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Studies in Chimpanzees of Live, Attenuated Hepatitis A Vaccine Candidates (41570)

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Abstract. Human hepatitis A virus was attenuated in virulence for chimpanzees by passage in FRhK6 and human diploid lung fibroblast cell cultures. A number of variants were developed by passage in cell cultures which showed different levels of virulence/attenuation for chimpanzees. These results were compared to those obtained with marmosets and reported previously. In general, most variants behaved similarly in the two animal types. Two chimpanzees which gave vaccine-like responses following inoculation with HAV cell culture variants were challenged with virulent HAV. Both animals were immune to HAV infection. These findings provide further evidence for the feasibility of developing live, attenuated vaccines against human hepatitis A.

The growth of human hepatitis A virus (HAV) in cell culture was reported in 1979 (1) and has since been confirmed and extended (2–5). Serial passage of HAV in cell cultures produced variants attenuated in virulence for marmosets (6). Marmosets inoculated with the attenuated variants developed anti-HAV and were immune to virulent HAV challenge (6), showing that the attenuated variants had properties of live, attenuated vaccines. The present report demonstrates that certain cell culture-passaged HAV variants also have attenuated, vaccine-like properties in chimpanzees.

Materials and Methods. *Passage of virus in cell culture.* The CR326 strain of HAV was adapted to grow in low passage fetal rhesus kidney (FRhK6) cell cultures at 35°C, after 31 serial *in vivo* marmoset passages, as described previously (1). More than 30 serial passages of the virus were carried out in FRhK6 cells. The virus was subsequently adapted to grow in human diploid lung fibroblast cell cultures (WI-38, MRC5) after different numbers of passages in FRhK6 cells. The virus was adapted to grow at 32°C in human diploid fibroblasts after 5, 10, 15, 20, and 25 passages in FRhK6 and at 35°C in human diploid fibroblasts after 11, 16, 20, and 25 passages in FRhK6 (6, 7). Details of adaptation and passage of two of these variants in cultures of human diploid fibroblasts were previously described (6). The methods used are applicable to all the variants described in the present paper. In general, following a successful first passage in human diploid fibroblasts, an additional three to four serial passages were carried out. These were followed by three serial limit dilution passages plus a final ordinary passage to prepare virus seed used for the tests in animals.

HAV derived directly from the stool of patient 033-03, without marmoset passage, was isolated in FRhK6 cell culture (5). This was the same human patient (033-03) whose blood had yielded the marmoset-adapted HAV strain CR326. This stool-derived HAV was subsequently adapted to grow in MRC5 cell cultures at 32°C after 10 serial passages in FRhK6 cells and in MRC5 cells at 35°C after 15 serial passages in FRhK6. The incubation periods on first passage in MRC5 cells were 55 and 63 days, respectively. These MRC5-adapted variants were serially passed with decreasing incubation periods a total of seven and eight times respectively, including three limit dilution passages, by methods comparable to those described for the marmoset-passaged CR326 variants (6).

Viral growth in cell cultures of all variants was monitored by direct immunofluorescence microscopy of acetone-fixed coverslip cultures (1) and by radioimmunoassay (RIA) for HAV antigen in homogenates of total cultures (cells plus fluid) prepared by freeze–thawing

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TABLE I. SUMMARY OF PROPERTIES OF CELL CULTURE-PASSAGED HAV VARIANTS IN CHIMPANZEES AND MARMOSETS

No. passages in				Chimpanzee		Marmoset	
Marmoset	FRhK6	Human diploid (°C)	$TCID_{50}$ per ml	SGPT elev.	Anti-HAV	SICD elev.	Anti-HAV
31	5	0	$10^{6.0}$	ND[a]	ND	+	+
31	10	0	ND	ND	ND	+	+
31	15	0	ND	ND	ND	+	+
31	20	0	ND	ND	ND	+	+
31	25	0	$10^{8.0}$	−[b]	+[c]	−	+
31	30	0	ND	ND	ND	−	+
31	10	9 (32)	$10^{7.3}$	−	+	+/−[d]	+
31	15	9 (32)	$10^{6.8}$	−	−	−	+/−[e]
31	20	9 (32)	$10^{8.2}$	ND	ND	ND	ND
31	25	9 (32)	$10^{7.2}$	−	−	−	−
31	16	8 (35)	$10^{6.6}$	−	+	−	+
31	20	8 (35)	$10^{6.8}$	−	+/−[f]	−	+
31	25	8 (35)	$10^{7.5}$	−	+	−	+/−[g]
0	10	7 (32)	$10^{6.0}$	−	−	−	−
0	15	8 (35)	$10^{7.3}$	−	+	+/−[h]	+

[a] ND indicates not done.
[b,c] The use of (−) indicates that none of the animals in the group gave the indicated response. The use of (+) indicates that all animals in the group gave the indicated response.
[d] 2/6 marmosets gave SICD values ≥1500 units.
[e] 1/5 marmosets developed anti-HAV.
[f] 1/2 chimpanzees developed anti-HAV.
[g] 3/6 marmosets developed anti-HAV.
[h] 1/6 marmosets gave SICD values ≥1500 units.

and sonication (1). The final passage viral seed preparations at the level tested in animals were assayed for infectivity titers ($TCID_{50}$/ml) in MRC5 cells as described previously (6). The titers are shown in Table I.

Animal studies—marmosets. Most of the studies in marmosets given in Table I were reported earlier (6) and are repeated here for comparative purpose. The human diploid-adapted variants had been inoculated into six marmosets each. Those preparations which had been passed only in FRhK6 cells, prior to human diploid cell adaptation and purification by limit dilution, had each been inoculated into two marmosets. The marmoset studies of MRC5-adapted variants isolated from the stool of patient 033-03 are new. They were performed in the same manner as those reported earlier. Briefly, 1 ml of 1:10 dilution of each variant was inoculated iv into each of six marmosets per variant. Weekly bleedings through 12 weeks postinoculation were assayed for serum isocitric dehydrogenase (SICD) and for anti-HAV by RIA (HAVAB) and immune adherence hemagglutination (IAHA) (8). A final bleeding at 17–18 weeks was similarly assayed. HAVAB assays for anti-HAV were used only qualitatively. Anti-HAV titers were measured by IAHA. In our laboratory normal marmoset plasmas give mean SICD values of about 500 units. Virulent HAV infections in marmosets invariably induce SICD values greater than 1500 units. In assessing the activity of HAV cell culture variants in the present work, SICD values ≥ 1500 units, occurring within a week of anti-HAV onset, were considered elevations.

Chimpanzees. Young adult chimpanzees were used. They were housed in open cages in individual isolation rooms maintained under negative pressure. Phencyclidine hydrochloride or ketamine hydrochloride were used to anesthetize the animals at weekly intervals. Each human diploid cell-adapted viral variant

was inoculated iv into two chimpanzees as 1 ml of undiluted virus seed per animal. At weekly intervals through 12 weeks postinoculation, blood samples and 16-gauge percutaneous needle liver biopsy specimens were obtained. An additional and final bleeding was done at 17–18 weeks. Stool samples were collected daily and stored at −20°C. The virus preparation which had been passed 25 times in FRhK6 cells prior to adaptation to human diploid cells was tested in only one chimpanzee.

Assays of chimpanzee specimens. Sera were assayed for alanine aminotransferase (SGPT) (9) and for anti-HAV by HĀVAB. SGPT values ⩾ 20 mU/ml were considered probable elevations. Sera which were positive by the HĀVAB assay were also assayed by IAHA. Liver needle biopsy specimens were fixed in 10% Formalin, embedded, sectioned, stained with hematoxylin and eosin by standard methods, and microscopically examined by Dr. André Phelps (Merck Sharp & Dohme Research Laboratories). Every other day stool specimens were thawed and homogenized to 20% extracts in phosphate-buffered saline by mechanical shaking with glass beads. After clarification by centrifugation, the extracts were assayed for HAV antigen by the same RIA procedure used to assay HAV antigen in cell cultures (1), but with the use of normal chimpanzee stool extract as negative control.

Virulent HAV challenge. A preparation of virulent HAV from infected marmoset liver (passage 13 of HAV strain CR326 in marmoset) with an infectivity titer for marmosets of about 10^9 ID_{50}/gm was diluted to 10^{-3} in phosphate-buffered saline and each chimpanzee given 1 ml iv. Thus the challenge inoculum represented about 10^6 marmoset ID_{50}. Material from the same source had been used at 10^{-5} dilution to challenge marmosets immunized with live, attenuated HAV variants (6). Two nonimmunized chimpanzees (061, 104) and two chimpanzees (058, 103), who had given immune responses to inoculation with attenuated HAV variants, were challenged. Bleedings, liver biopsies, stool collection, and assays were as described earlier.

Results. Table I is a summary of the tissue culture infectivity titers and behavior in chimpanzees of nine cell culture-passaged HAV variants, seven with a history of marmoset passage and two isolated directly from human stool. For comparison, the table also includes the behavior in marmosets of 12 variants which were previously described (6) as well as that of the two stool-derived HAV variants which are described for the first time.

As noted in Table I, none of the HAV variants tested in chimpanzees induced SGPT elevations, indicating that all were somewhat attenuated for these animals. Most of these variants also did not induce SICD elevations in marmosets. However, variants 31 + 10 + 9 (32°C) and 0 + 15 + 8 (35°C) did induce slight elevations in a portion of the test marmosets. Two of six marmosets given 31 + 10 + 9 (32°C) had 1-week SICD elevations of 1770 and 2080 units, respectively, coincident with the onset of anti-HAV. This variant was designated variant No. 1 (32°C) in an earlier report (6). One of six marmosets given variant 0 + 15 + 8 (35°C) also had a 1-week SICD elevation of 1620 at the time of anti-HAV onset. Other than for these and the SICD elevations induced by the nonlimit diluted, nonhuman diploid cell-passaged virus preparations (31 + 5 + 0, 31 + 10 + 0, 31 + 15 + 0, 31 + 20 + 0), the HAV variants tested in marmosets did not induce SICD elevations greater than 1500 units.

Though not shown in Table I, weekly needle liver biopsies of all test chimpanzees given HAV cell culture variants were free of evidence of viral hepatitis, with the possible exceptions of the one animal given 31 + 25 + 0 and the two animals given 0 + 15 + 8 (35°C). The livers of these animals showed brief traces of mononuclear cell inflammatory activity and liver cell necrosis at the time of anti-HAV onset. Needle liver biopsies were not performed in the marmoset studies, but some marmosets given variants 31 + 10 + 9 (32°C) and 31 + 16 + 8 (35°C) in our earlier report (6) had been killed and their liver tissue examined. Variant 31 + 16 + 8 (35°C) was also designated variant No. 2 (35°C) in this report. Histopathologic changes ranged from none to moderate with the former, while changes were uniformly minimal from the latter inoculum. These inocula produced no detectable liver damage in chimpanzees of the present study.

Assays (RIA) for HAV antigen in stool ex-

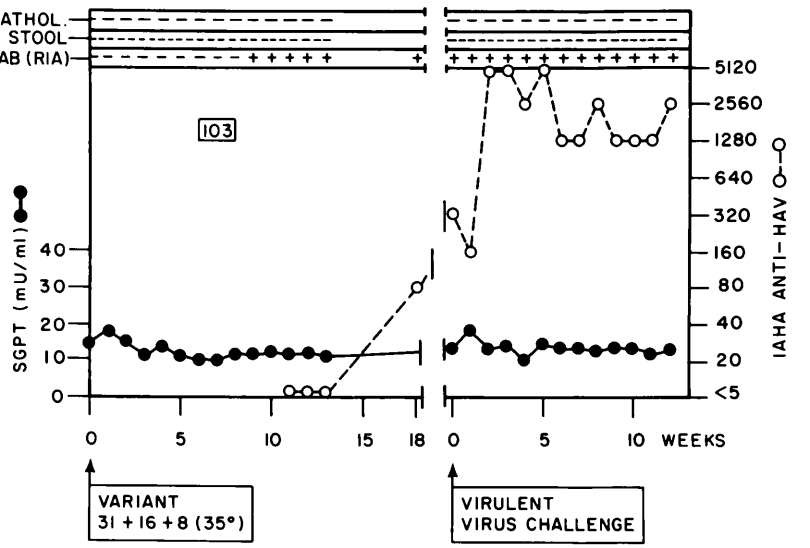

FIG. 1. Responses of chimpanzee No. 103 showing immunization with variant 31 + 16 + 8 (35°C) and resistance to subsequent challenge with virulent HAV.

tracts of chimpanzees given the HAV cell culture variants, including those from animals given 31 + 25 + 0 and 0 + 15 + 8 (35°C), were uniformly negative. This further substantiated the attenuated nature of these virus preparations. HAV antigen assays were not performed on marmoset stools.

Table I also indicates that some of the HAV cell culture variants were overattenuated, as indicated by the failure of these variants to induce anti-HAV in test animals. This was most pronounced in variants adapted to MRC5 cells at 32°C. Variant 31 + 25 + 9 (32°C) failed to induce anti-HAV in both marmosets and chimpanzees. Variant 31 + 15 + 9 (32°C) failed to induce anti-HAV in chimpanzees and seroconverted only one of five marmosets. The anti-HAV in the one positive marmoset was detectable by HĀVAB but was too weak to be measured by IAHA. These 32°C-adapted variants were most likely unable or minimally able to propagate in the test animals. Variant 31 + 10 + 9 (32°C), however, induced anti-HAV in six of six marmosets and two of two chimpanzees. All animals were positive by HĀVAB assay. By IAHA testing, however, only four of six marmosets had measurable titers (geometric mean, 1:120). Both chimpanzees were positive by IAHA (mean titer, 1:30). Although this variant as noted earlier retained a slight virulence for marmosets, the IAHA anti-HAV mean titer of 120 is well below that of ≥2560 which is characteristic of virulent HAV infection in the marmoset.

The HAV variants adapted to MRC5 cells at 35°C showed much less tendency to overattenuation, as also indicated in Table I. The most highly passaged variant (31 + 25 + 8, 35°C) retained the capacity to induce anti-HAV in chimpanzees. Two of two chimpanzees seroconverted by HĀVAB assay and both were also positive by IAHA testing (mean titer, 1:120). In marmosets, however, this variant was minimally active, seroconverting three of six animals by HĀVAB assay but none of six by IAHA testing. Variant 31 + 20 + 8 (35°C) was more active in marmosets. It seroconverted six of six marmosets in both HĀVAB and IAHA testing (geometric mean IAHA titer, 1:533). It was also active in chimpanzees, although it induced anti-HAV in only one of two test animals (IAHA titer, 1:20). Variant 31 + 16 + 8 (35°C) was also active in both types of animals. Two of two chimpanzees responded by both HĀVAB and IAHA testing (mean IAHA titer, 1:60), and six of six marmosets were also positive for anti-HAV in both assays (geometric mean IAHA titer, 1:320). Virus preparation 31 + 25

+ 0, which was grown in FRhK6 cells at 35°C but was neither adapted to MRC5 cells nor carried through limit dilution, was tested in only one chimpanzee. This animal seroconverted by both HĀVAB and IAHA testing (IAHA titer, 1:160). This virus preparation also induced anti-HAV in two of two marmosets by both assays (mean IAHA titer, 1:1280).

The differences between 32°C- and 35°C-adapted variants of HAV were especially distinct in tests of the stool-derived variants 0 + 10 + 7 (32°C) and 0 + 15 + 8 (35°C). As given in Table I, the former was totally inactive in both types of test animals. The latter, as discussed earlier, retained a trace of virulence for both types of animals. In terms of anti-HAV induction, this variant converted six of six marmosets by both HĀVAB and IAHA testing (geometric mean IAHA titer, 1:293). It also seroconverted two of two chimpanzees by both tests (mean IAHA titer, 1:160).

Two chimpanzees which showed vaccine-like responses following inoculation with variants 31 + 16 + 8 (35°C) and 31 + 25 + 8 (35°C) were challenged with 10^6 marmoset ID_{50} of virulent HAV. Figure 1 shows the response of chimpanzee 103 to inoculation with 31 + 16 + 8 (35°C). Through 12 weeks postinoculation, the animal showed no SGPT elevation, no histopathologic evidence of viral hepatitis, and no HAV antigen in its stool. Anti-HAV became positive at Week 9 by HĀVAB and the IAHA anti-HAV titer was 1:80 at Week 18. Approximately 8 months later, as also seen in Fig. 1, the animal had an IAHA titer of 1:320 and was challenged. Two weeks following challenge, the IAHA titer boosted to 1:5120 and stabilized at 1:1280 to 1:2560 throughout the following 10 weeks. There was no SGPT elevation, no evidence of viral hepatitis in the liver, and no HAV antigen excretion in the stool of this animal through 12 weeks postinoculation.

Similar data were obtained with chimpanzee 058 which also gave a vaccine-like response after inoculation with variant 31 + 25 + 8 (35°C). It gave no evidence of HAV infection other than an HĀVAB response beginning at Week 9 and an IAHA anti-HAV titer of 1:160 at Week 17 postinoculation. Approximately 7 months later, this animal still had an IAHA anti-HAV titer of 1:160 and was given the challenge virus. It showed a rapid rise in anti-HAV titer to 1:5120 at 2 weeks postchallenge. This titer stabilized at 1:1280 over the following 10-week period. As was the case with chimpanzee 103, this animal also showed no SGPT elevation, no evidence of viral hepatitis in the liver, and no excretion of antigen in the stool over the 12-week period following challenge.

In Figure 2 are recorded the responses of two chimpanzees (061 and 104) without anti-HAV which were also challenged with viru-

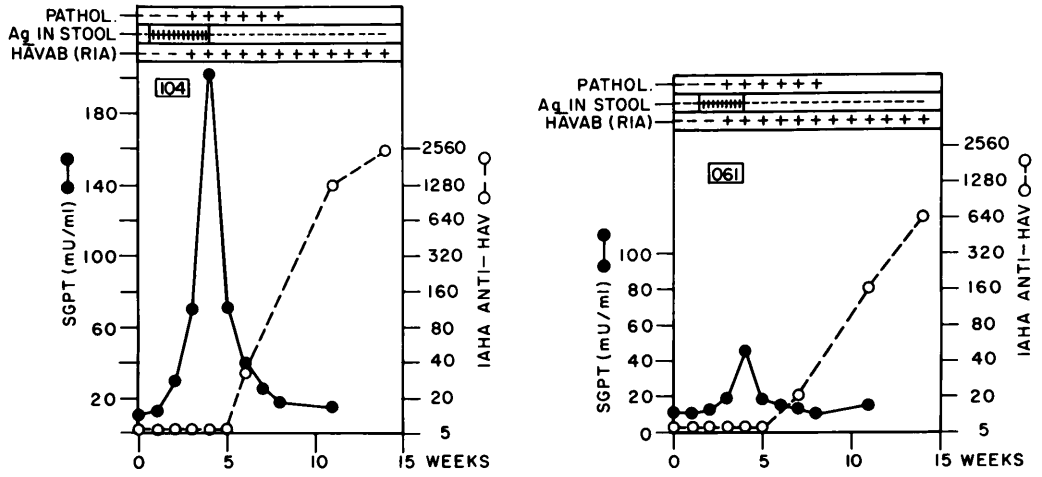

FIG. 2. Responses of nonimmunized chimpanzees 104 and 061 to virulent HAV challenge.

lent HAV in the same dose as was given to 058 and 103, to prove the nature of infection to be expected in nonimmunized chimpanzees. Both animals showed SGPT elevations. Animal 104 peaked at 204 mU/ml at Week 4. Animal 061 peaked at 46 mU/ml at Week 4. Both also showed HAV antigen excretion in stool extracts. Animal 104 was positive from Day 4 through Day 26. Animal 061 was positive from Day 10 through Day 26. Both animals showed evidence of viral hepatitis in liver biopsies starting at Week 3 and continuing through Week 8 when biopsying was discontinued. HAVAB onset occurred in both animals starting at Week 3 and both also showed IAHA anti-HAV responses commencing at Weeks 6 and 7 and reaching titers of 1:640 and 1:2560, several fold higher than those induced by any of the HAV cell culture variants. Thus, by every parameter of HAV infection, these two chimpanzees showed the nature of virulent HAV infection, in contrast to the attenuated infections induced by HAV cell culture variants.

Discussion. As was previously shown in marmosets (6), this report establishes that variants of HAV can be produced by passage in FRhK6 and human diploid lung fibroblast cell cultures which have live, attenuated vaccine-like properties for chimpanzees. Also in the present work we see evidence that HAV isolated directly from human stool is modified by a similar cell culture passage regimen to that which was effective with the marmoset-adapted CR326 strain of HAV. In general, marmosets gave greater evidence than chimpanzees of minor enzyme elevations and slight liver damage when inoculated with some viral variants, but overall there was a considerable similarity of behavior of the viral variants in the two animal types.

Most importantly, as was previously shown in marmosets (6), the present work establishes that chimpanzees are able to be fully protected against virulent HAV challenge by immunization with typical attenuated HAV variants. When challenged, immunized chimpanzees showed no enzyme elevation, no antigen excretion in stool, and no evidence of viral hepatitis in liver biopsies. However, in contrast to our earlier studies in marmosets (6, 10), the chimpanzees showed a boost in anti-HAV titer on challenge. This may be due to the fact that we used 100 times more viral mass in our chimpanzee challenge than had been used to challenge marmosets, and the excess antigen may have been adequate in amount to give a booster response. Alternatively, the booster response in anti-HAV may represent a difference in the immune capacity of the chimpanzee as compared to the marmoset.

In the present study, we have noted no excretion of detectable HAV antigen in the stools of chimpanzees infected with HAV variants. Chimpanzees given virulent HAV, in contrast, showed HAV antigen excretion detectable by RIA in stool extracts, commencing many days prior to histopathologic change in the liver and ceasing at the time of peak enzyme elevation and shortly after the onset of anti-HAV and histologic liver disease. The excretion of viral antigen for a considerable time period prior to liver damage is consistent with the noncytopathic nature of HAV, as has been noted with virus growing in cell cultures (1, 5). The absence of antigen excretion in stools of chimpanzees given attenuated variants does not necessarily imply that viral excretion does not occur, as the lowest detection level of the RIA is on the order of 2 ng of antigen/ml. Studies are in progress to determine if infectious virus is shed in the stools of these chimpanzees by inoculation of stool extracts into cell cultures and examination for viral replication.

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