Rh immune globulin: an interfering substance in compatibility testing

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Immunoglobulin therapy that interferes with pretransfusion testing may complicate the interpretation of test results and adversely affect patient management. Rh immune globulin (RhIG) should be considered an interfering immunoglobulin therapy when it is detected in an antibody detection test of a sample from a patient who has been treated with RhIG. Frequently, detection occurs in mother’s or newborn’s plasma. Because an antenatal injection of RhIG is indicated for pregnant Rh-negative women, anti-D is detected frequently by today’s highly sensitive antibody screen methods when the mother’s plasma is tested subsequently at delivery. Ascertainment of the source of anti-D is complicated by the inability of routine clinical laboratory methods to distinguish anti-D due to RhIG from alloimmune anti-D. A combination of qualitative and quantitative test methods, as well as a complete clinical history, is necessary for accurate diagnosis and patient management. Immunohematology 2019:35:51–60.

Key Words: RhIG, passive, immune, titration, antenatal, ITP

Introduction

Rh immune globulin (RhIG) is administered routinely to Rh-negative,* pregnant women to prevent hemolytic disease of the fetus and newborn (HDFN), to Rh-negative patients who have received Rh-positive blood components, and to patients as a treatment for immune thrombocytopenic purpura (ITP). The result of RhIG administration may be the presence of anti-D in the plasma of antepartum and postpartum Rh-negative women, occasionally in their newborns, and in patients with ITP. The number of therapeutic indications for RhIG has expanded over the more than 50 years since RhIG was introduced in clinical practice. Additionally, the sensitivity of laboratory methods for the detection of blood group antibodies has increased. The likelihood of encountering interference from RhIG has increased significantly, often creating additional work both serologically and administratively. This review will cover three topics related to RhIG: how RhIG is manufactured, clinical aspects of RhIG in therapeutic use, and the impact of RhIG on serologic testing.

Manufacture of Rh Immune Globulin

The manufacturing processes used to prepare RhIG have evolved since the introduction of Rh immune globulin in 1968 (Kedrion Biopharma, Melville, NY). Many processes used for the manufacture of RhIG have been based on methods developed by Cohn et al. and Oncley et al. to purify plasma proteins, particularly albumin and immunoglobulin G (IgG). Today, manufacturers of therapeutic immunoglobulins continue to use the previously developed cold alcohol precipitation process, but the process has progressed to optimize protein purification for multiple different proteins, including RhIG, and to enhance safety, such as pathogen testing.

Plasma fractionation starts with the collection of human plasma, typically by plasmapheresis. Plasma units, referred to as source plasma, are stored at –20°C or below and tested for the absence of blood-borne infectious agents, including hepatitis B virus, hepatitis C virus, hepatitis A virus, and human immunodeficiency virus. In the case of source plasma for RhIG, the plasma contains high titers of anti-D from healthy Rh-negative donors (males or females without childbearing potential) who have been immunized with allogenic donor Rh-positive red blood cells (RBCs).

In the typical traditional fractionation process, frozen plasma is thawed at 2°C to 8°C, pooled, and centrifuged to remove cryoprecipitate. The plasma is then treated stepwise with varying concentrations of alcohol and buffers with adjustments to pH and ionic strength to selectively precipitate specific proteins. This process is performed at temperatures below 0°C to prevent protein denaturation. At each step, either the precipitate or supernatant containing IgG is retained until the final step, which contains essentially pure

*To be consistent with clinical terminology, the authors use “Rh positive” and “Rh negative” when applying the results of laboratory typing tests to clinical applications. Conventional terminology of “D+” and “D−” are used for laboratory typing results, as is the style of Immunohematology.
IgG containing anti-D. Some manufacturers of RhIG have modified the traditional fractionation process, eliminating some or all of the precipitation stages and replacing them with ion-exchange chromatography to purify the IgG. Traditional alcohol fractionation and some chromatography processes purify all IgG in the plasma including anti-D, whereas other chromatography processes are designed to specifically isolate the anti-D IgG.

All manufacturers of RhIG incorporate at least two virus removal or inactivation steps into the manufacturing processes. The most common processes are:

1. **Virus filtration:** Virus filters work by size exclusion and are effective at removing both enveloped and non-enveloped viruses. Effective filters will generally have the capability to provide a 4-log (10,000-fold) reduction of typical bloodborne viruses that could be present.\(^4\)

2. **Solvent/detergent treatment:** The addition of a combination of a solvent (typically tri-n-butyl phosphate) and detergent (typically Triton X-100) to the manufacturing process has been shown to be very effective in inactivating lipid enveloped viruses.\(^5\) Downstream processing will remove these chemicals after treatment.

3. **Process partitioning:** Certain specific fractionation or chromatography steps used in the manufacturing process have been shown to remove viruses, although effectiveness may vary depending on the type of virus.

4. **Heat treatment:** Elevated temperatures, typically 60°C for 10 hours, for defined periods have been shown to effectively inactivate viruses. Stabilizers, such as amino acids or sugars, may be added temporarily to the product to prevent protein degradation.\(^6\)

The final step in the manufacturing process is sterile filtration of the product in the final container, which is generally a single-dose prefilled syringe. Most RhIG products are supplied as a dose of 300 µg (1500 IU) of anti-D, which is sufficient to suppress the immune response to an exposure of up to 15 mL Rh-positive RBCs. Some manufacturers provide a 50-µg (250-IU) dose to be used in cases of spontaneous or induced termination of pregnancy through 12 weeks’ gestation.

After the manufacturing process, RhIG products are extensively tested. Anti-D potency is measured compared with a standard traceable to the World Health Organization standard anti-D immunoglobulin.\(^7\) Several methods are typically used to ensure product purity, including electrophoresis and high-performance liquid chromatography. Microbiologic testing ensures that the product is sterile and non-pyrogenic. This testing, together with extensive screening of the source plasma and viral treatments, ensures that the RhIG is safe and effective.

### Clinical Aspects of RhIG in Therapeutic Use

We present three clinical scenarios in which a patient’s prior treatment with RhIG may result in an unexpected, “interfering” laboratory finding of anti-D. In one scenario, an Rh-negative woman received RhIG for antepartum or postpartum immunophrophylaxis, the laboratory was not informed, and the laboratory report of anti-D states or infers D alloimmunization. In the second scenario, anti-D was detected in the plasma/serum of an Rh-negative patient, suggesting D alloimmunization. However, the patient had received RhIG for immunophrophylaxis after transfusion of Rh-positive RBCs or transfusion of whole blood-derived platelets containing a relatively large content of donor’s Rh-positive RBCs. In the third scenario, an Rh-positive patient was treated with intravenous (IV) RhIG for ITP, the laboratory was not informed, and the laboratory reports a positive antibody detection test, with or without identification of anti-D and a positive direct antiglobulin test (DAT). The laboratory report states or infers that the patient has autoimmune hemolytic anemia. In each of these scenarios, a positive antibody detection test without a history of prior administration of RhIG may delay emergency release of RBCs, including RBC units released through an electronic crossmatch process.

### Resolving an “Interfering” Laboratory Finding

Sometimes, an unexpected “interfering” laboratory finding of anti-D can be resolved clinically, that is, without additional laboratory testing. In the first scenario, namely, a laboratory finding of anti-D in an Rh-negative woman of childbearing age, the unexpected detection of anti-D may be clarified by asking the patient if she is, or was recently, pregnant and received an injection of RhIG. A negative response may not be accurate, because not all patients are reliable historians. A negative response should be followed up by asking about a possible visit to an obstetrician or emergency department. The woman’s blood sample may have been collected at an outpatient facility. Determining whether she received a recent injection of RhIG may require a review of her electronic medical record, communication with her obstetrician, or communication with an outside ambulatory facility where she received prenatal care.\(^8\)

In the second scenario, the transfusion service detected anti-D, unexpectedly, in the plasma/serum of an Rh-negative woman...
patient. Unknown to the transfusion service, RhIG that is inventoried and dispensed by the hospital’s pharmacy was administered because of concern that a unit of whole blood–derived platelets from an Rh-positive donor appeared to contain an excessive content of RBCs (pink-colored plasma). In some hospitals, RhIG may be administered by policy if whole blood–derived platelets are transfused to an Rh-negative patient, especially to a female patient of childbearing potential or to a candidate for a progenitor cell transplant.

In the third scenario, the unexpected detection of anti-D in an Rh-positive child or adult may be clarified by asking if the patient had received an injection of IV RhIG to treat ITP, a low platelet count, bruises, or spontaneous bleeding.

The following discussion expands on the scientific basis and clinical presentations of these three scenarios in which administration of a therapeutic product may interfere with the correct interpretation of a serologic test result in a transfusion service.

A study conducted between 2006 and 2008 at the Canadian Blood Services, Edmonton, identified 91 women with anti-D in perinatal serum samples in the absence of a report of RhIG prophylaxis or previously identified anti-D.9 The authors distributed a survey to the women’s physicians asking about RhIG administration and for a report of sensitizing events, including prior transfusions. Of 91 responses, 44 (48.3%) of the identified D antibodies were due to passive RhIG; 36 (39.6%) were due to immune anti-D; and 11 (12.1%) were undetermined. The causes of immune anti-D were varied, raising concern for failure to adhere to specific guidelines and protocols, including failure to treat with RhIG at the time of delivery or at the time of amniocentesis or abortion, or failure of a single dose of RhIG to protect against transplacental hemorrhage. The study revealed the difficulty the perinatal laboratory encountered in obtaining accurate and reliable patient information. The authors recommended that each new prenatal patient with anti-D be followed up at the time of antibody discovery to obtain more accurate patient information. They also recommended periodic quality audits or surveys to monitor adherence to policies for RhIG administration.

### Determining RhIG Dosage Postpartum

In the United States, standard obstetrical practice requires an injection of RhIG for all non-alloimmunized Rh-negative women who deliver an Rh-positive newborn, including a newborn with a serologic weak D phenotype.10–12 Clinical trials established that immunoprophylaxis with RhIG within 72 hours of an Rh-negative woman delivering an Rh-positive newborn can prevent alloimmunization to D.10 A standard 300-µg dose of RhIG will prevent alloimmunization in an Rh-negative woman whose delivery of an Rh-positive newborn is associated with a fetomaternal hemorrhage (FMH) of ≤30 mL fetal whole blood or ≤15 mL fetal RBCs.10 The dose (i.e., the number of 300-µg vials/syringes of RhIG administered for immunoprophylaxis) is determined by the outcome of the following four steps.10

#### Screen Mother’s Peripheral Blood for Fetal RBCs

The first step in determining the postpartum dose of RhIG in an Rh-negative woman is to perform a rosette fetal blood screen test, which is a qualitative serologic assay used to detect fetal Rh-positive RBCs in maternal Rh-negative blood. A suspension of maternal RBCs is incubated with reagent anti-D, which will bind to D+ fetal RBCs, if present. Indicator D+ RBCs are added, which bind to anti-D–coated fetal D+ RBCs in a rosette pattern. Although there are alternative laboratory methods for screening for the presence of fetal RBCs in a sample of the mother’s peripheral blood, most laboratories in the United States use a commercially marketed kit for this function.13 A negative rosette fetal blood screen test indicates the absence of, or only a minimal, FMH (≤10 mL) requiring no more than the standard injection of one 300-µg dose of RhIG for immunoprophylaxis. A positive rosette fetal blood screen test indicates the likelihood of a greater FMH and requires further testing by a quantitative assay, typically an acid elution (Kleihauer-Betke) or, uncommonly, a flow cytometric assay.

#### Determine the Percentage of Fetal RBCs in Maternal Blood

If the initial rosette fetal screen test indicates a greater FMH than warrants a single dose of RhIG, the second step would be to determine the percent of fetal RBCs in a sample of the mother’s peripheral blood. In the United States, nearly all laboratories quantify FMHs by testing the mother’s postpartum peripheral blood sample using one of several commercially marketed kits for an acid-elution assay (Kleihauer-Betke).13 The acid-elution assay is based on the principle that hemoglobin A (HbA, adult Hb) can be distinguished from HbF (fetal Hb) on a peripheral blood smear that has been immersed in citric acid. Citric acid elutes HbA from RBCs, whereas HbF is acid-resistant and remains within the RBC. Staining a sample of the mother’s postpartum blood on a peripheral blood smear will color fetal RBCs deep red, because they retain their full content of HbF. In contrast, the mother’s RBCs, from which HbA has been eluted, stain light pink (“ghosts”). Acid elution is a
simple test that can be performed without specialized training in general laboratories, but it is tedious and has a subjective endpoint. The percent of fetal cells in the mother’s circulation is determined by counting the number of darkly staining fetal RBCs in a 2000-RBC scan of a peripheral blood smear. The acid-elution assay cannot be used in women whose RBCs have an increased content of HbF, which occurs if the mother has a coexistent hemoglobinopathy, thalassemia, aplastic anemia, or stress erythropoiesis.

Flow cytometry for HbF or D+ RBCs offers a more accurate and reproducible method to quantify an FMH in an Rh-negative woman. However, few hospitals can afford flow cytometric testing for FMHs because of the high cost for a low-volume service that requires a flow cytometer as well as skilled and proficient personnel to be available 24/7.

**Calculate the Volume of an FMH**

The third step in this process is to use the results of the acid-elution test to calculate the volume of the FMH in the mother’s total blood volume using the following formula:

\[
\text{FMH (mL fetal whole blood)} = \frac{\text{(number of fetal cells counted ÷ number of RBCs counted)}}{\text{maternal total blood volume (mL)}}
\]

*Most laboratories count a total of 2000 RBCs and assign an arbitrary total blood volume of 5000 mL.*

**Estimate the Dose (Number of Vials) of RhIG**

Once the FMH volume is determined, the final step of estimating the dose of RhIG can be performed. One 300-µg dose of RhIG administered within 72 hours after delivery of an Rh-positive newborn will protect an Rh-negative mother from D alloimmunization if there has been an FMH of ≤30 mL whole blood. Based on that formula, most laboratories in the United States will calculate the dose of RhIG using the method of the AABB Technical Manual, as follows:

\[
\text{Number of 300-µg vials/syringes of RhIG} = \frac{\text{(volume of FMH [mL of whole blood] ÷ 30 mL)}}{30 \text{ mL}}
\]

Recognizing the inherent imprecision of the acid-elution assay and the pitfalls in the calculation, the editors of the AABB Technical Manual provide the following precautionary adjustment in this formula:

• When the number to the right of the decimal point is 5 or greater, round up to the next number and add one dose of RhIG (e.g., if the calculation comes to 2.8, give 4 doses).

**Detecting Anti-D After Postpartum RhIG Administration**

Often, we are asked how long after a postpartum injection of RhIG will anti-D be detectable in the mother’s peripheral blood. This answer depends on the size of the dose, the volume of the FMH, and the sensitivity of the laboratory’s test method. A dose of multiple vials/syringes of RhIG will persist longer than a dose of one vial/syringe. A large-volume FMH consisting of many D+ fetal RBCs will adsorb more anti-D from the circulation than a small-volume FMH. A highly sensitive solid-phase automated analyzer will detect anti-D in a mother’s postpartum plasma/serum sample up to 6 months after a large dose of RhIG. An antibody detection test performed by a relatively insensitive manual tube method may fail to detect anti-D only a few weeks after an injection of only one 300-µg dose of RhIG.

**Anti-D After Routine 28- to 30-Week Antepartum RhIG Administration**

Clinical trials have demonstrated that adding an antepartum injection of RhIG at 28–30 weeks’ gestation to a postpartum injection of RhIG in an Rh-negative woman who has delivered an Rh-positive newborn will reduce the risk of D alloimmunization from approximately 16 percent to 0.1 percent. In the United States, it is standard practice to administer a 300-µg antepartum dose of RhIG at 28–30 weeks’ gestation to all Rh-negative pregnant women. However, only approximately 38 percent of fetuses of an Rh-negative mother who were fathered by an Rh-positive man will be Rh-negative, and the mother does not require D immunoprophylaxis. For this reason, many obstetricians practicing outside of the United States avoid the cost and injections of RhIG, relying on a determination of the fetus’ D type by testing for maternal cell-free DNA (cfDNA). In the United States, a prospective observational study for cfDNA in 520 non-alloimmunized Rh-negative pregnant women had one false-negative result (0.32%). Analysis of the results revealed that the one error was not in cfDNA testing but instead was caused by a mislabeled blood sample tube. Correcting for the mislabeling, the adjusted false-negative RHD result was reported to be 0.0 percent (95% CI 0.00–1.22%). Advocates of cfDNA testing are likely to cite the false-negative result as 0.0 percent. Others,
including these authors, who experience the practicality of day-to-day hospital operations, recognize that adding a step in the procedure that requires collecting, labeling, and reporting the cfDNA result for an additional blood sample is more likely to include the operational error in the results and consider the comprehensive false-negative result to be 0.32 percent. If RHD is not detected in the mother’s cfDNA, the fetus is assumed to be Rh-negative, and RhIG is not administered.

In the Netherlands, a prospective cohort study of a nationwide program compared results of fetal RHD testing with serologic cord D typing results for 25,789 pregnancies. Sensitivity for detection of fetal RHD was 99.94 percent and specificity was 97.74 percent. There were nine false-negative results, of which two were due to technical failures. Those authors concluded that RHD testing at week 27 of pregnancy as a part of an antenatal screening program is highly reliable and can be used to determine antenatal and postnatal immunoglobulin administration. In the United States, most obstetricians assume that the current practice of a routine 28-week antenatal injection of RhIG is 100 percent effective, although there are neither studies nor data to support this assumption. Obstetricians hesitate to change from a practice that is believed to be 100 percent effective in preventing D alloimmunization to a new practice that relies on cfDNA testing, which has been demonstrated to have false-negative results, no matter how few.

In our Transfusion Service laboratory, when a routine highly sensitive solid-phase antibody detection test is performed when an Rh-negative mother is admitted for delivery, we regularly detect residual anti-D in her plasma/serum, which is 8–10 weeks after a conventional 28- to 30-week injection of RhIG. If the newborn is Rh-negative, it is not unusual for a manual tube test to detect residual anti-D in maternal plasma with a 2–3+ graded reaction using R,R, reagent screen RBCs. There are reports that highly sensitive column gel and solid-phase antibody detection tests have detected residual anti-D from a one-dose injection of RhIG as long as 95 days and possibly 180 days after routine antepartum immunoprophylaxis. In a study using an indirect antiglobulin test (IAT, by tube method) to detect anti-D after antepartum injections of RhIG, 35.6 percent of samples had detectable anti-D at delivery, 54 percent at <76 days after injection, and only 2.4 percent had anti-D detected 76–95 days after injection of RhIG.

**Anti-D After RhIG Immunoprophylaxis During Pregnancy**

During an Rh-negative woman’s pregnancy, an injection of RhIG may be administered for D immunoprophylaxis for a suspected or proven FMH as the result of termination of pregnancy (abortion), placenta previa, amniocentesis, chorionic villus sampling, percutaneous umbilical blood sampling (PUBS), other obstetrical manipulative procedure (e.g., version), or abdominal trauma. Vials/syringes of RhIG, containing only 50 µg (250 IU) of anti-D, are commercially available in the United States and will suppress the immune response to an FMH of ≤2.5 mL Rh-positive RBCs. However, many hospital pharmacies stock only 300-µg doses of RhIG, avoiding the cost of maintaining two inventories and, more importantly, avoiding the risk of issuing the wrong RhIG product because 50- and 300-µg doses of RhIG may have similar brand names (e.g., MICRhOGAM versus RhoGAM; Kedrion Biopharma). Using the larger 300-µg dose of RhIG increases the likelihood that anti-D will be detected in the mother’s plasma by an antibody detection test performed any time before delivery.

**Anti-D After Post-Transfusion RhIG Administration**

Anti-D may be detected in an Rh-negative patient’s plasma after RhIG was administered to prevent D alloimmunization after exposure to transfused Rh-positive RBCs. That situation may occur if a unit or part of a unit of Rh-positive RBCs was transfused in error. Also, RhIG may have been administered if a unit of whole-blood–derived platelets from an Rh-positive donor containing a significant volume of residual RBCs was transfused to an Rh-negative recipient. Although RhIG is frequently administered in this situation, the number of Rh-positive RBCs is considered too few to require routine administration of prophylactic RhIG. In these situations, IV RhIG is the preferred product because a relatively large therapeutic dose can be administered in a small volume without concern for the discomfort of a large intramuscular or subcutaneous injection, which is the usual mode of administration of RhIG.

**Anti-D After IV RhIG Administration for ITP**

IV RhIG may be administered to induce a temporary and reversible “medical splenectomy” to treat thrombocytopenia in Rh-positive adults or children with ITP. The rationale is that an IV infusion of only 50–75 µg anti-D can coat autologous D+ RBCs, inducing phagocytosis and causing a
The clinical applications for RhIG, described in the previous section of this review, show that the serologic detection of anti-D due to RhIG may vary depending on the application. D+ RBCs in the circulation adsorb antibody, decreasing the concentration of antibody available, affecting the reactivity observed in serologic tests. In pregnancy, using the standard dose of 300 µg, the presence of detectable anti-D once maximum concentration is achieved is frequently detected in antibody detection tests within several weeks of its administration. In the antenatal application, the amount of antibody given is intended to provide protection against infrequent and generally small-volume bleeds that may occur over a 12-week period. There is little likelihood that all of the antibody will disappear serologically unless FMH bleeds that occur are more than of a very minor volume. If a potential for FMH occurs (any trauma, termination, amniocentesis, or PUBS) before the 28- to 30-week mark for receiving the standard dose, the patient will require continued protective support with an additional dose(s) of RhIG every 12 weeks after the incident until term delivery. Testing of a patient with RhIG who has been treated earlier than normal in the pregnancy could result in an unexpected detection of anti-D. This scenario causes a significant increase in error potential.

Serologic Detection of Anti-D Due to RhIG

The encounter of true alloimmunization due to D+ RBCs resulting in anti-D is generally considered an unusual event in today's pretransfusion testing setting, especially with current practices in place in transfusion therapy and obstetrical services to prevent the development of the antibody. Nevertheless, the detection of anti-D often prompts questions and actions by the transfusion service to determine whether the presence of anti-D is related to an immune response to D+ RBCs via transfusion or pregnancy or autoantibody formation or for another non-immunologic reason. When the patient is a female of child-bearing age, the initial explanation that comes to mind is whether this is a passively acquired antibody associated with pregnancy due to RhIG administration. Often when anti-D is encountered in other patient populations, the initial explanation is that the antibody is one of an immune response by the patient, whether alloantibody or autoantibody.

There are many factors that contribute to the detection of RhIG passively acquired through its use, as described under Clinical Aspects of RhIG in Therapeutic Use in this review. These factors include the following:

1. Concentration of RhIG given
2. Mode of administration (intramuscular, IV, or subcutaneous)
3. Pharmacokinetics of the formulation of RhIG
4. Half-life of RhIG
5. Clinical reason for administration
6. Timing of testing after dose(s)
7. Antibody detection test method

In the initial serologic findings of a sample in which RhIG is a factor, the reactivity of the tests can range from negative to barely detectable weak reactivity to strong reactivity. Consideration must be given to the listed factors and their role in the serologic picture that may be presented. The dose plays a significant role in how long the anti-D could be detected. Pharmacokinetics of the formulation, generally demonstrated by flow cytometric methods, confirms that a maximum concentration is achieved within hours to up to 10 days depending on route of administration. IV administration delivers anti-D at its highest concentration in the shortest amount of time. The antibody's in vivo half-life may vary from 20 to 31 days, with IV administration having a shorter half-life than intramuscular administration. This difference is likely due to the adsorption dynamics from interstitial tissue.
due to the assignment of an immune status to the identified anti-D when, in fact, the anti-D is passive.

On occasion, newborns will demonstrate a weak positive DAT or a positive antibody detection test as the result of RhIG crossing the placenta from the mother who received it antenatally.

The detection of alloantibodies of clinical relevance is highly desirable in the prevention of potential hemolytic transfusion reactions and prediction of possible HDFN. Unfortunately, the ability to distinguish between the antibody present in RhIG and that of a patient sensitized and producing allo–anti-D is, in practicality, impossible by current basic antibody detection tests. Because RhIG is made from polyclonal anti-D from multiple donors (as discussed in the Manufacture of Rh Immune Globulin section of this review) and is essentially the same antibody formed by a patient who is alloimmunized to D, the ability to determine the source of antibody is challenging.

Clearly, having a reliable and detailed patient history can be invaluable for interpreting the laboratory result when an Rh-negative patient presents with anti-D. When there is a history of administration of RhIG, laboratory practice, particularly in obstetrical patients, is to avoid the presence of the passively acquired anti-D using a D– set of selected reagent RBCs to complete antibody detection/selected cell antibody identification to rule out the presence of any other potentially clinically significant antibodies. The results reported by laboratories typically would state that the “presence of anti-D is likely passively acquired from RhIG.” Even with the knowledge of RhIG administration, the question remains, “Is there a possibility that the anti-D that is present has an alloimmune component?” Without clarity on patient history, however, making this supposition needs to be considered carefully, and further investigation may be required.

**Methods for Distinguishing Passive Versus Immune Anti-D**

Recommendations and suggested methods that attempt to distinguish passive versus immunized anti-D exist. The AABB Technical Manual notes that if there are questions about the origin of the anti-D, titration studies may provide information. Generally, the titer result when only passive anti-D is present rarely exceeds 4. Titer endpoints of 16 and above are indicative of the possibility of sensitization to D. However, the titer result should be followed by a cautionary statement that the result may be affected by the test method, RBC phenotype used in the test, technical variability, timing of RhIG dose, and concentration of the dose, therefore it must be carefully evaluated. Because the development of a recently formed anti-D is often predominantly IgM, and RhIG is IgG, the presence of reactivity at the saline phase of testing is indicative of active sensitization. This reactivity could be confirmed by IgM inactivation using sulfhydryl compounds (2-mercaptoethanol or dithiothreitol).

In Australia and New Zealand, the recommended dose of RhIG is 100–125 µg (500–525 IU) for antenatal RhIG prophylaxis administered at 28 and 34 weeks of pregnancy. The Australian and New Zealand Society of Blood Transfusion Guidelines for testing in the antenatal and perinatal setting suggest additional test protocols to distinguish passive versus active sensitization and are based on the detection of anti-D when both a confirmed history of RhIG administration within 6 weeks of the test and the reactivity strength and agglutination scores meet certain criteria. For those results showing a reaction grade of <2 or hemagglutination score <8, the assumption is made that the presence of anti-D is passive. The clinical report typically states that the results suggest that this is likely passive, although the possibility of an early immune response cannot be excluded using serology. Anti-D present within 6 weeks of the RhIG administration with reaction grade ≥2 or score ≥8 indicates that further study including titer, quantitation, and repeat testing should be considered. For those tests that show anti-D presence more than 6 weeks after the administration of antenatal RhIG or when there is no evidence of RhIG administration, further study by titration, quantitation, and repeat testing are indicated. Titers of ≥32 are considered a clinically relevant level in the guidelines and warrant quantitation to determine the IU/mL concentration of anti-D present.

The British Standards in Hematology–Transfusion Task Force established guidelines related to distinguishing between passive and immune anti-D. Like the Australian and New Zealand guidelines, the UK guidelines offer a twodose RhIG antenatal regimen at 28 and 34 weeks of pregnancy. The focus of recommendations by the British Standards in Hematology to ascertain passive versus immune anti-D presence is to apply a quantitative approach using a continuous flow analyzer with reference to a known standard. For continuous flow, rarely does passively administered RhIG exceed 0.4 IU/mL unless doses exceeding 1500 IU have been administered. Results between 4 and 15 IU/mL are considered a moderate risk for HDFN, and those higher than 15 IU/mL indicate a high risk for HDFN.
Several studies have been undertaken to determine the capability of serologic testing to distinguish passive versus active anti-D. Each of the studies has used time of injection, dose, gestational week, reaction strength, and various test methods (polyethylene glycol tube test [PEGT], gel, and solid phase [SP]) in an attempt to reach an analytical approach to establish the presence of active immunization. Two of the studies suggested that the PEGT was potentially more reliable as a predictive test for active immunization than gel and SP. The potential that alloimmunization should be considered based on strong reactivity in PEGT and gel during the later weeks (weeks 36–40) of pregnancy was more reliable compared with SP testing because both PEGT and gel would be expected to show less reactivity if only passive anti-D is present. The SP method was not possible to use because of the variability of the test method and the lack of triation of test results over time to establish the threshold criteria. One of the studies suggested in their conclusion, “However, it should be noted that it is not possible to definitively determine whether the anti-D detected is active or passive in this manner. If there is any doubt, RhIG should always be administered to patients who would otherwise qualify. Nevertheless, considering anti-D reaction strength may be of value when devising reporting comments and requesting repeat testing.”

Another study by Irving et al. used a slightly different approach involving testing with enzyme-treated RBCs to identify a serologic way to distinguish passive from active sensitization. Samples (N = 273) with known anti-D detected by column agglutination tests and clear histories of the origin of the anti-D were selected for testing. This group then tested each sample by a low-ionic-strength saline–IAT and enzyme–IAT (both by tube method) and compared the score change between the methods. A change (0–12 score range) of ≥2+ was considered significant (Table 1).

### Table 1. Score differences of immune anti-D and passive anti-D by LISS–IAT tube versus enzyme–IAT tube methods (N = 273)

<table>
<thead>
<tr>
<th>Score difference</th>
<th>Immune (n = 68)</th>
<th>Passive (n = 213)</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥2+ Positive</td>
<td>TP = 19</td>
<td>FP = 0</td>
</tr>
<tr>
<td>≤1+ Negative</td>
<td>FN = 41</td>
<td>TN = 213</td>
</tr>
</tbody>
</table>

Compiled from Irving et al.

LISS = low-ionic-strength saline; IAT = indirect antiglobulin test; TP = true positive; FP = false positive; FN = false negative; TN = true negative.

Sensitivity and specificity as well as predictive value were calculated. The sensitivity of the test algorithm was 59 percent, with specificity of 100 percent; the positive predictive value was 100 percent and the negative predictive value was 84 percent. The use of the enzyme test with a score change criteria was viewed as a potential stronger mechanism for possible alert to alloimmunization. However, this test could not be used to exclude the possibility of alloimmunization to D.

The serologic picture in clinical cases that involve the use of RhIG for treatment to prevent anti-D formation as the result of transfusion of Rh-positive RBCs in error or in Rh-positive platelets to an Rh-negative individual demonstrate reactivity that is influenced by the concentration of RhIG given and the amount of Rh-positive RBCs transfused. A positive antibody detection test and a typically weakly positive DAT result may be encountered when there are sufficient D+ RBCs and antibody present to elicit a positive DAT.

Although the use of RhIG in ITP treatment has waned in use because of the black box warning and the availability of other solutions, the serologic picture is likely similar to that seen with transfused Rh-positive RBCs treated with RhIG. Additionally, beyond anti-D, other passively acquired antibodies may be seen in patients soon after treatment with IV RhIG.

In both situations, this finding can lead to a perplexing picture, without evidence that the patient has been treated with RhIG. In this situation, identification of anti-D or potentially other passively acquired antibodies can result in erroneous conclusions and the patient being burdened with an antibody (allo or auto) that may have to be honored into the future. Therefore, we see the need to pursue the various information channels—communication with clinicians, health care records, and even outside health care entities are necessary to gain resolution to these uncertain cases.

### Conclusion

Anti-D collected from stimulated donors used in the manufacture of RhIG is indistinguishable by standard immunohematologic tests from anti-D formed by patients exposed to D by transfusion or pregnancy. Unfortunately, the expectation to serologically solve with total confidence the source of the presenting anti-D without some level of additional investigative work is a precarious position. Some serologic methods are of value in identifying patients who are sensitized to D. Using test methods that provide a quantitative picture versus a qualitative picture are important, in some situations, to make the distinction between passive and immune anti-D. Nevertheless, great care is necessary in labeling a patient as sensitized, especially if there is any chance...
that a misinterpretation may have occurred. Additionally, avoiding misinterpretation of test results and collecting clinical patient information, such that proper treatment to prevent sensitization in the patient is achieved, is just as critical to consider.

Ultimately, having the right information about the patient—transfusion history and clinical history paired with the serology and other testing methods—to call upon whenever the interference of RhIG is encountered in basic serology is critical to determine the proper course of patient treatment. If in doubt, especially in patients in whom prevention of the development of an immune anti-D is the goal, it is better to err on the conservative side and provide prophylaxis to the patient.

References


