

Meeting the transfusion needs of a patient with anti-En^a requires an international effort

Stella T. Chou,^{1,2,x} Sandra T. Nance,^{3,4} Paul Mansfield,³ David F. Friedman,^{1,2} and Margaret A. Keller^{4,5}

¹ Division of Hematology, Department of Pediatrics, Children's Hospital of Philadelphia, Philadelphia, PA

² Division of Transfusion Medicine, Department of Pathology and Laboratory Medicine, Children's Hospital of Philadelphia, Philadelphia, PA

³ Immunohematology Reference Laboratory, American Red Cross, Philadelphia, PA

⁴ American Rare Donor Program, American Red Cross, Philadelphia, PA

⁵ National Molecular Laboratory, American Red Cross, Philadelphia, PA

^x Corresponding author: CHOUS@chop.edu

This extraordinary case showcases the identification of a rare anti-En^a specificity that was assisted by DNA-based red blood cell antigen typing and collaboration between the hospital blood bank in the United States, the home blood center in Qatar, the blood center Immunohematology Reference Laboratory, as well as the American Rare Donor Program (ARDP) and the International Society for Blood Transfusion (ISBT) International Rare Donor Panel. En^a is a high-prevalence antigen, and blood samples from over 200 individuals of the extended family in Qatar were crossmatched against the patient's plasma with one compatible En(a-) individual identified. The ISBT International Rare Donor Panel identified an additional donor in Canada, resulting in a total of two En(a-) individuals available to donate blood for the patient. *Immunohematology* 2024;40:10–14. DOI: 10.2478/immunohematology-2024-003.

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Case Report

A 21-year-old female patient with pediatric-onset amyotrophic lateral sclerosis with severe lumbar hyperlordosis presented from Qatar for spinal fusion and vertical expandable prosthetic titanium rib placement at the Children's Hospital of Philadelphia. She had two previous surgeries for hip dislocations in Germany, for which she received blood transfusions uneventfully. The family history was notable for a younger brother with a similar progressive neurological disorder. The parents were healthy and were known to be first cousins.

The patient underwent an uncomplicated surgery with an estimated blood loss of 250 mL. Intraoperative cell salvage was attempted (Cell Saver; Haemonetics, Braintree, MA), but an insufficient amount was recovered for re-transfusion. The postoperative hemoglobin (Hb) was 9.1 g/dL, decreased from

12.2 g/dL preoperatively (normal range for adult female is 12.1–15.1 g/dL). Her postoperative course was complicated by fever and decreased alertness on postoperative day (POD) 3. She was initially treated with broad-spectrum antibiotics, but blood and urine cultures showed no growth. At that time, her Hb was 9.8 g/dL but gradually decreased to 7.1 g/dL by POD 5, prompting a red blood cell (RBC) transfusion. Her blood typed as group O, D+, and the gel indirect antiglobulin test (IAT) was negative. A single group O, D+ unit of RBCs was transfused, and the following day her Hb had increased to 9.0 g/dL.

On POD 13, the patient was again febrile and required an emergent operative exploratory procedure, drainage of a suspected infected surgical site, and the potential removal of the stabilizing hardware. The preoperative Hb was 7.9 g/dL. The blood bank received a new blood specimen and an urgent order for 2 units of RBCs. The antibody detection test was positive; all reagent RBCs were 3+ reactive by the gel IAT. The direct antiglobulin tests (DATs) performed with anti-IgG, anti-C3, and polyspecific antihuman globulin (AHG) were all negative. A specimen was sent to the American Red Cross Penn-Jersey region for antibody identification and for phenotype prediction using a genotyping panel. Given the urgency of surgery and expected surgical blood loss, a protocol waiver was approved to issue 2 units of crossmatch-incompatible group O, D– RBCs. The estimated blood loss was 300 mL; a total of 270 mL RBCs was transfused during surgery. The postoperative Hb was 6.5 g/dL.

The day after this second surgery, the blood bank received a grossly hemolyzed specimen that typed as group O, D+ with no mixed-field reactivity in the D typing, suggesting complete hemolysis of the D– RBCs transfused 1 day before (Fig. 1). An acute hemolytic transfusion reaction (HTR) was suspected although no specific symptoms were documented

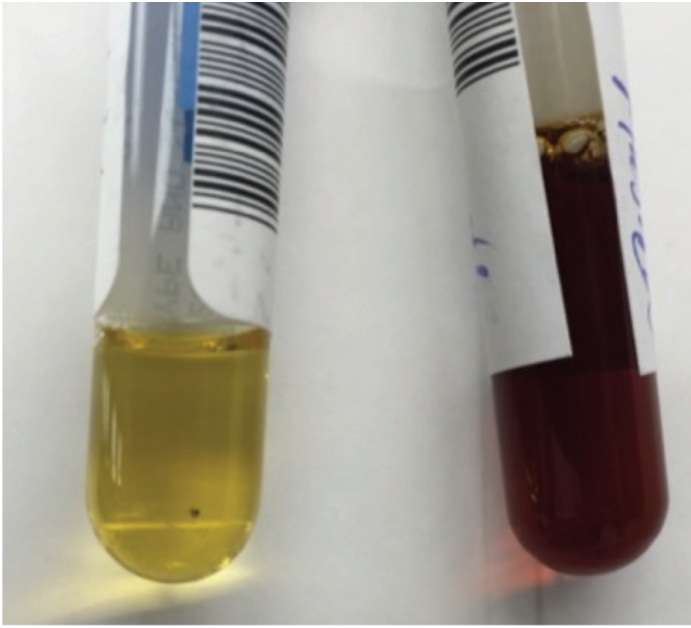


Fig. 1 Pre- and post-transfusion patient plasma. Pre-transfusion (left) and post-transfusion (right) patient plasma after a protocol waiver was approved for the transfusion of 2 units of crossmatch-incompatible group O, D- RBC units for emergent surgery.

by the primary team aside from the fever and tachycardia. The patient had no hemodynamic instability and no acute renal function deterioration. The patient was treated with methylprednisolone, erythropoietin, and intravenous iron. While the American Red Cross Immunohematology Reference Laboratory (IRL) attempted to identify the antibody specificity, the patient's Hb reached a nadir of 4.9 g/dL on the third day after the drainage procedure before it began to rise and subsequently reached 10 g/dL without RBC support.

Serologic Investigation

The new finding of a plasma antibody reactive with all reagent RBCs tested 8 days after RBC transfusion prompted a referral to the American Red Cross IRL in Philadelphia. The IRL found an antibody that was 4+ reactive with all reagent RBCs in all testing phases including immediate spin, room temperature, and 37°C using albumin (Immucor, Norcross, GA), albumin IgG, PEG (Polyethylene Glycol; American National Red Cross, Diagnostic Manufacturing Division, Gaithersburg, MD) IgG, gel (Ortho Clinical Diagnostics, Raritan, NJ) IAT, and ficin-treated RBCs. The patient's plasma was nonreactive with her own RBCs, suggesting hemolysis of almost all the transfused RBCs; further, the DAT was negative using anti-IgG, anti-C3d, and polyspecific AHG. RBC phenotyping was performed on a post-transfusion sample

Table 1. Serologic phenotype and genotype-predicted phenotype

Blood group system	Serologic phenotype	Genotype-predicted phenotype by PreciseType HEA Molecular BeadChip
RH	D+ C+ E+ c+ e+	C+ E+ c+ e+ V- VS-
KEL	K- k+	K- k+ Kp(a-b+) Js(a-b+)
JK	Jk(a+b+ ^w)	Jk(a+b-)
FY	Fy(a-b+)	Fy(a-b+)
MNS	M- N+ S+ s- U+	M(LS) N(LS) S+ s- U+
LU	NT	Lu(a-b+)
DI	NT	Di(a-b+)
CO	NT	Co(a+b-)
DO	NT	Do(a+b+) Hy+ Jo(a+)
LW	NT	LW(a+b-)
SC	NT	SC:1,-2
P1PK	P ₁ +	NT
VEL	Vel+*	NT
YT	Yt(a+)*	NT
GE	GE:2,3*	NT
IN	In(b+)*	NT

*Unlicensed reagents (stored sera) were used to type for these high-prevalence antigens.

LS = low signal; NT = not tested.

(Table 1) and showed that the patient's RBCs lacked K, Fy^a, and s as well as M and Le^a.

Adsorptions were performed with enzyme-treated R1, R2, and rr allogeneic adsorbing RBCs; the antibody reactivity was reduced but was not removed after five adsorptions. Four 37°C adsorptions with R1, R2, and rr untreated allogeneic adsorbing RBCs removed the reactivity that then allowed specificities to common RBC antigens to be ruled out. The reactivity was not removed when neat sample was tested with ficin-treated reagent RBCs; 4+ reactivity remained at 37°C and at the AHG phase with anti-IgG. Hemolysis was noted when the sample was tested with 0.2 M dithiothreitol-treated reagent RBCs that demonstrated 2+ reactivity at immediate spin. Reactivity remained when tested by the prewarmed technique at 37°C and at the AHG phase with anti-IgG.

Evaluation for an antibody to a high-prevalence antigen showed the patient's serum was reactive with RBCs lacking the following antigens: PP₁^{Pk}, H, I, Vel, Lu^a and Lu^b, U, U^{VAR+}, MAM, Rh_{null}, MER2, K_{mod}, Yt^a, Gy^a, Hy, At^a, and Cr_{null}, as well as cord blood-derived RBCs. The patient's RBCs from a pre-transfusion sample were also reactive with antisera to the following antigens: k, Ge2, Ge2,3, and In^b.

Genetic Evaluation

As the serologic evaluation was ongoing, the patient's DNA was used for RBC genotyping using the PreciseType HEA Molecular BeadChip (Immucor, Norcross, GA) at the National Molecular Laboratory. The assay returned a "No Type Determined" result due to "low signal" for M and N. Immucor was contacted and released the remainder of the genotype-predicted phenotype classified for these panel samples as "for investigational use only" (Table 1). Note: M and N are encoded by glycophorin A (*GYP*A), and the PreciseType HEA Molecular BeadChip panel interrogates a single nucleotide polymorphism (SNP) in exon 2 of *GYP*A to determine M and N status (Fig. 2). The low fluorescence signal for M and N suggested possible deletion of *GYP*A exon 2, which, if the deletion spanned exons 2–7, would predict the absence of glycophorin A and thus an M– N– En(a–) phenotype.

A whole-genome (WG) SNP microarray had been performed previously on the patient and parents as part of the diagnostic evaluation of the underlying neurologic condition. After receiving the PreciseType HEA Molecular BeadChip results, the clinical team requested that the genetics clinical consultant review the *GYP*A locus from existing WG SNP array. This review revealed that the affected and unaffected siblings shared many regions of homozygosity. Furthermore, a small region of signal dropout at 4q31.21 was found within a larger region of homozygosity, indicating a homozygous deletion of a portion of the *GYP*A gene.

Subsequent DNA analysis performed by the National Molecular Laboratory demonstrated that the genomic DNA sample failed to support polymerase chain reaction (PCR)

amplification of *GYP*A exons 2–7. The exon 1 product that was amplified and subsequently analyzed by Sanger sequencing could not be differentiated from the homologous region of the *GYPE* gene. These findings supported deletion of most or all of the *GYP*A gene and were concordant with the findings of the WG SNP array at 4q31.21.

Further Serologic Evaluation

Given the preliminary DNA array results, the patient's serum was tested against rare RBCs that were characterized as "En(a–)FS (ficin sensitive), possible M^kM^k." M^kM^k RBCs are null for MNS because of the deletion of the *GYP*A and *GYP*B genes. These RBCs were found to be nonreactive with the patient's serum.

The patient's RBCs using a post-transfusion sample were typed for the common antigens of the MNS system and gave results as follows: M–, N+ (1+), S+ (2+), s–, and U+ (2+) with no mixed-field reactivity. The patient's RBCs also reacted with the *Glycine soja* lectin, indicating decreased sialic acid content on the RBC surface¹ which was consistent with other reports of reactivity of En(a–) RBCs with *Glycine soja* lectin. Next, the patient's RBCs were typed with several patient sources (noncommercial) of anti-En^a. Testing was negative with one of two available sources of anti-En^a and with a serum from an anti-En^aFS individual but showed 1+ reactivity with the second source of anti-En^a. With the assistance of another IRL, the patient's serum was tested against En(a–) RBCs from a rare RBC reagent library and was found to be nonreactive by the PEG-AHG method. Further classification of the En^a antigen based on the level of enzyme sensitivity and resistance was

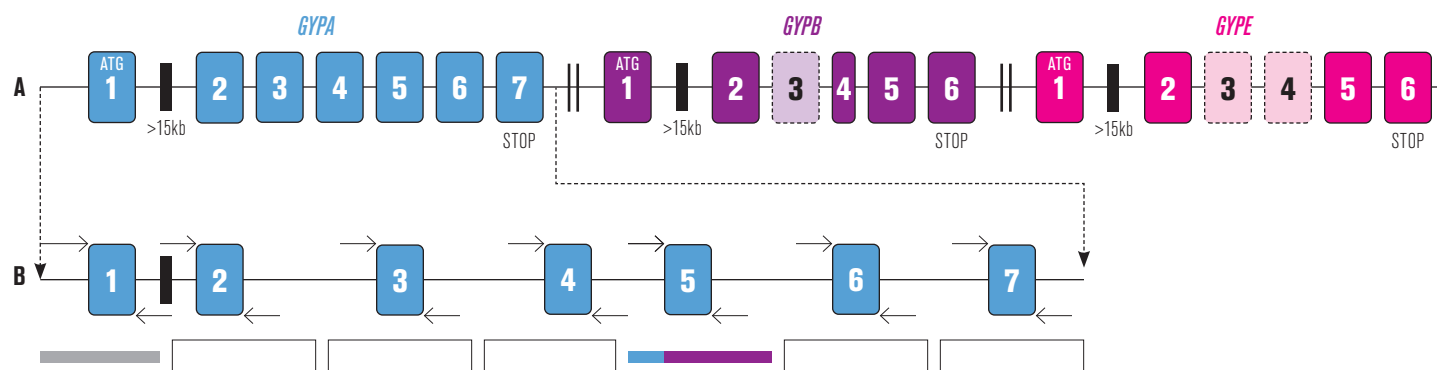


Fig. 2 Schematic of GYP gene locus, PCR amplifications performed, and products generated. (A) The GYP locus with *GYP*A, *GYP*B, and *GYPE* genes. ATG indicates start codon, and STOP indicates stop codon. Exon 3 of *GYP*B and exons 3 and 4 of *GYPE* are considered "pseudo-exons" because they are homologous to exons 3 and 4 of *GYP*A but are not included in the mRNA transcripts. Double dashed lines indicate a large distance between genes; black vertical rectangles indicate a large distance between exons 1 and 2 in each gene locus. (B) The *GYP*A gene with forward and reverse primers (black arrows) used to amplify exons 1–7. Amplification of exons 2, 3, 4, 6, and 7 failed to generate a product (open boxes). Products amplified by exons 1 and 5 (solid color lines) were sequenced; exon 1 aligned with glycophorin genes but could not differentiate *GYP*A, *GYP*B, or *GYPE*, while exon 5 corresponded *GYP*B sequences with *GYP*A intronic sequences.

not performed because of the limited availability of reagents. This information would not be helpful in finding suitable donors, since the En^a status of En(a–) donors is rarely subclassified.

Based on these findings, the IRL reported a preliminary antibody identification of anti-En^a 3 days after receiving the specimen on the night of the patient's emergent surgery. There were no En(a–) donors registered with the American Rare Donor Program (ARDP). The laboratory made inquiries to other reference laboratories for reagent RBCs lacking En^a to confirm the identification. None were found.

Identification of En(a–) RBCs for Transfusion

Given the extreme rarity of En(a–) RBCs, a request to the International Society for Blood Transfusion (ISBT) International Rare Donor Panel was made that initiated testing of family members, hoping to identify an En(a–) relative who could donate blood. The patient's mother and brother had samples drawn immediately, but the samples were 4+ incompatible with the patient's serum. Three members of the extended family in the United States were also 4+ incompatible.

In Qatar, the blood center typed the patient's father and a sister who were both group O, D+. After approval from the U.S. Food and Drug Administration Center for Biologics Evaluation and Research (FDA CBER) and U.S. Immigration, courier-transported blood specimens from the father and sister in Qatar were sent on a commercial flight. The flight was met by hospital representatives, who brought the package through U.S. customs to the hospital blood bank. Unfortunately, both of these family members' RBCs were 4+ incompatible with the patient's serum.

The blood bank shipped existing patient serum samples to the central blood center in Qatar to perform screening of the extended family. Registration and collection of specimens from 200 extended family members were performed using their ISBT128-compliant donor system. A serial dilution titer of the patient's serum was carried out with group O RBCs and was found to have an agglutinating antibody titer of 1024. Testing En(a+) RBCs with a serum diluted 1:32 in saline demonstrated 3+ reactivity, sufficient to exclude incompatible donors. The blood center in Qatar repeated the titer with their routine testing reagents and ultimately used a patient serum dilution of 1:16 for screening potential En(a–) donors. Screening of 155 donor samples continued up until 10 days after the patient's surgery and HTR, but no compatible donor was found. The patient's Hb was now 6.8 g/dL with a reticulocyte count of 20.4 percent (normal range 0.8–1.5%), but there remained

a high probability that she would require additional surgical intervention.

In hopes that some additional testing might be used to find a compatible donor from these samples, the entire collection of 200 donor samples was sent to the hospital blood bank. The specimens were labeled with assigned unique donation identifiers bar-coded in compliance with ISBT128 standards but were otherwise labeled in Arabic. The Qatar central blood center communicated that 45 of the donor samples had not been tested. When these were crossmatched with patient serum diluted 1:16, a single specimen was found to be compatible, and this one donor remained compatible with the neat serum of the patient, identifying a presumptively En(a–) family member. A blood specimen from this family member was used for DNA testing (PreciseType HEA Molecular BeadChip and *GYP*A exon amplification), and the results were consistent with the family member having the same *GYP*A deletion as was detected in the patient.

Twenty days after the initial request to the International Rare Donor Panel, a response was received from the Canadian Blood Services that a known En(a–) donor had been identified and was willing to donate blood for the patient. Since there are several distinct En(a–) phenotypes, a specimen of the patient's serum was sent to the Canadian Blood Services via the American Red Cross, and the donor proved to be crossmatch-compatible. At this point, the patient's Hb had risen to 7.3 g/dL with a reticulocyte count of 14.1 percent, but additional surgery and need for blood was anticipated. A request to import a nonconforming RBC unit was made to FDA CBER. After a total of 26 days from the initial rare unit request, permission to import the RBC unit was granted by FDA CBER, and the unit was shipped from Edmonton, Canada. This liquid unit was confirmed to be crossmatch-compatible with the patient upon receipt in the blood bank. By this time, the patient's Hb had risen to 9.0 g/dL.

The patient underwent an additional surgical procedure for drainage of the infection with minimal blood loss and did not require transfusion. With erythropoietin, iron, and treatment of her infection, the patient's Hb rose to a maximum of 11.3 g/dL without transfusion and was 10.5 g/dL on the day that the En(a–) unit reached expiration.

Discussion

Anti-En^a is very rare, occurring in individuals who lack glycophorin A, linking this antibody to the MNS blood group system.² One prior case similarly described a severe

acute HTR in the 24 hours after transfusion of En(a+) RBCs, which ultimately led to the patient's death.³ Unlike previous case reports and despite an extensive immunohematology evaluation for an antibody directed at a high-prevalence antigen, molecular assays for M and N provided the key information that led to the identification of the antibody specificity. Here, molecular typing using a commercial assay provided evidence of a deletion in the *GYPA* gene, and further DNA-based testing confirmed the En(a–) phenotype.

While the En(a–) phenotype involves deletion of the *GYPA* gene, the M^kM^k phenotype involves deletion of both the *GYPA* and *GYPB* genes. Both the En(a–) and M^kM^k phenotypes are considered “exquisitely rare” by the ARDP and would be registered by the International Rare Donor Panel.⁴ Donors who are identified as “exquisitely rare” and who are invited to join the ARDP are informed of the exceptional rarity of their RBC phenotype and encouraged to work with their physician and blood center to donate RBC units to build an inventory for themselves and others. A later survey of ARDP members performed in 2021 found that ARDP members had two active group O, D+ En(a–) donors and no M^kM^k donors.⁵ Currently, neither the ARDP, with nearly 100,000 active rare donors, nor the International Rare Donor Panel has registered En(a–) or M^kM^k donors.

When the FDA CBER approves an emergency Investigational New Drug (eIND) application for importation of a blood component, that component is restricted for use for only the specific patient for whom it was imported. In this case, when the imported blood unit was not used, the ARDP requested approval for the unit to be converted to a non-transfusable component that was aliquoted to create rare reagent RBCs. Liquid aliquots were distributed to IRLs to be used for antibody identification as part of the Serum, Cells, and Rare Fluid (SCARF) Exchange.

This extraordinary case of a rare anti-En^a specificity was assisted by DNA-based RBC antigen genotyping and exemplary collaboration between the hospital blood bank in the United States, the home blood center on the Arabian peninsula, the ARDP, and the ISBT International Rare Donor Panel. These groups collaborated to crossmatch samples from over 200 members of the extended family against the patient's serum; this testing successfully identified two En(a–), crossmatch-compatible RBC units. In addition, an En(a–) donor from the Canadian Blood Services was also identified. All compatible individuals donated blood to be available to the patient.

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