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Physiochemical and immunological characterization of hepatitis A virus nucleocapsids expressed in a vaccinia virus/T7/EMCV system

Brief Report

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Summary. Expression of the entire open reading frame of hepatitis A virus was achieved in a vaccinia virus/T7/EMCV hybrid system. The expressed antigens were characterized as particles with buoyant densities and sedimentation coefficients typical of emtpy capsids and pentamers of HAV. This was further confirmed by electron microscopy. All capsid proteins were determined as components of the particles which raised neutralizing antibodies in an immunized rabbit.

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Hepatitis A virus (HAV) is classified as a member of the family *Picornaviridae*. Its positive-stranded genomic RNA with its 3′ end polyadenylated is 7478 nucleotides long, among which the first 734 nucleotides belong to the 5′-nontranslated region (5′NTR). The morphogenesis process of HAV, like the other picornaviruses, commonly begins with the translation of a polyprotein, followed by cleavages made by proteinases provided by itself or its host [1]. The structural proteins processed by nonstructural ones are then assembled into pentamers, procapsids, or pre-provirions, provirions and finally into virions [2]. Therefore, it was assumed that the entire open reading frame (ORF) of HAV would be necessary for assembly of nucleocapsid.

We have achieved the expression of the entire open reading frame (ORF) of HAV in a vaccinia virus/T7/EMCV system which contains bacteriophage T7 promoter followed by the 5′NTR of encephalomyocarditis virus (EMCV). A recombinant vaccinia virus vT7HAV-4 was constructed by insertion of the entire coding region of HAV, with the 5′ nontranslated region (5′NTR) totally excluded, in the downstream of PT7 and EMCV sequences. The high expression level of this system resulted from both the transcription efficiency of bacterio-

phage T7 promoter and the cap-independent translation capability offered by the 5′NTR segment of EMCV [4, 5]. In this report, we describe the physio-chemical and immunological characterization of the expressed antigens.

Expression of hepatitis A virus antigens (HAAg) was achieved by coinfecting CV-1 cells with vT7HAV-4 and vTT7 (MOI = 0.01) [3]. The former contained the entire coding region of hepatitis A virus, while the latter expresses T7 RNA polymerase to provide the basis for the expression of genes in mammalian cells under the control of T7 promoters. Cells were harvested in PBS 48 h post-infection and lysate was prepared by sonication for further manipulations and analyses.

To investigate the ability of particle formation, cell lysate first went through a 20% sucrose cushion. The resuspended pellet was then layered on CsCl step gradients (5% to 45%, w/w) and centrifuged for 16 h at 35000 rpm. Fractions (150 μl) were collected from the bottom and assayed for HAAg. A direct ("sandwich") 3-step enzyme immunoassay (ELISA) was employed for the detection of HAAg as described in the legend of Fig. 1. Antigen profile in CsCl

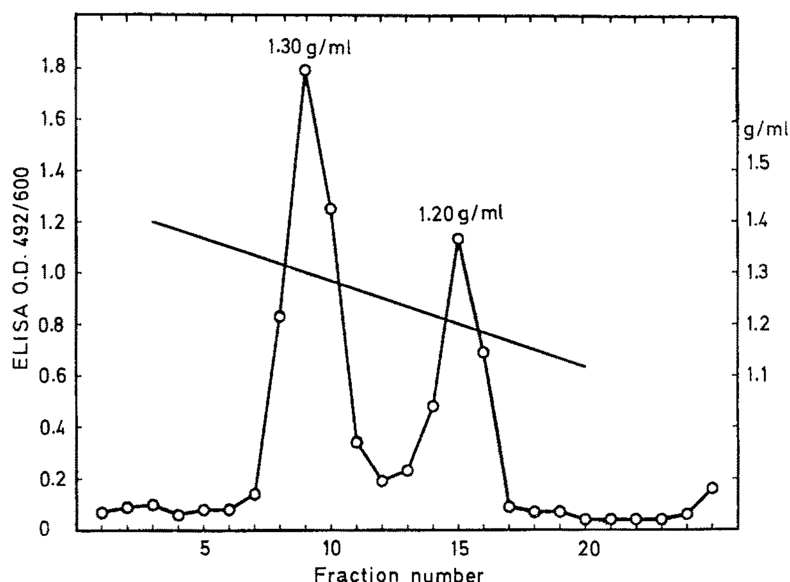

Fig. 1. Banding of expressed HAV particles in a step CsCl gradients. 2×10^7 coinfected CV-1 cells (MOI = 0.01) were harvested at 2 days post-infection. The cell lysate was first centrifuged through a 20% sucrose cushion at 35000 rpm for 24 h. The resuspended pellet was then layered on CsCl step gradients (5 to 45% w/w, 1.04 g/ml to 1.51 g/ml) which were centrifuged at 35000 rpm and 4 °C for 16 h in an RPS-65T rotor (Hitachi). Fractions (150 μl) were collected from the bottom and assayed for HAAg by ELISA. To each well 100 μl of suitable diluted polyclonal antibody solution (0.05 M Na_2CO_3–$NaHCO_3$, pH 9.5) was added and kept at 4 °C overnight. The wells were then filled with blocking buffer (3% BSA in PBS containing 0.02% sodium azide) for 20 min at room temperature. Fractions were diluted to 1:10 in PBS and incubated in the wells for 2 h at 37 °C. The next few steps followed those of HAVAB (Abbott). The typical incubation volume was 100 μl. Fraction 9 (1.30 g/ml) and fraction 15 (1.20 g/ml) presented two HAV antigen peaks

gradients is shown in Fig. 1. Two ELISA positive peaks were identified. A major peak banded at a density determined as 1.30 g/ml, while a minor peak banded at 1.20 g/ml. These two densities were reported as those typical of empty capsids [6].

For sedimentation analyses, cell lysate was layered on 10 to 30% (w/w) sucrose gradients, which were centrifuged for 2 h at 35 000 rpm. Fractions (250 μl) were collected from the bottom. As shown in Fig. 2, 80 S particles dominated in the expressed products. Some 15 S particles were also present. Judging from the S values, the 80 S and 15 S particles represented empty capsids (EC) and pentamers of HAV respectively [7, 8].

To further investigate the expressed particles, the major peak fractions of sucrose gradients were pooled and negatively stained with 2% uranyl acetate prior to observation in an electron microscope. Virus particles of approximately 25–30 nm were found, most of which appeared empty (Fig. 3).

Cell adaptation of HAV was reported by several groups [9]. Mature virions and some of noninfectious particles designated as empty capsids were found in such cell cultures. Mature virions have a density of 1.33 g/ml and a S values

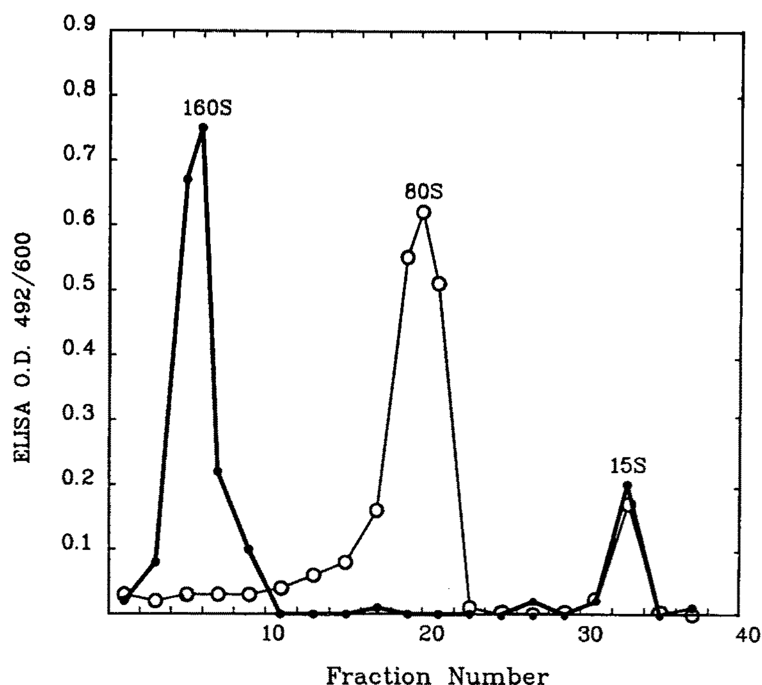

Fig. 2. Sedimentation of expressed HAV particles. The cell lysate was layered on a linear 10 to 30% (w/w) sucrose gradients in NT (50 mM NaCl, 10 mM Tris, pH 6.8, 0.2% Triton X-100), which were centrifuged for 2 h at 35 000 rpm in an RPS-40T rotor (Hitachi). Fractions (250 μl) were collected from the bottom and assayed for HAAg by ELISA as described in Fig. 1. For the expressed products (○), a dominant peak representing 80 S and a minor peak corresponding to 15 S were identified. The cell cultured HAV (●) were analysed parallelly as control. A dominant 160 S peak and minor 15 S peak were shown. The calculation of S values was according to the method of Applications Data by Beckman Instruments INC

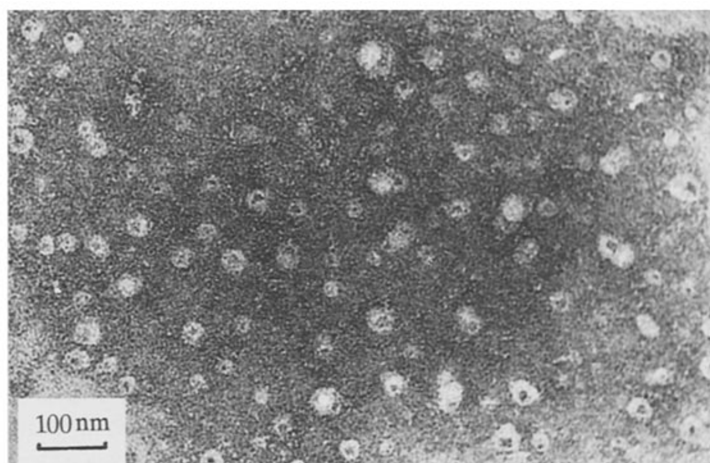

Fig. 3. Electron micrograph of the particles made by the Recombinant vaccinia virus vT7HAV-4 (×90000). 30 μl of sucrose purified particles were incubated with 10 μl of anti-HAV IgG in 200 μl of PBS-tween at 37 °C for 1 h. Centrifugation was performed at 16000 **g** and 4 °C for 30 min. The pellet was resuspended in water and negatively stained with 2% uranyl acetate prior to observation in an electron microscope (Hitachi)

of 160 S, while empty capsids banded at normal density (1.30 g/ml) and light density (1.20 g/ml) with S values of either 76 S or 59 S [8].

In our experiments, cell cultured HAV synthesized particles mostly with a density of 1.33 g/ml (data not shown) and a sedimentation coefficient of 160 S (Fig. 2). The data conformed exactly to the already established values representative of mature HAV virions [10]. Such particles were not detected in our expressed products.

Immunoblot analyses with various guinea pig antipeptide sera (VP1 10–30 a.a., VP2 40–60 a.a., VP3 31–47 a.a., VP4) were carried out to determine the specific protein components of the expressed particles purified by sucrose gradients (Fig. 4). Anti-VP1 serum recognized a 33 kDa protein and a 40 kDa protein. The former protein comigrated with the capsid protein VP1, while the latter one corresponded to a postulated VP1 precursor PX [2]. One 29 kDa band, which reacted with both anti-VP2 and anti-VP4 sera, should represent VP0. The cleavage of VP0 into VP2 and VP4 is believed to take place only after RNA encapsidation in mature virions. A 27 kDa protein which accorded to the molecular weight of the reported VP3 of HAV was detected when anti-VP3 serum was used.

A morphology model postulated from cell cultured HAV indicated that empty capsids were composed of VP1, VP0 and VP3 while pentamers were constructed of VP0, VP3 and PX [2]. Immunoblot analysis indicated that the expressed particles also contained similar protein components as empty capsids and pentamers.

We previously described the immunization of rabbit with the cell lysate of recombinant vaccinia virus vT7HAV-4. One rabbit was immunized by

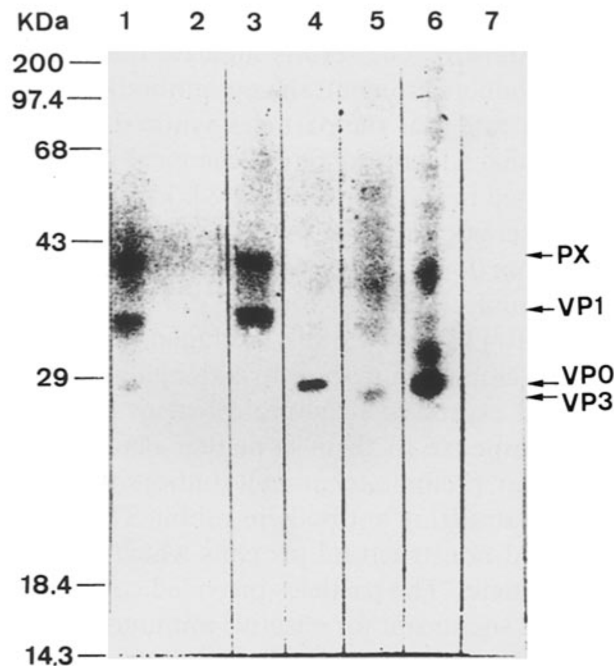

Fig. 4. Immunoblot analysis of expressed HAV proteins. After going through a 80% glycerol cushion, the cell lysates were precipitated by methanol. The pellet was dissolved in the loading buffer and resolved by a 12.5% SDS-PAGE. Immunoblot was performed by transferring proteins to nitrocellulose filters. Guinea pig antipeptide sera of VP1 (10–30 a.a.), VP2 (40–60 a.a.), VP3 (31–47 a.a) and VP4 were used as primary antibodies directed against target proteins. Horseradish peroxidase coupled protein A (Sigma) was used as secondary reagent and was detected with the substrate 3,3′-diaminobenzidine. *1,3–6* were loaded with samples prepared from cell lysates coinfected with vTT7 and vT7HAV-4. *2* and *7* were loaded with samples prepared from cell lysates infected with vTT7 alone. In *1* and *2*, a mixture of anti-VP1, -VP2 and -VP3 guinea pig antipeptide sera was used. *3–5* were incubated with anti-VP1, -VP2 and -VP3 sera respectively. *6* and *7* were probed with anti-VP4 serum

vT7HAV-4 and another was by vTT7 as a control. Anti-HAV sera were detected for the rabbit immunized by vT7HAV-4 four weeks post immunization [3]. In order to determine the neutralizing ability of the antiserum, we now further performed a neutralization assay by employing a TCID50 test. Dilutions of antisera from both rabbits were heat inactivated at 56 °C for 30 min. Then series diluted antisera were mixed in equal volume with 100TCID50 of HAV. 2BS (Human diploid fibroblast) cells were inoculated for each dilution. 40 days post-infection, the dilution of the antiserum at which giving 50% neutralization, according to tissue culture infected (TCI) ratio, was defined as the neutralization titre. The titer of the neutralizing antibody in the antisera immunized by vT7HAV-4 was thus determined as 1:256 upon results of both immunofluorescent assay (IFA) and ELISA. However, serum of the rabbit immunized by vTT7 did not show any neutralizing ability. Human anti-HAV neutralizing serum

and anti-HAV negative serum were used as positive and negative controls, respectively (data not shown). The results indicate that the antibodies raised from the expression products are neutralizing antibodies.

Our results demonstrate that the particles synthesized in the recombinant vaccinia virus system share identical physiochemical characteristics with the empty capsids as reported in the literature [6–8]. From the results of both the physiochemical characteristics and the protein components, we conclude that the expressed particles are dominantly empty capsids. In addition, the expressed particles should be noninfectious, since the 5′ NTR of HAV genome, which is required for infectivity [11], was totally excluded in our constructs.

In an attempt to develop immune-prophylaxis against hepatitis A virus, synthetic polypeptides and expressed structural proteins appeared disappointing because the immune response to them is neither neutralizing nor protective [12]. In our case, however, preliminary animal studies showed that the expressed products could raise neutralizing antibody in rabbit. This should be due to the expressed structural and nonstructural proteins which were further processed and assembled into particles. The particles provided conformational structures and epitopes, which are significant for effective immunogenicity and are absent in synthetic polypeptides or any expressed single structural proteins.

It has been reported that the neutralizing antibody responses to HAV and EC in rabbits are at the same level [8]. This seems very encouraging to us, although the data were from cell cultured HAV. We are now furthering our experiments to investigate the immunogenicity of the expressed particles after purification. The purification of empty capsids from our expression system should be simpler and safer than from cell cultured HAV which also contain infectious mature virions. Recombinant vaccinia viruses generally produce antigens within two days, while it takes weeks for HAV to replicate in cell culture [13]. All our results arrived at a conclusion that the expressed empty capsids are a most promising candidate for a safe and efficacious HAV vaccine. Entire HAV open reading frame has also been expressed in vaccinia viral system using vaccinia P7.5 promoter. Both empty capsids and pentamers were identified, but with more pentamers [14].

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