Recombinant blood group proteins facilitate the detection of alloantibodies to high-prevalence antigens and reveal underlying antibodies: results of an international study

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BACKGROUND: Alloantibodies to high-prevalence red blood cell (RBC) antigens are not easily identified by routine serologic techniques. This multicenter study was conducted to test the effectiveness of recombinant blood group proteins (rBGPs) at regional and international RBC reference laboratories.

STUDY DESIGN AND METHODS: Single or mixed soluble rBGPs (Lu, Yt, Kn, JMH, Sc, Rg, Ch, Do, and Cr) were assessed for their ability to inhibit the reactivity of antibodies to specific antigens. Initially, the effect of rBGPs was validated by testing panels of well-characterized patient serum samples containing antibodies to high-prevalence antigens in the hemagglutination inhibition assay. Subsequently, the rBGPs were prospectively used for routine antibody identification and the results were compared to those obtained with RBC-based diagnostics.

RESULTS: Panels of predefined antibodies to high-prevalence antigens were completely and specifically neutralized by the corresponding rBGP specificities. For prospective identification, antibodies to high-prevalence antigens (n = 62) were specifically inhibited by the corresponding rBGP specificities except for some Complement Receptor 1–related antibodies, which may be directed to epitopes not expressed on the truncated recombinant Kn. In 14 cases, additional clinically relevant alloantibodies were identified. In cross-matching, the rBGPs were successfully used to inhibit the reactivity of clinically irrelevant antibodies to high-prevalence antigens to determine compatibility between donor and recipient.

CONCLUSION: rBGPs enable the identification of antibodies to high-prevalence antigens without the need for rare RBC reagents, which are often unavailable. Underlying antibodies can be reliably detected and cross-matching results validated, resulting in a more efficient blood supply for immunized patients.

In current blood transfusion practice, red blood cell (RBC) antibodies are identified using panels of human RBCs pretyped for the most common blood group antigens. The specificity of a given antibody is identified based on the pattern of reactivity observed when serum is tested with the cell panel. Since many antigens are expressed on RBCs, antibody identification in RBC-based assays relies on nonreactivity of an antibody with panel cells lacking the corresponding antigen. This method of antibody identification is challenged if autoantibodies, multiple antibodies, or antibodies to high-prevalence antigens are present. In such cases, rare antisera and cells as well as specially trained personnel not available to routine laboratories are required for

ABBREVIATIONS: CR1 = Complement Receptor 1; HIA = hemagglutination inhibition assay; IBGRL = International Blood Group Reference Laboratory; LHR = long homologous repeat; MAEIA = monoclonal antibody–specific immobilization of erythrocyte antigens; rBGP(s) = recombinant blood group protein(s).

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antibody identification. This often results in the referral of samples to specialized reference laboratories, ultimately leading to a delay in antibody identification and a possible delay in patient care.

The availability of recombinant blood group proteins (rBGPs) enables the development of new antibody detection assays based on the use of defined antigens for each reaction.[1] Unlike current procedures, this novel approach directly indicates antibody specificities when an antibody reacts with its respective recombinant antigen. Moreover, it allows detection and identification of blood group antibodies in a single step, without the need for time-consuming additional tests. In particular, rBGPs may have wide applications in blood group serology as they can be used with different technical platforms, such as enzyme-linked immunosorbent assay, color-coded microspheres, and protein microarrays.[2-5] Previous studies have shown that rBGPs may be of great value for identification of alloantibodies to high-prevalence blood group antigens, which is still a major challenge for immunohematology laboratories.[2-12] If rBGPs are used as soluble protein reagents in the hemagglutinin inhibition assay, their implementation in blood group serology is straightforward, even in routine laboratories. Such reagents would enable serologists to identify antibodies directed against a host of distinct high-prevalence blood group antigens.

In this study, a panel of rBGPs was assessed by national and international blood group serology reference laboratories for their ability and usefulness to detect and identify antibodies to high-prevalence blood group antigens. First, the inhibitory effect of rBGPs was validated using predefined antisera. Subsequently, the recombinant proteins were prospectively used for routine antibody identification, and the results were compared to those obtained with RBC-based diagnostics.

MATERIALS AND METHODS

Study design

Over a 6-month period, single soluble rBGPs (Lu, Yt, Sc, Do^a, Do^b, Rg, Ch, Cr, Kn, and JMH) or cocktails thereof were assessed for their ability to inhibit the reactivity of antibodies to the corresponding antigens. Two national reference laboratories in Germany (the German Red Cross Blood Service Lower Saxony, Saxony-Anhalt, Thuringia, Oldenburg, and Bremen (NSTOB)/Institute Springe in Springe and the German Red Cross Blood Service Baden-Württemberg–Hessen/Institute for Transfusion Medicine and Immunohematology in Baden-Baden), a national reference laboratory in Ireland (National Blood Centre, Irish Blood Transfusion Service in Dublin), and an international reference laboratory in the United Kingdom (International Blood Group Reference Laboratory [IBGRL], National Health Service Blood and Transplant Filton Blood Centre in Bristol) participated in the study.

Initially, the inhibitory effect of rBGPs was validated by testing panels of well-characterized patient serum samples containing antibodies to high-prevalence antigens in the hemagglutination inhibition assay (HIA). A total of 40 antiserum samples with predefined antibodies to high-prevalence antigens obtained either from immunized in-house patients or from Serum, Cells and Rare Fluid (SCARF) exchange program members were used to test the specificity and activity of the single rBGP specificities and the rBGP cocktail. Patient serum samples containing RBC alloantibodies of other common or rare specificities (e.g., anti-K, anti-D, anti-c, anti-Jk^a, anti-Fy^a, anti-Fy^b, anti-S, anti-Vel, and anti-Lan) were used as reference samples.

Subsequently, the rBGPs were prospectively used by all participating laboratories for routine antibody identification, and the results were compared to those obtained with RBC-based diagnostics. At the two laboratories in Germany, where pretransfusion serologic cross-matching is mandatory, the rBGPs were used to inhibit the reactivity of clinically irrelevant high-prevalence antibodies to determine compatibility between donor and recipient.

Recombinant RBC proteins

Ten soluble rBGPs derived from eukaryotic expression systems (Imusyn GmbH, Hannover, Germany) were used in the study (Table 1). In earlier published and unpublished studies, they were designed and validated to express the most relevant polymorphic and high-prevalence antigens of the respective blood group systems.[3-5,8,10] A cocktail of five different rBGP specificities (rPromix) containing Sc, Rg, Ch, Kn, and JMH was prepared by mixing equal volumes of the respective single-rBGP solutions. The single-rBGP and rPromix products were supplied in storage buffer (150 mmol/L NaCl, 7 mmol/L sodium phosphate, pH 7.8, 0.1% ProClin 300, Sigma-Aldrich, St Louis, MO) at a concentration of 0.5 mg/mL, with a purity of more than 85% and a shelf life of 6 months. All protein solutions were stored at 2 to 8°C throughout the study. The single and mixed rBGPs were stored from 0 to 7 months before the start of validation testing.

Antibody detection procedures

Single and mixed rBGPs were used in the HIA for antibody detection, which is based on the principle that reactions between RBC antibodies and test cells carrying the corresponding antigens can be neutralized by incubating test samples with reagents containing soluble antigenic proteins. The ability of rBGPs to inhibit patient serum reactivity was assessed in an HIA using RBCs that tested positive for the respective high-prevalence antigens. If present, the corresponding RBC antibody is neutralized by

TABLE 1. Recombinant blood group antigens used in this study

rBGP name*	Common protein name†	Antigen(s)‡
Lu	B-CAM	Lu^{b},§ Lu3, Lu4, Lu5, Lu6, Lu8, Lu11, Lu12, Lu16, Lu17, Lu20, Lu21, LURC
Yt	AChE	Yt^{a}§
Sc	ERMAP	Sc1,§ Sc3,§ STAR,§ SCER, SCAN
Do^{a}	Dombrock	Do^{a},§ Gy^{a}, Hy, Jo^{a}, DOYA, DOMR, DOLG
Do^{b}	Dombrock	Do^{b},§ Gy^{a}, Hy, Jo^{a}, DOYA, DOMR, DOLG
Rg	C4A	Rg1,§ Rg2
Ch	C4B	Ch1,§ Ch2, Ch3, Ch4, Ch5, Ch6
Cr	DAF	Cr^{a},§ Tc^{a}, Dr^{a},§ Es^{a}, IFC, WES^{b}, UMC, GUTI, SERF, ZENA, CROV, CRAM, CROZ
Kn	CR1	Kn^{a},§ McC^{a},§ Sl^{a},§ Yk^{a},§ KCAM
JMH	Semaphorin 7A	JMH,§ JMHK,§ JMHL,§ JMHG,§ JMHM,§ JMHQ

* The rBGPs were named using the traditional initials of the respective blood group systems. The two Dombrock rBGPs were named according to the polymorphic Do^{a} and Do^{b} antigens.[13]

† The rBGPs were expressed as soluble forms of the listed proteins. For Lu, only the first three N-terminal IgSF domains and part of the fourth IgSF domain of the Lutheran glycoprotein were expressed. For CR1, only the homologous repeat D (LHR-D) domain was expressed.

‡ The antigens were called by their traditional names.

§ Those antigens shown in earlier studies to be correctly expressed and capable of inhibiting antibodies with the corresponding specificities.[3-5,8,10]

AChE = acetylcholinesterase; B-CAM = basal cell adhesion molecule; C4A = complement component isotype C4A; C4B = complement component isotype C4B; DAF = decay-accelerating factor; ERMAP = erythroid membrane-associated protein.

(i.e., reacts with) the recombinant protein during incubation and cannot cause a positive reaction in the HIA. If absent, the RBC antibody cannot be neutralized by (i.e., react with) the recombinant protein, and agglutination of the test RBCs is evident. Thus, a negative HIA indicates that an antibody to an antigen carried by the rBGP is present, and a positive HIA indicates either the absence of such an antibody or the presence of an additional antibody masked by the presence of the (inhibited) antibody to the rBGP in question. Therefore, a panel of test cells with different common phenotypes was used in the HIA to distinguish between positive reactions due to noninhibition of antibodies to high-prevalence antigens and positive reactions due to the presence of an additional antibody.

Gel agglutination–based systems providing standardized serologic procedures for measurement of antibody reactivity (ID-Card low-ionic-strength saline and antiglobulin test, Bio-Rad Laboratories, Hercules, CA, and ScanGel antiglobulin test anti-IgG, -C3d, Bio-Rad Laboratories, Munich, Germany) and a glass tube technique were used in the indirect antiglobulin test (IAT) for antibody detection. For antibody inhibition, aliquots of 0.5 µg or more of soluble rBGP were added to 25 µL (gel system) or 50 µL (glass tube technique) of patient serum and incubated for 30 minutes at room temperature. At one laboratory (Springe), 2.5 µg of soluble rBGP was alternatively mixed with 80 µL of plasma and incubated for 15 minutes at 37°C. After incubation, the serum-rBGP mixture was tested by IAT in each case. To distinguish antibody inhibition from a simple dilution effect, the antisera were also tested after addition of the same volume of phosphate-buffered saline (PBS) or inert AB serum instead of recombinant protein. Titration studies were performed with doubling dilutions of the respective antibody in PBS (pH 7.3) containing 6% bovine serum albumin. Agglutination reactions were assessed by IAT. Positive agglutination reactions were scored from 0.5+ (very weak) to 4+ (very strong), and negative agglutination reactions were assigned a score of "0," reflecting complete inhibition or a lack of reactivity. Agglutination reactions were scored visually in all four laboratories and additionally read microscopically in one laboratory (Bristol).

In selected cases, the IBGRL in Bristol used the monoclonal antibody–specific immobilization of erythrocyte antigens (MAIEA) technique for detection of antibodies to Knops system antigens on Complement Receptor 1 (CR1, CD35) and to Cromer antigens on decay-accelerating factor (CD55). This immunoassay technique is designed primarily to locate blood group antigens on specific RBC membrane proteins and does not allow for precise localization of antigens on proteins.[14]

RESULTS

Validation of the recombinant proteins

Single proteins

Each soluble rBGP specifically inhibited all antibodies to the corresponding high-prevalence antigen but did not affect antibodies to antigens not expressed by the recombinant protein (Table 2). Unfortunately, the performance of the Do constructs could not be tested in this study as samples containing anti-Do^{a} or anti-Do^{b} were not available. However, our previous validation studies have shown that antibody inhibition by the two polymorphic recombinant Do proteins is efficient and highly specific (Table 2).

TABLE 2. Single-protein inhibition study

rBGP	Antisera tested* Specificity	Number	Titer†	Inhibition§	Comment‡
Lu	Anti-Lu^a	2	ND	N	Seltsam *et al.*[3]
	Anti-Lu^b	3	ND	Y	
Yt	Anti-Yt^a	3	ND	Y	
Sc	Anti-Sc1	2	4 and 32	Y	Seltsam *et al.*[8]
Do^a	Anti-Do^a	2	4 and 8	Y	Results from an unpublished study
	Anti-Do^b	2	4 and 4	N	
Do^b	Anti-Do^a	2	4 and 8	N	Results from an unpublished study
	Anti-Do^b	2	4 and 4	Y	
Rg	Anti-Rg	4	64-640	Y	
	Anti-Ch	2	4 and 64	N	
Ch	Anti-Rg	4	64-640	N	
	Anti-Ch	2	4 and 64	Y	
Cr	Anti-Cr^a	2	ND	Y	
Kn	Anti-Kn^a	11	8-512	Y	See Table 3 for detailed results
	Anti-Yk^a	1	164	Y	
	Anti-McC^a	1	128	Y	
	Anti-Kn^a/McC^a	1	16	Y	
JMH	Anti-JMH	2	ND	Y	Seltsam *et al.*[5]

* Reactivity of patient serum samples containing RBC alloantibodies of other common or rare specificities (e.g., anti-K, anti-D, anti-c, anti-Jk^a, anti-Fy^a, anti-Fy^b, anti-S, anti-Vel, anti-Lan) were not inhibited by each of the soluble rBGPs.
† A titer range is given if more than 2 antisera were tested. ND = not determined
‡ References refer to additional validation studies published previously.
§ Y = successful inhibition; N = no inhibition; with exception of the Kn^a antibodies, 1 μg of soluble rBGP was sufficient to achieve complete inhibition.

TABLE 3. Kn inhibition study

Kn antisera Number	Specificity	Titer	Recombinant Kn dose (μg)* 0.5	1	1.5
1	Anti-Kn^a	512	0		
2	Anti-Kn^a	512	0		
3	Anti-Kn^a	64	0		
4	Anti-Kn^a	8	0		
5	Anti-Kn^a	512	0		
6	Anti-Kn^a	64	0		
7	Anti-Kn^a	8	0		
8	Anti-Kn^a	128	0		
9	Anti-Kn^a	512	0		
10	Anti-Kn^a	32	2+	0.5+	0
11	Anti-Kn^a	8	2+	0.5+	0
12	Anti-Yk^a	164	0		
13	Anti-McC^a	128	0		
14	Anti-Kn^a/McC^a	16	0		

* Serologic results were determined by IAT. Positive agglutination reactions were scored from 0.5+ (very weak) to 4+ (very strong), and negative agglutination reactions were assigned a score of "0," reflecting complete inhibition.

Activity of the recombinant Kn protein was further analyzed by the IBGRL in Bristol using an extended panel of Kn antisera with different specificities and titers (Table 3). In this study, a panel of RBCs (six donors) with high levels of the respective high-prevalence Kn antigens were tested by IAT. As demonstrated by gel agglutination–based IAT, 12 of 14 Kn antisera completely lost their ability to agglutinate test cells after incubation with 0.5 μg of soluble recombinant Kn protein. With the remaining two antisera, reactivity decreased with increasing amounts of soluble Kn and was completely inhibited after incubation with 1.5 μg of Kn protein. Interestingly, there was no correlation between the antibody titer and the inhibitory effect of soluble Kn protein. Whereas Kn antibodies with titers of up to 512 were completely inhibited by the lowest dose of protein, the two antibodies requiring three times as much recombinant protein for complete inhibition had relatively low titers of 8 and 32, respectively.

Inhibition of anti-Kn^a by soluble recombinant Kn protein allowed the detection of admixed clinically relevant antibodies present in the same sample. Figure 1 shows that detection and identification of anti-Jk^a was possible in a single step without the need for a panel of rare Kn(a–) RBC samples. Comparable results were obtained with serum samples containing mixtures of anti-Kn^a plus anti-E, anti-K, or anti-Fy^a after inhibition of anti-Kn^a with soluble Kn protein (data not shown).

Protein mixture

The cocktail (rPromix) of five recombinant proteins containing the specificities Sc, Rg, Ch, Kn, and JMH was additionally analyzed by the IBGRL in Bristol using an extended panel of antisera with different specificities and titers of antibodies (Table 4). RBCs with high levels of the respective high-prevalence antigens were used in the IAT. A quantity of 0.5 μg of rPromix completely inhibited antibodies to Sc1, Rg, Ch, and Kn^a, independent of antibody titers. The two JMH antibodies required three times more cocktail (1.5 μg) for full inhibition in the IAT.

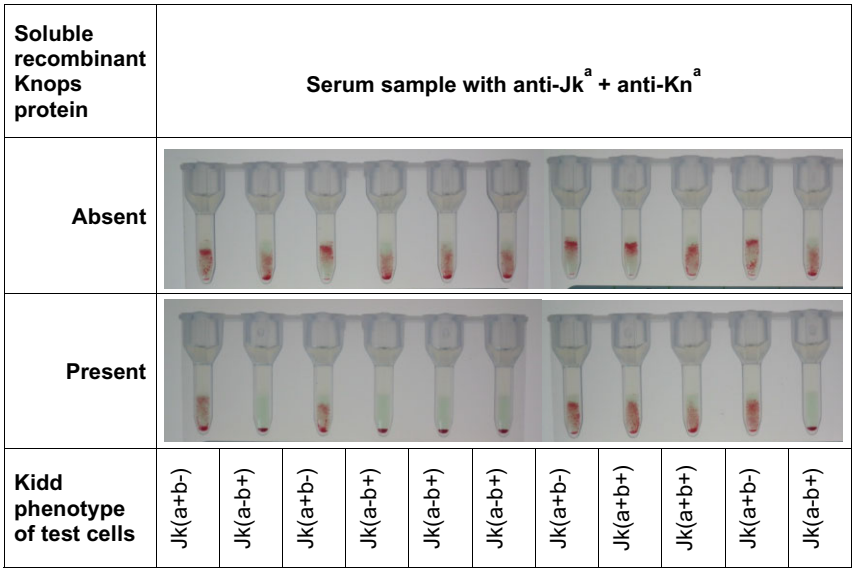

Fig. 1. Specific detection of admixed alloantibodies in the presence of anti-Kn[a]. As all RBC samples were agglutinated by mixtures of anti-Kn[a] and anti-Jk[a] (top panels), the admixed antibodies with common specificities were masked. The addition of soluble recombinant Kn protein inhibited the anti-Kn[a] component of the serum, making the clinically relevant alloantibody to Jk[a] easily identifiable (bottom panels).

TABLE 4. rPromix inhibition study

Antisera			rPromix dose (μg)*		
No.	Specificity	Titer	0.5	1	1.5
1	Anti-Sc1	32	0		
2	Anti-Sc1	4	0		
3	Anti-Rg	160	0		
4	Anti-Ch	640	0		
5	Anti-Ch	160	0		
6	Anti-Kn[a]	512	0		
7	Anti-Kn[a]	64	0		
8	Anti-Kn[a]	8	0		
9	Anti-JMH	16	1	0.5+	0
10	Anti-JMH	16	1	0.5+	0

* Serologic results were determined by IAT. Positive agglutination reactions were scored from 0.5+ (very weak) to 4+ (very strong), and negative agglutination reactions were assigned a score of "0," reflecting complete inhibition.

Prospective use of recombinant proteins for antibody identification

The rBGPs were used to identify RBC antibodies to high-prevalence antigens in in-house patient samples or patient samples submitted to the participating laboratories from external laboratories for antibody determination during the 6-month study period. For prospective identification, the HIA was performed using 1 μg or more of rBGP. This amount was used as the initial dose because data from our previous studies with well-characterized antisera had shown that 1 μg of protein is sufficient to inhibit the vast majority of antibodies to high-prevalence antigens. The amount was increased only if inhibition was unsuccessful or incomplete. The rBGPs showed no loss of activity when stored as a 0.5 mg/mL solution at 4°C for 6 months.

A total of 81 cases with antibodies to high-prevalence blood group antigens were included in the analysis (Table 5). In 62 (77%) of 81 cases, antibodies to high-prevalence antigens were specifically inhibited by the corresponding rBGP specificities. Of these 62 cases, five required 2 μg or more of rBGP for full neutralization of Ch (n = 1), Rg (n = 1), and CR1-related (n = 3) antibodies, six contained one additionally clinically relevant alloantibody, and eight contained multiple additional clinically relevant alloantibodies.

The remaining 19 (24%) of the 81 cases did not show specific inhibition. Of these samples, six contained CR1-related antibodies diagnosed by traditional RBC serology using rare blood samples and could not be inhibited by recombinant Kn, even when up to 10 μg of the protein was used for inhibition. The remaining 13 cases demonstrated antibodies to high-prevalence antigens unrelated to the rBGPs (n = 10), warm-reactive antibodies (n = 4), or indefinable reactions (n = 3).

Compatibility between donor and recipient was clearly identified by the two German Red Cross laboratories that used rBGPs for serologic cross-matching to inhibit the reactivity of clinically irrelevant antibodies to high-prevalence antigens. Negative reactions of patient sera with donor cells in the IAT performed after incubation of the samples with the rBGPs excluded the presence of clinically relevant antibodies to low-prevalence antigens that might have been missed in the preceding antibody identification process.

Only the protein specificities but not the exact antigenic epitopes of 21 successfully inhibited antibodies could be determined. These antibodies were consequently classified as CR1-related (n = 20) and Cromer-related (n = 1) antibodies. One CR1-related antibody and the Cromer-related antibody were additionally verified using the MAIEA assay.[14] The six CR1-related antibodies that could not be inhibited by recombinant Kn as well as the Cromer-related antibody were suspected of having novel antigen specificities.

For prospective identification, the recombinant proteins were generally used in parallel with conventional serology using blood samples with rare phenotypes and/or plasma neutralization in the case of Chido/Rodgers antibodies. In the majority of the cases, the recombinant proteins were used to confirm the results

TABLE 5. Prospective use of rBGPs for antibody identification

Specificity of antibody to high-prevalence antigen	Number of cases	Underlying antibodies: Number of cases	Underlying antibodies: Specificities	Comment
Cases in which neutralization was successful				
Anti-Ch	18	4	Anti-E plus anti-Fy[a] Anti-c plus anti-Kp[a] Anti-C plus anti-Jk[a] Anti-Fy[b] plus anti-Jk[b] plus anti-Au[b]	In one case, 2 μg of protein was required for neutralization
Anti-Rg	8	1	Anti-D plus anti-S	In one case, 5 μg of protein was required for neutralization
Anti-Kn[a]	8	2	Anti-K Anti-Fy[a] plus anti-S	
Anti-Kn[a]/McC[a]	1	0		
Anti-CR1-related	20	5	Anti-K Anti-Fy[a] Anti-U plus anti-N Anti-S Anti-Jk[a]	In three cases, more than 2 μg of protein was required for neutralization One antibody was confirmed in the CR1-MAIEA*
Anti-Yt[a]	2	2	Anti-K Anti-c plus anti-Jk[a]	
Anti-Lu8	1	0		
Anti-JMH	1	0		
Anti-Cr[a]	1	0		
Anti-Cromer related	1	0		DAF-MAIEA positive,† novel specificity suspected
Anti-WES[b]	1	0		
Cases in which neutralization was unsuccessful				
Anti-CR1 related	6	0		
Anti-Cs[a]	1	0		
Anti-Fy3	1	0		
Warm-reactive autoantibodies	4	0		
Undetermined reactions	7	0		

* CR1-MAIEA = MAIEA test for detection of antibodies to Knops antigens on the CR1 (CD35)[14]
† DAF-MAIEA = MAIEA test for detection of Cromer antibodies on the decay-accelerating factor (DAF, CD55).[14]

obtained with rare blood samples or plasma neutralization. However, at two laboratories (Dublin and Springe), recombinant proteins were also used first. The Irish laboratory used the recombinant Kn protein on two occasions before confirming the presence of a CR1-related antibody with rare cells. Both occasions involved an urgent request for blood for African patients in two different Dublin maternity hospitals. The laboratory in Springe used the rBGPs before applying conventional serology in more than half of its cases. There, the decision whether to use recombinant proteins before or after conventional antibody diagnostics was mainly dependent on the scientist's and/or technician's individual experience with the use of recombinant proteins.

DISCUSSION

This study demonstrates that rBGP-based diagnostics can easily be implemented in RBC serology and used for the detection and identification of alloantibodies to high-prevalence blood group antigens. With this technology, patient sera containing the underlying clinically significant alloantibodies were readily recognized without the need for a panel of rare blood samples. Moreover, rBGPs were successfully used to inhibit several clinically insignificant antibodies to high-prevalence antigens in pretransfusion cross-matching, allowing for determination of compatibility between donors and recipients.

Alloantibodies to high-prevalence blood group antigens can be problematic in transfusion medicine, and their identification requires advanced diagnostic procedures. The subsequent provision of transfusion support for patients with antibodies to high-prevalence antigens can be challenging due to the scarcity of compatible units, complex logistics, and the lack of experience on the part of many physicians in treating these patients.[15] Although most antibodies to high-prevalence antigens are not hemolytic and do not reduce the survival of transfused incompatible RBCs, they can cause an incompatible cross-match and must be identified before they can be dismissed as clinically insignificant.[16] They can also mask the presence of additional more clinically significant antibodies. Therefore, having an easy tool to improve diagnostics in these cases would be a significant improvement in the provision of suitable RBCs to patients immunized against high-prevalence antibodies.

In a Caucasian population, there were only a handful of antibody specificities that accounted for more than

two-thirds of cases with clinically relevant and irrelevant alloantibodies to high-prevalence antigens; these included anti-Kp^b, anti-Lu^b, anti-Yt^a, and anti-Vel as clinically relevant antibodies and anti-Ch, anti-Rg, and anti-Kn^a as antibodies that are generally considered as clinically irrelevant, although hemolytic transfusion reactions associated with Kn antibodies have been described.[15-17] The panel of rBGPs used in this study makes it possible to identify the majority of these antibodies as well as additional antibody specificities (Sc, Do^a, Do^b, Cr, and JMH). Among those protein specificities that will further complement the panel of soluble rBGP for detection of alloantibodies to high-prevalence antigens significantly are k, Kp^b, and Xg^a, which all have single extracellular domains and are therefore good candidates for expression as soluble recombinant molecules.

The inhibition of an antibody to a high-prevalence antigen by a single rBGP directly indicates the specificity of this antibody if only one high-prevalence antigen is described for this protein (e.g., Yt^a). However, as most recombinant proteins express more than one high-prevalence antigen, inhibition by a single rBGP indicates the protein specificity but not the antigen specificity of the antibody. In these cases, two "antithetical" recombinant proteins differing only in the expression of the antigen of interest are needed for identification of the exact antigen specificity of the antibody. This means that one construct must carry the high-prevalence antigen (e.g., Lu^b) while the other expresses a low-prevalence antigen (e.g., Lu^a) at the same protein position. In cases with clinically insignificant antibodies to high-prevalence antigens, it is generally sufficient to determine the protein rather than the antigen specificity. When dealing with clinically relevant antibodies, however, exact knowledge of the antibody specificity is absolutely essential for the provision of compatible blood units. Thus, if rBGPs are used solely for the identification of clinically relevant antibodies to high-prevalence antigens rather than as a complement to traditional serology, an rBGPs-based approach must include "antithetical" recombinant protein pairs, particularly for those antibodies for which compatible RBC units are available.

As the clinical relevance of an antibody appears to be determined to some extent by blood group protein specificity, a pragmatic and straightforward approach is to limit diagnostics of clinically insignificant antibodies to determination of the target protein. Once these antibodies have been recognized, they can be ignored in further blood provision for immunized patients. The use of recombinant proteins not only speeds up the identification of clinically insignificant antibodies, but also increases blood safety by facilitating the detection of underlying clinically relevant antibodies. This added benefit prompted two of the participating laboratories to use the rBGPs in first-line diagnostic testing.

The rationale behind using a cocktail of soluble rBGPs is that one can inhibit several clinically insignificant antibodies to high-prevalence antigens in a single test, which essentially eliminates the need to determine such specificities in routine serology. Without the need to identify the exact antibody specificity, the patient's serum can be treated with the neutralizing cocktail, which allows detection of any clinically significant alloantibody present and facilitates safer compatibility testing. In addition, it is predicted that a panel of protein cocktails containing several different sets of rBGPs (e.g., for different ethnic groups) can be used to identify or exclude clinically significant antibodies to high-prevalence antigens.[1]

Correct antigen presentation is a crucial factor to consider when developing reliable and sensitive recombinant protein-based antibody detection systems. The recombinant proteins used in this study were produced in a eukaryotic expression system that allows for posttranslational modifications, such as glycosylation and disulfide bonding. Both the high capacity and the specificity of antibody inhibition by the rBGPs in this and previous studies strongly suggest accurate folding of the proteins and correct formation of the blood group antigens carried by the recombinant proteins. Although neither Lu8 on the Lutheran glycoprotein nor WES^b on the Cr protein had been tested before, these antigens efficiently neutralized the corresponding antibodies in the inhibition assay used in this study. In general, this study and earlier studies have demonstrated that the tested rBGPs remain stable for 6 months or longer when stored at 2 to 8°C, independent of the specificity and source of the protein.[3-5,8] This also holds true when the proteins are used in highly concentrated cocktails. Their shelf life may be extended by cryopreservation, which would even make it possible for smaller laboratories to keep small stocks of rBGPs for antibody identification.

The long-lasting conformational integrity of the recombinant proteins suggests that the failure of the Kn protein to inhibit some of the CR1-related antibodies is more likely due to these antibodies being directed to epitopes not expressed on the truncated recombinant CR1 than to a problem of protein quality. The Kn protein used in this study consisted of the long homologous repeat (LHR)-D domain, on which all known antigens of the Knops blood group system are localized. Consequently, these CR1-related antibodies may be directed to Kn^a-like epitopes on the LHR-C domain or may have as yet undefined antigen specificities.[13,18] Sequence analysis of the complete *KN (CR1)* gene of immunized patients may reveal new mutations encoding for novel antigens that reside on the other LHRs of the CR1 molecule.

In most cases, 1 µg of rBGP was sufficient to completely or partly inhibit the corresponding antibodies. The rBGP solutions allowed us to increase the protein dose without significant dilution effects in samples requiring

more protein for antibody removal. Interestingly, successful inhibition with the Kn protein did not seem to correlate with the Kn^a antibody titers. This phenomenon may be due to a differential reactivity of some of these high-titer, low-avidity antibodies with proteins in solution and membrane-anchored proteins and warrants further investigation.

The results of this and previous studies have shown that the rBGPs have the same activity and specificity in single-protein solutions as in protein mixtures, indicating that the recombinant proteins are not affected when stored in a cocktail.[8] The reason why, in this study, two JMH antibodies required three times more cocktail for full inhibition in the IAT than the other antibody specificities is unclear. Unfortunately, these two JMH antibodies were not tested in parallel with the single JMH protein solution. Some JMH antibody samples may exhibit special characteristics similar to what has been observed for Kn^a antibodies in this study.

In conclusion, this study demonstrates that the use of recombinant proteins in immunohematology laboratories is feasible and facilitates the detection of antibodies to high-prevalence antigens. By converting advanced RBC antibody diagnostics from a "mystical art" to a rational procedure available to all routine laboratories, this novel technology could help to improve the blood supply for patients with rare RBC antibodies.

CONFLICT OF INTEREST

AS is a member of the scientific advisory board of Imusyn. DG and CS are employees of Imusyn. RB is a shareholder of Imusyn. The other authors report no conflicts of interest or funding sources.

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