Parameters influencing the attachment of hepatitis A virus to a variety of continuous cell lines

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We have investigated the interactions of purified radiolabelled hepatitis A virus (HAV) with a variety of continuous cell lines. Virus labelled either in vitro with radiolabelled iodine or in vivo with radiolabelled uridine bound to cells with similar efficiency. Attachment to BS-C-1 cells was calcium ion-dependent and this correlated with infectivity assay results. The cell tropism of HAV attachment was examined using cell suspensions and confluent cell monolayers at both 4 °C and 37 °C. The maximum level of attachment was observed at 4 °C with cells in suspension, but was severely inhibited by 2% foetal calf serum; these results again correlated with infectivity assays. The components of serum which inhibit attachment have been characterized by gel filtration chromatography, sucrose density gradient analysis, immunoprecipitation and Western blotting. The data show that such components are of high Mr and that the serum glycoprotein, α₂-macroglobulin, can partly mimic the inhibitory effect of whole serum.

Introduction

Hepatitis A virus (HAV) is a member of the Picornaviridae, but is unique in this family in its tropism for liver cells and its ability to cause liver injury (Lemon, 1985). Although the virus can be grown in a number of different cell types, it replicates slowly and produces a low final yield. In vitro culture often results in persistent infection, although this has been documented only rarely in vivo (Fagan et al., 1990).

In recent years the attachment of picornaviruses to susceptible cells has been the subject of considerable research. The cellular receptors have now been isolated for both poliovirus (Mendelsohn et al., 1986, 1989; Shepley et al., 1988) and the major group rhinoviruses (Greve et al., 1989; Staunton et al., 1989); however, the attachment of HAV has not been characterized extensively (Seganti et al., 1987, 1989). Viruses can interact with cells in a variety of ways (Dimmock, 1982; Hsu et al., 1988; Marsh & Helenius, 1989; Reagan et al., 1984); in addition to attaching to specific receptors, it has been shown that viruses can bind by indirect mechanisms, for example the attachment of dengue virus to cells bearing Fc receptors is enhanced by antibody raised to the virus (Halstead & O'Rourke, 1977), whereas cytomegalovirus exploits β₂-microglobulin in its interaction with cells (Grundy et al., 1987).

The natural route of HAV infection is faecal/oral, but whether virus is transported directly to the liver or undergoes primary replication at distal sites is still unclear. It is known that during viraemia, virus can be isolated from serum in association with immune complexes (Margolis et al., 1988; Margolis & Nainan, 1990). Virus grown in vitro is associated with host cell-derived material (Lemon & Binn, 1985) and has been shown to interact with the serum protein fibronectin (Seelig et al., 1984). It is possible that virion-bound host components play a role in virus attachment and dissemination.

In this paper we show that purified HAV will attach to a wide range of cultured cells and that attachment is affected by the cell type, the presence of calcium, temperature and the presence of serum. We also show that inhibitory factor(s) present in serum reside in the high Mr fraction obtained by gel filtration and that this inhibition can be partly mimicked by α₂-macroglobulin (α₂M).

Methods

Cell culture. Continuous cell lines were obtained from the European Collection of Animal Cell Cultures (ECACC, Porton Down, U.K.) or the ATCC, except Raw 264.7 and Hep G2 cells, which were kindly provided by Dr S. Lemon, University of North Carolina, Chapel Hill, NC., U.S.A., and propagated by standard techniques. Of the cells used in this study, only Hep G2 and BS-C-1 cells have been demonstrated to support the growth of HAV. The susceptibility of the other lines to HAV (175-18f) is unknown.
Virus growth and purification. The virus used was the tissue culture-adapted 18F isolate of the HM175 variant of HAV (kindly provided by Dr S. Lemon). This is a 'rapidly growing' cytolytic strain of virus which has an antigenic phenotype apparently identical to that of HM175.

Confluent BS-C-1 cell monolayers, between passages 65 and 85, were infected with HAV at an approximate multiplicity of 1 radioimmuno-focus-forming unit (r.f.u.) (Lemon et al., 1983) per cell for 1 h at 34 °C. Following adsorption, cells were maintained in Eagle's MEM (EMEM) containing 2% foetal calf serum (FCS). After incubation for 13 days at 34 °C, cells were subjected to three cycles of freezing and thawing. Cell debris was pelleted by low-speed centrifugation and resuspended in 10 mM-Tris-HCl, 10 mM-NaCl, 1.5 mM-MgCl2, 1% (v/v) NP40, 0.5% alkyl dimethyl amine betaine (Empigen; Albright and Wilson Chemicals) pH 7.6. The suspension was incubated at room temperature for 1 h, followed by sonication; the resulting suspension was clarified by low-speed centrifugation. An equal volume of saturated ammonium sulphate was added to the tissue culture supernatant obtained from the frozen and thawed cells. After 1 h at 4 °C, the precipitate was collected by centrifugation and resuspended in the supernatant obtained from detergent treatment of the cell debris. One third of a volume of 100 mM-Tris–HCl, 100 mM-NaCl, pH 7.6 was added and the resulting solution was adjusted to 3 mM-EDTA and 1% (w/v) sodium lauryl sarcosinate (Sarkosyl); when necessary, the pH was maintained at 7.6 by the addition of drops of 1.5 M-Tris-HCl pH 8.8. The mixture was incubated for 1 h at 37 °C, clarified by low-speed centrifugation and the virus was pelleted by centrifugation at 100000 g for 16 h at 5 °C in a Beckman Ti60 rotor. The resulting pellet was resuspended in 50 mM-Tris-HCl, 50 mM-NaCl, 0.1% Sarkosyl, pH 7.6 at 4 °C for 72 h and then extracted with an equal volume of chloroform. The chloroform layer was extracted with half a volume of 50 mM-Tris–HCl, 50 mM-NaCl, 0.1% (w/v) SDS. The aqueous layers were pooled, made 1% (w/v) SDS and applied to a linear 15% to 45% (w/v) sucrose gradient. Gradients were centrifuged at 100000 g for 4 h in a Beckman SW28 rotor.

Gradient fractions were assayed for the presence of viral antigen by radioimmunoassay (RIA) (Lemon et al., 1982). Purified virus was stored at −70 °C.

Infectivity assay. HAV infectivity was determined by radioimmuno-focus assay (RIFA) (Lemon et al., 1983). Virus was adsorbed to BS-C-1 cells as for attachment assays. After washing the monolayers, the cells were overlaid in EMEM supplemented with 2% FCS and containing 0.5% agarose, and incubated under 5% CO2 for 7 days at 35 °C. After incubation, the cells were pelleted by centrifugation at 90 s in an Eppendorf 5414 microfuge and washed twice in 0.5 ml of buffer, prewarmed or precooled to the temperature of incubation. The cell washings were pooled and the amount of radioactivity in the washings and cell pellet was determined by gamma counting or scintillation counting. Infected cell monolayers were fixed with acetone prior to transfer. Infected cell sheets, foci of virus replication were eluted with the buffer to be used in the assay.

Radioiodination of HAV. Approximately 2 μg of purified HAV was dialysed to 5 ml in 100 mM-Tris–HCl, 100 mM-NaCl, pH 7.6. Virus was pelleted by centrifugation at 210000 g for 2 h at 15 °C in a Beckman SW28 rotor, the pellet was resuspended in 15 μl 100 mM-borate buffer pH 8.2, containing 0.1% Sarkosyl, and virus was radioiodinated using 500 μCi 125I-labelled Bolton and Hunter reagent (Bolton & Hunter, 1973) (Amersham). Following iodination, the virus was applied to a Sephadex G-100 column and eluted in 100 mM-Tris–HCl, 100 mM-NaCl, 0.1% Sarkosyl, pH 7.6. The first peak of radioactivity, eluting in the column void volume, was pooled and stored at −70 °C.

Aliquots of radioiodinated HAV (125I-HAV) were applied to linear 15% to 45% (w/v) sucrose gradients containing 0.001% bovine serum albumin (BSA), fraction V, (BD Chemicals) and centrifuged to 200000 g for 2 h at 15 °C in a Beckman SW40 rotor; fractions were assayed for the presence of 125I by gamma counting. Radiolabelled HAV antigen was detected by antibody-capture RIA. Fractions containing the viral antigen peak were pooled and stored at −70 °C. Prior to their use in attachment assays, sucrose was removed from the fractions by applying the 125I-HAV to a Sephadex G-100 column and eluting with the buffer to be used in the assay.

Growth of metabolically radiolabelled HAV. BS-C-1 cells were infected with HAV at high multiplicity (approximately 25 r.f.u./cell) for 1 h at 35 °C. After a further 6 h incubation in EMEM containing no FCS, the cell monolayers were washed, the medium was replaced, and [3H]thymidine was added (250 μCi, specific activity 40 Ci/mmol) immediately and at approximately 24 h intervals thereafter. Cells were incubated at 35 °C for 7 days and virus was purified as described above except that 0.001% BSA was included in the sucrose gradient.

Attachment assays

(i) Cell suspension assays. Confluent cell monolayers were washed and mechanically removed (scraped) into cold buffer. The cells were washed twice by low-speed centrifugation, resuspended in fresh cold buffer and 106 cells were added to a 1.5 ml Eppendorf tube. Assays were started by the addition of between 600 and 1000 c.p.m. radiolabelled HAV to give a final volume of 1 ml. The tubes were gently agitated during the incubation period.

After incubation, the cells were pelleted by centrifugation for 90 s in an Eppendorf 5414 microfuge and washed twice in 0.5 ml of buffer, prewarmed or precooled to the temperature of incubation. The cell washings were pooled and the amount of radioactivity in the washings and cell pellet was determined by gamma counting or scintillation counting. U937, THP 1 and HL60 cells are non-adherent and could be transferred directly to an Eppendorf tube for attachment assays.

(ii) Monolayer attachment assays and infectivity assays. Confluent cell monolayers grown in 60 mm dishes were washed twice in cold buffer and inoculated with 0.2 ml HAV. After incubation, the cells were washed twice in several millilitres of buffer at the appropriate temperature. For attachment assays, the cell monolayers were dissolved in formic acid and dried onto glass fibre filter discs prior to scintillation counting, or dissolved in 1% SDS, prior to gamma counting, and the amount of radioactivity in the pooled washings and cellular material was measured. Virus attachment assay results are expressed as the percentage of cell-associated virus (%CAV). This figure represents the proportion of total recovered radioactivity (cells plus combined supernatants) associated with cellular material.

Gel chromatography. HAV antibody-negative human serum and FCS were separated on a Supersose 12HR (10/30) column connected to a Pharmacia FPLC system. The column was equilibrated in complete PBS and eluted at a flow rate of 0.9 ml/min. The protein concentration was monitored by measuring the absorbance at 280 nm. Fractions were stored at −70 °C.

Western blot analyses. Serum protein samples were adjusted to 0.1% SDS and electrophoresed on 6% polyacrylamide gels, or boiled for 1 min in 2% SDS, 2% 2-mercaptoethanol prior to gel electrophoresis (Laemmli, 1970; Mischak et al., 1988). The proteins were electrophoretically transferred to nitrocellulose membranes for 1 h at 40 mA and the membranes were blocked with 2% BSA, 1% Tween 20 (Sigma) in complete PBS for 75 min. Following blocking, membranes were incubated for 75 min with unlabelled HAV diluted in complete PBS containing 1% BSA, washed three times in blocking buffer and then incubated for 75 min with approximately 107 c.p.m. 125I-labelled anti-HAV monoclonal IgG (BS83, a kind gift from Dr R. Tedder, Middlesex Hospital Medical School, London, U.K.) or a human monoclonal antibody supplied by Dr N. R. Parry, Virology R&D, Wellcome Biotechnology, Beckenham, U.K.), diluted in complete PBS, 1% BSA. After further washing in blocking buffer, the membrane was air-dried and autoradiographed.

In some experiments virus was pretreated with excess human polyclonal anti-HAV IgG or a control (i.e. HAV-negative) IgG before being applied to membranes.
Assays for protein association with virus. \(^{125}\)I-HAV was mixed with serum proteins, including purified human \(\alpha_1\)M, FCS and the FCS Superose 12 column void volume fraction (fraction 1); samples were diluted to 0.4 ml in complete PBS, 0.5% BSA. As a control, HAV was mixed with buffer plus BSA alone. After incubating for 90 min at 4 \(^\circ\)C, the mixtures were applied to 15 to 45% linear sucrose gradients containing 0.001% BSA and centrifuged at 200,000 \(g\) for 2 h at 4 \(^\circ\)C. Fractions of approximately 0.5 ml were collected and aliquots assayed for radioactivity by gamma counting; peak fractions were pooled.

For radioimmunoprecipitation analysis (RIPA), 0.4 ml aliquots of the pooled sucrose gradient peak fractions were added to 0.1 ml samples of dilutions of anti-human \(\alpha_1\)M antisera in PBS, 0.1% BSA. After incubation for 18 h at 4 \(^\circ\)C, 0.1 ml 1% washed staphylococcal ghosts (Pansorbin; Calbiochem) was added to each mixture. After 75 min at room temperature the precipitates were collected by centrifugation for 30 s in an Eppendorf 5414 microfuge. Pellets were washed twice in 50 mM-Tris-HCl, 50 mM-NaCl, 1 mM-EDTA, 0.1% NP40 and radioactivity in the pellets and supernatants was determined.

Protein assays. The protein concentration in serum samples and gel chromatography fractions was determined by a BCA protein assay, used according to the manufacturer's instructions (Pierce and Warriner).

Results

Antigenic components of HAV

Purification of HAV on sucrose gradients resulted in two major peaks of antigenicity, representing mature virions and procapsids (Fig. 1a) (Gauss-Muller et al., 1986). Following in vitro radiiodination of virions, both radioactive counts and most of the antigenicity recycled to the same positions in sucrose gradients, showing that the iodination procedure had not significantly damaged the antigenic structure of the virus (Fig. 1b). Following purification of virus radiolabelled metabolically with \(^3\)H-uridine (\(^3\)H-HAV), only the virion peak contained radioactivity, although two peaks were detected by RIA (Fig. 1c).

HAV attachment and infectivity

The dependence of HAV attachment and infectivity upon divalent cation species and their concentration is shown in Fig. 2. Results of infectivity assays, in which HAV was adsorbed to cells in buffer containing varying concentrations of Mg\(^{2+}\) or Ca\(^{2+}\), correlated with attachment assay results, although infectivity appeared more sensitive to ion concentration. Magnesium ions did not affect HAV attachment or infectivity significantly, whereas calcium ions at various concentrations enhanced both approximately 10-fold. The cells used in these assays were viable as assessed by trypan blue dye exclusion.

The level of attachment of HAV to a variety of continuous cell lines is shown in Table 1. The general trends can be summarized as follows. (i) \(^{125}\)I-HAV and \(^3\)H-HAV attached to similar extents to suspended cells; (ii) the level of attachment was usually greater at 4 \(^\circ\)C than at 37 \(^\circ\)C; (iii) in general, the amount of attachment to suspended cells was greater than to confluent monolayers; (iv) serum usually inhibited attachment; (v) the inhibition of attachment due to serum was usually greater at 4 \(^\circ\)C than at 37 \(^\circ\)C and was more pronounced.
Fig. 2. Ion dependence of virus attachment and infectivity. BS-C-1 cells were suspended and mixed with $^{125}$I-HAV for attachment assays (○, ■) or incubated as monolayers with dilutions of unlabelled virus for infectivity assays (○, □). In both cases virus and cells were incubated for 1 h at 4 °C in 0.85% saline, 0.5% BSA, 20 mM-HEPES pH 7.3 containing various concentrations of CaCl$_2$ (○, ○) or MgCl$_2$ (□, □). Suspended cells were then sedimented to allow the assay of bound virus; monolayer cells were overlaid with agar and incubated at 35 °C for infectivity assays.

Table 1. Attachment of HAV to cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Suspended cells</th>
<th>Monolayers</th>
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<tr>
<td></td>
<td>FCS (%)</td>
<td>125I-HAV</td>
</tr>
<tr>
<td></td>
<td>4 °C † 37 °C 4 °C</td>
<td>37 °C</td>
</tr>
<tr>
<td>BS-C-1</td>
<td>0 33.5† 6.1</td>
<td>33.5 15.4</td>
</tr>
<tr>
<td>(African green monkey</td>
<td>2 0 † 0.7 0 0.7</td>
<td>80</td>
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<tr>
<td>kidney)</td>
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<td></td>
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<tr>
<td>Hep G2</td>
<td>0 11.6 7.0</td>
<td>12.3 10.1</td>
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<tr>
<td>(human hepatocellular</td>
<td>2 1.0 1.9 1.7 0.1</td>
<td>16</td>
</tr>
<tr>
<td>carcinoma)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WR-168</td>
<td>0 10.2 2.7</td>
<td></td>
</tr>
<tr>
<td>(human embryo liver)</td>
<td>2 0.5 0.7</td>
<td></td>
</tr>
<tr>
<td>Chimp</td>
<td>0 7.2 6.4</td>
<td></td>
</tr>
<tr>
<td>(chimpanzee liver)</td>
<td>2 1.3 2.8</td>
<td></td>
</tr>
<tr>
<td>Chang</td>
<td>0 32.5 8.6</td>
<td></td>
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<tr>
<td>(human liver)</td>
<td>2 0 0.2</td>
<td></td>
</tr>
<tr>
<td>Raw 264.7</td>
<td>0 50.7 2.3</td>
<td>47.6 4.8</td>
</tr>
<tr>
<td>(mouse leukaemia</td>
<td>2 1.9 0 0 0</td>
<td>8.4 17.2</td>
</tr>
<tr>
<td>monocyte/macrophage)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HeLa</td>
<td>0 17.3 1.1</td>
<td>80</td>
</tr>
<tr>
<td>(human cervical</td>
<td>2 0 0</td>
<td></td>
</tr>
<tr>
<td>carcinoma)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDCK</td>
<td>0 46.3 2.9</td>
<td></td>
</tr>
<tr>
<td>(Canine kidney</td>
<td>2 5.7 0</td>
<td></td>
</tr>
<tr>
<td>epithelium)</td>
<td></td>
<td></td>
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<tr>
<td>L cells</td>
<td>0 45.5 5.2</td>
<td></td>
</tr>
<tr>
<td>(mouse lung</td>
<td>2 5.9 0</td>
<td></td>
</tr>
<tr>
<td>fibroblasts)</td>
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</tr>
<tr>
<td>U937</td>
<td>0 42.4 2.8</td>
<td></td>
</tr>
<tr>
<td>(human histiocytic</td>
<td>2 0.4 0</td>
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<tr>
<td>lymphoma)</td>
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</tr>
<tr>
<td>THP-1</td>
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<td>(human monocyte)</td>
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</tr>
<tr>
<td>HL-60</td>
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<td></td>
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<tr>
<td>(human lymphoblast)</td>
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</table>

* FCS present during incubation.
† Temperature of incubation.
‡ All results are expressed as %CAV.

Fig. 3. Temperature- and serum-dependence of HAV infectivity. Virus was diluted in complete PBS (rows 1) or PBS containing 2% FCS (rows 2), and adsorbed to BS-C-1 cells at 4 °C (a) or 37 °C (b). Infectivity was assayed by RIFA. For adsorption at 4 °C, the results obtained using 10$^{-5}$ and 10$^{-6}$ dilutions of HAV are shown, for adsorption at 37 °C, results obtained using 10$^{-4}$ and 10$^{-5}$/dilutions of HAV are shown. The final titres are also indicated.

with suspended cells than with confluent monolayers, in which the presence of serum usually slightly enhanced attachment; (vi) virus attached to all of the cells used.

Infectivity assays, in which HAV was adsorbed to BS-C-1 cells at 4 °C or 37 °C in the presence or absence of 2% FCS, are shown in Fig. 3. The highest titre was obtained at 4 °C in the absence of serum and was reduced approximately fivefold in the presence of serum; at 37 °C, serum slightly enhanced the infectivity titre. Thus, attachment and infectivity results correlated and showed that serum had a marked inhibitory effect on virus/cell association at both 4 °C and 37 °C with suspended cells, and at 4 °C with cell monolayers.
Attachment of HAV to continuous cell lines

Fig. 4. The effects of temperature shift on HAV attachment. Cell suspension attachment assays were carried out at 4 °C for 1 h using 125I-HAV. After this period the cells were pelleted, washed twice in cold buffer, resuspended in 1 ml of cold complete PBS and then transferred to 37 °C for various times. Following this, the cells were pelleted and washed at 37 °C in complete PBS. The vertical axis represents the percentage of virus attached at 4 °C that remained bound after transfer to 37 °C. (●) BS-C-1 cells; (■) U937 cells; (▲) THP-1 cells; (○) HL60 cells. The %CAV at 4 °C is shown in Table 1.

Elution of attached virus

Following attachment to a range of cell lines at 4 °C, 40% to 60% of 125I-HAV eluted when the temperature was raised to 37 °C for 90 min (Fig. 4).

Characterization of inhibitory components in serum

The inhibitory effect of 2% serum on the attachment of 125I-HAV to suspended BS-C-1 cells at 4 °C was not duplicated by 10% BSA, 10% ovalbumin or an Escherichia coli cell lysate (unpublished results). However, chloroform-extracted FCS and a lipoprotein-deficient FCS (Sigma) still inhibited HAV attachment very efficiently at a concentration of 2%. Both fibrinogen and α2M (Sigma) inhibited HAV attachment by 50% at concentrations of approximately 3 mg/ml and 400 μg/ml respectively; the levels of these components in normal serum are approximately 3 mg/ml and 2.5 mg/ml respectively. At this concentration α2M inhibited attachment by approximately 90% (Fig. 6).

To investigate further the inhibitory components of serum, FCS and HAV antibody-negative human serum were separated by gel filtration chromatography, and the eluted fractions were assayed for their effects on HAV attachment; inhibitory components eluted in the column void volume (fraction 1) for both FCS and human serum. A lower level of inhibitory activity also eluted in the second fraction of human serum (Fig. 5).

Fig. 5. Gel filtration of serum. Samples (0.5 ml) were applied to a Superose 12 column and eluted in complete PBS to give the elution profile of FCS (a), or HAV antibody-negative human serum (b). The elution position of size markers is indicated. The effect of each fraction on the attachment of 125I-HAV to BS-C-1 cells was determined by mixing cells and virus at 4 °C with a 10% (v/v) dilution of each fraction. The %CAV obtained is shown.
The inhibitory effects of different concentrations of serum, fraction 1 and purified human α2M on virus attachment are shown in Fig. 6. In all cases the inhibition caused by serum or fraction 1 was much greater than that caused by an equivalent concentration of purified α2M alone. The components eluted in fraction 1 were more efficient at inhibiting HAV attachment than whole serum. Fraction 1 contained approximately 500 μg/ml protein and the major component comigrated with α2M on 6% SDS-polyacrylamide gels or 1% agarose gels (data not shown).

Effects of attachment inhibitors on the sedimentation coefficient of HAV

Sucrose gradient profiles of 125I-HAV mixed with α2M, FCS or FCS fraction 1 are shown in Fig. 7. Treatment of the virus with α2M resulted in a slight reduction in the sedimentation rate, whereas FCS caused the virus to ‘smear’ towards the top of the gradient. Virus mixed with fraction 1 remained near the top of the gradient; similar effects were observed with 3H-HAV and human serum (data not shown). Shown also in Fig. 7 is the percentage of the radioactivity in the pooled peaks which could be precipitated with anti-human α2M antiserum. Whereas 30 to 40% of 125I-HAV which had been mixed with α2M
Attachment of HAV to continuous cell lines

Fig. 8. Binding of HAV to serum protein and α2M immobilized on nitrocellulose, following separation on denaturing or non-denaturing 6% SDS–polyacrylamide gels. (a) Coomassie blue G250-stained non-denaturing gel. (b to e) Western blots of serum proteins reacted with HAV. Parts (b), (d) and (e) were from non-denaturing gels, (c) was from a denaturing gel. In (d) virus was reacted with control IgG, and in (e) with anti-HAV polyclonal IgG, prior to being applied to the blot. Bound virus was detected by autoradiography following reaction with 125I-labelled anti-HAV monoclonal antibody. Lanes 1, FCS; lanes 2, α2M; lanes 3, FCS fraction 1.

was immunoprecipitated, 125I-HAV alone and 125I-HAV which had been mixed with FCS or FCS fraction 1 were not immunoprecipitated. The interaction of fraction 1 with 125I-HAV appeared to be weak because, after storage at 4 °C for 16 h, approximately half of the radioactivity in fractions from the top of the gradient recycled to the expected position for free virus (data not shown). This result also shows that the virus/fraction 1 mixture sediments near the top of the gradient because of the interaction between the two components, and not because of virus degradation.

Western blotting of serum inhibitory components

The ability of serum proteins, including α2M, to bind virus after separation on reducing or non-reducing 6% SDS–polyacrylamide gels and transfer to nitrocellulose membranes was investigated. Under these gel separation conditions, α2M again comigrated with the major component of fraction 1 from FCS (Fig. 8a). After blocking of the membranes with BSA and Tween 40, virus mixed with neutralizing or control human IgG was added to the nitrocellulose membranes. Bound virus was detected by autoradiography following reaction with 125I-labelled anti-HAV MAb.

Immobilized proteins separated from whole serum, FCS fraction 1 or purified human α2M were capable of interacting with virus (Fig. 8b); virus bound to α2M and the major components of FCS and FCS fraction 1, which comigrated with α2M with an $M_r$ in excess of 200K. When the samples were boiled in SDS and 2-mercaptoethanol prior to electrophoresis, virus still bound to the immobilized proteins. Under these conditions the major reacting species in the FCS lane had an $M_r$ of approximately 170K, which corresponds to the reported size of the α2M monomer (Barrett et al., 1979). A similar protein could be seen in the fraction 1 sample but virus bound more extensively to a band migrating at approximately 110K, the reported $M_r$ of a proteolytic fragment derived from α2M (Barrett et al., 1979). The band in purified α2M binding virus most strongly also had an $M_r$ of 110K, suggesting that this material had been extensively cleaved.

Although pretreatment of the virus with non-immune human IgG did not affect its association with the immobilized proteins (Fig. 8d), IgG from convalescent serum severely reduced virus binding (Fig. 8e).

Discussion

Although radioiodination caused some virus particle breakdown, the labelled material that sedimented to the position of virus in sucrose gradients retained virus antigenicity and attached to cells in a similar extent as metabolically labelled virus. It therefore seemed reasonable to use virus labelled in vitro to examine virus–cell interactions.

We have examined a wide range of cells for their ability to attach virus and were surprised that all allowed attachment. However this does not imply that all the cells are permissive for virus growth nor that all cells contain a specific virus receptor. The attachment to all cells was temperature-dependent, the level of attachment being greater at 4 °C than at 37 °C. The reason for this is unclear; viruses are known to 'slough' from cells at 37 °C (Joklik & Darnell, 1961; Fenwick & Cooper, 1962; Crowell & Philipson, 1971), but experiments in which virus was allowed to attach to cells at 4 °C after which the temperature was increased to 37 °C indicated that, although virus was released at the increased temperature, the magnitude of the release was insufficient to account totally for the difference observed in the level of attachment at the two temperatures. It is also possible that at 4 °C virus receptors are spread evenly over the cell surface, thus allowing more virus to attach than at 37 °C, at which temperature receptor recruitment (capping) might lead to a depletion in the number of available virus-binding sites. In the absence of serum, the level of attachment to suspended cells at 4 °C was greater than that to cell monolayers. However, at 37 °C the extent of attachment depended more on cell type than whether the
cells were in suspension or as monolayers. This effect may be due to a differential, polarized distribution of receptors on the basal and apical surfaces of different cells (Rodriguez-Boulan & Nelson, 1989).

The levels of attachment and infectivity of HAV were low in the absence of divalent cations, and were enhanced by the addition of calcium, but not magnesium ions, a result also observed by Stapleton et al. (1990). It is well known that calcium is required for the attachment of many viruses and, for HAV, may act as a ligand in the formation of the virus–cell receptor complex. Alternatively, calcium ions may increase the level of attachment by altering the capsid structure, as has been observed for other small RNA viruses (Robinson & Harrison, 1982).

A surprising result was the finding that 2% FCS inhibited the attachment of virus to most cell lines, particularly at 4°C. The inhibition of attachment correlated with reduced infectivity titres when virus was adsorbed at 4°C in the presence of FCS. This suggests that FCS does not simply block non-specific and non-productive binding of the virus to cells. Gel filtration studies revealed that the components of both FCS and human sera which inhibited attachment were of high Mr and eluted in the void volume (fraction 1) of the column (Fig. 5); this ‘purified’ material had a higher specific inhibitory activity than the original serum. Only fraction 1 of FCS, and fraction 1 and, to a lesser extent, fraction 2 of human serum had inhibitory activity. BSA, ovalbumin and an E. coli lysate extract were negative, indicating that inhibition was not simply due to non-specific binding of proteins to the virus. The major protein component of fraction 1 comigrated in gel electrophoresis with α₂M; however, whole serum and fraction 1 both block virus attachment at much lower concentrations than purified α₂M. α₂M is a plasma glycoprotein which has the property of binding and inhibiting many endopeptidases, these complexes are then rapidly cleared from the circulation, thus controlling extracellular proteolytic activity. It is interesting to speculate that HAV could make use of this mechanism to be transported to the liver, because no other primary site of replication has been convincingly demonstrated.

The interactions of the various inhibitory components with HAV were demonstrated by their effects on the sedimentation of the virus in sucrose gradients. The greatest effect was observed with fraction 1, which caused most of the virus to sediment near the top of the gradient, probably due to virus interaction with lipoprotein or other serum components to form loose aggregates. The reaction with α₂M was less dramatic, resulting in only a slight reduction of the sedimentation coefficient; however, a significant amount of this virus was immunoprecipitated with anti-human α₂M IgG. The lack of any comparable precipitation of virus mixed with FCS or fraction 1 could be due to either antigenic differences between the human and bovine α₂M molecules, or the antigenic masking of α₂M with other proteins present in FCS. The latter explanation is plausible because Seelig et al. (1984) have demonstrated that fibronectin inhibits the detection of HAV by solid-phase RIA and that HAV may complex with a number of serum components to give immune complexes in vivo (Margolis et al., 1988; Margolis & Nainan, 1990).

Non-denaturing Western blot analysis showed that large Mr serum proteins and α₂M can interact with virus and that this interaction is prevented by preincubation of the virus with neutralizing IgG. When the proteins were fully denatured and reduced prior to electrophoresis, the reaction with the immobilized proteins was still observed, but at a reduced level. However the electrophoretic migration of the reactive species under these conditions corresponded to an Mr of approximately 170,000, consistent with the size of the α₂M subunit and with a smaller Mr protein which may represent a protease ‘nicked’ form of α₂M (Barrett et al., 1979). Thus it appears that the reaction of HAV with α₂M or FCS is not dependent on the quaternary structure of the protein.

It is unlikely that α₂M is the only serum protein which interacts with HAV and there are probably important differences between the situation in vivo and in vitro. However, it is possible that the HAV/serum complex may act as a vehicle for the transport of virus from the intestine to its primary site of replication in hepatocytes. It is known that α₂M can interact with macrophages, fibroblasts and hepatocytes (Debanne et al., 1976), and therefore HAV may attach to such cells via bound α₂M. The virus may also infect secondary organs of replication such as the oropharyngeal tract (Cohen et al., 1989) using such a surrogate receptor-binding mechanism. Hopefully future work will clarify the biological significance of the interaction between HAV and serum proteins.

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References


