Vertical Transmission of the Hepatitis C Virus to Infants of Anti-Human Immunodeficiency Virus-Negative Mothers: Molecular Evolution of Hypervariable Region 1 in Prenatal and Perinatal or Postnatal Infections

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In a prospective study of 33 infants born to hepatitis C virus (HCV)-positive human immunodeficiency virus-negative mothers the vertical transmission of HCV occurred in 6.8%. The evolution of HCV infection in two babies was studied from birth up to 5 or 6 years of age, and the sequencing of the hypervariable region (HVR) of the putative envelope-encoding E2 region of the HCV genome was performed. The HVR1 sequence variability and the different serological profiles during follow-up could reflect the differences in HCV transmission routes, HCV genotypes, and clinical evolution of infection.

While the estimated rate of vertical transmission of hepatitis B virus from Hbe antigen-positive mothers is nearly 100% in the absence of immunoprophylactic measures, the rate of hepatitis C virus (HCV) vertical transmission is still very widely debated in the literature (estimates range from 0 to 100%), and to date no specific study has investigated differences among prenatal, perinatal, and postnatal infections (12, 13). Many studies have confirmed that the risk of transmission may be enhanced by coinfection with the human immunodeficiency virus (HIV) (10, 15, 17).

The follow-up of infection in newborns may elucidate some aspects of virus evolution; the HCV variant detected at birth may be considered the starting viral sequence for a particular host, allowing a more reliable analysis of sequence variability over time.

This study evaluates the rate of HCV transmission from HCV RNA-positive anti-human immunodeficiency virus (HIV) antibody-negative mothers to their offspring and the clinical evolution of acquired infection. We carried out a longitudinal study of 33 anti-HCV-positive infants for 24 months, and two infected children belonging to this cohort were followed up until 5 or 6 years of age by evaluating several clinical and virological parameters and sequencing hypervariable regions (HVR) of the putative envelope-encoding E2 region of the HCV genome (5, 7, 8).

Longitudinal study. In our study 2,263 anti-HIV antibody-negative pregnant women were screened for anti-HCV antibodies between June 1992 and June 1995; anti-HCV antibody positivity was found in 56 cases (2.4%), and 33 women were enrolled in a prospective longitudinal study of HCV transmission. Their ages ranged between 19 and 42 years (mean age, 28 years); 20 women (60%) had an acknowledged history of in-

travenous drug use, 1 (3%) was a health care worker with professional exposure (3%), 4 (12%) had an acknowledged history of anti-HCV antibody-positive sexual partners, and 8 (24%) had no risk factor. No woman was in interferon therapy. Only 2 pregnant women were diagnosed as chronic hepatitis sufferers based on histological findings by liver biopsy, and 26 were asymptomatic. Four women breast fed up to 10 to 12 months, and two breast fed up to 30 days. All but 3 of 33 babies were delivered vaginally.

Serum samples were stored at -80°C within 3 h of collection in a day hospital. Testing for anti-HCV antibodies was done by a commercially available third-generation enzyme immunoassay (EIA; Abbott); positive results obtained by EIA were confirmed by a third-generation recombinant immunoblot assay (RIBA) (Ortho Diagnostic), and serum alanine aminotransferase (ALT) levels were determined. The test for anti-E2 antibodies was carried out by HCV E2 immunoglobulin G (IgG) kit EIA (Nuclear Laser Medicine) in cases of transmission of the infection.

Total RNA was extracted from 100 μl of serum by using the guanidine thiocyanate method and was detected by reverse transcriptase PCR and nested PCR by using two sets of oligonucleotide primers deduced from the highly conserved 5' untranslated region of the HCV genome (1–3).

Quantification of HCV RNA in serum was performed by the Amplicor HCV Monitor (Roche Diagnostic Systems, Branchburg, N.J.), and HCV genotypes were determined through a line probe assay (INNO-LiPA; Innogenetics, Nucleic Laser Medicine).

A 447-bp sequence of the E2 region encompassing HVR1 and HVR2 of the HCV genome (from nucleotide [nt] 1324 to nt 1771) was amplified by heminested reverse transcriptase PCR. E2 cDNA was obtained with an E2A antisense primer (5'-TTCATCCAGGTGCASCCGSAA-3'; nt 1986 to 1966) and amplified with E2A and E2S (5'-CCYGGTTGCTCYTT YTCTATCTT-3'; nt 848 to 870) primers. A second amplifica-

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FIG. 1. Longitudinal evaluation of viremia, ALT levels, and production of IgG anti-C33 antibodies in a serial serum sample from C13.

tion was performed with E2A and E2NA (5'-GGTGGGTAG TGCCAGCAATA-3'; nt 1813 to 1794) primers.

Crude PCR products were used as templates for a cycle sequencing reaction with the Thermo Sequenase cycle kit (Amersham, Little Chalfont, United Kingdom) and an infrared IRD 800-labeled E2-specific sequencing MB primer (5'-GCA TGGCTTGGGATATGATG; nt 1291 to 1310). Termination products were electrophoresed and analyzed in a model 4000L Licor DNA sequencer (Molecular Biotechnology) (18).

Computer analysis of the sequence data was performed with Chromas, version 1.51 (Technelysium Pty. Ltd.). Nucleotide and deduced amino acid sequences were aligned with the CLUSTALW program, and synonymous and nonsynonymous nucleotide mutations were evaluated. Mother-child distances were computed by DNADIST of the Philip, version 3.57, package according to the Kimura model.

The *t* test for equality of means of two independent samples and the Wilcoxon signed rank test were used for statistical analysis (program SPSS, version 8.0, for Windows).

At birth, serum samples from mother-child pairs showed similar antibody titers in EIAs and similar RIBA profiles. HCV RNA was detected in serum samples at delivery of all mothers but four. Both viremia and the HCV genotype were determined for 29 HCV RNA-positive mothers. HCV genotypes 1a (35.2%), 3a (47%), and 4c (17.6%) were most represented in the intravenous drug user risk group, and 1b and 2c were most represented in the group with no risk factors (sporadic hepatitis) risk group (57.1 and 28.5%, respectively).

The EIA serological follow-up study of 31 noninfected newborns showed the clearance of anti-HCV antibodies within 9 months in 50% of the babies; in five children anti-HCV antibodies were detectable up to 15 months (14.7%). No infant was anti-HCV antibody positive at 18 months.

There was a correlation between anti-HCV antibody titers in mothers and the time of serum reversion in their babies: multiple anti-HCV antibody reactivity, as determined by immunoblotting, in sera of mothers was associated with a longer per-

sistence of passively acquired anti-HCV antibodies in their children ($P < 0.0005$), and, in particular, there was a direct correlation between the anti-C22 titers of mothers and the time of serum reversion in their infants ($P < 0.005$). Abnormal ALT levels in the serum of infants did not generally correspond to the persistence of anti-HCV antibodies or HCV RNA; only two babies (C13 and C32) acquired the HCV infection, as shown by persistent viremia with HCV RNA levels of up to 1.2×10^6 copies/ml and production of specific anti-HCV antibodies (Fig. 1 and 2). A *t* test for equality of means of two independent samples detected a significant correlation between high levels of viremia ($>10^6$ copies/ml) and frequency of vertical transmission ($P < 0.0005$). No infant born to HCV RNA-negative mothers had fluctuating or persistent viremia.

Clinical evolution of acquired infection. C32 exhibited highly abnormal ALT levels starting from 6 months, and C13 showed a slight alteration of ALT levels starting from 9 months (Fig. 1 and 2). The HCV genotypes in C13 and C32 were 2c and 1b, respectively, and those in mother-infant pairs were identical. RIBA was indeterminate for C13 and M13, the only pair which expressed positivity for the anti-C22 antibody alone.

A longitudinal study was performed to follow up the two cases (C13 and C32, born to M13 and M32, respectively) of vertical transmission up to 5 to 6 years of age in order to evaluate the evolution of the HCV infection. Both HCV-infected babies were breast fed for 12 months.

In C13 the titer of HCV RNA at the birth was 4.1×10^4 copies/ml. The viral genome in C32 was absent at birth and became detectable at 6 months of age, with a titer of 5.1×10^4 copies/ml. HCV RNA was detected only in the colostrum ($<1.0 \times 10^3$ copies/ml) of M32.

Both C13 and C32 showed persistent viremia during the entire longitudinal study. The production of specific anti-C33 antibodies, absent in the mother, occurred at 9 months of age in C13, and specific production of anti-C22 and anti-C33 antibodies was observed in C32 at 15 and 18 months of age, respectively. Both mother and child were anti-E2 antibody

FIG. 2. Longitudinal evaluation of viremia, ALT levels, and production of IgG anti-C22 and anti-NS3 antibodies in a serial serum sample from C32.

negative (Nuclear Laser Medicine) at the time of birth, and only C13 showed seroconversion (anti-E2 positive) during a follow-up (Fig. 1).

The highest HCV RNA titer in C13, 1.3×10^6 copies/ml, was at 9 months; after the production of specific anti-HCV antibodies, the HCV RNA titer decreased to low levels ($\leq 1.0 \times 10^3$ copies/ml) up to 4 years of age. Except for a brief period (at 55 months of age) when HCV RNA was not detectable, C13 has shown a fluctuating viremia to date. Borderline ALT levels were observed throughout the follow-up period (Fig. 1). Except for a slight splenomegaly, C13 has shown no important clinical symptoms of infection.

C32 was not viremic at birth; HCV RNA was detected at 6 months of age. Viremia fluctuated throughout the follow-up period and peaked (1.2×10^6 copies/ml) at 18 months of age. In contrast to C13, C32 has persistently altered ALT levels (250 to 350 U/liter). After the production of specific anti-C33 IgG antibodies at 18 months of age, ALT levels remained elevated and viremia began to decrease; the HCV RNA titer remained low

between 45 and 54 months of age but increased thereafter (Fig. 2). C32 showed no clinical symptoms of infection.

Comparison of E2 region encompassing HVR sequences in mother-infant pairs. Sequencing of a portion of the E2 region allowed us to evaluate the mutation rate in HVR1 by comparing serial samples of C13 and C32 with those of their mothers at delivery.

Deduced amino acid sequence alignments of the M13 sample at delivery and nine samples of C13 are shown in Table 1. Nonsynonymous amino acid mutations occurred, particularly in HVR1, starting from 9 months of age.

Deduced amino acid sequence alignments of the M32 sample at delivery and of eight C32 samples are shown in Table 2. There are two distinct HVR (HVR1 and HVR2) typical of HCV genotype 1b in the C32-M32 pair. Starting from 46 months of age, nonsynonymous amino acid mutations occurred in HVR1 but not in HVR2.

The DNA distance matrix for the nucleotide sequence of the newborn began to differ from that for the mother at 9 months

TABLE 1. Amino acid sequence alignments of HVR1 and DNA distance matrices for C13 during follow-up compared with the amino acid sequence of the M13 sample collected at delivery

Sample	HVR1 sequence ^a	DNA distance matrix	No. of mos
M13	HTRTVGGQAA QTARGLTSLF SPGPQQS		
C13.1		0.0000	0
C13.2		0.0000	3
C13.3	V...F.....	0.0250	9
C13.4	NV...F.....	0.0381	18
C13.5	RNV...F.....K..	0.0649	30
C13.6	..L.....RNV...F.....K..	0.0791	40
C13.7	..L.....RNV...F.....K..	0.0791	47
C13.8	N..L.....RNV...F.....K..	0.1089	62
C13.9	N..L.....RNV...F.....K..	0.1089	72

^a Bullets, identical amino acids.

TABLE 2. Amino acid sequence alignments of HVR1 and DNA distance matrices for C32 during follow-up compared with the amino acid sequence of the M32 sample collected at delivery

Sample	HVR1 sequence ^a	DNA distance matrix	No. of mos
M32	HTRTVGGQAA QTARGLTSLF SPGPQQS		
C32.1		0.0000	6
C32.2		0.0000	12
C32.3		0.0000	18
C32.4		0.0000	24
C32.5		0.0000	32
C32.6		0.0000	40
C32.7	...I.....A.....	0.0257	46
C32.8	...V.....N...A.....	0.0525	54

^a Bullets, identical amino acids.

in C13 and at 46 months in C32, with values of 0.0791 and 0.025, respectively, for HVR1 at 47 to 48 months of age (Tables 1 and 2).

Discussion. Our data show that, in HCV RNA-positive, HIV-negative mothers, HCV infection is transmitted to 6.8% of newborns.

Although intravenous drug use itself, as well as coinfection with HIV, may be a major predisposing factor in perinatal transmission of HCV, none of the 20 drug-addicted women of our cohort study transmitted the infection to their babies; moreover, we found no link between active liver disease in mothers and enhanced risk of viral transmission in the absence of nursing (9, 16). Although the mothers of both infected babies were asymptomatic and didn't belong to any risk group, they had high levels of circulating HCV RNA at delivery ($\geq 10^6$ copies/ml) and breast fed up 10 months.

Genotypes 1a and 3a were more frequent among the intravenous drug users of our cohort study than among other groups, but none of these genotypes was transmitted.

Although HCV RNA can be present in breast milk, most studies reveal that infection through breast feeding is apparently infrequent. In this study in only one case was breast feeding possibly responsible for HCV transmission.

According to our results, C32 possibly acquired HCV infection via perinatal or postnatal transmission by nursing (data not shown), as suggested by several factors: (i) absence of HCV RNA in serum at birth, (ii) ALT level increase and presence of HCV RNA at 6 months of age, (iii) delayed production of anti-C22 (at 15 months of age) and anti-C33 (at 18 months of age) IgG antibodies, and (iv) presence of HCV RNA in the M32 colostrum (1.0×10^3 copies/ml).

The presence of HCV RNA at birth (1.0×10^4 copies/ml) in C13 suggests fetal infection; this assumption is supported by quasispecies analysis of the core E1 region in a previous study, where nucleotide variability was considerably greater at the time of birth than 6 months after birth (11). Our results reveal interesting differences in the serological profiles of the two infants; the presence of anti-E2 antibodies could explain the high variability of HVR1 in C13, a region found within the E2 envelope protein which was shown to be a major site for the genetic evolution of HCV after the onset of hepatitis and which might escape the host immune surveillance system (14).

By sequencing from crude PCR products of the E2 region in sequential serum samples from HCV-infected newborns, it was found that nonsynonymous mutations occurred in C13 and C32; their greatest frequency was detected after 3 months of age in C13 and 2 years after specific seroconversion in C32.

Differences in the HVR1 distance matrices for C13 and C32 with respect to those for their mothers could reflect different types of vertical transmission (intrauterine transmission or perinatal or postnatal transmission), different genotypes, and different serological profiles. Different selective pressures acting on HCV in C13 and C32 may have produced different clinical outcomes, although they were both asymptomatic at the end of the study (6).

Long-term follow-up of HCV-infected newborns is clearly necessary to evaluate the impact of these aspects over time.

We conclude that HCV infection can be vertically transmitted by HIV-negative HCV RNA-positive mothers with asymptomatic infection it was significant in our statistical study that

a high level of HCV RNA ($>1.0 \times 10^6$ copies/ml) in serum is an important risk factor ($P < 0.0005$). Further investigation is necessary to understand the role of the type of delivery (vaginal or caesarean), breast feeding, and familial contact in HCV vertical transmission, and only by studying more cases of vertical transmission will it be possible to correlate routes of transmission with HVR1 variability.

Compared to traditional methods of examining viral complexity involving sequencing of viral quasispecies, too cumbersome to be applied to large numbers of individuals and in a diagnostic routine, the sequencing of HVR1 with crude PCR products represents a simple, economic routine diagnostic tool, which also provides important information on persistent infections and on the evolution of vertically acquired viruses. To date, the frequencies of the different types of HCV infection (prenatal, perinatal, and postnatal) transmitted vertically are still not known; we suggest that the new procedure be used to address this topic.

The study of the effects of neonatal infection on the evolution of the HCV envelope protein could help elucidate some aspects of intrahost virus evolution: the early detection of the HCV variant may be considered the starting sequence for a particular host, allowing easier and better analysis of sequence diversification over time and clarification of viral pathogenicity mechanisms (4). The evolutionary analysis of virus populations in HCV-infected newborns (i.e., in a host with immature humoral and cytotoxic immune responses) may provide insight into the natural history of HCV infection, viral pathogenic potential, and virus-host interplay.

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REFERENCES

1. Caudai, C., I. Bastianoni, M. Padula, P. E. Valensin, V. Shyamala, J. H. Han, C. A. Boggiano, and P. Almi. 1998. Antibody testing and RT-PCR results in hepatitis C virus (HCV) infection: HCV-RNA detection in PBMC of plasma viremia-negative HCV-seropositive persons. *Infection* **26**: 151-154.
2. Caudai, C., M. G. Padula, V. Bettini, and P. E. Valensin. 1998. Detection of HCV and HGV infection by multiplex PCR in plasma samples of transfused subjects. *J. Virol. Methods* **70**: 79-83.
3. Chomczynski, P., and N. Sacchi. 1987. Single step method of RNA isolation by acid guanidinium thiocyanate phenol-chloroform extraction. *Anal. Biochem.* **162**: 156-159.
4. Choo, Q. L., K. H. Richman, J. H. Han, K. Berger, C. Lee, C. Dong, C. Gallegos, D. Coit, A. Medina-Selby, P. J. Bart, A. J. Weiner, D. W. Bradley, G. Kuo, and M. Houghton. 1991. Genetic organization and diversity of the hepatitis C virus. *Proc. Natl. Acad. Sci. USA* **88**: 2451-2455.
5. Kudo, T., Y. Yanase, M. Oshiro, M. Yamamoto, M. Morita, M. Shibata, and T. Morishima. 1997. Analysis of mother to infant transmission of hepatitis C virus: quasispecies nature and buoyant densities of maternal virus population. *J. Med. Virol.* **51**: 225-230.
6. Manzin, A., L. Solforosi, M. Debiaggi, F. Zara, E. Tanzi, L. Romano, A. R. Zanetti, M. Piazza, and M. Clementi. 2000. Dominant role of host selective pressure in driving hepatitis C virus evolution in perinatal infection. *J. Virol.* **74**: 4327-4334.
7. Manzin, A., L. Solforosi, E. Petrelli, G. Macarri, G. Tosone, M. Piazza, and M. Clementi. 1998. Evolution of hypervariable region 1 of hepatitis C virus in primary infection. *J. Virol.* **72**: 6271-6276.
8. Ni, Y. H., M. H. Chang, P. J. Chen, H. H. Lin, and H. Y. Hsu. 1997. Evolution of hepatitis C virus quasispecies in mothers and infants infected through mother-to-infant transmission. *J. Hepatol.* **26**: 967-974.
9. Ni, Y. H., H. H. Lin, P. J. Chen, H. Y. Hsu, D. S. Chen, and M. H. Chang. 1994. Temporal profile of hepatitis C virus antibody and genome in infants born to mothers infected with hepatitis virus but without human immunodeficiency virus coinfection. *J. Hepatol.* **20**: 641-645.
10. Novati, R., V. Thiers, A. D. Monforte, P. Maisonneuve, N. Principi, M. Conti, A. Lazzarin, and C. Brechot. 1992. Mother to child transmission of hepatitis

- C virus detected by nested polymerase chain reaction. *J. Infect. Dis.* **165**: 720–723.
11. **Rapicetta, M., C. Argentini, E. Spada, S. Dettori, M. P. Riccardi, and M. Toti.** 2000. Molecular evolution of HCV genotype 2c persistent infection following mother-to-infant transmission. *Arch. Virol.* **145**: 965–977.
 12. **Sternek, M., T. Kalinina, S. Otto, S. Gunther, L. Fisher, M. Burdelski, H. Greten, C. E. Broelsch, and H. Will.** 1998. Neonatal fulminant hepatitis B: structural and functional analysis of complete hepatitis B virus genome from mother and infant. *J. Infect. Dis.* **177**: 1378–1381.
 13. **Stevens, C. E., R. P. Beasley, J. Tsui, and W. C. Lee.** 1975. Vertical transmission of hepatitis B antigen in Taiwan. *N. Engl. J. Med.* **292**: 771–774.
 14. **Taniguchi, S., H. Okamoto, M. Sakamoto, M. Kojima, F. Tsuda, T. Tanaka, E. Munekata, E. E. Muchmore, D. A. Peterson, and S. Mishihiro.** 1999. A structurally flexible and antigenically variable N-terminal domain of the hepatitis C virus E2/NS1 protein: implication for an escape from antibody. *Virology* **195**: 297–301.
 15. **Tovo, P. A., E. Palomba, G. Ferraris, N. Principi, E. Ruga, P. Dallacasa, and A. Maccabruni.** 1997. Increased risk of maternal-infant hepatitis C virus transmission for women coinfecting with human immunodeficiency virus type 1. *Clin. Infect. Dis.* **25**: 1121–1124.
 16. **Weintrub, P. S., G. Veereman-Wanters, M. J. Cowan, and M. Thaler.** 1991. Hepatitis C virus infection in infants whose mothers took street drugs intravenously. *J. Pediatr.* **119**: 869–874.
 17. **Zanetti, A. R., E. Taanzi, S. Paccagnini, N. Principi, G. Pizzocollo, M. L. Caccamo, E. D'amico, G. Cambiè, L. Vecchi, and The Lombardy Study Group on Vertical HCV Transmission.** 1995. Mother to infant transmission of hepatitis C virus. *Lancet* **345**: 289–291.
 18. **Zazzi, M., M. L. Riccio, G. Venturi, M. Catucci, L. Romano, A. De Milito, and P. E. Valensin.** 1998. Long-read direct infrared sequencing of crude PCR products for prediction of resistance to HIV-1 reverse transcriptase and protease inhibitors. *Mol. Biotechnol.* **10**: 1–8.