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Large-Scale Culture of Hepatitis A Virus in Human Diploid MRC-5 Cells and Partial Purification of the Viral Antigen for Use as a Vaccine

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Abstract A large-scale culture of hepatitis A virus in human diploid MRC-5 cells was conducted. In a roller bottle culture, the virus was grown to a maximum titer in 3 weeks after infection. Over 95% of the cell-associated virus was excreted after culturing the infected cells in suspension media without fetal bovine serum for 3 days. The cultured virus was inactivated with formalin, concentrated by ultrafiltration, and partially purified by ultracentrifugation in a non-ionic gradient medium of Renocal. Two separate peak fractions showing high anti-HAV ELISA titer were pooled and about 40% of HAV antigen was recovered by this purification procedure. Of the partially purified vaccine, the protein pattern in SDS-PAGE and immunogenicity in mice were compared with a commercial HAV vaccine. In SDS-PAGE, the purified vaccine in this study and the commercial vaccine showed almost the same protein pattern. The seroconversion rate of the purified vaccine in mice was not different from that of the commercial vaccine. Therefore, we could prepare a good grade of HAV vaccine by a simple purification procedure although the purification itself was not completed.

Key words: Hepatitis A virus, cell culture, purification, immunogenicity

Hepatitis A virus (HAV) is a major cause of acute hepatitis and is responsible for significant morbidity worldwide. It is responsible for about 30% of acute hepatitis cases in the United States each year [1]. HAV is a member of the *Picornaviridae* family and has been classified as an enterovirus until recently [17, 22]. Because of its unique genetic and physical characteristics, HAV has now been re-assigned to a distinct genus, hepatovirus, within the *Picornaviridae* family [23]. HAV is a 27 nm non-enveloped icosahedron. The capsid contains three large proteins (VP1, VP2, VP3) ranging from 25 kDa to 33 kDa and one small protein (VP4) of around 2.5 kDa, of which VP1 appears to be the dominant surface protein [10]. Only a single antigenic specificity has been associated with HAV, and no significant antigenic variation has been recognized among different HAV strains [20].

HAV was first detected in stool isolates in 1973 [16], and successfully adapted to growth in cultured cells by Provost and Hilleman [26]. Adaptation of wild-type HAV from fecal isolate to growth in cell culture is slow and intracellular viral antigens are detected after 1 to 3 months in most permissive cell types [5]. Once adapted, HAV grows well in cell culture like other picornaviruses. Although viral RNA is detectable at 24 h postinfection in individual cells, maximal titer of infectious particles is not attained until 12 to 14 days after infection [19]. Recently, some researchers have isolated more rapidly growing and even cytolytic variants of HAV whose replication cycles in specific co-selected cell lines are considerably shorter. In some instances, these cytopathic variants have emerged during continued passage of persistently infected cell cultures [2, 12, 29].

Several laboratories have tried to produce HAV in cell culture in a large scale to make a vaccine. HAV strain HM175 was isolated in Australia during an outbreak from October to November, 1976 and has been used for the study of physical properties and replicative strategies until now [18]. Its isolation in a primary African green monkey kidney (BS-C-1) cell culture and preparation for prototype vaccine have been described [7, 13]. Although the vaccine produced in this cell was safe and efficacious, the cell has not been employed for preparation of a viral vaccine for human use. The HM175 virus was adapted to the human diploid lung MRC-5 cells that have been extensively used in human vaccine production [6].

Roller bottles and cell factory systems have been used for a large-scale virus culture. Viruses associated with the infected cells have been harvested and extracted by freezing and thawing several times in dry ice-ethanol baths [3, 6]. The virus suspension was concentrated by

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polyethylene glycol precipitation or ultrafiltration, and further purified by gel filtration, ion-exchange chromatography [25], and gradient centrifugation [8, 10, 15]. However, the methods generally used for growth, harvest, and purification of slow growing viruses like HAV are expensive and time-consuming, which contributes to the high cost of vaccine production and may limit the total number of vaccine doses which can be produced. All strains of HAV which have been grown in cell culture are characterized by inefficient release of virus into the culture supernatant; less than 30% of infectious viruses are released into the culture supernatant. This is also true with cytopathic strains of HAV [11, 24]. Since antigens in culture supernatant are often undetectable, concentrations of large culture volumes also contribute to the difficulties in HAV purification accompanying the problem of virus release.

In this report, we describe a method of virus release using suspension media without fetal bovine serum and a purification method which makes use of ultrafiltration for virus concentration and gradient centrifugation in a non-ionic, high density reagent for virus isolation. We show that the inactivated and purified virus with this method has good immunogenicity in animal tests and that it can be formulated into a good vaccine for human use.

MATERIALS AND METHODS

Cells and Viruses

Hepatitis A virus strain HM175 (P6 in MRC-5) which had been adapted to MRC-5 cells after ten passages in African green monkey kidney (AGMK) cells was obtained from Walter Reed Army Institute of Research (WRAIR), Washington, D.C., U.S.A. The virus was amplified two times in MRC-5 cells to make a working seed virus and stored at -70°C. MRC-5 cells (PDL19) certified for safety by testing all relevant tests were also provided by WRAIR. Master Cell Bank (MCB) and Working Cell Bank (WCB) were established at PDL21 and PDL23, respectively, followed by testing sterility and safety in this laboratory.

Virus Culture and Harvest

Virus was cultured in MRC-5 cells of PDL 25-27 using 850 cm^2 plastic roller bottles. Cells from WCB were expanded into T180 culture flasks (Corning, U.S.A.) and amplified two times to make cell cultures in roller bottles. Growth medium was Eagle's Minimum Essential Medium (Gibco BRL, U.S.A.) supplemented with 10% fetal bovine serum (Gibco BRL, U.S.A.), 2 mM L-glutamine, 2.2 g/l of sodium bicarbonate, neomycin sulfate (50 μg/ml), and streptomycin sulfate (100 μg/ml). The cultures of roller bottles reached confluency at 5-6 days after being split and each culture was inoculated with the working seed virus at the MOI of 0.1. The infected culture was maintained in maintenance medium containing 2% FBS instead of 10% FBS in growth medium for 3 weeks with a media change every week. At the end of the culture, the cell monolayer was washed three times with 100 ml of Hank's balanced salt solution (HBSS, BioWhittaker, U.S.A.) per wash, and maintained for 3 more days in 100 ml of suspension media (Gibco BRL, U.S.A.) without FBS. The infected culture was harvested by swirling several times to detach all cells followed by centrifugation at 8,000 rpm for 20 min in a Beckman type 45Ti rotor (Beckman, U.S.A.) to remove cell debris. To the clarified supernatant prewarmed to 35°C, formalin was added to a 0.05% concentration to inactivate viruses. The inactivation was carried out by incubation for 12 days at 35°C.

Virus Purification

The inactivated bulk virus prepared above was concentrated to 1/20 of the starting volume with Ultrasette which has a molecular weight exclusion of 100 kDa (Filtron Technology Co., U.S.A.). For gradient centrifugation, a step gradient was prepared using a non-ionic material, Renocal (diatrizoate meglumine and diatrizoate sodium, Bracco Diagnostics, U.S.A.). In 100 ml centrifuge tubes, 10 ml of 30% (v/v), 15 ml of 40% (v/v), 15 ml of 50% (v/v), 15 ml of 60% (v/v), and 15 ml of 76% (v/v) Renocal was added in turn. The 20-fold concentrate of HAV was then loaded onto the step gradient and centrifuged at 40,000 rpm in a Beckman type 45Ti rotor for 18 h. After all 3-ml fractions were collected by puncturing the bottom of the tube, antigen detecting ELISA and protein assay by Bradford's method [9] were performed to determine antigen and total protein quantity in each fraction, respectively. Fractions containing antigen were pooled and diluted 1:3 with phosphate buffered saline (PBS). After precipitation of the diluted virus by centrifugation at 30,000 rpm for 6 h in a Beckman 45Ti rotor, the resulting pellet containing HAV was resuspended in 6 ml of PBS. The virus suspension was dialyzed against 100 volumes of PBS overnight at 4°C to minimize the contaminating gradient material and was analyzed in HPLC (Hewlett Packard series 1100, U.S.A.) with a gel chromatography column (TSK3000SW, TOSOH HAAS, Japan). The flow rate, injection volume, and buffer were 0.8 ml/min, 50 μl, and PBS (pH 7.0), respectively.

Growth Pattern of Virus

In order to determine the appropriate time for virus harvest, the growth pattern of the HAV was determined by assaying the virus quantity according to the incubation period after inoculation. The cell cultures in roller bottles were infected with the working seed virus at MOI of 0.1 and the infected cells were harvested each week for 5 weeks as described above. Each sample was tested for

HAV antigen with a quantitative ELISA and corresponding infectivity titer was calculated using a reference HAV with known titer (provided by WRAIR).

Indirect Immunofluorescence

Monolayers of MRC-5 cells in T75 flasks were inoculated with HAV at MOI of 0.1 and incubated for 2 weeks at 35°C accompanying uninfected controls. At the end of incubation, both cultures were washed two times with 20 ml of PBS solution and detached by scraping. The detached cells were then deposited by low-speed centrifugation and suspended in 1 ml of PBS. One drop of cell suspension was spotted onto a microscope slide, air-dried, and then fixed in cold acetone for 2 min. Anti-HAV IgG which was isolated from an acute hepatitis A patient by protein A column chromatography and FITC-conjugated anti-human IgG (Sigma, U.S.A.) were used to detect HAV antigens. Slides were incubated with anti-HAV IgG for 45 min at 37°C, washed with PBS, and incubated with anti-human IgG-FITC for 45 min at 37°C. After washing three times with PBS, the slides were mounted in a glycerol-PBS medium and examined with a Zeiss standard microscope equipped with an incident light condenser and HBO-50 light source. Cells with intracellular fluorescence were photographed.

ELISA and Protein Assay

HAV antigen was detected by a double sandwich ELISA. Wells of a 96-well microtiter plate (Nunc, Denmark) were coated for 3 h at 37°C with 100 μl of the monoclonal antibody K3-2F2 (Commonwealth Serum Laboratories, Australia) diluted to 1 μg/ml in 50 mM sodium carbonate buffer (pH 9.4) and were blocked with 1% (w/v) bovine serum albumin overnight at 4°C. After washing with PBS-T (PBS containing 0.2% (v/v) of Tween 20), virus dilutions in PBS were added to wells and incubated overnight at 4°C followed by vigorous washing. Anti-HAV-HRP conjugate was added to each well and incubated at 37°C for 2 h. After color reaction with *o*-phenylenediamine (OPD) substrate for 10 min, optical density at 492 nm was measured in a microtiter plate reader (Denley, Denmark). Patient serum with a high titer of anti-HAV antibody was obtained from Soonchunhyang University Medical Center and IgG was isolated by protein A column chromatography. Horse radish peroxidase conjugation was performed by the method of Avrameas and Ternynck [4].

Protein content was determined with a protein microassay kit (Biorad, U.S.A.) according to the manufacturer's instruction.

SDS-PAGE

Samples from gradient fractions were dialyzed against 200 volumes of PBS at 4°C for 20-24 h and mixed with 0.1 volume of 10× loading buffer followed by boiling for 2 min prior to electrophoresis in a gel containing 8-16% acrylamide and 3.5 M urea using the Excell system (Novex, U.S.A.). After electrophoresis, proteins were stained with silver stain (Rapid Ag stain; ICN Radiochemicals, U.S.A.) according to the manufacturer's instruction. Final vaccine and the commercial HAV vaccine (HAVRIX, SmithKlein Beecham Biologics, U.S.A.) were extracted with 0.1 volume of an extraction buffer containing 0.11% EDTA, 0.4% Tween20, 0.1% gelatin (pH 8.5) overnight at room temperature.

Immunogenicity in Mice

Immunogenicity tests were carried out in 4-5-week old female ICR mice weighing 250-300 g. The gradient purified HAV was adsorbed to alum hydroxide (Rehydrogel, REHEIS Inc., U.S.A.) at 0.5 mg/ml as an alum content, which was serialy diluted in PBS by two fold as inocula. Five mice were each inoculated intraperitoneally with 0.5 ml of each dilution and bled at 28 days post-inoculation. As a positive control, a commercial HAV vaccine (HAVRIX) was inoculated in the same manner. Sera were assayed for HAV antibodies by a commercial immunoassay kit (HAVAB-EIA, Abbot Laboratories, U.S.A.) and ED_{50} was calculated by the Reed-Muench equation [27].

Results and Discussion

Cell Culture and Harvest of Virus from Monolayer Cells

The overall virus culture and purification procedure is outlined in Fig. 1. In order to determine the appropriate incubation time for maximum antigen yield, samples from each week after virus infection were collected and the virus content was determined by ELISA. As shown in Fig. 2, antigen content reached a maximum at 3 weeks after inoculation and decreased slightly at week 4 and week 5. Therefore, crude lysate containing cells and viruses was harvested at 3 weeks after inoculation for purification of the viral antigen. HAV HM175 has been known to be incubated for 21-28 days in BS-C-1 cells and for 42 days in MRC-5 for vaccine production [6, 7]. HAV viral antigen is usually maximal during 9-14 days after virus infection and a small portion of virus was released into the culture medium after 14 days, but nearly all of the HAV remained cell-associated [14]. Viral antigens produced at the highest level seem to maintain an intact capsid detectable by ELISA until week 3, and the content of intact viral antigen is thought to decrease as time passes.

Since HAV is cell-associated, a virus releasing step must be included for purification. Minimum essential media for suspension culture were used to detach cells from roller bottles and to release the intracellular viruses into the

Cell culture inoculated with HAV at MOI of 0.1
(3 week culture at 35°C in EMEM+2% FBS)
↓
Wash cells 3 times with HBSS
↓
Detach cells and release the viruses
(incubation for 3 days in suspension media without FBS)
↓
Centrifuge (8,000 rpm, 20 min), discard debris
↓
Inactivate virus with formalin (0.05%, 35°C, 12 days)
↓
Concentrate to 1/20 of the original volume
(ultrafiltration, membrane of 100 kDa cut-off)
↓
Centrifuge in Renocal gradient (40,000 rpm, 18 h),
antigen detecting ELISA
↓
Collect antigen fractions and dilute with 3 vol of PBS,
precipitate by centrifuge (30,000 rpm, 6 h)
↓
Resuspend in PBS and dialyze against PBS

Fig. 1. Overall procedure for virus culture and purification.
Virus release by incubation of the infected cells in suspension media, inactivation, ultrafiltration, and partial purification in Renocal gradient centrifugation are major steps included in this procedure. Dialyzed sample was used for the immunogenicity test in mice.

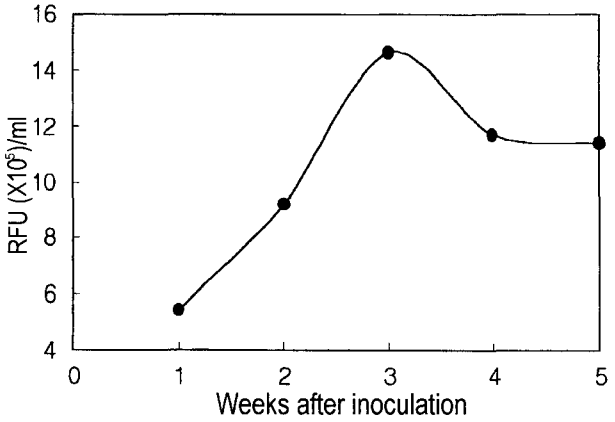

Fig. 2. Growth pattern of HAV in cell culture according to culture period.
Virus titer was determined by comparative ELISA, in which the standard curve of the reference virus with known infectivity titer was prepared and the corresponding titer of each sample was determined.

media. Virus content in the clarified supernatant and in the cell pellet resuspended in PBS was assayed by ELISA to determine the comparative quantity of remaining virus in the cell pellet. Over 96% of viruses were in the supernatant and a negligible amount of virus remained in the cell pellet (Table 1). Infected monolayer cells were detached by trypsin treatment or by freezing-thawing several times in a dry ice-ethanol bath and the intracellular virus was released by sonication for HAV harvest [6, 7]. This method is not a feasible method in a large-scale HAV harvest due to the laborious and time consuming process. Use of the suspension media is a very effective and simple method to release the cell-associated HAV for a large-scale culture.

Indirect Immunofluorescence

The presence of HAV antigen in infected cells was confirmed by anti-HAV IgG isolated from a patient sera and anti-human IgG conjugated with FITC. At 2 weeks after infection with HAV at MOI of 0.1, over 90% of cells were infected and detected with the anti-HAV antibody and FITC conjugate reflecting the spread of virus in this time (Fig. 3). Due to the lack of virus specific cytopathology, HAV infected cell cultures have to be identified by immunological techniques. End-point titration of HAV infectivity via the assay of HAV antigen in cellular extracts is time consuming and radioimmunofocus assay using I^{125}-labeled anti-HAV antibody is dangerous. Therefore, this method can be applied to fluorescent focus assay and to foci reduction assay for the neutralization antibody test.

Recovery of Viral Antigen from Gradients

Renocal was used to make gradients to isolate viral antigen. In antigen detecting ELISA, peaks were detected at fraction numbers 10 and 15, and in protein assay, a peak was detected at fraction number 17 (Fig. 4). Three antigen peaks were reported in CsCl gradient centrifugation of HAV and a major portion of the antigen was associated with non-infectious, empty particles, which banded at densities of 1.305 g/ml and 1.20 g/ml in CsCl gradient. An additional RNA-containing complete particle represents only a minor species characterized by a density of 1.32 g/ml. Both major and minor antigens had identical capsid patterns and could be used as an immunogen for vaccine

Table 1. Purification of hepatitis A antigen from the cell culture inoculated with HAV.

	Total activity (EU[a])	Total protein (mg)	Specific activity (EU/mg)	Yield (%)	Purification fold
Clarified sup.	15,000	250.8	59.8	100	1.0
Cell debris	600	117.2	5.1	4	-
Inactivation	14,400	250	57.6	96.0	1.03
Ultrafiltration	8,940	110.25	81.1	59.6	1.36
Gradient centrifugation	8,057	4.123	1954.2	53.7	32.67
Precipitation	6,015	2.5	2406.0	40.1	40.2

[a]ELISA unit.

Fig. 3. HAV antigen detected in cell culture by anti-HAV patient sera and indirect immunofluorescence with FITC conjugated anti-human IgG.
Over 90% of the cells are infected and show a bright color.

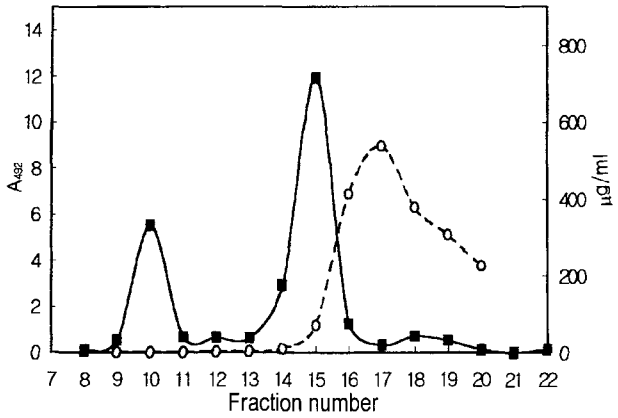

Fig. 4. Antigen and total protein content after gradient centrifugation in Renocal (30% to 76%).
Antigen content (■) was measured by ELISA and expressed as OD at 492 nm. For protein assay, each fraction was dialyzed overnight against PBS and protein content (○) was determined by Bradford microassay.

preparation [28]. When metrizimide, a non-ionic gradient material, was used for the separation of the same virus, a major antigen was detected at 1.31 g/ml and a less dense antigen at 1.22 g/ml [21]. Each antigen peak accompanying two near fractions were collected and precipitated by centrifugation after dilution with three volumes of PBS. The antigen and protein content were determined by ELISA and the protein assay at each purification step, of which results are summarized in Table 1. About 40% of the HAV antigen was recovered and the purification fold was 40.2. Since 38% of antigen was lost during concentration by ultrafiltration, this step should be the first target for the improvement of the purification yield. Specific activity was greatly increased from 81.1 EU/mg to 1954.2 EU/mg through gradient centrifugation in Renocal, suggesting that most contaminating proteins could be

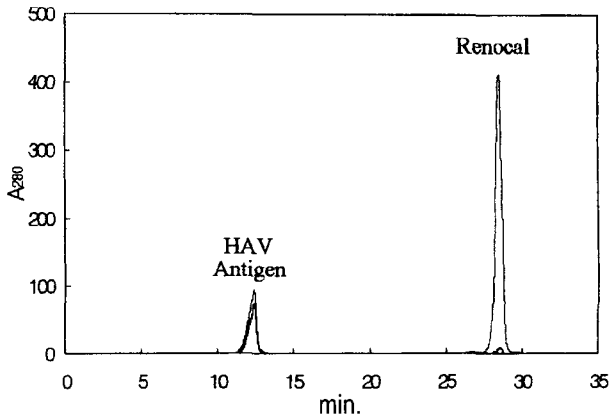

Fig. 5. Elimination of Renocal by dialysis.
Thin line and thick line stand for the samples before and after dialysis, respectively. Column chromatography in HPLC was carried out as described in Materials and Methods.

separated efficiently from the HAV antigen. After dialysis, the residual amount of Renocal was analyzed in HPLC using TSK 3000SW gel chromatography column (Fig 5). The first peak was confirmed to contain HAV antigen by ELISA and the second peak contained Renocal. Most of the Renocal was eliminated by dialysis without decreasing the antigen content in HPLC. Removal of the gradient material is desirable although it is a radiopaque contrast agent for intravascular use and proved to be safe for human use. Centrifugation in Renocal is a simple and efficient purification method for the recovery of acceptable amounts of HAV antigen. The low viscosity and high density of Renocal is ideal for the isolation of dense particles like HAV.

Characterization of the Partially Purified HAV Antigen

Gradient fractions were analyzed by SDS-PAGE to observe the protein pattern. The protein content in the first antigen peak of the fraction number 10 was hardly detectable by SDS-PAGE, while too many proteins were detected at the second peak of fraction number 15 (Fig. 6A). The two peak fractions were pooled to prepare a final HAV antigen for vaccine use. The protein pattern of the final HAV antigen was compared with that of the commercial HAV vaccine (Fig. 6B). Both samples showed exactly the same protein pattern, showing that the partially purified HAV antigen prepared in this study had a possible usage as a vaccine. However, HAV antigen was not confirmed in this experiment due to little protein at the first peak of the gradient centrifugation even with silver stain. Confirmation of HAV antigen requires western blot analysis using antibodies against 4 subunit proteins of the viral capsid, since HAV has a conformational epitope.

The immunogenecity of the final antigen in mice was compared with that of the commercial vaccine (Table 2).

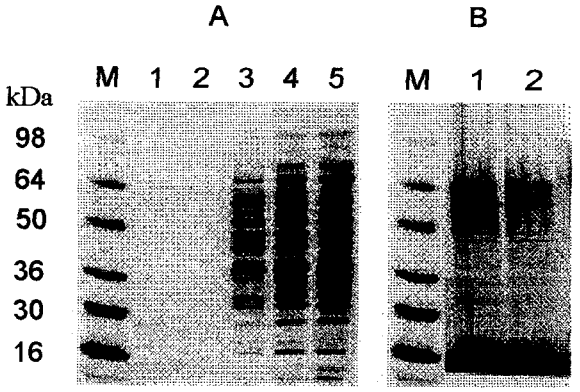

Fig. 6. Protein patterns of the fractions from Renocal gradient centrifugation (A) and comparison of the proteins extracted from a commercial HAV vaccine and the vaccine prepared in this study (B).

A. Fractions from the Renocal gradient were dialyzed against PBS overnight to remove interfering reagents. M: Molecular weight markers; lanes 1, 2, 3, 4, 5, proteins from fraction numbers 9, 10, 1 5, 16, 17 as shown in Fig. 4, respectively. B. Lane 1, extract of a commercial HAV vaccine (HAVRIX, SmithKlein Beecham Biologics); lane 2, extract of the vaccine prepared in this study.

Table 2. Comparison of antibody responses to the gradient purified vaccine and a commercial vaccine in mice.

Dilution rates	No. seropositive[a]/total	
	gradient purified vaccine	commercial vaccine[b]
4^{-1}	5/5	5/5
8^{-1}	4/5	3/5
16^{-1}	2/5	2/5
32^{-1}	0/5	1/5
ED_{50}	12.7^{-1}	12.9^{-1}

[a]Inhibition of ≥50% in enzyme immunoassay (HAVAB EIA, Abbott).
[b]HAVRIX (SmithKlein Beecham Biologics, 720 EU/dose).

All five mice were seropositive at 1/4 dilution rate and the percent seropositive decreased as the dilution rate increased. The ED_{50} calculated by the Reed and Muench equation was 12.7^{-1}, almost the same as that of the commercial vaccine, 12.9^{-1}. The similarity of the immunogenecity of the gradient purified vaccine with that of the commercial vaccine suggests that the partially purified antigen prepared by the procedure described above can be a good vaccine candidate against HAV for human use after requiring safety tests.

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REFERENCES

1. Alter, M. J., S. C. Hadler, and F. N. Judson. 1990. Risk factors for acute non-A, non-B hepatitis in the United States and association with hepatitis C. *JAMA* **264:** 2231-2235.
2. Anderson, D. A. 1987. Cytopathology, plaque assay, and heat inactivation of hepatitis A virus strain HM175. *J. Med. Virol.* **22:** 35-44.
3. Andre, F. E., A. Hepburn, and E. D. Hondi. 1990. Inactivated candidate vaccines for hepatitis A. *Prog. Med. Virol. Basel, Karger* **37:** 72-95.
4. Avrameas, S. and T. Ternynck. 1971. Peroxidase labelled antibody and Fab conjugates with enhanced intracellular penetration. *Immunochemistry* **8:** 1175-1179.
5. Binn, L. N., S. M. Lemon, R. H. Marchwicki, R. R. Redfield, N. L. Gates, and W. H. Bancroft. 1984. Primary isolation and serial passage of Hepatitis A virus strains in primate cell cultures. *J. Clin. Microbiol.* **20:** 28-33.
6. Binn, L. N., W. H. Bancroft, K. H. Eckels, R. H. Marchwicki, D. R. Dubois, L. V. S. Asher, J. W. Leduc, C. J. Trahan, and D. S. Burke. 1988. Inactivated hepatitis A vaccine produced in human diploid MRC-5 cells, pp. 91-93. *In* A. J. Zuckerman (ed.), *Viral Hepatitis and Liver Diseases*, New York, Alan R. Liss, 91-93.
7. Binn, L. N., W. H. Bancroft, S. M. Lemon, R. H. Marchwicki, J. W. Leduc, C. J. Trahan, E. C. Staely, and C. M. Keenan. 1986. Preparation of a prototype inactivated hepatitis A virus vaccine from infected cell culture. *J. Infect. Dis.* **153:** 749-756.
8. Bishop, N. E., D. L. Hugo, S. V. Borovec, and D. A. Anderson. 1994. Rapid and efficient purification of hepatitis A virus from cell culture. *J. Virol. Methods* **47:** 203-216.
9. Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248-254.
10. Coulepis A. G., S. A. Locarnini, E. G. Westaway, G. A. Tannock, and I. D. Gust. 1982. Biophysical and biochemical characterization of hepatitis A virus. *Intervirology* **18:** 107-127.
11. Crance, J. M., J. Passagot, E. Biziagose, and R. Deloince. 1987. Continuous production of hepatitis A virus PLC/PRF/5 cell cultures: Use of antigen for serology. *J. Biol. Methods* **18:** 193-203.
12. Cromeans, T., M. D. Sobsey, and H. A. Fields. 1987. Development of a plaque assay for a cytopathic, rapidly replicating isolate of hepatitis A virus. *J. Med. Virol.* **22:** 45-56.
13. Daemer R. J., S. M. Feinstone, I. D. Gust, and R. H. Purcell. 1981. Propagation of human hepatitis A virus in African green monkey kidney cell culture: Primary isolation and serial passage. *Infect. Immun.* **32:** 388-393.
14. de Chastonay, J. and G. Siegel. 1987. Replicative events in hepatitis A virus-infected cells. *Virology* **157:** 68-75.
15. Dubois, D. R., K. H. Eckels, J. Ticehurst, L. N. Binn, R. L. Timchark, D. A. Barvir, C. T. Rankin, and S. P. O'Neill. 1991. Large-scale purification of inactivated hepatitis A

virus by centrifugation in non-ionic gradients. *J. Virol. Methods* **32:** 327-334.
16. Feinstone, S. M., A. Z. Kapikian, and R. H. Purcell. 1973. Hepatitis A: Detection by immune electron microscopy of a virus-like antigen associated with acute illness. *Science* **182:** 1026-1028.
17. Gust, I. D., A. G. Coulepis, S. M. Feinstone, S. A. Locarnini, Y. Moritsugu, R. Najera, and G. Siegel. 1983. Taxonomic classification of hepatitis A virus. *Intervirology* **20:** 1-7.
18. Gust, I. D., N. I. Lehmann, S. Crowe, M. McCrorie, S. A. Locarnini, and C. R. Lucas. 1985. The origin of the HM175 strain of Hepatitis A virus. *J. Infect. Dis.* **151:** 365-367.
19. Harmon, S. A., D. F. Summers, and E. Ehrenfeld. 1989. Detection of hepatitis A virus RNA and capsid antigen in individual cells. *Virus Res.* **12:** 361-370.
20. Lemon, S. M. and L. N. Binn. 1983. Antigenic relatedness of two strains of hepatitis A virus determined by cross-neutralization. *Infect. Immun.* **42:** 418-420.
21. Lemon, S. M., R. W. Jansen, and J. E. Newbold. 1985. Infectious hepatitis A virus particles produced in cell culture consist of three distinct types with different buoyant densities in CsCl. *J. Virol.* **54:** 78-85.
22. Melnick, J. L. 1982. Classification of hepatitis A virus as enterovirus type 72 and hepatitis B virus as hepadnavirus type I. *Intervirology* **18:** 105-106.
23. Minor, P. D. 1991. *Picornaviridae. In* R. I. B. Franki, C. M. Fauquet, D. L. Knudson, F. Brown (eds.), Classification and Nomenclature of Viruses: The Fifth Report of the International Committee on Taxonomy of Viruses. Wien, Springer-Verlag, *Archives of Virology* Suppl. **2:** 320-326.
24. Nasser, A. M. and T. G. Metcalf. 1987. Production of cytopathology in FRhK-4 cells by BS-C-1 passaged hepatitis A virus. *Appl. Environ. Microbiol.* **53:** 2967-2971.
25. Pellegrini, V., N. Fineschi, G. Matteucci, I. Marsili, L. Nencioni, M. Puddu, H. Garelick, and A. J. Zuckerman. 1993. Preparation and immunogenicity of an inactivated hepatitis A vaccine. *Vaccine* **11:** 383-387.
26. Provost, P. J. and M. R. Hilleman. 1979. Propagation of human hepatitis A virus in cell culture *in vitro*. *Proc. Soc. Exp. Bio. Med.* **160:** 213-221.
27. Reed, L. J. and H. A. Muench. 1938. A simple method of estimating fifty percent endpoints. *Am. J. Hyg.* **27:** 493-497.
28. Ruchti, F., G. Siegel, and M. Weitz. 1991. Identification and characterization of incomplete hepatitis A virus particles. *J. Virol. Meth.* **72:** 2159-2166.
29. Venuti, A., C. D. Russo, N. Del Grosso, A. M. Patti, F. Ruggeri, P. R. De Stasio, M. G. Martinello, P. Pagnotti, A. M. Degener, M. Midulla, A. Pana, and R. Perez-Bercoff. 1985. Isolation and molecular cloning of a fast-growing strain of a human hepatitis A virus from its double-stranded replicative form. *J. Virol.* **56:** 579-588.