Incomplete Neutralization of Hepatitis A Virus in vitro due to Lipid-associated Virions

By STANLEY M. LEMON* AND LEONARD N. BINN

547 Burnett-Womack CSB, 229H, Division of Infectious Diseases, Department of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27514 and 1Department of Virus Diseases, Walter Reed Army Institute of Research, Washington, D.C., U.S.A.

(Accepted 25 July 1985)

SUMMARY

Hepatitis A virus (HAV) released from infected BS-C-1 cells was incompletely neutralized when incubated with a variety of convalescent sera (non-neutralizable fraction of 17 to 32%). Chloroform extraction of virus resulted in a substantial reduction of the non-neutralizable fraction (to less than 1%), suggesting that non-neutralizable virions might be associated with lipids. Non-neutralizable HAV recovered from untreated cell culture supernatant fluids sedimented heterogeneously and less rapidly than normal virus in rate-zonal sucrose gradients and also banded at a lower density in CsCl (1.14 to 1.18 g/ml) than normal, neutralizable virus (1.32 g/ml). This bimodal distribution of HAV in CsCl gradients was confirmed by cDNA–RNA hybridization. Together, these observations suggest that a substantial proportion of HAV particles released from infected cells are lipid-associated and imply an important role for cell membranes in the assembly and release of HAV in vitro.

The primary isolation and passage of hepatitis A virus (HAV) in cell culture was first described in 1979 (Provost & Hilleman, 1979), and has since been achieved in many laboratories (Frosner et al., 1979; Flehmig, 1980; Daemer et al., 1981; Binn et al., 1984). Although the infection is invariably non-cytopathic, reports from various laboratories differ concerning the degree to which virus is released from cells. In some systems, virus appears to be tightly cell-associated (Frosner et al., 1979; Daemer et al., 1981) while in others there is substantial release of virus into culture medium (Flehming, 1980; Binn et al., 1984). These differences remain unexplained, as is the mechanism by which HAV may be released in the absence of cytopathology. However, the titre of virus found in supernatant culture fluids may approximate that of cell-associated virus (Binn et al., 1984), suggesting that release of virus is not related to non-specific degeneration of infected cells or apparent cell death. During studies on the neutralizing antibody response to HAV, we encountered a large, non-neutralizable virus fraction in preparations made from cell culture supernatant fluids (Lemon & Binn, 1983). In this report, we describe experiments which indicate that non-neutralizable HAV virions are tightly associated with lipids, and may be largely eliminated by extraction with chloroform. While of practical importance, these observations also suggest that cell membranes play an important role in the assembly and release of HAV from infected cells.

The HM-175 strain of HAV used in these experiments had been adapted to growth in continuous green monkey kidney (BS-C-1) cells (55th to 65th passage level) as described previously (Lemon et al., 1983; Binn et al., 1984). Virus was harvested by collection of supernatant culture fluid (Eagle’s MEM with Earle’s salts and 2% foetal bovine serum) 21 days after inoculation of cells, followed by clarification at 7000 g for 30 min. This virus had been passed 10 times in African green monkey kidney (AGMK) to cells, and 1 to 4 times in BS-C-1.
Table 1. Residual, non-neutralized virus following 60 min incubation of HAV with immune serum

<table>
<thead>
<tr>
<th>Virus</th>
<th>Serum*</th>
<th>log₁₀ Inoculum r.f.u./ml</th>
<th>log₁₀ Residual r.f.u./ml</th>
<th>% Non-neutralized</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stock virus</td>
<td>GR002</td>
<td>2.17</td>
<td>1.42</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>GR002</td>
<td>3.80</td>
<td>3.31</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>GR002 + anti-IgG</td>
<td>3.80</td>
<td>3.27</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>CH953</td>
<td>4.26</td>
<td>3.76</td>
<td>31</td>
</tr>
<tr>
<td>CHCl₃-extracted</td>
<td>GR002</td>
<td>2.42</td>
<td>0.30</td>
<td>0.77</td>
</tr>
<tr>
<td></td>
<td>GR002</td>
<td>3.38</td>
<td>0.30</td>
<td>0.83</td>
</tr>
<tr>
<td></td>
<td>CH953</td>
<td>4.10</td>
<td>2.07</td>
<td>0.92</td>
</tr>
</tbody>
</table>

*GR002 and CH953 are late convalescent human and chimpanzee sera respectively; each was tested at a dilution of 1:10. Where indicated, anti-human IgG was added to virus-serum mixtures.

Attempts to neutralize HAV present in supernatant fluids of infected cell cultures with a variety of immune primate sera repeatedly demonstrated a large, non-neutralizable fraction of virus (up to 32%) (Table 1). This fraction persisted after incubation with high concentrations of immune serum (1:10), when either early, acute or late convalescent sera were tested, and after extending the time of the neutralization reaction to 2 h (data not shown). The addition of affinity-purified goat anti-human IgG (Kirkegaard and Perry, Rockville, Md., U.S.A.) to mixtures of HAV and immune human sera, followed by incubation for an additional 30 min at 37°C, did not result in more complete neutralization of the virus. However, two equal-volume extractions of the HAV preparation with chloroform eliminated over 97% of non-neutralizable virus (reduction of this fraction from between 17 and 32% to <1%) (Table 1).

The substantial reduction of the non-neutralizable fraction following extraction with chloroform suggested that non-neutralizable virus might have been protected from antibody by association with chloroform-sensitive lipids. We therefore examined the sedimentation characteristics of neutralizable and non-neutralizable HAV. Virus (0.2 ml) was layered onto a 5 ml 10 to 30% linear sucrose gradient which was centrifuged at 160000 g for 120 min at 4°C. Fractions (0.25 ml each) were collected from the bottom of the gradient and replicate 1:10 dilutions of each were immediately mixed with either a 1:10 dilution of immune chimpanzee serum or foetal calf serum. Mixtures were incubated for 60 min at 37°C and assayed for residual virus by radioimmunofocus assay (Fig. 1). Non-neutralizable virus sedimented less rapidly and more heterogeneously than the predominant population of neutralizable virions. Approximately 10% of the virus present in fractions 16 to 20 was not neutralized following incubation with antibody, whereas less than 0.2% of the virus present in the peak fractions 12 to 14 was non-neutralizable. Were the non-neutralizable HAV fraction primarily related to aggregation of virus (Wallis & Melnick, 1967), non-neutralizable virus would have sedimented more rapidly than single virions and should have been found in the lower regions of the gradient. Instead, the observed distribution of non-neutralizable virus suggested that it was associated with very low density material, possibly lipids.

We next compared the buoyant density of neutralizable and non-neutralizable virus in CsCl. Stock virus was layered onto a pre-formed step gradient consisting of 1.4, 1.3, 1.2 and 1.1 g/ml aqueous solutions of CsCl (1 ml each). Gradients were spun at 165000 g for 16 to 22 h at 4°C, and fractions (0.25 ml each) were collected from the bottom. Selected fractions, chosen on the basis of density estimated from the refractive index, were assayed immediately after collection for virus titre and percent non-neutralizable virus. Two broad but discrete bands of infectious virus...
were identified in this gradient (Fig. 2). The major population of infectious HAV particles banded at a density of approximately 1.32 g/ml. This banding density is similar to that which we have observed previously for the major component of cell culture-derived virus in self-forming CsCl gradients (1.325 g/ml) (Lemon et al., 1985), and is somewhat less than that previously reported for virus derived from faeces. Virus found at this density was completely neutralized by antibody (less than 0.1% non-neutralizable virus), even in the absence of chloroform extraction (Fig. 2). However, a second, broad band of infectious virus was found between 1.14 g/ml and 1.18 g/ml, and a substantial proportion of this virus was found to be resistant to antibody-mediated neutralization (52% non-neutralizable virus in fraction 13). Virus found in the first peak (1.32 g/ml) was detectable by solid-phase radioimmunoassay [maximum positive: negative (P/N) ratio 5.3; positive defined as P/N > 2.1] (Lemon et al., 1982). The second virus peak was not detected by this method, possibly because it contained lesser amounts of virus (maximum P/N 1.7). Previously described dense (1.40 to 1.44 g/ml) and light (1.27 g/ml) infectious HAV particles (Lemon et al., 1985) were not identified in this gradient because of the small quantity of virus applied and the relatively low resolution achieved with this broad step gradient.

cDNA–RNA hybridization (Lemon et al., 1985) confirmed the presence of two broad peaks of viral RNA within CsCl gradients containing virus which had not been treated with chloroform (Fig. 3). Virus in both peaks could be detected by an immunoaffinity cDNA–RNA hybridization procedure (R. W. Jansen, J. E. Newbold & S. M. Lemon, unpublished results) in which virus in each gradient fraction was first adsorbed to and then eluted from monoclonal anti-HAV antibody immobilized on PVC microtitre plates (data not shown). These results, and the partial neutralization of virus banding between 1.14 g/ml and 1.18 g/ml (Fig. 2), suggest that at least a portion of the lighter, lipid-associated virus is available for binding to antibody. However, whether or not those virions escaping virus neutralization bind any antibody at all remains uncertain.

These experiments demonstrate that a substantial proportion (as much as 30%) of HAV released into supernatant fluids by non-cytopathically infected BS-C-1 cells is not neutralized by immune sera. That this non-neutralizable virus fraction is largely due to the association of lipid with some virions is suggested by the slower sedimentation of non-neutralizable virus in rate-zonal sucrose gradients (Fig. 1), the substantially lower buoyant density of non-neutralizable compared with neutralizable virus in CsCl (Fig. 2), and the marked reduction of the non-neutralizable fraction following extraction with chloroform (Table 1). Other workers (Hughes et
Fig. 2. CsCl gradient centrifugation of non-chloroform-extracted HAV (10 passages AGMK, 1 passage BS-C-1) released into cell culture supernatant fluids. Fractions were collected from the bottom of the gradient and assayed for total HAV (▲) and non-neutralizable HAV (◇); density (○) was estimated from the refractive index.

Fig. 3. cDNA-RNA hybridization of fractions from a second CsCl step gradient containing non-chloroform-extracted HAV (10 passages AGMK, 4 passages BS-C-1) released from infected cells. Gradient fractions were blotted directly to nitrocellulose, and hybridized as described previously (Lemon et al., 1985).

al., 1984) have also noted that neutralization of HAV is significantly enhanced if virus is first treated with low concentrations of detergent. Thus, with respect to neutralization, HAV appears to be very similar to some other enteroviruses, notably echovirus 4 (Pesascek strain), echovirus 18, enterovirus 71, and some strains of rhinoviruses which are more effectively neutralized after treatment with sodium deoxycholate or chloroform (Gwaltney & Calhoun, 1970; Kapsenberg et al., 1979).

The apparent association of some HAV virions with lipids suggests that cellular membranes play an important role in the assembly and release of HAV from non-cytopathically infected cells. This hypothesis is supported by previous electron microscopic studies of HAV-infected hepatocytes which have demonstrated virus particles within cytoplasmic vesicles (Schulman et al., 1976; Shimizu et al., 1982). HAV antigen in infected chimpanzee liver has also been found to be approximately equally divided between soluble cytoplasmic and smooth microsomal fractions (Khan et al., 1984). These findings are consistent with abundant evidence that cellular membranes play a critical role in the assembly of other picornaviruses (Rueckert, 1976).
In early studies conducted by Provost et al. (1973a), infectious HAV present in acute-phase marmoset sera was found to band at two densities in CsCl (1·34 g/ml and 1·15 g/ml), and it was suggested that the less dense particles might represent lipid-associated virions. This banding pattern is remarkably similar to that which we found for virus released from BS-C-1 cells in vitro (Fig. 2). If the less dense virus found circulating in vivo is also largely non-neutralizable, it would explain the relatively high 'breakthrough' rate observed in early studies of HAV neutralization which utilized infectious sera as inoculum and susceptible marmosets for viral assays (Provost et al., 1973b). In addition, the presence of circulating non-neutralizable virus would reconcile the apparently contradictory observations that IgM antibody, presumably capable of viral neutralization (Lemon & Binn, 1983), and infectious virus may co-exist in some acute-phase serum specimens (Purcell et al., 1984).

We are indebted to J. R. Ticchurst for supplying Escherichia coli strains containing recombinant plasmids, and to J. E. Newbold for his gift of purified HAV plasmid DNA. We also acknowledge the expert technical assistance of R. W. Jansen and N. L. Gates.

REFERENCES


(Received 12 March 1985)