Evolution in the Hypervariable Region of Hepatitis C Virus in Infants after Vertical Transmission

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ABSTRACT

To elucidate the clonal evolution of hepatitis C virus (HCV) during mother-to-infant transmission, we prospectively analyzed HCV clones of the hypervariable region in four HCV RNA-positive infants and compared them with those of the mother. Cord blood samples from three of the four infants were positive for the HCV RNA ($\leq 10^3$ copies/mL), and all of the four infants had the HCV RNA titer of $>10^6$ copies/mL within 2 mo after birth. The hypervariable region clones detected in the infants were closely related to those in the respective mothers. The results suggest the perinatal transmission of HCV. The hypervariable region clones transmitted to infants were not a single selected clone or minor clones in the mother. None of the clones specific to the low-density fraction in the mother was transmitted to the infants. Moreover, the proportion of HCV in the low-density fraction was minimal in the first few months of life, but increased several months after birth in association with the elevation of alanine aminotransferase. These results suggest that the increase of HCV in the low-density fraction reflect the evolution of immune response in infants. We also demonstrated that the emergence of quasispecies in infants precedes the infantile antibody response. (*Pediatr Res* **48**: 450–456, 2000)

Abbreviations
HCV, hepatitis C virus
HVR, hypervariable region
RT-PCR, nested PCR coupled with reverse transcription
NTR, 5′-nontranslating region
LDF, low-density fraction
HDF, high-density fraction
ALT, alanine aminotransferase

Chronic HCV infection is associated with liver cirrhosis and hepatocellular carcinoma (1, 2). Approximately half of patients with chronic HCV infection have a history of blood transfusion (3). The incidence of mother-to-infant transmission of HCV is up to 10% of infants born to HCV-positive mothers (4–8). HCV infection after mother-to-infant transmission can be persistent (4, 5, 9), and the clinical course varies from asymptomatic, transient hepatitis to chronic hepatitis (10, 11). Because HCV infection associated with blood transfusion has essentially been eliminated in countries employing blood donor screening (12), the proportion of mother-to-infant transmission to overall new HCV infections should become greatly increased.

The HVR, the N-terminus region of glycoprotein 2 (E2) of HCV, is one of the major targets of neutralizing antibodies (4, 13, 14) and cellular immunity (15). Vertical transmission of HCV is confirmed by a close relationship between the HVR sequences of HCV in the mother and infant pair (5, 16, 17).

The major survival strategy used by HCV against humoral and cellular immunosurveillance systems by the host seems to be the continuous mutation of its HVR in an immunocompetent host to form subpopulations or quasispecies (14, 15, 18, 19). Spontaneous remission from chronic hepatitis C may occur in children with low HCV RNA load (20).

HCV particles in the serum can be divided into two populations based on their densities. Inoculation into chimpanzees revealed that HCV in the HDF was less infective than that in the LDF (21). Immunoprecipitation demonstrated that the HDF HCV consisted of immune-complexed particles and nucleocapsids (22, 23). In a tissue culture system, the immune complexed population was less infective (24). Patients with active hepatitis tend to have abundant HDF HCV (22, 23, 25, 26). If this observation could be extended for the mother-to-infant transmission of HCV, the HCV clone(s) responsible for infection in the infant would be preferentially present in the LDF of the maternal serum at delivery. To elucidate the clonal evolution of HCV in the mother-to-infant transmission, we analyzed the HCV HVR in the LDF and HDF of four HCV-infected infants infected *via* the RNA-positive mother.

METHODS

Patients. We screened 18,380 pregnant women for anti-HCV antibody from April 1992 to December 1997 using a second-generation assay based on passive hemagglutination (HCV PHA, Dainabot, Tokyo, Japan). Seropositive mothers were further screened for HCV RNA by nested RT-PCR targeting on the NTR. We prospectively followed 59 infants born to the HCV RNA-positive mothers. Sera of these infants were tested for anti-HCV antibody, HCV RNA, and liver function every 3 mo during the first year and every 6 mo thereafter. The RT-PCR for HCV RNA yielded positive signals in six infants on at least two occasions. The other infants were consistently negative. All six positive infants born to four HCV-positive mothers were positive for HCV RNA at the age of 1 mo. A pair of siblings born 2 y apart was excluded from this study because their HCV was genotype 2b reactive to the RT-PCR directed for NTR but inapplicable to that directed for HVR. The other four cases involved genotype 1b HCV. Cases 2 and 3 were born to the same mother. Each case was delivered vaginally and free from other viral infections, including hepatitis B virus and HIV. Cases 1–3 were bottle-fed, and case 4 was both breast- and bottle-fed. The maternal grandmother of case 1 was positive for HCV. Enrolled mothers consented to the study before the screening and after the delivery. This study has been approved by the ethics committee of the Faculty of Medicine, Tottori University.

Anti-HCV antibody titer. We used a third-generation kit for titration of anti-HCV antibody based on an enzyme immunoassay (anti-HCV 3rd, Sympep HCV-EIA II, Kyokuto Med. Corp., Tokyo).

Detection and titration of HCV RNA. For detection of the HCV RNA, a nested RT-PCR targeting on NTR of the HCV genome (26) was applied with the minimum detection level of 200 HCV RNA copies/mL of serum. The HCV RNA load was titrated by a sandwich hybridization assay kit using the bDNA amplifier molecules according to the method recommended by the supplier (Quantiplex Version 1.0, Chiron Corp., Emeryville, CA, U.S.A.). The HCV genotype was determined by a nested RT-PCR kit (Sumai Test, Tokusyu-Meneki-Kenkyusyo, Tokyo, Japan) using genotype-specific primers designed from the core region.

Differential flotation centrifugation. The density population of HCV in serum was analyzed by the method of Hijikata *et al.* (22) with minor modifications. A 50-μL aliquot of the serum was layered on top of an 8-mL NaCl solution with a density of 1.063 g/cm^3, and centrifuged in a Beckman 70.1 Ti rotor at 145,000 $\times$ g for 22 h at 14°C. RNA was extracted from 50 μL each of the top and bottom 1-mL fraction. Approximately 90% of the HCV RNA that was layered on was recovered in the top and bottom 1-mL fractions (26). After serial 10-fold dilutions, the end point dilution was determined by RT-PCR using quintuplicate tubes, and the HCV RNA titer was computed by the Behrens-Kärber method (27). To estimate the relative amount of virions in the LDF, the ratio of the HCV RNA in the top fraction to that in the combined top and bottom fractions was computed.

Amino acid sequence analysis of HVR. For sequencing the HVR, a nested RT-PCR directed for the E2/NS1 region (28) was applied with minor modifications (29). RNA was extracted as described above, and cDNA was transcribed using primer A1 (5′-G^{1692}CT CCG GGC ACC CGG ACG AGT TGA A^{1668}-3′, based on HCV-J (30). DNA was amplified for 50 cycles in the first PCR using a thermal cycler (PC-700, ASTEK, Fukuoka, Japan). Each cycle consisted of the following: 94°C for 1 min (3 min for the first cycle), 61°C for 1 min, and 72°C for 1 min (7 min for the last cycle). The reaction mixture in a volume of 50 μL contained 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM MgCl$_2$, 0.25mM each dNTP, 25 pmol each of the outer primer pair (Primer S1 (5′-G^{1261}CC ATT TAT CAG GTC ACC GCC ATG GC^{1285}-3′] and A1), 1.25 U of Taq polymerase (Pharmacia Biotech, Uppsala, Sweden), 0.005 U of Deep Vent DNA polymerase (New England Biolabs, Beverly, MA, U.S.A.), 5% DMSO, and 2% glycerol. The Deep Vent DNA polymerase containing a 3′–5′ proofreading exonuclease activity was used to enhance the fidelity of the PCR. A 5-μL aliquot was used for the second PCR with the inner primer pair of A2 (5′-G^{1634}GT GTG GAG GGA GTC ATT GCA GTT^{1611}-3′) and S2 (5′-G^{1284}CT TGG GAT ATG ATG ATG AAC TGG TC^{1309}-3′) for 35 cycles; each cycle consisted of 94°C for 1 min (3 min for the first cycle), 57°C for 1 min, and 72°C for 1 min (7 min for the last cycle). The amplified DNA fragment with the expected size was cloned into the *Eco*RV site of p-BlueScript. Fifteen clones from each fraction were sequenced in both directions by the dideoxynucleotide chain termination method using a DNA sequencer (ALFred, Pharmacia Biotech). Amino acid sequence of HVR was deduced from the DNA sequence. Clones were classified in the same group if there were less than three amino acid changes.

Statistical analysis. Dichotomous data were analyzed by Fisher's exact test.

RESULTS

Clinical background of the infants infected with HCV from their mothers. The peak ALT levels recorded in the four infants were 199, 43, 84, and 125 IU/L, respectively, which were noted between 3 and 8 mo of the age (Fig. 1, *A–D*). In case 1, the fluctuating ALT level was persistently elevated over 100 IU/L during the ages of 6–10 and 11–12 mo. In cases 2 and 3, the ALT level was under 100 IU/L until the age of 12 mo. In case 4, the ALT level remained over 100 IU/L between 3 and 5 mo of age. Antibody titers of each infant declined in the first several months and increased again starting from 3 to 6 mo, suggesting loss and dilution of maternal antibodies followed by *de novo* production of infantile antibody. ALT values peaked after the lowest antibody titer in each infant (Fig. 1, *A–D*).

Cord blood samples of cases 1–3 were positive for HCV RNA by RT-PCR. However, the HCV RNA titer ($\leq$10^3 copies/mL) was too low to be detected by the bDNA assay. The HCV RNA titer was already high at the age of 1 mo in each case (>10^6 copies/mL), and remained at that level for at least 5 mo. In cases 1–3, HCV RNA never disappeared throughout the
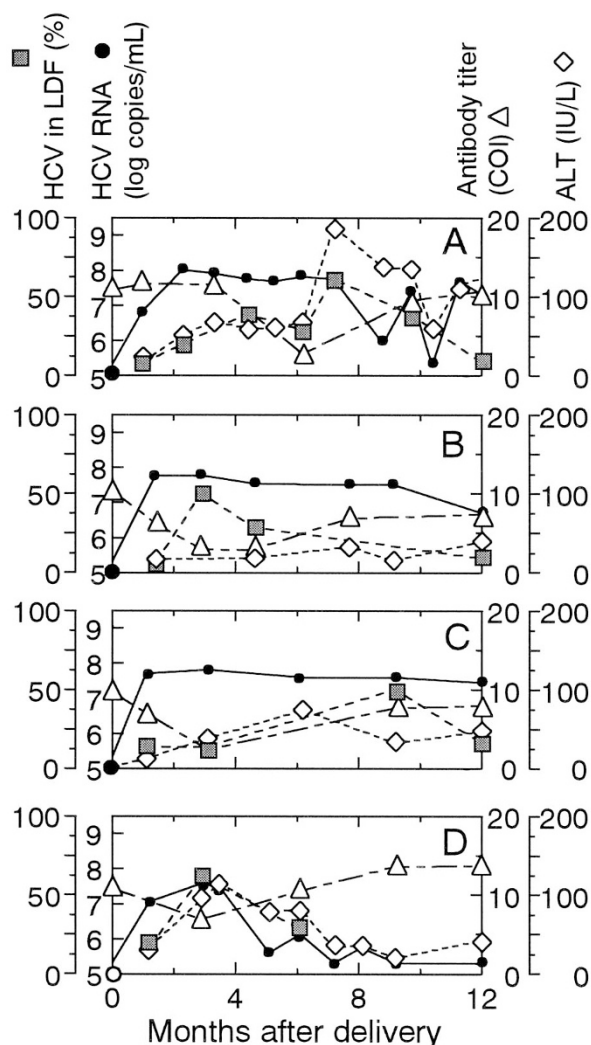
Figure 1. Clinical profiles of cases 1–4 (*A–D*). Proportion of HCV in LDF (%, *hatched rectangle*), HCV RNA titer determined by the bDNA assay (copies/mL in logarithm, *small closed circles*), anti-HCV antibody titer determined by the third-generation assay (COI: cut-off index, *triangle*), and serum ALT level (100 IU/L, *diamond*). HCV RNA in cord blood was positive in cases 1–3 (*large closed circle*) and negative in case 4 (*large open circle*) by RT-PCR (minimum detection level; 200 copies/mL).

observation period. In case 4, the titer became undetectable at the age of 9 mo by the bDNA assay and at the age of 16 mo by RT-PCR.

The ratio of HCV in the LDF was less than 20% in each case at 1–2 mo after birth. The ratio of the LDF virions increased approximately 2 mo after the increase of HCV RNA in cases 1, 2, and 4, but several months after in case 3. The increase of this ratio was followed by the elevation of ALT level at 1–4 mo and 6–7 mo in case 1, and at 1–3 mo in case 4. In cases 2 and 3, the increase in ratio was not accompanied by significant elevation of the ALT. The results suggest that the increase in ratio is not accompanied by the increase of HCV RNA titer in the serum.

HCV-HVR Evolution in case 1. The HVR clones detected in the case 1 mother and infant pair could be classified into seven groups (Gr): Gr101–Gr107 (Table 1). In the mother at delivery, two Gr101 and nine Gr105 clones appeared in the LDF, and nine Gr101 and four Gr105 clones in the HDF ($p = 0.019$). In the infant at the age of 2 mo, all of the 15 clones in the HDF were clone A of Gr101; the HCV RNA was undetectable in the LDF by RT-PCR for HVR. At the age of 4 mo, there were 4 Gr101 and 11 Gr105 clones in the LDF and 11 Gr101 and 4 Gr105 clones in the HDF ($p = 0.027$).

The profile changed at the age of 9 mo: six Gr101 and nine Gr105 clones in the LDF, and seven Gr101 and eight Gr105 clones in the HDF. It changed further at the age of 12 mo. There were only two Gr101 clones in the HDF, and all others in the LDF and HDF were in the Gr106 and Gr107, which were closely related with the Gr105. However, none of the Gr105 clones in the infant (clones I–L, O, and P) were identical to the Gr105 clones in the mother (clone E). Clones of the Gr102–Gr104 of the mother with the frequency of 2/15 each never appeared in the infant. By the age of 4 mo, new clones that were never detected in the mother emerged, occurring 2 mo before the lowest antibody titer.

HCV-HVR evolution in cases 2 and 3. The HVR clones of the mother at 6 mo before and at 1 mo after the delivery of case 2 could be classified into six groups, Gr201–Gr206 (Table 2). In the mother, Gr201 and Gr203 were not present at 6 mo before delivery and first appeared in the HDF at 1 mo after delivery. Because Gr205 and Gr206 were not detected in case 2 throughout the study, they may not have been transmitted at all. Eighteen months after the birth of case 2, the same mother gave birth to case 3, during which clones of groups Gr201–Gr203 persisted and of groups Gr304–Gr308 emerged (Table 3). The latter groups were similar to each other. Although

Table 1. *Evolution of HVR clones in case 1 by relative frequencies in the LDF and HDF*

Clone group*	AA sequence	Mother at delivery		Case 1 at the age of								Name of clones
				2 months		4 months		9 months		12 months		
		LDF	HDF	LDF	HDF	LDF	HDF	LDF	HDF	LDF	HDF	
Gr101	ATRTIGGEAGRTTGSFTALFNAGSSQK	2	9		15	4	11	6	7		2	A–D,M,N,Q,R,Y
Gr102	QS..........ASG.AG...P.....		2									H
Gr103	EAH.T..K........A..........	2										G
Gr104	.........A...R..VS..SP.....	2										F
Gr105	E.H.T....A...R..VG..SP.....	9	4			11	4	9	8			E,I–L,O,P
Gr106	E.H.T....A...RR.VG..SS.....									14	13	S–W
Gr107	.............R..VG..SS.....									1		X
Total		15	15	0	15	15	15	15	15	15	15	

* Clones were classified in the same group if there were fewer than 3 amino acid (AA) changes.

Table 2. *Evolution of HVR clones in case 2 by relative frequencies in the LDF and HDF*

| Clone group* | AA sequence | Mother at† | | | | Case 2 at the age of | | | | Name of clones |
| | | −6 months | | +1 month | | 1 month | | 3 months | | |
		LDF	HDF	LDF	HDF	LDF	HDF	LDF	HDF	
Gr201	ETHVTGGSAARTTMSFARFFAPGASQK				1	11	5	15	14	A,L–R
Gr202	................S.L.N......		4	3	2	4	10		1	C–E,S,T
Gr203	................SSL.T......				3					J,K
Gr204	..........Y.S....L.N......		4	12	9					B,H
Gr205	..........Y.SG..GL.T......		6							F
Gr206	.I..A..P..Y..SG..GL.T......		1							G
Total		0	15	15	15	15	15	15	15	

* See footnote to Table 1 for clone groups. Clone groups in italic indicate common to case 3.

† Mother before (−) and after (+) the delivery.

Table 3. *Evolution of HVR clones in case 3 by relative frequencies in the LDF and HDF*

| Clone group* | AA sequence | Mother at delivery | | Case 3 at the age of | | | | Name of clones |
| | | | | 1 month | | 9 months | | |
		LDF	HDF	LDF	HDF	LDF	HDF	
Gr201	ETHVTGGSAARTTMSFARFFAPGASQK	2		5	1		1	A,j
Gr202	................S.L.N......	3	2		6	6	6	C,D,p–t
Gr203	................SSL.T......		4					J,n,o
Gr304	..........H..S....L.K......	1	1					e,k
Gr305	..........A.S.LT.L......H.	2						f
Gr306	..........ASGLT.L.S......	1		4	2			b,u,v
Gr307	...A......H.AS.LT.L....P...		1					m
Gr308	..........HAASGLT..L...P...	6	7	6	6	9	8	g,h,w–z
Total		15	15	15	15	15	15	

* See footnote to Table 1 for clone groups. Clone groups in italic indicate common to case 2.

† Name of the clones in upper case indicates common clone to case 2, and in lower case distinct in case 3.

Gr204 was the major component of the mother at 1 mo after the delivery of case 2, only two clones were detected in Gr304 closely related to Gr204 in the mother at delivery of case 3. Although the Gr203 persisted in the mother at the delivery of case 3, the group was not transferred to case 3.

All of the clones isolated from case 2 at the ages of 1–3 mo were Gr201 and Gr202 (Table 2). At the age of 1 mo, the Gr201 was more abundant in the LDF, and the Gr202 in the HDF ($p = 0.066$). By the age of 3 mo, the Gr201 clones became dominant consisting of 15 (100%) and 14 (93%) clones in the LDF and HDF, respectively. Thus, in spite of the absence of the Gr201 in the mother before delivery, it probably existed in the mother as a minor component.

Case 3 at the age of 1 mo had four groups: Gr201, Gr202, Gr306, and Gr308, all detected in both LDF and HDF of the mother at time of delivery (Table 3). At the same age, all these groups were also detected in both LDF and HDF of the mother at delivery. However, the population in each group has been changed. For example, whereas the groups Gr304, Gr305, and Gr307 in the mother disappeared from the infant, the Gr306 in the infant had not been observed in the mother except in one clone. At the age of 9 mo, only a single clone in the Gr201, clone j, was still present. Although 40% of the clones belonged to the Gr202 in both LDF and HDF (clones D, r–t), the majority of clones were newly emerged derivatives in the Gr308 (clones h, w–z).

HCV-HVR evolution in case 4. Among five groups detected in case 4 at the age of 1 mo, Gr401 (clones A and B) and Gr402 (clones G and H) were both in the LDF and HDF (Table 4). The Gr410 clones were not detectable in the mother, which were fairly distant from other clones in the HDF of case 4 at the age of 1 mo. At the age of 3 mo, only two groups, Gr401 and Gr402, were detectable in the LDF and HDF, and Gr401 formed the majority. The Gr401 clones were not detectable in the mother at 3 mo after delivery, and only one Gr403 clone related to Gr402 was detected in the mother.

The profile markedly changed at the age of 6 mo. Whereas Gr404 became dominant in the LDF, Gr410 became dominant in the HDF. Both groups were detected at 1 mo but not at 3 mo. The former was detectable in the mother but not the latter. Interestingly, Gr404 and Gr409 clones present at the age of 1 mo, once disappeared at the age of 3 mo, reappeared at the age of 6 mo. The Gr401 and Gr402 clones formed the majority at the age of 3 mo after delivery but completely disappeared at the age of 6 mo. A variety of clone groups was observed in the mother at 3 mo after delivery that were never seen in the child: Gr405–Gr408, Gr411, and Gr412.

DISCUSSION

The mother-to-infant transmission of HCV is one of the several established pathways for HCV transmission, affecting up to 10% of infants born to mothers chronically infected with HCV but not with HIV (4–8). However, its mechanism remains to be elucidated. Both *in utero* (16) and perinatal (5) transmissions have been proposed.

Table 4. *Evolution of HVR clones in case 4 by relative frequencies in the LDF and HDF*

| Clone group* | AA sequence | Mother at† +3 months | | Case 4 at the age of | | | | | | Name of clones |
| | | | | 1 month | | 3 months | | 6 months | | |
		LDF	HDF	LDF	HDF	LDF	HDF	LDF	HDF	
Gr401	TTRVSGGTAGHTTMGFTSLFSSGAQQK			4	5	10	13			A–E
Gr402	S.Y......A.N.K..A...TL.SN.R			7	5	5	2			F–I
Gr403	S.Y......A.N.K..A....L.S...		1							J
Gr404	S.Y....TAYN.R.L.....L.S...	2	3	2				11	3	K–Q
Gr405	S.Y....A.A.N.R.L.....L.S...		1							R
Gr406	S.Y......ALN.R.L....PL.S...	8								S–U
Gr407	N.Y....S.....S.L....PL.S...	2								V,W
Gr408	D.Y....S.....S.LA...AP.QS..	2	4							X,Y
Gr409	D.Y....S..Y..S..A....P.QS..	1		2	1			4	1	a–c
Gr410	E.H.T..S.AR..SSLARFLAP..S..				4				11	Z
Gr411	..H....A.........P...F.SR.N		5							d–f
Gr412	D.Y....S..Y..S..A....F.SR.N		1							g
Total		15	15	15	15	15	15	15	15	

* See footnote to Table 1 for clone groups.
† Mother after (+) the delivery.

Previous studies suggested that HCV RNA is undetectable in the cord blood of infected infants (5, 31). In this study, whereas three out of four cord blood samples of infected infants possessed the HCV RNA at the titer of $\leq 10^3$ copies/mL, only one (4%) did out of 28 uninfected infants born to HCV RNA positive mothers (data not shown). Positive signals in the cord blood sample only detectable by nested PCR are not informative for *in utero* transmission of either HTLV-1 (32) or HIV (33). All four cases in this study had extremely high levels of viremia ($> 10^7$ copies/mL) at the age of 1–2 mo, although a previous report found the peak titer at 3–14 mo in 3 of 4 infants (5). Because the rapid boost of HCV RNA within a few months after delivery is hard to explain by the *in utero* transmission, the results strongly suggest the perinatal transmission.

We analyzed 15 HCV HVR clones each in mothers and infants at various points. Although we did not confirm the reproducibility of the test by repetition, we considered them acceptable because the similarity of clones at different time points of each patient and the detection of the identical clones in the sibling of cases 2 and 3 served as the internal control.

The close similarity of HVR clones in each mother-infant pair molecularly confirmed the mother-to-infant transmission of HCV in our cases. Weiner *et al.* (16) found that 10 HVR clones obtained from a baby at 24 h after birth were all the same but unique from 10 clones obtained from the mother suggesting selective transfer of HCV to the infant. Ni *et al.* (34) also reported the homogeneous population of HCV in infants at the age of 3 mo. In case of the mother-to-infant transmission of HIV, the transmission of both selective clones (35, 36) and multiple maternal clones (37–39) has been reported. However, several factors, such as intrauterine and cell-associated transmissions, may play additional roles in the mother-to-infant transmission of HIV.

In our cases, although the HVR population in case 1 at the age of 2 mo was homogeneous, those of cases 2–4 were heterogeneous. Moreover, clones that emerged beyond the first few months were in the same group as those in the mother. Because the titer of HCV in the mother was $> 10^6$ copies/mL and that in the cord blood was $< 10^3$ copies/mL in each case,

the HCV dose to the infant at the time of delivery was calculated as $\sim 10^4$ copies. Considering the presence of noninfectious particles and efficiency of transmission, the number of HCV particles precipitated in each infection would be minimal. Because we picked only 15 clones at each stage, minor clones would be undetectable and some clones might emerge later than the observation period. These results suggest that the number of HCV clones transmitted in each case is not necessarily limited to a few clones.

Kudo *et al.* (17) suggested that the clones transmitted to infants are minor clones in the mother, because the clones of infants were related, but not identical, to those of the mothers. Clone A appeared in 7/30 of the mother at delivery of case 1, clone C in 4/15 of the mother at 6 mo before delivery of case 2, and clone g in 12/30 of the mother at delivery of case 3. Our results suggest that the clones transmitted to the infants are not always the minor clones in the mother at delivery. This is not surprising as the mother-to-infant transmission is expected to depend on the mother's quasispecies complexity and status of antibody repertory at the time of infection.

Previous studies suggested that HCV virions in the LDF are more infective than those in the HDF (21, 22) and are responsible for the mother-to-infant transmission (17). However, in case 1 at 2 mo after delivery, all 15 A clones in the HDF were identical to 7 A clones in the HDF of the mother. In case 2 at the age of 1 mo, Gr201 and Gr202 were observed. The Gr201 was absent in the mother at 6 mo before delivery, but the Gr202 was in the HDF. In case 3 at the age of 1 mo, Gr202 was only in the HDF, and Gr201, Gr306, and Gr308 were both in the LDF and HDF. Of these, Gr201 and Gr306 were detected only in the LDF of the mother. In case 4 at the age of 1 mo, Gr401 and Gr402 were both in the LDF and HDF. These clones were absent in the mother at 3 mo after delivery, except for a single related clone in the Gr403. Gr410, which was fairly distant from others in the HDF at the age of 1 mo, was not detectable in the mother. The results suggest that clones transmitted to newborn babies do not always belong to the LDF of the mother. Because not all antibodies are capable of neutralization, we consider that at least some HDF HCV are still

infective. The selective replication of HCV in the first few months of life probably reflect the maternal antibody and the evolution of infantile immune response.

The proportion of HCV in the LDF increased a few months after the rise of HCV RNA in serum, and its peak coincided with the nadir of antibody titer in the infant. Our results suggest that the increase of HCV in the LDF reflects the loss of maternal antibody and the evolution of antibody generation in infants. Ni *et al.* reported the slower nucleotide substitution rate in infants (34), and Kudo *et al.* suggested the role of infantile immunity for the selection of new quasispecies (17). Clones I–N of case 1 (in Gr101 and Gr105) emerged at the age of 4 mo. Clones L–P (in Gr201 and Gr202) and u–v (in Gr306) of cases 2 and 3, respectively, emerged at the age of 1 mo. Furthermore, the majority of the LDF clones were not the newly emerged clones but preexisting clones. Because these preceded the lowest antibody titer in each infant, our results suggest that emergence of new quasispecies in infants reflect the evolution of cell-mediated immune response (15).

Although ALT level never exceeded 100 IU/L in cases 2 and 3, it surpassed that level in cases 1 and 4. Case 4 showed a single rise point of the ALT level above 100 IU/L at the age of 3 mo, and case 1 showed multiple bouts of ALT elevation. All cases had more than 10^7 copies/mL of HCV RNA at least in the first 3 mo, and only case 4 became free of HCV within the observation period. Fujisawa *et al.* (20) suggested that children with a low titer of HCV RNA tend to recover spontaneously. However, the HCV titers at the age of 2–4 mo in our data did not support the concept. Yamaguchi *et al.* (40) reported a patient who eventually became HCV RNA-negative after frequent substitutions in the HVR. Quasispecies emerged in all four cases. Although the mother of case 4 had the most diverse quasispecies among them, the clonal diversity of case 4 was not remarkably different from other cases. To provide more convincing evidence, a larger number of cases should be studied.

In summary, our study suggests that the major route of mother-to-infant transmission of HCV is established during the perinatal period, and that the HCV responsible for the transmission is not restricted to a virus in the LDF or a single minor clone. We also demonstrated that the emergence of quasispecies in infants precedes the infantile antibody response.

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