High anti-HLA response in women exposed to intrauterine transfusions for severe alloimmune hemolytic disease is associated with mother–child HLA triplet mismatches, high anti-D titer, and new red blood cell antibody formation

Esther P. Verduin, Henk Schonewille, Anneke Brand, Geert W. Haasnoot, Frans H.J. Claas, Irene T.M. Lindenburg, Enrico Lopriore, Dick Oepkes, Dave L. Roelen, and Ilias I.N. Doxiadis

BACKGROUND: Women whose fetuses were treated with intrauterine transfusions (IUTs) for alloimmune hemolytic disease are high responders to red blood cell (RBC) antigens. We investigated the risk for HLA alloimmunization.

STUDY DESIGN AND METHODS: Women and their children treated with IUT between 1987 and 2008 were included. Participants were HLA antigen typed and studied for the prevalence of HLA antibodies compared to age-matched parous nontransfused blood donors. Anti-D titer, the formation of new RBC antibodies after IUT, and the degree of fetomaternal HLA mismatches on HLA antibody formation and/or persistence were analyzed.

RESULTS: A higher prevalence of HLA Class I antibodies was observed in these women compared to controls (41% vs. 23%). Both a higher anti-D titer (>8000) and formation of new RBC antibodies after IUT were associated with increased HLA immunization. HLA antibody formation was associated with the number of fetomaternal triplet epitope mismatches. Antigens within HLA-Bw4, HLA-B35/51/52/53/18/78-complex and A1/A9, were higher and mismatches within HLA-C were less immunogenic than expected. HLA antibodies against the IUT-treated fetus were more persistent than other antibodies.

CONCLUSION: Women whose fetuses were treated with IUT had a high risk of developing and maintain fetal-specific HLA Class I antibodies. Factors associated with increased HLA immunization were a higher amount of fetomaternal HLA triplet mismatches, higher anti-D titer, and additional RBC antibody formation. We presume that the induction of HLA Class I antibodies is the result of increased fetomaternal hemorrhage during IUT, eliciting antibodies in women with an increased susceptibility to alloimmunization.

Pregnancy can lead to maternal antibody formation directed against mismatched paternal antigens expressed on fetal cells. Antibodies against red blood cells (RBC) can cause hemolytic disease of the fetus and newborn (HDFN). The mainstay treatment for severe HDFN is intrauterine transfusion (IUT).

Approximately 90% of all severe HDFN in need of IUT treatment is caused by anti-D.[1] During IUT there is increased fetomaternal hemorrhage (FMH) while transfused donor cells may also enter the maternal circulation.[2] Women whose fetus has undergone IUT treatment are high alloresponders to RBC antigens. In a cohort of more than 300 women who had undergone IUT treatment, 25% formed additional RBC antibodies and after pregnancy

ABBREVIATIONS: CDC = complement-dependent cytotoxicity; FMH = fetomaternal hemorrhage; HDFN = hemolytic disease of the fetus and newborn; IUT(s) = intrauterine transfusion(s); LUMC = Leiden University Medical Center.

From the Sanquin-LUMC Jon J. van Rood Center for Clinical Transfusion Research and the Department of Immunohematology and Blood Transfusion, the Department of Obstetrics, and the Department of Neonatology, Leiden University Medical Center, Leiden, the Netherlands.

Address reprint requests to: Esther P. Verduin, Sanquin-LUMC Jon J. Van Rood Center for Clinical Transfusion Research, Plesmanlaan 1a, 2333 BZ Leiden, the Netherlands; e-mail: e.verduin@sanquin.nl.

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more than 70% of women possessed multiple RBC antibodies.[3,4] This poses the question whether these women are high responders to all alloantigens present on blood cells to which they are exposed. Several studies have already reported that patients with RBC antibodies are more likely to possess antibodies against other blood cell antigens, such as HLA.[5-8]

HLA antibodies frequently occur after uncomplicated pregnancy with increased prevalence with higher parity, although the first two pregnancies seem the strongest immunizing events.[9-11] In samples taken during or shortly after anti-D–complicated pregnancies, HLA antibodies were reported in up to 85% of cases.[12-14] We questioned in women who received IUT for severe HDFN whether HLA antibodies occurred more often in high RBC antibody responders and/or in multi-RBC responders who had produced additional RBC antibodies after IUT.

MATERIALS AND METHODS

Study participants

The Leiden University Medical Center (LUMC) is the Dutch national referral center for pregnancies complicated by HDFN caused by maternal RBC alloimmunization. Yearly, 20 to 25 affected fetuses with severe anemia are treated with IUTs. All women and the children who have been treated with IUT for HDFN from 1987 to 2008 at the LUMC were asked to participate in a long-term follow-up cohort study (LOTUS; LOng Term follow-Up after intrauterine transfusionS). This study addresses putative mechanisms associated with blood group alloimmunization in these mothers. After informed consent was obtained, blood or saliva samples were taken from participating mothers and children. The full protocol of the study has been published previously.[15] The study was approved by the ethics committee of the LUMC (P08.080).

IUTs

IUTs were prepared from fresh (<3 days old) leukoreduced (prestorage filtration, white blood cell [WBC] count $<10^6$), irradiated (25 Gy), Parvo B19–safe (since 2002), and cytomegalovirus-seronegative RBCs adjusted to a hematocrit of 0.80 to 0.85 L/L with 0.9% saline. The donor RBCs were D, C, c, E, and e blood group compatible with the mother; K–; antigen negative with maternal antibodies; and complete crossmatch negative (including an antiglobulin fase) with maternal serum.

Historical antibodies

For all participating women historical RBC antibody data, including highest anti-D titer, and HLA antibody data, determined during pregnancy or shortly after delivery, were retrieved from hospital records. The presence and titer of RBC antibodies was routinely tested from 1987 to 2005 using the polyethylene glycol antiglobulin tube method and afterward using the low-ionic-strength saline (LISS)-direct antiglobulin test gel-microcolumn technique (LISS DiaMed ID gel system, DiaMed, Murten, Switzerland), both of which are of comparable sensitivity for the detection of clinically relevant antibodies.[16] Women were classified as single responders when antibodies were directed against antigens within one blood group system (e.g., Rhesus) and multiresponders when the antibodies were against antigens in multiple RBC blood group systems (e.g., Rhesus, Kell, Duffy, Kidd, MNS).

HLA antibodies were tested by an enzyme-linked immunosorbent assay (ELISA)-based assay (Lambda Antigen Tray, One Lambda Inc., Canoga Park, CA) and positive sera were further tested for immunoglobulin (Ig)G HLA antibody specificities by complement-dependent cytotoxicity (CDC) test and/or an antibody analysis system (DynaChip, Invitrogen, Life Technologies, Paisley, UK; discontinued).

HLA antibody testing

The presence of anti-HLA in maternal sera were initially analyzed by the Lambda Antigen Tray (One Lambda Inc.). The ELISA for HLA Class I and II was conducted according to manufacturer's protocol with OD readouts at 630 nm. Positive sera ($OD_{patient/control\ ratio} > 2.0$) were further tested for IgG HLA antibody specificities by CDC against a panel of peripheral blood cells from 54 different donors in the absence and presence of dithiothreitol that breaks down disulfide bonds of pentameric IgM, with minimal effects on IgG. The reactions were read in a semiautomatic system (Leitz, Wetzlar, Germany) using single-color readout as described by Bruning et al.[17] Additionally, positive sera were also analyzed using the DynaChip antibody analysis (Invitrogen, Life Technologies, Paisley, UK; discontinued), which has a high sensitivity for Class II antibodies.[18,19]

To assess whether the HLA antigens of the fetus corresponded with the specificity of the maternal HLA antibodies, all antigen specificities were translated into the corresponding broad antigen specificities. For example if a child was typed as A*24:02 and the mother had an anti A9, this antibody was considered as fetus induced.

Control population

To assess whether the HLA alloimmunization frequency differed from other previously pregnant women, the results were compared to those of a group of female blood donors recently described by Middelburg and colleagues.[20] From this cohort of 1094 never transfused, previously pregnant female blood donors, an age-matched control group consisting of 526 individuals was randomly

selected. The presence of Class I and Class II HLA antibodies in this cohort had been tested by a Luminex screen (Lifescreen deluxe and Lifecodes LSAI/II). In case of a positive HLA antibody screen result, antibodies were not further specified, nor were women tested for RBC antibodies in this study.

HLA typing

All women and children were typed for HLA Class I and II antigens with molecular methods. Medium resolution (first field) molecular typing techniques were applied, covering at least all serologic defined specificities. For HLA-A, -B, and -C a commercially available polymerase chain reaction (PCR) reverse hybridization line-probe assay was applied (RELI tm SSO, Invitrogen, Washington, DC). The HLA-DRB and -DQB typing was performed with a reversed approach of the PCR–sequence-specific oligonucleotide probe technique described previously.[21] HLA allele frequencies were tested for Hardy Weinberg equilibrium.[22,23]

Triplet mismatches

Triplet mismatches are defined by triplets of three sequential amino acid residues of HLA molecules expressed on antibody-accessible sites in the groove. The number of triplet mismatches between mother and child were calculated with computer software (HLA Matchmaker LB100, Version 2.1, http://www.hlamatchmaker.net/) to evaluate a possible correlation between the number of triplet mismatches between mother and child with HDFN and the production of HLA Class I antibodies against corresponding fetal antigens.[24-26]

To estimate the relative immunogenicity of mismatched triplets in our cohort, equal immunogenicity for each mismatched triplet was assumed. Next, the overall triplet immunogenicity was calculated by dividing the total number of observed antibodies by the total number of triplet mismatches. Then, the "expected" number of antibodies directed against a specific triplet was calculated by multiplying the assumed immunogenicity per triplet with the relative frequency of the specific triplet. Finally, the expected number of antibodies was compared to the "observed" number of antibodies. A Sidak[27] correction for multiple testing and a goodness-of-fit analysis with the chi-square test were used.

Statistical analysis

Software packages were used for data management and analysis (Access, Excel, SPSS 17 [SPSS, Inc., Chicago, IL]). Statistical analysis was performed using chi-square test for categorical variables and odds ratios (ORs) to calculate change differences. To assess the relationship between categorical variables Spearman's rank correlation coefficients were calculated. Differences between the medians were compared with the Mann-Whitney U test.

A multivariate logistic regression analysis was performed to assess the influence of the variables that showed a significant relationship in the univariate analysis on test outcome. Correction for multiple testing was performed.[27] Groups were assumed to differ significantly when the probability level was less than 0.05.

RESULTS

Transfusion and RBC antibody characteristics of LOTUS women

The antibodies primarily responsible for HDFN were anti-D (n = 209); anti-K1 (n = 33); anti-c (n = 18); and anti-E, -C^w, and -Kp^a in one case each. The highest anti-D titer in D induced HDFN varied between 32 and 2×10^6 (median, 8000). After a median of three IUTs (range, one to seven) the 263 mothers possessed a total of 539 RBC antibodies.

A single antibody remained present in 62 mothers and 201 developed multiple (two to five) specificities. In 130 women these additional antibodies were directed to other antigens of the Rhesus system only (single system responders) and 71 women possessed antibodies against antigens in two to four blood group systems (multisystem responders).

HLA antibodies in LOTUS and control women

Blood samples from all 263 women participating in the study were screened for HLA antibodies and compared to the results from 526 age-matched previously pregnant blood donors. In LOTUS women HLA antibodies were significantly more frequent compared to the controls (48 and 35%, respectively; $p < 0.001$). In particular HLA Class I antibodies were more frequent in the LOTUS women, whereas Class II antibodies were equally frequent (Table 1). Five fetuses had received one intrauterine platelet (PLT) transfusion and one woman had received seven PLT transfusions since delivery during treatment for B-cell acute lymphoblastic leukemia. Of the six women who underwent (intrauterine) PLT transfusions three had HLA antibodies, directed against Class I, Class II, and both HLA classes, one woman each.

To explore an effect of number of pregnancies (median, three in our cohort vs. two in the control group) and interval of follow-up since delivery ($p = 0.07$) between LOTUS and control group, a multivariate logistic regression was performed. None of the outcomes were influenced by the follow-up period. For the outcome "HLA antibody positive" a higher number of pregnancies was significantly associated ($p = 0.003$) and LOTUS women showed a trend ($p = 0.06$). The outcome "Class I" antibod-

ies was stronger correlated with the LOTUS group ($p < 0.001$) than with the number of pregnancies ($p = 0.011$). The formation of HLA Class II antibodies was not correlated with number of pregnancies ($p = 0.96$) and LOTUS women showed a trend ($p = 0.08$). A higher number of IUTs was not associated with more HLA-immunized women (Spearman's rho = 0.003, $p = 0.12$) nor did the median number of IUTs differ between women with and without HLA antibodies (three [range, one to seven] vs. three [range, one to six], respectively; not shown).

RBC and HLA antibody association in LOTUS women

Single and multiple (range, two to four) HLA antibody specificities were found in 52 and 75 women, respectively. These antibodies were directed against 152 Class I and 58 Class II antigens, for a total of 210 HLA antibody specificities.

The specificity of RBC antibodies primarily responsible for HDFN was not associated with the presence of HLA antibodies at follow-up, for example, 48% in both D and non-D hemolytic disease. However, among females with anti-D HDFN there was a correlation between the strength of the historical anti-D titer and HLA immunization rate (Spearman's rho = 0.141, $p = 0.043$).

The 71 women with antibodies against multiple RBC blood group systems had, compared to the 192 women with one or more antibodies against antigens of a single RBC blood group system, similar age, number of pregnancies, and follow-up period ($p \geq 0.34$) and equally often multiple HLA antibodies (15.5 and 15.1%, respectively). During IUT treatment 65 women (24.7%) formed 74 new RBC antibodies. Of these, 40 women (61.5%) had HLA antibodies at follow-up compared to 88 of 198 women (44.4%) who had not formed new RBC antibodies ($p = 0.022$; OR, 2.0; 95% confidence interval [CI], 1.1-3.5; Fig 2).

HLA antigens in mothers and children and HLA antibodies

The phenotypes of the separate HLA loci in 263 HLA-typed LOTUS women and 203 children from 200 mothers were in Hardy-Weinberg equilibrium except for higher frequencies of HLA-DQ2 and -DQ7 homozygotes in women. Only four of these women formed DQ antibodies. The total cohort was used for the HLA Class I triplet analysis.

From 92 of the 127 women who possessed HLA antibodies at follow-up, the HLA antigens of their 95 LOTUS children were available. The children carried the corresponding HLA antigens in 37% in case of Class I antibodies (against 115 specificities) and in 29% for Class II antibodies (42 specificities).

Triplet mismatches and HLA Class I antibody formation

From 198 mother–child combinations HLA types were available to analyze a possible correlation between HLA Class I antibody formation and the number of amino acid triplet mismatches between mother and child. The percentage of women having at least one Class I HLA antibody against fetal antigens increased from 5.0% in case of 0 triplet mismatches to approximately 25% when 10 or more triplet mismatches were present (Spearman's rho = 0.210, $p = 0.023$; Fig. 1).

The HLA Matchmaker program identified 2437 triplet mismatches between mother and child and 472 antibodies directed against these mismatches. Assuming equal immunogenicity of all mismatches, the expected number of antibodies per triplet mismatch was 0.193 (=472/2437). Comparison with observed antibodies revealed that, before correction for multiple testing, antibodies induced by five triplet mismatches (three different triplets for HLA-Bw4, one for the HLA-R complex [HLA-B35, -B51, -B52, -B53, -B18, and -B78], and one for the A1/A9 alleles) were significantly more frequent, while antibodies toward six other triplet mismatches (all HLA-C) were less frequent than expected (Table 2). After a Sidak[27] correction for multiple testing, significance disappeared, probably due to the low numbers in the comparison. The test for goodness of fit showed that the distribution of the number of observed antibodies against specific triplet mismatches in the LOTUS population significantly differed from the expected distribution (legend to Table 2).

The influence of number of triplet mismatches, highest anti-D titer, and new RBC antibody formation on HLA Class I immunization.

For 157 women with HDFN due to anti-D the combined effect of the number of maternal–child triplet mismatches

TABLE 1. Characteristics and HLA antibody frequency of 263 LOTUS women and 526 age-matched controls

Characteristics	LOTUS	Controls	p value	OR (95% CI)
Age (years)	42 (18-60)*	42 (19-60)	0.69	NA
Number of pregnancies	3 (1-14)	2 (1-7)	<0.001	NA
FU period (years)	8.6 (0-23)	8.0 (0-29)	0.07	NA
HLA antibody positive†	127 (48%)	184 (35%)	<0.001	1.74 (1.28-2.35)
Class I†	108 (41%)	120 (23%)	<0.001	2.36 (1.71-3.24)
Class II†	57 (22%)	125 (24%)	0.53	0.53 (0.62-1.27)

* Data are reported as median (range) or number (%).
† Percentages were calculated with total number of LOTUS women (n = 263) and controls (n = 526) as denominator.
NA = not applicable.

(≤10 and >10), the highest historical anti-D titer (≤8000 and >8000), and new RBC antibody formation during IUT treatment on the frequency of HLA Class I antibodies at follow-up was determined. The combination of high anti-D titer with new RBC antibody formation was associated with more HLA immunization at follow-up in both low- and high-number triplet mismatched cases (Fig. 2). Women with a high number of HLA triplet mismatches with their HDFN child, together with a high anti-D titer and new RBC antibody formation upon IUT, had a 10.4 times (95% CI, 1.8-61; p = 0.014) higher chance to have HLA antibodies compared to women with fewer than 10 triplet mismatches, lower anti-D titer who had not produced new RBC antibodies after IUT.

Persistence of HLA antibodies

During or soon after pregnancy, 55 (21% of the cohort) women had been screened for HLA antibodies for clinical indications (e.g., fetal or neonatal thrombocytopenia) and 78% tested positive. From 27 women, antibody specificities had been determined: 36 were directed against HLA Class I and nine against Class II. Of 38 of these 45 antibodies the infant's HLA antigens were available and carried the corresponding HLA antigens in circa 30% of the antibodies. At follow-up of these 27 women, after an interval of median 10 years (range, 2-16 years), two-thirds of these antibodies had persisted, of which all antibodies against the HDFN children (13/13), while 32% (8/25) of the other antibodies had disappeared (p = 0.034; OR, 13.1; 95% CI, 0.7-248).

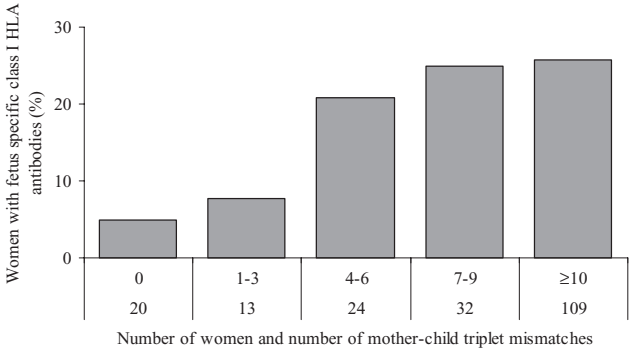

Fig. 1. Percentage of HLA Class I–immunized women in relation to number of mother–child triplet mismatches.

DISCUSSION

During pregnancy women can form antibodies against paternal RBC and HLA antigens present on fetal cells. While the rate of pregnancy-induced RBC alloimmunization, depending on whether RhD prophylaxis is implemented, generally does not exceed 6%, HLA antibodies are formed in up to 60% depending on higher number of parities.[9-11,28-30] It has been postulated that the higher HLA alloimmunization rate is due to higher polymorphism of the HLA antigens leading to an increased chance for incompatibility.[31] Furthermore, different immunization pathways by WBCs providing direct and indirect antigen presentation to helper T cells in contrast to less polymorphic RBC antigens presented via the indirect pathway only may play a role.[31,32] Few studies are available on the combined appearance of both types of antibodies after transfusion and most reported higher HLA antibody rates in RBC immunized compared to non-RBC–immunized organ transplant, sickle cell disease, and other multiply transfused patients.[5-7,33] Pregnant women, tested during pregnancy or at delivery, showed a higher HLA immunization incidence in D-immunized compared to non-D–immunized and D+ women.[12-14] Our study evaluating RBC and HLA antibodies almost 9 years after a pregnancy complicated by IUTs confirms a higher incidence (48% vs. 35%) of HLA antibodies in patients with RBC antibodies compared to a control parous population of blood donors, albeit RBC antibodies had not been tested in these con-

TABLE 2. Immunogenicity of specific HLA Class I triplet mismatches in 198 mother–child combinations

Triplet mismatch	HLA allele*	Amino acid position	Number of mismatches	Number of antibodies		p value
				Expected	Observed	
AA in triplet			2437		472	
RIA	Bw4	79	36	6.97	15	0.0024
REN	Bw4	75	32	6.20	12	0.0198
RKL	C	79	32	6.20	1	0.0368
RVN	C	75	32	6.20	1	0.0368
QIF	R-complex†	65	31	6.00	12	0.0144
EHP	C	183	31	6.00	1	0.0411
ALR	Bw4	81	30	5.81	12	0.0102
EPP	C	183	30	5.81	1	0.0460
HPL	C	192	30	5.81	1	0.0460
QNY	C	65	30	5.81	1	0.0460
VDG	A1-A9	165	27	5.23	10	0.0370

* A total of 45 HLA alleles were analyzed and only HLA alleles with a p value of less than 0.05 between expected and observed are shown.
† R-complex consists of HLA-B35, -B51, -B52, -B53, -B18, and -B78 HLA antigens. Goodness-of-fit test for triplet mismatch immunogenicity analysis: The overall Hardy-Weinberg equilibrium assumption failed (p = 0.0007).

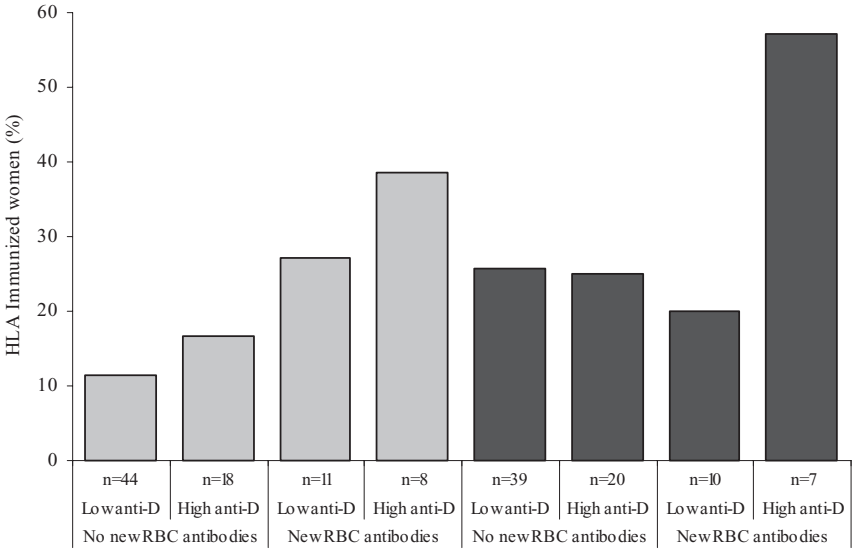

Fig. 2. Risk of fetus-specific HLA Class I immunization with increasing fetomaternal HLA triplet mismatches, increasing anti-D seroresponsiveness, and RBC multiresponsiveness. The values on the *X*-axis represent the number of women. (□) Cases with not more than 10 mother–child triplet mismatches; (■) cases with more than 10 mother–child triplet mismatches. The median anti-D titer (= 8000) from the total cohort of 157 women was used as the cutoff value for low versus high anti-D titer.

trols. However, the frequency of RBC antibodies in more than 28,000 Dutch female blood donors is only 0.6% (unpublished Sanquin Blood Supply Donor Registry data). The HLA antibody rate in published cohorts of parous blood donors varies, which may depend on the different techniques of determining HLA antibodies. Using both Luminex platforms, the prevalence of HLA antibodies in the age-matched parous control cohort that we selected is in line with the REDS II study, albeit we found slightly higher antibody prevalence (35%) than the REDS II study found in parous woman (24.4%). A different cutoff value to discriminate positive and negative results may explain this, because the Dutch blood donor cohort also revealed a higher proportion of HLA antibodies in nontransfused males (9% vs. 1%, respectively).[11] Densmore[8] and colleagues reported that HLA antibodies declined after 5 years after delivery using a functional test as the CDC, while Powers and coworkers[10] and Gatault and coworkers[34] reported longer persistence of HLA antibodies using more sensitive Luminex techniques.[35] Although in comparative assays, Luminex-based techniques are most sensitive compared to ELISA, FlowPRA, and CDC, the cutoff is not equivocal.[18] In the current study HLA antibodies in the LOTUS population were screened by multiplex ELISA and CDC, while in the control group by Luminex and it may well be possible that antibody prevalence in the LOTUS women will even be higher when tested with a more sensitive technique.

This higher incidence in females with HDFN came mainly on account of increased HLA Class I antibodies. We explored three possible causes that may play a role in enhancing HLA immunization: first, the number of pregnancies, which were higher in LOTUS women; second, the number of IUTs, because the IUT treatment is associated with excess fetomaternal hemorrhage (FMH); and third, whether these women were high responders, by comparing HLA immunization with the anti-D titer and the formation of new RBC antibodies after IUT.

Multivariate logistic regression analysis revealed the number of pregnancies indeed as independent risk factor for HLA antibodies. However, this association was weak and the presence of HDFN (and IUT) was a strong independent risk factor associated with anti-HLA Class I antibodies, much stronger than the number of pregnancies.

Second, the IUT procedure increases FMH, exposing the mother to fetal and donor antigens.[4,12] The number of IUTs was not associated with HLA immunization, but this does not completely preclude this cause. The first and to a lesser extend a second IUT will lead to increased hemorrhage of fetal RBC, whereas in later IUTs the child's erythropoiesis is completely suppressed and replaced by donor RBCs. A FMH of incompatible RBCs as is the case in the first IUT, to which the mother has strong antibodies likely induces cytokine release that may enhance other immunizations.[36,37] Why mainly Class I antibodies are induced is not explained. It is conceivable that especially during the first IUT, when a FMH contains a more young fetal RBC population of erythroblasts and reticulocytes, expressing HLA Class I antigens, plays a role. In addition, mature RBCs from both the fetus and the IUT donor express low levels of HLA Class I antigens and may contribute to immunization.

Third, to unravel a possible relationship with high responsiveness we studied the association between HLA antibodies in D– individuals who produced strong antibodies against the D antigen as well as the occurrence in women who produced one or more additional RBC antibodies after IUT. Although the antibody specificity primarily responsible for HDFN was not associated with HLA immunization, women with higher maximum anti-D titers and who had formed additional RBC antibodies after IUT treatment, indications of hyperseroresponsiveness and multiresponsiveness, showed a higher risk for HLA alloimmunization.

A previous study showed that the number of amino acid triplet mismatches between mother and child were associated with Class I antibody formation shortly after

delivery.[26] In the current study, the same correlation between the number of triplet mismatches and HLA antibodies was observed years after HDFN complicated pregnancy. Assessing the relative immunogenicity of specific mismatches revealed five triplet mismatches with significantly higher antibody formation rates than others. All these triplets corresponded with known higher immunogenic HLA specificities: Bw4, A1/A9, and the "R" complex consisting of HLA-B35, -B51, -B52, -B53, -B18, and -B78. Clinical outcomes underline the possible higher immunogenicity of certain HLA triplets: HLA-Bw4 and its correlation with HLA DR has shown to be prevalent in many antibody formers,[38] HLA A1/A9 is a well known cross reacting antigen combination,[39,40] and the R-complex is a broad cross-reacting group.[41,42] The triplet mismatches for HLA-C alleles were shown to be less immunogenic, confirming observations in mismatched kidney transplantation[43] probably because of the low expression on HLA-C on the cell surface.[43,44]

In a subgroup of women, the persistence of HLA antibodies could be analyzed and it was revealed that all antibodies directed against fetal antigens expressed by the child with HDFN had persisted for at least 10 years, whereas other HLA antibodies more often disappeared, a situation also described for RBC antibodies.[9,10,45-49] Factors involved in the antibody persistence may be fetal chimerism, environmental antigen exposure, and lack of formation of anti-idiotypic antibodies against anti-HLA.[50,51] Since we had only the HLA type of the IUT-treated children and not from their father, siblings, and blood donors, the exact other HLA-immunizing events could not be defined. Persisting antibodies not directed against antigens of the IUT-treated fetus might therefore have been induced by a sibling and could be fetus-induced antibodies as well. The fact that all antibodies against the child had persisted for 10 years pleas for the larger FMH possibly associated with establishment of fetal chimerism. It would therefore be of interest to investigate fetal chimerism levels in women who have undergone IUT treatment. Although this is one of the largest follow-up studies ever conducted on HLA antibody prevalence and persistency after RBC antibody complicated pregnancy, for some subanalyses the numbers were relatively low, which makes it difficult to form definitive conclusions for, for example, HLA antibody persistency and specific triplet mismatch immunogenicity.

In conclusion, this study showed that women whose fetuses were treated with IUT for HDFN had a high risk of developing and maintaining antibodies against fetal HLA Class I antigens. A high (anti-D) antibody titer during pregnancy together with new RBC antibody formation after IUT treatment were associated with a higher HLA immunization rate, suggesting a relationship between hyperseroresponders and multiresponders against RBC antigens and HLA immunization. The induction of persisting HLA Class I antibodies against fetal antigens is likely related to the greater FMH during IUT and the immunogenicity of HLA mismatches between mother and child and less to the number of pregnancies.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest relevant to the manuscript submitted to **TRANSFUSION**.

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