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Evaluation of Antisera for Bloodstain Grouping II. Ss, Kell, Duffy, Kidd, and Gm/Km

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ABSTRACT: Thirty-one different examples of commercially available blood grouping antisera specific for the S, s, K, k, Fy^a, Fy^b, Jk^a, and Jk^b antigens and anti-human globulin sera were serologically evaluated with red cells and in absorption-elution tests to determine their applicability to bloodstain antigen determinations. Nineteen examples of commercially available antisera specific for various Gm and Km antigens and their corresponding anti-D reagents were likewise evaluated in inhibition tests with sera and bloodstains. Elution tests with the blood grouping antisera and inhibition tests with the Gm/Km antisera on a series of aging bloodstains on cotton cloth, and on bloodstains on a number of different substrata, demonstrated that properly evaluated commercial antisera are useful reagents for bloodstain grouping in forensic serology.

KEYWORDS: pathology and biology, genetic typing, antigen systems, blood groups, serum groups, bloodstains, MNSs system, Kell system, Duffy system, Kidd system, Gm system, Km system, absorption-elution, antiserum evaluation

Methods of grouping dried bloodstains as a means of partial individualization are as old as the recognition of blood groups as genetic markers in human blood [*1,2*]. As new blood group systems have been discovered and shown to be independent of previously known systems, forensic science and medicolegal investigators have initiated studies to devise methods for their determination in dried bloodstains [*3*]. Inhibition methods have usually been developed and utilized earlier than elution methods. Although an elution procedure was first described for dried blood grouping more than 50 years ago [*4*], the method was not widely employed in forensic serology until 1960, when Kind described a technique [*5,6*] that was quickly shown to be sensitive, reliable, and reproducible [*3*]. All the bloodstain grouping procedures in common use today employ heat for the elution of the bound antibodies, a technique first described by Landsteiner and Miller [*7*].

Up to 1940, only three blood group systems, ABO, MN, and P, had been described, and the Rh system antigens were beginning to be recognized. The simultaneous finding by Race

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[8] and Wiener [9] in 1944 that certain blood group antibodies were "incomplete," that is, that they could be bound to the antigenic receptors on the cells but fail to bring about agglutination in saline media, opened up a new era in blood group serology. Methods and reaction media that enabled or enhanced agglutination reactions with the "incomplete" antibodies were soon described.

These included albumin and other protein and colloidal media [10-12], anti-human globulin serum (Coombs serum) [13,14], and the treatment of red cells with certain enzymes to render them more agglutinable by incomplete blood group antibodies [15,16]. The availability of these methods and the knowledge on which they were based led to the discovery of a number of new blood group antigens and systems, including Ss, Kell, Duffy, and Kidd. More recently, the ionic strength of the medium has been shown to have a significant effect on agglutinin-agglutinogen binding, especially with incomplete antibodies. Low ionic strength solutions (LISS) can substantially enhance the agglutination of cells by these antibodies. This effect, first demonstrated with anti-D [17], can be observed with a variety of different antibodies [18-22]. In addition, LISS enhancement may be used in conjunction with other enhancement procedures, such as the use of papain-treated cells [23,24]. LISS procedures have been shown to be applicable to the detection of eluted antibodies, and thus to bloodstain grouping by elution techniques [25,26].

The S antigen was recognized in 1947 [27-29]. The antigen s, coded for by the allele of the gene coding for S, was found in 1951 [30], and the *Ss* locus was found to be closely linked to that of *MN* [31]. The serological characteristics of many Ss antisera are more like those against Kell, Duffy, and Kidd antigens than those against MN. For this reason, we have included the Ss studies here rather than with those on MN [32].

The K antigen of the Kell system was found independently in two different laboratories [33-36]. The antigenic product of the Kell gene's allele, denoted k, was described soon afterward [37]. The Kell system has since been found to be more complex [3], but these complexities are not important to the present work.

The original Duffy system antigens, Fy^a and Fy^b, were described within a year or two of one another [38-41] and shown to be the products of allelic genes. It was soon found that the phenotype Fy(a-b-), which is extremely rare in white populations, is very common in black populations [42]. The observation is accounted for by the occurrence of a common silent Duffy allele, *Fy*, in the latter.

The common antigens of the Kidd system, Jk^a and Jk^b, were likewise described in the 1950s [43,44] and shown to be the products of allelic genes.

The Gm system is a complex group of inherited antigenic receptors located on the γ heavy chains of human immunoglobulin G (IgG) molecules. The Gm system is not a blood group system, although serological methods have traditionally been employed in detecting Gm antigens. The first Gm antigen, called Gm^a, was described by Grubb [45] and shown to be inherited [46]. Many additional factors have since been described [3,47,48]. The Gm factors have been designated by lowercase letters, or by lowercase letters with numbers, or by numbers, and the nomenclature history of Gm is quite involved. There is international agreement on the nomenclature [49], which takes into account the recognized subclasses of IgG. Both numerical and alphameric designations are acceptable under the conventions; we use numerical designations.

The first Km antigen, originally called Inv but now called Km(1), was described in 1961 [50], and several other Km factors have since been found. The Km antigens reside on the κ light chains of the immunoglobulins.

The background and serology of the Ss, Kell, Duffy, and Kidd blood group systems have been extensively reviewed elsewhere [3,51-53], as have those of Gm and Km [3,47,48,54-57]. Sensitive and successful absorption-elution procedures for the determination of S [58,59], s, K, Fy^a, Fy^b, and Jk^a [59] in dried bloodstains have been available for a number of years. Some of the antigens can be detected in stains more than a year old. The use of LISS

technique could improve the sensitivity of elution tests for S, thus permitting determination of the antigen in older stains, and manual techniques were found to be more satisfactory for S and K than parallel autoanalyzer detection procedures [*25,60*]. Serological inhibition techniques are commonly employed for the determination of Gm antigens. With minor adaptations, they may be used for the identification of Gm antigens in dried bloodstains. Methods for Gm typing in bloodstains were devised more than 20 years ago [*61*], and their reliability and usefulness in forensic serology have been widely confirmed [*62-69*]. The presence of Km(1) can be determined in dried blood as well [*70*]. An informative and useful review of Gm and Km typing procedures in stains was given by Kipps [*71*].

Most forensic science laboratories have no access to blood grouping or to Gm/Km antisera other than those available commercially, and it is widely agreed among knowledgeable investigators that antisera intended for bloodstain grouping require careful prior evaluation. It was decided, therefore, to undertake an extensive study of commercially available antisera for blood group and Gm/Km antigens to evaluate their applicability to bloodstain typing. In a previous paper, we reported the results of these investigations on ABH, MN, and Rh antisera [*32*]. The absorption-elution procedure and the optimization of its variables were covered as well; those considerations are equally applicable to the blood group antiserum studies in the present work. Here, we report our results with antisera to the Ss, Kell, Duffy, and Kidd blood group antigens and to selected Gm and Km serum group antigens.

Materials and Methods

Antisera and Red Cells

Antisera to S, s, K, Fy^a, and anti-human globulin sera were obtained from Ortho Diagnostics, Raritan, NJ (Ortho), American Dade through American Scientific Products, Boston, MA (Dade), and Dr. Molter GmbH, Heidelberg, West Germany (Molter). Antisera to S, s, K, Fy^a, and Jk^a were obtained from Biological Corp. of America, West Chester, PA (BCA).[3] Hyland anti-s was obtained from Accugenics, Costa Mesa, CA (Hyland), and antisera to K and Jk^a were obtained from Pfizer Diagnostics Division, Irvine, CA (Pfizer).[3] Additional examples of anti-Jk^a were obtained from Dade and Molter, and an anti-Jk^b was procured from Ortho. Anti-Gm/Km sera of eight different specificities and their corresponding anti-D coats were obtained from four different suppliers as follows: anti-G1m(1), -G1m(2), -G1m(3), -G3m(10), and -Km(1) from Molter; anti-G1m(1), -G1m(2), -G1m(3), -G3m(5), and -Km(1) from Calbiochem-Behring, La Jolla, CA (Behring); anti-G1m(1), -G1m(2), and -G3m(11) from Biotest-Serum-Institut, Dreieich, West Germany, through Folex-Biotest-Schleussner, Inc., Fairfield, NJ (Biotest); and finally, anti-G1m(1), -G1m(2), -G1m(3), -G3m(5), -G3m(21) and -Km(1) from Dr. E. Fresenius, Borkenberg, West Germany (Fresenius). The Gm/Km serum controls were from Behring.

Antisera were stored at 4°C when not in use. For longer storage, some antisera were divided into small aliquots of 0.3 to 1 mL in polypropylene tubes and kept at −85°C until required. Antisera stored frozen were thawed only once and used immediately. Antisera supplied lyophilized were kept at −15°C and reconstituted as needed. Those requiring lengthy storage after reconstitution were divided into small aliquots and stored at −85°C, as indicated above for liquid antisera. Some control and donor sera were stored at −85°C. Those to be used within two weeks were kept at 4°C.

Identigen I and II and Resolve A and B cell panels were obtained from Ortho. Data Cyte

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

Reagent Red Blood Cell panels and Coombs positive (sensitized) red cells were from Dade. Some panel cells, as well as some red cells from donors, were frozen in glycerol in 1 mL aliquots at −85°C, as described by Boorman et al [*53*]. Frozen cells were recovered by dialysis against several changes of a large volume of saline and washed three times in saline before use.

Other Materials and Solutions

Phosphate-buffered saline was prepared by first making 50m*M* of sodium phosphate buffer, pH 7, and then dissolving 8.5 g of sodium chloride (NaCl) in each litre of buffer. Bovine albumin was obtained from Ortho or Dade as a 22% solution. Saline-albumin (sometimes designated as "albumin") is saline containing a final concentration of 0.5% albumin. The AB serum medium used (AB serum) was human AB serum diluted 1 in 10 with saline; the serum was obtained for these studies from Molter or Accugenics. Papain was from Sigma Chemical Co., St. Louis, MO, and papain solution was prepared according to the method of Löw [*72*]. LISS was either prepared according to the method described by Löw and Messeter [*73*] or obtained from Ortho.

Bloodstains were prepared from fresh whole blood on clean cotton cloth, or on other indicated substrata, dried completely, and stored at room temperature.

Procedures

Titrations were carried out in 6- by 50- or in 12- by 75-mm tubes, using 0.05 or 0.025 mL as one volume, by making doubling dilutions of antisera such that each tube in the row finally contained one volume. One volume of cells (0.05 to 1.0%) was added to each tube. After appropriate incubation and usually a brief centrifugation step to pack the cells, the contents of each tube were transferred to Boerner slides for rotation and microscopical reading. For titrations by indirect anti-human globulin technique, sensitization was carried out essentially as described for setting up titrations, except that 2 to 3% cell suspensions were employed for antisera and 0.5 to 1% cell suspensions for eluates. After incubation at 37°C for 45 min (or longer as necessary), the cells were washed three times in saline. One volume of 0.5% cells was then added to one volume of appropriately dilute AHG serum, mixed well and placed in a Boerner slide well for rotation and reading.

Anti-human globulin (AHG) sera were titrated with 0.5% sensitized cells. For routine use, AHG sera were employed at the dilution giving the last 4+ agglutination reaction in the titration series. Agglutination was scored from 4+ to w (weak) or − (negative). A titration score was obtained by assigning an arbitrary number to each degree of agglutination and summing the values over the titration series. The scoring system, modified from Issitt and Issitt [*74*], was 4+ = 12, 3+ = 10, 2+ = 8, 1+ = 5, w = 2, and negative = 0. Scores are usually given in parentheses following the titer value.

Red cell concentrations were determined on the basis of packed cells as 100%. Papain treatment of red cells followed the procedure of Boorman et al [*53*].

Absorption-elution tests were carried out in 12- by 75-mm tubes, following the optimized procedures described in detail in our previous work [*32*]. Three bloodstained cotton threads were covered with appropriately high titered antisera for 17 h at 37°C. Excess antiserum was removed and the threads washed six times in ice-cold saline, allowing 15 min at 4°C per wash. After the final wash and removal of essentially all liquid, saline, saline-albumin, or another diluent equivalent to two volumes was added and elution carried out at 56°C for 20 min. Eluates were quickly removed to a previously prepared row of tubes of titration, either directly or by indirect anti-human globulin technique. Titration of eluates, as pointed out previously [*32*], permits the estimation of the relative quantity of antibody eluted from the stain sample. Eluted antibodies were detected with group O test cells homozygous for the corresponding antigen.

Anti-Gm/Km sera were titrated by doubling dilutions in the usual way. The test cells for these titrations, and for the detection of anti-Gm/Km antibodies in general, were group O, Rh+ cells previously sensitized with an anti-D containing the corresponding Gm or Km antigen. Determination of the optimal concentration of anti-D and Rh+ cells was accomplished by preparing a series of mixtures of 50% washed red cells and anti-D in volume ratios from 1:1 to 1:6, incubating at 37°C for 45 min, washing the cells three times in saline, and then titrating the corresponding anti-Gm/Km serum with 0.5% suspensions of the sensitized cells in saline-albumin. The ratio of 50% cells to anti-D giving the highest titer and score with a given anti-Gm/Km is the optimal sensitizing ratio, and the titer and score of the anti-Gm/Km reagent have been determined in the process.

The dilution of anti-Gm/Km serum to be employed for typing was taken to be that giving the last 3+ reaction in the titration series with optimally sensitized test cells. Antisera that gave 3+ reactions only when neat, or that were weaker than 3+ when neat, were used neat for typing.

To type a serum sample for Gm/Km, three sample tubes and four control tubes were used for each specificity. Two of the sample tubes received one volume of 1:10 dilute serum; the third, a volume of 1:20 dilute serum. Two control tubes received one volume each of 1:10 and 1:20 dilute antigen-positive serum and two other control tubes were similarly arranged with antigen-negative serum. A volume of appropriately dilute anti-Gm or anti-Km serum was added to each control tube, to the 1:20 serum sample tube, and to one of the 1:10 serum sample tubes. To the other 1:10 serum sample tube was added a volume of saline (saline control). After incubation of the tubes at 4°C for 17 h, one volume of 0.5% optimally sensitized test cells was added to every tube, after which the contents were mixed and then transferred to a Boerner slide well. The Boerner slide was rotated for 15 or 20 min or until the antigen-negative controls showed definite agglutination. All wells were then read microscopically.

Antigen-positive controls must be completely negative, antigen-negative controls must show definite agglutination, and both the 1:10 and 1:20 serum sample tubes must be completely inhibited (negative agglutination) for a convincing positive. If the 1:20 serum sample showed inhibition but the 1:10 did not, the test was repeated using more dilute anti-Gm/Km serum or each tube was titrated out completely. Finally, the saline control must be negative; agglutination in this tube indicates some kind of anti-Gm/Km activity in the test serum, and the results cannot be interpreted.

For stain typing, three 1-cm-long cotton threads of bloodstained material was the usual sample. More sample can be taken with older stains or stains that appear to be dilute or thin. A similar quantity of cloth (substratum) control must be taken along with a third sample to serve as a saline control. Positive and negative control stains should be set up in each test. The tests were carried out in tubes. Appropriately dilute anti-Gm/Km sera was placed in the tubes in sufficient quantity to cover the threads. One volume was adequate for a "one-step" test; two volumes were required if the tests are to be titrated out. The saline control sample received the same quantity of saline in place of antiserum. Tubes were incubated at 4°C for 17 h. The bloodstained samples were then carefully removed, taking care to remove as little liquid as possible. A volume of optimally sensitized 0.5% test cells was added to every tube, and the remainder of the procedure was the same as that described for serum typing above.

Results

Anti-Human Globulin Reagents

Many Ss and all Kell, Duffy, and Kidd antisera were Coombs-reactive and thus required the use of AHG. Table 1 indicates the properties of a number of commercial Coombs sera. All were titrated against 0.5% test cells. Most of the reagents had titers of 128 to 256. The

TABLE 1—*Serological properties of representative anti-human globulin sera.*

Manufacturer	Test Cells	Reciprocal Dilution Giving Last		Titer (Score)
		4+ reaction	1+ reaction	
Ortho	K+, strongly sensitized	1	256	256 (85)
Ortho	K+, moderately sensitized	...	64	64 (...)
Ortho	K+, weakly sensitized	...	2	2 (...)
Ortho	K+, unsensitized	...	...	0 (...)
Ortho	Dade, sensitized	8	256	256 (91)
Dade	Dade, sensitized	8	256	256 (87)
Molter	Dade, sensitized	4	128	128 (81)

last dilution in the titration series giving a 4+ reaction was chosen as the working dilution for routine use. Those sera that gave less than a 4+ reaction when neat were used undiluted.

Ss, Kell, Duffy, and Kidd Antisera

Table 2 shows the titers and scores of a number of representative antisera to S, s, K, k, Fy^a, Fy^b, Jk^a, and Jk^b under several different serological test conditions. Titrations were carried out using an indirect Coombs technique, except in the case of the first two anti-S sera, which were saline-reacting (designated "sal" in the table). Titers (or scores) were usually higher with cells homozygous for the corresponding antigen, especially in albumin. Enhancement in AB serum compared with albumin was seen with some Ss and Kell antisera, but this effect was slight or absent with Duffy and Kidd reagents. Many of the reagents were more reactive in LISS, some of them significantly so. Kidd reagents were significantly more reactive with papain-treated cells.

Absorption-Elution Tests with Ss, Kell, Duffy, and Kidd Antisera in Aging Bloodstains

A number of Ss, Kell, Duffy, and Kidd antisera were employed in a series of elution tests on bloodstains representing both homozygous and heterozygous phenotypes over the course of a year's time.

Eluates were titrated to provide a relative measure of antibody yield. In some cases, antisera were employed under more than one serological condition to provide a comparison. Table 3 shows the results of these studies. Along a given row in the table, the same bloodstain was followed for the entire course of the aging period. In the 24-week-old and older stains, the effects of AB serum or LISS media enhancement, particularly the latter, could be seen in a number of the cases. The antigen was detected in some stains in enhancing media where it was undetected in comparably aged stains in saline-albumin. Antisera did not always show better eluate antibody yields in older stains with enhancement media, however, even in some cases where enhancement was apparent with red cells. There was no apparent correlation between antiserum cell titers or scores and eluate antibody titers in older bloodstains. Some antisera of every specificity yielded convincing amounts of antibody in eluates from 24-week-old bloodstains aged at room temperature. Year-old bloodstains yielded measurable eluate antibody with one anti-S, two anti-s, two anti-K, and two anti-k antisera. Duffy and Kidd antisera did not give convincing antibody yields in the year-old stains shown in Table 3. In a few stains one to several years old that are not shown in the table, however, detectable eluate antibody was obtained with selected examples of antisera to S, s, k, Fy^a, and Jk^a.

TABLE 2—*Serological characteristics of Ss, Kell, Duffy, and Kidd antisera.*

Specificity	Manufacturer	Cell Phenotype	Titer (Score)			
			Albumin	AB Serum	LISS	Papain
Anti-S	Molter (sal)[a]	SS	256 (75)	256 (76)	256 (90)	
		Ss	128 (70)	256 (76)	256 (84)	
Anti-S	BCA (sal)	SS	64 (58)	64 (54)	64 (60)	
		Ss	32 (48)	32 (51)	64 (54)	
Anti-S	Ortho	SS	32 (50)	32 (43)	32 (51)	
		Ss	32 (46)	32 (41)	32 (50)	
Anti-S	Dade	SS	32 (51)	64 (63)	64 (65)	
		Ss	16 (38)	64 (61)	64 (54)	
Anti-s	Ortho	ss	32 (52)	...	...	
		Ss	16 (38)	...	...	
Anti-s	Dade	ss	128 (63)	...	128 (65)	
		Ss	32 (43)	...	32 (43)	
Anti-s	Molter	ss	64 (65)	256 (77)	128 (70)	
		Ss	64 (60)	128 (72)	64 (65)	
Anti-s	BCA	ss	128 (63)	128 (67)	128 (77)	
		Ss	32 (43)	64 (61)	64 (65)	
Anti-s	Hyland	ss	64 (58)	64 (56)	...	
		Ss	32 (51)	64 (54)	...	
Anti-K	Ortho	KK	64 (64)	128 (67)	64 (65)	
		Kk	8 (40)	32 (40)	64 (67)	
Anti-K	Dade	KK	64 (54)	64 (64)	64 (65)	
		Kk	32 (41)	64 (58)	64 (58)	
Anti-K	Molter	KK	64 (51)	64 (56)	64 (61)	
		Kk	64 (51)	32 (54)	64 (58)	
Anti-K	BCA	KK	128 (59)	128 (56)	128 (69)	
		Kk	64 (54)	64 (56)	64 (56)	
Anti-K	Pfizer	KK	256 (71)	256 (76)	128 (68)	
		Kk	128 (68)	128 (63)	64 (65)	
Anti-k	Ortho	kk	64 (58)	64 (58)	64 (63)	
		Kk	32 (48)	64 (56)	64 (50)	
Anti-k	Dade	kk	64 (60)	64 (63)	128 (66)	
		Kk	32 (48)	32 (51)	64 (60)	
Anti-k	Molter	kk	64 (47)	64 (58)	64 (63)	
		Kk	32 (45)	64 (51)	64 (55)	
Anti-Fy[a]	Ortho	Fy(a+b−)	64 (60)	64 (57)	128 (78)	
		Fy(a+b+)	32 (48)	32 (49)	64 (75)	
Anti-Fy[a]	Dade	Fy(a+b−)	64 (51)	128 (63)	128 (75)	
		Fy(a+b+)	64 (49)	64 (60)	64 (65)	
Anti-Fy[a]	Molter	Fy(a+b−)	128 (69)	128 (58)	128 (74)	
		Fy(a+b+)	64 (64)	64 (56)	128 (65)	
Anti-Fy[a]	BCA	Fy(a+b−)	64 (55)	64 (58)	128 (68)	
		Fy(a+b+)	32 (47)	32 (50)	64 (58)	
Anti-Fy[b]	Molter	Fy(a−b+)	64 (53)	64 (60)	256 (73)	
		Fy(a+b+)	32 (45)	32 (52)	128 (61)	
Anti-Fy[b]	Dade	Fy(a−b+)	32 (57)	...	128 (70)	
		Fy(a+b+)	32 (38)	...	128 (65)	
Anti-Jk[a]	Molter	Jk(a+b−)	8 (35)	8 (37)	64 (57)	64 (56)
		Jk(a+b+)	8 (33)	8 (37)	64 (55)	32 (58)
Anti-Jk[a]	Dade	Jk(a+b−)	16 (37)	16 (33)	64 (60)	64 (52)
		Jk(a+b+)	16 (32)	8 (28)	64 (60)	32 (47)
Anti-Jk[a]	BCA	Jk(a+b−)	8 (30)	4 (24)	128 (62)	128 (60)
		Jk(a+b+)	4 (27)	4 (22)	64 (55)	32 (53)
Anti-Jk[a]	Pfizer	Jk(a+b−)	4 (12)	4 (17)	128 (58)	64 (47)
		Jk(a+b+)	4 (10)	2 (10)	64 (53)	32 (41)
Anti-Jk[b]	Ortho	Jk(a−b+)	2 (10)	...	128 (51)	64 (46)
		Jk(a+b+)	2 (10)	...	32 (41)	32 (41)

[a]sal = saline-reacting.

TABLE 3—*Absorption-elution tests with Ss, Kell, Duffy, and Kidd antisera in aging bloodstains.*

Specificity	Manufacturer	Titer (Score) with Red Cells		Stain Phenotype	Titer (Score)[a] of Eluate from Bloodstains, Age in Weeks								
					Fresh	1	2	4	6	8	12	24	52
Anti-S	Ortho	SS	32 (50)	SS	...	8 (25)	...	4 (18)	4 (18)	8 (26)	4 (15)	2 (10)	...
		Ss	32 (46)	Ss	...	8 (28)	...	8 (20)	4 (20)	4 (20)	2 (12)	1 (10)	...
Anti-S	Molter[b]	SS	256 (75)	SS	...	128 (53)	...	4 (18)	8 (28)	4 (17)	8 (20)	4 (15)	...
		Ss	128 (70)	Ss	...	64 (49)	...	8 (20)	8 (22)	8 (23)	4 (17)	2 (10)	...
Anti-S	Molter[b,c]	SS	256 (76)	SS	...	...	...	32 (48)	...	...	...	4 (12)	0
		Ss	256 (76)										
Anti-S	Molter[b,d]	SS	256 (90)	SS	...	...	...	16 (36)	...	...	...	4 (25)	4 (17)
		Ss	256 (84)										
Anti-S	Dade	SS	32 (51)	SS	...	8 (23)		NFT[e]					
		Ss	16 (38)	Ss	...	8 (20)							
Anti-S	BCA[b]	SS	64 (58)	SS	...	64 (48)	...	4 (15)		NFT			
		Ss	32 (48)	Ss	...	64 (47)	...	8 (30)					
Anti-s	Ortho	ss	32 (52)	ss	...	16 (33)	...	...	...	1 (7)	...	0	
		Ss	16 (38)	Ss	...	8 (30)	...	...	...	1 (5)	...	0	
Anti-s	Dade	ss	128 (63)	ss	...	64 (48)	...	16 (31)	8 (25)	8 (20)	4 (17)	1 (7)	
		Ss	32 (43)	Ss	...	32 (40)	...	8 (25)	4 (15)	4 (17)	2 (12)	1 (7)	
Anti-s	Molter	ss	64 (65)	ss	...	16 (40)		NFT					
		Ss	64 (60)	Ss	...	8 (33)		NFT					
Anti-s	BCA	ss	128 (63)	ss	...	64 (48)	...	16 (25)	4 (20)	8 (28)	8 (20)	2 (15)	
		Ss	32 (43)	Ss	...	64 (46)	...	4 (15)	4 (23)	4 (20)	4 (15)	1 (7)	
Anti-s	BCA[c]	ss	128 (67)	ss	...	...	...	8 (33)	...	...	...	4 (20)	1 (7)
		Ss	64 (61)										
Anti-s	BCA[d]	ss	128 (77)	ss	...	...	...	16 (43)	...	...	...	4 (17)	2 (12)
		Ss	64 (65)										
Anti-s	Hyland	ss	64 (58)	ss	...	32 (43)		NFT					
		Ss	32 (51)	Ss	...	16 (33)		NFT					
Anti-K	Ortho	KK	64 (64)	Kk	64 (41)		NFT						
		Kk	8 (40)										
Anti-K	Dade	KK	64 (54)	Kk	64 (46)	...	...	...	...	...	...	0	
		Kk	32 (41)										
Anti-K	Molter	KK	64 (51)	Kk	128 (50)	16 (33)	8 (35)	8 (30)	8 (28)	8 (20)	2 (12)	1 (2)	
		Kk	64 (51)										

Anti-K	Molter[c]	KK	64 (56)	Kk	...	...	32 (55)	...	...	...	...	4 (15)	1 (5)
		Kk	32 (54)										
Anti-K	Molter[d]	KK	64 (61)	Kk	...	...	32 (41)	...	...	...	...	8 (25)	1 (5)
		Kk	64 (58)										
Anti-K	BCA	KK	128 (59)	Kk	64 (41)		NFT						
		Kk	64 (54)										
Anti-K	Pfizer	KK	256 (71)	Kk	64 (50)	32 (38)	8 (30)	8 (31)	8 (23)	8 (20)	2 (15)	2 (10)	
		Kk	128 (68)										
Anti-k	Ortho	kk	64 (58)	kk	128 (60)	32 (40)	8 (35)	8 (28)	8 (31)	4 (21)	4 (20)	2 (10)	...
		Kk	32 (48)	Kk	32 (43)	8 (28)	8 (35)	4 (27)	4 (23)	2 (10)	4 (18)	1 (7)	
Anti-k	Ortho[c]	kk	64 (58)	kk	...	...	...	8 (31)	...	...	...	2 (15)	2 (15)
		Kk	64 (56)	Kk	...	...	...	8 (31)	...	...	...	1 (9)	1 (7)
Anti-k	Ortho[d]	kk	64 (63)	kk	...	...	...	8 (28)	...	...	...	4 (15)	2 (10)
		Kk	64 (50)	Kk	...	...	...	4 (26)	...	...	...	2 (10)	1 (5)
Anti-k	Dade	kk	64 (60)	kk	128 (55)	32 (36)	16 (35)	8 (28)	8 (30)	4 (21)		NFT	
		Kk	32 (48)	Kk	32 (40)	8 (27)	8 (28)	4 (23)	8 (28)	2 (12)		NFT	
Anti-k	Molter	kk	64 (47)	kk	64 (55)		NFT						
		Kk	32 (45)	Kk	16 (33)		NFT						
Anti-Fy[a]	Ortho	a+b−	64 (60)	a+b−	16 (35)		NFT						
		a+b+	32 (48)	a+b+	4 (25)		NFT						
Anti-Fy[a]	Dade	a+b−	64 (51)	a+b−	16 (36)	16 (35)	8 (28)	8 (23)	8 (26)	4 (26)	4 (15)	0	
		a+b+	64 (49)	a+b+	16 (31)	8 (28)	8 (23)	2 (10)	2 (12)	4 (18)	2 (10)	0	
Anti-Fy[a]	Dade[c]	a+b−	128 (63)	a+b−	...	...	8 (28)	4 (10)	...	2 (12)	...	1 (7)	0
		a+b+	64 (60)										
Anti-Fy[a]	Dade[d]	a+b−	128 (75)	a+b−	...	...	16 (25)	8 (18)	...	2 (10)	...	1 (7)	0
		a+b+	64 (65)										
Anti-Fy[a]	Molter	a+b−	128 (69)	a+b−	16 (35)	...	...	...	...	...	0 (2)		
		a+b+	64 (64)	a+b+	8 (29)	...	...	...	...	...	0		
Anti-Fy[a]	BCA	a+b−	64 (55)	a+b−	32 (36)	8 (32)	16 (33)	4 (20)	4 (18)	4 (23)	2 (10)	1 (7)	
		a+b+	32 (47)	a+b+	8 (25)	8 (25)	16 (33)	4 (20)	4 (17)	2 (13)	2 (12)	1 (7)	
Anti-Fy[a]	BCA[c]	a+b−	64 (58)	a+b−	...	...	4 (20)	2 (15)	...	1 (7)	...	0 (2)	0
		a+b+	32 (50)										
Anti-Fy[a]	BCA[d]	a+b−	128 (68)	a+b−	...	...	8 (31)	4 (23)	...	2 (20)	...	1 (7)	0 (2)
		a+b+	64 (58)										
Anti-Fy[b]	Molter	a−b+	64 (53)	a−b+	8 (30)	8 (23)	8 (25)	4 (22)	4 (23)	4 (18)	2 (12)	1 (7)	
		a+b+	32 (45)	a+b+	4 (20)	8 (23)	8 (23)	2 (14)	4 (15)	2 (12)	2 (12)	1 (5)	
Anti-Fy[b]	Dade	a−b+	32 (57)	a−b+	8 (23)	8 (28)	16 (36)	4 (22)	4 (20)	...	...	1 (7)	
		a+b+	32 (38)	a+b+	8 (26)	8 (25)	16 (30)	4 (20)	4 (20)	...	...	1 (5)	

TABLE 3—*(Continued)*

Specificity	Manufacturer	Titer (Score) with Red Cells	Stain Phenotype	Titer (Score)[a] of Eluate from Bloodstains, Age in Weeks								
				Fresh	1	2	4	6	8	12	24	52
Anti-Jk[a]	Molter	a+b− 8 (35) a+b+ 8 (33)	a+b−	32 (35)	16 (25)	8 (25)	2 (10)	4 (17)	2 (10)	2 (10)	0	
Anti-Jk[a]	Molter[d]	a+b− 64 (57) a+b+ 64 (55)	a+b−	...	...	8 (25)	8 (23)	...	...	2 (10)	1 (5)	
Anti-Jk[a]	Dade	a+b− 16 (37) a+b+ 16 (32)	a+b−	64 (44)	64 (41)	8 (23)	8 (26)	8 (23)	4 (20)	2 (10)	1 (7)	
Anti-Jk[a]	Dade[c]	a+b− 16 (33) a+b+ 8 (28)	a+b−	...	...	8 (30)	4 (20)	...	...	1 (7)	0 (2)	
Anti-Jk[a]	BCA	a+b− 8 (30) a+b+ 4 (27)	a+b−	16 (33)	...	...	...	...	0	NFT		
Anti-Jk[a]	Pfizer	a+b− 4 (12) a+b+ 4 (10)	a+b−	32 (38)	8 (20)	8 (23)	4 (15)	4 (20)	4 (15)	2 (10)	2 (12)	
Anti-Jk[a]	Pfizer[f]	a+b− 64 (47) a+b+ 32 (41)	a+b−	...	...	...	...	...	...	8 (20)	...	

[a]Albumin-saline medium and AHG technique unless otherwise indicated.
[b]Saline-reacting; direct testing.
[c]AB serum.
[d]LISS.
[e]NFT = not further tested.
[f]Papain technique.

Detection of k and Fy^a in Bloodstains on Different Substrata

Cellano and Fy^a were arbitrarily selected as representative antigens from the group in the present study. Tests for their detectability were conducted with bloodstains on various substrata, including cotton blends, synthetic fabrics, linen, wool, silk, suede, leather, Teflon®, wax, ceramic tile, and linoleum. The bloodstains used were two to four weeks old and eluates were not titrated in this series. Samples from textile materials could be cut out and subjected to direct testing. With materials where direct sampling was not possible, the dried blood was dissolved in minimal saline and transferred to cotton threads that were allowed to redry before testing. Both antigens were convincingly detected on the majority of stains tested. The Fy^a antigen was not as strongly detected on a number of stains as k. No false positive results were seen with any of the antigens on cotton or other substrata in these studies.

Gm/Km Reagents and Optimization of Gm/Km Test Parameters

Data were presented in previous work showing that blood grouping antisera and red cells retained activity well under cryogenic storage conditions [*32*]. Table 4 shows that several anti-G1m(1) reagents likewise retained activity well after storage at −85°C when thawed and tested immediately. Other anti-Gm sera and anti-D coats behaved similarly.

Gm and Km antisera were titrated by doubling dilutions using Group O, Rh+ cells sensitized with an incomplete anti-D which possessed the corresponding Gm or Km factor (the coat) as test cells. The optimal sensitizing volume ratio of anti-D to 50% Rh+ cells along with the titers of the anti-Gm/Km sera with optimally sensitized cells can be determined in one operation by using the two-dimensional titration scheme shown in Fig. 1. The results of these determinations for a representative selection of Gm/Km reagents is shown in Table 5.

Optimal sensitizing volume ratios varied but were generally in the 1 : 2 to 1 : 4 range. Titers of anti-Gm/Km reagents at optimal sensitizing volume ratios tended to be 32–64 for G1m(1) and G1m(2) antisera. Other specificities, of which we had fewer examples, varied. The working dilution of an anti-Gm/Km serum for routine inhibition testing is arbitrary within certain limits. In the present work, the dilution representing the last tube in the titration series (at the optimal sensitizing ratio) that gave 3+ agglutination was chosen. Table 6 shows the results of determining the working dilution for several representative reagents.

Table 7 shows a time course for the sensitization of R_1R_1 cells by an anti-D/Gm(2). Sensitization times of 45 min were essentially optimal. There appeared to be little advantage in sensitizing for longer periods. A number of different Rh+ cell phenotypes can be used as test cells for Gm/Km typing. Most workers have recommended R_2R_2 or R_1R_1 cells, some preferring the latter. The recommendations are probably based on studies of the number of measurable anti-D binding sites on the different Rh+ cells [*75*], those cells with greater numbers of sites being preferred. Table 8 shows titers and scores for three anti-G1m(1) sera with cells of three different Rh+ phenotype appropriately sensitized with anti-D/Gm(1). The differences between the cell types are relatively small. The R_1R_1 cells were used routinely in the

TABLE 4—*Activity of anti-Gm(1) before and after cryogenic storage.*

	Titer (Score) with Appropriately Sensitized R_1R_1 Cells	
Anti-G1m(1)	Fresh	After One Thaw
1	64 (60)	64 (57)
2	32 (52)	16 (49)
3	64 (52)	32 (48)

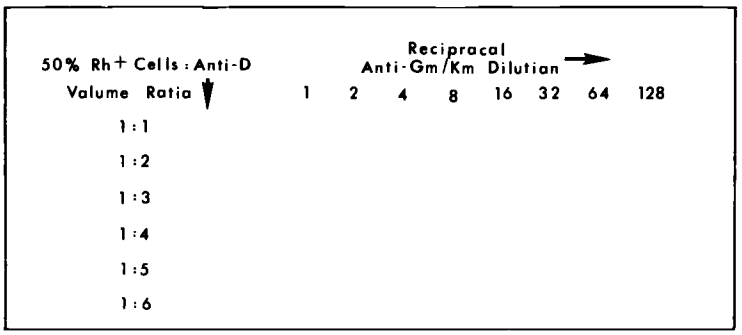

FIG. 1—*Two-dimensional titration scheme for determination of optimal sensitizing anti-D to red cells volume and anti-Gm/Km titers.*

TABLE 5—*Titration of representative anti-Gm/Km sera at different red cells to anti-D volume ratios.*

Anti-Gm/Km Reagent [Manufacturer]	Titer (Score) with 50% R_1R_1 Cells: Anti-D Volume Ratio					
	1:1	1:2	1:3	1:4	1:5	1:6
Anti-G1m(1) [Molter]	32 (52)	32 (54)	64 (60)	32 (50)	32 (48)	32 (46)
Anti-G1m(1) [Fresenius]	4 (15)	16 (52)	32 (52)	16 (47)	16 (43)	...
Anti-G1m(1) [Biotest]	32 (42)	32 (46)	64 (52)	32 (48)	32 (44)	...
Anti-G1m(1) [Behring]	4 (18)	32 (56)	32 (52)	64 (65)	32 (51)	16 (43)
Anti-G1m(2) [Molter]	32 (48)	32 (52)	32 (55)	32 (50)	32 (50)	32 (43)
Anti-G1m(2) [Fresenius]	32 (41)	64 (56)	32 (50)	64 (49)	32 (41)	...
Anti-G1m(2) [Biotest]	64 (49)	64 (56)	64 (60)	64 (56)	64 (56)	...
Anti-G1m(2) [Behring]	16 (33)	32 (48)	128 (68)	...	16 (41)	8 (33)
Anti-G1m(3) [Molter]	8 (28)	16 (33)	8 (28)	8 (28)	8 (33)	8 (26)
Anti-G1m(3) [Fresenius]	2 (10)	2 (12)	4 (18)	2 (12)	2 (10)	...
Anti-G1m(3) [Behring]	32 (51)	32 (51)	64 (64)	64 (60)	32 (41)	16 (41)
Anti-G3m(5) [Fresenius]	2 (12)	4 (21)	4 (21)	4 (23)	2 (13)	...
Anti-G3m(5) [Behring]	...	4 (15)	2 (12)	8 (23)	4 (20)	...
Anti-G3m(10) [Molter]	32 (55)	64 (60)	128 (72)	64 (55)	32 (52)	32 (50)
Anti-G3m(11) [Biotest]	32 (43)	32 (44)	32 (46)	16 (38)	16 (38)	...
Anti-G3m(21) [Fresenius]	32 (52)	32 (57)	16 (49)	32 (52)	16 (38)	...
Anti-Km(1) [Fresenius]	32 (41)	64 (56)	64 (55)	32 (46)	16 (45)	...

TABLE 6—*Determination of optimal anti-Gm/Km dilutions for representative reagents.*

Anti-Gm/Km Specificity [Manufacturer]	1	2	4	8	16	32	64	128	Dilution to Be Used for Typing
Anti-G1m(1) [Molter]	4+	3+	3+	3+	2+	1+	–	–	1:8
Anti-G1m(1) [Behring]	4+	3+	3+	3+	3+	2+	1+	–	1:16
Anti-G1m(2) [Fresenius]	3+	3+	3+	2+	2+	1+	1+	–	1:4
Anti-G1m(3) [Molter]	2+	2+	1+	1+	1+	w	–	–	neat
Anti-G3m(11) [Biotest]	4+	3+	2+	1+	1+	1+	w	–	1:2
Anti-Km(1) [Fresenius]	4+	3+	3+	2+	1+	1+	1+	–	1:4

TABLE 7—*Time course of sensitization of R_1R_1 cells by an anti-D/Gm(2).*

Time of Sensitization, min	Titer (Score) with Sensitized Cells Using Anti-G1m(2)
15	1 (5)
30	4 (21)
45	4 (23)
60	4 (23)
90	4 (23)
120	4 (23)
150	8 (28)

TABLE 8—*Effect of Rh cell phenotype on anti-Gm titers.*

	Titer (Score) with Sensitized Rh+ Cells		
Anti-G1m(1)	R_1R_2	R_1R_1	R_2R_2
1	128 (77)	128 (75)	128 (68)
2	128 (72)	256 (78)	128 (64)
3	128 (69)	128 (73)	256 (72)

present studies. Anti-Gm/Km reagents and their corresponding anti-D coats are generally supplied together, but reagent pairs from different sources can be mixed. Table 9 shows the properties of two anti-G1m(1) sera, each tested with its paired anti-D and with the anti-D intended for use with the other.

Gm and Km Antigen Determination in Aging Bloodstains

Bloodstains of a number of different Gm/Km antigen compositions were tested for the Gm and Km antigens for which reagents were available over the course of 48 weeks. Table 10 indicates the results of these studies. All the Gm and Km factors present were detected in the stains aged up to six months. All Gm factors present were detected in all but one of the stains aged up to eleven months. Km(1) was detected in the 26-week-old stain in which it was present, but not at 39 weeks. In a 39-week-old stain, the IgG1 markers present and the G3m(5) were detected, but G3m(21) was not. In another stain, all the markers present were detected at eleven months. In a few older stains not shown in the table, G1m(3) and G3m(5), (10), (11), and (21) were not detected in a year-old stain, and G1m (1) and (2) were not detected in stains two to five years old. Detection of immunoglobulin markers in stains by

TABLE 9—*Effect of different anti-D/Gm(1) on different anti-G1m(1) serum titers.*

	Titer (Score) of Sensitized Test Cells	
Anti-G1m(1)	Anti-D/Gm(1) 1	Anti-D/Gm(1) 2
1	64 (67)	64 (65)
2	16 (45)	16 (38)

TABLE 10—*Gm/Km typing results in aging bloodstains.*

Stain Phenotype	Gm/Km Typing Results[a] in Bloodstains,[b] Age in Weeks			
	Fresh	2	4	8
−1,−2,3;10		(−1,−2,3;10)	(−1,−2,3;10)	(−1,−2,3;10)
1,2,−3;−10	(1,2,−3;−10)	(1,2,−3;−10)		(1,2,−3;−10)
−1,−2,3;5,10,11,21	(−1,−2,3;5,10,11,21)		(−1,−2,3;5,10,11,21)	
−1,2,−3;−5,−10,−11,21			(−1,2,−3;−5,−10,−11,21)	
1,2,−3;−5,−10,−11,21	(1,2,−3;−5,−10)			
1,−2,3;5,10,11,21	(1,−2,3;5,10)			
1,2,−3;5,−10,−11,21	(1,2,−3;5,−10)			
Km(1)	Km(1)	Km(1)		
Km(−1)	Km(−1)	Km(−1)	Km(−1)	
	12	26	39	48
−1,−2,3;5,10,11,21				(−1,−2,3;5,10,11,21)
1,2,−3;−5,−10,−11,21	(1,2-3;−5,−10)	(1,2-3;−5,−10,−11,21)		
1,−2,3;5,10,11,21	(1,−2,3;5,10)	(1,−2,3;5,10,11,21)		
1,2,−3;5,−10,−11,21		(1,2,−3;5,−10)	(1,2,−3;5,−10,−11,−21)	
Km(1)	Km(1)	Km(1)	Km(−1)	
Km(−1)	Km(−1)	Km(−1)	Km(−1)	

[a] Shown for specificities actually tested, in many cases with a number of different stains and with a number of antisera of the same specificity; the prefix "Gm" has been omitted.

[b] Cotton cloth.

inhibition tests depends in part on the concentration of the IgG subclass molecule that carries the marker. Accordingly, limited experiments were conducted to determine the maximal serum dilution at which Gm factors present in the sample could be detected. The results are shown in Table 11. The IgG1 factors were detectable to serum dilutions of 1:1000, while G3m(10) was detected at 1:100 but not at 1:1000 dilutions.

It was noticed in the aging stain experiments that Gm factors present seemed to be detectable longer in stains made from drawn whole blood than in stains made from finger sticks. A possible explanation was that stains obtained from finger sticks might contain less serum than those prepared with drawn whole blood. Several pairs of comparably aged bloodstains were thus tested for serum content by using albumin as a marker. Each pair was from a different person, one member of the pair having been obtained by finger stick and the other from drawn whole blood. A radial immunodiffusion system was used to quantitate albumin, using a high titered anti-albumin and essentially the procedure described by Mancini and collaborators [*76*]. Comparable quantities of the stains prepared by finger stick contained about 50 to 65% of the albumin seen in stains made from drawn whole blood.

Detection of Gm Antigens in Bloodstains on Various Substrata

Bloodstains two to eight weeks old on a number of different substrata, including cotton and cotton blends, synthetic fabrics, wool, linen, various plastics, ceramic tile, wood, and glass, were tested from Gm(1), (2), (3), and (10) and Km(1) factors. The antigens present could be detected in all the bloodstains. Stains on substrata other than textile materials were handled by dissolving the dried blood in minimal saline and transferring to cotton threads for testing after the transferred material had redried.

Discussion

AHG, Ss, Kell, Duffy, and Kidd Antisera

The limited number of AHG reagents tested showed relatively uniform titers. Most antisera to Ss, Kell, Duffy, and Kidd antigens were Coombs-reactive, except two anti-S sera, which were active in saline. The titer of these reagents varied from 2 to 256 in albumin. All the Ss, Kell, and Duffy antisera except one had titers of at least 16 in the absence of enhancement media. Kidd antiserum titers were for the most part lower. A number of the reagents were enhanced in LISS media. LISS enhancement is generally expected to be more significant with incomplete AHG reacting antibodies. LISS may increase both the amount and the rate of antibody binding at the sensitization stage of an indirect Coombs test [*17*]. Kidd reagents were significantly more reactive with papain-treated cells. Papain technique is not commonly employed with Ss or Kell antisera and cannot be used with Duffy reagents be-

TABLE 11—*Detection of Gm factors in serum dilutions.*

Gm Factor Tested	Gm Phenotype of Serum	Reciprocal Serum Dilution					
		n	10	100	1000	2000	4000
G1m(1)	Gm(1)	−	−	−	w	+	++
G1m(2)	Gm(2)	−	−	−	−	+	+
G1m(3)	Gm(3)	−	−	−	−	+	+
G3m(10)	Gm(10)	−	−	w	+	+	++
All	negative controls	++	++	++	++	++	++

cause of its destructive effect on the antigenic receptors [77]. Without the use of an enhancement medium or technique, the titers of the Kidd antisera were quite low.

Ss, Kell, Duffy, and Kidd Antigen Determination in Bloodstains

Eluate titers decreased as expected as the bloodstains aged. However, most antisera of every specificity yielded convincing amounts of antibody in eluates from 24-week-old bloodstains aged at room temperature. With several antisera, antibody was not detected in 24-week-old stains, even though it had been detected previously. One anti-Fy^a gave no eluate antibody with the heterozygous stain and virtually none with the homozygous stain at twelve weeks, and one anti-Jk^a was inactive with the eight-week-old stain.

With a few bloodstains one to several years old, convincing antibody yields were obtained with particular examples of S, s, k, and Jk^a antisera. In the older stains, the effects of AB serum or LISS media enhancement could be seen in a number of the cases. The antigen was detected in some stains in enhancing media where it was undetected in comparably aged stains in saline-albumin. In many cases where the antigen was detected in albumin and in enhancing media, the antibody yield was higher with the latter. Antibody binding at the sensitization stage of a direct Coombs test with cells is increased in LISS [17]; the same effect has been observed in the absorption and detection stages of absorption elution tests on bloodstains [25].

It was thought at the outset that there might be a consistent correlation between the titers of the antisera with red cells and the antibody yield in aging stains. While this relationship seemed to exist in some instances, in others it did not. With the BCA anti-s reagents, for example, scores with ss cells were 63 in albumin, 67 in AB serum, and 77 in LISS; higher antibody yields were obtained in the 24-week-old ss stains with the enhancement media than in the albumin alone. Similarly, Pfizer anti-Jk^a had a significantly better titer and score with papain technique than in albumin, and a better antibody yield was obtained in the twelve-week stain with papain. With the saline-reacting Molter anti-S, there was little AB serum effect in SS cell titers, and correspondingly little difference between antibody yield from SS stains aged 24 weeks. On the other hand, a BCA anti-Fy^a in albumin gave better eluate titers with 12- and 24-week-old stains than a comparable Molter reagent that had a higher cell titer.

Similarly, comparable or identical stain eluate titers were seen with Pfizer, Dade, and Molter anti-Jk^a sera in albumin in 12- and 24-week-old stains, even though they had quite different cell titers. The titer of these antisera with red cells could not always be used, therefore, as a predictor of eluate antibody yield. Data from other studies indicate, however, that eluate antibody yields tend to improve within limits as the absorption antiserum titer is increased [32, 78, 79], and that enhancement procedures sometimes significantly improve the detection of certain antigens in older bloodstains [25, 26]. The latter effect is apparent in the present studies as well.

These results are generally in accord with those of previous studies. Burke and Tumosa [80] detected both K and Fy^a in four-year-old bloodstains using the procedures of Lincoln and Dodd [59]. McDowall and collaborators [25] detected K and s in 7- to 10-month-old stains, and S in a 64-week-old stain. In some older stains, the use of LISS permitted convincing detection of an antigen where the results were negative or ambiguous in its absence. The AB serum diluent was also shown to be of value in enhancing eluate yields with selected antisera. Maeda and collaborators [81, 82] found that the Ss, k, and Duffy antigens could be detected in all the stains they studied up to 42 weeks old, and all except k could be detected in all the 2-year-old stains. The k antigen was detected in some two-year-old stains. Denault and collaborators [83] found s to be detectable in stains up to 26 weeks old regardless of humidity, but S was not detected in stains more than 4 weeks old. Kell, Duffy, and Kidd antigens were not detected in stains older than two weeks.

The Japanese investigators [81, 82] used somewhat larger samples of bloodstain than were

used in our studies, and the stains were made from packed cells rather than from whole blood. Denault and collaborators [*83*] used stain samples larger than ours, but smaller than those used by Maeda et al. It is not clear why some of the antigens were not detected in older stains by that group. Cellano and Fy^a were arbitrarily selected as antigens representative of the group in the present study to be examined for detectability in bloodstains on various different substrata. Eluates were tested without titration. The two antigens could be detected in the majority of stains examined. Denault and collaborators looked at S, s, Fy^a, K, and Jk^a on a number of substrata, and there were no great differences in detectability of the antigens.

The results of these studies indicate that many commercially available blood grouping antisera for the Ss, Kell, Duffy, and Kidd antigens can be employed successfully in bloodstain antigen determinations. The reagents should be evaluated with cells and known control bloodstains to determine serological characteristics. Some are significantly more reactive in LISS or AB serum media, by papain technique, or with combinations of enhancement procedures. These characteristics may be used to select conditions that would seem to be most favorable for the typing of older stains. No false positive results were observed in any of the tests performed, although antigens present in stains can fail to be detected. As long as interpretation is based upon antigens actually detected, false negative results will not be the cause of any problems.

The Ss, Kell, Duffy, and Kidd antigens can provide additional individualizing information from bloodstains, supplementing that obtained by ABO, M, and Rh antigen [*24,25,32, 58,59*] and isoenzyme and serum group system marker [*3,51,54*] determinations, in laboratories equipped to carry out the typing procedures.

Gm/Km Reagents and Gm/Km Determination in Bloodstains

Nineteen Gm/Km antisera and their corresponding anti-D coats were obtained and tested. The optimal sensitizing volume ratio of anti-D to 50% R_1R_1 cells was 1:2 to 1:4 for most of the reagents, and the titers of anti-Gm/Km sera (at the optimal ratio) varied from 4 to 128. Suppliers of anti-Gm/Km reagents usually make recommendations about the optimal sensitizing volume of anti-D to red cells and the dilution of the anti-Gm or anti-Km for routine inhibition testing. It is preferable, however, to carry out the two-dimensional titration under one's own laboratory conditions. The working dilutions of the anti-Gm/Km sera determined by titration were generally the same as or within two dilutions of the supplier's recommendations.

The Gm and Km typing tests are inhibition tests. Accordingly, they are made more sensitive to smaller quantities of antigen by the use of increasingly dilute antisera. The recommended working dilution gave convincing results in the great majority of sera and bloodstains tested, but it can be adjusted as necessary for particular samples. Sensitization of R_1R_1 cells by anti-D coats was found to be essentially optimal after 45 min of incubation, differences in anti-Gm titer were minor with red cells of three common Rh(D+) phenotypes, and anti-Gm reagents and anti-D coats could be interchanged with little effect on the anti-Gm titer.

A series of bloodstains with several different Gm/Km antigen compositions were tested for specificities for which reagents were available over a period of 48 weeks. Several additional stains from one to a few years old were tested as well. Generally, all the factors present could be detected in stains up to six months old, and some but not all were detected in older stains. The ability to detect these markers in stains by inhibition tests is a function of a number of variables, including the concentration of the IgG subclass molecule that carries the marker, the quantity of serum in the stain and its uniformity of distribution with respect to the sample selected, the quantity of stain taken for testing, the test procedure, and the reagents used.

It has been reported that certain Gm antigens could be detected in dried blood up to 30

years old. Fairly large samples of dried blood were tested in these instances, however. Planques and collaborators [*61*] detected IgG1 markers in seven- to ten-year-old dried bloodstains testing 20 mg of dried blood. Görtz [*66*] detected several factors in extracts of fairly large quantities of stains. In one case where the test failed to detect a marker known to be present, he could show that the stain contained comparatively little serum protein (and hemoglobin). Hoste and collaborators [*84*] reported detection of Gm factors in 30-year-old dried blood by extracting 250 mg of blood powder and testing the extract. The sample used for testing for a particular factor represented an extract of about 50 mg of dried blood.

In this context, we found that IgG1 factors were detectable in serum dilutions of up to 1:1000, while G3m(10) was detected at 1:100 but not at 1:1000. This result indicates that something on the order of 0.4 to 0.8 μg IgG is required to detect the G1m factors, and some tenfold more to detect G3m factors, in fresh serum (assuming that serum contains an average of about 12-mg/mL IgG, range 8 to 17, and a 50-μL serum sample size). Assuming that whole blood has a specific gravity of about 1.02, that it is about 80% water, and that three 1-cm threads represent 20% of the area occupied by a 50-μL drop of blood on cotton cloth, then three 1-cm threads of stain would contain about 2 mg of dried blood, and perhaps 40 to 85 μg of IgG. Our tests were routinely carried out with three 1-cm threads, a significantly smaller sample than was used in the studies reporting detection of Gm factors in old stains. The crude calculations suggest that a sample of three 1-cm threads would contain about 100 times as much IgG as necessary to detect G1m markers and 10 times as much as required to detect G3m markers in fresh serum. There is undoubtedly some loss of detectable activity, however, as blood dries, and as a stain ages, consistent with what is known about many other genetic markers. In addition, serum proteins may not be uniformly distributed in bloodstains. If they are not, detectability results could vary depending upon the part of the stain sampled. The Gm antigens do seem to exhibit considerable intrinsic stability in older bloodstains, but relatively larger sample sizes must be employed to detect the factors present by inhibition tests.

The observation that comparable quantities of bloodstains made by finger stick and by deposition of drawn whole blood from the same person differed in albumin content (presumably a reflection of differences in serum content) may have implications for the preparation of control bloodstains for serum group markers and deserves additional study.

All the Gm and Km antigens present could be detected in the bloodstains on all the different substrata that were tested. A final noteworthy point is that Gm antigens provide virtually the only genetic marker system of current significance in forensic serology in which it is possible under certain circumstances to interpret negative inhibition results in stains. Ordinarily, a negative result can be interpreted to mean that the antigen was never present in the stain, or that it is present but was not detected. With Gm, however, several antigens are carried on one group of molecules, subclasses IgG1 or IgG3. Thus, detection of one or more factors can be used as an indication of the IgG content of the stain. Khalap and Divall [*85*] noted the value of G3m(5) determination in stains that were Gm(-1,-2) because many of the Gm(-1,-2) stains encountered in their work were from Gm(-1,-2;5) persons. Since serum contains more IgG1 than IgG3, detection of an IgG3 factor strongly suggests that sufficient IgG1 is present to detect any IgG1 markers present. Shaler [*86*] has noted that detection of a factor on a given IgG subclass would permit the essentially unambiguous interpretation of a negative result with another factor on that same subclass.

The allotypic antigens of immunoglobulins have been recognized as valuable genetic markers in bloodstains for a number of years. Although the commercially available antisera tested here yielded good results in typing stains, these reagents have traditionally been inconvenient or difficult to obtain in this country. Further, not all the specificities one could want are available.

The age of monoclonal antibodies having arrived, however, it is possible that plentiful supplies of antisera for any desired specificity will be available in the future. Evaluation of

monoclonal anti-G1m(3), both in traditional inhibition tests [*87*] and in enzyme-linked immunosorbent assay (ELISA) procedures [*88,89*], have shown very promising results.

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