Investigation of whether the acute hemolysis associated with Rh_o(D) immune globulin intravenous (human) administration for treatment of immune thrombocytopenic purpura is consistent with the acute hemolytic transfusion reaction model

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BACKGROUND: Immune thrombocytopenic purpura and secondary thrombocytopenia patients treated with Rh_o(D) immune globulin intravenous (human; anti-D IGIV) have experienced acute hemolysis, which is inconsistent with the typical presentation of extravascular hemolysis—the presumed mechanism of action of anti-D IGIV. Although the mechanism of anti-D-IGIV–associated acute hemolysis has not been established, the onset, signs/symptoms, and complications appear consistent with the intravascular hemolysis of acute hemolytic transfusion reactions (AHTRs). In transfusion medicine, the red blood cell (RBC) antigen-antibody incompatibility(-ies) that precipitate AHTRs can be detected in vitro with compatibility testing. Under the premise that anti-D-IGIV–associated acute hemolysis results from RBC antigen-antibody–mediated complement activation, this study evaluated whether the incompatibility(-ies) could be detected in vitro with a hemolysin assay, which would support the AHTR model as the hemolytic mechanism.

STUDY DESIGN AND METHODS: Seven anti-D IGIV lots were tested to determine the RBC antibody identities in those lots, including four lots that had been implicated in acute hemolytic episodes. Hemolysin assays were performed that tested each of 73 RBC specimens against each lot, including the RBCs of one patient who had experienced acute hemolysis after anti-D IGIV administration.

RESULTS: Only two anti-D IGIV lots contained RBC antibodies beyond those expected. No hemolysis endpoint was observed in any of the hemolysin assays.

CONCLUSION: Although the findings did not support the AHTR model, the results are reported to contribute knowledge about the mechanism of anti-D-IGIV–associated acute hemolysis and to prompt continued investigation into cause(s), prediction, and prevention of this potentially serious adverse event.

ABBREVIATIONS: AHTR(s) = acute hemolytic transfusion reaction(s); anti-D IGIV = Rh_o(D) immune globulin intravenous (human); IRB = institutional review board; ITP = immune thrombocytopenic purpura.

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TRANSFUSION **;**:**-**.

The Food and Drug Administration (FDA) initially licensed Rh_o(D) immune globulin intravenous (human; anti-D IGIV) as a lyophilized formulation (then WinRho, currently WinRho SDF;[1] Cangene Corporation, Winnipeg, Manitoba, Canada) in March 1995 and as a liquid formulation (WinRho SDF Liquid[1]) in March 2005. Both formulations (hereinafter referred to as WinRho unless otherwise noted) are approved for treatment of immune thrombocytopenic purpura (ITP) in Rh_o(D)-positive, nonsplenectomized children with acute ITP, children and adults with chronic ITP, and children and adults with ITP secondary to human immunodeficiency virus (HIV) infection as well as for suppression of Rh isoimmunization.[1] WinRho is also used "off-label" to an unknown extent for treatment of secondary thrombocytopenia.

The presumed mechanism of action of WinRho in ITP involves extravascular hemolysis of anti-D–sensitized red blood cells (RBCs) by splenic macrophages.[1] In patients who respond therapeutically, this mechanism results in decreased splenic sequestration of autoantibody-sensitized platelets (PLTs), which results in an increased PLT count.[1] In what appears contradictory to the presumed extravascular hemolysis mechanism of action and its typical clinical and laboratory findings, two cases of "acute-onset hemoglobinuria consistent with intravascular hemolysis" were observed during the WinRho ITP clinical trials.[2] After licensure, additional reports of acute hemolysis after WinRho administration for ITP or secondary thrombocytopenia were (and continue to be) submitted to the FDA.

Most patients treated with WinRho for ITP or secondary thrombocytopenia do not experience signs/symptoms of acute hemolysis,[1,3-13] and not all who experience signs/symptoms of acute hemolysis experience hemolysis-related complications[14] or require medical intervention for any complications experienced.[13-16] Nonetheless, the acute hemolysis–associated complications that have been reported to date include clinically significant anemia, the need for RBC transfusion(s), acute or exacerbated renal failure, the need for dialysis, disseminated intravascular coagulation, and death secondary to these complications.[14,17] The complications may occur singly or in combination,[14,17] were previously reported in two case series of patients,[14,17] and are listed in the WinRho professional package insert.[1]

If the acute hemolysis that occurs in some patients treated with WinRho for ITP or secondary thrombocytopenia is consistent with the acute hemolytic transfusion reaction (AHTR) mechanism and could be detected in vitro with a hemolysin assay, this assay could conceivably be used to identify patients at risk for acute hemolysis with specific WinRho lots. For such patients, those lots might be contraindicated. The hemolysin assay might also allow identification of WinRho lots that appear to pose no risk of acute hemolysis and could presumably be safely administered.

Such testing could be performed prior to administration of WinRho, borrowing from the historical precedent for the manufacture and distribution of two-vial packaging of an FDA-licensed Rh_o(D) immune globulin for intramuscular administration—one vial for preadministration testing of product and patient RBCs and one vial for subsequent patient administration (RhoGAM, then Ortho Diagnostic Systems, now Ortho-Clinical Diagnostics, Raritan, NJ[18]). Although what prompted the two-vial packaging that was previously used with RhoGAM was unrelated to either acute hemolysis or ITP, we recognized that this packaging precedent could apply to the performance of a hemolysin assay as a screening procedure before administration of anti-D IGIV for treatment of ITP or secondary thrombocytopenia.

We report the results of a hemolysin assay study that we designed to evaluate whether the acute hemolysis associated with WinRho administration for treatment of ITP is consistent with the AHTR model. We also report an additional case of WinRho-associated acute hemolysis in a patient with a history of ITP.

CASE REPORT

A 51-year-old female with ITP secondary to HIV infection and concurrent medical problems that included hepatitis C, liver failure, splenomegaly, and insulin-dependent diabetes mellitus was admitted to the hospital for evaluation of abdominal pain and thrombocytopenia. She had been treated successfully for ITP with steroids in the past. However, due to concern for immunosuppression, among other steroid-related side effects, her hematologist elected to treat her ITP with WinRho SDF. She received 27.7 μg per kg WinRho SDF (Lot 0240501), and within 1 hour of administration, she experienced severe rigors, back pain, headache, and chills. She developed hemoglobinuria, as evidenced by "tea-colored" urine that was collected the following day and yielded a urine reagent strip reading of "large" blood and a microscopic examination showing 0 RBCs per high-power field. She subsequently developed acute renal failure, presumed secondary to hemoglobinuria-induced acute tubular necrosis. Her serum creatinine level peaked at 2.7 mg per dL 2 days after WinRho SDF, increased from a baseline of 0.9 mg per dL; however, she did not undergo dialysis. By 3 days after WinRho SDF, her hemoglobin (Hb) level had decreased 3.7 mg per dL, from a baseline of 11.2 to 7.5 g per dL, and she was transfused with 1 unit of RBCs 6 days after WinRho SDF. She was subsequently discharged from the hospital 12 days later in stable condition without apparent sequelae.

Pre–WinRho SDF testing revealed that her RBC phenotype was A; D+C+E–c–e+; K–; Jk(a–b+); Fy(a+b+);

M–N+S+s+; Le(a+); P1+. She had a negative direct antiglobulin test (DAT) but a positive indirect antiglobulin test (IAT) that revealed anti-c and anti-M due to alloimmunization from previous blood transfusions. Four hours after WinRho SDF, her DAT was 3+ positive for anti-immunoglobulin G (IgG) but negative for anti-C3; an acid eluate revealed anti-D. Her IAT remained unchanged when tested against c– and M– RBCs. At 4 months after WinRho SDF, her DAT remained weakly positive for anti-IgG but negative for anti-C3, although no eluate was performed. Her IAT test results again revealed anti-c and anti-M. Also after WinRho SDF, her RBCs were genotyped and tested for Rh antigen density. The results of that testing revealed her genotype as R_1R_1 (DCe/DCe) and her antigen density "as expected for a [D antigen] homozygote."

MATERIALS AND METHODS

Institutional review board clearances

The National Institutes of Health Institutional Review Board (IRB) approved the testing of blood specimens from normal blood donors for this study. The Beth Israel Deaconess Medical Center IRB approved the testing of blood specimens obtained from the patient who experienced acute hemolysis after administration of WinRho SDF. The patient gave verbal consent, in accordance with local IRB policy, for blood specimens to be collected for this study.

Testing procedures

Overview

This study consisted of two separate sets of testing procedures. We tested each of the seven WinRho lots for RBC antibodies. We then performed hemolysin assays in which we tested each of those WinRho lots against each of 73 RBCs (i.e., 511 hemolysin assays).

WinRho lots tested

We sought to include both WinRho lots that had been implicated in cases of acute hemolysis and those that had not been reported as having been associated with acute hemolysis. We tested seven lots of WinRho for RBC antibodies, which consisted of four purposively selected lots that had been implicated in three separate cases of acute hemolysis (one patient received a dose pooled from two lots) and three nonimplicated lots that were selected by convenience sampling (i.e., availability). WinRho SDF lots were 0240501, 1460402, 1460501, 1470403, and 1480410; WinRho SDF Liquid lots were 0110501 and 0120502.

Three of the WinRho SDF lots had passed their expiration dates at the time of testing. Implicated Lot 1480410 was 1 month beyond its dating period; nonimplicated Lots 1460402 and 1470403 were 7 and 8 months beyond their dating periods, respectively. All of the lots beyond their expiration dates were lyophilized and would not have been expected to exhibit significant declines in antibody titers or integrity between the expiration and testing dates.[19,20] The remaining WinRho SDF lots were within their dating periods, as were both WinRho SDF Liquid lots included in this study.

WinRho is not equivalent to serum, and we had no frame of reference for determining what WinRho dilutions should be used in this study, other than wanting to replicate the in vivo environment (i.e., "physiologic" conditions) as much as possible in the hemolysin assays. If RBCs were hemolyzed by WinRho under conditions that reflected physiologic conditions, we believed that this finding could provide important clues as to the mechanism of the acute hemolysis that occurs in vivo.

WinRho SDF lots were reconstituted with the manufacturer-supplied diluent according to the professional package insert instructions before testing. WinRho SDF Liquid lots were tested as distributed. All lots were subsequently diluted with phosphate-buffered saline (PBS; Cellgro, Mediatech, Inc., Herndon, VA) to both 1-in-10 and "physiologic" dilutions. The 1-in-10 dilution was tested to detect low-titer RBC antibodies that might be diluted beyond the limits of detection in the physiologic dilutions. Physiologic dilution was defined and calculated to approximate the in vivo dilution of a recommended initial 50 μg per kg dose of WinRho in the blood volume of an average adult patient. We chose to test a physiologic dilution under the assumption that this dilution would approximate the RBC antibody titers at which acute hemolysis occurs in vivo after WinRho administration. The physiologic dilutions of WinRho lots ranged from 1 in 40 to 1 in 100, depending on the labeled international unit (IU) dosage of anti-D in the vials.

All lots were tested for RBC antibodies by an IAT method using column agglutination (MTS anti-IgG, Micro Tying Systems, Inc., Pompano Beach, FL) and a tube technique using polyethylene glycol additive (PeG, Immucor Gamma, Gamma Biologicals, Inc., Houston, TX) and anti-IgG anti-human globulin (Ortho-Clinical Diagnostics). Tests to differentiate anti-G from anti-D and anti-C were not performed.

RBCs tested

We sought to include RBCs that varied in both ABO blood group and Rh antigen phenotypes. The rationale was that patients who have experienced acute hemolysis after WinRho administration have ranged across ABO blood groups and Rh phenotypes. We used 73 RBC specimens in our study, which included RBCs from normal blood donors (n = 40), commercially available RBC reagents (n = 32), and RBCs from the patient who experienced acute hemolysis after WinRho SDF administration (n = 1)

and from patient "facsimiles" (n = 4, from among the 40 normal blood donor and 32 reagent specimens).

The normal blood donor RBCs were selected by convenience sampling, which we presumed would yield a range of both ABO blood groups and Rh phenotypes/genotypes. The reagent RBCs were selected by purposive sampling to provide an array of Rh phenotypes/genotypes that we presumed corresponded to different D and other Rh antigenic densities,[21,22] which has been proposed as a potential factor in whether acute hemolysis occurs after administration of WinRho.[13,21-25] The patient facsimiles were selected by purposive sampling to express the equivalent non-ABO RBC antigen–positive and antigen-negative phenotype as the patient. Two facsimiles were of the same ABO blood group as the patient; two were of another ABO blood group.

The normal blood donor specimens were collected in ethylenediaminetetraacetate (EDTA) and tested within 7 days of collection. The ABO blood groupings and Rh typings for these specimens were excerpted from blood donor records. The reagent RBCs (Immucor, Inc., Norcross, GA; and Ortho-Clinical Diagnostics, Inc.) were washed with pH 7.4 PBS before testing but were otherwise tested as supplied by the manufacturers. If the Rh genotype of a reagent RBC was not specifically noted by the manufacturer, we assigned the most probable genotype based on the reported Rh phenotype.

We deliberately opted not to use enzyme-treated RBCs in the hemolysin assay to more closely approximate in vivo conditions. Had we tested enzyme-treated RBCs, we would have been unsure that we could generalize our findings to physiologic conditions. Furthermore, although we realized that using enzyme-treated RBCs may have increased the sensitivity of the hemolysin assay, that was not the purpose of this study.

The ABO blood groups of the normal blood donor and reagent RBCs were as follows: O (n = 32), A (n = 27), and B (n = 13). The Rh types of these RBCs included the following distribution: R_1R_1 (DCe/DCe; n = 16), $R_1^wR_1$ (DC^we/DCe; n = 3), R_2R_2 (DcE/DcE; n = 15), R_1R_2 (DCe/DcE; n = 7), R_1r (DCe/dce; n = 16), rr (dce/dce; n = 3), R_oR_o (Dce/Dce; n = 6), R_2r'' (DcE/dcE; n = 1), R_1R_z (DCe/DCE; n = 2), R_2R_z (DcE/DCE; n = 2), and $R=NR_2$ (D(C)(e)/DcE; n = 1).

For the patient who experienced acute hemolysis, we excerpted the RBC phenotyping, DAT, and IAT results of her pre-, 4-hour post-, and 4-month post–WinRho SDF specimens from her medical records. Another set of 4-month post–WinRho SDF blood specimens was collected for this study. These specimens were collected in EDTA or without anticoagulant, respectively, and were tested within 7 days of collection. The 4-month post–WinRho SDF time frame was selected under the presumption that this blood specimen would be representative of her indigenous RBC population, without the potentially confounding influence of residual transfused RBCs.

Hemolysin assay

We used a standardized hemolysin assay[26] that is designed to detect the presence of IgM and IgG RBC antibodies that are capable of binding complement and initiating complement-dependent hemolysis. We used this assay for testing each of the seven WinRho lots against each of the 73 normal blood donor, reagent, patient, and patient facsimile RBCs. RBCs were tested as 10 to 20 percent concentrations suspended in 0.9 percent (wt/vol) isotonic saline (Fisher Diagnostics, Fisher Scientific Co., Middletown, VA). We performed the assay at what approximated an in vivo physiologic dilution by diluting the WinRho lots in fresh Group AB serum according to the following calculation:

(50 μg WinRho/1 kg [recommended initial dose of WinRho]) × (1 kg/40 mL [plasma volume of an average adult]) × (mL/[IUs of anti-D per vial]).

We selected fresh group AB serum as the test system diluent because it lacked confounding ABO blood group antibodies and provided a source of complement required for complement-dependent antigen-antibody–mediated hemolysis. We collected the group AB serum as white blood cells (WBCs) and plasma by an apheresis procedure using anticoagulant citrate-dextrose solution Formula A. The WBC product was issued for other research purposes, and the plasma was centrifuged at 4900 × *g* for 6.5 minutes shortly after collection, 100 mL of which was transferred to a 600-mL bag. Approximately 100 μL of thrombin and 1 mL of 10 percent $CaCl_2$ were added to the plasma; the bag was mixed vigorously and placed into a 2 to 8°C monitored refrigerator overnight. The bag was centrifuged the next day at 4900 × *g* for 6.5 minutes and the supernatant (serum) was transferred to a 300-mL bag. Serum was then aliquoted into 2-mL vials and placed into a monitored –30°C freezer. Serum was kept at –30°C until the day of testing, when it was removed from the freezer, thawed at 20 to 24°C, and used in the test system shortly after thawing.

In performing the hemolysis assays, we added 1 drop of the RBC suspension to 200 μL of WinRho diluted in group AB serum. We mixed and incubated the tubes for 1 hour at 37°C and then mixed, centrifuged at 1000 RCF (1000 × *g*) for 2 minutes, and examined the tubes for the hemolysin assay endpoint of macroscopically visible supernatant hemolysis.[26] We suspended the RBCs for both the positive and the negative controls in group AB serum and followed the mixing, incubation, mixing, centrifugation, and examination procedure that was used with the hemolysin assays. For a positive control, we used group O penicillin-coated RBCs, coated according to a standardized procedure,[26] and an IgG anti-penicillin. The anti-penicillin was well-characterized donor plasma that had demonstrated 3+ agglutination with penicillin-coated

TABLE 1. RBC antibody identities in WinRho lots tested

WinRho lots tested		RBC antibodies	
WinRho lot	Expiration date	1-in-10 dilution	Physiologic dilution
0110501	July 2007	Anti-D, -C, -Js[a]*	Anti-D, -C
0120502	September 2007	Anti-D, -C, -A, -B, -Jk[a]	Anti-D, -C, -A, -B
0240501	January 2008	Anti-D, -C, -A	Anti-D, -C
1460402	April 2006	Anti-D, -C	Anti-D, -C
1460501	March 2007	Anti-D, -C, -A	Anti-D
1470403	February 2006	Anti-D, -C, -A, -B	Anti-D, -A
1480410	November 2006	Anti-D, -C	Anti-D, -C

* Antibody could not be ruled out.

RBCs in previous testing and hemolyzed penicillin-coated RBCs when a source of complement was added (see above). For a negative control, we used group O unsensitized RBCs.

RESULTS

RBC antibody identities in WinRho lots

In Table 1, we list the RBC antibodies identified in the WinRho lots. We confirmed the presence of anti-D in all lots at both the 1-in-10 and the physiologic dilutions. In five lots, we detected other RBC antibodies in the 1-in-10 dilutions that were not detected in the physiologic dilutions. However, in only two lots did we detect RBC antibodies other than the expected anti-D, anti-A, anti-B, anti-C, and anti-E,[1] and we detected those other RBC antibodies in only the 1-in-10 dilutions.

Hemolysin assays

We observed no hemolysis endpoints with any of the hemolysin assays in which each of the seven WinRho lots was tested against each of the 73 normal blood donor, reagent, patient, and patient facsimile RBCs. We observed a hemolysis endpoint in the positive control and no hemolysis endpoint in the negative control. Because the positive control showed a hemolysis endpoint, we concluded that the test system indirectly validated that there were adequate amounts of complement and calcium ions in our converted plasma/complement source and that the anti-penicillin was not diluted beyond the limits of detection of our hemolysin assay. The negative control yielded the expected result, which we interpreted as further validation of our test system.

DISCUSSION

Mechanism of acute hemolysis in AHTRs compared to anti-D IGIV

Both the presumed mechanism of action of WinRho in the treatment of ITP and the established mechanism of AHTRs involve RBC antigen-antibody–mediated hemolysis. In most ITP patients treated with WinRho, the "expected" hemolysis is presumed attributable to IgG anti-D,[1] which typically causes extravascular hemolysis[23,27,28] in D+ patients. In both textbooks and the literature, extravascular hemolysis has traditionally been characterized by delayed onset, indirect hyperbilirubinemia, and urobilinogenuria and seldom results in morbidity or clinical sequelae.[23,26,27,29,30] In contrast, the hemolysis in AHTRs is generally due to IgM anti-A or anti-B and typically results in complement-mediated intravascular hemolysis in correspondingly antigen-positive patients.[23,26-30] That hemolysis is generally characterized by acute onset, hemoglobinemia (and hemoglobinuria), and potentially life-threatening complications of shock, renal failure, and disseminated intravascular coagulation.[23,26,27,29,30]

The mechanism of acute hemolysis associated with WinRho administration has not yet been definitively established as "intravascular hemolysis" in terms of RBC antigen-antibody–mediated complement activation, or any other mechanism(s) of hemolysis,[1,14,17] and remains unexplained. If attributable to anti-D, the mechanism of the acute hemolysis is perplexing, given the generally non–complement-fixing behavior of anti-D;[23,28-30] the relatively limited number of D antigens per RBC;[23,24] the distance between these antigens, which exceeds the span of IgG anti-D;[23,24] and the lack of complement activators in WinRho.[2,19]

However, WinRho contains multiple RBC antibodies[1,31] that, at least in theory, could collectively sensitize a critical mass of RBC antigens in correspondingly antigen-positive patients, precipitate RBC antigen-antibody–mediated complement activation, and present with the time of onset, signs/symptoms, and complications of AHTRs.[14,17,28] RBC antibodies contained in WinRho include high-titer IgG anti-D as well as low-titer IgG anti-A, anti-B, anti-C, and anti-E, all of which can be passively acquired.[1] The manufacturer quantitatively assays these antibodies before release of lots for market distribution to ensure compliance with FDA-recommended specifications. WinRho may also contain other low-titer RBC IgG antibodies (e.g., anti-Fy[a], anti-Jk[a]),[31] some of which have been implicated in AHTRs.[23,27,29,32] These other RBC antibodies exhibit lot-to-lot variability in identities and titers[31] and can likewise be passively acquired,[31] but are not routinely assayed before lot release for market distribution.

Thus, the complete inventory of RBC antibody identities and titers in WinRho lots in market distribution is unknown, as is the extent of variation among lots. Given the diversity in patient RBC antigen phenotypes, the

potential lot-to-lot variability in RBC antibody identities and titers could explain why only some patients experience acute hemolysis with seemingly random WinRho lots.[14] Similarly, the potential lot-to-lot variability could explain why a given patient experiences an uneventful administration with a WinRho lot yet experiences acute hemolysis with another WinRho lot administered on another occasion.[14,17]

Explanations for the negative results of the study

The negative result for the hemolysin assay that was performed with RBCs from the patient who experienced acute hemolysis and the WinRho SDF lot administered to her did not support the premise that her acute hemolysis was consistent with the AHTR model. If, however, the AHTR model is the mechanism of the WinRho-related acute hemolysis, future research could consider other variables that were beyond the scope of, and not addressed in, this study. Other technical variables could include modifications of the hemolysin assay procedure used in this study (e.g., other dilutions of the WinRho lots, use of enzyme-treated RBCs) or use of another procedure. Although few of the case series patients who experienced WinRho-associated acute hemolysis had been recently transfused, we cannot rule out that recent transfusion history may account for the acute hemolysis of some patients. Recent transfusion with ABO-incompatible plasma from PLTs[22,30,33] could sensitize patient RBCs with IgG and/or complement,[26,33,34] which, in conjunction with passively acquired RBC antibodies from WinRho,[23] might initiate RBC antigen-antibody–mediated complement activation.

If the AHTR model is not the mechanism of the WinRho-related acute hemolysis, future research could investigate other immune-mediated and alternative mechanisms of hemolysis. The role of complement regulatory proteins CD55 (decay accelerating factor), CD59, and complement receptor type 1 (CR1, CD35) that may inactivate complement on RBCs and prevent complement-mediated hemolysis[23,26,33-36] might warrant further evaluation. Although not often cited in textbooks and the literature, extravascular hemolysis has been associated with hemoglobinemia and hemoglobinuria in what has been termed an "acute extravascular hemolytic transfusion reaction." [23,26,30] By this mechanism, the acute hemolysis of anti-D–sensitized RBCs is due to splenic macrophage lysozymes and/or fragmentation of anti-D–sensitized RBCs during phagocytosis by splenic macrophages with release of free Hb into the circulation.[23,26,30] Another potential immune-mediated mechanism of RBC hemolysis that could be considered involves cell-mediated immune hemolysis by "armed macrophages," in which bound antibody reacts with nonsensitized RBCs that possess the corresponding antigen and leads to more extensive hemolysis than would be seen with untreated macrophages alone.[26,37,38] Finally, the possibility of circulating lymphocyte-mediated cytotoxicity cannot be excluded as a cause of, or contributory factor to, the acute hemolysis.[26,39]

Regardless of the mechanism(s) by which acute hemolysis may occur after WinRho administration, other patient characteristics (e.g., degree of splenomegaly) beyond those previously examined[14,15,17,40-48] could be evaluated as potential predictors of, and factors related to, the acute hemolysis. In terms of other characteristics of WinRho composition, environmental conditions of WinRho storage, or anomalies of WinRho reconstitution or administration, we assess as unlikely that these could be implicated as causative factors in the acute hemolysis in the absence of lot-related clusters of cases.

Limitations of this study

We acknowledge several notable limitations of this study. We are unaware of any reference(s) or data that cite or discuss the sensitivity, specificity, or predictive power of the hemolysin assay used in this study for detecting RBC antigen-antibody–mediated complement activation. Neither can we judge the predictive power of the hemolysin assay as an in vitro corollary to in vivo physiologic conditions. We recognize that anti-D-IGIV–associated acute hemolysis could, in fact, model the AHTR mechanism but that the hemolysin assay used in this study did not possess the power to test our premise. We tested only 1-in-10 and physiologic dilutions of WinRho. Although other dilutions of WinRho could have been included, we elected to test only the 1-in-10 and physiologic dilutions, given that the purpose of our study was to determine the mechanism by which WinRho causes acute hemolysis, not if WinRho could be made to hemolyze RBCs.

We tested only seven WinRho lots for RBC antibody identities (although four of those lots had been implicated in cases of acute hemolysis). We have no means to assess how representative the RBC antibody identities and titers of those lots were when compared to other WinRho lots. We tested only 73 RBCs in the hemolysin assays, 40 (55%) of which were from normal blood donors, who were otherwise healthy, and 32 (44%) of which were reagent RBCs. Whether there were relevant qualitative differences between these RBCs and the RBCs of ITP or secondary thrombocytopenic patients is indeterminate. If the AHTR model is applicable, but given the low estimated reporting rates for WinRho-associated acute hemolysis,[14,17] we would have needed to test an impracticably large number of normal blood donor and reagent RBCs to randomly encounter an RBC phenotype/genotype and WinRho lot RBC antibody combination that would have yielded a hemolysis endpoint in our hemolysin assay.

We were able to test RBCs from only a single patient who experienced acute hemolysis after WinRho SDF administration for ITP. Had it been feasible, we would have elected to test RBCs from the patient that had been collected immediately before and/or at the time of her acute hemolytic episode. We cannot be certain whether relevant qualitative differences existed in her RBCs at the time that she received WinRho SDF and 4 months afterward. In fact, there was a qualitative difference in that her pre-WinRho SDF DAT was negative while her 4-month post–WinRho SDF DAT was weakly positive (presumably due to anti-D but not confirmed). However, the significance, if any, of that difference cannot be assessed. Neither can we be certain whether there were relevant qualitative differences between her RBCs and the patient facsimile RBCs tested. Of note, the WinRho SDF lot implicated in her acute hemolytic episode contained no other RBC antibodies beyond those expected to be present. Also of note, she had experienced acute hemolysis post-WinRho SDF, yet her RBCs were only of an antigen density "as expected for a [D antigen] homozygote."

The other two patients who had experienced acute hemolysis after administration of the other purposively sampled WinRho lots had died. Consequently, we were unable to obtain blood samples from those patients for testing in this study or locate RBC phenotype data in their medical records that would have allowed for patient facsimile testing. Testing of RBCs from other patients who have experienced acute hemolysis post-WinRho administration may be informative in future research.

We tested only WinRho because it was the only anti-D IGIV that was FDA-licensed for treatment of ITP at the time that this study was initiated. However, cases of acute hemolysis involving other brands of anti-D IGIVs administered for treatment of ITP or secondary thrombocytopenia have been reported in clinical studies[49] and in the literature.[15,43,50] After we initiated our study, the FDA licensed Rhophylac (ZLB Bioplasma AG, Bern, Switzerland) in February 2004 for suppression of Rh isoimmunization[49] and in March 2007 for treatment of chronic ITP in Rh_o(D)-positive, nonsplenectomized adults.[49] Other anti-D IGIVs that are produced by other manufacturers are licensed in other countries. Future research into the mechanism of the anti-D-IGIV–associated acute hemolysis should include other brands of anti-D IGIV as well.

Summary

Based on the negative results obtained with the hemolysin assay used for this study, we doubt that the acute hemolysis after WinRho SDF administration in the patient whose RBCs were tested is consistent with the AHTR model. Just as more than one mechanism may account for PLT responses in anti-D IGIV–treated ITP patients, multiple mechanisms may contribute to anti-D-IGIV–associated acute hemolysis and could account for the acute hemolysis experienced by the patient whose RBCs were tested as well as for differences among patients. Understanding the mechanism(s) of the acute hemolysis might allow us to develop contraindications or strategies for anti-D IGIV treatment of ITP and secondary thrombocytopenia patients that could minimize the risk of acute hemolysis–associated morbidity and mortality. Any and all potential causes or mechanisms of anti-D-IGIV–associated acute hemolysis warrant further evaluation with targeted studies unless and until other patient risk factors or predictive tests for the acute hemolysis can be identified.

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