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Serum albumin inhibits echovirus 7 uncoating

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Echoviruses induce a wide spectrum of diseases in man, the most severe being meningitis. In neonates, however, a severe systemic infection can be observed, leading to death. Serum albumin is the most abundant protein in plasma and most interstitial fluids, and its functions include osmoregulation and transport and delivery of hydrophobic molecules such as fatty acids and steroids. The results of cold-synchronized one-step growth analysis of echovirus 7 infection and sucrose-gradient analysis of A-particles suggest that physiological concentrations of albumin block echovirus 7 infection by inhibiting uncoating. The blockage was reversible and was still effective when albumin was added 30 min after virus adsorption. Inhibition of uncoating was confirmed by using rhodanine, a known specific inhibitor of echovirus uncoating. After removal of the albumin blockage, addition of rhodanine perpetuated the inhibition. Serum and interstitial albumin concentrations may limit echovirus infection *in vivo* and thereby act as an extracellular determinant for echovirus tropism.

Introduction

Echoviruses are small, non-enveloped, positive-strand RNA viruses belonging to the genus *Enterovirus* of the family *Picornaviridae*. They are human pathogens that infect mainly young children, causing a wide spectrum of clinical symptoms including aseptic meningitis, encephalitis, paralysis, myocarditis, respiratory and gastro-intestinal disorders, muscular disability, exanthema and Reye's syndrome. However, the majority of infected individuals are asymptomatic. After transmission via the faecal–oral route, echoviruses are thought to establish themselves in primary sites of replication in the alimentary tract before spreading to other secondary target organs. However, little is known about the actual course of echovirus infections *in vivo*, except that viraemia may occur from 6 to 30 days prior to the onset of illness. There are 28 echovirus serotypes and most are associated with multiple clinical symptoms (Melnick, 1996).

Recent experiments have began to elucidate some aspects of the use of cellular receptors by echoviruses. Serotypes 1 and 8 use the integrin VLA-2 (Bergelson *et al*., 1992). Types 6, 7, 13, 21, 29 and 33 use decay-accelerating factor (DAF, CD55), a regulator of autologous complement activity (Ward *et al*., 1994; Bergelson *et al*., 1994), and may require secondary receptors for uncoating (Powell *et al*., 1997, 1998). The identity of the receptors used by the other echoviruses is not yet known. Binding of poliovirus and the major-group rhinoviruses to their specific receptors, PVR and ICAM-1, respectively, is sufficient to mediate the formation of 135S altered A-particles that are believed to represent disassembly intermediates in infection (Greve *et al*., 1991; Kaplan *et al*., 1990). The virus disassembly process for picornaviruses is thought to involve several stages. The first step involves binding of a specific receptor sequence in a hydrophobic depression on the viral surface. Displacement by the receptor of a stabilizing hydrophobic molecule, called 'pocket factor', from a hydrophobic pocket within the β-barrel of VP1 is then thought to occur (Rueckert, 1996). The loss of the pocket factor is thought to facilitate movement of the capsid proteins, including the loss of VP4 from the viral capsid, and the extrusion of the hydrophobic N-terminal domains of VP1. Pocket factors include a sphingosine-like molecule in poliovirus types 1 and 3, myristate in bovine enterovirus (Smyth *et al*., 1995) and an uncharacterized but smaller pocket factor in rhinovirus 1A and 16 (Rueckert, 1996). Echoviruses are also likely to posses stabilizing pocket factors, though these have not been characterized.

We have recently shown that binding of echovirus 7 (EV7) to DAF may not be sufficient for infection and uncoating and we suggested that EV7 may require interaction with a secondary factor on cells (Powell *et al*., 1997). DAF is a glycosylphosphatidylinositol-anchored protein and may

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sequester virus into caveolae (Bergelson *et al*., 1994). It seems likely, therefore, that if secondary factors are required for echovirus uncoating, they are probably located in caveolae.

Our recent findings show that β_2-microglobulin (β_2m), possibly in association with HLA class I antigens, may be required as a secondary factor during the early stages of echovirus infection (Ward *et al*., 1998). Significantly, class 1 antigens have also been located in caveolae (Stang *et al*., 1997). Whilst working on β_2m, we noted that 10% foetal calf serum (FCS) enhanced the blockage of EV7 infection by antibodies to β_2m (unpublished data), and this suggested to us the presence of a factor(s) in serum that inhibited echovirus infection. In this paper, we show that EV7 uncoating is inhibited by serum albumin. In parallel experiments, albumin had no effect on poliovirus. The possible mechanism of the inhibition and the relevance of these results to echovirus infections *in vivo* are discussed.

Methods

■ **Cell culture and virus propagation.** Rhabdomyosarcoma (RD) cells and Ohio HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS unless otherwise stated. All echovirus serotypes (prototype strains) were obtained from the Public Health Laboratory Service (Colindale, UK) and were propagated in RD cells in serum-free DMEM. Poliovirus 3 (PV3, Leon) was propagated in Ohio HeLa cells. The titres of stock virus preparations were determined as $TCID_{50}$ in RD cells.

■ **Antibodies and albumins.** The goat anti-mouse immunoglobulin (Ig)–β-galactosidase conjugate used in the viral antigen immuno-assays was from Harlan Sera-lab and was used at a dilution of 1/400. The anti-enterovirus MAb 5-D8/1 was from Dako and was used at a dilution of 1/200. In the immuno-assays, antibodies were used after dilution in PBS containing 0·5% BSA (fraction V, Sigma). All albumin preparations and other chemicals were purchased from Sigma. Bovine albumins used were BSA (catalogue number A-8918), globulin-free BSA (A-7638), essentially fatty acid-free BSA (A-7511), globulin- and fatty acid-free BSA (A-0281), dimeric BSA (A-9039) and monomeric BSA (A-1900). Human serum albumins (HSA) used were globulin-free HSA (A-8763), essentially fatty acid-free HSA (A-1887) and globulin- and fatty acid-free HSA (A-3782). Chicken albumin (A-3014) was also used. All albumins were solubilized in serum-free DMEM with a final pH of 7·4.

■ **Virus-blocking assays.** Plates (96-well) of RD or HeLa cells at 80% confluence were washed with serum-free DMEM and were then incubated with 100 µl DMEM with or without BSA or other albumins, or milk protein, at a variety of concentrations, containing EV7 or PV3. Plates were then incubated at 37 °C in a 5% CO_2 atmosphere for 24 h. Cells were then fixed and stained with pre-filtered Giemsa crystal violet for 5 min. The stain was then removed and washed away thoroughly, and cell survival was assessed by measuring the degree of cell staining by use of a 96-well plate reader (MRX, Dynatech) at 560 nm.

■ **Measurement of EV7 antigen production.** Plates (24-well) of RD cells at 80% confluence were washed with serum-free DMEM and were then incubated with 200 µl EV7 at room temperature for 30 min to permit virus adsorption to the cells. Unbound virus was then washed away with three 1 ml DMEM washes and cells were then incubated in DMEM with or without BSA (A-7638, globulin free) under standard tissue culture conditions (37 °C, 5% CO_2 in a humidified atmosphere). Cell monolayers were then fixed with 0·5 ml acetone/methanol (1 : 1) at 6–10 h post-infection, washed three times with 1 ml volumes of PBS and then incubated successively with 200 µl volumes of MAb 5-D8/1, goat anti-mouse Ig–β-galactosidase conjugate and 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal), as described previously for blue cell assays (Ward *et al*., 1998). After 2 h in X-Gal, cells were washed three times with PBS, solubilized overnight in 200 µl 1% SDS, 0·2 M NaOH and 150 µl aliquots of the solubilized cells were then transferred to 96-well plates. The level of capsid antigen in the samples was determined by colorimetry at 560 nm with a 96-well plate reader.

■ **One-step growth experiments.** Flasks (25 cm^2) of RD cells at 90% confluence were washed with serum-free DMEM and then inoculated with virus at 1 $TCID_{50}$ per cell. After 30 min adsorption at room temperature, cells were washed three times with 5 ml volumes of serum-free DMEM and then covered with 3 ml DMEM with or without 2% BSA (A-7638, globulin free). Cell were then either stored at −20 °C immediately or incubated for 2–10 h in tissue culture to allow virus replication. Virus was then recovered from the cells by three cycles of freeze–thawing and titrated by $TCID_{50}$ on RD cells.

■ **Sucrose gradient analysis.** Approximately 2×10^7 RD cells were incubated on ice for 1 h with ^{35}S-labelled virus (approx. 10^5 c.p.m. of each virus) in 200 µl serum-free DMEM. Cells were then pelleted by centrifugation at 1000 ***g*** for 4 min and washed twice with 1 ml volumes of DMEM to remove non-adsorbed virus. Cells were then resuspended in 1 ml DMEM, divided into two 500 µl aliquots in 1·5 ml plastic tubes, pelleted and then resuspended in 1 ml either serum-free DMEM or DMEM containing 2% BSA (A-7638, globulin free). EV7-treated cells were then incubated in a 37 °C warm room and tubes were rotated for 2 h (6 r.p.m.) to keep the cells aerated and in suspension. PV3-treated cells were incubated in this manner for 1 h. Cells were then pelleted as before and the supernatants containing the eluted A-particles were harvested. The cells were then resuspended in 1 ml DMEM and solubilized by the addition of NP-40 (0·2% final concentration). Insoluble cell debris was removed by centrifugation at 13 000 r.p.m. for 1 min in a microfuge. The ^{35}S-labelled virus particles in the eluted fractions and solubilized cells were then sedimented through parallel 15–45% sucrose gradients for 4·5 h at 25 000 r.p.m. in a Beckman SW28 rotor. Radioactivity in fractions was then quantified by scintillation counting.

Results

During our previous work on β_2m (Ward *et al*., 1998), we noted that 10% FCS enhanced the blockage of EV7 infection provided by anti-β_2m antibody and here we suggest that a factor(s) was present in serum that inhibited echovirus infection. To investigate this, we infected RD cells with EV7 in the presence of 0·9% BSA, which is the protein concentration equivalent to 10% FCS. After 24 h of incubation, we observed a significant reduction in EV7-induced cytopathic effect (CPE). This result prompted a further investigation into the effects of albumin.

Serum albumin inhibits EV7 but not PV3 infection

RD cells were infected with either EV7 or PV3 in the presence of varying concentrations of BSA up to 2%, the level found in most interstitial fluids (Rose *et al*., 1989). CPE was then

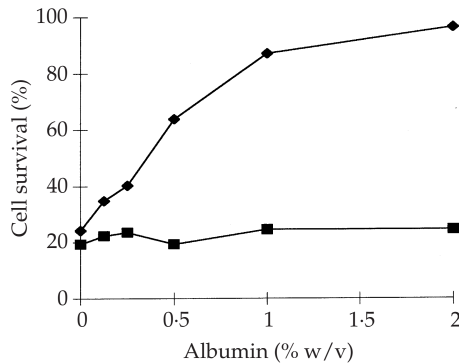

Fig. 1. Plates (96-well) of RD cells at 80% confluence were washed with serum-free DMEM and were then incubated with or without BSA (A-8918; 0–2%) containing 2×10^6 $TCID_{50}$ EV7 (◆) or PV3 (■). After incubation in tissue culture for 24 h, cells were fixed and then stained with Giemsa crystal violet for 5 min. Cell survival was measured by colorimetry at 560 nm ($n \geqslant 8$).

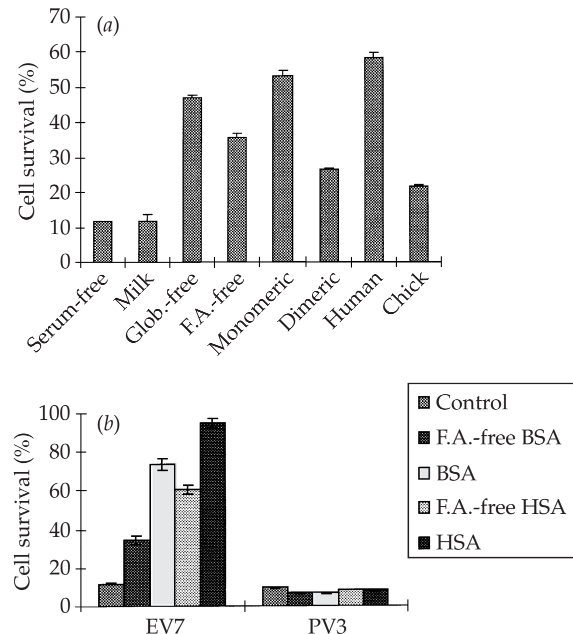

Fig. 2. (*a*) RD cells in 96-well plates were infected with EV7 (as described in Fig. 1) in the presence of a variety of albumins or milk protein, all at 1%. (*b*) Cells were infected with EV7 or PV3 in the presence of 2% globulin (glob.)-free BSA or HSA or globulin-free and essentially fatty acid (F.A.)-free BSA or HSA. After incubation in tissue culture for 24 h, cells were fixed and then stained with Giemsa crystal violet for 5 min. Cell survival was measured by colorimetry at 560 nm ($n \geqslant 8$).

assessed 24 h later by staining the infected cell monolayers with Giemsa violet. Surviving, attached cells were stained, whereas the dead cells were washed away. At all concentrations of BSA used, PV3-induced CPE was unaffected, whereas infection of cells by EV7 was inhibited (Fig. 1). A similar result was also observed with HeLa cells (data not shown). Having shown blockage of EV7 infection by reagent-grade fraction V BSA, we then investigated other commercial preparations of BSA and HSA. These included globulin-free BSA and HSA, to rule out the possibility that the blockage could have been caused by antibodies in the albumin preparation. We also used chicken albumin, milk protein, monomeric and dimeric forms of BSA and fatty acid-free BSA and HSA. All forms of albumin were found to block EV7 infection, whereas milk protein had no effect (Fig. 2*a*). Notably, HSA was found to block EV7 infection most efficiently and chicken albumin was least able to block infection; these differences probably reflect protein sequence differences between bovine, human and chicken albumins. The amino acid sequences of human and bovine albumins are 76% identical and share 47% and 44% identity, respectively, with the amino acid sequence of chicken albumin. Highly pure monomeric BSA was also found to inhibit EV7 infection effectively, whereas the dimeric form, at an equivalent w/v concentration, was only approximately half as effective. Monomeric albumin is found in serum, whereas dimers form during purification (Atmeh & Shabsoug, 1997). The fatty acid-depleted forms of BSA and HSA blocked infection less efficiently than globulin-free BSA or HSA (Fig. 2*b*). Nevertheless, the blockage was substantial at the single concentrations tested. This result may reflect variations between different batches of BSA or may relate to the ligand-binding status of albumin. The conformation of albumin is known to be modified by ligand binding (Kragh-Hansen, 1981) and the further purification required to remove the fatty acid may lead to a greater degree of dimerization. Since depletion removes 99% of fatty acid and substantial inhibition was nonetheless observed with the fatty acid-depleted forms, the inhibition is probably not due to a lipid component in the albumin preparations.

The albumin blockage is rapid and reversible

Having determined that serum albumin inhibits EV7-induced CPE, we next investigated the position of the blockage in virus replication, by using an immuno-assay to measure viral antigen production. When BSA was added at the same time as virus, or 30 min post-infection, viral antigen production was reduced significantly. Addition of BSA at 1 h post-infection was less effective and at 2 h there was no effect (Fig. 3*a*). We then pre-treated the RD cells for various times and removed the BSA prior to the addition of virus. The inhibitory effect was largely reversible, although some slight protective effect was observed following removal of albumin after several hours pre-treatment (Fig. 3*b*). As expected, if the BSA was not removed, there was a complete inhibition of antigen production as measured at 6 h post-infection (ANR in Fig. 3*b*).

Finally, we adsorbed virus to RD cells and then incubated the cells in BSA for various times up to 4 h, before washing followed by further incubation in serum-free DMEM. Antigen was then measured at 10 h post-infection. Removal of the BSA after 1 or 2 h allowed infection to proceed to produce a level of antigen comparable with that observed in the untreated

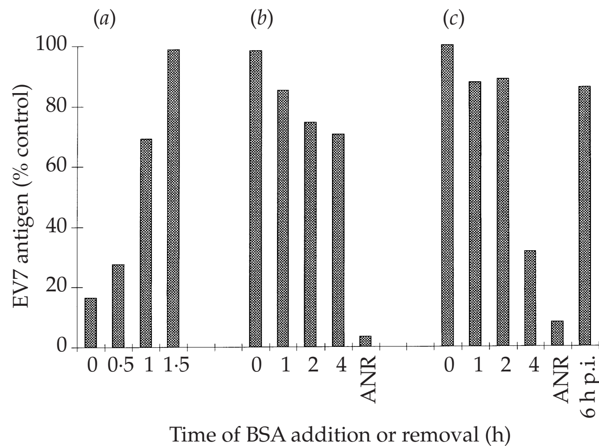

Fig. 3. (*a*) BSA added after EV7 adsorption. RD cells at 80% confluence were incubated with EV7 (5 $TCID_{50}$ per cell) at room temperature for 30 min to permit virus adsorption and were then incubated in tissue culture. At 0–90 min post-adsorption, the serum-free DMEM was removed and BSA (2%, A-7638, globulin free) was added. Cells were then incubated in tissue culture and at 6 h post-adsorption, they were fixed. (*b*) BSA removed before EV7 adsorption. Cells were pre-incubated with 2% BSA for 0–4 h before being washed. EV7 was added and at 6 h post-adsorption, cells were fixed. Control cells were pre-treated with BSA for 4 h, washed, incubated with virus for adsorption and then re-incubated with BSA for a further 6 h (ANR). (*c*) BSA removed after EV7 adsorption. Cells were incubated with EV7 at room temperature for 30 min to permit virus adsorption and were then incubated with or without addition of BSA (2%, A-7638, globulin free) before being incubated in tissue culture. At 0–4 h post-adsorption, the BSA was either removed by washing or left on. Cells were then incubated in tissue culture again and at 10 h post-adsorption, they were fixed. Cells were also infected for 6 h only (6 h p.i.), so that the level of EV7 viral antigen at 6 h could be compared with the amount produced at 10 h post-adsorption. In (*a*)–(*c*), fixed cell monolayers were assayed for EV7 viral antigen as described in Methods. Results shown represent the means of duplicate experiments.

control (Fig. 3*c*). After 4 h treatment with BSA, the infection did not recover completely (compare antigen production at 6 h with no treatment), but this could be due to virus degradation over the 4 h period rather than to a lasting blockage of infection.

Taken together, the results presented in Fig. 3 suggest that the albumin-induced inhibition of EV7 infection is rapid and is reversible up to 2 h post-adsorption of virus. A similar pattern has been observed for inhibition of EV12 infection by rhodanine, a chemical that blocks EV12 uncoating (Eggers, 1977).

Albumin inhibits uncoating of EV7 but not PV3

Having established the selective, rapid and reversible nature of the albumin-induced inhibition, we then investigated EV7 replication by using cold-synchronized one-step growth analysis (Fig. 4). During the first 2 h of infection, the measurable adsorbed titre of enteroviruses normally decreases by up to 90% due to viral capsid uncoating and release of viral RNA into the cytoplasm, prior to active replication (eclipse phase). In control RD cells treated with serum-free DMEM, we observed an eclipse phase in EV7 replication; the virus titre was reduced by 90% at 2 h post-infection, prior to a burst in replication activity. However, in cells treated with BSA, the eclipse phase at 2 h post-infection was absent. The final titre of virus at 10 h post-infection in the presence of BSA was reduced by 2 $\log_{10}$ compared with control cells (Fig. 4*a*). In contrast, no reduction in titre was observed in comparable experiments with PV3 (Fig. 4*b*). These results confirm the specificity of the albumin blockage against EV7 and are consistent with the conclusion that albumin blocks an early step in the infection cycle.

We showed that the albumin blockage is highly effective even 30 min after virus adsorption (Fig. 3*a*). It is therefore unlikely that albumin inhibits EV7 attachment to DAF. To confirm this, ^{35}S-labelled virus was incubated with BSA in binding assays on RD cells; neither EV7 nor PV3 binding was affected (data not shown). After receptor attachment, the next step is uncoating: the conversion of 160S infectious virus to 135S altered A-particles and 80S empty capsids accompanies productive uncoating and infection (Rueckert, 1996). We investigated whether albumin affected the uncoating process, by monitoring the fate of sucrose gradient-purified, ^{35}S-labelled EV7 and PV3 (Fig. 5). For PV3-infected cells, we found high levels of eluted A-particles, comparable to published results (Kaplan *et al*., 1990). Albumin had no effect on the levels

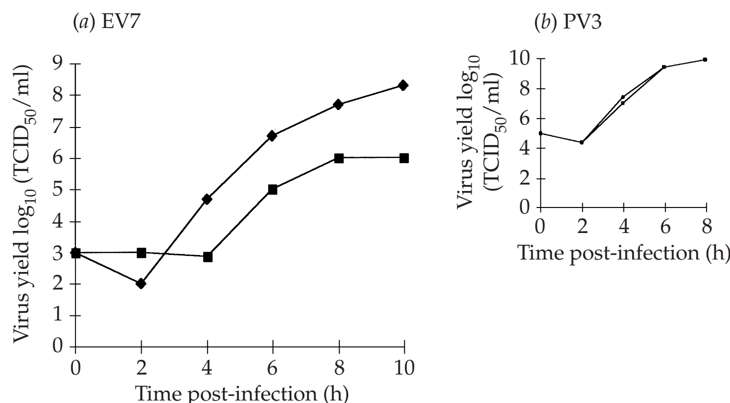

Fig. 4. Flasks (25 cm^2) of RD cells at 90% confluence were washed with serum-free DMEM and then inoculated with virus at 1 $TCID_{50}$ per cell. After 30 min adsorption at room temperature, cells were washed and then incubated with (■) or without (◆) 2% BSA (A-7638, globulin free) for 0–10 h in tissue culture to allow virus replication. Virus was then recovered from the cells by three cycles of freeze–thawing and titrated by $TCID_{50}$ on RD cells. Growth curves of EV7 (*a*) and PV3 (*b*) are shown.

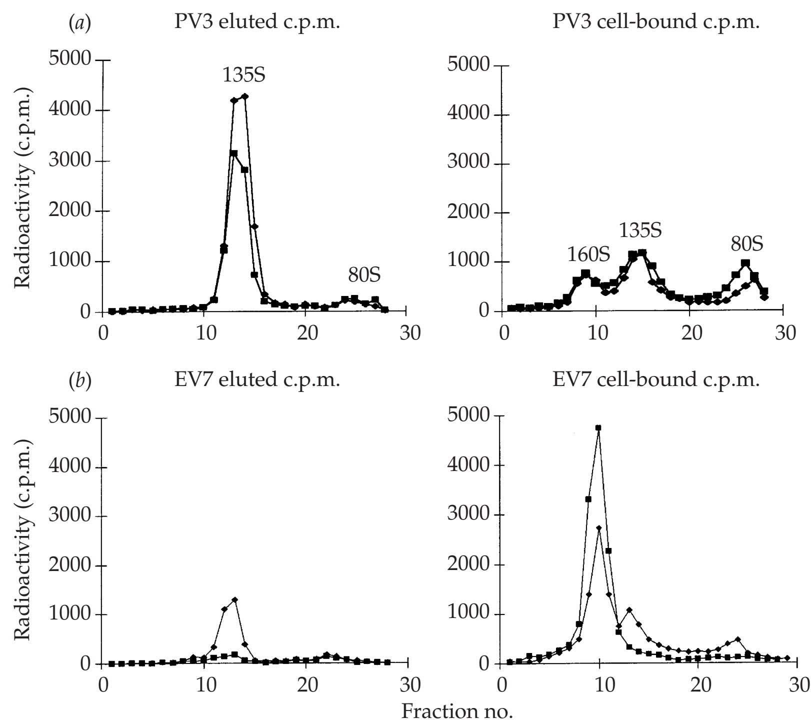

Fig. 5. RD cells were incubated on ice for 1 h with ^{35}S-labelled EV7 or PV3 (approx. 10^5 c.p.m. of each virus), as described in Methods, to permit adsorption. After washing, cells were either resuspended in serum-free DMEM (◆) or DMEM containing 2% BSA (■) (A-7638, globulin free). (*a*) PV3-treated cells were incubated for 1 h at 37 °C. (*b*) EV7-treated cells were then incubated for 2 h at 37 °C. Cells were pelleted and supernatants containing eluted A-particles were harvested. Cell pellets were then solubilized with NP-40. ^{35}S-labelled virus particles in the eluted fractions and in NP-40-solubilized cells were then sedimented through parallel 15–45% sucrose gradients for 4·5 h. Radioactivity in fractions was quantified by scintillation counting.

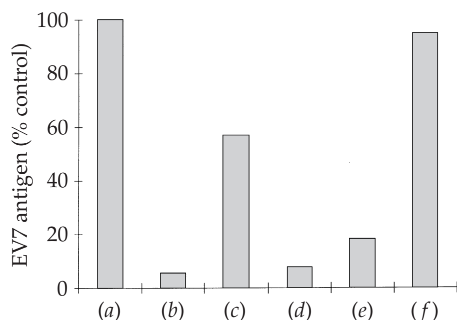

Fig. 6. RD cells at 80% confluence were incubated with EV7 (5 $TCID_{50}$ per cell) at room temperature for 30 min to permit virus adsorption and were then washed and incubated in tissue culture for 6 h in the presence of: (*a*) serum-free DMEM; (*b*) 2% BSA (A-7638, globulin free); (*c*) 2% BSA for 2 h followed by serum-free DMEM for 4 h; (*d*) 2% BSA for 2 h followed by rhodanine (200 μg/ml) for 4 h; (*e*) rhodanine; or (*f*) serum-free DMEM for 2 h followed by rhodanine for 4 h. Cells were then fixed and assayed for viral antigen using an immuno-assay as described in Methods.

of PV3 135S A-particles or 80S empty capsids, which accumulate within the cell (Fig. 5 *a*). For EV7-infected cells, the number of eluted A-particles was generally lower than for PV3 (Fig. 5 *b*), as previously observed (Powell *et al*., 1997). However, albumin completely blocked the release of EV7 A-particles. Moreover, cell-associated virus remained completely as 160S particles in the presence of albumin. In other experiments, in which EV7 viral RNA was transfected into RD cells by electroporation, albumin did not inhibit EV7 antigen production (data not shown). These results indicate clearly that EV7 uncoating, and not later stages of replication, is inhibited by albumin. PV3 uncoating, in contrast, is not affected.

This conclusion was confirmed by the results of experiments using rhodanine, a chemical that inhibits uncoating of EV12 completely, and of EV7 partially (Eggers *et al*., 1970; Eggers, 1977). Cells were infected with EV7 in the presence of albumin. After 2 h, the albumin was removed and replaced with rhodanine at 200 μg/ml. This treatment produced a blockage of infection at least as great as rhodanine alone (Fig. 6 *d*, *e*). Since the albumin blockage is reversible after 2 h treatment (Fig. 3 *c*), and since rhodanine alone has no effect if added 2 h after adsorption (Fig. 6 *f*) (Eggers, 1977), these results indicate that the stage of infection blocked by BSA is not later in infection than that blocked by rhodanine.

Collectively, the results suggest that albumin, like rhodanine, inhibits uncoating of the virus. The fact that albumin can block infection when added 30 min after adsorption suggests that EV7 uncoating is not completed within this time.

Discussion

Albumin blocks echovirus infection

We have shown that serum albumins, of both bovine and human origin, inhibit echovirus infection of RD and HeLa cells. Albumin preparations contain fatty acids and are contaminated with other proteins such as globulins (antibodies). Our results with globulin- and fatty acid-free albumins and highly pure monomeric BSA suggest that albumin per se is responsible for

the observed blockage of echovirus infection. The concentrations of albumin used in these studies (1–2%) might seem high, and it may be argued that the inhibition of EV7 infection by albumin was due to a non-specific effect of concentrated protein. However, 1–2% approximates to physiological concentrations of albumin (Rose *et al*., 1989) and no effect was seen on poliovirus infection. Furthermore, milk protein had no effect on EV7 infection and the level of the blockage by albumin was dependent on the species type used. Chicken albumin was less efficient than BSA and HSA; this result is probably due to species differences. We therefore suggest that albumin blocks echovirus infection by a specific mechanism. The possibility exists that albumin may also inhibit other viruses. Notably, a virus-neutralizing substance has been found in bovine and human serum that inhibits bovine enterovirus and not poliovirus (McFerran *et al*., 1968). Interestingly, this inhibitor was found in the albumin peak when serum was fractionated with Sephadex G-200. However, further work on the identity of this protein has not been reported. Infection by other viruses may also be modulated by albumin. For example, hepatitis B virus surface proteins (Krone *et al*., 1990) and the N-terminal peptide of human immunodeficiency virus type 1 gp41 (Gordon *et al*., 1993) interact with albumin. Furthermore, albumin blocks the binding of bovine respiratory syncytial virus to cells (Fassi Fihri *et al*., 1993).

Albumin inhibits uncoating

Our results show that albumin inhibits echovirus infection by a readily reversible mechanism and suggest that the molecular interactions involved are weak; the high concentrations of albumin required also support this. We have found that albumin prevents the eclipse phase of the echovirus replication cycle and inhibits 135S A-particle and 80S empty capsid formation. The albumin blockage, although reversible, was also found to be continued, after the removal of BSA, by rhodanine, a chemical that is known to inhibit echovirus uncoating (Eggers, 1977). Based on these observations, we suggest that albumin itself blocks EV7 infection by inhibiting the uncoating step. The mechanism of this blockage, however, remains unclear.

Albumin may bind to a secondary cellular factor required for virus uncoating. Against this hypothesis are the observations that albumin binds tightly to specific receptors on the cell surface and cannot be removed by simple washing (Wang *et al*., 1994). We have shown that the echovirus blockage is rapidly reversible upon removal of albumin by simple washing. However, we cannot rule out the possibility that albumin may be binding to the putative secondary factor through a weak hydrophobic interaction; this may be plausible, given the high concentrations of protein required to block infection. Alternatively, albumin may change the lipid:cholesterol ratio of caveolae or lipid rafts, the membrane sites where echoviruses are thought to be sequestered after binding to DAF, prior to uncoating (Bergelson *et al*., 1994). Albumin transports cholesterol to and from cells (Zhao & Marcel, 1996) and may increase the lipid:cholesterol ratio of cholesterol-rich caveolae and lipid rafts and thereby alter their interactions with DAF and/or echoviruses. Another possible hypothesis is that albumin directly stabilizes viral capsids. This is based on known protein-stabilizing properties of albumin; it protects thermolabile proteins, probably via hydrophobic interactions (Chang & Mahoney, 1995; Tessier *et al*., 1996). Moreover, the degree of surface hydrophobicity of proteins is thought to be a major determinant of this stabilizing effect. Where receptor binding leads directly to uncoating, such as for polioviruses and rhinoviruses, the receptor binding sites are surface hydrophobic depressions called canyons. All of the enteroviruses are structurally similar and are thought to possess some form of canyon. On binding of receptor at the canyon, the stabilizing hydrophobic pocket factor is thought to be displaced from the pocket situated on the floor of the canyon within the β-barrel of VP1, initiating uncoating (Rueckert, 1996). That binding of DAF to EV7 does not induce uncoating (Powell *et al*., 1997) suggests that DAF is an attachment protein that does not bind at the putative canyon, but somewhere else on the viral surface. The secondary factor may actually bind at the canyon and then induce uncoating. In support of our hypothesis that DAF-binding viruses require additional cell surface receptors for uncoating (Powell *et al*., 1997), coxsackievirus A21 and coxsackie B virus serotypes 1, 3 and 5 also use DAF for attachment, but require secondary receptors for infection (Shafren *et al*., 1997*a*, *b*). It is therefore probable that after attachment to DAF, the hydrophobic canyons on echoviruses may be unoccupied. We speculate that albumin may interact with these and possibly other viral surface depressions by weak hydrophobic interactions, and in doing so may stabilize the capsids or prevent the binding of the putative secondary factor.

Implications for echovirus infection *in vivo*

Serum albumin regulates osmotic pressure and the transport and delivery of fatty acids, steroids and amino acids (Kragh-Hansen, 1981). Albumin also maintains the selective permeability of the endothelial cell barrier. It is the most abundant protein in blood plasma (normal levels are 3·5–4·9%) and interstitial fluids (up to 2%) (Long, 1968; Rose *et al*., 1989) and is found at much lower levels in cerebrospinal fluid (CSF) (Long, 1968). During viral meningitis, high concentrations of albumin are found in CSF (Hung *et al*., 1995); this influx of albumin occurs because of virus-induced membrane damage. We speculate that prior to the albumin influx, the meninges are probably highly susceptible to infection by echoviruses. However, the rise in CSF albumin concentrations may actually reduce virus replication in the meninges and neighbouring tissues. The alimentary tract is also permissive for these viruses; we suggest that this may be due to the near-absence

of albumin in intestinal fluids. Conversely, we speculate that tissues such as muscle, bathed in albumin, may be less permissive for echoviruses.

Neonates and infants have lower serum albumin levels than adults (Long, 1968) and are particularly prone to systemic echovirus infections (Melnick, 1996). It will be interesting to investigate whether serum albumin levels and echovirus-associated pathology in neonates are linked. The possibility that albumin replacement therapy (Storch, 1993) could have an impact on systemic echovirus infections in infants is intriguing.

In humans, more than 50 different genetic variants of HSA have been found and shown to have different ligand-binding properties (Nielsen *et al*., 1997). The albumins used in our study constitute pooled material and therefore we have no information on whether all genetic variants of albumin block echovirus infection. If albumin does have an anti-echovirus property *in vivo*, it would be of great interest to test the blocking ability of the genetic variants of albumin. The concentrations of free fatty acids and other ligands that bind to albumin also vary in serum under different circumstances. For example, the demand for fatty acid-binding is increased during stress, fasting and in certain disease states, such as diabetes (Nielsen *et al*., 1997). Our results show that fatty acid-depleted albumin blocks echovirus infection. However, the ability of fatty acid-rich albumin to block infection has not yet been studied.

Preliminary results indicate that albumin inhibits other echovirus serotypes to varying degrees (unpublished data). Perhaps the range of associated diseases caused by echoviruses is modulated by receptor usage, interstitial albumin concentrations and serotype sensitivity to albumin.

The results of this work demonstrate that serum albumins, BSA and HSA, specifically block echovirus and not poliovirus infection by inhibiting the uncoating step in the virus replication cycle. This inhibition is rapid and reversible and suggests that weak hydrophobic interactions occur with either a secondary receptor or surface depressions on virions. The *in vivo* implications of this work, although speculative, may be significant. We suggest that, in man, echovirus infection, susceptibility, tropism and associated pathology and disease may be modulated by serum albumin.

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