HIERARCHY OF BABY-LINKED IMMUNOGENETIC RISK FACTORS IN THE VERTICAL TRANSMISSION OF HEPATITIS C VIRUS

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Mother-to-infant transmission of Hepatitis C Virus (HCV) represents the major cause of pediatric HCV infection today. Immunogenetic influence has been poorly investigated and mainly confined to HLA-class II serological polymorphisms. Among 290 parities, 135 from Pavia and 155 from Bergamo, of HCV-RNA-infected Italian women, 21 babies (7.24%) were HCV-RNA positive at birth and steadily positive over 20 months of life. All the 21 infected babies and 44 randomly selected uninfected ones, born to HCV-RNA+ mothers but steadily negative for HCV-RNA during a follow-up of 2 years, and their mothers were investigated for HLA-G, -C, -DRB1, -DQA1 and -DQB1 genomic polymorphisms. Among the different covariates, HLA-Cw*07, -G*010401, -DRB1*0701, -DRB1*1401 and homozigosity for HLA-G 14bp deletion can be considered as risk factors for HCV vertical transmission. On the contrary, protection was conferred by the HLA-DQB1*06, -G*0105N, -Cw*0602, DRB1*1104 and -DRB1*1302 alleles. Our initial question was: has the immunogenetic profile any role in the protection of the fetus growing in an infected milieu and, if so, is it independent from the other non-immunogenetic parameters? The answer to both questions should be yes.

In children, given the decline of post-transfusion HCV hepatitis after the introduction of molecular testing for viral RNA in all blood units, the mother-to-child HCV transmission has become of great epidemiological relevance and nowadays accounts for 5% of HCV pediatric infections (1-5). Discussion still exists concerning the route of HCV vertical transmission (*in utero* or perinatal) and regarding the influence of viral, fetal and maternal factors on its occurrence (6-7). Several studies in horizontally-infected people indicated that specific HLA class II alleles might modulate hepatitis C severity (8-12), and preliminary evidence also envisaged a role of HLA class II molecules in vertical infection (13-14).

We extended our previous immunogenetic investigation (13) to the telomeric class I (G and C loci) region because HLA-G and HLA-C are the earliest HLA molecules expressed on the invasive extravillous trophoblast in the human placenta (15-20). The possibility of a function as protecting factors of the fetus from the maternal rejection as part of an allogenic response and in defending the

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fetus from pathogens coming from the mother are interesting hypotheses strengthened by the evidence of their interaction with the inhibitory receptor of natural killer cells (KIR) (21-24). This inhibition could serve to protect the embryo from maternal aggression playing a fundamental role in maternal-fetal tolerance (25).

Recently, it was demonstrated that a homozygous deletion of 14 bp in the exon 8 of HLA-G increases product stability and expression and influences the level of soluble forms (26). We identified a genomic trait in the telomere of the HLA region containing gene(s) associated with, or protective against, HCV infection *in utero*.

MATERIALS AND METHODS

Mothers

Between January 1991 and June 2000, 109 HCV infected women, aged from 25 to 41 years, with their 135 children were followed up at the Institute of Infectious Diseases of IRCCS Policlinico S.Matteo of Pavia. Informed consensus was obtained from each mother, and the study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinky, as reflected in prior approval by the Institution's human research committee. Anamnestic information was collected from each patient; clinical examinations and laboratory tests (biochemical and virological) were performed every 3 months during and after pregnancy.

The mothers were subdivided into 2 main categories: those positive for HCV alone (group I; N=66) and those positive for HCV and HIV (group II; N=43). 73.4% of the women from group I and 80.9% from group II presented a persistent alanine transaminase (ALT) increase twice exceeding the upper normal limit (normal value: <40 IU/L). 86% and 88% of mothers, from group I and group II respectively, resulted steadily positive for HCV-RNA. We also included 6 mothers of children who were HCV-RNA persistently positive at 1 year of life, followed up at the Infectious Disease Unit of Ospedali Riuniti in Bergamo (Northern Italy) and belonging to a larger panel of 155 infected women whose clinical data are published elsewhere (27).

Babies

We retrospectively analyzed 135 babies born to 109 HCV infected mothers followed up in Pavia Hospital, IRCCS Policlinico S.Matteo. All were checked at birth and every 3 months during the first 2 years of life. All were positive for anti-HCV antibodies at birth and 17 (12.6%) resulted HCV-RNA carriers. In particular, the vertical transmission rate was 14.1% (12/85) in group I (i.e. HCV positive mothers) and 10% (5/50) in group II (i.e. HCV-HIV co-infected mothers). All HCV-RNA infected children remained persistently asymptomatic as demonstrated by the absence of pathologic alterations at the abdomen ultrasound scanning performed almost every year. Two out of 17 HCV-infected children had abnormal ALT levels (more than 30 x normal value) with no symptoms of acute liver disease; ALT levels became spontaneously normal within 6 months.

Virological tests

The diagnosis of HCV infection in vertically exposed children was based on the persistence of specific antibodies beyond 18 months of age and on the detection of viral RNA in serum by PCR on at least 2 separate occasions. Anti-HCV antibodies were tested by second-generation enzyme-linked immunosorbent assay (Ortho Diagnostic System Inc., Raitan, NJ) and HCV RNA was detected by nested reverse transcriptase-polymerase chain reaction using conserved primers in the 5' non-coding region of viral genome (11). HCV typing was performed by amplification of core region sequences with universal and 5 subtype-specific primers.

HLA genomic typing

Sixty-four babies and 55 HCV infected mothers were included in the immunogenetic study: 1) 21 vertically infected infants, 15 from Pavia and 6 from Bergamo, steadily positive for anti-HCV-antibodies and HCV-RNA over 20 months of life, 11 female and 10 male, and 2) 43 randomly selected uninfected infants, 27 male and 16 female, steadily negative for HCV-RNA during a follow-up of 2 years, who were born to HCV-infected mothers matched for clinical conditions with those of infected babies.

HLA-G typing was performed by PCR-RFLP (polymerase chain reaction-restriction length polymorphism technique), using 3 restriction enzymes (MspI, AcyI and HindI) according to the protocol of the XII International Histocompatibility Workshop (28). HLA-C (exons 2 and 3), -DRB1, -DQA1 and -DQB1 (exon 2) polymorphism was defined by PCR-SSP (sequence specific primers) genomic techniques as described elsewhere (29).

Controls

The reference group was constituted by ethnically matched blood donors: 75 typed for HLA-G genomic polymorphisms, 128 for HLA-Cw* alleles at medium resolution level and 179 for HLA-DRB1*, -DQA1*, -DQB1* alleles at 4 digits level.

Statistical Analysis

Comparisons of HLA allele frequencies found in controls, mothers and HCV-RNA positive and HCV-RNA negative children, were made by means of χ^2 or Fisher exact

test as appropriate. To identify which of two risk factors showed the strongest association, a method described by Svejigaard and Ryder was followed (30).

Linkage disequilibrium between HLA-C and G alleles was evaluated by means of D'_{ij} statistics. Correspondence analysis (31) was performed to graphically show the relationships between the immunogenetic or non-immunogenetic variables and the different groups under study: mothers, babies, controls. Multiple logistic regression was used to establish a hierarchy among the different risk factors.

RESULTS

Non immunogenetic factors

Maternal history of drug abuse, especially if associated with HIV co-infection, elevated maternal ALT levels and HCV genotype 1, represented the most important risks of infection for the Pavia babies (Table I). First-born children seem to have an increased risk in respect to the other parities (34.7% vs 17.6%) as well as male sex (60% vs 40%).

Immunogenetic factors

As shown in Table II, the gene frequency of HLA-G*010401 was significantly higher in HCV positive children than in negative ones (22.5% vs 6.8%, p=0.014, OR 3.97, 95% CI 1.14-14.59). On the contrary, the HLA-G*010101, HLA-G*010103 and HLA-G*0105N alleles were more frequent in HCV-negative children. The HLA-G genotypes homozygous for the 14 bp deletion in the exon 8 was higher in positive babies than in negative ones, although not significantly (45% vs 27%). The gene frequency of HLA-Cw*07 was higher in HCV positive children than in negative ones (35% vs 19.8%, p=0.05, OR 2.7) (Table II). On the contrary, the HLA-Cw*0602 and Cw*15 alleles were more frequent in HCV-negative children (16.3% vs 10% and 7% vs 0% respectively).

As far as HLA- DRB1*, DQA1* and DQB1* class II genes are concerned, the distribution of alleles (defined to a four digit level of resolution) in all the categories under study demonstrated an involvement only in the mother. HLA-DRB1*1102 allele and the haplotype HLA-DRB1*0701, DQA1*0201, DQB1*0202 resulted significantly more frequent in HCV-RNA positive mothers than in controls (3.7% vs 0.3% p=0.0026, 19.4% vs 10% p=0.014 and 18.5% vs 8.7% p=0.007 respectively).

Comparing HCV negative versus positive children, we observed that HLA-DRB1*1104 allele and the haplotype HLA-DRB1*1302, DQA1*0102,

Table I. *Influence of maternal risk factors on HCV vertical transmission in 135 parities from Pavia.*

Maternal risk Factors	GROUP I HCV infected mothers N = 66 mothers infected babies/total mothers	GROUP II HIV and HCV co-infected mothers N = 43 mothers infected babies/total mothers
HCV genotype 1		
YES	6/17 (35.3%)	1/19 (5.3%)
NO	6/49 (12.2%) **	4/24 (16.7%)
Intravenous drug user		
YES	3/15 (20.0%)	5/37 (13.5%)
NO	9/51 (17.6%)	0/6 (0%)
Elevated ALT YES NO	 11/48 (22.9%) 1/18 (5.5%)	 4/34 (11.7%) 1/9 (11.1%)

** *genotype 1 vs others 35.3% vs 12.2%: p=0.04*

Table II. *Susceptibility markers of HCV vertical infection (HLA-G* and –C*) and horizontal infection (HLA-DRB1*, DQA1* and DQB1*).*

HLA alleles	Controls	HCV positive mothers	HCV negative children	HCV positive children
G*010401	13/150 (8.7%)	9/110 (8.0%)	6/88 (6.8%)	9/40 (22.5%) *
Cw*07	62/256 (24.2%)	24/104 (23.1%)	17/86 (19.8%)	14/40 (35.0%) **
DRB1*0701	36/358 (10.1%)	21/108 (19.4%)°	14/88 (15.9%)	9/42 (21.4%)
DRB1*1102	1/358 (0.3%)	4/108 (3.7%)°°	3/88 (3.4%)	1/42 (2.4%)
DQA1*0201	36/358 (10.0%)	21/108 (19.4%)$	14/88 (15.9%)	9/42 (21.4%)
DQB1*0202	31/358 (8.7%)	20/108 (18.5%)$$	13/88 (14.8%)	8/42 (19.1%)

** HCV+ vs HCV- children p=0.014*
*** HCV+ vs HCV- children p=0.05*
° Mothers vs Controls p=0.014
°° Mothers vs Controls p=0.003
$ Mothers vs Controls p=0.014
$$ Mothers vs Controls p=0.007

Table III. *Analysis of independence of HLA-G and C risk factors according to Svejgaard and Ryder statistical method.*

		Individual Association		Combined Association	Is A associated independently from B ?		Is B associated independently from A ?		Different association with B and A	Linkage Disequilibrium	
Factor A	Factor B	A vs noA	B vs noB	++ vs --	++ vs -+	+- vs --	++ vs +-	-+ vs --	+- vs -+	Associa-tion A,B in HCVneg	Associa-tion A,B in HCVpos
CW*07	G*010401	2.7 0.05	3.5 0.04	**6,3** 0.03	0.8 n.s.	4.5 0.02	1.4 n.s.	**8,4** 0.02	0.5 n.s.	0.5 n.s.	2.9 n.s.

The first 2 tests investigated the individual association of two factors, i.e. A and B; in test 3 the combined action of the two factors A and B was compared relatively to their absence (++ vs --); tests 4 and 5 investigated whether factor A was deviated in B-positive (++ vs -+) or in B-negative (+- vs --) subjects; contrariwise, tests 6 and 7 investigated whether factor B was deviated in A-positive (++ vs +-) or A-negative subjects (-+ vs --); test 8 investigated whether A and B association differed (+- vs -+). Finally in tests 9 and 10 the linkage disequilibrium between the two factors was determined in HCV positive and negative groups respectively.

Table IV. *Logistic regression.*

Exposure variable	OR	95% CI	LR test	p
HLA-Cw* 07	3.32	1.01- 10.84	4.15	0.042
First-born child	2.50	0.54-11.44	1.51	ns
HLA-G*010401	2.46	0.61-9.84	1.55	ns
Viral genotype 1b	2.43	0.56-10.73	1.59	ns
HLA-G 14bp--	1.80	0.48-11.00	1.47	ns

DQB1*0604 were more frequent in HCV-negative children than in positive ones (14.8% vs 7.1% and 4.6% vs 0%). On the contrary, the haplotypes HLA-DRB1*0301, DQA1*0501, DQB1*0201 and HLA-DRB1*0701, DQA1*0201, DQB1*0202 and HLA-DRB1*1401, DQA1*0104, DQB1*0503 were more frequent in HCV-positive children (11.9% vs 5.7%, 21.4% vs 15.9% and 11.9% vs 4.6%; respectively).

At a low level of resolution, a significant increase of HLA-DQB1*06 allele was found among negative children: 12.5% vs 0%, p=0.022. HLA-DRB1*13 also resulted protective (7.9% in HCV negative vs 2.3% in HCV positive) while HLA-DRB1*14 seemed to define susceptibility (4.5% in HCV negative vs 14.3% in HCV positive) but without any statistical significance. We analyzed the independence of HLA-G and HLA-C contributions to the vertical transmission. We demonstrate that HLA-Cw*07 and G*010401 alleles act independently and their effect increases when both are present rather than one alone (OR=6.3), as shown in Table III.

Correspondence analysis showed that HLA-Cw*07, -G*010401, -DRB1*0701, -DRB1*1401, maternal viral genotype 1b, male sex, first birth and breast feeding may be considered risk factors for HCV vertical transmission. On the contrary, protection was conferred by the HLA-DQB1*06, -G*0105N, -Cw*0602, -DRB1*1104 and -DRB1*1302 (Fig. 1).

Logistic regression scores the exposure variables: the major risk factor was represented by the presence of the HLA-Cw*07 allele (OR= 3.32), followed by being first-born, carrier of HLA-G*010401 allele and having a mother with HCV genotype 1b (Table IV). Contrariwise, the highest protection was ascribable to HLA-DQB1*06 allele (OR=n.d.; p=0.022).

DISCUSSION

The fetus is usually protected against circulating maternal HCV quasispecies: intrauterine infection is a rare event (7.24% of infections in our experience) and represents the result of a complex interplay of viral, maternal and fetal risk factors. HCV may enter the placenta in two ways: 1) actively, crossing the villous trophoblastic layer or 2) passively, nested into nucleated maternal cells (32-33). This two-way traffic has been demonstrated with the PCR-based technique in umbilical cord blood.

Among 290 parities of HCV-RNA infected women, 21 babies (7.24%) were HCV-RNA positive at birth and steadily positive over 20 months of life; none developed severe HCV-associated clinical signs or symptoms, except for a transient increased ALT concentration in 2 of them, thus confirming that primary HCV infection, acquired from the mother before or during delivery, is usually milder than infection acquired when older (3, 34). A history of maternal intravenous drug abuse (IVDU) and coinfection with HIV has been definitely associated with an increased probability of HCV vertical transmission because of a further depression of the immune system already compromised by HIV (35-36). In this study, we confirm that the drug abuse, together with the copresence of HIV, exerts a worse effect (Table I).

Although ALT activity is a poor surrogate marker

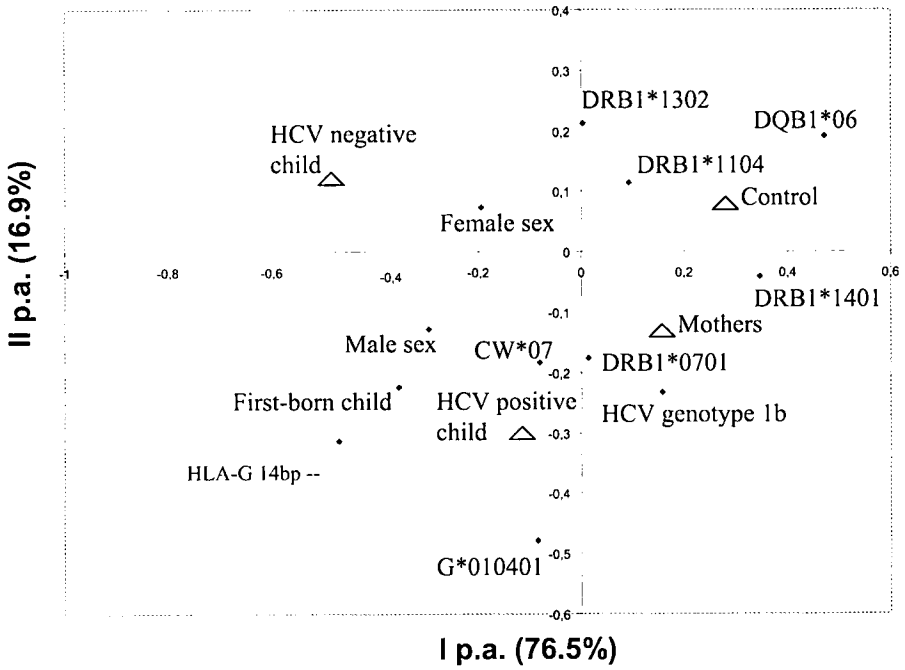

Fig. 1. *Correspondence analysis. A unique picture is represented here of all the relationships among all the various parameters studied. Horizontal axis (explaining 76.5% of total variation) separates the protective covariates, on the upper side, from the permissive (susceptible) ones, on the lower part of the plot. The nearer they are to the symbol of "HCV negative child" or to the symbol of "HCV positive child", the higher is their relevance in determining protection against or permissivity to fetus infection. HLA-Cw*07, -G*010401, -DRB1*0701, -DRB1*1401, homozogosity for 14bp deletion in HLA-G, maternal viral genotype 1b, male sex and first birth may be considered risk factors for HCV vertical transmission. On the contrary, protection was conferred by the HLA-DQB1*06, -G*0105N, -Cw*0602, -DRB1*1104 and -DRB1*1302.*

of disease evolution, in our study we found that HCV-infected mothers with more elevated ALT levels transmitted HCV more frequently. However, this factor seems to be less important for co-infected mothers. Maternal genomic viremia is one of the most important risk factors as it has been demonstrated that HCV infected, but RNA negative, mothers didn't transmit HCV infection to the newborn (32, 37). With regards to the patterns of maternal HCV quasispecies, we observed that the 1a and 1b and 3a types were the most common in our sample and that the 1b was very frequently transmitted to the fetus. In logistic regression analysis, it is evident that HCV 1b variant confers "*per se*" a risk of vertical infection (OR=2.43) (Table IV). This is in accordance with the high load and aggressiveness of this viral type reported in the literature (38) and therefore explains its spread and persistence in fetal circulation. The subtype 3a was peculiar to IVDU mothers, consistent with reports on the high prevalence of this viral isolate in drug users (39).

Evaluating whether the birth order was a risk factor for HCV vertical transmission, a susceptible risk of 2.5 to the first parity was found (Table IV). Similar results have been observed in babies born to HIV infected mothers where fetal protection is in parallel with the increase of parity; firstborns are more frequently HIV infected than the second or third conceptuses (40). Immunological mechanisms may be claimed to explain these results.

Searching for baby-linked immunogenetic factors, which is the primary scope of this work, we completed our previous study (13) by defining the HLA class II genes at high resolution level and we explored the telomeric region of HLA. In particular, we are now investigating the involvement of HLA-G and C fetal polymorphisms in self defense. Our question was: do these molecules have any role for the fetus growing in an infected milieu?

With regard to the HLA-class II alleles, we confirm our previous suggestion that the course of HCV

intrauterine infection evolves differently in the presence of particular MHC class II alleles (13) and that both the susceptibility to and the protection against HCV infection are associated with the same sequence motifs of HLA-DRB1 and DQB1 genes as in horizontal infection (11-12). We also agree with a recent publication (14) suggesting that HLA-DR13 is a protective immunogenetic factor against vertical infection. In this work, we split the HLA-DR13 serological variant into four genomic subtypes, demonstrating that they were very infrequent among infected babies. Interestingly, we observed that the HLA-DQB1*06 allele, in linkage disequilibrium with DRB1*1301 and DRB1*1302, was absent in viremic neonates (12.5% vs 0%: p=0.02). Probably, neither DRB1*13 nor DQB1*06 are directly involved in protection from transplacental infection but they possibly mark a subregion of interest in the control of the immune response.

HLA-G*010401 and HLA-Cw*07 were significantly more frequent in infected babies than in uninfected ones, as reported in the plot of correspondence analysis (Fig.1). Since these alleles are not in linkage disequilibrium with each other (D'<0), we can speculate on two different and independent HLA subregions encoding factors facilitating the viral entry in the fetal circulation. HLA-Cw*07 has recently been claimed as one of the susceptibility genes for hepatitis B and C viruses horizontal infection in a representative cohort of Italian patients, by favoring viral spread and the development of hepatocarcinoma (41).

The importance of HLA-C surface molecules has been emphasized since the discovery of their physical interaction with the inhibitory receptors of natural killer cells (KIRs) which render them able to control and switch off their lytic activity (42). In particular, homozygosity for HLA-Cw*07 has been related to an intrinsically reduced protection from viral infection due to a reduced size of NK group 1 ligands which hampers the host in mounting a vigorous response against the virus (43).

Three different scenarios could account from this study: 1) either HLA-Cw*07 and HLA-G*010401 alleles are passively involved, hitchhiking with other truly putative genes or 2) their products are directly involved in binding crucial viral peptides or KIR inhibitory receptors and therefore they control the immune response or 3) they are survival alleles. In this regard, it is known that intrauterine pathogens may be selective agents of fetuses carrying alleles which confer characteristics of resistance (21).

A fourth scenario is open for HLA-G whose expression in embryo cells is mandatory: low levels of HLA-G expression induce a Th1 cytokine response and are associated with miscarriages and preeclampsia (44). It was recently proven that the insertion or deletion of 14bp (base pairs) in the exon 8 of the HLA-G gene has some significance in fetal survival. In fact, the mRNA expression levels of membrane bound HLA-G isoforms of alleles lacking the 14bp sequence are significantly higher than those of alleles with 14bp insertion (44). HLA-G*010401 is a high secretor allele and this may be an advantage for the fetus growing in an infected milieu (45-46). The conclusions reached above are solely related to the vertical transmission mechanisms of HCV and are not automatically applicable to other transmission routes. To confirm this further studies need to be done.

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