Properties of a Hepatitis A Virus Candidate Vaccine Strain

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SUMMARY

This paper describes the biophysical and biochemical properties as well as electron microscopical studies of a candidate hepatitis A vaccine strain propagated in human fibroblast cells. Our results indicated that, in CsCl, the density of hepatitis A virus (HAV) from cell culture supernatant and of HAV extracted from infected cells was influenced by the quantity of lipid material associated with HAV. Antigenicity of untreated HAV, therefore, was detected primarily in low density CsCl fractions (1.11 g/ml, 1.21 g/ml). After lipid reduction with NP40 detergent or chloroform/Genetron, antigenicity and infectivity were primarily detected in high density CsCl fractions (1.31 g/ml). Electron microscopy demonstrated a strong association between membranous material and virus particles of low density in CsCl as well as virus-like particles in ultrathin sections of HAV-infected human fibroblast cells. The uncovered virus particles banded in the 1.31 g/ml dense CsCl fraction lacked lipid material. The s value was 79 for 1.19 g/ml to 1.22 g/ml dense HAV and 147 for 1.29 g/ml to 1.33 g/ml HAV. Autoradiography of the radioiodinated dense HAV revealed some proteins with high Mr (120K to 67K) and others with low Mr (37K to 15K).

Hepatitis A virus (HAV) was propagated in human fibroblast (HFS) cells (Flehmig et al., 1981) using MEM with Earle's salts and 5% foetal calf serum and stored at 37 °C in a 5% CO₂ atmosphere. HAV antigen was detected by radioimmunoassay (RIA) (Flehmig et al. 1978). Infective HAV was titrated by inoculation of microplate wells containing a dense monolayer of HFS cells with 200 µl of each virus dilution, followed by incubation at 37 °C in a 5% CO₂ atmosphere. After 3 weeks incubation the cells were frozen and thawed three times and HAV in the supernatant (supernatant-HAV) was determined by RIA.

HAV was first concentrated by ammonium sulphate precipitation and then, after dialysis against phosphate-buffered saline (PBS) pH 7.2, in a second step, by high speed ultracentrifugation for 2 h at 200 000 g. The pellet was suspended in PBS and sonicated for 3 min. Resuspended HAV was purified by high speed centrifugation (180 000 g) through a 10% to 30% sucrose gradient for 60 min at 5 °C. Fractions (0.5 ml) were taken from the upper part of the tube, dialysed against PBS and examined for HAV by RIA. Antigen peak fractions were pooled and pelleted for 2 h at 200 000 g. The resuspended and sonicated pellet was then layered onto a CsCl gradient (1.1 g/ml to 1.5 g/ml) and centrifuged for 20 to 22 h at 12 °C. Fractions (0.5 ml) were taken from the upper part of the tube, dialysed against PBS, and tested for HAV by RIA. Cell-bound HAV was extracted by three freeze–thaw cycles, sonication for 3 min and low speed centrifugation. After pelleting (15 h, 180 000 g), the virus was extracted five times with equal amounts of Genetron and five times with equal amounts of chloroform. Organic and aqueous phases were separated by low speed centrifugation. The interphases were re-extracted five times with PBS. All aqueous phases were combined and dialysed against PBS. HAV was then concentrated by ultracentrifugation at 180 000 g for 15 h. The virus pellet was resuspended in PBS and purified by sucrose and CsCl gradient centrifugation. The NP40 treatment of HAV...
extracted from infected cells (cell-HAV) was carried out prior to CsCl gradient centrifugation: NP40 (1% final concentration) was added and the cells were incubated for 1 h at 37°C.

The sedimentation coefficient (s) for HAV was determined by comparing its sedimentation with that of markers (poliovirus, tobacco mosaic virus, haemocyanin) after simultaneous sucrose gradient (10% to 30%) centrifugation (80 min, 180000 g, 5°C). HAV was labelled with 125I by the chloramine-T method described by Coulepis et al. (1980). SDS-PAGE was carried out according to Laemmli (1970) in a 10% discontinuous gel system. Electrophoresis was done at a constant current (20 mA/gel). Autoradiography was performed according to Coulepis et al. (1980).

The supernatant-HAV with an antigen RIA titre of 1:7, which corresponds roughly to a specific amount of 0.26 μg/ml virus antigen, was harvested weekly from the HAV-infected cell cultures. The harvested HAV suspension with a TCID₅₀ titre of 10⁶.⁵/ml was concentrated 50-fold by ammonium sulphate precipitation (TCID₅₀ 10⁷.⁵/ml, antigen RIA titre 1:440) and then, in a second step, 6500-fold by ultracentrifugation (TCID₅₀ 10⁹.⁵/ml, antigen RIA titre 1:22 000). The total protein concentration increased from 0.6 mg/ml in the starting material to 40 mg/ml after ultracentrifugation; the virus proteins were concentrated from 0.26 to 2800 μg/ml.

The concentrated supernatant-HAV was purified by sedimentation in a 10% to 30% sucrose gradient. The two antigen peaks of the gradient were pooled (pool 1, fractions 5 to 15; pool 2, fractions 16 to 25) and centrifuged in a CsCl gradient. In pool 1, two antigen peaks were detected by RIA in CsCl fractions with densities of 1.19 g/ml to 1.22 g/ml and 1.29 g/ml to 1.33 g/ml (Fig. 1 a) and, in pool 2, two antigen peaks with densities of 1.21 g/ml and 1.29 g/ml to 1.33 g/ml (Fig. 1 b). The highest titres of infectivity, however, were detected in fractions with densities lower than 1.21 g/ml in which only low quantities of HAV antigen were demonstrated by RIA (Fig. 1 c, d). After treatment with NP40 detergent (0.2% NP40) for 2 h at 37°C, the highest quantities of previously masked HAV antigen were detectable by RIA in the fractions corresponding to the infectivity peaks (Fig. 1 e, f).

The quantity of non-viral proteins and lipid-like substances associated with the virus, demonstrated by electron microscopy, was reduced by extracting the supernatant-HAV, which had been purified by sucrose gradient centrifugation, with chloroform/Genetron. Subsequent CsCl gradient centrifugation showed one antigen peak of 1.308 g/ml dense HAV particles (Fig. 2a) that were not associated with lipid-like substances (Fig. 4f). Infectivity was primarily detected in fractions corresponding to the antigen peak (Fig. 2b).

Since contamination by calf serum protein of the cell culture supernatant could be avoided by using cell-HAV instead of supernatant-HAV, the virus was extracted from HFS cells. The cell-HAV, centrifuged in a CsCl gradient without prior sucrose gradient centrifugation, showed one peak for the HAV antigen in the 1.11 g/ml dense fraction (Fig. 3a). Electron micrographs of the peak fraction demonstrated aggregated virus particles that were covered and surrounded by membranous material (Fig. 4a). Such virus aggregates were also found in HFS cells infected with HAV (Fig. 4 g, h). This material was reduced by treating the cell-HAV with 1% NP40 followed by centrifugation in a CsCl gradient. The peaks of HAV antigen in CsCl at a density of 1.106 g/ml (fraction 5), 1.174 g/ml (fraction 10) and 1.27 g/ml (fraction 15) are presented in Fig. 3(b). Electron microscopical examination showed many virus particles associated with membranous material in fraction 5 (1.106 g/ml) (Fig. 4 b) and fraction 10 (1.174 g/ml; Fig. 4 c) as well as single, uncovered virus in fraction 15 (1.27 g/ml; Fig. 4 d).

Since the reduction of lipid material after NP40 treatment was not sufficient, stronger treatment of cell-HAV was performed with chloroform/Genetron. CsCl gradient centrifugation revealed HAV antigen mainly in fraction 16 at a density of 1.312 g/ml (Fig. 3c). Electron microscopy showed individual and uncovered virus particles in this fraction (Fig. 4 e).

The s value was 79 for the 1.19 g/ml to 1.22 g/ml HAV and 147 for the 1.29 g/ml to 1.33 g/ml HAV.

Autoradiography of the polypeptides of the latter HAV demonstrated some proteins with high Mr (120K to 67K) and others with low Mr (37K to 15.5K) (Fig. 5). Our results indicated that proteins of high Mr (67K to 120K), which may be non-specifically virus-associated, were detectable even after thorough purification. The low Mr proteins (37K, 30K, 27K, 15.5K)
Fig. 1. CsCl gradient of pooled sucrose gradient fractions, sucrose pool 1 (a, c, e) and sucrose pool 2 (b, d, f). Determination of antigenicity (fractions diluted 1:100) (a, b) and infectivity (c, d). Detection of antigenicity after NP40 treatment (fractions diluted 1:100) (e, f).
were similar to the virus proteins described by other investigators (Coulepis et al., 1980; Tratschin et al., 1981; Gerlich & Frösner, 1983).

From our results we conclude that, after effective purification procedures, HAV particles are left at primarily only one density, 1.32 g/ml. In agreement with Provost et al. (1975) we assume that the lower density value for HAV represents an association with lipids. Lemon et al. (1985), however, reported contradictory data: after extraction with chloroform, the buoyant density of light HAV particles (1.27 g/ml) propagated in BS-C-1 cells was unchanged. Lemon et al. (1985), therefore, concluded that the low density was not due to association with lipids. Lemon & Binn
Fig. 4. Preparations of CsCl gradient peak fractions of cell-bound HAV (a to e) and supernatant-HAV (f) negatively stained with uranyl acetate. (a) Untreated virus (see Fig. 3a, fraction 5, 1.11 g/ml), virus particles enclosed by membranous bodies. (b to d) NP40-treated virus (see Fig. 3b); (b) fraction 5 (1.106 g/ml), virus particles associated with membranes; (c) fraction 10 (1.174 g/ml), virus particles associated with membrane-like material; (d) fraction 15 (1.27 g/ml), individual virus particles lack membranous material; (e) chloroform/Genetron-treated virus (see Fig. 3c, fraction 16, 1.312 g/ml), individual uncovered virus particles; (f) supernatant-HAV treated with chloroform/Genetron (see Fig. 2a, fraction 17, 1.308 g/ml). (g, h) Ultrathin sections of Epon-embedded HFS cells, persistently infected with HAV. Arrowheads indicate vesicle-bound virus-like particles. Bar marker in (a) represents 100 nm for (a) to (f). Bar marker in (g) represents 100 nm for (g) and (h).

(1985), however, demonstrated that HAV of cell culture supernatant cannot be completely neutralized because the lipid is tightly bound to the virus; this was also our experience (unpublished findings). In addition to finding that the lipid association of HAV lowers the density of HAV particles, Gauss-Müller et al. (1986) established that relatively higher quantities of virus protein p30, which probably represents VP0, are present in low density particles, a factor that could influence the buoyant density of HAV. The origin of the membranous material associated with HAV is unknown. It may well be that HAV has a high, but non-specific, affinity
for any lipid-containing material or that this high affinity results from virus assembly and release. Our electron microscopy study of HAV-infected HFS cells (Fig. 4g, h) demonstrated vesicle-associated virus-like particles similar to those reported in infected human liver (Shimizu et al., 1982).

Cell-HAV with a density of 1.32 g/ml and lacking both lipid material and large quantities of foetal calf serum from the cell culture supernatant is well suited for use in vaccination studies.

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REFERENCES


Short communication


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