Red cell antibody identification by solid phase red cell adherence utilizing dried RBC monolayers

D.L. Stone, R.A. Eatz, S.D. Rolih,
S.J. Farlow, G.S. Hudson, L.T. Sinor

Abstract: Recent technological advances in the immobilization and drying of red cell monolayers for use in solid phase red cell adherence (SPRCA) assays have resulted in the development of reagent red cells for antibody screening and identification that are stable at room temperature. Panels consisting of twelve different RBC samples dried onto individual microplate wells were evaluated with 176 samples whose antibody specificities had previously been determined by conventional hemagglutination techniques. Identification tests performed with dried SPRCA panels proved to be more sensitive and less time consuming than hemagglutination tests. The red cell antigens of dried membranes were shown to be stable and reactive following storage for 120 days at room temperature.

First-generation solid phase red cell adherence (SPRCA) assays require preparation of monolayers of intact red cells in individual microplate wells just before testing.[1–3] Before exploring new applications for second-generation solid-phase testing, an SPRCA assay using monolayers of dried donor cells prepared in advance in microplate wells was tested. Two advantages were evident. The microplate could be leisurely prepared and stored until needed. Hemoglobin-free dried monolayers were transparent, which facilitated reading and interpretation of test results.

Several studies were initiated using the dried SPRCA assay. Specificity of 176 sera containing red cell antibodies that had previously been determined using a hemagglutination method were tested in microplates containing panels of dried red cell monolayers. Selected antibodies were diluted and tested in parallel by hemagglutination and by solid phase using dried monolayers. In addition, the stability of red cell monolayers dried to microplate wells and stored at room temperature for up to 120 days was evaluated.

Materials and Methods

First-generation SPRCA assays were prepared by adding one drop of a 1 percent (v/v) suspension of red cells in isotonic saline to antibody-coated or chemically modified (Immucor, Inc., Norcross, GA) microtiter wells. U-bottom microplates (Dynatech Laboratories, Chantilly, VA) containing 96 wells or stripwells (Nunc, Roskilde, Denmark) containing 16 wells were used. Red cell monolayers were formed by centrifuging the microplates or stripwells in a Sorval GLC-2B (E.I. duPont de Nemours & Co., Wilmington, DE) at 200 × g for five minutes. Following a saline wash to remove excess unbound red cells, the microplates or stripwells were maintained wet in saline prior to use. Second-generation SPRCA assays contained red cell monolayers prepared on chemically modified microtiter wells as described above and then dried onto the microwell surface (Immucor, Inc.).

Antibody identification assays were performed by adding one drop of serum or plasma plus two drops of 1.9 percent glycine (low ionic potentiator) to 12 wells containing the dried red cell membranes from selected group O donors. Following a 15-minute incubation at 37°C, the wells were washed with an automated microplate washer (BioTek Model EL402, BioTek Instruments, Winooski, VT; or Handiwash 110, Flow Labs, McLean, VA). One drop of monoclonal anti-IgG-coated indicator red cells was added to each well and the microplate or stripwell was centrifuged in a Sorval GLC-2B at 900 × g for 1 minute. Positive reactions were characterized by adherence of the indicator red cells to part or all of the well surface, whereas negative tests appeared as tight buttons of indicator red cells at the bottom of the test well, with no area of adherence. The pattern of reactivity obtained with the dried SPRCA panel was compared with the results on an antigen profile sheet to determine if the specificity of each antibody was identified correctly.

Hemagglutination tests were used to determine antibody specificities. The hemagglutination tests were performed in duplicate by adding one drop of a 4 percent (v/v) suspension of single donor commercial panel cells to glass test tubes plus two drops of plasma or serum. One set of tubes was centrifuged immediately; the strength of agglutination and degree of hemolysis observed with each panel cell was recorded. Low ionic strength solution (LISS) was added to the second set of tubes according to the manufacturer's instructions. Following a 10- to 15-minute incubation at 37°C, the tubes were centrifuged and the reaction mixture examined for agglutination and hemolysis. The red cells were then resuspended as completely as possible and washed with saline, and commercial antihuman globulin was added according to the manufacturer's instructions. The tubes were centrifuged, agglutination results recorded, and specificity determined. The commercial panel cells used for hemagglutination tests were different from those employed in dried SPRCA assays.

Sensitivity of dried red cell monolayers used in SPRCA assays was compared to intact red cells used in a conventional tube test by titration using master dilutions of anti-D, -Fy^a -Jk^a, and -M. Three work sites involved in this study compared the sensitivity of the two methods, using the same red cells carrying a single dose of the respective antigen. Titration endpoints were defined as the last dilution producing visible positive reactions in each test system.

Stability of the dried SPRCA red cell antigenic determinants was evaluated in tests with serial dilutions of select antisera (anti-D, -c, -K, -Fy^a, -Fy^b, -M, -N, -S, -s, -Jk^a, -Jk^b, -Lu^a, and -Lu^b). Evaluations were performed at days 14, 67–71, and 108–120 following storage of dried red cell monolayers at room temperature. On each test date, results obtained with dried red cells were compared to those obtained in parallel using solid phase tests incorporating intact red cell monolayers prepared from freshly drawn samples of the same donors. The results obtained in titration tests were assigned a score value based on a grading system (Fig. 1). The final score of each titration was calculated by adding the score values of all reactive test wells. The titration value was calculated as the last serial dilution giving a positive reaction (score value of ≤ 3).

A total of 176 samples obtained from patients or commercial reagents that contained antibodies of known specificity by hemagglutination methods were evaluated on dried SPRCA panels (Table 1). These samples were

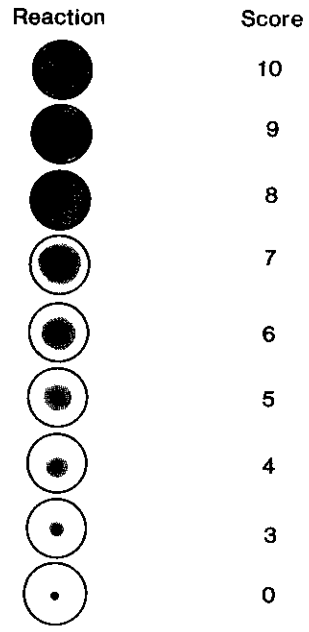

Fig.1. SPRCA scoring system. Scoring values range from a 10 for a strong positive reaction to a 0 for a negative reaction.

Table 1
Comparison of antisera tested by dried SPRCA panels and hemagglutination panels

Anti-	No. tested	SPRCA reactions agreeing with hemagglutination	SPRCA reactions differing from hemagglutination
D	28	28	0
C	5	4	1
c	5	4	1
E	14	13	1
e	9	8	1
V	4	3	1
C^w	5	5	0
K	18	18	0
k	4	4	0
Kp^a	5	3	2
Kp^b	5	5	0
Js^a	2	2	0
Js^b	4	4	0
Fy^a	7	6	1
Fy^b	6	5	1
Jk^a	7	6	1
Jk^b	7	5	2
Le^a	1	0	1
Le^b	1	0	1
P_1	3	0	3
M	7	5	2
S	4	2	2
s	6	5	1
Lu^a	4	4	0
Lu^b	4	4	0
Xg^a	6	5	1
Yt^b	1	1	0
Co^b	2	2	0
Totals	176	151	25

stored at −20°C before use. Included in the evaluation were antibodies to antigens of the Rhesus, Kell, Duffy, Kidd, Lewis, P, MNSs, Lutheran, Xg^a, Cartwright, and Colton systems.

Results

Of the 176 samples tested, 151 produced results in agreement with hemagglutination tests (Table 1). With 25 samples, results obtained in dried SPRCA assays differed from those of hemagglutination tests and were evaluated further (Table 2). Of the 25 samples, 10 (samples 1–10 in Table 2) failed to react with any red cell sample by dried SPRCA assay but were reactive by hemagglutination; 12 (samples 11–22) produced positive reactions with all panel cells, including red cells lacking the appropriate antigen; and three (samples 23–25) produced reactivity patterns suggesting that antibody(ies) in addition to those detected by hemagglutination were present.

Of the 10 samples that were nonreactive by the dried SPRCA assay (Table 2), eight were found to contain IgM antibodies (anti-Le^a, -Le^b, -P_1, and -S). Such antibodies are not detected by the anti-IgG indicator cells used in the dried SPRCA assay. When these samples were retested using anti-IgM-coated indicator cells, they all produced the expected reactions with dried red cells. The eight antibodies were detected predominately at room temperature. These reactions were abolished by reduction with 2-mercaptoethanol, confirming that these samples contained IgM antibodies. Two additional samples (anti-c and anti-Kp^a) that failed to react by the dried SPRCA assay displayed gross bacterial contamination. Repeat testing of the contaminated samples by hemagglutination confirmed the loss of reactivity.

When samples were diluted, specificity was restored for 11 of 12 samples (samples 11, 13–22) initially reactive with all panels cells by dried SPRCA assay. Repeat testing of undiluted samples by hemagglutination demonstrated a single antibody specificity. One sample from a patient (sample 12) who initially demonstrated anti-E specificity by hemagglutination was reactive with all dried panel cells regardless of dilution. Absorption and elution of the sample with cDE/cDE red cells and repeat testing on the dried SPRCA panel produced the desired anti-E specificity. Further testing on the dried SPRCA panels failed to determine the cause of the additional reactions.

Table 2
Twenty-five samples producing different results between hemagglutination and dried SPRCA panels

No.	Sample source	Hemagglutination result	Reason for unexpected result
		Samples initially nonreactive with all dried panel cells	
1	Patient	anti-Le^a	IgM antibody
2	Reagent	anti-Le^a	IgM antibody
3	Patient	anti-Le^b	IgM antibody
4	Reagent	anti-Le^b	IgM antibody
5	Patient	anti-c*	Deterioration of sample
6	Patient	anti-Kp^a*	Deterioration of sample
7	Patient	anti-P_1	IgM antibody
8	Patient	anti-P_1	IgM antibody
9	Patient	anti-P_1	IgM antibody
10	Patient	anti-S	IgM antibody
		Samples initially reactive with all dried panel cells	
11	Reagent	anti-C	Specific at 1:40 dilution
12	Patient	anti-E	Non-specific at all dilutions
13	Reagent	anti-e	Specific at 1:20 dilution
14	Reagent	anti-Kp^a	Specific at 1:40 dilution
15	Reagent	anti-Fy^a	Specific at 1:20 dilution
16	Reagent	anti-Fy^b	Specific at 1:10 dilution
17	Reagent	anti-Jk^a	Specific at 1:10 dilution
18	Reagent	anti-Jk^b	Specific at 1:10 dilution
19	Reagent	anti-Jk^b	Specific at 1:20 dilution
20	Reagent	anti-M	Specific at 1:20 dilution
21	Reagent	anti-s	Specific at 1:20 dilution
22	Reagent	anti-Xg^a	Specific at 1:20 dilution
		Samples containing additional antibodies	
23	Patient	anti-V	Weak anti-C also present
24	Patient	anti-M	Weak anti-D also present
25	Patient	anti-S	Weak anti-D also present

*Nonreactive by hemagglutination on repeat testing

For samples 23–25, the presence of multiple alloantibodies was shown to be the cause of unexpected reactions by dried SPRCA assay. The sera displayed a single specificity using hemagglutination, but additional antibodies were found in these sera when the dried SPRCA assay was used.

The sensitivity of the dried SPRCA panels was more directly compared to that of hemagglutination by parallel titration studies using the same red cells in each test system. The red cells carried a single dose of the particular antigen. As seen in Table 3, of the antibodies tested (anti-K, -D, -Jk^a, -Fy^b), all reacted to higher dilutions in the dried SPRCA assay than with hemagglutination. The dried SPRCA assay titration end points were typically one to two dilutions higher than those obtained with hemagglutination.

Since the sensitivity of the dried solid phase system is dependent on the stable expression of the red cell antigens, the expressions of the antigens on dried red cell membranes were compared to those found on the surface of the same red cells collected immediately before each test date. As shown in Table 4, antigens that are carried on dried red cell membranes are stable for 108–120 days when stored at room temperature.

Using a 12-cell panel, a comparison of the average time needed to complete antibody identification on eight samples from patients by hemagglutination and by dried SPRCA assay was performed. For tube/plate preparation, reagent/specimen addition, and the test procedure itself (including incubation, centrifugation, washes, and reagent additions), identification tests incorporating dried SPRCA panels took less than one-third of the time needed to perform similar tests by hemagglutination [27 minutes and 99 minutes, respectively (Table 5)].

Discussion

The use of dried SPRCA panels provides several advantages over identification tests incorporating red cell suspensions and hemagglutination. These advantages include increased sensitivity, prolonged stability, and simplicity.

The sensitivity of dried SPRCA assays was typically one to two dilutions higher than those obtained by hemagglutination. This could be due in part to the replacement of polyclonal rabbit antihuman IgG-coated indicator cells, used in first-generation solid-phase assays, with murine monoclonal antihuman IgG (heavy chain specific). Previous studies have demonstrated the increased sensitivity of monoclonal indicator cells compared to polyclonal indicator cells in solid phase antibody detection assays (unpublished data). In our study, the monoclonal antihuman IgG allowed easier standard-ization of the coating of the indicator cells and provided better lot-to-lot consistency of the finished indicator cells.

The dried red cell monolayers were found to be stable for up to 120 days when stored at room temperature. Additional studies have determined that the dried red cell antigens were stable when subjected to extremes of temperature and humidity (data not shown). Room temperature stable panels could help alleviate reagent storage problems by freeing up space in cramped laboratory refrigerators. Furthermore, the future availability of dried monolayers of rare red cells could eliminate the need for freezer storage and the accompanying loss of valuable red cells when they are reconstituted.

Previous first-generation solid-phase assays required the preparation of monolayers of intact red cells prior

Table 3
Determination of sensitivity between hemagglutination and the dried SPRCA assay by titration

		Titration end point	
Antibody	Test work site	Hemagglutination	Dried SPRCA
Anti-K	1	8	16
	2	4	16
	3	8	16
Anti-D	1	4	32
	2	4	32
	3	4	16
Anti-Jk^a	1	4	32
	2	2	16
	3	8	16
Anti-Fy^b	1	8	16
	2	4	16
	3	8	16

Table 4
Stability of antigens on dried red cell monolayers stored at room temperature compared to antigens on fresh red cell monolayers from the same donors

		Test dates (by day)		
		14	67–71	108–120
Antibody	RBC monolayer	Titer/Score	Titer/Score	Titer/Score
Anti-D	Dried	32/51	32/52	32/48
	Fresh	32/52	32/52	16/39
Anti-c	Dried	32/60	32/58	128/79
	Fresh	16/45	32/55	128/79
Anti-K	Dried	8/25	4/19	4/16
	Fresh	8/27	4/21	2/17
Anti-Fy^a	Dried	64/55	128+/58+	64/58
	Fresh	32/51	64/54	64/67
Anti-Fy^b	Dried	16/45	128+/57+	128+/68+
	Fresh	32/56	32/49	64/70
Anti-M	Dried	8/22	8/18	4/11
	Fresh	1/5	4/4	<1/<1
Anti-N	Dried	128+/36+	16/40	16/42
	Fresh	<1/<1	2/20	8/40
Anti-S	Dried	32/64	16/46	64/50
	Fresh	8/37	8/31	4/30
Anti-s	Dried	32/42	32/41	16/50
	Fresh	8/39	32/48	32/59
Anti-Jk^a	Dried	16/21	16/21	8/12
	Fresh	2/9	4/14	1/3
Anti-Lu^b	Dried	64/45	64/40	64/62
	Fresh	16/38	16/36	64/60

Table 5
Average time needed to complete antibody identification on eight samples using the same 12-cell panel

	Test	
Step	Hemagglutination test (minutes)	Dried SPRCA assay (minutes)
Tube/plate preparation	5	1
Reagent/specimen addition	6	4
Test procedure	87	21
Reading/recording results	1	1
Total for eight samples	99	27

to testing. The automated reading and interpretation of test results using the intact monolayers proved difficult because of reduced sensitivity associated with the high absorbance of hemoglobin. A benefit of the drying process was the removal of hemoglobin from membrane layers, resulting in a transparent reaction surface. The transparent background makes it easier to differentiate positive and negative reactions visually and with inexpensive spectrophotometric devices.

Several possible explanations could account for the additional reactions detected with samples 11–22 in the dried SPRCA assay (Table 2). Since different panel cells were utilized in hemagglutination tests, the dried SPRCA panels could have detected underlying antibody specificities to antigens not present on the panel cells used in the hemagglutination tests. In addition, the drying of red cell monolayers for the SPRCA assay could expose or produce antigenic determinants not present on intact red cells. The 11 serum examples would then have to contain antibodies to these cryptantigens. Both explanations are highly unlikely. Another possibility, since the majority (11/12) of the samples producing

discrepant results were of commercial origin, was that potentiators used in the commercial antisera contributed to the nonspecific reactions. The causes of the nonspecific reactions are currently being investigated.

Many of the special test procedures utilized by reference laboratories to help identify the specificity of RBC specific antibody(ies) could theoretically be performed on dried SPRCA panels. For example, the dried immobilized red cell monolayers can easily be treated with enzymes in the microtiter wells. Dried SPRCA panels are amenable to incubations at various temperatures, including 4°C, room temperature, and 37°C. The inclusion of uncoated, chemically modified microtiter wells could allow the immobilization of autologous red cells to help determine whether alloantibody, autoantibody, or both are present in the sample. Furthermore, the utilization of anti-IgM and anti-IgA indicator cells in addition to the anti-IgG indicator cells would allow the identification of any immunoglobulin heavy chain class of alloantibody.

The solid phase scoring system (Fig. 1) also provides a means of grading differences in the strengths of reactions on solid phase. Frequently, different antibodies will produce similar strong adherent reactions in solid phase tests, even though such antibodies react +w, 1+, 2+, 3+, or 4+ in hemagglutination tests.[1–3] Some antibodies tested by SPRCA assay also produce differing degrees of adherence in positive wells. Therefore, the method of scoring (grading) used in this study (Fig. 1) provided help in selecting reactions due to different antibodies in three of the sera evaluated (samples 23–25, Table 2).

This study showed that the use of the dried SPRCA assay for antibody identification was as accurate as and more sensitive than hemagglutination testing. In fact, a timed study showed a saving of 72 minutes in completing antibody identifications using the dried SPRCA assay versus the hemagglutination method. In addition, the microplates containing panels of dried red cell monolayers obtained from selected donors can be stored at room temperature, ready for use, for up to 120 days.

References

1. Plapp FV, Sinor LT, Rachel JM, et al. A solid phase antibody screen. Am J Clin Pathol 1984;82:719–21.
2. Rolih SD, Eisinger RW, Moheng MC, et al. Solid phase adherence assays: alternatives to conventional blood bank tests. Lab Med (USA) 1985;16:766–70.
3. Mayr WR, Gassner H, Kempkes A, et al. Erste erfahrungen mit einem festphasen-immunoassay fur erythrozytare IgG-antikoper. Lab Med (Germany) 1989;13:6–7.

Darryl L. Stone, PhD., Immucor, Inc., PO Box 5625, Norcross, GA 30091, Ralph A. Eatz, MS, Susan D. Rolih, MS, Seaborn J. Farlow, BS, Gordon S. Hudson, BA, Lyle T. Sinor, PhD., Immucor, Inc., Norcross, GA.