Received: 10 October 2019 | Revised: 25 May 2020 | Accepted: 11 June 2020

DOI: 10.1111/trf.15965

IMMUNOHEMATOLOGY

TRANSFUSION

Prevalence of natural and acquired antibodies to amustaline/glutathione pathogen reduced red blood cells

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Abstract

Background: The INTERCEPT™ Blood System for Red Blood Cells (RBCs) utilizes amustaline (S-303) and glutathione (GSH) to inactivate pathogens and leukocytes in transfused RBCs. Treatment-emergent low titer non-hemolytic antibodies to amustaline/GSH RBC were detected in clinical trials using a prior version of the process. The amustaline/GSH process was re-formulated to decrease S-303 RBC adduct formation.

Study Design and Methods: A standard three-cell antibody screening panel was modified to include reagent red cells (RRC) with high (S-303H) or low (S-303L) S-303 adduct density as assessed by flow cytometry, representative of the original and current amustaline/GSH treatment processes, respectively. General hospital and RBC transfusion-dependent patients never exposed, and clinical trial subjects exposed to amustaline/GSH RBC were screened for antibodies to amustaline/GSH RBC using a standardized agglutination assay.

Results: Twelve (0.1%) of 10,721 general hospital and 5 (0.5%) of 998 repeatedly-transfused patients not previously exposed to amustaline/GSH RBCs expressed natural, low titer (2-32) IgM and/or IgG (non-IgG_1 or IgG_3 isotype) antibodies with acridine (a structural element of amustaline) (n = 14) or non-acridine (n = 3) specificity. 11 of 17 sera reacted with S-303L panel RRCs. In clinical studies 81 thalassemia and 25 cardiac surgery patients were transfused with a total of 1085 amustaline/GSH RBCs and no natural or treatment-emergent S-303 antibodies were detected.

Conclusion: Standardized RRC screening panels are sensitive for the detection of natural and acquired S-303-specific antibodies. Natural low titer antibodies to amustaline/GSH RBC are present in 0.15% of naïve patients. The clinical relevance of these antibodies appears minimal but is under further investigation.

1 | INTRODUCTION

The INTERCEPT Blood System for Red Blood Cells (RBCs) is an investigational device that uses amustaline (S-303) and glutathione (GSH) in an ex vivo process to inactivate a broad spectrum of viruses, bacteria, protozoa, and leukocytes, to reduce the risks of transfusion-transmitted infection (TTI) and transfusion associated-graft versus host disease (TA-GVHD).[1-4] Amustaline/GSH RBCs have been shown to be safe and effective in cardiac surgery and repeatedly transfused thalassemia patients and are undergoing further clinical study.[5,6]

RBCs prepared with a prior version of the process were evaluated in two Phase III clinical trials that were halted following discovery of low titer S-303 antibodies in two transfused subjects, but without evidence of hemolysis. The antibodies were low-titer, transient, and had characteristics unlikely to cause hemolysis in vivo based on evaluation of phagocytosis in a monocyte monolayer assay (MMA).[78] Further investigation showed some patients had pre-existing natural antibodies to RBC membrane-bound S-303 adducts.[9] To lower the density of membrane bound adducts, the pathogen reduction (PR) process was reformulated. The current treatment system utilizes an increased pH and concentration of GSH to quench extracellular amustaline to minimize unwanted reactions with RBC surface molecules[3] Following treatment, the pathogen reduced (PR) RBCs are exchanged into fresh additive solution to reduce the concentration of GSH and amustaline breakdown products.

We describe the design and use of amustaline/GSH treated reagent red cell (RRC) screening and confirmatory panels for the detection and differentiation of S-303-specific and RBC alloantibodies. A serology surveillance study to determine the prevalence of natural S-303 antibodies was conducted using patient samples collected from two distinct populations: general hospital patients in a large German academic medical center; and repeatedly transfused thalassemia and sickle cell disease patients from multiple medical centers in Europe and the US. The characteristics of these natural antibodies are described. Subsequently, the amustaline/GSH RRC panels were utilized to screen for pre-existing natural S-303 antibodies and treatment-emergent antibodies in two clinical trials involving complex cardiac surgery patients receiving amustaline/GSH RBCs during or shortly after surgery,[6] and transfusion dependent thalassemia patients receiving regular repeated amustaline/GSH RBC transfusions.

2 | METHODS

2.1 | Reagents for antibody screening

Three-cell primary and six-cell secondary reagent red cell (RRC) panels were constructed by selection of antigen-phenotyped, group O donors representing a cross section of relevant RBC alloantigens. Packed RBC concentrates from each donor were aliquoted into three parts (Figure 1) and treated with processes similar to either (a) the original amustaline/GSH treatment process (S-303H) using 0.2 mM S-303/2 mM GSH, or (b) the current amustaline/GSH process (S-303L) prepared with 0.2 mM S-303/20 mM GSH, or (c) left untreated (Control RRC).[6,9,10] The panels

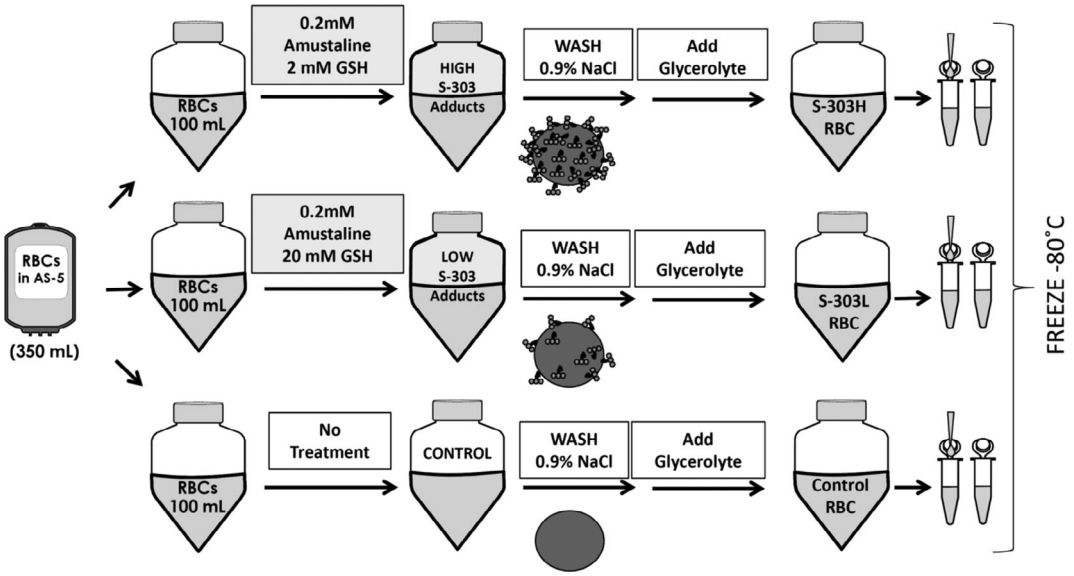


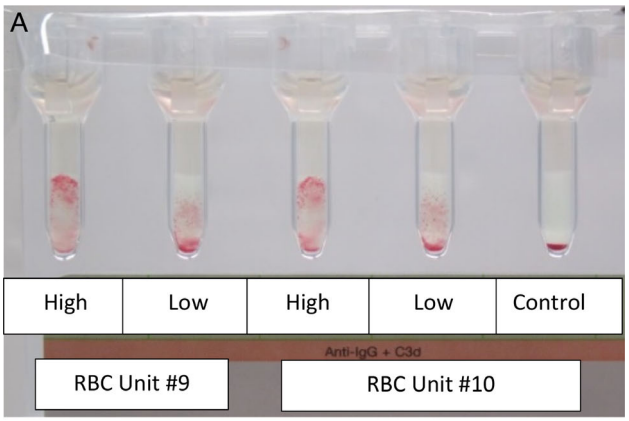
FIGURE 1 Manufacturing scheme for RRC screening panel cells

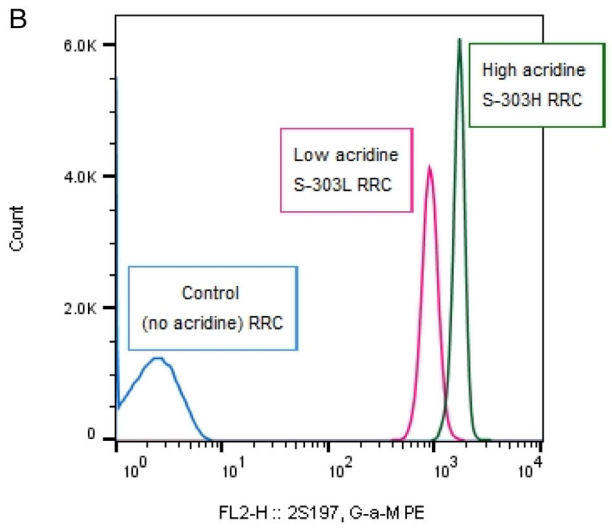
were suspended in Glycerolyte 52 (Fenwal Inc., Lake Zurich, IL), frozen in 1 mL aliquots at −80°C for up to 2 years, and were used within 24 hours of thaw and stored at 4°C until use. As a positive control, a high titer acridine-specific mouse monoclonal antibody (2S197-2M1) was developed against S-303 by immunizing mice with keyhole limpet hemocyanin (KLH)-S-197. S-197 is a stable analog of S-303, facilitating the production of antibody reactive to acridine, a component of the S-303, S-197, and S-300 (the primary S-303 reaction by-product) molecules.

Immune reactivity was assessed by indirect antiglobulin test (IAT) with thawed and washed RRCs using gel column agglutination (ID-Card anti human gamma globulin [AHG] plus complement [IgG + C3d] LISS/Coombs gel cards: BioRad Laboratories GmbH, Munich, Germany). Frozen RRC were thawed and deglycerolized by washing to prepare RRC at 0.8% hematocrit in ID-Diluent 2 (modified LISS). The mouse monoclonal antibody 2S197-2M1, together with an isotype matched negative control was used to validate assay sensitivity and as positive and negative agglutination controls to ensure consistency of the frozen RRC panels. For each RRC panel sample, serological reactivity with amustaline/GSH treated RRC (S-303H and/or S-303L) with a negative reaction to the corresponding untreated control was considered indicative of S-303 antibody specificity (Figure 2A).

2.2 | Quasi-quantitative flow cytometric assessment of RBC S-303 surface adducts

A quasi-quantitative flow cytometric assay for S-303 RBC surface adducts was validated using the unconjugated 2S197-2MI antibody and a polyclonal goat-anti-mouse IgG (isotype matched) phycoerythrin (PE) conjugated secondary antibody (Molecular Probes, Eugene, OR).[11] A commercial CD55 monoclonal antibody (BD Biosciences, San Jose, CA) was used as a comparator. Quantification was accomplished using calibrated beads (QuantiBRITE™ PE beads, BD Biosciences, San Jose, CA) to generate a standard curve. Antibody labelling of a deglycerolized frozen 1% solution of RRC was achieved with previously titrated concentrations of each antibody with incubation for 30 minutes on ice. Cells were washed in ice-cold buffer and antibody binding was detected utilizing a secondary antibody incubated for 30 minutes on ice. Cells were fixed in 1% formaldehyde solution before analysis. Florescence labelling was assessed on a FACSCalibur (488/635) flow cytometer (BD Biosciences, San Jose, CA) calibrated for quantitative analysis with QuantiBRITE PE beads and CellQuest Pro or FlowJo v10 (BD Biosciences, San Jose, CA) software.





SAMPLE ID	PE Molecules/Cell
Control RRC	42
S-303L RRC	15643
S-303H RRC	29721

FIGURE 2 A) Gel agglutination assay analysis of Patient #7 demonstrating S-303 specificity on two representative RRC panel cells (Unit #9 or #10), with reactivity on S-303H and S-303L RRC and no reactivity on untreated Control RRC. B) Overlaid representative quantitative flow cytometry histograms of Control, S-303L and S-303H RRC with 2S197-2M1, an S-303 specific monoclonal antibody [Color figure can be viewed at wileyonlinelibrary.com]

2.3 | Patient samples for screening of natural S-303 antibodies

Residual clinical laboratory plasma (EDTA-anticoagulated) and sera from 10,721 consecutive hospital patients at the Goethe University Hospital, Frankfurt, Germany submitted for routine blood grouping or pretransfusion compatibility testing were stored at 4°C and screened for S-303 antibodies within a week of phlebotomy. Samples from chronically-transfused patients were obtained from one US and five European study sites

(Table S1). Serum samples were divided into 1 mL aliquots, frozen and stored at −70°C. All samples were de-identified by study specific codes to provide subject confidentiality. This study was conducted in accordance with the internationally accepted Guidelines on Good Clinical Practice, ICH E6 (R1) and legal requirements that are consistent with the Declaration of Helsinki. Local Ethics Committee approval with exemption from informed consent was obtained and documented for each site.

2.4 | Serologic testing of patient samples

A strategy was designed to test each patient sample against a single donor RRC cell panel (untreated [Control]; S-303H; and S-303L RRC) which in case of accompanying irregular RBC alloantibodies did not express the corresponding antigen. A Tecan automated Freedom Evo robotic pipettor (Tecan Group Ltd., Männedorf, Switzerland) loaded RRC and patient samples onto the gel cards, which were incubated at 37°C for 15 minutes and centrifuged at room temperature for 10 minutes (BioRad ID-Microtyping system). Scoring of agglutination was performed using an automated gel card reader (Saxo reader, DiaMed Diagnostika GmbH, Munich, Germany) with manual confirmation. The monoclonal antibody 2S197-2M1 was included as a positive control each time the assay was performed. A positive reaction in the primary screen was confirmed using two other RRC screening cells (control, S-303H and S-303L). Due to sample limitations only a preliminary antibody characterization could be performed for some patients.

In the SPARC and STARS clinical studies, screening for S-303 antibodies was performed as previously described.[5,6]

2.5 | Determination of antibody titer

Antibody titer was established by serial two-fold sample dilution in ID-Diluent and incubation with S-303H or S-303L RRC. The titer was recorded as the inverse of the highest dilution that gave an observable, reproducible positive agglutination result.

2.6 | Confirmation of antibody specificity by hapten inhibition

A hapten inhibition test was performed using S-300, a soluble stable analog of S-303, incubated with patient plasma prior to addition to the test RRCs.[1] Ten-fold serial dilutions of S-300 were prepared in ID-Diluent to yield final concentrations ranging from 0.01 to 0.0001 mM. The 2S197-2M1 antibody was utilized as a positive control. The specificity evaluation also included inhibition studies with GSH if there was no inhibition with S-300. All patient and control samples were incubated in the presence of S-300 for 15 minutes at 37°C. Gel agglutination cards were prepared, each with 50 μL of 0.8% RRCs (S-303H and/or S-303L) per gel micro-column. After the incubation period, 25 μL of plasma or control antibody plus S-300 was added to the RRCs, which were incubated for 15 minutes at 37°C followed by centrifugation for 10 minutes in gel cards. All results were scored with the end point designation with the highest concentration of S-300 at which visible agglutination of the cells was observed. Acridine-specificity required inhibition of agglutination with S-300 in a dose-dependent manner.

2.7 | Determination of antibody class and subclass

A commercial monospecific gel card was used to determine immunoglobulin type ([monospecific AHG] [ID Card DC Screening I]). This card consists of five monospecific rabbit AHG reagents to anti-IgG, anti-IgA, anti-IgM, anti-C3c, and anti-C3d (monoclonal cell line C139-9) with a saline control. The IgG subclass was determined using a monospecific anti-IgG_1 and anti-IgG_3 gel card (ID-Card DAT IgG_1/IgG_3). An IgG antibody with a negative result with IgG_1 and IgG_3 reagents was presumed to be subclass anti-IgG_2 or anti-IgG_4. The presence of pure IgG_4 anti-S-303 antibodies was evaluated in a subset of samples where volume allowed. S-303H, S-303L and control RRC were utilized as target cells in the Immucor Capture -R Select assay (Immucor, Atlanta, GA) performed on an Immucor Neo Iris platform. Capture -R Ready Indicator cells coated with Gamma-clone (Immucor, Atlanta, GA), a monoclonal IgM mouse antihuman IgG (clone 16H8), that detects all IgG subclasses except IgG_4 and 2 of 15 IgG_3 isoallotypes. Lack of reactivity in this assay might suggest a pure IgG_4 antibody of those isoallotypes.[12] IgM antibody was confirmed using dithiothreitol (DTT) pre-incubation and ID-Card NaCl (no AHG reagent). Patient samples (25 μL) were pre-incubated with an equal volume (25 μL) of 0.01 M DTT (37°C for 30 min) before performing the gel card test with RRCs.

2.8 | Determination of thermal amplitude

Thermal amplitude testing was performed by incubating the RRC and patient plasma in IgG + C3d LISS/Coombs gel cards at three different temperatures: 4°C, 20°C, and 37°C for 15 minutes followed by gel card centrifugation.

2.9 | Statistics

Data are described descriptively throughout. The serologic survey study was designed to have sufficient sensitivity to detect the prevalence of S-303 antibodies with an upper bound of a 95% confidence interval of at least 0.03%, if no positive reactions were observed in 10,000 subjects.[13] The one sided 95% Clopper-Pearson exact confidence interval for binomial proportion was calculated using SAS software, Version 9.4 of the SAS System.

3 | RESULTS

RRC panels were constructed for the detection and discrimination of RBC allo- and S-303- specific antibodies, by treatment of selected RBC units in a process similar to that used in the original and current amustaline/GSH processes (Figure 1). Flow cytometry and gel agglutination assays confirmed the presence of S-303 adducts using the acridine-specific monoclonal antibody 2S197-2M1. An approximate 2- to 4-fold higher level of adducts was consistently found when comparing S-303H with S-303L RRCs (Figure 2B). Preliminary studies revealed that RRC surface adduct levels declined with prolonged (>48 hours) storage at 4°C but were stable when frozen at -80°C. RRC panels were therefore frozen in 1 mL aliquots, stored at -80°C and used within 24 hours of thaw.

The stability of S-303 adducts on the reagent RRC during the gel-column assay incubation conditions was further evaluated using S-303H and S-303L RRC with room temperature (RT) or 37°C incubation after the addition of positive control 2S197-2M1 monoclonal antibody. When tested immediately after thaw, S-303H and S-303L RRC performed as expected and treatment adducts were stable at RT for up to 6 hours (Table 1). In contrast, S-303L RRC showed reduced sensitivity when incubated for >15 minutes at 37°C and both S-303H and S-303L RRC show reduced sensitivity when incubated for 30 mins at 37°C. It was concluded that S-303 adducts on the RRC surface are stable for at least 6 hours when stored at RT and should be stored at 4°C after thaw and before use.

To confirm that the amustaline/GSH process does not alter expression of RBC alloantigens, RRC phenotyping with commercial blood grouping reagents was assessed. Reagents were titered using either freshly prepared RRCs, or RRCs frozen and stored for 15 months at -80°C.[14] RRC phenotyping was conducted with commercial polyspecific antibodies and gel card agglutination or monospecific antibodies with traditional tube agglutination techniques (Table S2). Comparison of reaction strengths (measured in titers), with RRC from five donors and antibodies to 20 clinically-relevant antigens showed that fresh and frozen Control untreated RBC and those treated with amustaline/GSH (both S-303H and S-303L) were similar (ie, were within ±1-2 doubling dilutions). There were no false positives, false negatives, or discordant agglutination results. These results demonstrate that clinically relevant RBC antigens are not affected by amustaline/GSH treatment and amustaline/GSH RBCs accurately reflect the RBCs serological phenotype.[14]

TABLE 1 Stability of S-303 adducts on S-303H and S-303L RRC frozen for various periods, after incubation at room temperature (RT) or 37°C, as detected with an acridine-specific monoclonal antibody (2S197-2M1)

		Agglutination score		
Preincubation conditions		**April**	**May**	**September**
Control RRC	Baseline	0	0	0
	RT, 3 h	0	0	0
	RT, 6 h	0	0	0
	37°C, 15 min	0	0	0
	37°C, 30 min	0	0	0
S-303H RRC	Baseline	4+	4+	4+
	RT, 3 h	4+	3+	4+
	RT, 6 h	4+	4+	4+
	37°C, 15 min	4+	4+	4+
	37°C, 30 min	1+	Weak	2+
S-303L RRC	Baseline	3+	3+	3+
	RT, 3 h	3+	2+	3+
	RT, 6 h	3+	3+	3+
	37°C, 15 min	1+	1+	1+
	37°C, 30 min	0	0	0

A serology surveillance study was conducted to evaluate the prevalence of natural immune reactivity to amustaline/GSH RBCs using samples from 10,721 general hospital patients at the Institute of Transfusion Medicine and Immunohaematology, Frankfurt, Germany and from 998 multiple transfused patients with hemoglobinopathies treated at five international study centers (Table 2).[15,16]

The Institute of Transfusion Medicine and Immunohaematology hospital population cohort had a mean age of 67 years, were 57% male and included patients with a wide variety of medical and surgical disorders. In 1691 individuals of this cohort (16%, 1691/10,721), RBC alloantibodies (eg, anti-K, anti Fy(a) or other) were present. The multiple-transfused patients with hemoglobinopathies (eg, thalassemia or sickle cell disease) had a mean age of 30 years and were 51.5% male (Table S1).

TABLE 2 Serological profile of natural S-303 specific antibodies in patients without prior exposure to amustaline/GSH RBC

	Reactivity, initial screen			Antibody titer			Temperature reactivity						Patient characteristics		
							Reactivity at 37°C		Reactivity at 20°C		Reactivity at 4°C				
Patient number	S-303H RRC	S-303L RRC	Antibody class/subclass	S-303H RRC	S-303L RRC	Specificity by inhibition assay	S-303H RRC	S-303L RRC	S-303H RRC	S-303L RRC	S-303H RRC	S-303L RRC	Sex (M/F)	Age (y)	Disease status
General hospitalized patients															
1	++	−	IgG, non $G_{1,3}$/non G_4	4	neg	Acridine	+	−	+++	−	++	−	M	75	Cardiovascular surgery
2	−	++	IgG, non $G_{1,3}$	neg	8	Unidentified	nt	nt	nt	nt	nt	nt	M	84	Hematology/oncology
3	++	−	IgG, non $G_{1,3}$/non G_4[a]	4	neg	Acridine	++		++		−		F	35	Infectious disease
4	++++	+++	IgG, non $G_{1,3}$/non G_4	8	8	Acridine	+++	++	++	+	+/−	−	M	84	Hematology/oncology
5	+	−	IgG, non $G_{1,3}$	1	neg	Acridine	+/−	−	+/−	−	+	−	F	21	Pregnancy
6	+++	+	IgG, non $G_{1,3}$/non G_4	4	neg	Acridine	+	−	−	−	−	−	M	76	Unknown
7	++	+	IgG, non $G_{1,3}$	2	neg	Acridine	+	−	++	−	++	−	M	85	Urology (bladder cancer)
8	++	−	IgG, non $G_{1,3}$	2	neg	Acridine	+/−	nt	+/−	nt	−	−	F	32	Pregnancy
9	+	++	IgG, non $G_{1,3}$/non G_4	4	4	Acridine	+/−	++	nt	++	−	−	M	75	Unknown
10	+++	−	IgM	16	neg	Acridine	+/−	−	+++	++	+++	−	F	76	Unknown
11	+/−	+	IgG, non $G_{1,3}$/ possible G_4	4	nt	Acridine	+/−	+	+/−	+/−	++	+	M	51	Orthopedic surgery
12	+/−	−	IgG, non $G_{1,3}$/non G_4	4	neg	Acridine	+/−	−	+/−	−	−	−	M	87	Unknown
Hemoglobinopathy patients															
13	++++	+++	possible IgM	8	neg	Acridine	++++	+++	nt	nt	nt	nt	unk	unk	Thalassemia
14	+++	++	probable IgM	8	neg	Acridine	++++	++	nt	nt	nt	nt	unk	unk	Thalassemia
15	−	++	IgG, non $G_{1,3}$	neg	1	unidentified	−	+	nt	nt	nt	nt	unk	unk	Thalassemia
16	++++	+++	IgG, non $G_{1,3}$	32	neg	acridine	+++	++	nt	nt	nt	nt	F	27	Thalassemia
			IgM	16	neg		++++	++	nt	nt	nt	nt			
17	+/−	+++	IgG, non $G_{1,3}$	nd	nt	unidentified	+/−	++	+	+	++	++	unk	unk	Thalassemia

Abbreviations: nt, not tested; unk, unknown.

[a]Capture-R Select assay showed positive reactivity on both S-303H and S-303L cells.

Overall, 17 (0.15%) plasma samples from subjects naïve to amustaline/GSH RBCs contained antibodies with specificity for either S-303H and/or S-303L RRC, including 12 (0.1%) general hospital patients and 5 (0.5%) chronically transfused hemoglobinopathy subjects (Table 2). All five hemoglobinopathy samples were from thalassemia patients from Greece, that contributed 326 of the 998 (26%) samples tested (Table S1). Subjects were of various ages and either sex, with thalassemia as the most common diagnosis. Of the 17 reactive samples, nine reacted with both S-303H RRC and S-303L RRC, six sera reacted only with S-303H RRC and not with S-303L RRC, and two reacted only with S-303L RRC. Those reacting with S-303L RRC included 6 (0.06%) general hospital patients and 5 (0.5%) hemoglobinopathy patients. Of note, overall five sera reacted more strongly with S-303L than S-303H RRC.

Initial characterization identified 13 antibodies as IgG, three as IgM and one an IgG/IgM combination. In 14 cases, the antibodies were inhibited by the addition of S-300, confirming specificity for the acridine moiety of S-303. In three cases, reactivity was not inhibited by the presence of high concentrations of either S-300 or GSH, and these sera did not react with S-303H RRC. These data suggest a specificity not directed to either acridine or GSH. All amustaline/GSH RRC specific sera were either IgM or IgG (non $IgG_{1,\ 3}$) isotype and of low titer (<32). Eight sera were tested in the Capture-R Select assay (Table 2) and the results generally confirmed the findings with the other technologies. Two samples were unreactive for S-303 antibodies, one (patient #10) had a known IgM antibody, and the other (patient #11) represented a possible pure IgG_4 S-303 antibody.

Where tested, the thermal amplitude of the sera was variable, often reacting most strongly at room temperature. The reactions with four sera with acridine specificity were noted as weak (±) at 37°C.

The RRC panels were used to screen patients for eligibility in two Phase 3 clinical trials, in which the presence of natural S-303 specific antibodies was an exclusion criterion. In STARS, – a randomized controlled trial of amustaline/GSH RBC in complex cardiac surgery (Table 3), 87 patients were screened[6] and in SPARC—a randomized, controlled trial of amustaline/GSH RBC in thalassemia patients, 86 thalassemia patients were screened.[5] All patient sera were found to be non-reactive for S-303 antibodies. Nine of the 87 thalassemia subjects had pre-existing RBC alloantibodies. All subjects were re-screened for S-303-specific and RBC alloantibodies before each transfusion and at the end of study (~90 days after exposure in the STARS study and >42 days after last exposure in SPARC). All results were non-reactive for treatment-emergent S-303-specific or RBC alloantibodies.[5,6]

To assess how the S-303 epitope densities of the transfused RBCs in the SPARC and STARS studies compared to each other and to the S-303H and S-303L RRC, quantitative flow cytometry was performed. Freshly treated amustaline/GSH RBC from both the DRK Frankfurt and Izmir, Turkey clinical trial sites were different, but both had lower S-303 epitope densities (mean 9064 and 13,151 epitopes/RBC, respectively) than S-303L RRC (mean 15,643 epitopes/RBC: Figure 2B), and adduct density on RBC from both clinical trial sites decreased by ~45% by Day 35 of storage (Table 3).

4 | DISCUSSION

Amustaline/GSH treatment of RBCs inactivates a broad spectrum of bacteria, enveloped and non-enveloped viruses, protozoa and leukocytes, reducing the risk of TTI and TA-GVHD.[3,9] S-303 is a labile alkylating agent derived from a novel class of frangible anchor-linker effector (FRALE) compounds. When added to concentrated RBC

TABLE 3 Patient characteristics in clinical studies using the current generation of amustaline/GSH RBC concentrates

Diagnosis	STARS[6] Cardiac surgery	SPARC[5] Thalassemia	Total
Number screened for natural S-303 antibody	87	86	173
Patients transfused with amustaline/GSH RBC concentrates in test arm	25	81	106
Mean (range) exposure to amustaline/GSH RBC (# of concentrates)	2.9 (1-7)	12.5 (3-18)	(1-18)
Mean (SD) S-303 epitopes/RBC (n = 12) on Day 2 post manufacture[a]	9064 (1005)	13151 (919)	-
Mean (SD) S-303 epitopes/RBC (n = 12) on Day 35 post manufacture	5033 (841)	7035 (639)	-
Patients with natural S-303 specific antibodies at screening	0	0	0
Patients with S-303-specific antibodies at Day ~90 post transfusion	0	0	0

[a]Epitope density assessed by flow cytometry on amustaline/GSH RBC manufactured at Ege University, Izmir, Turkey and DRK Frankfurt Blutspendedienst, Frankfurt am Main, Germany.

preparations, it passes rapidly through cell and nuclear membranes and targets tertiary structures in DNA and RNA by intercalation of an acridine ring structure.[10] Single adducts and nucleic acid strand cross-linkages are formed through a bischloroethylamine effector moiety and these serve to prevent DNA/RNA replication. After the effector group reacts, the intermediates decompose at the frangible alkylester linker sites, resulting in a major degradation product which is a negatively charged, acridine-ring moiety, designated S-300, which is no longer reactive with nucleic acid.[1] The native S-303 compound has a propensity to react with other nucleophiles, such as water, phosphates and proteins. These reactions are minimized by the addition of relatively high concentrations of GSH, a quenching compound that is restricted to the extracellular compartment and does not inhibit pathogen and leukocyte inactivation.

The original amustaline/GSH technology for RBCs was evaluated in two Phase III clinical trials that were halted when 2 of 17 patients in the chronic transfusion study, with a history of exposure to amustaline/GSH RBCs, formed antibodies to treated RBCs.[9] Although no adverse clinical outcomes or evidence of hemolysis were evident, the sponsor terminated both trials to elucidate the mechanism of this unexpected reaction. The antibodies were low titer (2 and 8) and scored ≤5% in a MMA, indicating a low risk of immune mediated RBC clearance.[7,8,17,18] Samples from 127 patients (64 test and 63 controls) in the acute anemia trial were tested for antibodies to amustaline/GSH RRCs, and the majority were negative, however two control patients demonstrated S-303 antibodies 535 days and 56 days after the study began, respectively.[9] Both were inhibited by free S-300. Two Test subjects had indeterminate results: One test patient who had received 3 amustaline/GSH RBC had weak (1+) S-303 RBC reactivity on 2 of 3 amustaline/GSH RRC on Day 28 that was not inhibited by free S-300. Another test patient who received a single amustaline/GSH RBC transfusion was reactive with 2 of 10 amustaline/GSH RRC units at baseline before transfusion and 2 of 11 amustaline/GSH RRC units on Day 47 (maximum strength 1+) and did not react on Day 350. S-300 inhibition studies were not performed.[9] These studies demonstrated the existence of both natural antibodies in subjects never previously exposed to amustaline/GSH RBC, as well as acquired S-303-specific antibodies, and formed the rationale for the need to develop a standardized assay to screen for S-303 antibodies to use in clinical studies and to evaluate the prevalence of natural S-303 antibodies.

In the current study, we demonstrate the use of a standardized assay for S-303 antibodies using RRC screening panels labeled with either S-303H or S-303L adducts. Using an acridine-specific monoclonal antibody 2S197-2M1 as a positive control, we show that S-303 adducts are labile on the RBC surface, requiring storage under frozen conditions. Upon thaw, the adducts are stable for at least 6 hours at room temperature, but not at 37°C, where S-303L RBC begin to lose their reactivity after a 30 minute incubation. Reactivity with conventional RBC alloantibodies in the gel agglutination assay is not affected by amustaline/GSH treatment.

Using these RRC panels, we observed natural S-303 antibodies in 0.1% of general hospital patients in Germany and 0.5% of multiple transfused hemoglobinopathy patients. Antibodies were generally of low titer (<32) and of either IgM or IgG (non-IgG_1 or IgG_3). The IgG antibodies are presumed to be IgG_2 or IgG_4 subclass. Seven (of 14) patients with IgG S-303 antibodies were tested and one was unreactive in a Capture-R Select assay that does not detect IgG_4 antibodies. The one is presumably a pure IgG_4 antibody. The other six patients presumably have IgG_2 antibodies. The IgG_2 and IgG_4 isotypes are likely to be of lesser clinical relevance due to limited complement activation and Fc receptor binding. IgG_2 antibody effector function is particularly sensitive to epitope density and is the major subclass formed against repetitive T cell-independent polysaccharide structures found on encapsulated bacteria.[19]

At least two specificities are evident: one inhibited by high concentrations of the acridine analogue S-300; the other is neither inhibited by S-300 or GSH and is expressed on detectable levels on S-303L RRC and not on S-303H RRC. The specificity is unknown but may represent novel epitopes formed by a combination of S-303 and GSH on the RBC surface. Six antibodies reacted only with S-303H RRC and not S-303L RRCs, and four of the acridine-specific sera reacted only weakly at 37°C, both characteristics suggesting limited potential for in vivo hemolysis with the current therapeutic version of amustaline/GSH RBC.

The origin of natural antibodies to S-303 is unknown, but environmental exposure to acridine seems likely. Acridine was first purified from coal tar extracts in the 1800s and then synthesized as a historical base for commonly-used dyes. These are no longer in general use. The acridine structure is also a component of proflavine, acroflavine, and 9-amino acridine, all of which were commonly used in topical antiseptic medications, and are still available in many countries for human and veterinary use.[20,21] Interestingly, atopic dermatitis is a known complication of these antiseptics, indicating potential immunogenicity.[21] That all five hemoglobinopathy sera with S-303 specificity came from one country (Greece) suggests that local exposure to a cross-reactive antigen is possible.

The description of natural and acquired S-303 antibodies raises the question how they may impact the utility of amustaline/GSH RBC in clinical practice. Current clinical studies exclude patients that have S-303 antibodies at

enrollment and currently any subject that develops S-303 antibodies is withdrawn from receiving amustaline/GSH RBC. In these cases, subjects will be evaluated for evidence of intravascular or extravascular hemolysis and the antibodies will be characterized for hemolytic potential to predict their clinical significance.[22] An in vitro investigation will characterize the isotype, titer, thermal spectrum, complement activating capacity and reactivity in a MMA. These data will assist in determining the clinical relevance of any treatment-emergent S-303 antibody. Prevailing assumptions using the current amustaline/GSH process is that S-303 antibodies will be infrequent, and when detected, will be of low clinical relevance.

As noted by Garratty,[22] hospital blood banks are routinely faced with transfusing patients with pre-existing RBC alloantibodies, and it is expected that S-303 antibodies will behave in a similar fashion to RBC alloantibodies. Antibodies which are poorly reactive at 37°C and/or negative (≤5% reactivity) in an MMA assay may be less clinically important and may not preclude transfusion with careful monitoring. Our finding that pre-incubation of S-303L RRC at 37°C leads to loss of S-303 reactivity; and that amustaline/GSH RBC after 35 days of storage have ~45% lower S-303 epitope expression than fresh RBC, suggests that the S-303 epitope is labile, especially at body temperatures (~37°C). The behavior of S-303 epitopes in vivo after transfusion is unknown and requires further investigation, but their density is likely to be reduced. Lower epitope density is known to affect IgG_2 antibody effector functions, in particular.[19] Transfusion of "least incompatible" amustaline/GSH RBC near the end of their storage life may be considered under careful supervision. The alternative, that patients who develop S-303 antibodies receive non-PR RBC is less attractive, as many of the advantages of PR RBC, such as allowing some infectious disease testing to be discontinued and relaxation of donor selection criteria, rely on universal application of the technology.

In summary, RRC panels that can detect and differentiate RBC alloantibodies and S-303 antibodies are sensitive reagents to protect patients who receive amustaline/GSH RBC in clinical trials, and in routine practice once marketed. Further characterization of natural and acquired S-303 antibodies in clinical studies will help inform decisions with regards to the nature and extent of the risk in clinical practice.

CONFLICT OF INTEREST

A.N., M.v.G., L.C., R.J.B., N.M. are employees of Cerus Corporation. C.G., L.B., V.B. and E.S. have no conflicts to declare.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Geisen C, North A, Becker L, et al. Prevalence of natural and acquired antibodies to amustaline/glutathione pathogen reduced red blood cells. *Transfusion*. 2020;60: 2389–2398. https://doi.org/10.1111/trf.15965