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Propagation of Human Hepatitis A Virus in African Green Monkey Kidney Cell Culture: Primary Isolation and Serial Passage

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Human hepatitis A virus (HAV) was propagated in primary African Green Monkey (*Cercopithecus aethiops*) kidney (AGMK) cell cultures. Three strains of HAV were used: MS-1, SD-11, and HM-175. Cells were inoculated with marmoset-passaged material or human clinical specimens and were stained by direct immunofluorescence to establish the identity of the virus. Both clinical samples and marmoset-passaged material produced immunofluorescence. HAV antigen was found scattered throughout the cytoplasm of inoculated cultures. The HM-175 strain produced the most intense immunofluorescence. This strain of HAV had been serially passaged in cell culture seven times. Blocking experiments with paired human sera from naturally acquired HAV infections and hyperimmune chimpanzee serum from an experimentally infected animal established that the immunofluorescence was specific. The viral antigen was found to be exclusively intracellular. The interval to maximum HAV antigen expression was decreased by serial passage. The HAV strain described herein, which was recovered directly from the stool specimen of a patient with HAV in primary AGMK cell culture, may prove useful as a source of antigen for serological tests and as a candidate vaccine strain.

Hepatitis A virus (HAV) was only recently propagated in cell culture for the first time (13). HAV most closely resembles the picornaviruses, especially the subgroup of enteroviruses (3, 4, 7, 14, 16, 17; Y. Moritsugu, T. J. W-K Shih, T. Kakefuda, S. M. Feinstone, J. L. Gerin, and R. H. Purcell, submitted for publication). It may share properties with many of these viruses, such as the coxsackievirus A group, some of which are also difficult to propagate in vitro.

Since HAV does not seem to be produced in large quantities from tissue culture cells (see below) nor to produce recognizable cytopathic effect, the development of in vitro culture systems could not have occurred until after the development of an adequate detection system, in this case, immunofluorescence (IF). By utilizing the recently developed HAV-specific IF system (12), Provost et al. (13) demonstrated that HAV passaged 31 times in marmosets could replicate in cell culture. Since it was not clear if the important step in the in vitro cultivation of HAV was the adaptation to marmosets, the cell culture substrate used, or the IF detection system, we have attempted the in vitro cultivation of HAV obtained directly from human clinical specimens as well as after serial passage in marmosets.

MATERIALS AND METHODS

HAV. Three strains of HAV were employed for direct isolation attempts. Two of these strains (MS-1 and SD-11) were derived from clinical specimens obtained, respectively, from experimental infections (2, 9) and a foodborne epidemic (5) in the United States, and the third strain (HM-175) was obtained from an outbreak in Australia. Stool suspensions were prepared either as 0.2, 2, or 20% extracts in phosphate-buffered saline, pH 7.4, clarified by low-speed centrifugation at 3,000 rpm for 30 min, and stored at −70°C.

Marmoset-passaged material was prepared as 20% liver homogenates in phosphate-buffered saline. Livers from infected animals were frozen and thawed once, homogenized for 30 s in a Colworth 80 Stomacher, and sonicated for 2 min (model 185, Heat Systems Ultrasonics, Plainview, Long Island, N. Y.). Homogenates were clarified by low-speed centrifugation. Supernatants were stored at −70°C. All inocula were known to contain infectious virus as determined by chimpanzee or marmoset inoculations or both. Specimens used for tissue culture inoculation included the following: HM-175, 20% stool suspension; HM-175, marmoset passage (20% liver homogenate); SD-11, 2% stool suspension; SD-11, serum; SD-11, marmoset passage (20% liver homogenate); MS-1, 0.2% stool suspension (filtered); MS-1, serum; MS-1, marmoset passage (20% liver homogenate). These inocula were used for the following reasons. They represented inocula from different parts of the world and were from different epidemiological

settings. In some cases, they represented virus strains which were previously well characterized and well known. They were derived from stool samples with large amounts of HAV antigen (HAV Ag), as determined by serological techniques. As such, they were among the more potent in terms of the quantity of antigen present but were not necessarily the most potent. Also, they were all infectious for chimpanzees and marmosets. The inocula titers for primates are being determined but are not available for inclusion in this paper.

Marmoset passage. Two marmoset species, *Suguinus mystax* and *Saguinus labiatus*, were used for serial passage of HAV. Inocula for first passage in marmosets were HM-175 20% stool suspension (undiluted), SD-11 2% stool suspension (10^{-1} dilution), and MS-1 serum (undiluted). Marmosets were inoculated intravenously with 1 ml of the appropriate virus sample. Livers used for serial passage were removed from sacrificed animals within 1 day of the time when weekly biopsy specimens were found to contain near maximal HAV Ag by IF. Maximal HAV Ag was judged by previous experience with animals that received the same inoculum and that were studied by weekly percutaneous biopsies for the entire course of HAV infection. Serial passage in marmosets was maintained by intravenous inoculation with 20% liver extracts from infected marmosets from the previous passage.

Cell cultures. Primary cell cultures of African Green Monkey (*Cercopithicus aethiops*) kidneys (AGMK) (Microbiological Associates, Bethesda, Md. or Flow Laboratories, Rockville, Md.) were used for attempts at virus propagation. AGMK cells were grown in cover slips in Leighton tubes, in 75 cm^2 flasks, and 850 cm^2 roller bottles. The maintenance medium was Eagle minimal essential medium with Earle salts and 25 mM *N*-2-hydroxyethylpiperazine-*N*'2-ethanesulfonic acid (HEPES) buffer. Medium was supplemented with 2% inactivated fetal bovine serum (GIBCO Laboratories, Grand Island, N.Y.), 50 μg of gentamicin (Microbiological Associates) per ml, 2 μg of amphotericin B (Flow Laboratories) per ml, and 2 mM glutamine (GIBCO Laboratories). Cultures were maintained at 37°C for many weeks under these conditions.

Virus propagation. (i) Primary isolation. Leighton tube cultures of AGMK cells were washed twice with Hanks balanced salt solution and inoculated with 0.1 ml of either a 10^{-1} or 10^{-2} dilution of the serum or stool material. Serum-free medium containing antibiotics was used to dilute stock virus preparations. Adsorption was for 2 h, after which the cells were refed with 1 ml of maintenance medium and incubated at 37°C. Cover slips were assayed by direct IF for HAV Ag at weekly intervals.

(ii) Marmoset-passaged material. Leighton tube cultures of cells were handled in a manner similar to that for primary isolation. Cells were inoculated with 0.1 ml of a 10^{-1} or 10^{-2} dilution of 20% liver homogenate.

(iii) Serial passage. Cells in Leighton tubes were initially inoculated with 0.1 ml of a 1:20 dilution of stool containing strain HM-175. Flasks were inoculated with 1 ml of the same virus suspension. Flasks were refed at weekly intervals with 15 ml of medium. For serial passage of cells from flasks and Leighton tubes, cells were harvested by treatment with trypsin-ethylenediaminetetraacetic acid (GIBCO Laboratories), pelleted by low-speed centrifugation, and suspended in 4 ml of maintenance medium. Leighton tubes were inoculated with 0.1 ml of a 1:2 dilution of the whole cell suspension, and flasks were inoculated with 1 ml of the same cell suspension.

Roller bottles (850 cm^2) were initially inoculated with 5 ml of a 10^{-1} dilution of sixth-marmoset-passaged liver homogenate. The flasks contained 25 to 30 ml of medium. Cells were refed weekly. Cells were harvested at 3- and 8-week intervals, resuspended in 5 ml of Hanks balanced salt solution, frozen-thawed three times, and sonicated for 1 minute. These harvests (1:20 dilution) were then used for passage 2 in AGMK cells. For subsequent passages in AGMK cells, we utilized the same procedure as that for cultures inoculated with stool suspensions. All manipulations of marmoset-passaged inocula and cells inoculated with marmoset-passaged materials were performed in a separate biohazard hood in a separate room from that used for handling inocula and cells for primary isolation.

IF. Cover slips were rinsed with phosphate-buffered saline and fixed in acetone for 1 min. Direct IF was performed as previously described by Mathiesen et al. (12). Fluorescein-conjugated immunoglobulin G prepared from a hyperimmune serum from a chimpanzee (753) that had been experimentally infected with the MS-1 strain of HAV was used at a 1:200 dilution for staining. Cover slips were examined immediately with a Zeiss Photomicroscope III fluorescence microscope, using a Xenon XBO 75-W light source. Photographs were made with a Zeiss 35-mm camera and Kodak ETP-135-36 film. The film was processed by the Kodak ESP-1 method.

Blocking tests. To confirm the specificity of the IF reactions, blocking tests were performed under code with paired sera from humans naturally infected with HAV and paired sera from a chimpanzee experimentally infected with the MS-1 strain of HAV (6). Blocking experiments were performed by preincubating the cover slips for 15 min with a 1:5 dilution of the serum to be tested. The slides were then stained with the conjugated chimpanzee serum at a final dilution of 1:200.

RIA. The solid-phase radioimmunoassay (RIA) and RIA-blocking tests were performed by the method of Purcell et al. (15). Positive/negative (P/N) ratios were expressed as the ratio of the counts per minute in the test wells to the mean counts per minute of the negative control. Values of 2.1 or greater were considered to be positive for antigen; 50% or greater suppression of binding of the radioactive ligand by a sample was considered to be positive for antibody.

RESULTS

Propagation in vitro of HAV after serial passage in marmosets. In Table 1 attempts to recover HAV in AGMK cells by using as the inocula infected livers from marmosets at different passage levels are summarized. Data represent results obtained with 10^{-2} dilutions of in-

ocula unless stated otherwise in the table. Cells inoculated with the marmoset-passaged MS-1 strain did produce weak but detectable antigen. The fluorescence was cytoplasmic and resembled that seen in HAV-infected hepatocytes of marmoset liver biopsies: small, discrete granules of fluorescence scattered throughout the cytoplasm of infected cells (11). Cells inoculated with the marmoset-passaged SD-11 strain of HAV, although positive at 7 days (passages 2 and 3), were negative by 14 days. The greatest amount of fluorescence was detected in the culture inoculated with the sixth-marmoset-passaged HM-175 strain. That inoculum produced intense fluorescence in almost 100% of the AGMK cells after 21 days in culture.

Primary isolation of HAV directly from clinical specimens into AGMK cells. In Table 2 attempts to isolate HAV in AGMK cells from clinical specimens that contained one of three different strains of HAV are listed. No antigen was detected in cultures inoculated with stool or serum containing strain MS-1 by 21 days. Cells inoculated with stool containing strain SD-11 were positive for HAV Ag by day 21, although fluorescence was very weak. Cells inoculated with serum containing strain SD-11 were positive by day 14. In the cell cultures inoculated with stool extract containing strain HM-175, HAV Ag was observed in a few cells 7 days postinoculation. Both the number of cells involved and the intensity of staining increased with time. By 21 days postinoculation, scattered, single fluorescing cells and foci of intensely fluorescing cells could be seen.

Serial passage of HAV in AGMK cells. Because the best results in the initial experiments were obtained with the HM-175 stool- and marmoset-passaged HAV, subsequent experiments were confined to strain HM-175. Roller bottle cultures of AGMK cells inoculated with strain HM-175, sixth-marmoset-passaged material were harvested at weeks 3 and 8 postinoculation and assayed for antigen by RIA. No viral antigen was detected in the cell culture supernatants. However, in the cell lysates (5× concentration, frozen-thawed three times, sonicated) P/N values of 22 and 39 in cultures from weeks 3 and 8, respectively, were observed. Therefore, additional cell culture inoculations were performed with both cell harvests, as well as with the HM-175 stool extract.

Results for cells inoculated with the 3- and 8-week cell harvest of the first AGMK passage are summarized in Table 3. For both cell harvests, the time period for maximum HAV Ag expression decreased with increasing cell passage. At passage 3, both harvests required 11 weeks for the greatest HAV Ag expression. By passage 5 the time for greatest HAV Ag had decreased to 4 weeks. Subsequent passages (6 and 7) also achieved greater HAV Ag levels in a shorter time period. Figure 1 shows fluoresence in the 8-week harvest at week 4 of passage 2. At this time, 80 to 90% of the cells were positive for HAV Ag. At week 6, these cells were harvested. RIA of this cell harvest (3× concentration) yielded a P/N of 8.4.

Inoculation of cells with the stool extract containing HM-175 was repeated, and the cells were maintained for a longer period of time (Table 3). Intense granular fluorescence was observed in a few cells after week 1. Fluorescence was not observed in cover slips during weeks 2 and 3 but could be observed again after week 4, when very few cells were positively stained but focal fluorescence was observed. At week 8, intensely

TABLE 1. *HAV in AGMK cell culture: isolation from marmoset-passaged inocula*

Strain	Marmoset passage no.	IF by wk[a]		
		1	2	3
MS-1	6	–	1	1[b]
	7	1	2	–
	8	2	2	2
SD-11	2	2	–	–
	3	1	–	–
	4	–	–	–
HM-175	2	–	2	3
	5	2	3	2
	6	2	3	4

[a] Fluorescein-labeled anti-HAV was applied to acetone-fixed cell culture cover slips. The amount and distribution of HAV Ag was estimated on the basis of a scale of 1 to 4, indicating increasing intensity of staining and the proportion of cells fluorescing. In general, 4 indicates that the cell sheet contained almost 100% intensely staining cells.

[b] A 10^{-1} dilution of inoculum; nonspecific cell deterioration evident with 10^{-2} dilution.

TABLE 2. *HAV in AGMK cell culture: primary isolation*

Strain	Inoculum	IF by wk[a]		
		1	2	3
MS-1	Stool	—	—	—
MS-1	Serum	—	—	—
SD-11	Stool	±	—[b]	±
SD-11	Serum	—	1	2
HM-175	Stool	1	1	3

[a] Fluorescein-labeled anti-HAV was applied to acetone-fixed cell culture cover slips. The amount and distribution of HAV Ag was determined on a scale of 1 to 4. In general, 4 indicates that the cell sheet contained almost 100% intensely staining cells.

[b] A 10^{-1} dilution of inoculum; nonspecific cell deterioration evident with 10^{-2} dilution.

TABLE 3. *HAV in AGMK cell culture: cell culture passage (HM-175)*

Marmoset passage no.	AGMK passage no.	IF by wk[a]						
		1	2	3	4	6	8	11
0	1	1	—[b]	±	1	1	2	4
	2						4[b]	
	3	—	±	3	4[b]			
	4	2	2	4	4[b]			
	5	1	2	3	4[b]			
	6	1	1	2	3[b]			
	7	1	2	2	2[b]			
6	1	2	3	4[b]			(RIA+)[b]	
	2 (3)[c]	—	4	4	4	1[b]		
	(8)	1	1	4	4	4[b]		
	3 (3)	—	1	—	—	—[b]	—	4
	(8)	1	1	1	1	±[b]	—	4
	4 (3)	—	—	1	2	2[b]		
	(8)	—	—	1	2	2[b]		
	5 (3)	2	3	3	4[b]			
	(8)	2	3	3	4[b]			
	6 (3)	1	2	3[b]				
	(8)	1	2	3[b]				
	7 (3)	2	2	2	2			
	(8)	±	1	3	3			

[a] The amount and distribution of HAV Ag was evaluated on a scale of 1 to 4.

[b] Used for passage.

[c] The second passage was made from 3- and 8-week harvests. These were then maintained and passaged separately.

staining focal areas of rounded cells were observed (Fig. 2). Positive fluorescence continued through the final observation at week 12. At week 2 postinoculation, whole cells harvested from the first AGMK cell passage of HM-175 stool (flasks) were used for passage-2 inoculations of cells in T-75 flasks. At week 8, cells scraped from these flask cultures from passage 2 were strongly positive for antigen, and almost all cells were stained. RIA gave a P/N value of 14.3 for the cell extract. RIA of the supernatant was negative. Cells from passage 2 were used for passage 3. Almost 100% of the cells from passage 3 exhibited intense staining by week 4 postinoculation, and the P/N value of the cell extract was 23.7 by RIA. RIA of the supernatant was again negative. At week 2 after inoculation of passage 4, intense fluorescence was observed in approximately 10% of the cells. By week 3, intense staining was observed in approximately 60% of the cells. At week 4, almost 100% of the cells were stained, and the RIA P/N value was 22.4 on the extract of the cell pellet, but negative on the supernatant. Blocking experiments with hyperimmune chimpanzee serum at this passage level confirmed the specificity of the fluorescence: convalescent but not preinfection serum completely blocked fluorescence. Cultures from passage 5 were positive after week 1, and by week 3, 80 to 90% of the cells were intensely stained. Blocking experiments at this passage level were performed with paired human preinfection and convalescent sera and preexposure and hyperimmune chimpanzee sera. Only the sera containing anti-HAV blocked fluorescence. Cells from passages 6 and 7 were also positive by week 1, and the number of positive cells increased with time.

The specificity of the tissue culture-derived HAV Ag was further proven by RIA blocking experiments: when used as the source of antigen for a solid-phase RIA blocking test (15), the HAV Ag reactivity was blocked by convalescent sera from a chimpanzee and a marmoset experimentally infected with the HM-175 strain of HAV and by convalescent sera from three humans who were naturally infected with the SD-11 HAV strain. None of the preinfection sera blocked the HAV Ag reactivity, nor did preinfection and convalescent sera from one human case and two chimpanzee cases of hepatitis B virus infection.

DISCUSSION

In addition to confirming the findings of Provost et al. (13), this study indicates that HAV can be isolated and serially propagated in AGMK cell culture directly from human clinical specimens. Our results are similar to those of Frösner et al. (8), who reported the isolation of HAV directly from human stool specimens into the hepatitis B surface antigen-producing Alexander hepatoma cell (1), a cell that contains the complete hepatitis B virus genome (10). In both the studies of Frösner et al. and our own, the virus isolated from stool specimens was found to undergo an eclipse phase of approximately 4 weeks, after which the amount of HAV Ag increased. Our results indicate that, with serial passages, the interval to maximum intracellular HAV Ag expression was substantially decreased. An additional similarity between our findings and those of Frösner is the failure to detect any HAV Ag in the cell culture medium. All antigen, and presumably most virus, appears to be cell-associated.

These results also suggest differences among strains in their ability to grow in vitro. The HM-175 strain, whether isolated directly or after marmoset passage, consistently produced more viral antigen than either the MS-1 or SD-11 strains. Whether this is the result of biological differences or differences in titer must be determined after the completion of infectivity titrations of these inocula in primates.

The specificity of the IF staining was proven

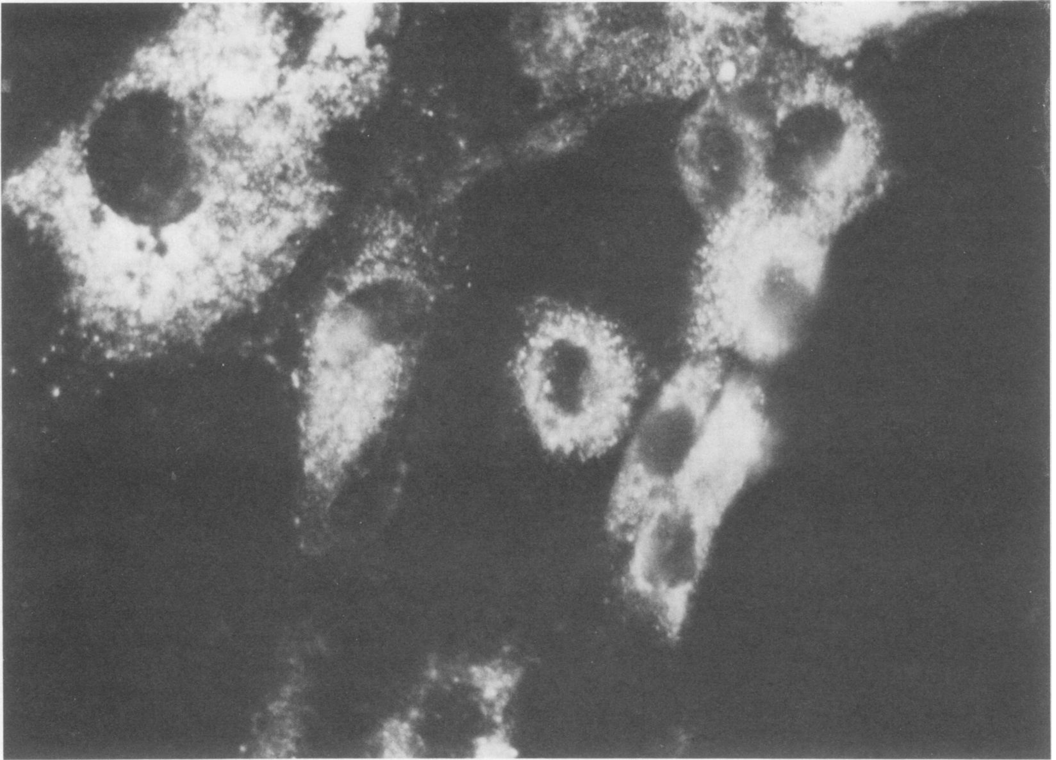

FIG. 1. *HAV Ag expression in AGMK cells at week 4 of the second serial passage. Cells were inoculated with cells from strain HM-175 marmoset passage 6 and AGMK passage 1; 8-week cell harvest. Staining is by direct IF. Large numbers of fluorescent granules are present in the cell cytoplasm.*

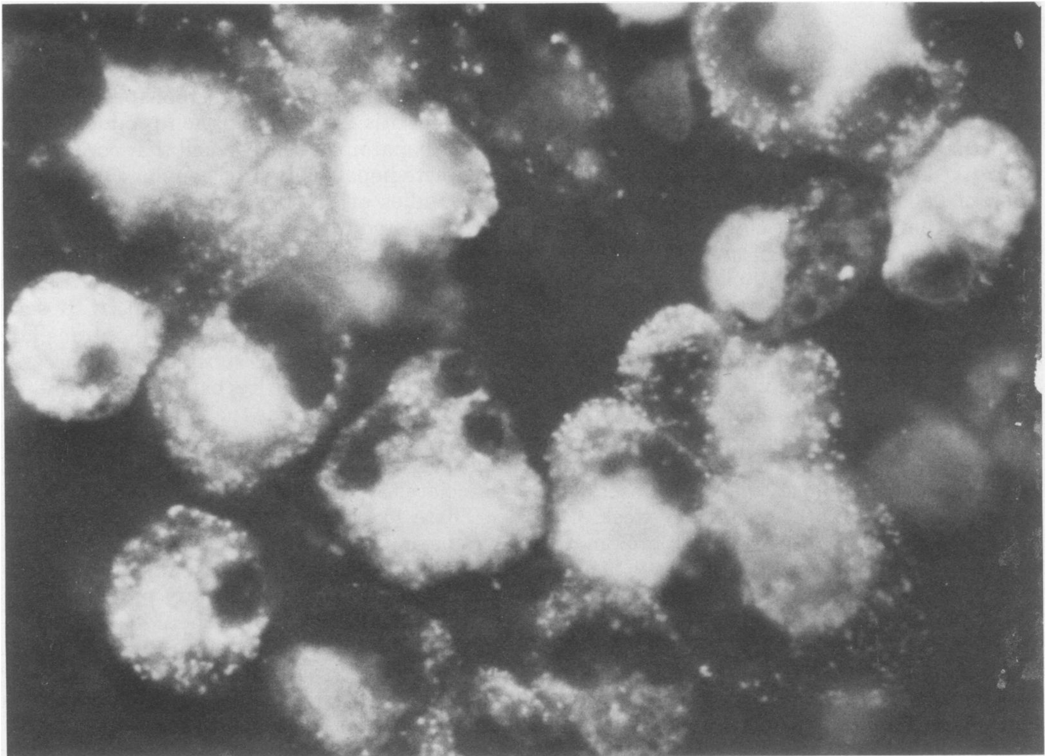

FIG. 2. *HAV Ag expression in AGMK cells at week 8 of the first cell passage. Cells were inoculated with the HM-175 stool extract. Staining is by direct IF. Intense staining of large numbers of granules and focal rounding of cells are present.*

by blocking experiments in which only convalescent serum from a chimpanzee inoculated with the MS-1 HAV strain and two convalescent human sera blocked the IF. Specificity was also proven by RIA blocking experiments. Chimpanzee and marmoset convalescent sera from animals experimentally infected with the HAV HM-175 strain and three human convalescent sera from naturally acquired infections with HAV strain SD-11 blocked HAV Ag reactivity. Convalescent sera from cases of hepatitis B virus did not block reactivity.

At this time, marmosets are the major source for production of HAV and HAV Ag. However, the decreasing availability of these animals and the high cost of acquiring and maintaining them has accelerated the search for an alternative source of antigen for serological tests. We have confirmed in this report the suitability of tissue culture-grown HAV Ag as an antigen for RIA detection of anti-HAV.

HAV isolation directly from clinical specimens into a cell line suitable for vaccine production (primary AGMK cells) raises the hope that additional in vitro cultivation of the virus will yield a strain suitable for vaccine development.

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