastra *et al.*, 2000). It was therefore hypothesized that the various DAF homologs were functioning as receptors for EV70.

In order to test this hypothesis, receptor-negative murine embryonic fibroblast cells (NIH 3T3) which do not express any mouse DAF (data not shown) were transfected with the plasmid pDR2EF1α encoding the GPI form of each of the non-primate DAF homologues as well as human DAF. Cell populations expressing the desired surface protein were selected using the antibiotic hygromicin B and were gown from a single clone. Cells transfected with empty plasmid DNA were named F1 and used as negative control cells. Expression of the homologues was verified by flow cytometry using mAbs specific for each of the DAF molecules. These antibodies and stably transfected cells were obtained from Dr. B. Spiller. The level of expression of each DAF molecule is depicted in figure 3, with the mean fluorescence intensities summarized in table 4. These

Figure 3. Expression of mammalian DAF homologues on stably transfected NIH/3T3 cells. Cells were washed with PBS-azide and incubated with the corresponding primary mAb for 15 minutes at room temperature. Cells were washed a second time and then incubated with FITC-conjugated secondary antibody for 15 minutes at room temperature. Mean fluorescence intensities (**purple histogram**) are compared to background levels of fluorescence due to non-specific binding of the secondary antibody (**blue histogram**) or autofluorescence (**red histogram**).

A. Human DAF detected with mouse anti-human DAF mAb MBC1 (1.0 μg/mL)
B. Pig DAF detected with mouse anti-pig DAF mAb MBC-PD1 (concentration unknown, dilution recommended by Dr .B. Spiller)
C. Mouse DAF detected with rat anti-mouse DAF mAb IC6 (1.0 μg/mL)


A


B

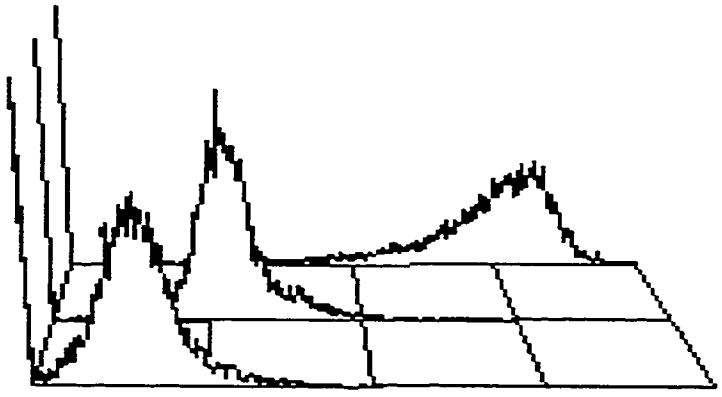

C

Table 4. Expression of hDAF, mDAF, rDAF, and pDAF on the surface of transfected NIH/3T3 cells.[a]

	Antibodies				
Cell line	**2Y[b]**	**MBC1[c]**	**2C6[c]**	**RDIII7[c]**	**MBC-PD1[c]**
F1	1.01[d]/1.44[d]	1.11	1.68	0.659	1.26
3T3/hDAF	1.08	6.53	--	--	--
3T3/mDAF	1.50	--	85.9	--	--
3T3/rDAF	0.743[e]	--	--	4.08	--
3T3/pDAF	1.47	--	--	--	13.4

[a] data expressed as mean fluorescence intensities (MFI)
[b] MFI with anti-mouse (or rat) FITC-conjugated Ig only
[c] antibodies MBC1, 2C6, RDIII7 and MBC-PD1 are specific for hDAF, mDAF, rDAF, and pDAF, respectively
[d] MFI with anti-mouse/anti-rat FITC-conjugated Ig
[e] flow cytometry on 3T3/rDAF cells was done on a different day, hence the lower value for the secondary control

data show that significant amounts of hDAF, mDAF, and pDAF were expressed on the surface of the selected transfectants.

The various DAF-expressing cells were then inoculated with EV70 and virus replication was assessed by plaque assay. As shown in figure 4, only human DAF transfectants were capable of supporting EV70 replication, producing titres approaching three orders of magnitude higher than background, and similar to what was observed for HeLa T4 cells, which express large amounts of DAF (see figure 12 later). Whereas virus production in HeLa T4 cells seemed to have reached a peak before 20 hours post infection, for 3T3/hDAF cells it did not plateau until after 30 hours. Cells expressing either pig, mouse, or rat DAF showed no virus production above background levels.

Although EV70 does not seem to replicate in the latter cells, it is possible that virus is binding to the cells but is unable to initiate a productive infection. Consequently, the ability of the DAF-expressing cells to bind radiolabelled EV70 was tested. As shown in figure 5, cells expressing non-human DAF did not bind virus above background binding to control cells (F1). 3T3 cells expressing human DAF, on the other hand, bound almost as much virus as HeLa T4 cells, the positive control.

Taken together, these results indicate that as opposed to human DAF, the mouse, rat, and pig homologues of DAF expressed on NIH 3T3 cells neither bind EV70 nor render these cells susceptible to infection, suggesting that they do not function as receptors for EV70. Similar observations have been reported by Spiller *et al.* (2000) who demonstrated that echoviruses 7, 11, and 12 as well as coxsackieviruses B1, B3, and B5 bind human but not mouse or rat DAF. Furthermore, mouse L-929 cells were found to support replication of EV70 (figure 6) even though no DAF was detected on their surface

Figure 4. EV70 replication in NIH 3T3 cells stably expressing mammalian DAF homologues. Negative control cells (F1) were transfected with the empty pEF6 vector. HeLa T4 cells were used as a positive control. Cells were grown to confluency in 12-well tissue culture dishes, counted prior to infection and infected with EV70 at a MOI of 5 for 1 hour at 33°C. Cells were then washed twice with culture medium and left incubating for the different time periods indicated before being subjected to two cycles of freezing and thawing to release virus. Virus production at each time point was determined by plaque assay, and is expressed relative to titres observed for the negative control cells. Results for F1 cells are expressed relative to virus present in these cells at time 0. The data are presented as means + standard deviation for two to four independent experiments. For each experiment, plaque assays were performed in duplicate for each sample.

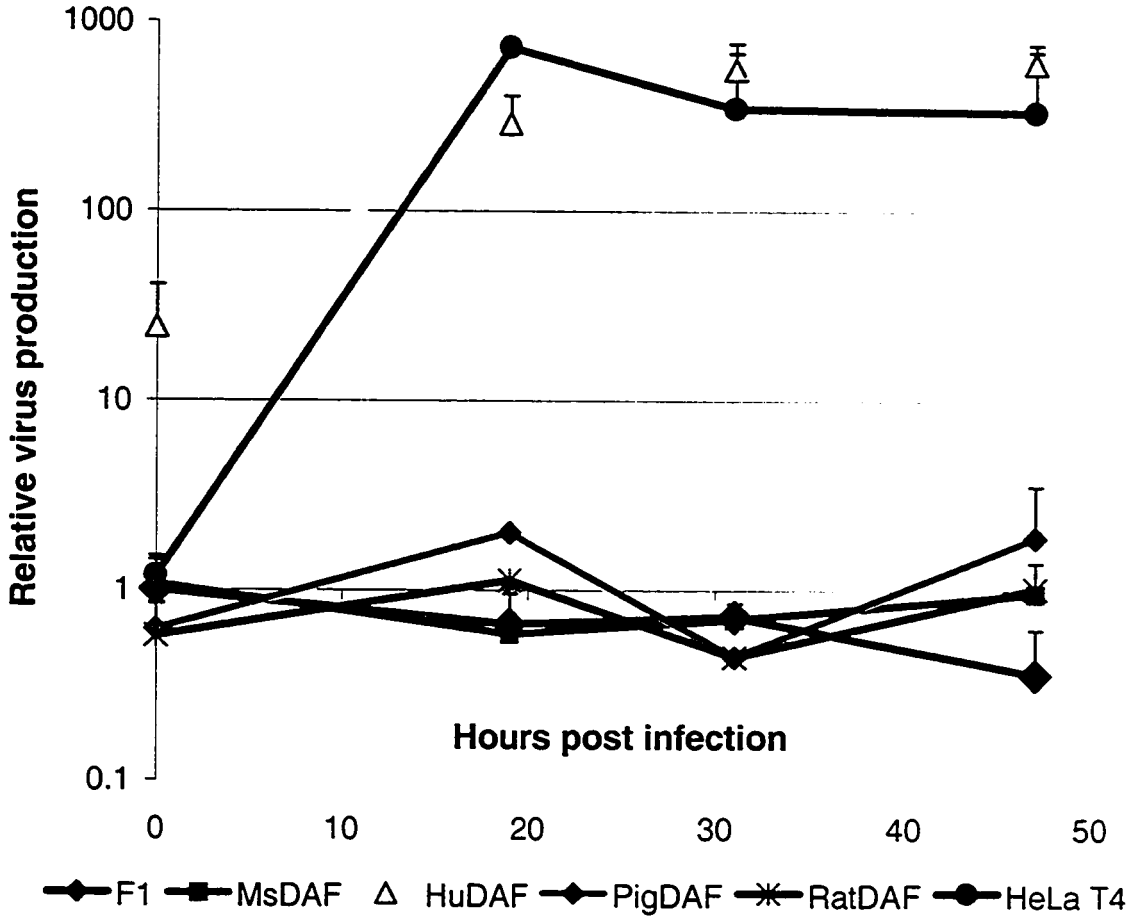

1000
100
10
1
0.1
Relative virus production
Hours post infection
0
10
20
30
40
50
F1
MsDAF
HuDAF
PigDAF
RatDAF
HeLa T4

Figure 5. EV70 binding to NIH 3T3 cells expressing mammalian DAF homolgues. Negative control cells (F1) were transfected with the empty pEF6 vector. HeLa T4 cells were used as a positive control. Cells grown to confluency in 12-well tissue culture dishes were incubated in serum-free medium containing 3-6x10^3 cpm of radiolabelled virus for 1 hour at 33°C. Bound and unbound fractions of EV70 were separated and their proportions were determined by scintillation counting. The results are presented as mean percentages of input virus bound ± standard deviation for two to four independent experiments, each performed in triplicate.

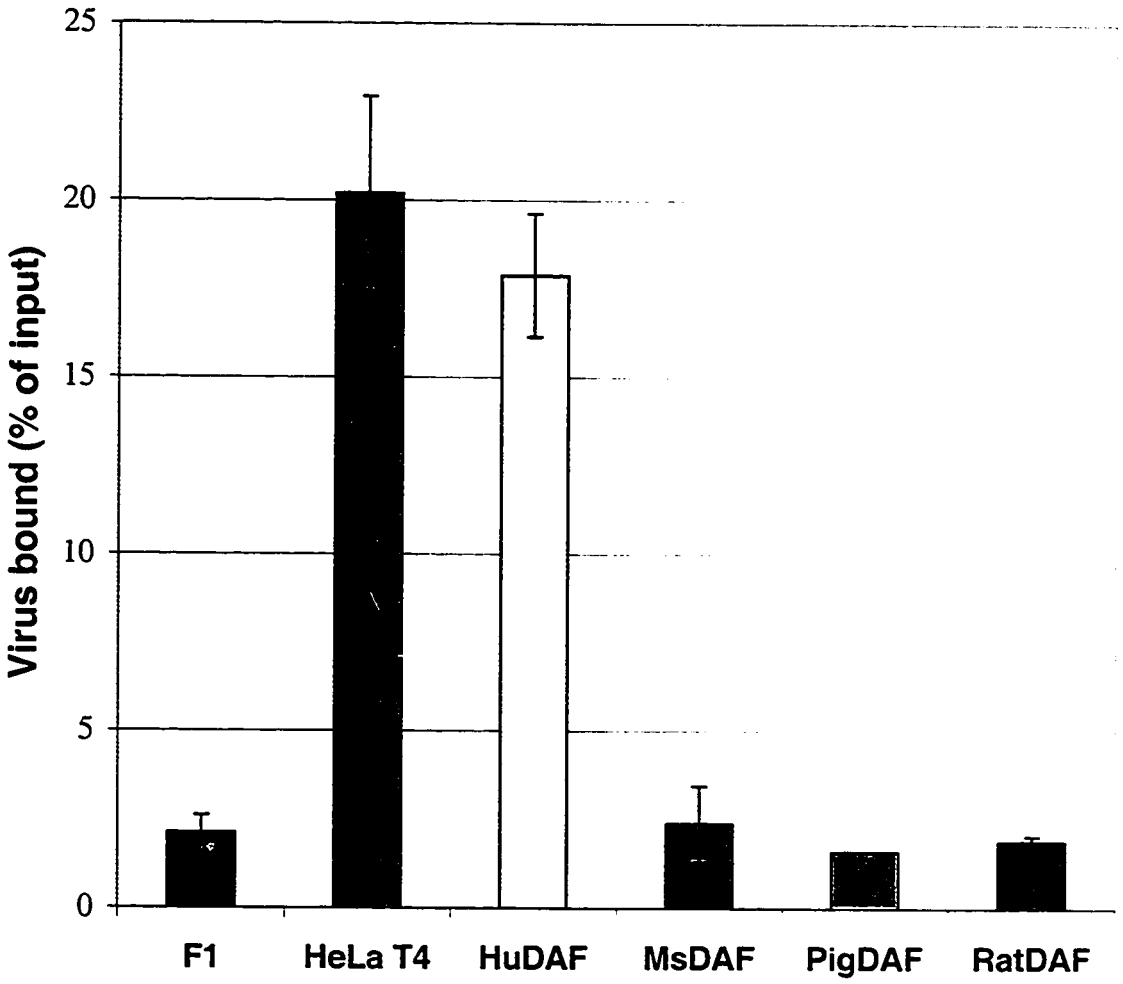
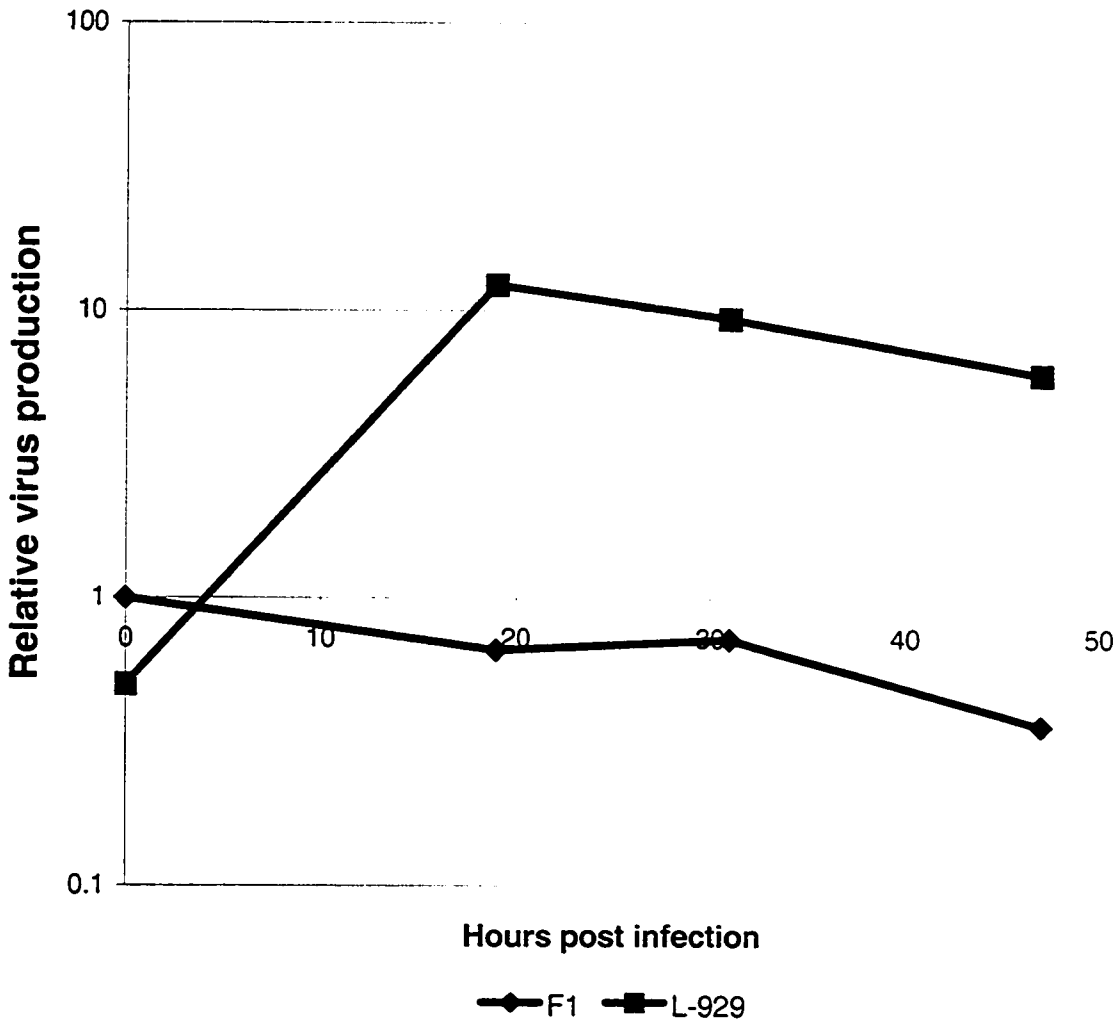

25
20
15
10
5
0
Virus bound (% of input)
F1
HeLa T4
HuDAF
MsDAF
PigDAF
RatDAF

Figure 6. EV70 replication in mouse L-929 cells. Negative control cells (F1) were transfected with the empty pEF6 vector. Cells were grown to confluency in 12-well tissue culture dishes, counted prior to infection and infected at a MOI of 5 for 1 hour at 33°C. Cells were then washed twice with culture medium and incubated for the different time periods indicated before being subjected to two cycles of freezing and thawing to release virus. Virus production at each time point was determined by plaque assay, and is expressed relative to titres observed for the negative control cells. Results for F1 cells are expressed relative to virus production at time 0. The data presented derive from one experiment performed in duplicate.


100
10
1
0.1
Relative virus production
0
10
20
30
40
50
Hours post infection
F1
L-929

(figure 7). Virus replication in L-929 cells was not as extensive as in HeLa T4 cells or NIH 3T3 cells expressing human DAF (figure 4), but seemed to correlate with the degree of binding (figure 8). This suggests that EV70 utilizes a receptor other than DAF on mouse and presumably other non-human cells.

II. EV70 utilizes a receptor other than DAF on human leukocyte cell lines

The next objective was to confirm that DAF was functioning as a receptor not only on HeLa cells but also on other human cell lines. Previous experiments in our laboratory had shown that the following leukocyte cell lines could be infected by EV70 (appendix I): Jurkat (T-cells), Daudi and Raji (B-cells), THP-1 (monocytes), U-937 (hystiocytes), and HL-60 (promyelocytes). In order to assess the functional role of DAF in this process, binding inhibition assays using mAb EVR1 were perfomed. This mAb is specific to CCP1 of DAF and has been shown to block EV70 binding to HeLa cells (Karnauchow *et al.*, 1996). Figure 9 shows that incubation of HeLa T4 cells with 110 μg/mL of EVR1 inhibited virus binding by 43%. In contrast, no significant change in the virus binding to U-937, Daudi, Jurkat, or THP-1 cells was observed. However, there was a concern that the high antibody concentration used in these experiments may have resulted in a decreased avidity of the antibody for DAF. Hence, saturation analysis (figure 10) for EVR1 binding to Jurkat and U-937 cells was therefore carried out. Cells were incubated with serial dilutions of EVR1 and antibody binding was assessed by flow cytometry. This analysis indicated that for both U-937 and Jurkat cells the optimal

Figure 7. Expression of mouse DAF on L-929 cells. Cells were washed with PBS-azide and incubated with the mDAF-specific mAb IC6 (1.0 μg/mL) for 15 minutes at room temperature. Cells were washed a second time and then incubated with FITC-conjugated secondary antibody for 15 minutes at room temperature. Mean fluorescence intensities (**purple histogram**) are compared to background levels of fluorescence due to non-specific binding of the secondary antibody (**blue histogram**) or autofluorescence (**red histogram**).

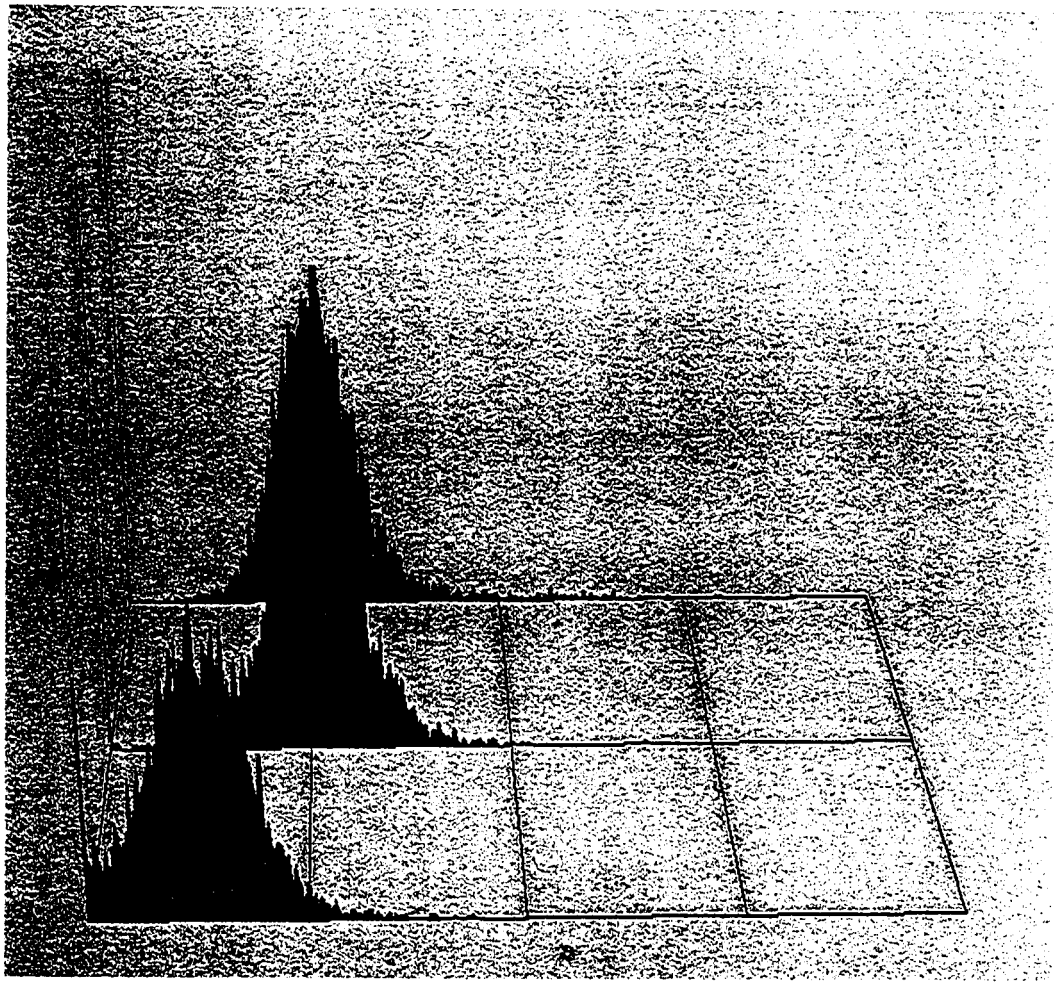


Figure 8. EV70 binding to mouse L-929 cells. Negative control cells (F1) are NIH 3T3 cells transfected with empty vector DNA. Cells grown to confluency in 12-well tissue culture dishes were incubated in serum-free medium containing 3-6x10^3 cpm of radiolabelled virus for 1 hour at 33°C. Bound and unbound fractions of EV70 were separated and their proportions were determined by scintillation counting. The results are presented as mean percentages of input virus bound ± standard deviation for one experiment performed in duplicate.

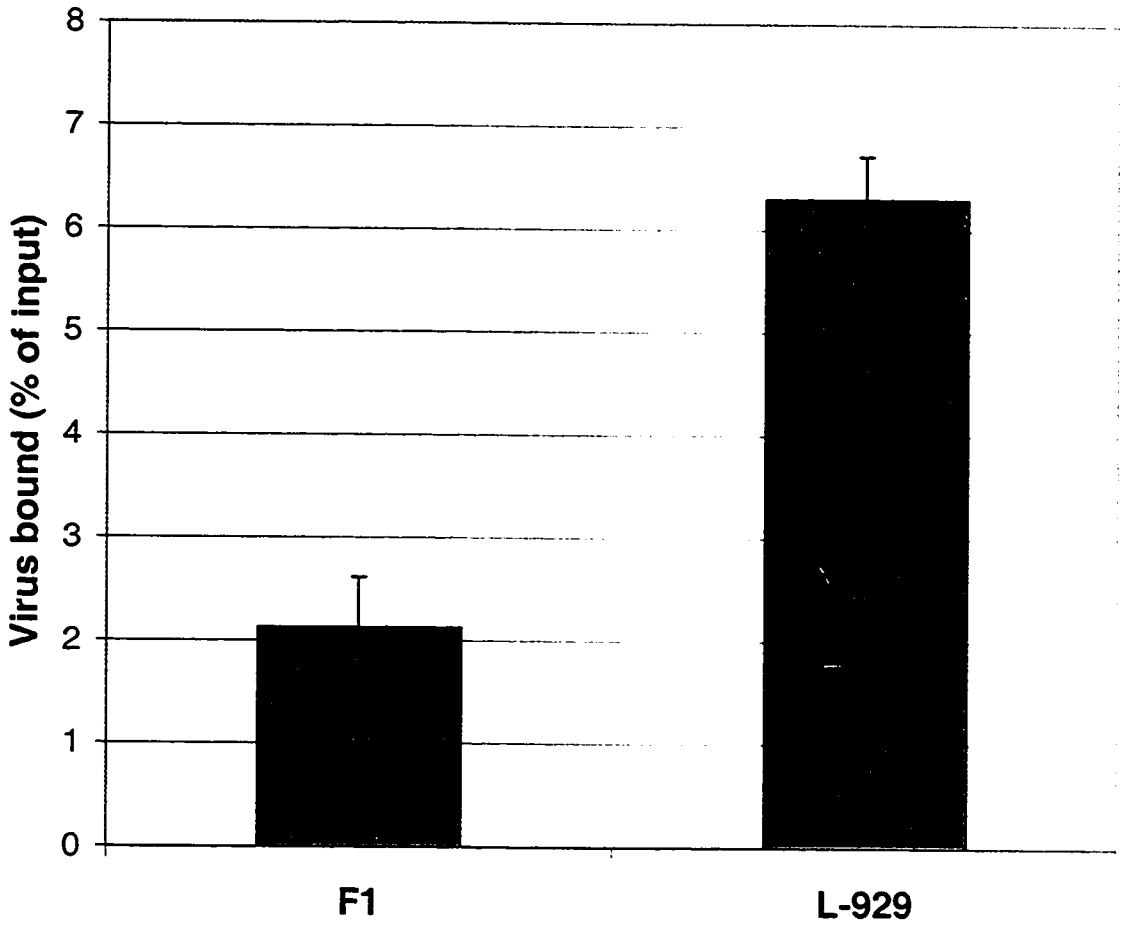

Virus bound (% of input)
8
7
6
5
4
3
2
1
0
F1
L-929

Figure 9. Inhibition of EV70 binding by the hDAF-specific mAb EVR1. Cells were pelleted and incubated in serum-free medium containing or lacking 110 μg/mL of EVR1 for 1 hour at 37°C. Cells were then washed once and incubated in serum-free medium containing 3-6x10^3 cpm of radiolabelled virus for 1 hour at 33°C. Bound and unbound fractions of EV70 were separated and their proportions were determined by scintillation counting. The results are presented as the amount of virus bound as compared to virus bound in the absence of antibody, ± standard deviation for one (HeLa T4) to three independent experiments, each performed in triplicate.

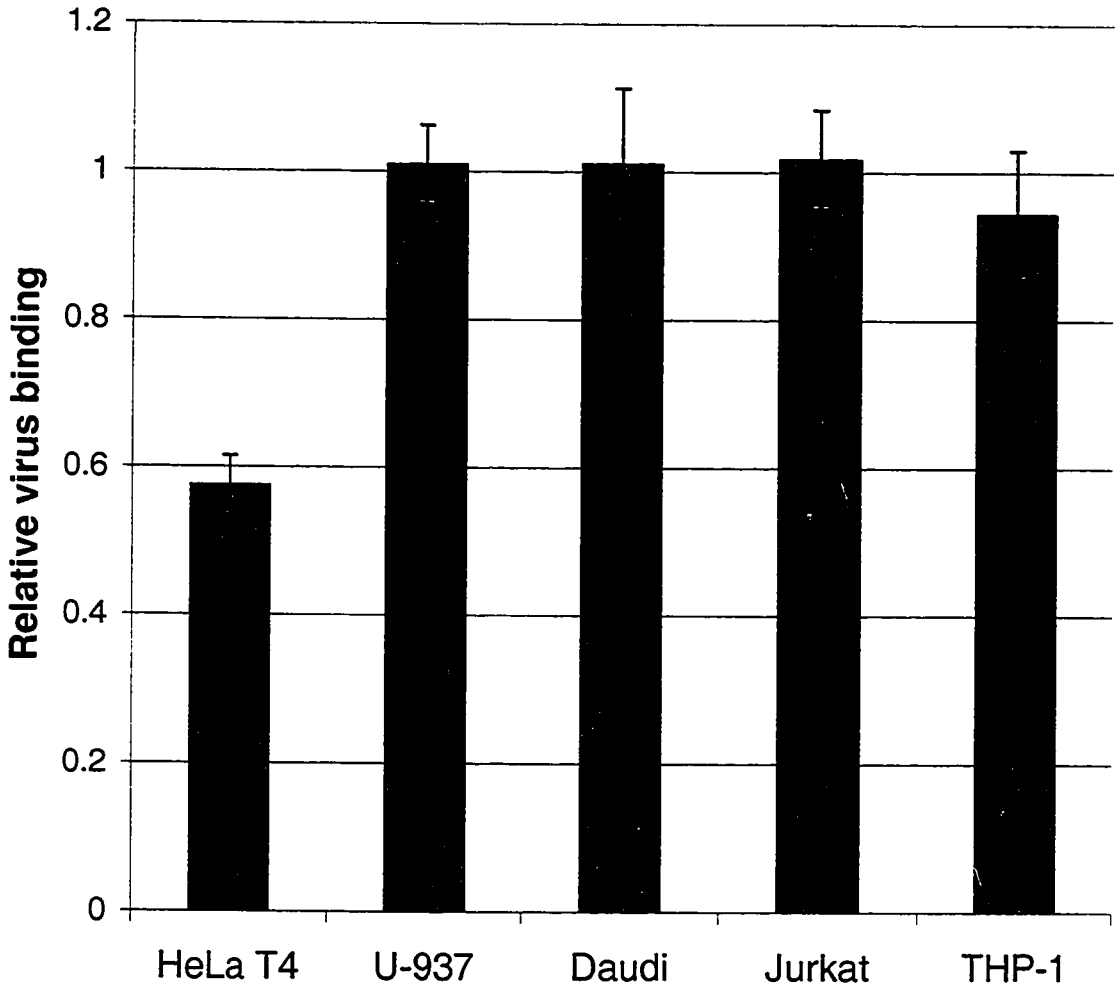
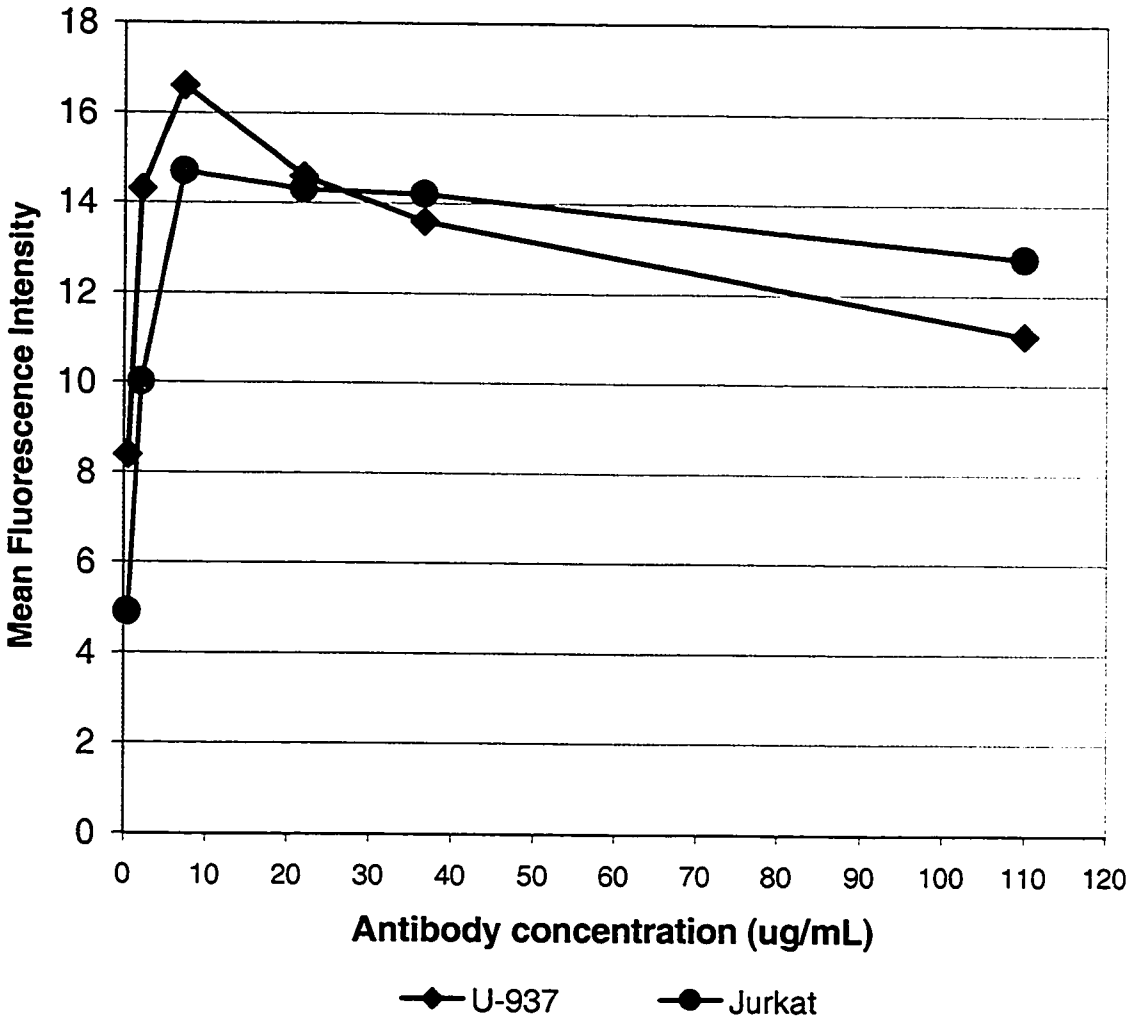

Relative virus binding
1.2
1
0.8
0.6
0.4
0.2
0
HeLa T4
U-937
Daudi
Jurkat
THP-1

Figure 10. EVR1 saturation curve on U-937 and Jurkat cells. Cells were pelleted and incubated in serum-free medium containing different dilutions of the EVR1 mAb for 1 hour at 37°C. Cells were then washed once and incubated in PBS-azide containing FITC-conjugated secondary antibody for 15 minutes at room temperature. The relative amount of EVR1 binding was assayed by flow cytometry. Results shown are presented as mean fluorescence intensities obtained with the different concentrations of primary antibody.


18
16
14
12
10
8
6
4
2
0
Mean Fluorescence Intensity
0
10
20
30
40
50
60
70
80
90
100
110
120
Antibody concentration (ug/mL)
U-937
Jurkat

concentration of EVR1 lay between 7 and 22 μg/mL. The binding inhibition experiments were repeated using a concentration of 11 μg/mL and yielded results (figure 11) that were similar to what was observed at the higher concentration of EVR1. EVR1 at concentrations less than 11 μg/mL were previously shown to inhibit binding of EV70 to HeLa cells (K. Dimock, personal communication). With Raji cells, binding inhibition was inconsistent. In four experiments performed with these cells, two resulted in variable degrees of binding inhibition (17-43%) whereas two others indicated a complete lack of inhibition. HL-60 cells did not express any DAF detectable by flow cytometry (figure 14) and thus were not included in this analysis.

Although virus binding to HeLa T4 cells is clearly inhibited by EVR1, these experiments suggest that EV70 is not binding to DAF on the leukocyte cell lines. However, it is conceivable that a population of DAF molecules on the leukocytes is masked from this mAb and is binding virus. Hence, phosphatidylinositol-specific phopholipase C (PI-PLC) digestion was used as an alternative to evaluating the role of DAF in EV70 binding to the leukocyte cell lines. PI-PLC is an enzyme which releases surface proteins anchored by a glycosyl-phosphatidylinositol moiety by cleaving at the base of the anchor (see figure 2), between the phosphate moiety and the glycerol which is attached to the fatty acids embedded in the plasma membrane (Roberts *et al.*, 1988, Udenfriend and Kodukula, 1995).

All cells were treated with PI-PLC prior to virus binding, and the extent of DAF cleavage was monitored by flow cytometry using mAb 8D11, as depicted in figure 12. These results are summarized in Table 5 which indicates that a significant proportion of DAF was removed by this treatment from the surface of all cell lines. The efficiency of

Figure 11. Inhibition of EV70 binding by the hDAF-specific mAb EVR1. Cells were pelleted and incubated in serum-free medium containing 11 μg/mL of EVR1 for 1 hour at 37°C. Cells were then washed once and incubated in serum-free medium containing 3-6x10^3 cpm of radiolabelled virus for 1 hour at 33°C. Bound and unbound fractions of EV70 were separated and their proportions were determined by scintillation counting. The results are presented as the amount of virus bound as compared to virus bound in the absence of antibody, ± standard deviation for one experiment performed in triplicate.

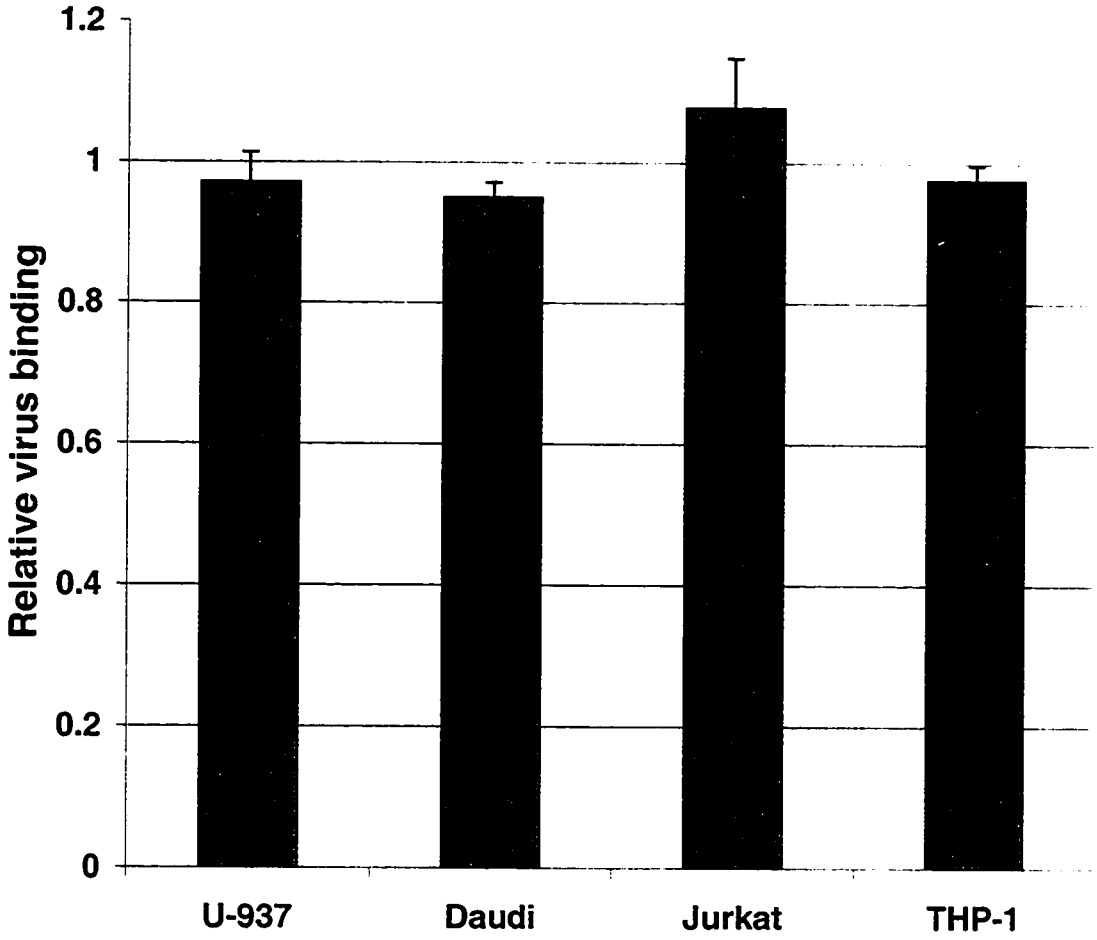
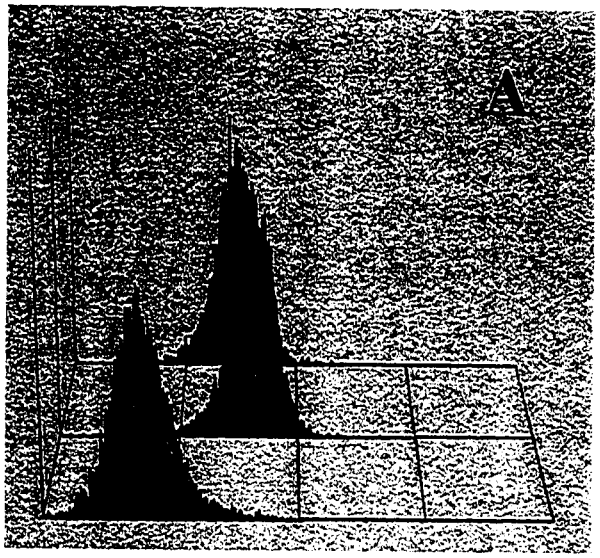
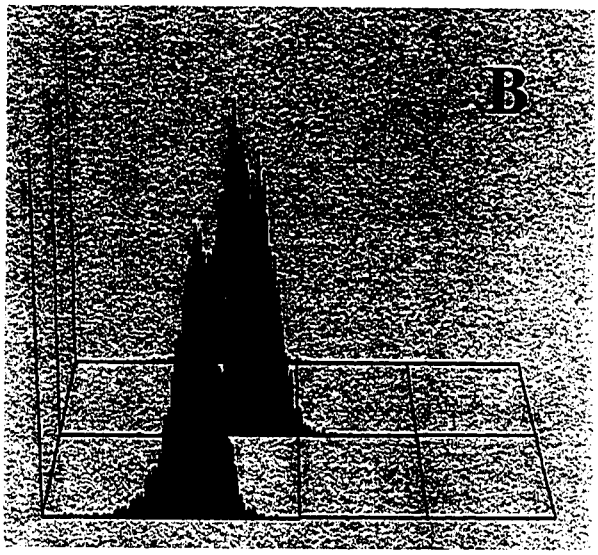
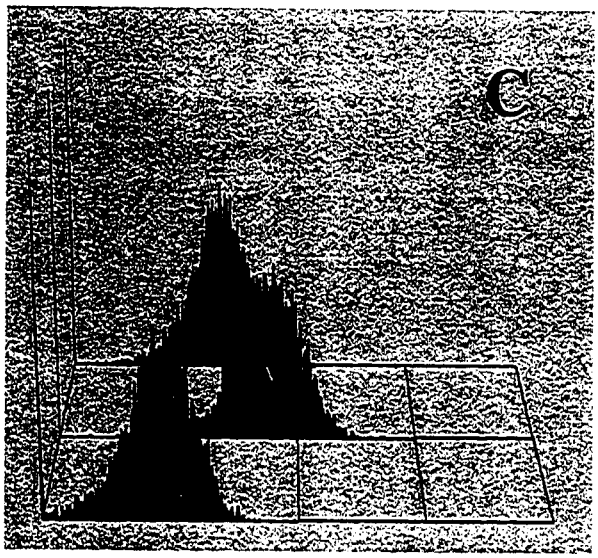

1.2
1
0.8
0.6
0.4
0.2
0
Relative virus binding
U-937
Daudi
Jurkat
THP-1

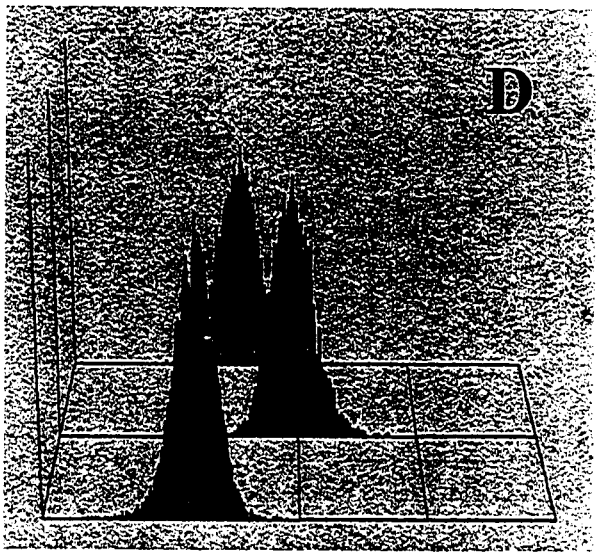
Figure 12. PIPL-C treatment removes DAF from the surface of cultured cells. Cells were washed three times and then incubated in buffer in the presence or absence of PIPL-C (6 units/mL) for 90 minutes at 37°C. After two additional washes, cells were incubated in DAF-specific mAb 8D11 for 15 minutes at room temperature, washed, and incubated with FITC-conjugated secondary antibody for another 15 minutes at room temperature. Mean fluorescence intensities of treated (**purple histogram**) and untreated samples (**blue histogram**) are compared with background levels due to non-specific binding of the secondary antibody (**red histogram**).

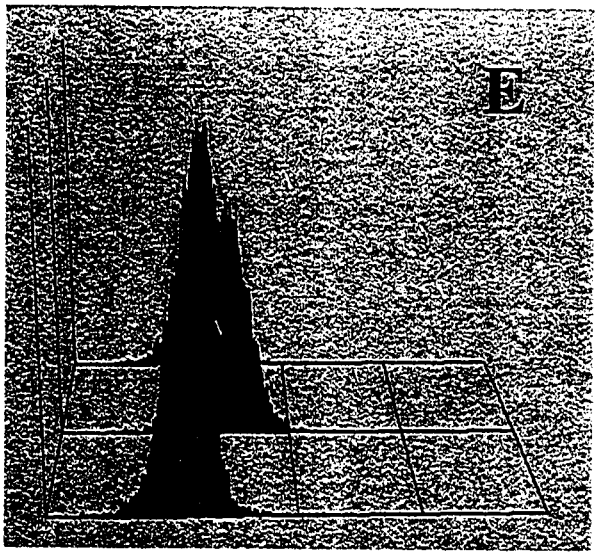
A: HeLa T4
B: Jurkat
C: U-937
D: Daudi
E: THP-1
F: Raji


A


B


C


D


E

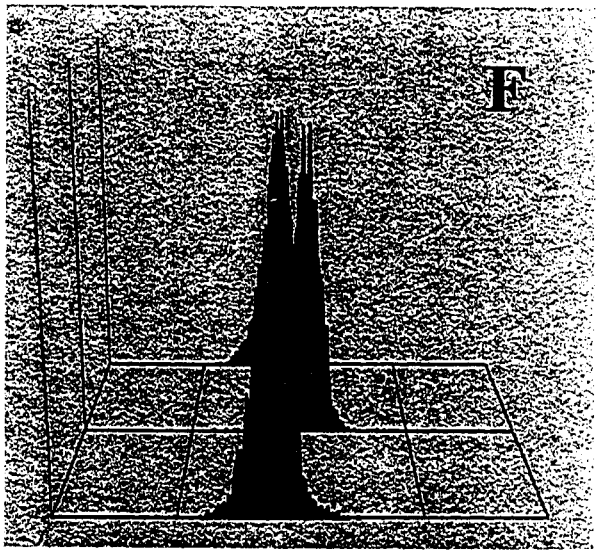

F

Table 5. Loss of DAF from cells following treatment with PI-PLC[a]

	DAF removal (%)	Average ± S.D.[b]
HeLa T4	37-55	47.6 ± 6.8
U-937	60-88	75.8 ± 8.4
Jurkat	60-69	64.3 ± 3.7
THP-1	65-76	72.0 ± 5.0
Raji	86-93	90.1 ± 3.3
Daudi	65-68	66.7 ± 1.2
A.201	92	-

[a] determined by flow cytometry using the mAb 8D11 (2.0 μg/mL)
[b] standard deviation; based on three to five independent experiments

the PI-PLC digestion was dependent on the cell type, with the loss of surface DAF varying between 37 and 93%. Some correlation can be made between this percentage and the amount of DAF expressed on the cells. For instance, PI-PLC treatment of Raji cells, which express the least amount of DAF, consistently released most of the DAF molecules whereas for HeLa T4 cells, which express the highest amount of DAF, 37 to 55% of DAF was removed. Other factors, such as steric hindrance, must also play a role since U-937 cells which express almost as much DAF as HeLa T4 cells lost 75.8±8.4% of this protein following PI-PLC treatment.

The ability of each cell line to bind radiolabelled EV70 following PI-PLC treatment is shown in figure 13. The removal of most of the surface DAF molecules from Daudi, Jurkat, THP-1, or U-937 cells did not result in any significant change in EV70 binding levels. Conversely, binding to HeLa T4 cells was very sensitive to PI-PLC, decreasing by approximately 54%. In fact, extending the enzymatic cleavage to 4 hours resulted in almost complete abrogation of binding to HeLa T4 cells (a 97% decrease, figure 15) correlating with a 90% loss of DAF (data not shown). An extended incubation of U-937 cells with PI-PLC, however, resulted in a small but not significant decrease in EV70 binding (figure 15).

These observations confirm the results obtained from the antibody inhibition experiments and are consistent with the idea that DAF is not required for EV70 binding to the human leukocyte cell lines. This interpretation is supported by results obtained with two other human leukocyte cell lines. A.201 T-cells do not bind EV70 (figure 13), even though low to moderate levels of DAF are expressed on their surface (figure 14). HL-60 cells, on the other hand, do not express any detectable DAF (figure 14) but are

Figure 13. PI-PLC treatment has little effect on EV70 binding to susceptible leukocyte cell lines. Cells were washed three times and then incubated in buffer in the presence or absence of PI-PLC (6 units/mL) for 90 minutes at 37°C. Cells were washed again and incubated in serum-free medium containing 3-6x10^3 cpm of radiolabelled virus for 1 hour at 33°C. Bound and unbound fractions of EV70 were separated and their proportions were determined by scintillation counting. The results are presented as mean percentages of input virus bound ± standard deviation for two or three independent experiments, each performed in triplicate.

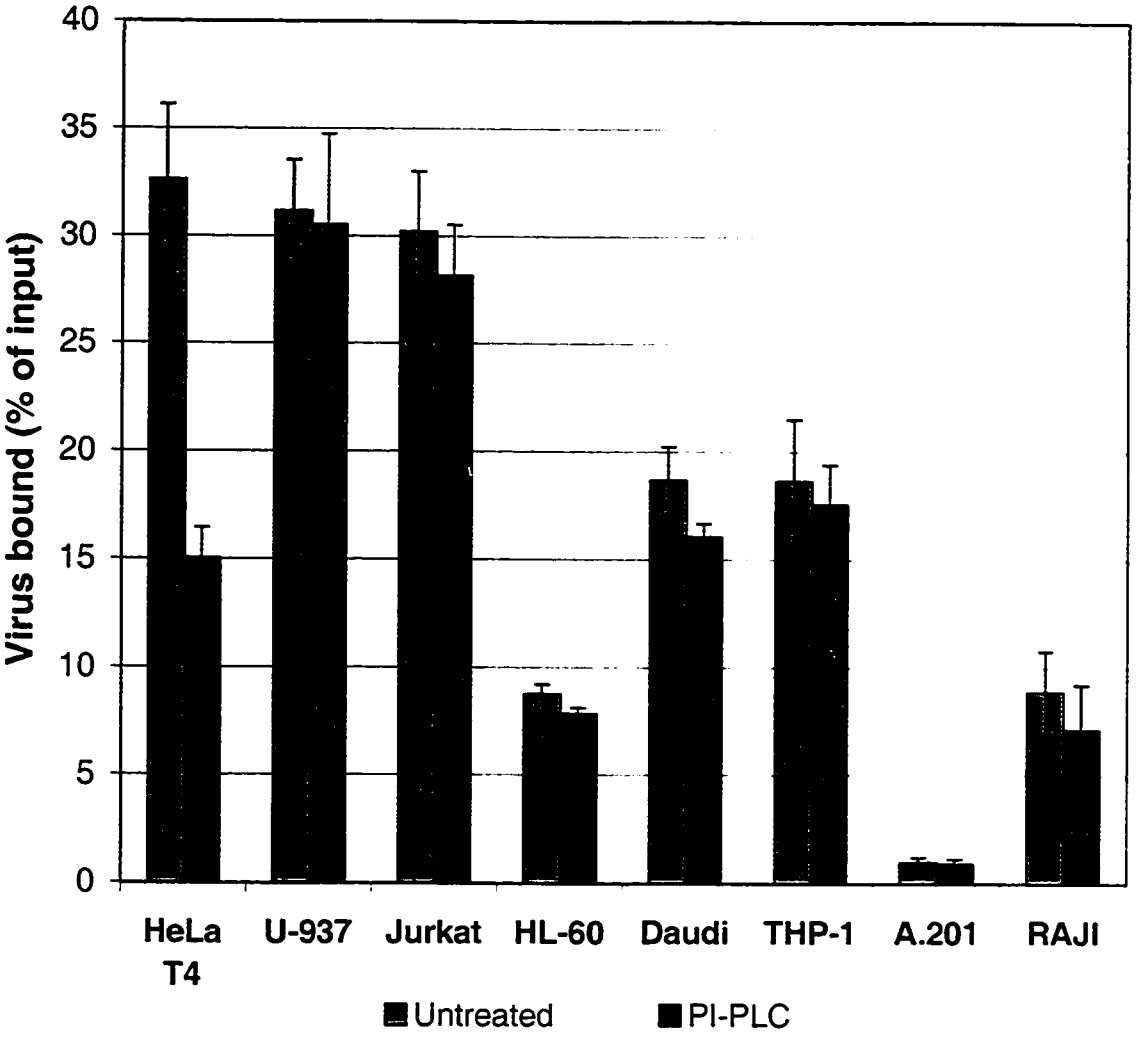
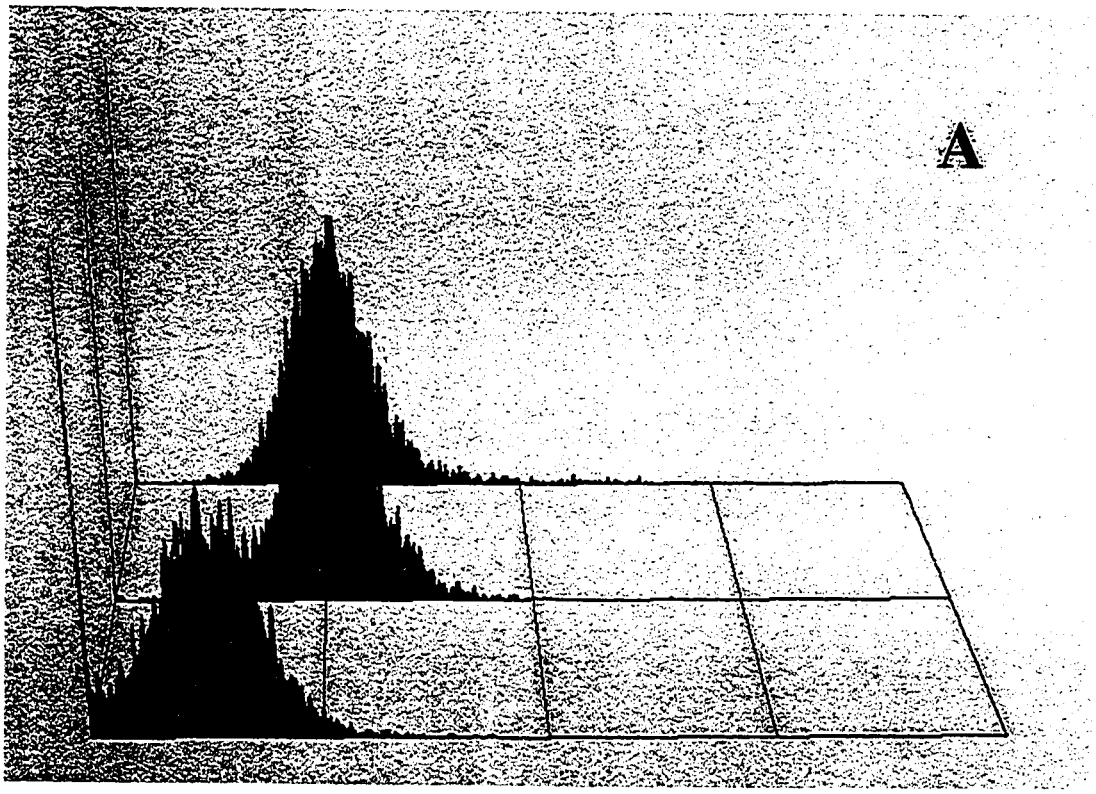

40
35
30
25
20
15
10
5
0
Virus bound (% of input)
HeLa T4
U-937
Jurkat
HL-60
Daudi
THP-1
A.201
RAJI
Untreated
PI-PLC

Figure 14. Expression of hDAF on HL-60 and A.201 cells. Cells were washed with PBS-azide and incubated with the DAF-specific mAb 8D11 (2.0 μg/mL) for 15 minutes at room temperature. Cells were washed a second time and then incubated with FITC-conjugated secondary antibody for 15 minutes at room temperature. Mean fluorescence intensities (**purple histogram**) are compared to background levels of fluorescence due to non-specific binding of the secondary antibody (**blue histogram**) or autofluorescence (**red histogram**).

A. HL-60 cells.
B. A.201 cells.


A

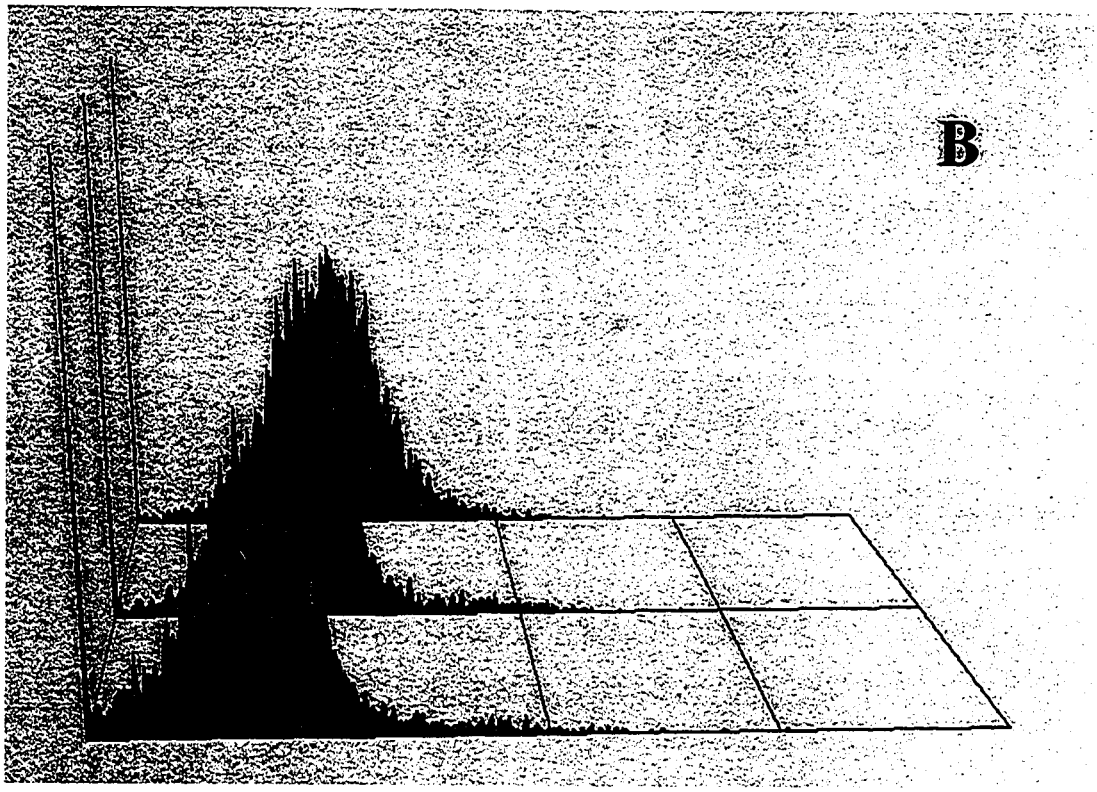
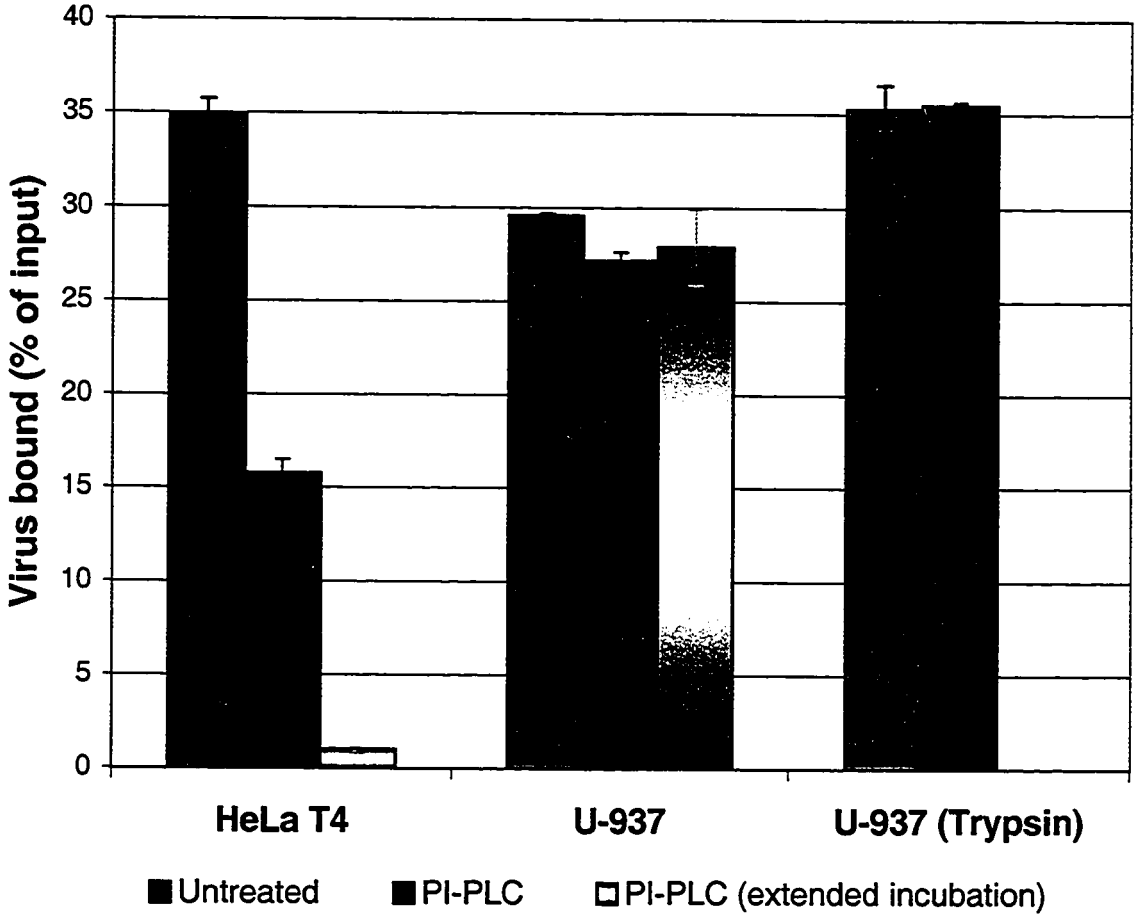

B

Figure 15. Effects of extended PI-PLC incubation on EV70 binding to HeLa T4 and U-937. Cells were washed three times and then incubated in buffer in the presence or absence of PI-PLC (6 units/mL) for 90 minutes at 37°C or for four hours for the extended incubations. Some samples were treated with trypsin-EDTA for 10 minutes at 37°C prior to the three washes. Cells were washed again and incubated in serum-free medium containing 3-6x10^3 cpm of radiolabelled virus for 1 hour at 33°C. Bound and unbound fractions of EV70 were separated and their proportions were determined by scintillation counting. The results are presented as mean percentages of input virus bound ± standard deviation for one experiment performed in triplicate.


40
35
30
25
20
15
10
5
0
Virus bound (% of input)
HeLa T4
U-937
U-937 (Trypsin)
Untreated
PI-PLC
PI-PLC (extended incubation)

capable of binding radiolabelled virus, even following PI-PLC treatment (figure 13), and are susceptible to infection by EV70 (appendix I).

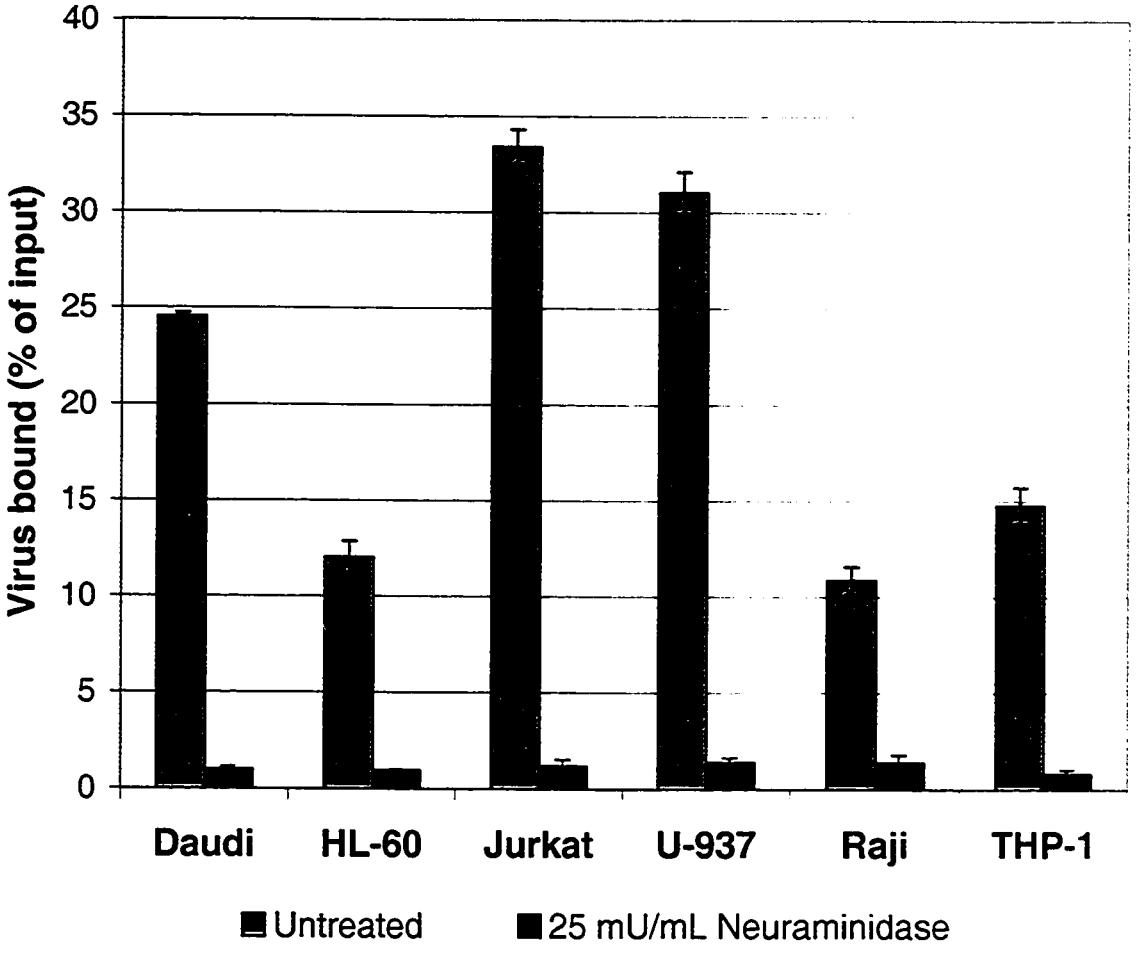
III. The EV70 receptor on human leukocytes is neuraminidase-sensitive

As a first step to characterizing the cell surface molecule responsible for EV70 binding to the leukocyte cell lines, these were treated with neuraminidase prior to virus binding. Utagawa *et al.* (1982) reported that the receptor for EV70 on erythrocytes is sensitive to neuraminidase treatment. As shown in figure 16, cleavage of sialic acid from the surface of all cell types tested almost completely abrogated binding of EV70, thereby suggesting that a sialylated moiety or receptor is required for binding of EV70 on leukocyte cell lines. An identical treatment of both the HeLa T4 and 3T3/hDAF cell lines also results in complete abrogation of binding (data not shown; D. Alexander, personal communication).

IV. HCAR and ICAM-1 are not receptors for EV70

The results described above indicated that at least one molecule other than DAF is involved in EV70 binding to susceptible human leukocyte cell lines. This observation prompted the search for another receptor(s). Two surface molecules that serve as receptors for several other human enteroviruses were considered as potential candidate for another EV70 receptor.

Figure 16. Neuraminidase treatment inhibits EV70 binding to human leukocytes. Cells were incubated in serum-free medium in the presence or absence of neuraminidase (25 mU/mL) for 30 minutes at 37°C. Cells were then washed twice and incubated in serum-free medium containing 3-6x10^3 cpm of radiolabelled virus for 1 hour at 33°C. Bound and unbound fractions of EV70 were separated and their proportions were determined by scintillation counting. The results are presented as mean percentages of input virus bound ± standard deviation for two independent experiments, each performed in triplicate.

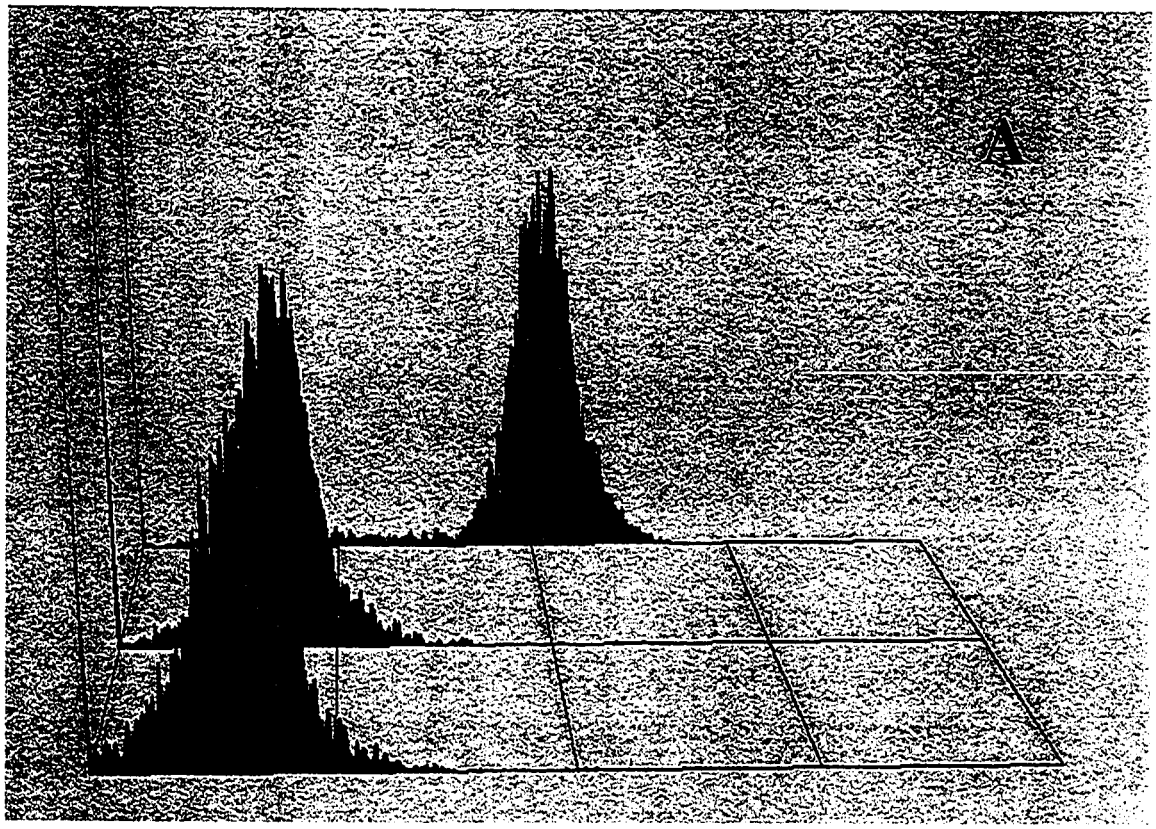

40
35
30
25
20
15
10
5
0
Virus bound (% of input)
Daudi
HL-60
Jurkat
U-937
Raji
THP-1
Untreated
25 mU/mL Neuraminidase

HCAR is a protein whose surface expression is required for CVB3 and CVB4 infection in CHO-transfected cells (Bergelson *et al.*, 1997a). DAF has been shown to function as an attachment protein for some of the group B coxsackieviruses including CVB3 (Shafren *et al.*, 1995), although its expression on receptor-negative cells was not sufficient for infection. However, antibodies to both proteins are required for complete inhibition of CVB3 infection (Shafren *et al.*, 1997c). Taken together, these observations led to the suggestion that DAF may function as a sequestration site for CVB3 thereby enhancing presentation of the virus to hCAR, which is essential for CVB3 entry. A similar relationship was proposed for CVA21, DAF, and ICAM-1, whereby DAF was found to function as an attachment protein possibly leading to a more productive infection of CHO cells by enhancing viral presentation to ICAM-1 (Shafren *et al.*, 1997b). DAF was also shown to physically interact with ICAM-1. Moreover, previous experiments in our laboratory (S. Dawe, unpublished results) demonstrated that ICAM-1 was expressed on all the susceptible leukocyte cell lines that were used for this study. For these reasons, it was decided to investigate the possibility that hCAR and ICAM-1 function as receptors for EV70.

The coding sequences for hCAR and ICAM-1 were amplified by PCR and were cloned into pEF6/V5-His TOPO so that transcription of the coding sequence was under the control of the EF-1α promoter. Nucleotide sequencing analysis confirmed correct coding sequence and plasmids were transfected into murine hDAF-negative NIH 3T3 cells. Cells resistant to the antibiotic Blasticidin were selected and then sorted by FACS to isolate the populations expressing high levels of ICAM-1 and HCAR. Expression of these proteins was monitored by flow cytometry and is shown in figure 17.

Figure 17. Expression of ICAM-1 and hCAR on stably transfected NIH/3T3 cells. Cells were washed with PBS-azide and incubated with the corresponding primary mAb for 15 minutes at room temperature. Cells were washed a second time and then incubated with FITC-conjugated secondary antibody for 15 minutes at room temperature. Mean fluorescence intensities (**purple histogram**) are compared to background levels of fluorescence due to non-specific binding of the secondary antibody (**blue histogram**) or autofluorescence (**red histogram**).

A. 3T3/ICAM : ICAM-1 detected using mAb 84H10 (1.0 μg/mL)
B. 3T3/CAR: hCAR detected using mAb RmcB (concentration unknown, dilution recommended by Dr. J. Bergelson)


A

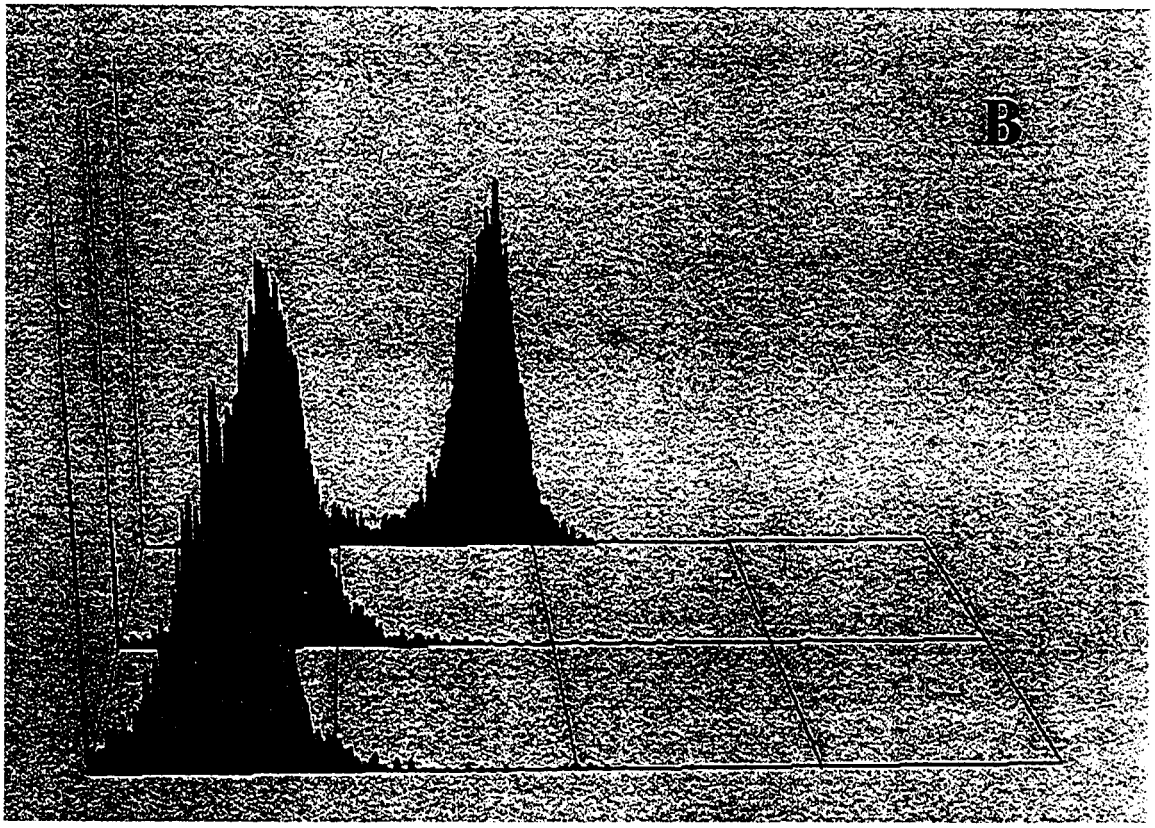

B

Figure 18. EV70 does not replicate in 3T3/hCAR or 3T3/ICAM cells. HeLa T4 cells were used as a positive control and 3T3/pEF6 cells as a negative control. Cells were grown to confluency in 12-well tissue culture dishes, counted prior to infection and infected at a MOI of 5 for 1 hour at 33°C. Cells were then washed twice with culture medium and incubated for the different time periods indicated before being subjected to two cycles of freezing and thawing to release virus. Virus titres at each time point were determined by plaque assay. The data presented are representative of two independent experiments. For each experiment, plaque assays were performed in duplicate for each sample.

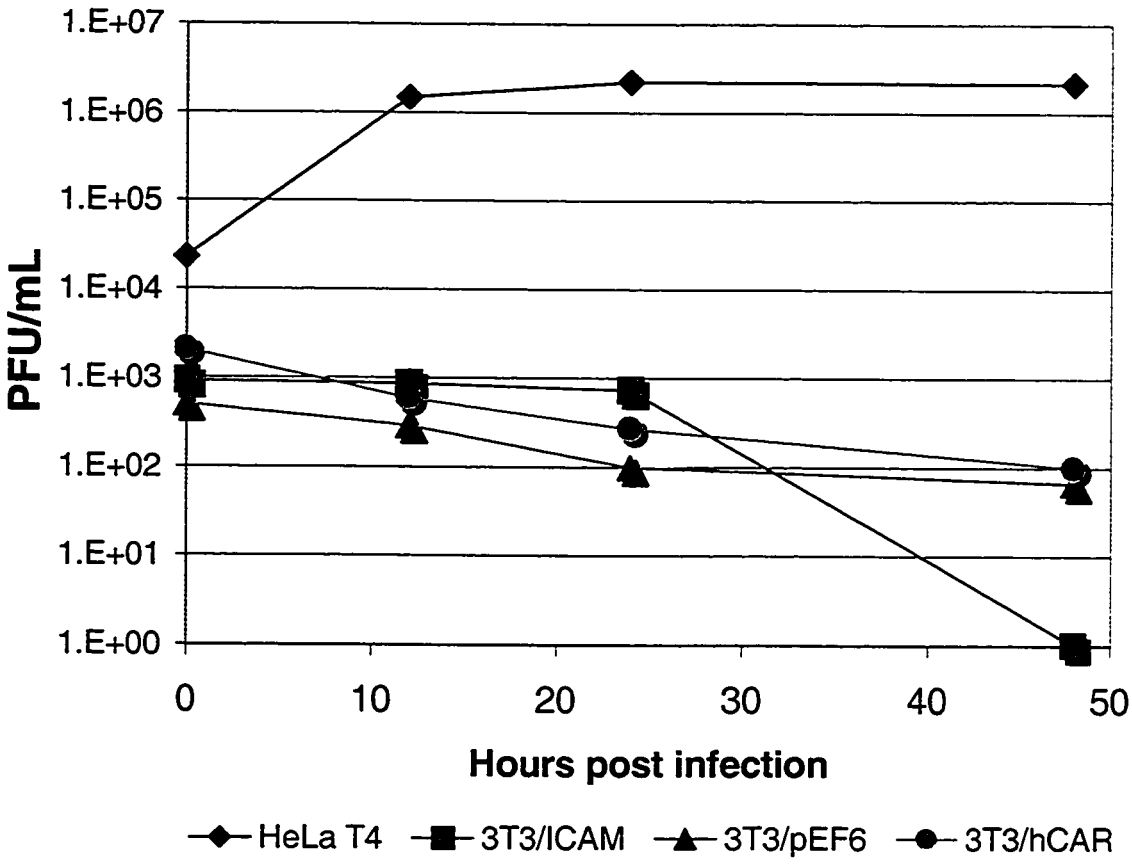
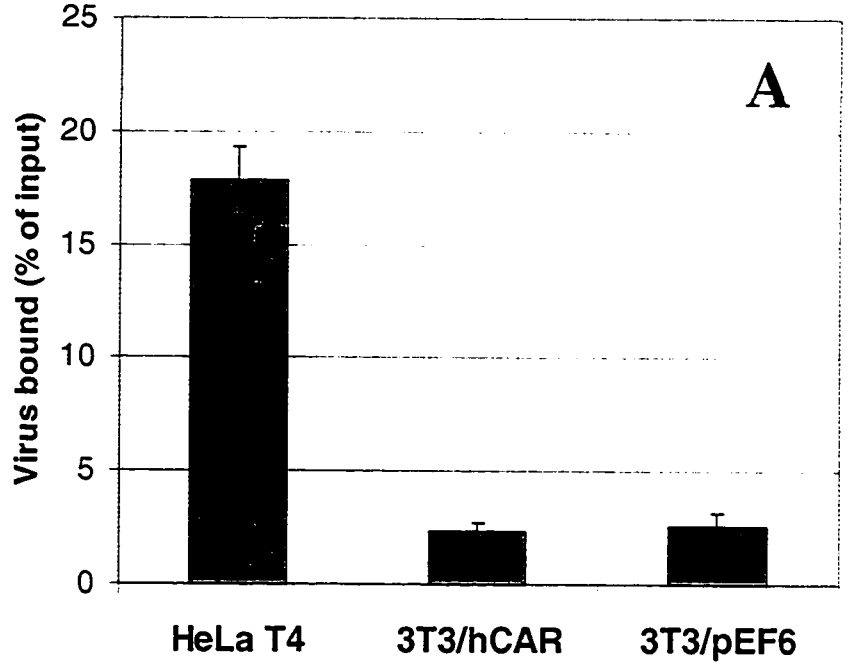
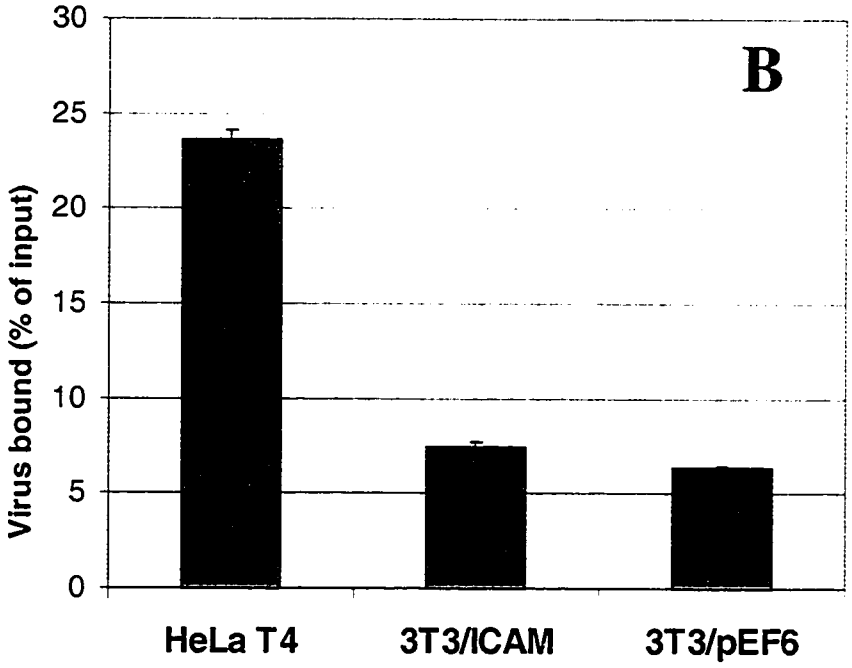

1.E+07
1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
1.E+01
1.E+00
PFU/mL
0
10
20
30
40
50
Hours post infection
HeLa T4
3T3/ICAM
3T3/pEF6
3T3/hCAR

Figure 19. EV70 binding to NIH 3T3 cells stably transfected with either ICAM-1 or hCAR. 3T3/pEF6 cells were used as negative controls. HeLa T4 cells were used as a positive control. Cells grown to confluency in 12-well tissue culture dishes were incubated in serum-free medium containing 3-6x10^3 cpm of radiolabelled virus for 1 hour at 33°C. Bound and unbound fractions of EV70 were separated and their proportions were determined by scintillation counting. The results are presented as mean percentages of input virus bound ± standard deviation for two independent experiments, each performed in triplicate.

A. 3T3/hCAR
B. 3T3/ICAM


A
Virus bound (% of input)
25
20
15
10
5
0
HeLa T4
3T3/hCAR
3T3/pEF6


B
Virus bound (% of input)
30
25
20
15
10
5
0
HeLa T4
3T3/ICAM
3T3/pEF6

Infection and plaque assays were then performed. The results of these experiments (figure 18) indicated that neither 3T3/hCAR nor 3T3/ICAM-1 cells were able to support EV70 replication. To confirm that this was not due to a block in virus entry, virus binding assays were also carried out. Figure 19 indicates that this was not the case as EV70 could not bind either hCAR- or ICAM-1-expressing cells.

Even though both hCAR and ICAM-1 were detected on the surface of transfected 3T3 cells, it was important to ensure that these molecules were biologically active. To confirm that hCAR expressed on the surface of 3T3 cells was functional, infection and binding assays using CVB3 were performed. Figure 20 shows that replication of CVB3 was significantly higher in the 3T3/hCAR cells than in cells transfected with the empty vector (3T3/pEF6). Binding of radiolabelled CVB3 was also enhanced in the 3T3/hCAR cells (figure 21). Similarly, an attempt was made to assess the functionality of ICAM-1 by infecting 3T3/ICAM cells with HRV14. Figure 22 indicates that although 3T3/ICAM cells were not permissive to HRV14 replication like Hep-2 cells, the amount of HRV14 bound to 3T3/ICAM cells at 0 time was significantly higher than in 3T3/pEF6 cells. This observation suggested that ICAM-1 expressed on 3T3 cells was functioning as an attachment protein for HRV14. It was then concluded that neither hCAR nor ICAM-1 functioned as receptors for EV70.

Figure 20. Replication of CVB3 in NIH 3T3 cells stably transfected with hCAR. 3T3/pEF6 cells were used as negative controls. Cells were grown to confluency in 12-well tissue culture dishes, counted prior to infection and infected with CVB3 at a MOI of 5 for 1 hour at 37°C. Cells were then washed twice with culture medium and incubated for the different time periods indicated before being subjected to two cycles of freezing and thawing to release virus. Virus production was determined by plaque assay. The data are presented as means ± standard deviation for two independent experiments. For each experiment, plaque assays were performed in duplicate for each sample.

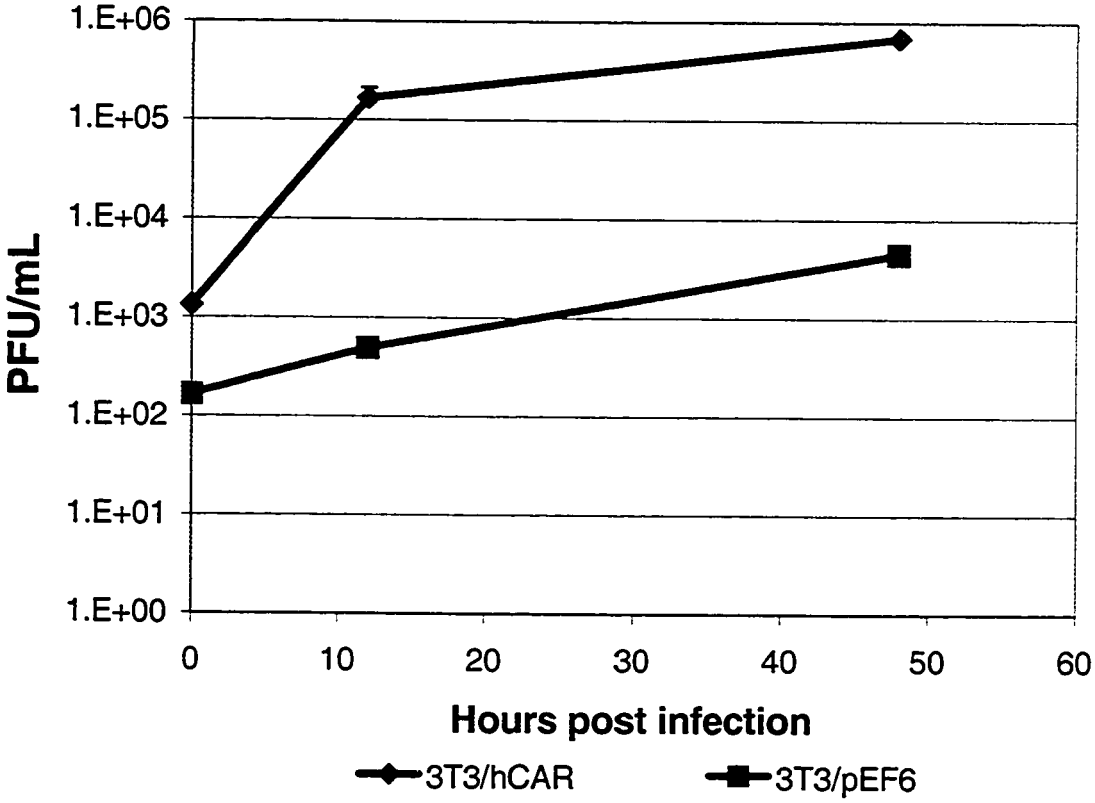

1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
1.E+01
1.E+00
PFU/mL
0
10
20
30
40
50
60
Hours post infection
3T3/hCAR
3T3/pEF6

Figure 21. CVB3 binding to NIH 3T3 cells stably transfected with hCAR. 3T3/pEF6 cells were used as negative controls. Cells grown to confluency in 12-well tissue culture dishes were incubated in serum-free medium containing 3-6x10^3 cpm of radiolabelled virus for 1 hour at 37°C. Bound and unbound fractions of EV70 were separated and their proportions were determined by scintillation counting. The results are presented as mean percentages of input virus bound ± standard deviation for two independent experiments, each performed in triplicate.

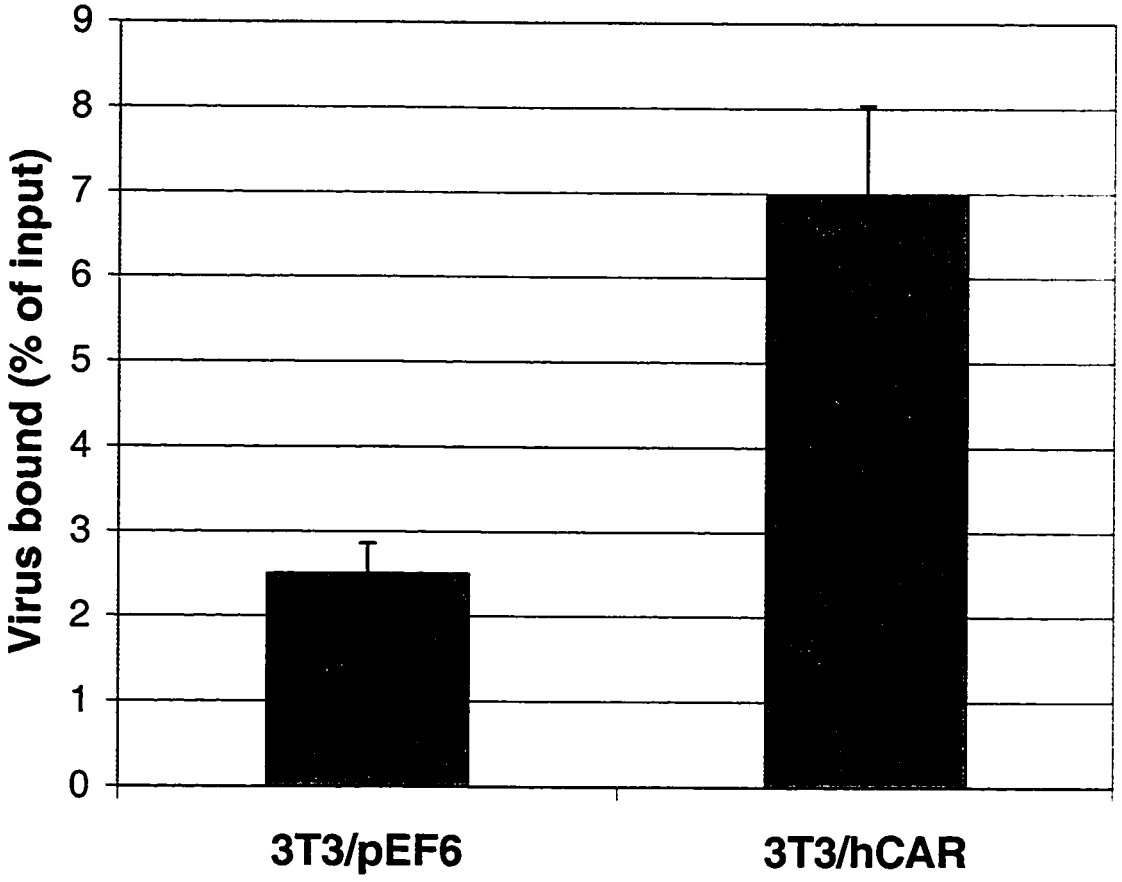
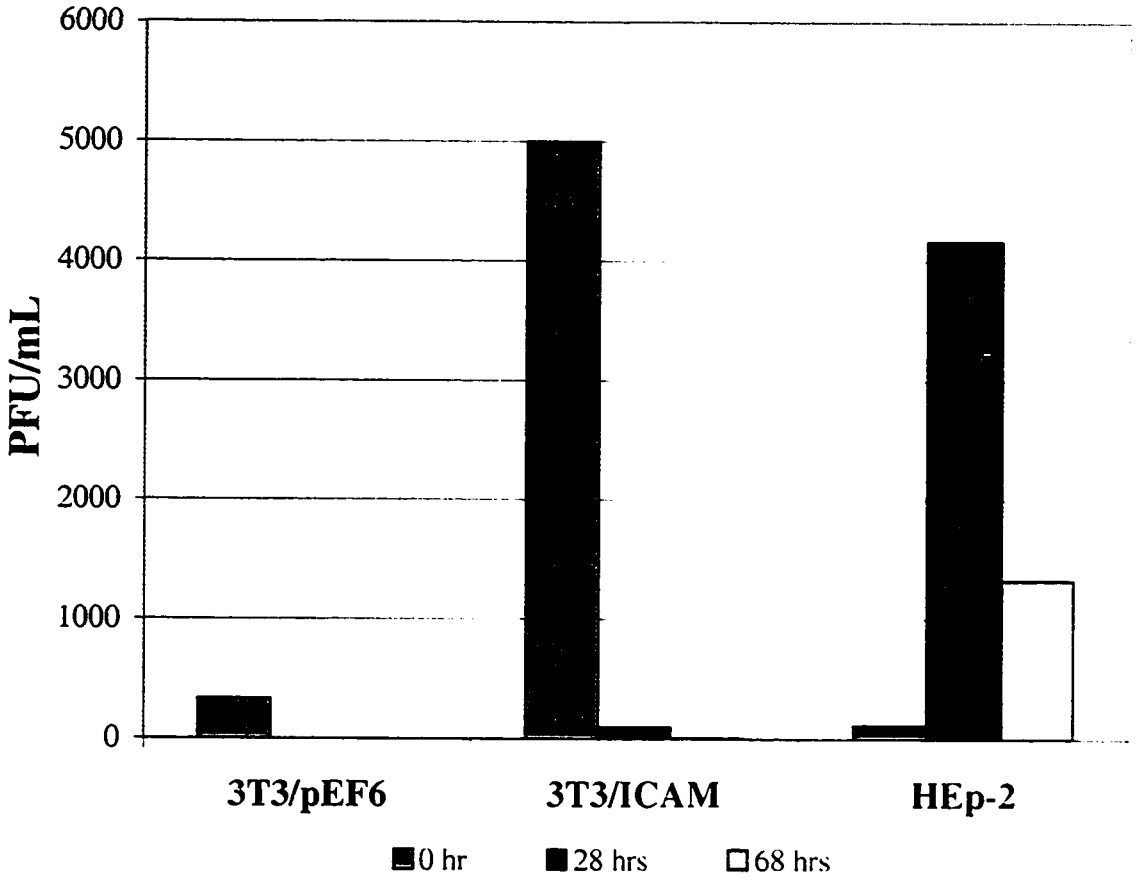

Virus bound (% of input)
9
8
7
6
5
4
3
2
1
0
3T3/pEF6
3T3/hCAR

Figure 22. Binding of HRV14 to NIH 3T3 cells stably transfected with ICAM-1. 3T3/pEF6 cells were used as negative controls. HEp-2 cells were used as a positive control. Cells were grown to confluency in 12-well tissue culture dishes and infected at a MOI of 5 for 1 hour at 33°C. Cells were then washed twice with culture medium and incubated for the different time periods indicated before being subjected to two cycles of freezing and thawing to release virus. Virus production was determined by plaque assay. The data presented derive from a single experiment performed in duplicate.


6000
5000
4000
3000
2000
1000
0
PFU/mL
3T3/pEF6
3T3/ICAM
HEp-2
0 hr
28 hrs
68 hrs

DISCUSSION

I. Non-primate DAF homologues are not receptors for EV70

A wide *in vitro* host range is fairly uncommon among human enteroviruses (Melnick, 1996). EV70 is quite unique among the human enteroviruses in its ability to infect cell lines originating from several non-primate mammals including a variety of murine, crecitine (hamster), leporine, porcine, and bovine kidney cell lines (Yoshii *et al.*, 1977).

The receptor for EV70 on human HeLa cells was identified as DAF (Karnauchow *et al.*, 1996). This complement regulatory protein is not only found on numerous human cell types (Caudwell *et al.*, 1990; Cervoni *et al.*, 1993; Kinoshita *et al.*, 1985; Lass *et al.*, 1990; Medof *et al.*, 1987a; Nicholson-Weller *et al.*, 1985; Vuorinen *et al.*, 1999) but homologues have also been identified in many rat (Hinchliffe *et al.*, 1998), mouse (Kameyoshi *et al.*, 1989), pig (Pérez de la Lastra *et al.*, 2000), rabbit (Sugita *et al.*, 1987), and guinea pig (Wang *et al.*, 1998) tissues.

Based on these observations, it was of interest to determine if non-primate homologues of DAF were functioning as receptors for EV70. This was the first objective of this thesis. A series of murine NIH 3T3 cell lines which expressed hDAF, or the mouse, rat, or pig homologues of DAF were tested for their ability to support EV70 replication and bind EV70. Results revealed that only hDAF rendered the cells permissive to EV70 infection. In addition, none of the other mammalian homologues

tested were able to bind virus. It was concluded that mDAF, rDAF, and pDAF do not function as receptors for EV70 when expressed on murine NIH 3T3 cells.

Although disappointing, these results are not surprising considering the low level of similarity between the different homologues and their human counterpart. The overall protein sequence of mDAF is 47% identical to that of hDAF (Spicer *et al.*, 1995). Although sequence identity is higher in the CCPs involved in complement function (CCPs 2,3, and 4), it is only 44% for the amino-terminal CCP1 which has previously been shown to be essential for binding of EV70 to hDAF (Karnauchow *et al.*, 1998). The similarity between hDAF and the rat homologue is even lower, with an amino acid identity of only 40% through the four CCPs and the S/T-rich region (Hinchliffe *et al.*, 1998). Pig DAF is more similar to hDAF with an average of 64% amino acid identity for CCPs 1-3 (Pérez de la Lastra et al., 2000). However, pDAF contains only three CCPs making the molecule shorter than hDAF. For this reason, the results reported here do not exclude the possibility that a binding site for EV70 is present on pDAF, but is not accessible to EV70. It would be of interest to test chimeric molecules containing pDAF CCPs 1-3 and CCP from hDAF, for example, for EV70 receptor function. Single nucleotide variations between pDAF and hDAF CCPs may also be useful in further characterizing the interaction between EV70 and hDAF.

The low level of amino acid similarity likely explains the inability of mammalian homologues of hDAF to function as a receptor for EV70. Similar observations have been reported for other human enteroviruses and receptor homologues, including other enteroviruses that use DAF as an attachment protein. CHO cells transfected with plasmids encoding hDAF were found to bind radiolabelled EV serotypes 7, 11, and 12, as

well as CVB serotypes 1, 3, and 5, whereas CHO cells expressing either mouse or rat DAF could not bind any of these viruses (Spiller *et al.*, 2000). A search for a murine homologue of the poliovirus receptor (PVR) yielded a protein (MPH) which was 51% identical to PVR (Morrison and Racaniello, 1992). MPH, however, was unable to function as a receptor for poliovirus. On the other hand, there is at least one example of a murine homologue of a human enterovirus receptor that functions. Murine CAR was shown to mimic its human counterpart by functioning as a receptor for coxsackieviruses B3 and B4 as well as for adenoviruses 2 and 5 (Bergelson *et al.*, 1998; Tomko *et al.*, 1997). This homologue, however, is 91% similar to hCAR (Bergelson *et al.*, 1997a). It will be of considerable interest to test the ability of DAF from non-human primates such as the rhesus macaque or the patas monkey to function as a receptor for EV70. These DAF homologues may be good candidates as they are 83% identical to hDAF (Kuttner-Kondo et al., 2000). Work in the laboratory has shown that rhesus monkey kidney cells, LLC-MK_2, bind EV70 and are permissive to infection.

In addition to the results described above, the fact that murine L-929 cells do not express mDAF but support EV70 replication further indicates that a cell surface molecule other than DAF must act as a receptor for EV70 on mouse L-929 cells and presumably for cells of other mammalian species. L-929 cells may be a good tool for identification of the murine receptor for EV70.

Transmembrane and secreted isoforms of the mammalian DAF homologues tested have been reported (Hinchliffe *et al.*, 1998; Pérez de la Lastra *et al.*, 2000; Spicer *et al.*, 1995). According to Pérez de la Lastra *et al.* (2000), the divergence between the sequences of pDAF-TM and pDAF-GPI is caused by splicing and occurs several residues

downstream of the predicted sites of GPI addition. This means that sequence variation is not in the regions predicted to be involved in EV70 binding. Similar observations were reported for the secreted form of rDAF (Hinchliffe *et al.*, 1998). One exception to this is mDAF whose transmembrane version is encoded by a gene distinct from the one encoding the GPI isoform (Spicer *et al.*, 1995). Although these two proteins are highly similar (>85%), several amino acid variations are located in the CCPs, which may be sufficient for the transmembrane version to behave differently from mDAF-GPI. Although unlikely, the possibility that mDAF-TM could function as a receptor for EV70 is currently being investigated in our laboratory.

II. EV70 utilizes a receptor other than DAF on human leukocyte cell lines

The mechanism by which EV70 infection spreads to the central nervous system is unknown. One possibility is direct spread through the optic nerve (Higgins, 1982). Involvement of the preauricular lymph node in EV70 infection has been reported (Sasagawa *et al.*, 1982, Kono and Uchida, 1977) and suggests that spread may occur via circulating leukocytes. In addition, previous experiments in our laboratory have shown that EV70 can infect several different leukocyte cell lines, although it was unknown whether EV70 was using DAF on these human cell lines. For these reasons, the second objective of this thesis was to assess DAF's potential role as a receptor on these cells.

Inhibition of EV70 binding to any of the leukocyte cell lines could not be demonstrated using EVR1, a monoclonal antibody (mAb) specific for hDAF CCP1, at the concentrations that decreased binding of radiolabelled EV70 to HeLa T4 cells. Binding of EV70 to U-937 cells was also not inhibited by the CCP3-specific mAb 1H4. Even

though CCP3 is not required for EV70 binding, Karnauchow *et al.* (1998) reported that 1H4 inhibited EV70 binding to HeLa cells by more than 60% at the concentration used in the experiment described here.

DAF was also ruled out as an attachment protein for EV70 on leukocyte cell lines by treatment with PI-PLC, an enzyme that cleaves GPI-anchored proteins from the surface of cells (Roberts *et al.*, 1988; Udenfriend and Kodukula, 1995). Since DAF is linked to the cell surface by a GPI moiety (Davitz *et al.*, 1986; Medof *et al.*, 1986), treatment of cells with PI-PLC should result in a decrease in the ability of cells to bind EV70 if DAF is functioning as a receptor. Treatment of HeLa T4 cells did have a marked effect on EV70 binding. In contrast, binding of virus to the other human cell lines tested was unaffected by PI-PLC treatment.

Since resistance to PI-PLC digestion has been reported for at least one other GPI-anchored protein (acetylcholinesterase, Roberts *et al.*, 1988), it was important to confirm that PI-PLC treatment removed hDAF from cells. This was achieved by monitoring the extent of mAb binding to PI-PLC-treated or untreated cells by flow cytometry. DAF on all cells was found to be sensitive to PI-PLC treatment. In fact, although Daudi cells have been reported to lack GPI-anchored proteins (Morelon *et al.*, 2001), we were able to detect changes in surface DAF expression on these cells.

Although treatment of cells with PI-PLC released the majority of DAF from the cell surface, it is conceivable that whatever molecules remained were sufficient to bind the levels of radiolabelled virus observed. However, taken together with binding inhibition experiments and experiments performed on HeLa T4 cells, results from the PI-PLC experiments suggest this possibility to be very unlikely.

The results discussed above provide strong evidence that EV70 must utilize at least one molecule other than DAF as a receptor on several human and non-primate mammalian cell lines. Also consistent with this interpretation is the observation that HL-60 cells can be infected by EV70 even though DAF could not be detected on these cells by flow cytometry. Since EV70 binding to these cells, as well as to the other leukocyte cell lines examined, was resistant to PI-PLC treatment, EV70 must be binding to a receptor that is not a GPI-anchored protein. The data also suggest that this molecule is not expressed on the surface of HeLa T4 cells since loss of DAF is sufficient to inhibit EV70 binding to these cells (figure 15). Furthermore, treatment of U-937 cells with trypsin prior to PI-PLC treatment did not affect EV70 binding, suggesting that whatever other receptor(s) EV70 is using is not trypsin-sensitive (figure 15).

It is noteworthy to mention that these data, and other data from our lab, confirm that hDAF is a receptor for EV70 on HeLa cells. Expression of hDAF on receptor-negative murine cells rendered them permissive to this virus. Moreover, partial release of DAF from the surface of these transfected cells with PI-PLC (data not shown), as well as from HeLa T4 cells, resulted in decreased EV70 binding. However, a universal feature for EV70 binding to the cell lines examined to date seems to be a requirement for sialic acid. This carbohydrate plays a role in the attachment of several viruses (Evans and Almond, 1998; Young, 2001), including the picornaviruses bovine enterovirus and rhinovirus 87. Neuraminidase treatment of both the HeLa T4 and 3T3/hDAF cell lines, as well as the leukocyte cell lines tested here, results in complete abrogation of binding (figure 16; D. Alexander, personal communication). Other work performed in our laboratory has demonstrated that the N-linked carbohydrate moeity on DAF is not

important for EV70 binding (D. Alexander). It is possible that the sialylated moeity required for binding to the leukocyte cell lines is found on the other receptor(s).

III. HCAR and ICAM-1 are not receptors for EV70

DAF has been shown to associate with ICAM-1 and form a receptor complex for CVA21 (Shafren *et al.*, 1997b). Although there have not been any reports of DAF interacting with hCAR, both proteins can be utilized by CVB3 (Shafren *et al.*, 1997c). In both cases, DAF was suggested to play a sequestration role thereby enhancing the availability of CVB3 and CVA21 to hCAR and ICAM-1, respectively (Shafren *et al.*, 1997b, 1997c). Based on these observations, ICAM-1 and hCAR were examined as candidate receptors for EV70.

The coding sequences of these genes were cloned and stable NIH 3T3 transfectants expressing each of these surface proteins were produced. Unlike hDAF, ICAM-1 and HCAR were not sufficient to support EV70 binding or to render NIH 3T3 cells susceptible to EV70 infection. It was concluded that neither ICAM-1 nor HCAR function as receptors for EV70 when expressed on NIH 3T3 cells.

IV. Concluding remarks

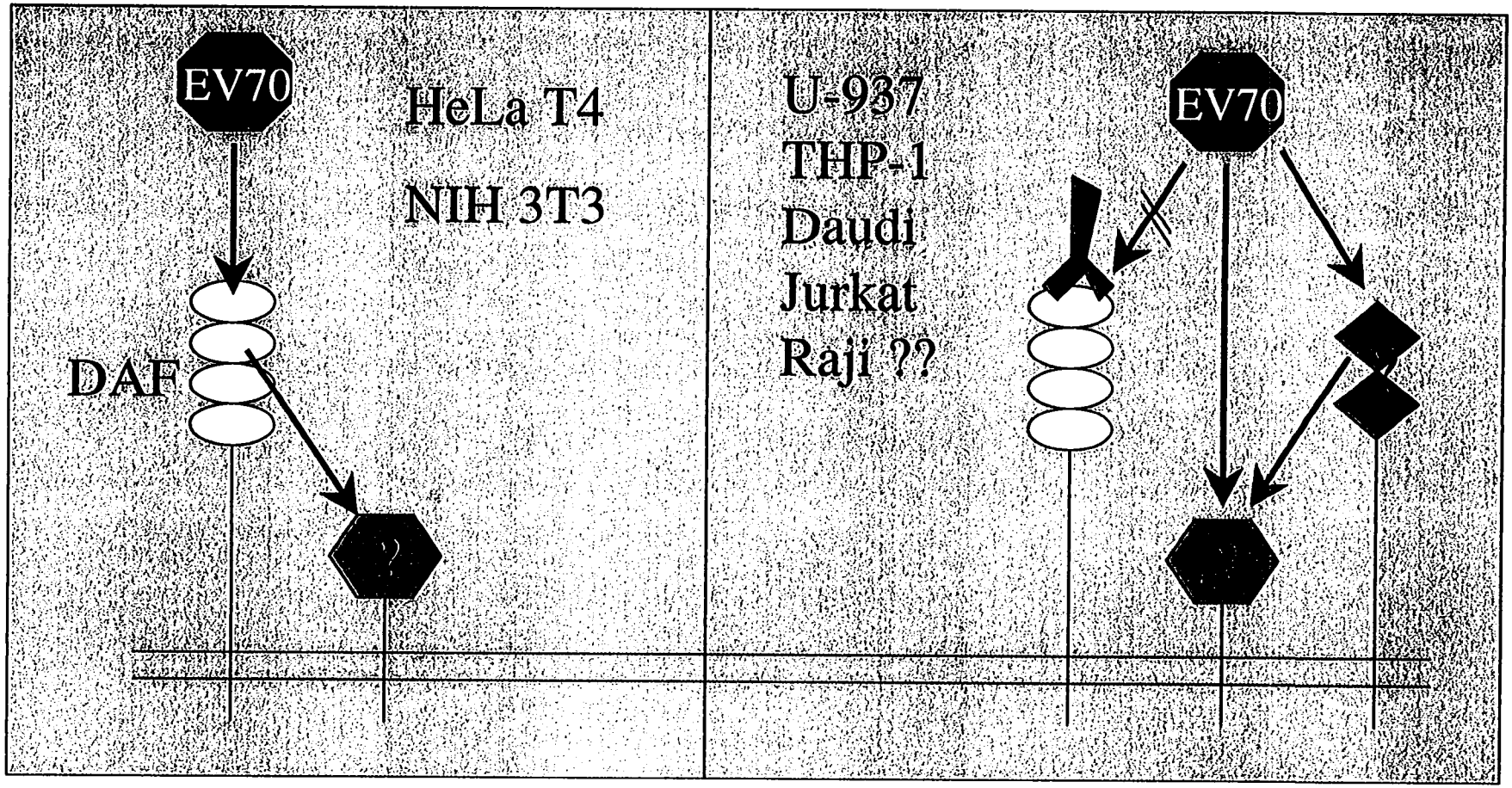
The results presented in this thesis suggest that EV70 utilizes more than one molecule as a human cell surface receptor, and that receptor usage may be cell line-dependant. This is different from what has been observed for CVB3. This enterovirus can bind to two different molecules, hDAF and hCAR, but only hCAR appears to

mediate entry and infection (Shafren *et al.*, 1997c). Similarly, for CVA21, both hDAF and ICAM-1 bind virus, but only ICAM-1 is essential for virus entry and infection (Shafren *et al.*, 1997b). A model for EV70 binding to the various cell lines used in this study is depicted in figure 23. On HeLa cells and 3T3/hDAF, hDAF would appear to be essential for EV70 infection. But it appears that the removal of DAF from the leukocyte cell lines does not affect EV70 binding, suggesting that another molecule must function as an attachment molecule on leukocytes. Binding to non-human cell lines must also be mediated by a molecule other than DAF. It is possible that homologues of the 'other' human EV70 receptor may be present on these susceptible cells.

The results described also demonstrate that regardless of cell type, binding of EV70 is sensitive to neuraminidase. Hence, one possible model for EV70 infection may involve the use of either DAF or another molecule as a sequestration receptor. Interaction with DAF would provide access to or enhance the likelihood of virus interaction with a less available sialylated moiety that is involved in virus entry. This model would be consistent with the observations made with 3T3 cells, where no virus binding occurs until DAF is expressed, and binding, as for all cell lines that have been examined, is neuraminidase sensitive.

As for the leukocytes, the 'other' molecule may function similarly to DAF but may be favored by EV70, and the need for DAF would be bypassed. It is also conceivable that the 'other' receptor and the sialylated moiety are one and the same molecule.

Figure 23. Model for EV70 binding and entry. hDAF is essential for EV70 binding and entry on HeLa T4 and NIH 3T3 cells, acting as a sequestration molecule allowing the virus to bind to a less accessible but also essential sialylated moiety. Leukocyte cell lines, however, seem to be able to bind virus without the use of hDAF. They may either express another molecule on their surface which behaves similarly to hDAF, or they may express the sialylated moiety in a manner which makes it directly accessible by the virus.


EV70
HeLa T4
NIH 3T3
DAF
?
U-937
THP-1
Daudi
Jurkat
Raji ??
EV70

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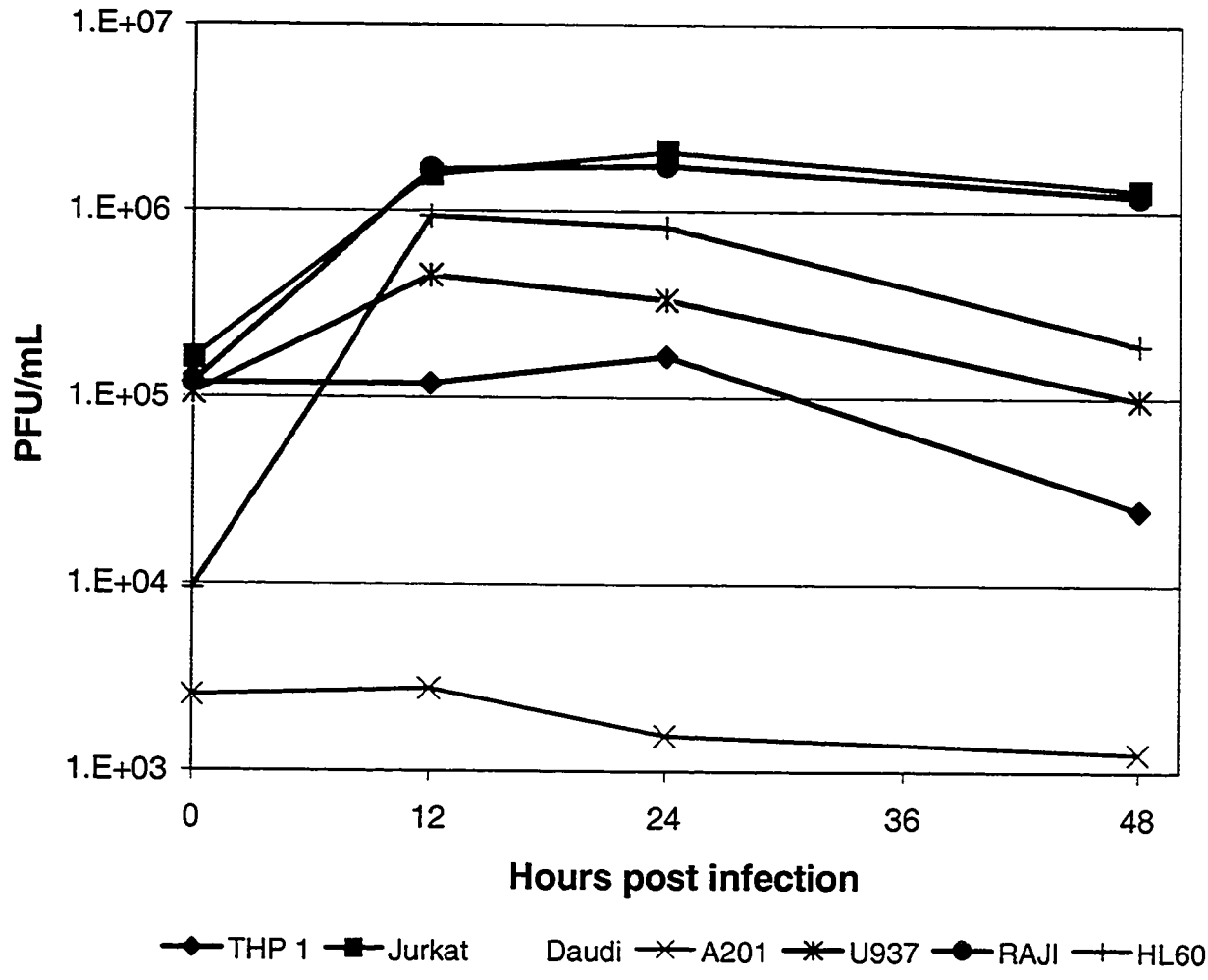
Appendix I. EV70 replication in U-937, Jurkat, Daudi, Raji, THP-1, HL-60, and A.201 cells. Cells were infected with EV70 at a MOI of 5 for 1 hour at 33°C. Cells were then washed three times with culture medium and aliquots were withdrawn immediately and at various times after infection. Cells were subjected to two cycles of freezing and thawing to release virus, and virus production at each time point was determined by plaque assay, in duplicate. The data presented are for one experiment and were performed by C. Grisé.


1.E+07
1.E+06
1.E+05
1.E+04
1.E+03
PFU/mL
0
12
24
36
48
Hours post infection
THP 1
Jurkat
Daudi
A201
U937
RAJI
HL60

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