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Evaluation of Antisera for Bloodstain Grouping I. ABH, MN, and Rh

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ABSTRACT: Sixty-eight different commercially available blood grouping antisera and lectins with ABH, MN, and Rh D, C, E, c, and e specificities were serologically evaluated for their applicability to bloodstain antigen determination. The characteristics of the antisera were determined with red cells, with fresh bloodstains, and with series of aging bloodstains. The Rh antisera were tested under a variety of serological conditions and with bloodstains on various substrata. Additionally, studies on optimization of absorption-elution procedure variables were carried out, and some data on the storage characteristics of red cells and blood grouping antisera were gathered.

KEYWORDS: pathology and biology, genetic typing, antigen systems, blood groups, bloodstains, ABO system, MN system, Rh system, absorption-elution, antiserum evaluation

The potential value of blood grouping in dried bloodstains as a means of partial individualization was recognized immediately by Landsteiner in the laureate account of the ABO system [*1*] and soon afterward made the subject of a separate study [*2*]. Efforts to group ABO in dried blood and bloodstains focused first on the serum isoantibodies [*1-4*] but were later directed toward the more stable red cell antigens using inhibition techniques [*5-8*]. Bloodstain antigen determination by an elution procedure was first described in 1923 [*6*] but did not come into widespread use until the description of a method by Kind in 1960 [*9,10*], which was found to be reproducible and reliable in many laboratories [*11*].

As other blood group systems were found, forensic science and medicolegal investigators attempted to devise inhibition procedures for the detection of the various antigens in dried bloodstains [*11,12*]. The MN system was described in 1927 [*13,14*] and the original Rh antigen in 1939 [*15-18*]. Landsteiner and Levine themselves showed in one of the original papers that M and N could be determined in dried blood by an inhibition method [*14*] and confirmatory reports from other laboratories followed [*11,12*]. Similarly, inhibition procedures for the Rh antigens in dried bloodstains were described [*19,20*].

Since 1960, most studies on blood group antigen determinations in dried stains have employed the sensitive elution technique. Although there are a number of different procedures

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for the elution of absorbed agglutinating antibodies from the corresponding cellular or stromal antigens, all the bloodstain grouping procedures in common use employ the heat elution procedure of Landsteiner and Miller [*21*]. Many variations of the absorption-elution procedures have been described; these have been reviewed elsewhere [*11,12*]. Some of these reports have to do with sample handling, while others were designed to take into account the nature and properties of the blood group antibodies for the system of interest.

Most of the reports on ABH typing in bloodstains have not been particularly concerned with antiserum evaluation or selection, although some studies have been devoted in whole or in part to the subject [*22-24*]. There is agreement that relatively highly titered antisera, free of antibodies to other blood group system antigens and containing antibodies that elute readily at 56°C, are to be preferred in elution tests [*22-26*]. Commercially available anti-A and anti-B sera contain complete antibodies and do not vary greatly in their essential properties from one to another. *Ulex europaeus* anti-H lectin preparations require somewhat more attention, but acceptable examples are relatively easy to prepare or obtain [*27*]. Commercially available MN antisera are significantly less consistent in properties. MN antigen determination in bloodstains using an elution technique was described by Pereira in 1963 [*28*]; a number of other laboratories have extended and confirmed the results of the initial studies [*26,29-32*]. The careful evaluation and selection of antisera for MN antigen typing in bloodstains has been stressed by all the investigators who have looked into it. Since the earliest studies on MN, it has been known that anti-N can react with M cells. The finding that anti-N can react with type M bloodstains was thus not surprising. This phenomenon has been called "cross-reactivity." Not every example of anti-N shows it, and an anti-N that "cross-reacts" with one example of M cells or bloodstain does not necessarily do so with other M cells or stains. Accordingly, much of the attention given to MN antiserum evaluation and selection has focused on selecting or preparing "non-cross-reacting" anti-N [*28,31,32*]. More recently, the biochemical basis for the so-called anti-N cross-reactivity has been investigated and revealed [*33-36*].

Determination of Rh antigens in bloodstains by the elution technique was first described in 1962 [*37*]. Various modifications of the technique have subsequently appeared, and there is general agreement on the value and reliability of Rh antigen determination in bloodstains [*38-44*]. Antisera for the Rh system antigens can exhibit great variability in serological characteristics. Further, Rh antisera differ from the usual ABH and MN reagents in that they contain predominantly IgG antibodies. There are a variety of techniques for bringing about or enhancing agglutination with these antibodies. Accordingly, a number of procedures and conditions that differ from those employed with ABH or MN antisera are used in Rh serology, and the characteristics of different antisera must be considered in this light. All of the investigators who have studied Rh antigen grouping in bloodstains have pointed out and emphasized the importance of careful selection and evaluation of antisera.

Since the majority of forensic laboratory caseworkers have no access to antisera other than those commercially available, it was decided to undertake a careful evaluation of a large number of such reagents in terms of their applicability to bloodstain grouping. We report here and in a subsequent paper [*45*] the results of those investigations. A detailed monograph based upon these studies has been submitted to the National Institute of Justice [*46*].

Methods and Materials

Antisera and Red Cells

Anti-A, anti-B, and anti-human globulin (AHG) antisera were obtained from Ortho Diagnostics, Inc., Raritan, NJ (Ortho); American Dade through American Scientific Products, Boston, MA (Dade); and Dr. Molter GmbH, Heidelberg, West Germany (Molter). Anti-H lectin (*Ulex europaeus*) was obtained from Dade and from In Vitro Research Sources, Inc.,

Bel Air, MD (IVRS). Anti-H lectin was also prepared from *Ulex europaeus* seeds (obtained from F. W. Schumacher Co., Sandwich, MA) following the method of Kind [*47*]. MN antisera, both human and rabbit, lectins, and a variety of different types of Rh antisera of the five major specificities, were obtained from Ortho, Dade, Biological Corp. of America, West Chester, PA (BCA), Molter, and Pfizer Diagnostics Division, Irvine, CA.[3] Travenol or Hyland products were obtained from Accugenics, Costa Mesa, CA.[3] Affirmagen, Identigen, and Resolve cell panels and A_2 cells were obtained from Ortho; Data-Cyte cell panels and BBQA Reference Red Blood Cells were obtained from Dade; and Referencells of groups A_1, A_2, B, and O were obtained from Immucor, Norcross, GA.

Antisera were kept at 4°C when not in use. For longer storage, some examples of antisera were divided into small aliquots of 0.3 to 1.0 mL in polypropylene tubes and stored at −85°C until required. Antisera frozen in this way were thawed only once and used immediately. Antisera supplied lyophilized were stored at −15°C and reconstituted as needed for use. Those requiring storage for any significant length of time following reconstitution were divided into small aliquots and stored at −85°C as indicated above for liquid antisera.

Some examples of red cells from donors as well as from cell panels were frozen in glycerol in 1-mL aliquots at −85°C following the procedure described by Boorman et al [*48*]. Frozen cells were recovered by dialysis for several hours against several changes of a large volume of saline and the recovered cells were washed three times in saline prior to use.

Other Materials and Solutions

Saline was prepared by dissolving 8.5 g sodium chloride (NaCl) in distilled water. Phosphate buffered saline (PBS) was prepared by first making a 50-m*M* sodium phosphate buffer, pH 7, and then dissolving 8.5 g of NaCl in each litre of buffer. Bovine albumin was obtained from Dade or from Ortho as a 22% solution. Saline-albumin (sometimes designated simply as "albumin") is saline containing a final concentration of 0.5% bovine albumin. AB serum medium ("AB serum") is AB serum diluted 1 in 10 with saline. Papain was obtained from Sigma Chemical Co., St. Louis, MO and papain solution was prepared according to the method of Löw [*49*]. Human AB serum was obtained from Molter or from Accugenics. Some low ionic strength solution (LISS) was prepared according to the procedure of Löw and Messeter [*50*], and some was obtained from Ortho. Bloodstains were made from fresh whole blood on clean cotton cloth or other substrata as indicated, dried completely, and stored at room temperature.

Procedures

Titrations were carried out in 6- by 50-mm or 12- by 75-mm test tubes, using 0.05 or 0.025 mL as one volume, by making doubling dilutions of antisera. Each tube finally contained one volume of antiserum and one volume of test cells.

After appropriate incubation, and usually a brief centrifugation step to pack the cells, the contents of the tubes were transferred to Boerner slides for rotation and microscopical reading. Titrations of antisera by indirect anti-human globulin (Coombs) technique were carried out in three stages. Sensitization was done essentially as described for titrations above, using 2 to 3% cell suspensions for antisera and 1% cell suspensions for eluates. After incubation at 37°C for 45 min, the cells were washed three times in large volumes of saline. One volume of cells (adjusted to 0.5%) was then added to one volume of AHG serum, mixed well, and

[3]Products of BCA and of Accugenics are currently handled by BCA-Accugenics, Malvern, PA. Pfizer's blood banking and blood products are no longer available; the responsible division was acquired by Immucor, Inc., of Norcross, GA. Immucor products are sold through Chasma Scientific, Cambridge, MA.

transferred to a Boerner slide for 15 min (or longer as necessary) of rotation before reading. For LISS titrations, LISS was used as the antiserum diluent and as the test cell suspending medium.

Agglutination was scored from 4+ to w (weak) or − (negative). A titration score was obtained by assigning arbitrary values to the different degrees of agglutination and summing the values over a titration series. The scoring system, modified from Issitt and Issitt [*51*], was 4+ = 12; 3+ = 10; 2+ = 8; 1+ = 5; w = 2; and − = 0. The titration score is given in parentheses following the titer value in the data.

Red cell suspension concentrations were calculated on the basis of packed cells as 100%. Papain treatment of red cells was carried out according to the method described by Boorman et al [*48*]. Absorption-elution was carried out in test tubes (12 by 75 mm). After the final wash, saline or other specified diluent equivalent to two volumes was added and elution carried out in the tubes. Eluates were then quickly removed into a previously prepared row of tubes for titration, after which the procedures were the same as for titrations. Titration of eluates permits the estimation of the relative quantity of antibody that was eluted from the dried blood sample. Group A_1 cells, B cells, and O cells were used to detect eluted anti-A, anti-B, and anti-H, respectively. Eluted anti-M and anti-N were detected with homozygous M or N cells, and eluates containing Rh antibodies were detected with group O, R_1R_1 cells (for D, C, and e) or group O, R_2R_2 cells (for c and E).

Results

Stability of Cells and Antisera Under Different Storage Conditions

Since both antisera and cells were to be stored for varying lengths of time, the retention of serological reactivity under various storage conditions was investigated. Table 1 shows the results obtained with three blood grouping antisera. Although some activity was lost upon freezing and thawing, the losses were small if the reagent was subjected to only one freeze-thaw cycle. The difference between storage at −15 and −85°C was not significant. An antiserum first frozen and thawed and then kept at 4°C for a week lost a little activity compared with that seen when it was first thawed. Antisera kept at 4°C for six months showed about the same activity as a reagent that had been frozen and thawed once. Neither glycerol nor dimethylsulfoxide (DMSO), both of which can protect red cells during cryogenic storage,

TABLE 1—*Effect of various storage conditions on antiserum reactivity.*

Conditions, °C	Anti-B[a]	Anti-c[b]	Anti-k[c]
Fresh	512 (92)	1000 (97)	64 (51)
−15° 1× thaw	256 (87)	512 (88)	32 (44)
−15° 2× thaw	256 (75)	256 (74)	8 (30)
−85° 1× thaw	256 (87)	512 (88)	16 (39)
−85° 2× thaw	256 (75)	256 (76)	8 (28)
−15° 1:10 glycerol	256 (84)	512 (88)	...
−85° 1:10 glycerol	256 (84)	512 (88)	...
−15° 1:10 DMSO	256 (82)	512 (88)	...
−85° 1:10 DMSO	256 (82)	256 (80)	...
4° 6 months	256 (86)	512 (90)	32 (44)
−15°, then 4° 1 week	256 (82)	...	...
−85°, then 4° 1 week	256 (82)	...	...

[a] Saline technique, B cells.
[b] Papain technique, rr cells.
[c] AHG technique, kk cells.

showed any protective effect on antisera through a freeze-thaw cycle at the concentrations tested.

Table 2 shows the reactivity of red cells with five examples of antisera after storage at 4°C in saline or modified Alsever's diluent (Ortho) for seven to ten days and frozen at −85°C in glycerol for several weeks to several months. The cells retained more activity at 4°C in modified Alsever's diluent than in saline, and this mode of storage was suitable for short periods. Red cells retained reactivity well under cryogenic storage conditions.

Optimization of Absorption-Elution Variables

The effect of absorption antibody concentration, as indicated by antiserum titer, on eluate antibody yield is shown in Table 3. The optimal absorption titer was 256 with an anti-B and 512 with an anti-D. Antibody yield in eluates was greatly affected by the titer of the absorbing antiserum.

Anti-D yield was increased 128-fold by increasing the absorbing anti-D titer from 50 to 256 or 512, and anti-B yield was increased eightfold by increasing the absorption titer from 16 to 256. With the anti-B used here, the B antigen was undetected using an anti-B with a titer of 4 for absorption. At the upper end of the concentration spectrum, anti-D with an absorption titer higher than 512 not only failed to increase antibody yield in eluates from stains containing the antigen but could give false reactions with antigen-negative control stains. Elution tests in these studies were carried out by using antisera adjusted to titers of 512 for absorption. Antisera which had titers lower than 512 initially were used undiluted for absorption.

The effect of absorption time on antibody yield with three antisera is shown in Table 4. With anti-A and anti-B, the antigens were detected in stains with absorption times as short as 15 min, but it is also clear that antibody yield in the eluate was increased 8 to 16 times by increasing the absorption time to 17 h. With anti-D, no significant antibody was detected in the eluate until absorption had been carried out for 4 h, and antibody yield was increased 64-fold by absorbing for 17 h. Optimal absorption time was 17 h. There was no advantage in

TABLE 2—*Antiserum titers and scores with fresh and stored red cells.*

Antiserum	Titer (Score) with Red Cells[a]			
	Fresh	4°C Saline	4°C Diluent[b]	Cryogenic
Anti-A[c]	256 (82)	64 (69)	256 (82)	128 (69)
Anti-c No. 1				
Saline	128 (94)	64 (58)	64 (58)	64 (62)
Papain	2000 (114)	2000 (100)	2000 (109)	2000 (104)
Anti-c No. 2				
Saline	4 (31)	2 (18)	2 (20)	2 (18)
Papain	2000 (112)	1000 (92)	1000 (101)	1000 (91)
Anti-e				
Saline	128 (70)	64 (61)	128 (66)	64 (61)
Papain	1000 (100)	512 (83)	512 (88)	1000 (89)
Anti-k[d]	64 (43)	32 (41)	32 (43)	32 (41)

[a]Red cells of type A_2, rr, kk titrated at 0.1% cell concentration when fresh, after three times washing in saline, or after indicated storage and three times washing in saline; cryogenic storage in glycerol and recovery by dialysis (see "Methods and Materials").

[b]Ortho modified Alsever's diluent.

[c]Saline technique.

[d]AHG technique.

TABLE 3—*Effect of antibody concentration on antibody recovery in eluates.*

	Titer of Absorbing Antiserum[a]	Titer of Eluates[a] from Stains of Type	
		R_1R_2	rr
Anti-D with Rh+ and Rh− stains	1000	64	1
	512	256	0
	256	256	0
	128	64	0
	100	32	0
	50	2	0
		B	Non-B
Anti-B with B and other ABO type stains	256	8	0
	128	4	0
	64	2	0
	16	1	0
	4	0	0

[a]Anti-D by papain technique; anti-B by saline technique.

TABLE 4—*Effect of absorption time on eluate antibody yield.*

Absorbing Antiserum	Titer in Saline (Papain)	Stain Phenotype	Absorption Time, h	Papain Titer (Score) of Eluate from 1-mm^2 Stain
Anti-D	16 (512)	R_1R_2	1	0 (0)
			2	0 (2)
			4	2 (12)
			6	4 (15)
			8	16 (31)
			10	32 (45)
			17	128 (63)
			24	128 (66)
Anti-A	64 (512)	A	0.25	8 (28)
			1	16 (36)
			4	32 (41)
			17	64 (52)
			24	64 (57)
Anti-B	512 (1000)	B	0.25	2 (9)
			1	4 (23)
			4	8 (23)
			17	32 (41)
			24	32 (43)

absorbing for longer periods (up to 24 h), although there was no apparent disadvantage either.

The effect of antigen concentration, as altered by varying the quantity of bloodstained sample taken for testing, is seen in Table 5. There was a slight increase in eluate antibody yield with 0.5 or 1.0 cm^2 stain samples as compared with three 1-cm threads, but thorough washing is more difficult with the larger samples. Except where indicated otherwise, the studies in the present work were carried out on samples consisting of three 1-cm threads.

TABLE 5—*Effect of bloodstain sample quantity on eluate antibody recovery.*

Antiserum	Quantity of Bloodstain[a] Used for Absorption	Titer (Score) of Eluates[b] from Rh C+D+ Stain
Anti-D[c]	1 cm^2	128 (69)
	0.5 cm^2	128 (67)
	1 thread (1 cm)	64 (51)
	3 threads (1 cm)	64 (58)
Anti-D[d]	1 thread (1 cm)	64 (53)
	3 threads (1 cm)	128 (68)
Anti-C[e]	1 thread (1 cm)	16 (28)
	3 threads (1 cm)	16 (33)

[a]Cotton cloth, less than 1 week old.
[b]By same technique as antiserum titer.
[c]Papain titer 1000.
[d]Albumin titer 512.
[e]Saline titer 64.

Lund [*52*] found many years ago that the sensitivity of agglutination reactions with anti-A and anti-B was significantly increased by decreasing the cell concentrations. Table 6 shows the effect of cell suspension concentration on agglutination reaction sensitivity at several different cell and antibody concentrations. The lower cell concentrations gave higher titers and thus greater sensitivity. This effect was more apparent at lower antibody concentrations such as would be expected in eluates.

Serological Characteristics of Commercial Antisera

The titers of representative anti-A and anti-B sera and anti-H lectins are shown in Table 7. Titers of anti-A and anti-B were relatively uniform. Anti-H lectin preparations may vary not only in their titers with O and A_2 cells, but also in the extent to which they react with A_1 and B cells.

The titers of representative MN sera and lectins are indicated in Table 8. These showed more variability than ABH reagents. Anti-M sera raised in rabbits usually had higher titers

TABLE 6—*Effect of concentration of R_1R_1 cell suspension on the titer of an anti-D at different dilutions.*

Dilution of Anti-D	Concentration of Cell Suspension, %	Titer (Score) of Anti-D[a]
1:8	3	1000 (102)
	1	2000 (109)
	0.1	4000 (106)
	0.05	4000 (106)
1:32	3	64 (67)
	1	128 (67)
	0.1	256 (71)
	0.05	256 (76)
1:512	3	16 (41)
	1	16 (40)
	0.1	32 (48)
	0.05	128 (56)

[a]Papain technique in AB serum diluent.

TABLE 7—*Serological characteristics of ABH antisera and lectins.*

Specificity	Manufacturer	Titer (0.1% Cells)[a]	
		Saline	Papain
Anti-A	Ortho	512 (A_1) 256 (A_2)	
Anti-A	Molter	512 (A_1) 256 (A_2)	
Anti-A	Dade	256 (A_1) 128 (A_2)	
Anti-A	Biotest	1T (A_1) 128 (A_2)	32T (A_1) 4T (A_2)
Anti-B	Molter	512	
Anti-B	Ortho	512	
Anti-B	Dade[b]	256	512
Anti-B	Dade[b]	512	
Anti-B	Biotest	512	1T
Anti-H	Seeds[c]	32 (O) 16 (A_2) 2 (B) 1 (A_1)	
Anti-H	Seeds[c]	128 (O) 32 (A_2) 8 (B) 4 (A_1)	4T (O)
Anti-H	Seeds[c]	32 (O) 16 (A_2) 8 (B) 0 (A_1)	128 (O)
Anti-H	Dade	16 (O) 8 (A_2) 8 (B) 0 (A_1)	
Anti-H	IVRS	128 (O) 32 (A_2) 32 (B) 4 (A_1)	
Anti-H	Polysciences[d]	128 (O) 32 (A_2) 1 (B) 16 (A_1)	8T (O)

[a]T means "thousand."
[b]Different lots.
[c]Method of Kind [*46*].
[d]Affinity purified *Ulex europaeus* lectin, protein concentration adjusted to 0.625 mg/mL.

TABLE 8—*Serological characteristics of MN antisera and lectins.*

Specificity	Manufacturer and Type	Saline Titer (Score) with Cells of Type		
		M	MN	N
Anti-M	Ortho (rabbit)	128 (70)	64 (58)	0
	Molter "komplett" (rabbit)	256 (88)	128 (76)	0
	BCA (human)	64 (59)	32 (42)	0
	Pfizer (human)	64 (62)	32 (38)	0
	Travenol [Hyland] (rabbit)	128 (72)	64 (57)	0
	Travenol [Hyland] (human)	64 (60)	32 (43)	0
	Dade (human)	16 (42)	8 (37)	0
Anti-N	Ortho (rabbit)	1 (7)	8 (33)	16 (47)
	Molter "komplett" (rabbit)	2 (13)	128 (59)	256 (94)
	BCA (rabbit)	0	8 (34)	34 (44)
	Pfizer (*V.g.* lectin)[a]	0	16 (47)	64 (59)
	Travenol [Hyland] (rabbit)	1 (5)	16 (33)	32 (40)

[a]*Vicia gramineae* lectin.

than those of human origin, and titers were always lower with heterozygous cells. Three of the five anti-N cross-reacted to some extent with M cells. The cross-reactivity was not correlated with the titer of the reagent against N cells.

The titers of representative Rh antisera in saline-albumin, AB serum, and LISS media as well as with papain treated cells are shown in Table 9. Manufacturers ordinarily indicate by a label designation the serological conditions for which the antiserum is intended. The designations are often "saline," "high protein," or a trade name or other indication that the IgG molecules have been modified to make the antibodies more saline-reactive (such as "Novaserum" from Ortho or "modified IgG" from Dade).

All the reagents are marketed for use as cell grouping reagents. The Rh antisera showed considerable variability in titer under various conditions. Some were more reactive in AB serum than in albumin and a few exhibited LISS enhancement as compared with albumin. All the Rh antisera showed modest to significant increases in titer with papain treated cells.

Bloodstain Antigen Determination and Persistence

Antisera of different specificities which showed varying serological properties with red cells were employed in elution tests with bloodstains, antibody yield in the eluates being estimated by titration. Antisera were selected for the various series of tests on the basis of their serological properties or behavior with cells or fresh stains, or because they were representative of a type of antiserum.

Table 10 shows the eluate titers from a number of stains subjected to elution with various representative anti-A and anti-B sera and anti-H lectins. Similar data indicating the effect of absorbing antiserum titer on eluate antibody yield were shown for an anti-B serum in Table 3. Eluate antibody and lectin titers do vary with different reagents, different stain samples, and different serological conditions, even at comparable absorption titers. Eluates are not ordinarily titrated in absorption elution tests on casework samples. Test cells are usually simply added to the eluate and agglutination results read after appropriate incubation. Dif-

TABLE 9—*Serological characteristics of Rh antisera.*

Specificity	Manufacturer	Type	Cell Phenotype	Titer (Score)			
				Albumin	AB Serum	LISS	Papain
Anti-D	Ortho	saline	R_1R_1	16 (40)	64 (60)	32 (43)	512 (86)
			R_1r	16 (37)	64 (56)	16 (35)	512 (84)
	Ortho	high-protein	R_1R_1	16 (35)	64 (57)	8 (34)	512 (69)
			R_1r	4 (27)	64 (53)	8 (34)	256 (64)
	Ortho	Novaserum	R_1R_1	64 (59)	256 (76)	64 (53)	512 (86)
			R_1r	16 (35)	128 (68)	32 (42)	256 (79)
	Molter	high-protein	R_1R_1	64 (66)	256 (77)	128 (69)	2000 (109)
			R_1r	32 (50)	64 (55)	64 (64)	512 (89)
	Dade	high-protein	R_1R_1	128 (72)	64 (67)	16 (42)	512 (94)
			R_1r	64 (67)	64 (63)	16 (36)	512 (89)
	Dade	saline	R_1R_1	256 (78)	256 (76)	256 (80)	1000 (111)
			R_1r	256 (72)	256 (66)	128 (56)	512 (95)
	BCA	saline	R_1R_1	64 (57)	64 (55)	32 (34)	512 (89)
			R_1r	32 (50)	32 (48)	32 (32)	256 (85)
	BCA	high-protein	R_1R_1	64 (58)	64 (60)	64 (51)	512 (96)
			R_1r	64 (55)	64 (53)	32 (48)	256 (89)
	Pfizer	saline	R_1R_1	32 (57)	64 (63)	64 (63)	512 (103)
			R_1r	16 (35)	32 (42)	32 (50)	128 (68)
	Hyland	high-protein	R_1R_1	128 (62)	128 (68)	...	512 (109)
			R_1r	64 (58)	128 (60)	...	256 (87)
	Ortho	AHG	R_1R_1	2000 (95)	4000 (102)	4000 (102)	4000 (100)
			R_1r	1000 (90)	4000 (100)	2000 (97)	4000 (100)

TABLE 9—*(Continued)*

Specificity	Manufacturer	Type	Cell Phenotype	Titer (Score)			
				Albumin	AB Serum	LISS	Papain
Anti-C	Ortho	saline	R_1R_1	64 (76)	64 (60)	64 (72)	128 (86)
			R_1r	64 (69)	64 (58)	64 (68)	128 (85)
	Ortho	Novaserum	R_1R_1	32 (47)	32 (55)	64 (50)	512 (104)
			R_1r	32 (40)	32 (53)	32 (40)	256 (99)
	Molter	saline	R_1R_1	128 (66)	128 (70)	128 (63)	1000 (118)
			R_1r	128 (61)	128 (67)	64 (53)	512 (103)
	Molter	high-protein	R_1R_1	32 (55)	512 (85)	64 (60)	2000 (92)
			R_1r	32 (53)	512 (80)	32 (55)	1000 (90)
	Dade	modified IgG	R_1R_1	64 (53)	256 (77)	32 (53)	1000 (107)
			R_1r	32 (45)	256 (70)	32 (47)	512 (99)
	BCA	high-protein	R_1R_1	64 (50)	64 (60)	64 (52)	512 (105)
			R_1r	32 (43)	64 (58)	32 (45)	512 (95)
	Pfizer	high-protein	R_1R_1	64 (55)	64 (60)	64 (58)	512 (92)
			R_1r	32 (45)	32 (52)	64 (50)	256 (84)
Anti-E	Ortho	Novaserum	R_2R_2	16 (49)	64 (57)	16 (46)	1000 (109)
			R_2r	8 (28)	32 (54)	8 (30)	64 (54)
	Molter	saline	R_2R_2	64 (67)	512 (87)	64 (65)	512 (113)
			R_2r	64 (63)	256 (80)	64 (63)	512 (104)
	Molter	high-protein	R_2R_2	64 (60)	512 (94)	...	512 (99)
			R_2r	64 (58)	512 (90)	...	512 (94)
	Dade	saline	R_2R_2	64 (60)	128 (68)	64 (60)	512 (90)
			R_2r	32 (49)	128 (63)	64 (58)	512 (84)
	Dade	modified IgG	R_2R_2	16 (48)	64 (60)	32 (48)	512 (94)
			R_2r	16 (46)	32 (50)	32 (48)	512 (92)
	BCA	high-protein	R_2R_2	16 (47)	32 (50)	...	128 (72)
			R_2r	16 (46)	32 (48)	...	128 (60)
	BCA	saline	R_2R_2	64 (67)	64 (67)	64 (65)	256 (89)
			R_2r	32 (49)	64 (62)	32 (52)	128 (86)
	Hyland	high-protein	R_2R_2	128 (63)	128 (66)	128 (60)	512 (92)
			R_2r	64 (55)	128 (63)	64 (53)	512 (90)
Anti-c	Ortho	Novaserum	rr	8 (27)	64 (60)	64 (45)	512 (99)
			R_1R_2	2 (14)	64 (58)	8 (37)	512 (97)
	Molter	saline	rr	64 (58)	256 (80)	64 (53)	2000 (102)
			R_1R_2	64 (52)	256 (76)	32 (37)	512 (87)
	Molter	high-protein	rr	128 (65)	512 (92)	128 (65)	2000 (104)
			R_1R_2	64 (58)	512 (92)	64 (60)	2000 (104)
	Dade	saline	rr	64 (67)	128 (74)	...	512 (85)
			R_1R_2	64 (56)	64 (71)	...	512 (74)
	Dade	high-protein	rr	64 (70)	64 (72)	64 (60)	512 (95)
			R_1R_2	64 (60)	64 (70)	32 (50)	512 (89)
	Dade	high-protein	rr	32 (48)	32 (52)	32 (45)	512 (94)
			R_1R_2	16 (40)	16 (42)	32 (43)	256 (77)
	Hyland	high-protein	rr	64 (55)	64 (62)	64 (60)	512 (106)
			R_1R_2	64 (53)	64 (57)	64 (55)	512 (100)
Anti-e	Ortho	saline	rr	128 (70)	128 (70)	64 (65)	512 (93)
			R_1R_2	32 (54)	64 (59)	64 (63)	512 (90)
	Ortho	Novaserum	rr	8 (32)	64 (60)	16 (50)	512 (95)
			R_1R_2	4 (29)	64 (47)	16 (38)	512 (86)
	Molter	saline	rr	64 (66)	256 (74)	64 (56)	1000 (97)
			R_1R_2	64 (54)	128 (60)	64 (56)	512 (92)
	Molter	high-protein	rr	64 (60)	512 (87)	64 (60)	2000 (104)
			R_1R_2	64 (58)	512 (85)	64 (60)	2000 (101)
	Dade	high-protein	rr	64 (57)	64 (58)	64 (60)	256 (92)
			R_1R_2	64 (55)	64 (58)	32 (46)	256 (86)
	BCA	high-protein	rr	32 (47)	...	32 (45)	256 (85)
			R_1R_2	16 (38)	...	32 (45)	128 (75)
	Hyland	high-protein	rr	16 (40)	64 (52)	32 (47)	512 (88)
			R_1R_2	16 (38)	64 (48)	16 (42)	512 (86)

TABLE 10—*Elution of ABH antibodies and lectins from bloodstains.*

Specificity	Titer[a]	Titer[a] (Score) of Eluate from Bloodstain[b]
Anti-A	512 saline	64 (52) papain
	1000 papain	
Anti-A	256	16 (31)
Anti-B[c]	512 saline	16 (38) saline
	1000 papain	32 (41) papain
Anti-B[c]	256	32 (42)
Anti-H	128 saline	4 (18) saline
	4000 papain	128 (65) papain
Anti-H	256 papain	16 (38) papain

[a] A_1 cells and stain with anti-A; B cells and stain with anti-B; and O cells and stain with anti-H; saline unless otherwise noted.
[b] Cotton cloth, 1 mm^2.
[c] See also Table 3.

ferences in antibody or lectin yield would not be as apparent without titration, and would be reflected as differences in agglutination reaction strength. Any eluate that would have a titer greater than 1 or 2 if titrated would give a completely convincing agglutination result in the usual eluted antibody detection test. General experience as well as the present data indicate that there should usually be little problem in obtaining satisfactory eluate antibody yields from bloodstains with anti-A and anti-B sera and anti-H lectins, and extensive studies on ABH antisera and lectins were not pursued.

Table 11 shows the titers of eluates of stains of all three MN types subjected to elution after absorption with anti-M and anti-N at time intervals over a five-month period. An anti-N that exhibited reactivity with M cells was selected for this study. The anti-N reacted with the M stain when it was fresh, but not after three or more weeks of aging. N was detectable in MN and N stains with the anti-N for the entire aging period. With the anti-M, the M antigen was also detectable in the M and MN stains for five months. As expected, eluate titers decreased in both cases as the stains aged.

A number of different examples of Rh antisera of the five major specificities were used in elution tests on relatively fresh bloodstains of several different Rh phenotypes and the papain titers of the eluates determined. The results are shown in Table 12. All the antigens were readily detected by all the antisera employed in all the stains tested, and papain titers of the eluates varied from 64 to 512. With anti-c and anti-e, eluates from homozygous stains tended to show higher scores than comparable ones from heterozygous stains, and eluate

TABLE 11—*Absorption-elution tests on bloodstains with selected MN antisera.*

Bloodstain Phenotype	Tested With	Titer (Score) of Eluates with Homozygous Test Cells			
		3 days	3 weeks	8 weeks	20 weeks
M	Anti-M[a]	64 (55)	16 (38)	8 (26)	2 (12)
MN		32 (48)	16 (33)	4 (20)	2 (10)
N		0	0	0	0
M	Anti-N[b]	1 (5)	0	0	0
MN		16 (36)	8 (23)	2 (12)	2 (10)
N		16 (38)	8 (28)	4 (18)	2 (12)

[a] Titer (score) with cells of type M, 256 (88); MN, 128 (76); N, 0 (0).
[b] Titer (score) with cells of type M, 2 (13); MN, 128 (59); N, 256 (94).

TABLE 12—*Absorption-elution tests on fresh bloodstains with Rh antisera.*

Specificity	Manufacturer/Type	Titer (Score) of Eluate from Stains of Phenotype				
		R_1R_2	R_2r	R_1r	rr	
Anti-D	Ortho/saline	128 (56)	256 (69)	256 (57)	0	
	Ortho/high-protein	128 (74)	128 (62)	128 (58)	0	
	Ortho/Novaserum	256 (80)	256 (75)	256 (79)	0	
	Molter/high protein	512 (79)	512 (80)	256 (73)	0	
	Dade/high-protein	256 (75)	256 (77)	128 (68)	0	
	Dade/saline	256 (82)	256 (85)	256 (71)	0	
	BCA/saline	64 (64)	64 (64)	64 (46)	0	
	BCA/high-protein	128 (65)	128 (65)	128 (63)	0	
	Pfizer/saline	256 (61)	128 (58)	64 (53)	0	
	Hyland/high-protein	128 (65)	128 (63)	64 (56)	0	
		R_1R_2	R_1R_1	R_1r	R_2r	rr
Anti-C	Ortho/saline	128 (55)	...	64 (50)	4 (10)	0
	Ortho/Novaserum	128 (71)	...	256 (82)	0	0
	Molter/saline	64 (54)	...	128 (63)	0	0
	Molter/high-protein	128 (79)	...	256 (80)	0	0
	Dade/modified IgG	128 (52)	128 (62)	64 (56)	0	0
	BCA/high-protein	128 (59)	128 (63)	128 (63)	0	0
	Pfizer/high-protein	128 (61)	128 (72)	128 (63)	0	0
	Hyland/high-protein	64 (56)	128 (61)	128 (61)	0	0
		R_2R_2	R_1R_2	R_2r	R_1r	
Anti-E	Ortho/Novaserum	128 (59)	128 (60)	128 (54)	0	
	Molter/saline	...	128 (62)	128 (61)	0	
	Molter/high-protein	128 (58)	128 (67)	256 (75)	0	
	Dade/saline	64 (51)	128 (43)	256 (56)	0	
	Dade/modified IgG	...	64 (43)	64 (38)	0	
	BCA/high-protein	128 (58)	128 (56)	128 (67)	0	
	BCA/saline	...	64 (60)	128 (58)	0	
	Hyland/high-protein	...	128 (55)	128 (68)	0	
		rr	R_1R_2	R_2r	R_1R_1	
Anti-c	Ortho/Novaserum	128 (65)	128 (46)	128 (50)	0	
	Molter/saline	128 (83)	64 (65)	128 (87)	0	
	Molter/high-protein	256 (82)	64 (62)	128 (86)	0	
	Dade/saline	128 (74)	64 (64)	128 (68)	0	
	Dade/high-protein	256 (91)	128 (60)	256 (82)	0	
	Dade/high-protein	512 (97)	128 (73)	512 (92)	0	
	Hyland/high-protein	512 (92)	128 (62)	128 (77)	0	
		rr	R_1R_2	R_2r	R_2R_2	
Anti-e	Ortho/saline	128 (59)	128 (57)	64 (38)	0	
	Ortho/Novaserum	128 (56)	256 (60)	128 (52)	0	
	Molter/saline	64 (65)	128 (61)	64 (53)	0	
	Molter/high-protein	128 (72)	64 (60)	64 (55)	0	
	Dade/high-protein	128 (61)	64 (53)	64 (46)	0	
	BCA/high-protein	256 (66)	128 (61)	128 (59)	0	
	Hyland/high-protein	64 (60)	128 (70)	64 (59)	0	

scores tended to be lower with anti-E and anti-e than with the other specificities. The titers of the eluates from stains are not necessarily tightly correlated with the red cell titers of the antisera used for absorption.

Control stains which lacked the corresponding antigen gave the expected negative results in every case except one. An anti-C reacted with an R_2r stain to a small but measurable extent, but was completely negative with an rr stain. This result indicates an unexpected antibody in the particular anti-C to an antigen in the R_2r stain. The unexpected antibody could be an anti-D or an anti-G [*53,54*], and the result illustrates the importance of evaluating each reagent with appropriate control bloodstains in elution tests. A number of examples of Rh antisera of each specificity were employed to follow the detectability of the corresponding antigens in a series of aging bloodstains of various phenotypes. The results of these studies are shown in Table 13. For the 26-week period shown in the table, the same bloodstain was followed with the same antiserum at each time interval.

All the antisera tested gave eluate titers ranging from 8 to 512 in all the week-old stains. The eluate titers decreased as expected as the stains aged, but in the majority of cases eluate titers from 2 to 8 were seen in 26-week-old stains. In several cases, the antigen was undetected at 26 weeks, and one anti-c failed to detect c in two 16-week-old stains.

Four anti-D, four anti-C, three anti-E, four anti-c, and three anti-e sera were additionally used in tests with stains from one and one half to five years old. Eluate titers of at least 1 with corresponding scores of at least 5 were observed in 16 stains with anti-D, 8 stains with anti-C, 8 stains with anti-E, 13 stains with anti-c, and 7 stains with anti-e. The D, C, E, and c antigens were convincingly detected in at least some of the five-year-old stains tested, but e was not detected in stains older than three years.

Limited tests were carried out with Rh antisera to compare papain technique with several other recognized agglutination enhancement procedures. The data are shown in Table 14. Papain enhancement was more effective with cells than the use of AB serum or LISS media in the absence of papain with most antisera. Some reagents were enhanced in AB serum or LISS relative to saline-albumin. All the antisera tested reacted with all the stains tested in varying degrees, regardless of the enhancement technique used. Stain eluate titers varied in the different media and were not necessarily correlated with the corresponding cell titers. One anti-e gave high eluate titers in AB serum, considering that its AB serum titer with cells was 64.

Some studies were carried out to determine the detectability of Rh, C, D, and e in relatively fresh (two to four weeks old) bloodstains on a variety of different substrata. Substrata that could be cut into threads or pieces of fabric were tested directly in elution tests. With stains on other substrata, such as wood or fixed surfaces that could not be tested directly, the bloodstain was dissolved in a minimal quantity of saline and transferred to cotton threads. The threads were then allowed to dry completely and subjected to testing in the usual manner. The transfer technique could be used for bloodstains on any substratum. With most of the substrata, all the expected antigens were readily detected in the stains. On some denim, suede, polyester, nylon, and rayon substrata, one or more expected antigens were not detected. In some cases, antigens that were not detected by direct testing could be detected by transfer technique.

Discussion

Retention of Activity by Stored Antisera and Red Cells

Both antisera and red cells may be stored for varying lengths of time, particularly if they are required only occasionally. The present data indicate that relatively simple 4°C and freezing storage procedures and conditions may be employed for cells and sera without unacceptable losses of serological activity.

Optimization of Absorption-Elution Variables

The principles underlying the absorption-elution procedure, which are based in turn on the principles underlying blood group antigen-antibody reactions and agglutination, had to be considered in arriving at procedures for the evaluation of the various blood grouping antisera. The absorption-elution procedure is made up of a series of stages, in each of which it is possible to vary the conditions and the concentrations of reacting components. The stages are absorption, washing, elution, and detection. The major variables include

(1) antibody concentration (antiserum titer),
(2) absorption time,
(3) antigen concentration (quantity of dried blood) at the absorption stage,
(4) washing time,
(5) wash fluid volume,
(6) wash fluid temperature at the washing state,
(7) eluate volume,
(8) elution temperature,
(9) elution time,
(10) speed of recovery of the eluate after elution at the elution stage,
(11) detection cell type,
(12) detection cell concentration, and
(13) serological technique used for detection of the eluted antibodies, especially with antibodies other than ABH and MN, at the detection stage.

Extensive previous studies on the variables in absorption-elution have been carried out by Lincoln and Dodd [*23,24*], with the objective of optimizing the overall process. Determining the effect of changing a variable parameter in the absorption-elution process on the eluate antibody titer represents a means of optimizing that parameter in terms of antibody yield. This approach may be used to determine the conditions under which elution tests on bloodstain antigens are most sensitive.

At the absorption stage, antiserum titer, absorption time, and the quantity of bloodstain may be varied. The titer of the absorbing antiserum has a significant effect on eluate antibody yield. Titers below the optimal value of 256 to 512 result in lower yields, thus reducing sensitivity, while higher absorption titers can give rise to detectable antibody in eluates from negative control stains. In previous studies on the effect of absorbing antiserum titer [*24*], a very potent anti-D (papain titer 32000) convincingly showed how much antibody can be eluted from a negative control stain with absorbing antisera that are too concentrated. Exposure to such high antiserum concentrations may make it difficult to wash out all the unbound antibody without making the washing step unreasonably involved. The observation that eluate antibody yield is lower at very high absorption titers could reflect the binding of a larger population of antibody molecules that do not elute readily at 56°C than occurs at the optimal absorption titer. Commercial anti-A, anti-B, anti-M, and anti-N sera and seed lectins with anti-H or anti-N activity seldom have saline titers exceeding 512. Commercial Rh antisera, with which enhancement procedures are commonly employed, can have titers significantly greater than 512 and may thus require dilution for use in elution tests with stains. Optimal absorption time was found to be 17 h, in agreement with previous studies [*24*]. Shorter absorption periods tend to decrease eluate antibody yield.

The effect of varying the quantity of bloodstained sample taken for testing was relatively small. The eluate titer doubled as the sample size was increased from one 1-cm thread to a 1-cm^2 piece of stained cloth, an increase of about 20-fold in sample. In previous studies [*24*] it was found the eluate antibody yield decreased quite significantly at the greatest concentration of antigen. However, the experiment was done in quite a different way and with an anti-A rather than an anti-D.

TABLE 13—*Absorption-elution tests with Rh antisera in aging bloodstains.*

Specificity	Type	Papain Titer (Score) with Red Cells	Stain Phenotype	Papain Titer (Score) of Eluate: Age of Stain in Weeks							
				1	2	4	6	8	12	16	26
Anti-D	Novaserum	R_1R_2 512 (86)	R_1R_2	256 (75)	128 (51)	16 (36)		16 (34)	16 (22)	8 (23)	8 (23)
		R_1r 256 (79)	R_1r	256 (66)	64 (47)	16 (31)		16 (31)	8 (25)	4 (15)	4 (23)
			R_2r	256 (64)	128 (51)	16 (33)		16 (39)	8 (23)	4 (20)	8 (26)
	AHG	R_1R_1 4000 (100)	R_1R_2		128 (53)	64 (44)		16 (40)	8 (25)	16 (34)	8 (23)
		R_1r 4000 (100)	R_1r		256 (54)	32 (45)		8 (26)	16 (36)	8 (30)	8 (25)
			R_2r		512 (74)	64 (45)		8 (33)	16 (33)	8 (32)	8 (28)
	high-protein	R_1R_1 512 (94)	R_1R_2	256 (68)	256 (56)	64 (48)		16 (33)	8 (25)	2 (10)	8 (20)
		R_1r 512 (89)	R_1r	128 (53)	32 (37)	64 (49)		16 (30)	8 (23)	8 (26)	4 (20)
			R_2r	256 (63)	128 (55)	64 (53)		16 (33)	2 (15)	2 (12)	4 (20)
	saline	R_1R_1 1000 (111)	R_1R_2	128 (55)	64 (41)	32 (43)		8 (25)	4 (20)	8 (23)	4 (18)
		R_1r 512 (95)	R_1r	64 (48)	64 (40)	64 (44)		8 (31)	8 (28)	8 (23)	4 (17)
			R_2r	128 (55)	64 (38)	64 (48)		16 (34)	8 (23)	4 (15)	4 (18)
	saline	R_1R_1 512 (89)	R_1R_2	64 (51)				4 (15)			1 (5)
		R_1r 256 (85)	R_1r	64 (48)				2 (12)			0
			R_2r	64 (53)				4 (17)			2 (15)
Anti-C	saline	R_1R_1 1000 (118)	R_1R_1	128 (53)		32 (43)	32 (36)	16 (36)	8 (22)		8 (23)
		R_1r 512 (103)	R_1R_2	64 (46)		16 (38)	16 (25)	8 (33)	4 (26)		4 (17)
			R_1r	128 (56)		32 (45)	16 (28)	8 (28)	8 (23)		4 (18)
	high-protein	R_1R_1 2000 (92)	R_1R_1	128 (56)		32 (47)	32 (38)	16 (45)	4 (20)		8 (25)
		R_1r 1000 (90)	R_1R_2	128 (56)		64 (51)	32 (38)	16 (31)	2 (12)		8 (20)
			R_1r	128 (61)		64 (57)	16 (28)	8 (30)	8 (23)		4 (20)
	modified IgG	R_1R_1 1000 (107)	R_1R_1	256 (63)		64 (46)	32 (36)	16 (43)	8 (23)		8 (26)
		R_1r 512 (99)	R_1R_2	64 (59)		64 (46)	32 (30)	16 (36)	8 (22)		4 (18)
			R_1r	128 (53)		64 (44)	32 (28)	16 (43)	4 (20)		4 (18)
	high-protein	R_1R_1 512 (105)	R_1R_1	128 (63)		64 (45)	32 (43)	32 (50)	4 (20)		8 (25)
		R_1r 512 (95)	R_1R_2	128 (56)		128 (46)	16 (28)	8 (30)	2 (12)		4 (20)
			R_1r	128 (68)		32 (45)	16 (31)	8 (35)	8 (23)		8 (25)
	saline	R_1R_1 128 (86)	R_1R_1	128 (55)							1 (5)
		R_1r 128 (85)	R_1r	64 (50)							1 (5)
Anti-E	Novaserum	R_2R_2 1000 (109)	R_2R_2	128 (59)		64 (46)		16 (43)	2 (12)		4 (17)
		R_2r 64 (54)	R_2r	128 (56)		64 (48)		4 (23)	2 (12)		4 (17)
			R_1R_2	64 (41)		16 (25)		8 (28)	2 (13)		4 (15)
	high-protein	R_2R_2 512 (99)	R_2R_2	128 (58)		8 (38)		8 (38)	8 (33)		4 (17)

		R_2r	512 (94)	R_2r	64 (55)	32 (48)		16 (38)	8 (28)		4 (17)
				R_1R_2	64 (48)	32 (47)		8 (35)	4 (17)		2 (10)
	saline	R_2R_2	512 (90)	R_2R_2	64 (51)	16 (33)		4 (18)	4 (20)		4 (18)
		R_2r	512 (84)	R_2r	32 (43)	16 (28)		2 (13)	2 (15)		4 (18)
				R_1R_2	8 (26)	4 (15)		2 (13)	2 (12)		2 (10)
	high-protein	R_2R_2	128 (72)	R_2R_2	128 (58)	32 (41)		16 (38)	2 (12)		
		R_2r	128 (60)	R_2r	64 (41)	32 (44)		8 (35)	4 (20)		
				R_1R_2	64 (41)	16 (25)		16 (40)	4 (15)		
	high-protein	R_2R_2	512 (92)	R_2R_2				4 (15)			1 (5)
		R_2r	512 (90)	R_2r	128 (68)			4 (18)			0 (2)
				R_1R_2	128 (55)			2 (12)			0
Anti-c	Novaserum	rr	512 (99)	rr	128 (68)	32 (43)	16 (46)	16 (45)	8 (33)		4 (23)
		R_1R_2	512 (97)	R_2r	128 (63)	16 (38)	16 (38)	16 (45)	4 (15)		2 (15)
				R_1R_2	128 (51)	16 (34)	8 (30)	8 (30)	4 (15)		2 (10)
	saline	rr	2000 (102)	rr	128 (66)	64 (62)	32 (51)	16 (41)	8 (30)		8 (33)
		R_1R_2	512 (87)	R_2r	64 (56)	64 (58)	32 (46)	16 (36)	8 (28)		8 (33)
				R_1R_2	32 (55)	32 (44)	16 (34)	4 (25)	4 (15)		4 (20)
	high-protein	rr	2000 (104)	rr	64 (56)	64 (67)	32 (48)	16 (45)			8 (28)
		R_1R_2	2000 (104)	R_2r	32 (47)	64 (65)	16 (36)	16 (43)			4 (25)
				R_1R_2	32 (51)	32 (46)	32 (41)	16 (38)			4 (23)
	high-protein	rr	512 (94)	rr	256 (76)	32 (55)	32 (48)	8 (37)	4 (23)		4 (23)
		R_1R_2	256 (77)	R_2r	256 (75)	16 (40)	16 (36)	8 (37)	4 (18)		4 (20)
				R_1R_2	128 (68)	16 (38)	8 (26)	8 (31)	2 (12)		4 (15)
	high-protein	rr	512 (106)	rr	512 (92)			2 (12)		0	
		R_1R_2	512 (100)	R_2r	128 (77)			2 (10)		2 (12)	
				R_1R_2	128 (68)			2 (10)		0	
Anti-e	saline	rr	512 (93)	rr	128 (61)	32 (48)	8 (23)		4 (20)		2 (18)
		R_1R_2	512 (90)	R_2r	64 (54)	16 (31)	4 (20)		4 (15)		2 (12)
				R_1R_2	64 (46)	16 (28)	2 (12)		4 (20)		4 (18)
	Novaserum	rr	512 (95)	rr	128 (61)	32 (45)	16 (33)	4 (25)	8 (28)		4 (18)
		R_1R_2	512 (86)	R_2r	128 (62)	8 (25)	16 (28)	4 (18)	4 (18)		4 (18)
				R_1R_2	64 (51)	16 (28)	4 (15)	2 (15)	8 (23)		2 (10)
	high-protein	rr	256 (85)	rr	64 (54)	32 (44)	8 (31)	16 (35)	8 (28)		4 (21)
		R_1R_2	128 (75)	R_2r	64 (51)	8 (31)	8 (20)	4 (28)	4 (17)		2 (15)
				R_1R_2	64 (46)	16 (38)	8 (26)	8 (30)	8 (28)		2 (13)
	high-protein	rr	256 (92)	rr	64 (60)						1 (7)
		R_1R_2	256 (86)	R_2r	64 (59)						0
				R_1R_2	128 (70)						0

TABLE 14—*AB serum and LISS enhancement effects with Rh antisera in cells and stains.*

Antiserum Specificity/Type	Cell Phenotype	Titer (Score) Against Cells Using				Stain Phenotype	Age[a]	Titer (Score) of Eluate With		
		Albumin	AB Serum	LISS	Papain			Papain	AB Serum	LISS
Anti-D (AHG)	R_1R_2	2000 (95)	4000 (102)	4000 (102)	4000 (100)	R_2r	6 m	8 (28)	2 (12)	2 (10)
	R_1r	1000 (90)	4000 (100)	2000 (97)	4000 (100)	R_1r	6 m	8 (26)	1 (7)	2 (16)
Anti-C (saline)	R_1R_1	128 (66)	128 (70)	128 (63)	1000 (118)	R_1R_2	1 m	16 (38)	16 (15)	16 (38)
	R_1r	128 (61)	128 (67)	64 (53)	512 (103)	R_1R_1	4 m	8 (22)	4 (20)	4 (18)
						R_1r	6 m	4 (18)	2 (15)	4 (17)
Anti-e (modified IgG)	R_1R_2	4 (29)	64 (47)	16 (38)	512 (86)	R_1R_2	6 w	16 (28)	32 (41)	8 (23)
	rr	8 (32)	64 (60)	16 (50)	512 (95)	R_2r	3 m	4 (15)	16 (33)	4 (18)
						rr	6 m	2 (18)	8 (20)	1 (7)
Anti-e (saline)	R_1R_2	32 (54)	64 (59)	64 (63)	512 (63)	R_1R_2	6 w	2 (12)	8 (30)	16 (25)
	rr	128 (70)	128 (70)	64 (65)	512 (65)	R_2r	3 m	4 (15)	4 (18)	4 (17)
						rr	6 m	2 (18)	2 (10)	2 (10)
Anti-c (high protein)	R_1R_2	64 (58)	512 (92)	64 (60)	2000 (104)	R_1R_2	6 w	32 (41)	64 (48)	16 (33)
	rr	128 (65)	512 (92)	128 (65)	2000 (104)	rr	6 m	8 (28)	2 (12)	2 (10)

[a]m = months; w = weeks.

At the washing stage, washing time and wash fluid volume relative to the quantity of sample are important in ensuring that all unbound antibody is removed. The procedure developed was to wash samples consisting of one to several threads or pieces up to 0.5 cm^2 in 12- by 75-mm test tubes. The tubes were completely filled with saline six times, with 15 min between changes. This procedure was found to be sufficient. Incomplete washing can cause false positive reactions at the detection stage, but the substratum and antigen-negative bloodstain controls included in every test reveal this potential problem should it occur. Washing fluid temperature should be 4°C at all times. It was found in previous studies [*24*] and confirmed here that a significant amount of antibody is eluted at 20 and 37°C. Although the quantity is less than that obtained at 56°C (by a factor from two to eight), the results show that considerable specifically bound antibody could be lost with washing fluids warmer than 4°C, especially during multiple washes.

At the elution stage, the eluate volume, the temperature, and the time may be varied. The volume of eluate need not exceed one recoverable volume unless a second recoverable volume is needed to carry out a titration of the eluate. Elution temperature is commonly 56°C [*21*]. Our studies indicate that 56 to 60°C is optimal for ABO antibodies. In previous studies with an anti-D [*24*], it was found that antibody yield increased at temperatures somewhat higher than 56°C, although it was completely satisfactory at 56°C. Elution times of 20 to 30 min yield maximal antibody from the samples. There is no advantage in eluting for longer periods, and evaporation of elution fluid can become a problem at longer times. The speed with which the eluate is separated from the sample after elution is complete is important because the antibodies can become bound again to stain receptors if the temperature decreases and thus be lost to the recovered eluate. It was shown in previous studies [*24*] that eluate anti-A yield from packed A cells decreased 8- to 32-fold if the samples were allowed to remain at room temperature for 5 min before removing the eluate, as compared with immediate removal.

At the detection stage, the phenotype of the test cells employed is important to the extent that antibodies often have higher titers with one type than with another. With anti-A, A_1 cells are more reactive than A_2; with most other antibodies, cells homozygous for the corresponding antigen give higher titers than heterozygous cells. Using test cells that give higher titers with the antibody increases the sensitivity of the procedure. In addition, the sensitivity of agglutination reactions may be increased by decreasing test cell concentrations. The effect is more apparent at lower antibody concentrations. The present data with an anti-D confirm previous findings with anti-A and anti-B [*52*] and anti-c [*24*].

Characteristics of Commercial Antisera

The titers of representative anti-A and anti-B are relatively uniform. Anti-H lectins exhibit greater variability. Anti-H preparations that have acceptable O (and A_2) cell titers but relatively low or nil titers with A_1 and B cells are preferable. Representative MN sera and lectins showed more variability than anti-A and anti-B reagents; three of the five anti-N reacted to some extent with M cells.

Representative Rh antisera showed considerable variability in titer under various conditions, and these require careful evaluation. While some showed AB serum and LISS enhancement as compared with albumin, all exhibited enhancement with papain-treated cells, and the papain technique may be regarded as the best enhancement procedure for routine use.

Bloodstain Antigen Determination and Persistence

Extensive studies on ABH antisera and lectins were not pursued. Problems encountered in ABH grouping of bloodstains are commonly at the level of interpretation. They arise from

the ubiquitous occurrence of the antigenic structures in nature, a matter that has been extensively discussed elsewhere [*11*], and not primarily because of problems with antisera. Anti-A and anti-B are among the best agglutinins in the spectrum of blood grouping antisera, and red cells have significantly more ABH receptors per cell than any other blood group antigen for which there is data [*55*]. As a result, there should be little problem in obtaining suitable elution results if proper technique is followed, if appropriately titered antisera and lectins are employed, and if the bloodstains have dried without undergoing some unexpected degradative change.

Anti-M behaved as expected with M and MN bloodstains, and anti-N detected N in N and MN stains over the five-month aging period. The anti-N used in these experiments was cross-reactive with M cells. It reacted with the M stain when it was fresh, but not after three or more weeks of aging. The data cannot be taken to mean that the cross-reacting N in an M stain disappears from every M stain upon aging, even though it did so in this one. The results with a different anti-N and a different M stain could be different. The biochemical basis for anti-N reactivity with M cells and stains is now known [*33–36*]. All red cells contain a sialoglycoprotein that carries the amino acid sequence defining N. The fact that anti-N reagents are specific for cells that are genotypically NN or MN is thought to have to do with the topography of arrangement of sialoglycoproteins on the red cell surface, and perhaps also with the complex carbohydrate structures attached to the amino acids. Since little is known about the changes these surface structures undergo as blood dries, the extent to which a given anti-N will show specificity is unpredictable, even if the reagent has been tested with red cells and other bloodstains. The reactivity of some anti-N with some M cells or stains is intrinsic and not attributable to a lack of antiserum specificity. For these reasons, N is not determined in casework bloodstains. A solution to the problem may lie in a biochemical approach, such as that suggested by Shaler [*56*]. As monoclonal antibody technology progresses for blood grouping reagents [*57,58*] and forensic science applications are pursued [*59*], it is possible that appropriately specific anti-N can be prepared. One recently described monoclonal anti-N, however, did not show the required specificity [*60*].

In the elution tests with Rh antisera on relatively fresh bloodstains of different phenotypes, the antigens were readily detected by all the antisera employed in the stains tested, with the papain titers of the eluates varying from 64 to 512. Stain eluate titers and scores do not necessarily correlate with the red cell titers of the absorbing antisera. Antisera of comparable red cell titer can give measurably different eluate titers when used in elution tests.

Several Rh antisera of each specificity were used in elution tests for the corresponding antigens in a series of aging bloodstains of various phenotypes, the eluates being titrated as a relative measure of antibody yield. Eluate titers decreased as the stains aged, but in the majority of 26-week-old stains the antigens were convincingly detected.

It was initially thought that the titer and score of an antiserum with cells and the eluate titer and score with fresh stains might serve as a basis for predicting the success with which the antiserum could be used to detect the corresponding antigen in older stains. The data on the limited number of stains studied, however, indicate that antisera that have higher titer and score values with cells do not necessarily yield the highest eluate titers with fresh stains. Further, those that yield comparatively high eluate titers with fresh stains do not necessarily give the highest eluate titers with older (six months to several years) stains. The majority of the antisera tested, however, gave convincing typing results for the corresponding antigen in six-month-old stains, and a significant number of antisera yielded convincing results with stains one to three years old. The results with a particular antiserum and a particular stain cannot be accurately predicted, and an antiserum can react convincingly with a three- or four-year-old stain, while failing to react with a different stain that is eighteen months old.

Tests carried out on Rh antisera to compare papain enhancement with other recognized agglutination enhancement procedures indicated that all the stains tested were readily typable. The different antisera gave variable eluate titers with the stains in the various media, not necessarily related to the titer and score value with cells in the same medium.

Studies to determine the typability of Rh, C, D, and e in relatively fresh bloodstains on different substrata showed that the expected antigens were readily detectable on most of them. In a few cases, antigens actually present were not detected by direct testing of stains. With a few substrata in which direct testing proved negative, the antigens in the stains could be detected by transfer technique. These Rh results are generally in accord with those of previous investigators [*39,41–44*]. Martin [*42*] could detect all the antigens present in stains up to six months old using procedures similar to those in the present work, except that eluates were not titrated. The Rh antigens c and D were more readily detected than C, E, and e in year-old stains in his studies. This effect was not apparent in the present studies, although few of the stains examined were a year or more old. Bargagna and his collaborators [*43*] found D, C, and c to be detectable in stains up to six months old, and E and e in stains up to four and two months old, respectively, using the papain technique. The use of LISS in conjunction with papain enabled detection of the antigens in still older stains, and the LISS effect was particularly noticeable in older stains.

McDowall and collaborators [*43*] had previously shown that the use of LISS increased the sensitivity of elution tests for detecting blood group antigens, including Rh antigens, in stains, and that LISS sometimes enabled detection of an antigen in an older stain which would have reacted weakly or gone undetected without it. The usefulness of LISS in conjunction with papain-treated cells in enhancing eluted antibody detectability has been demonstrated by Lincoln and Dodd [*61*]. Denault and collaborators [*62*] reported that C could be detected in 26-week-old stains, D, c, and E in 13-week-old stains, and e in 2-week-old stains on cotton cloth at normal humidity. Some antigens were not detectable for as long in stains kept at higher humidities. Their elution tests were carried out using saline-albumin media, and somewhat higher cell concentrations than in the present studies. Surprisingly, they said that no significant improvement in detectability was observed with the enzyme technique. The findings of Maeda and collaborators [*63,64*] indicated that all the Rh antigens except e could be detected in room temperature aged bloodstains ten to eleven months old. The D antigen was occasionally detected in two-year-old stains, but C, c, and E were generally difficult to detect in fifteen- to twenty-month-old stains. Their studies were carried out with commercially available antisera on bloodstains prepared from packed cells. Testing was carried out on 0.5-cm^2 stain samples, and the papain technique was employed with some of the antisera. The results of these studies on the applicability of commercially available grouping antisera to bloodstain antigen determination confirm previous findings on the optimization of elution procedure parameters and provide additional information on commercial antisera intended for stain grouping.

Some emphasis has been placed on Rh antigens in this paper. In a subsequent paper [*45*], we focus on Ss, Kell, Duffy, Kidd, and selected Gm/Km antigens.

Except for the circumstances in which an unexpected Rh antibody was encountered and in which the absorbing anti-D titer exceeded the optimal value, no false positive results were observed in the elution tests performed with Rh antisera in these studies. There is no doubt, however, that antigens actually present may not be detected, particularly in older stains. The false negative results should cause no problems as long as results are interpreted in terms of antigens actually detected. In those laboratories equipped to carry out the procedures, Rh and M antigen determination can provide valuable individualizing information from bloodstains in addition to that obtainable from ABO, isoenzyme, and serum protein typing.

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