ABO incompatibility and RhIG immunoprophylaxis protect against non-D alloimmunization by pregnancy

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BACKGROUND: Hemolytic disease of the fetus and newborn (HDFN) is caused by maternal antibodies against fetal red blood cell antigens, most often anti-D, -K, or -c. ABO incompatibility between mother and child and anti-D immunoprophylaxis (RhIG) are known to reduce the risk of D immunization and subsequent HDFN. However, no immunoprophylaxis has been developed to prevent non-D immunizations.

STUDY DESIGN AND METHODS: We evaluated whether ABO incompatibility has a preventive effect on formation of non-D alloantibodies, by performing a case-control study including pregnant women with newly detected non-D antibodies, identified within a nationwide data set, immunized during their first pregnancy and/or delivery. Subsequently, we assessed a possible protective effect of RhIG in a subgroup with non-Rh antibodies only. The proportions of previous ABO incompatibility and of RhIG administrations of these women were compared to the known rate of 19.4% ABO incompatibility and 9.9% RhIG administrations (D– women carrying a D+ child) in the general population of pregnant women.

RESULTS: A total of 11.9% of the 232 included immunized women had a possible ABO incompatibility in their first pregnancy (vs. expected 19.4%; 95% confidence interval [CI], 7.3-18.8; $p = 0.036$). Furthermore, 1.0% women with non-Rh antibodies were D–, delivered a D+ child, and had therefore received RhIG, whereas 9.9% was expected (95% CI, 0.18-5.50; $p = 0.003$).

CONCLUSION: We found that ABO incompatibility and RhIG reduce the risks not only for D, but also for non-Rh immunizations, suggesting that antibody-mediated immune suppression in this condition is not antigen specific.

Hemolytic disease of the fetus and newborn (HDFN) is a serious pregnancy complication, caused by maternal antibodies against fetal red blood cell (RBC) antigens. These antibodies may provoke fetal hemolysis, resulting in fetal anemia, hydrops, and even death if left untreated.[1,2] HDFN is most frequently caused by antibodies with anti-D specificity, followed by anti-K, anti-c, anti-E, other Rh antibodies, or exceptionally, anti-Fy (Duffy) or anti-Jk (Kidd).[1-4]

Already in 1943, Levine[5] made the pivotal observation that ABO incompatibility occurred less in patients with D immunization during pregnancy compared to couples

ABBREVIATION: HDFN = hemolytic disease of the fetus and newborn.

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without D immunization, indicating a preventive effect of ABO incompatibility on the formation of D antibodies. This observation was confirmed by others, of which Nevanlinna and Vainio[6] most widely studied the effect of mother–child ABO incompatibility on D immunization. These observations eventually led to the hypothesis that the development of anti-D immunoglobulin prophylaxis (RhIG) could prevent D immunization.[7]

Indeed, postnatal prophylaxis with RhIG, introduced in the 1960s, and additional antenatal prophylaxis in the 1990s, have drastically reduced the risk for D immunizations by pregnancy or birth.[8] As a consequence, RhIG is a very effective measure to prevent D immunizations. Several possible pathways have been hypothesized and thoroughly studied in the past decades, although the exact mechanisms of action of RhIG still remain unclear.[9-13]

Clinically relevant RBC alloantibodies directed against other RBC antigens (non-D RBC alloantibodies), in the absence of D antibodies, were found at screening in the first trimester of pregnancy in 0.33% of all pregnancies in the Netherlands between 2002 and 2004.[3] As mentioned, non-D antibodies might also cause HDFN, although to a lesser extent than anti-D.[3] To prevent non-D alloimmunization, women of reproductive age (<45 years) in need for RBC transfusions receive K-matched (from 2004 onward) and c- and E-matched (2011 onward) blood units in the Netherlands.[14] So far, no immunoprophylaxis has been developed to prevent non-D alloimmunization, although the clinical relevance of implementing anti-KEL[15] and anti-HPA-1a[16] immunoglobulin have been investigated in murine models.

It is not known whether the immunization against non-D RBC antigens might be preventable by administration of an immunoprophylaxis, like in D immunization. Therefore, we first assessed whether ABO mismatch in pregnancy also reduces the risk of immunization toward non-D RBC antigens. Subsequently, we investigated if the administration of RhIG to D– mothers protects for alloimmunization against non-Rh antigens.

MATERIALS AND METHODS

Study design

We performed a case-control study, comparing pregnant women with one previous delivery and non-D alloantibodies detected at first trimester screening that most likely were immunized by RBC antigens of their first child (cases), to the Dutch population of pregnant women (control population).

Study population

Previously, all women with non-D alloantibodies, but no D antibodies, found at first trimester screening in the Netherlands between September 1, 2002 and June 1, 2003, and between October 1, 2003 and July 1, 2004, were included in the prospective OPZI (Opsporing en Preventie Zwangerschapsimmunisatie/Detection and Prevention of Pregnancy Immunisation) study.[3] These cases were identified at Sanquin Diagnostics, the Dutch national reference laboratory, or BIBO (Bijzonder Instituut voor Bloedgroepen Onderzoek/Special Institute for Blood Group Investigation), where the specificity of all RBC alloantibodies found at first-trimester screening in regional laboratories is determined. All women with non-D alloantibodies from the OPZI study were initially included in this study as cases. To facilitate subgroup analyses of different antibody specificities, additionally, women with newly detected anti-E, anti-K, anti-Fy, or anti-Jk, and without D antibodies, identified at the laboratory of Sanquin Diagnostics between July 2012 and September 2015 and between January and September 2016, were included. Subsequently, to compose a group of women that was most likely immunized by one previous pregnancy or delivery, multiparous or nulliparous women were excluded, as well as women with blood transfusions after a negative antibody screen in their previous pregnancy and women with partners negative for the antigen against which the maternal antibodies were directed. The likelihood that part of the population was not immunized by their previous delivery, but by a miscarriage or abortion in between was considered nihil, as we previously found that these factors are not associated with an increased risk of alloimmunization.[4]

Cases were compared to the general pregnant Dutch population. If ABO incompatibility or RhIG administration would have a protective effect on any type of immunization, this would be indicated by a low incidence of ABO incompatibility or RhIG administrations in our case group compared to the general population. Therefore, we compared the probability of ABO incompatibility of the cases with the calculated proportion in the general population, based on the distribution of AB antigens in a Caucasian population.[17] Second, the proportion of cases that previously received RhIG was compared to the proportion of D– women with D+ fetuses in the general Caucasian population, assuming a 100% coverage of the national prevention program for pregnancy immunization.[18] We hypothesize that the preventive effect of RhIG on non-Rh immunizations is limited to D+ fetal RBCs and would be less profound or absent in pregnancies of D– women carrying a D– child. Therefore, we considered D– women with D– fetuses, who received untargeted antenatal prophylaxis before the introduction of fetal D typing in maternal blood in 2011, as not having received RhIG.

Data collection

From the OPZI database we collected laboratory data (antibody type; paternal antigen phenotype; blood group

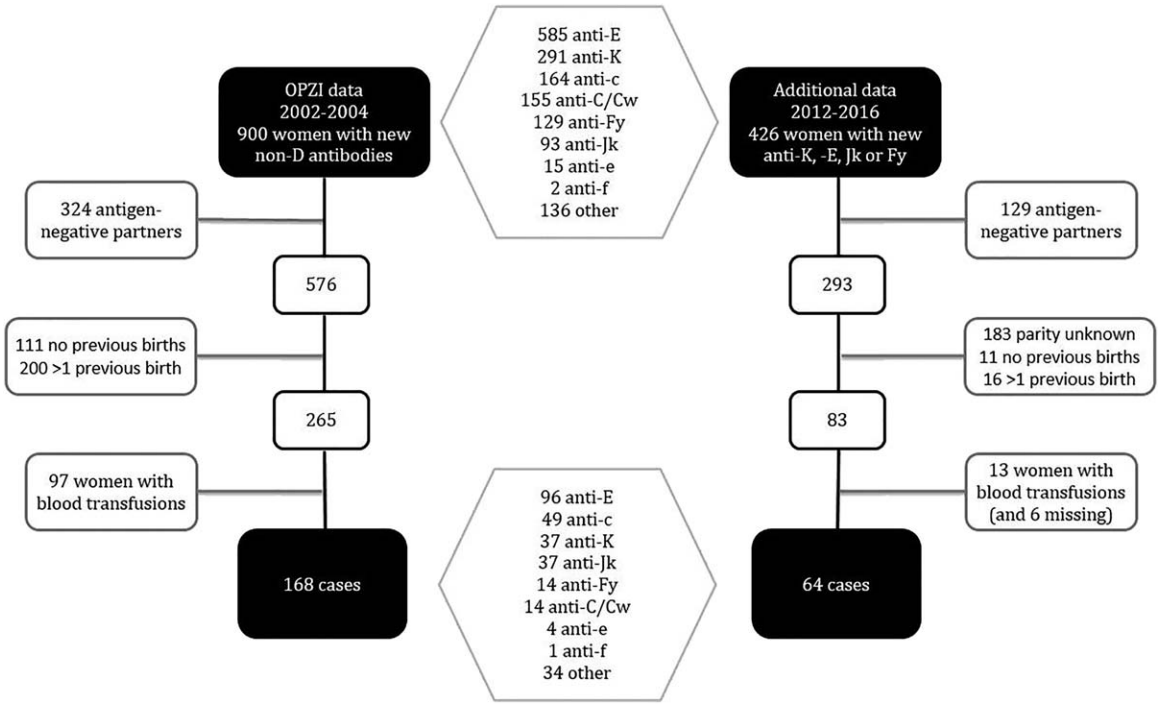

Fig. 1. Selection of cases. The total number of antibodies may differ from 232 as women may have developed more than one antibody.

of mother, father, and second child), data on the obstetric history, and data on blood transfusions after a previous negative antibody screen in the first pregnancy.[3] Laboratory data (antibody type, paternal antigen phenotype, ABO blood group of mother and father) concerning the additional cases were collected from the Sanquin database. After written informed consent from the women, additional clinical data were obtained from the patients' midwife, gynecologist, or general practitioner.

Ethical considerations

As patients were not subjected to additional interventions due to this study, formal ethical approval was not mandatory in the Netherlands and was therefore not obtained. All participants gave informed consent.

Statistical analysis

To compare proportions, 95% confidence intervals (CI) and concordant p values were obtained using the Wilson score, where a p value less than 0.05 was considered significant. The probability of ABO incompatibility in the first pregnancy in cases was estimated twice: 1) based on the ABO blood group of the cases and their partners and, more accurately, 2) based on the ABO blood group of the cases and their partners, as well as the ABO blood group of the children born from the pregnancy with alloantibodies, and compared to population probabilities on incompatibility using the same variables. All calculations are shown in Tables S1 through S6 (available as supporting information in the online version of this paper).

We assessed the comparability of cases and general population in respect to RhIG administrations by comparing the number of D– mothers in both groups. Subsequently, to compare the number of RhIG administrations in the cases and the general population, we planned to analyze two separate subgroups, the Rh (non-D, anti-C/Cw, and anti-E) and non-Rh antibodies, as the risk to develop anti-Rh antibody specificities is dependent on the D phenotype of the mother. Since there is a strong linkage between RHCE (e.g., RHce) and D, almost all D– women are c+ and e+. As a consequence, women who develop anti-c, anti-e, or anti-f are virtually always D+ and never receive RhIG. Therefore, these antibody specificities were excluded in the planned Rh subgroup analyses.

RESULTS

In total, 1326 women with new non-D antibodies were included (Fig. 1). After excluding women in their first ongoing pregnancy or with more than one previous birth, women with an antigen-negative partner, or with a history of RBC transfusion after a negative antibody screen in their previous pregnancy, 232 women remained and were included in the analysis. E antibodies were most frequently found, followed by anti-K, anti-c, anti-C, and anti-

TABLE 1. ABO incompatibility in previous pregnancy per maternal ABO blood group*

Maternal blood group	Probability of incompatible first pregnancy†			
	Cases, n/n‡	Cases, % (95% CI)	Population, %	p value
O	11.7/50	23.34 (13.78-36.70)	30.72	0.26
A	2/54	3.70 (1.02-12.53)	6.09	0.46
B	1.1/12	9.43 (1.83-36.68)	24.60	0.22
AB	0/8	0	0	1
All blood groups	14.8/124	11.94 (7.34-18.81)	19.36	0.04

* Wilson score used for 95% CI and concordant p values.
† Based on combination of ABO blood group of mother, father, and second child. See Tables S3, S4, and S6 for calculations.
‡ Possible number of incompatible cases/total number of cases per blood group with complete ABO data.

Jk. The median maternal age at first alloantibody detection was 32 (range, 19-40) years.

ABO incompatibility

Data on ABO blood group of all women with non-D antibodies were complete and for 201 of 232 partners (Table S5). Based on these data, we determined that the first pregnancy was surely compatible in at least 74.6% of the cases, whereas in the total population this is only in 66.5% ($p = 0.015$). The probability of an ABO-incompatible first pregnancy was 14.1% in cases (28/201) versus 19.4% in the general population (95% CI, 9.9-19.6; $p = 0.058$; Tables S1, S2, and S5). The accuracy to estimate whether the first pregnancy was ABO incompatible was increased by taking the blood group of the second child into account. These data were available for 124 of the 232 cases (Table S6) and this more accurate estimation showed that the first pregnancy had surely been compatible in 79.0% of the cases, compared to 66.5% in the general population ($p = 0.003$; Tables S3, S4, and S6). The probability of an ABO-incompatible first pregnancy in this specific group is shown in Table 1. In total, the first pregnancy might have been ABO incompatible in 11.9% of cases, significantly less than the 19.4% in the Dutch population (95% CI, 7.3-18.8; $p = 0.036$; Tables S3, S4, and S6). The group was too small to calculate a potential difference in protective effect between anti-A and anti-B.

RhIG administrations

In total, four of 232 cases received RhIG in their previous pregnancy and/or after their first delivery. In the general (Caucasian) population, this is 9.9%.[17] One D– woman received untargeted antenatal prophylaxis while carrying a D– child and was therefore considered as not having received RhIG prophylaxis. We planned to analyze Rh and non-Rh specificities separately. We found that, in the subgroup of women with anti-E or anti-C, the proportion of women being D– was far lower than expected (2/83 [2.4%] vs. 22.3% and 1/9 [11.1%] vs. 78.4%, respectively).[17] Therefore, we did not continue the planned separate analysis for Rh antibodies.

TABLE 2. Subgroup analyses of RhIG administrations in 99 cases with non-Rh antibodies compared to the population*

Antibody specificity	Number/proportion of RhIG administrations			
	Cases, n/total	Cases, %†	95% CI	p value
All non-Rh	1/99‡	1.0	0.18-5.50	0.003
Anti-K	1/37	2.7	0.48-13.82	0.14
Anti-Jk	0/37	0	0-9.41	0.04
Anti-Fy	0/14	0	0-21.53	0.21
Other	0/34	0	0-10.15	0.05

* Wilson score used for 95% CI and concordant p values.
† Compared to 9.9%, the calculated probability of D– women carrying a D+ fetus.[17]
‡ The sum of different antibodies may differ from 99 as women may have developed more than one antibody.

In cases with non-Rh antibodies, the percentage of D– women without necessity of RhIG prophylaxis was approximately as expected (5/99, 5.1% vs. 7.0% expected [16.9% D– women of whom 41.2% were carrying a D– child and therefore without an indication for prophylaxis[17]]). Table 2 therefore shows the results of this subgroup of cases with non-Rh antibodies and separate analyses for different non-Rh antibody specificities. Only one of 99 (1%) women with non-Rh antibodies received RhIG in her previous pregnancy and/or after her first delivery, significantly less often than the expected number of 10 women based on calculations for the general population (16.9% D– women of whom 58.8% were carrying a D+ child).

DISCUSSION

In this study, we assessed whether ABO mismatch in pregnancy may reduce the risk of immunization toward non-D RBC antigens. In 232 women with non-D alloantibodies due to their first ongoing pregnancy or delivery, we found a significantly smaller proportion of possible ABO-incompatible first pregnancies in cases than in general population, implicating a preventive effect of ABO incompatibility on non-D antibody formation.

Subsequently, we evaluated whether RhIG also prevents non-Rh immunizations and found that only 1% had

previously received RhIG prophylaxis, whereas approximately 10% was expected. This underrepresentation of D– pregnant women with previous RhIG prophylaxis indicates a possible protective effect of RhIG on formation of non-Rh alloantibodies. These findings also suggest that, in general, for all pregnant women, non-Rh immunizations might be preventable via a mechanism similar to prevention of D immunizations. The prophylactic effect of both ABO mismatch and RhIG is not absolute, as is also not the case for RhIG and ABO incompatibility in D immunization.[6,8,19]

Our finding that ABO incompatibility also protects against non-D immunizations is in line with early studies of Levine, reporting on a protective effect on c and K immunizations.[19,20] Later, Stern[21] also postulated an effect of ABO incompatibility on other types of immunization, although the possible influence of a previous blood transfusion was not completely clear in this study.

The found preventive effect on non-D immunizations may be clinically relevant, as severe HDFN may also be caused by anti-K (prevalence, 1.02/1000), anti-c (0.71/1000 pregnancies), and (rarely) by other Rh and non-Rh antibodies.[3,22] If anti-K or anti-c is present, this can lead to severe HDFN in 26%[3] to 53% of pregnancies with K+ (Y.M. Slootweg et al., unpublished observations) and in 10% of pregnancies with c+ children.[3] It was not possible to determine whether anti-D immunoprophylaxis might prevent c immunizations, as women at risk for development of c antibodies are virtually always D+ and therefore never receive RhIG.

The strength of this study is that we assessed only women with one previous birth and thereby we selected a group of women exposed to approximately the same amount of fetal RBCs. Furthermore, in this matter we effaced the possible immunosuppressive effect of RhIG administrations in pregnancies (ending in miscarriage or termination) before the previous pregnancy. We further specified our cohort by electing women with a high probability of being immunized by their previous pregnancy or delivery, as we excluded women with antigen-negative partners and those with blood transfusions after their first pregnancy or delivery.

We believe that the retrospective study design does not reduce this study's value, as RhIG coverage is more than 98% in the Netherlands[18] and therefore the comparison in RhIG administration between cases and Dutch population could well be made. Moreover, the cases were prospectively collected in the OPZI study. Another strong point is that, although the ABO blood group of the first child and therefore the true proportion of incompatible first pregnancies was unavailable, a distinct approximation could be made as in approximately 50% of cases, the ABO blood group of mother, father, and second child was known.

By not including women who developed D antibodies, part of the D– population (of which a considerable proportion might be "high-responders,"[11] very prone to develop additional antibodies) was excluded. This exclusion did not affect the found preventive effect of RhIG on the development of non-Rh antibodies, as we previously found that in primiparous women with newly detected D antibodies and without a previous blood transfusion, non-Rh antibodies in addition to D antibodies are rarely developed.[23]

However, a limitation of our study is that by not including women with D antibodies, we were not able to evaluate the effectiveness of RhIG in preventing the development of Rh antibodies. This is reflected by the observation that we found barely any D– women with anti-E and anti-C. Because of the linkage disequilibrium between RHD and RHCE alleles, anti-C and anti-E are mainly formed in pregnancies with D+ children. As the D antigen is a more immunogenic antigen than E or C, women in whom RhIG fails will make anti-E/C most likely in addition to anti-D. Possibly, in this manner, RhIG not only protects strongly against D immunizations, but also against anti-E or anti-C.

Furthermore, we are limited to a relatively small sample size in the subgroup analysis with non-Rh antibodies only, to assess a protective effect of RhIG. However, even in this small sample the difference between the expected (10) and observed (1) number of women who previously received RhIG is statistically significant.

Although several studies have previously addressed the possible mechanisms of action of RhIG, the exact mechanism(s) remain unclear.[11-13,24,25] Whereas the antigen masking or steric hindrance hypothesis appears to be the prevailing mechanism in the antibody-mediated immune suppression model with sheep RBCs in mice,[26] this mechanism insufficiently explains RhIG function in humans, based on the low level of opsonization sufficient to exert suppression.[9-11] Furthermore, antigen masking is an antigen-specific mechanism and if this was the main explanation for RhIG function, it would not prevent development of other RBC alloantibody specificities as found in our study. In agreement with our findings, in a mouse model it was shown that antibodies directed against a nonimmunogenic Fy antigen could mediate immune suppression toward the immunogenic antigen (HEL), although in these studies the Fy and HEL were expressed on the same protein (HOD).[27]

Furthermore, the recently postulated antigen-specific "antigen-modulation hypothesis," in which the preventive effect of anti-KEL sera on KEL immunization was attributed to the complete removal or substantial modulation of the KEL antigen, is not in line with our findings.[15,25] A possible explanation to this discrepancy is that antibody responses might function through different mechanisms for different antigens.

The rapid clearance hypothesis, in which macrophages in the red pulp of the spleen rapidly eliminate the

RhIG-coated RBCs before the antigen is noticed by the antigen-presenting cells has recently been widely questioned as a result of uncertainty of Fcγ-receptor involvement in RhIG function.[24,27,28] In general, a major drawback of all described animal studies is that in these models the immune response toward D is not studied, which makes the extrapolation from animal studies to the human setting even more questionable.[29] In this respect it is relevant that our observations as well as the previously observed suppression of anti-D response after administration of anti-K prophylaxis in healthy D–, K– men are in agreement with the rapid clearance hypothesis.[29,30] This mechanism of action is not antigen specific and could therefore also explain the currently found preventive effect of RhIG on the development of non-D antibodies. It should be clear that several mechanisms might act together in immunization prophylaxis.

In conclusion, we found that ABO incompatibility reduces the risk not only for D, but also for non-D immunizations. Furthermore, RhIG was associated with a reduced risk on non-Rh immunizations, both suggesting that antibody-mediated immune suppression is not antigen specific. These findings suggest that non-Rh immunizations might be prevented in a similar way as D immunizations. Therefore, universal prophylaxis against the fetal RBCs to prevent RBC alloimmunization might be achievable.

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CONFLICT OF INTEREST

The authors have disclosed no conflicts of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Table S1. Calculation of ABO incompatibility in general population based on the ABO blood group of mother and father
Table S2. Probability of incompatible pregnancy based on maternal and paternal ABO blood group in general population
Table S3. Calculation of ABO incompatibility in the first pregnancy in general population based on the ABO blood group of mother, father and second child
Table S4. Probability of incompatible first pregnancy based on the ABO blood group of mother, father and second child in general population
Table S5. Calculation of ABO (in)compatibility in cases and general population based on mothers and fathers
Table S6. Calculation of ABO (in)compatibility in first pregnancy of cases and general population based on mothers, fathers and second children