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Progress toward a Live, Attenuated Human Hepatitis A Vaccine (41387)

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Abstract. Human hepatitis A virus was attenuated in virulence for marmosets by passage in FRhK6 and human diploid lung fibroblast cell cultures. A number of variants were produced that showed different levels of virulence/attenuation for marmosets. Marmosets that had attenuated virus-like responses following injection of variant virus were challenged with virulent virus; all were solidly immune to infection. Antibody stimulated by the vaccine equated with protection. These findings show that human hepatitis A virus can be attenuated and show the feasibility for eventual development of a live, attenuated hepatitis A vaccine for human use.

Deinhardt and co-workers (1) provided evidence for the suitability of the marmoset as a host animal for human hepatitis A virus (HAV). We confirmed and extended this work and adapted the CR326 strain of HAV to *Saguinus mystax* and *S. labiatus* marmosets (2–4). HAV derived from infected marmoset liver provided antigen for the first practical serologic assays of anti-HAV (5, 6) and formalin-inactivated virus vaccine prepared using infected liver was shown to protect marmosets against virulent virus challenge (7). Also, HAV derived from infected marmoset liver was used as inoculum in the first successful propagation of HAV in cell culture (8). The virus was passaged serially in fetal rhesus monkey kidney cells (FRhK6) (9), and was subsequently adapted to grow in human diploid lung fibroblast (WI-38, MRC5) cell cultures (10). Attenuation of virulence of the virus was found both for marmosets and chimpanzees. This report summarizes experiments in development of an attenuated, live HAV vaccine and its evaluation in marmosets.

Materials and Methods. *Passage of virus in cell culture.* The CR326 strain of HAV, after 31 passages in the marmoset, was adapted to FRhK6 cell cultures incubated at 35°, as described earlier (8). More than 30 serial passages of the virus were carried out in FRhK6 cell cultures. 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 in human diploid lung fibroblast cells at 32° after 5, 10, 15, 20, and 25 passages in FRhK6 and at 35° after 16, 20, and 25 passages in FRhK6 (10).

The following describes the development of two particular HAV variants adapted to and serially passed in human diploid lung fibroblast cells. The methods are generally applicable to all the variants passed in human diploid lung fibroblast cells.

Variant No. 1 (32°): 31 marmoset passages (4) plus 10 FRhK6 passages (8) plus 9 passages in MRC5 cells at 32° (31 + 10 + 9 at 32°). Virus from FRhK6 passage 10 was diluted 1:5 in Williams medium E (Flow) with 0.5% fetal calf serum, 2 m*M* glutamine, and 50 μg/ml neomycin. One milliliter of virus was applied per drained, confluent monolayer of MRC5 cells in 25-cm² Falcon flasks. This was absorbed for 4 hr at 32° after which an additional 4 ml of culture medium was added. The cultures were incubated at 32° and medium was replaced at 7-day intervals thereafter. Comparable cultures that contained coverslips were also prepared and the removed coverslips were examined by direct immunofluorescence microscopy (8) at periodic intervals for the presence of HAV antigen. At the time that immunofluorescent cytoplasmic granules were clearly demonstrable

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in the coverslip cultures, the noncoverslip cultures were harvested by freeze–thawing twice, sonication, and low-speed clarification (8). The HAV antigen content in the product was estimated by radioimmunoassay (RIA) (8). Results of the radioimmunoassay were expressed as P/N values. These values were obtained by dividing the cpm of the test sample by the mean cpm obtained from five negative control specimens. Samples were considered positive when cpm values were three or more standard deviations greater than the mean negative control cpm. In passages 2 through 5, the inoculum was 1 ml of 1:20 dilution of seed, that was washed off after the 4-hr absorption period. These cultures were refed with 5 ml of culture medium and subsequently treated in the same manner as the first passage. Passages 6, 7, and 8 were limit dilution passages in which four 25-cm^2 MRC5 cultures were inoculated with 1 ml at each of eight successive half-logarithm dilutions of the virus inoculum, from $10^{-5.5}$ to $10^{-9.0}$. The 1-ml inocula were absorbed for 4 hr, were not removed, and all flasks were subsequently supplemented with 4 ml culture medium. These cultures were incubated 4–5 weeks with weekly medium replacement. Each flask was harvested individually and assayed for HAV antigen by RIA. The material derived from a single positive flask at the highest dilution of seed inoculum was used for the next passage. In this way, three successive limit dilution passages were carried out. The inoculum in the final passage (passage 9) was 1 ml of a 1:200 dilution of virus. This was absorbed for 4 hr and subsequently washed off. Virus growth was followed by immunofluorescence assay as in passages 1 through 5. The final viral harvest was assayed by RIA and by titration in MRC5 cell culture.

Variant No. 2 (35°): 31 passages in marmoset (4) plus 16 passages in FRhK6 cells (8) plus 8 passages in human diploid cells at 35° (31 + 16 + 8 at 35°). The first and second passages of this variant in human diploid cells were carried out in WI-38 cells. All subsequent passages (3 through 8) were in MRC5 cells. Passages 1 and 2 were carried out in the same manner, except for temperature, as passage 1 of variant No. 1 (32°), described earlier. Passages 3 and 4 were carried out at 35° in the same manner as passages 2 through 5 of variant No. 1 (32°). Passages, 5, 6, and 7 were limit dilution passages at 35° carried out in a manner comparable to the limit dilution passages of variant No. 1 (32°). The final passage (passage 8) was made at 35° in the same way as passage 9 of variant No. 1 (32°).

Titration of infectious virus in cell culture. The products of final viral passages were diluted in culture medium in 10-fold steps from 10^{-5} through 10^{-9} and were titrated in MRC5 cells by inoculation of 1 ml to each of four 25-cm^2 confluent monolayers per dilution. After 4 hr of absorption, the cultures were supplemented with 4 ml culture medium and incubated at appropriate temperature (32 or 35°). Culture medium was replaced weekly and all flasks were individually harvested after 35 days of incubation. The products were assayed for HAV antigen by RIA. The $TCID_{50}$ per milliliter was calculated by the Reed and Muench method.

Inoculation of marmosets. A number of variants that were prepared in cell culture were inoculated into *S. labiatus* marmosets to measure virulence/attenuation for these animals. In all cases, the animals were inoculated iv with 1 ml of a 1:10 dilution of the virus in phosphate-buffered saline. The animals were bled weekly and plasmas were assayed for isocitric dehydrogenase (ICD) values (2) and for anti-HAV by HAVAB (Abbott) and immune adherence hemagglutination (IAHA) methods (6). ICD values of ≥1500 Sigma units for one or more weeks were considered to be elevations. The HAVAB assay was used to give positive or negative anti-HAV results only, as described by the manufacturer. Anti-HAV titers were determined by the IAHA assay.

Virulent HAV challenge in marmosets. A preparation of virulent HAV from infected marmoset liver (passage 13 of CR326 agent in marmosets) had an infectivity titer for marmosets of about 10^9 fifty percent infectious doses per gram of liver. This same virus had been used previously at 10^{-6} dilu-

tion as challenge in marmosets that were immunized with killed HAV vaccine (7). In the present work, animals that were previously inoculated with variants No. 1 (32°) and No. 2 (35°) were subsequently challenged iv with 1 ml of a 10^{-5} dilution of the virulent HAV preparation. In addition, eight normal marmosets served as virus controls—four were inoculated iv with 1 ml of 10^{-5} dilution, and four were inoculated iv with 1 ml of 10^{-7} dilution. All animals were bled weekly and plasmas were assayed for ICD values and anti-HAV responses, as already described.

Histologic findings in livers of marmosets. Liver specimens were obtained from seven additional *S. labiatus* marmosets that had been inoculated as follows: three with 1 ml of 1:10 diluted variant No. 1 (32°), and four with 1 ml of 1:10 diluted variant No. 2 (35°). These animals were killed within 24 hr of the first detectable onset of anti-HAV as measured by HAVAB. Liver sections were fixed in 10% formalin, sectioned, stained by hematoxylin–eosin, and examined for histopathologic changes.

Results. HAV was usually more easily adapted to grow in human diploid fibroblasts at 32° than at 35° (10). However, once adaptation was achieved, the 32° and 35° strains of HAV had similar growth characteristics both in terms of incubation periods and viral yields. This was true of variants No. 1 (32°) and No. 2 (35°). Table I gives the incubation periods and viral yields at all passage levels of the two variants. At the final passages, variants No. 1 (32°) and No. 2 (35°) gave similar yields of total antigen (P/N values 7.1 and 5.3, respectively) and of infectious virus ($TCID_{50}$/ml $10^{7.3}$ and $10^{6.6}$, respectively).

The ICD and anti-HAV responses during a 224-day observation period of four marmosets (Nos. 1–4) injected with variant No. 1 (32°) are shown in Table II. A slight ICD elevation occurred in animal 4 at Day 35. Its occurrence just prior to anti-HAV appearance enhances the likelihood that it was related to HAV infection. Animal 2 experienced a suggestive rise in ICD value at Day 46. All four animals developed anti-HAV as measured by HAVAB (RIA). At intervals of approximately 1 to 4 weeks after onset of HAVAB, three of four marmosets became positive for anti-HAV by IAHA. Animal 3 failed to develop IAHA anti-HAV.

Table III shows the ICD and anti-HAV responses through a 138-day observation

TABLE I. PASSAGE HISTORIES OF HAV VARIANTS IN MRC5[a] CELL CULTURES

Variant							
No. 1 (31 + 10 + 9 at 32°)				No. 2 (31 + 16 + 8 at 35°)			
Pass. No.	No. days	RIA (P/N)[b]	$TCID_{50}$/ml	Pass. No.	No. days	RIA (P/N)[b]	$TCID_{50}$/ml
1	28	4.4		1	42	1.2	
2	28	4.2		2	42	3.4	
3	26	10.2		3	28	5.8	
4	21	8.0		4	20	5.0	
5	20	4.6		5	35	5.8[f]	
6	34	8.5[c]		6	34	3.7[g]	
7	31	6.8[d]		7	28	2.7[h]	
8	27	2.2[e]		8	20	5.3	$10^{6.6}$
9	19	7.1	$10^{7.3}$				

[a] All passages were made in MRC5 cells except passages 1 and 2 of variant 31 + 16 + 8 at 35° which were done in WI-38 cells.

[b] RIA (P/N) values were calculated by dividing the cpm of the test material by the mean cpm obtained from 5 negative control specimens.

[c,d,e,f,g,h] These were all limit dilution passages. P/N values are those of the single flask cultures at highest positive dilutions, which were used for passage. Respectively, the dilutions were $10^{-6.5}$, $10^{-7.5}$, $10^{-7.0}$, $10^{-7.0}$, $10^{-7.0}$, $10^{-6.5}$.

TABLE II. Responses of Marmosets 1, 2, 3, and 4 to Inoculation with Variant No. 1 (32°)

	Animal No.											
	ICD value				HĀVAB (RIA)				IAHA titer			
Days	1	2	3	4	1	2	3	4	1	2	3	4
0	640	690	600	670	–	–	–	–	<5	<5	<5	<5
6	300	480	290	390	–	–	–	–	<5	<5	<5	<5
13	460	530	400	400	–	–	–	–	<5	<5	<5	<5
20	570	850	670	610	–	–	–	–	<5	<5	<5	<5
27	340	740	590	400	–	–	–	–	<5	<5	<5	<5
35	460	570	370	1770	–	–	–	–	<5	<5	<5	<5
46	520	1040	500	570	+	–	–	+	<5	<5	<5	<5
55	260	720	300	320	+	+	–	+	<5	<5	<5	80
62	539	680	540	430	+	+	–	+	<5	<5	<5	40
69	630	620	450	370	+	+	–	+	<5	<5	<5	40
76	470	470	550	600	+	+	–	+	80	160	<5	640
83	610	450	590	460	+	+	–	+	80	160	<5	1280
90	400	850	650	400	+	+	+	+	160	320	<5	2560
97	290	580	630	500	+	+	+	+	160	320	<5	2560
103	290	390	580	340	+	+	+	+	80	320	<5	1280
112	450	710	630	400	+	+	+	+	80	320	<5	2560
224	340	540	380	280	+	+	+	+	320	1280	<5	5120

period of four marmosets (Nos. 5–8) given variant No. 2 (35°). None of the animals showed ICD elevations. All developed anti-HAV as measured by HĀVAB within 4–6 weeks. All four animals also developed IAHA anti-HAV at intervals of 4 to 8 weeks after onset of HAVAB.

At Day 224 postinoculation with variant No. 1 (32°), animals 1–4 were challenged with virulent virus at 10^{-5} dilution. The results through 12 weeks postchallenge are summarized in Table IV. None of the four animals showed ICD elevation. None showed an increase in anti-HAV titer. Even

TABLE III. Responses of Marmosets 5, 6, 7, and 8 to Inoculation with Variant No. 2 (35°)

	Animal No.											
	ICD value				HAVAB (RIA)				IAHA titer			
Days	5	6	7	8	5	6	7	8	5	6	7	8
0	350	440	430	430	–	–	–	–	<5	<5	<5	<5
5	420	480	440	500	–	–	–	–	<5	<5	<5	<5
12	410	480	430	450	–	–	–	–	<5	<5	<5	<5
18	270	350	280	250	–	–	–	–	<5	<5	<5	<5
25	410	440	500	580	–	–	–	+	<5	<5	<5	<5
33	760	620	550	660	–	–	+	+	<5	<5	<5	<5
40	710	660	540	500	+	+	+	+	<5	<5	<5	<5
47	330	490	360	320	+	+	+	+	<5	<5	<5	<5
53	520	490	490	400	+	+	+	+	<5	<5	<5	<5
61	520	560	640	520	+	+	+	+	<5	<5	<5	160
68	480	450	650	470	+	+	+	+	<5	<5	<5	160
75	400	450	400	330	+	+	+	+	<5	<5	<5	320
81	330	380	310	260	+	+	+	+	<5	<5	<5	160
88	340	370	400	350	+	+	+	+	320	<5	<5	320
95	370	400	420	490	+	+	+	+	320	160	160	320
117	300	370	330	340	+	+	+	+	320	160	160	320
138	310	380	320	300	+	+	+	+	1280	640	320	1280

TABLE IV. RESPONSES OF IMMUNIZED AND CONTROL MARMOSETS TO VIRULENT VIRUS CHALLENGE

Marmoset group	Animal No.	Antibody status prior to challenge: HĀVAB (RIA)	Antibody status prior to challenge: IAHA titer	Challenge dose	Postchallenge results: ICD[a] elev.	Postchallenge results: Antibody status: RIA	Postchallenge results: Antibody status: IAHA titer
Immunized;	1	+	320	10^{-5}	None	+	160
variant	2	+	1280	10^{-5}	None	+	320
No. 1 (32°)	3	+	<5	10^{-5}	None	+	<5
	4	+	5120	10^{-5}	None	+	1,280
Immunized;	5	+	1280	10^{-5}	None	+	640
variant	6	+	640	10^{-5}	None	+	1,280
No. 2 (35°)	7	+	320	10^{-5}	None	+	320
	8	+	1280	10^{-5}	None	+	1,280
Nonimmunized;	9	−	<5	10^{-5}	+/−[a]	+	2,560
virus controls	10	−	<5	10^{-5}	+	+	10,240
	11	−	<5	10^{-5}	+	+	10,240
	12	−	<5	10^{-5}	+	+	5,120
Nonimmunized;	13	−	<5	10^{-7}	+	+	1,280
virus controls	14	−	<5	10^{-7}	+	+	5,120
	15	−	<5	10^{-7}	+	+	2,560
	16	−	<5	10^{-7}	+	+	2,560

[a] Values of ≥1500 Sigma units for one or more weeks were considered ICD elevations. Animal no. 9 fell slightly short of this level, with values of 1070 on Day 41 and 930 on Day 48.

animal 3, without detectable IAHA antibody, appeared to be fully protected against the virulent challenge. At Day 138 postinoculation with variant No. 2 (35°), animals 5–8 were also challenged with virulent HAV at 10^{-5} dilution, with results as given in Table IV. All four animals were fully protected. No ICD elevations or boosts in IAHA anti-HAV were noted. The findings in challenge tests of marmosets that had not been vaccinated are also shown in Table IV. Virus control animals (Nos. 9–12) given the same challenge dose as the vaccinated animals, as well as control animals (Nos. 13–16) given 1/100 the challenge dose, all developed ICD elevations and strong and rapid anti-HAV responses.

Figure 1 shows graphically the ICD values, HĀVAB results, and IAHA anti-HAV titers of marmoset 7 that was given variant No. 2 (35°) and challenged subsequently with virulent HAV. The typical vaccine-like response and resistance to challenge are shown. Figure 2 shows the different response found in a nonvaccinated control marmoset (No. 12) after receiving virulent virus challenge. The ICD elevation was pronounced and a strong anti-HAV response was obtained.

The examination of liver specimens from three marmosets infected with variant No. 1 (32°) showed histopathologic changes varying from essentially nothing in one animal, to minimal periportal inflammation (mononuclear cells) with trace amounts of focal liver cell necrosis in the second animal, and to moderate periportal inflammation (mononuclear cells) with extension

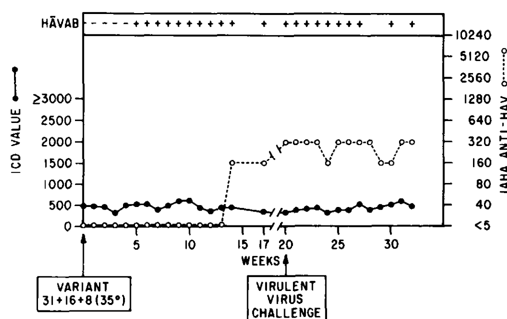

FIG. 1. Serum ICD and anti-HAV responses of marmoset No. 7 immunized with HAV variant No. 2 (31 + 16 + 8 at 35°) at zero time and subsequently challenged with virulent HAV at Week 20.

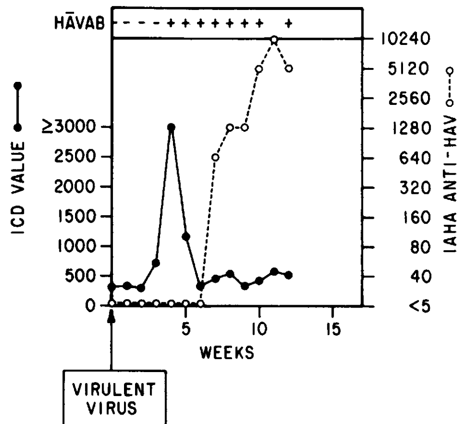

FIG. 2. Serum ICD and anti-HAV responses in a nonimmunized control marmoset (No. 12) given virulent HAV.

into the midzonal sinusoids and occasional necrosis in the third animal. The liver specimens of four marmosets infected with variant No. 2 (35°) showed a more uniform reaction consisting of minimal inflammatory mononuclear cell aggregates that were primarily periportal. In two of the specimens, there were occasional extensions of the inflammation into midzonal sinusoids with trace amounts of focal liver cell necrosis.

As stated under Materials and Methods, a number of other cell culture-passaged HAV variants were produced in addition to variants No. 1 (32°) and No. 2 (35°). Their behavior was also studied in marmosets, but virulent virus challenges were not done. The findings are summarized in Table V. HAV that had been passed in FRhK6 cells alone showed a clear tendency toward attenuation of virulence for marmosets at passages 25 and 30, but retained the ability to induce anti-HAV. Lower passage levels of the virus remained at least partially virulent for marmosets. Among the variants adapted to human diploid lung fibroblasts, all were either attenuated (little or no enzyme elevation with retention of anti-HAV induction) or overattenuated (i.e., showed loss of capacity to induce anti-HAV). Among the former, in addition to variants No. 1 (31 + 10 + 9 at 32°) and No. 2 (31 + 16 + 8 at 35°), were variants 31 + 15 + 9 at 32°, 31 + 20 + 8 at 35°, and 31 + 25 + 8 at 35°, although 31 + 15 + 9 at 32° and 31 + 25 + 8 at 35° induced anti-HAV in only a portion of recipients. Overattenuation was clearly noted in variant 31 + 25 + 9 at 32°. The $TCID_{50}$ values of these several variants ranged from about 10^6 to 10^8/ml.

Discussion. The present work provides evidence that HAV can be attenuated in virulence for marmosets by passage in cell culture, either in FRhK6 cells alone or in FRhK6 cells followed by passage in human diploid lung fibroblast cultures. As compared to virulent HAV, the marmoset responses to attenuated variants in the pres-

TABLE V. SUMMARY OF BEHAVIOR OF CELL CULTURE-PASSAGED HAV VARIANTS IN MARMOSETS

No. passages in				Marmoset testing	
Marmoset	FRhK6	Human diploid (Temp.)	$TCID_{50}$/ml	ICD elevation	Anti-HAV
31	30	0	ND[a]	−	+
31	25	0	$10^{8.0}$	−	+
31	20	0	ND	+	+
31	15	0	ND	+	+
31	10	0	ND	+	+
31	5	0	$10^{6.0}$	+	+
31	25	9 (32°)	$10^{7.2}$	−	−
31	20	9 (32°)	$10^{8.2}$	ND	ND
31	15	9 (32°)	$10^{6.8}$	−	+/−
31	10	9 (32°)	$10^{7.3}$	+/−[b]	+
31	25	8 (35°)	$10^{7.5}$	−	+/−
31	20	8 (35°)	$10^{6.8}$	−	+
31	16	8 (35°)	$10^{6.6}$	−	+

[a] Not done.
[b] Where used, +/− indicates that only a portion of the animals in the test group gave a positive response.

ent work were characterized by little or no serum ICD elevation, by delayed onset of anti-HAV, and by generally lower anti-HAV titers, consistent with less viral replication in the host animal. Liver histopathologic changes were minimal with variant No. 2 (35°). With variant No. 1 (32°), the pathologic findings in the liver were more variable, in one case showing a moderate degree of inflammation. This would appear consistent with the greater tendency of this variant to induce slight serum ICD elevations.

Overattenuated variants were also produced by passage of HAV in cell culture. These were characterized by failure to induce anti-HAV in marmosets and occurred especially rapidly on passage at 32° in human diploid lung fibroblasts, probably resulting from an inability to multiply in the marmoset. The overattenuated variants had $TCID_{50}$ titers equal to or greater than those of the attenuated variants, indicating that the mass of antigen present in the inoculum was insufficient to induce anti-HAV in the absence of viral replication.

All marmosets that were "vaccinated" with the attenuated variants were resistant to virulent virus infection as evidenced by the stability of their ICD values and anti-HAV titers. This indicates that the challenge virus did not multiply in these animals and that antibody equated with protection. This finding is consistent with the observation (7) that marmosets given inactivated hepatitis A virus vaccine also showed stable enzyme values and antibody titers on challenge with virulent virus.

We are in the process of carrying out similar studies in chimpanzees. The findings of these studies in marmosets and chimpanzees are being used as guides to attaining appropriate levels of attenuation of HAV for studies in man.

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