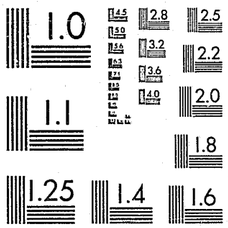
If you have issues viewing or accessing this file contact us at NCJRS.gov.

National Criminal Justice Reference Service

ncjrs

This microfiche was produced from documents received for inclusion in the NCJRS data base. Since NCJRS cannot exercise control over the physical condition of the documents submitted, the individual frame quality will vary. The resolution chart on this frame may be used to evaluate the document quality.



MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS-1963-A

Microfilming procedures used to create this fiche comply with the standards set forth in 41CFR 101-11.504.

Points of view or opinions stated in this document are those of the author(s) and do not represent the official position or policies of the U. S. Department of Justice.

National Institute of Justice
United States Department of Justice
Washington, D.C. 20531

9/27/84

94227

9117

9117

PROCEDURES AND EVALUATION OF ANTISERA FOR THE TYPING OF ABH, Rh, MNSs, KELL, DUFFY AND KIDD BLOOD GROUP ANTIGENS AND Gm/Km SERUM GROUP ANTIGENS IN BLOODSTAINS

R. E. Gaensslen, Ph.D.
Henry C. Lee, Ph.D.

Forensic Science Laboratories
University of New Haven
West Haven, Connecticut

and

Connecticut State Police
Forensic Science Laboratory
Meriden, Connecticut

1983

NCJRS
JUN 14 1984
ACQUISITIONS

Procedures and Evaluation of Antisera for the Typing of ABH, Rh, MNSs, Kell, Duffy and Kidd Blood Group Antigens and Gm/Km Serum Group Antigens in Bloodstains

R. E. Gaensslen, Ph.D.
Henry C. Lee, Ph.D.

with the technical assistance of
Elaine M. Pagliaro, M.S.
Julie K. Bremser, M.S.

Forensic Science Laboratories
University of New Haven
West Haven, Connecticut

and

Connecticut State Police
Forensic Science Laboratory
Meriden, Connecticut

Final Report, Grant 79-NI-AX-0125
U.S. Department of Justice
National Institute of Justice
Washington, D.C.

1983

This work was supported in part by grants from the National Institute of Justice, U.S. Department of Justice, Washington, D.C. [79-NI-AX-0125] and the Faculty Research Fund, University of New Haven, West Haven, CT. The financial assistance provided does not necessarily indicate the concurrence of the U.S. Government, the Department of Justice, the National Institute of Justice, nor the University of New Haven with the statements made or the conclusions reached herein. Mention of materials or processes by generic, trade or brand names is for purposes of information and does not constitute an endorsement or recommendation by the authors, the U.S. Government, the National Institute of Justice or the University of New Haven.

Acknowledgements

We gratefully acknowledge the financial assistance provided for this work by the National Institute of Justice and the University of New Haven Faculty Research Fund. We thank our government project monitors, the late John O. Sullivan and Mr. Joseph Kochanski for their continuing support and encouragement.

Dr. Henry Graham of Ortho Diagnostics generously provided us with a potent, incomplete anti-D reagent for which we are most grateful. In addition, we would like to thank our colleagues Dr. C.S. Tumosa, Philadelphia, PA, and Dr. R.C. Shaler, New York, NY, for their helpful discussions during the course of the work. In addition, Dr. Tumosa generously contributed a number of useful reagents and bloodstains for the project.

U.S. Department of Justice
National Institute of Justice

This document has been reproduced exactly as received from the person or organization originating it. Points of view or opinions stated in this document are those of the authors and do not necessarily represent the official position or policies of the National Institute of Justice.

Permission to reproduce this ~~copyrighted~~ material has been granted by
Public Domain/NIJ
~~U.S. Dept. of Justice~~
to the National Criminal Justice Reference Service (NCJRS).

Further reproduction outside of the NCJRS system requires permission of the ~~copyright~~ owner.

CONTENTS

LIST OF TABLES

I. Introduction

A. Genetic Markers in Human Blood

The first blood group system, ABO, was discovered by Landsteiner and his collaborators at the turn of the century (Landsteiner, 1901; von Decastello and Sturli, 1902). The red cell antigens defining this system are inherited, and are stable characteristics of an individual's blood throughout life. The discovery of ABO provided the first example of a genetic marker system in human blood, and thereby revealed the possibility of distinguishing different people on the basis of inherited blood characteristics.

Subsequent discoveries and developments in serology, biochemistry and genetics have shown that human blood contains numerous genetic markers, which may be grouped into five major categories: (1) Blood groups; (2) Red cell isoenzymes; (3) Serum groups; (4) Hemoglobin variants; and (5) HLA system antigens. Each of the first three categories contains a number of different systems, distinguished from one another on the basis of being inherited independently. The enormous literature which has developed on the different genetic marker systems has been periodically reviewed and organized (see, for example Prokop and Uhlenbruck, 1969; Giblett, 1969; Brinkmann, 1971; Culliford, 1971; Dodd, 1972; Race and Sanger, 1975; Harris and Hopkinson, 1976; Issitt and Issitt, 1976; Prokop and Göhler, 1976; Boorman, Dodd and Lincoln, 1977; Giblett, 1977; MPFSL, 1978; Beckman, 1978; Gaensslen and Camp, 1981; Sensabaugh, 1981; Lee, 1982; Gaensslen, 1983). This monograph is concerned with the blood groups, and with the Gm and Km serum group systems.

The applicability of the genetic marker systems to blood individualization in forensic science requires procedures for the determination of the markers in dried bloodstains. Landsteiner (1901) immediately recognized this possibility, and described experiments on determining ABO system antibodies in serum stains on linen. The initial studies were soon extended (Landsteiner and Richter, 1903). Efforts to group bloodstains were focused on ABO for many years, because it was the only blood group system known until 1927. As other blood groups were found, attempts were made to find methods for their determination in bloodstains. Methods for blood group antigen determination in bloodstains were developed first for ABO antigens, and were later modified and adapted for other blood group system antigens.

B. Development of Techniques for Grouping Bloodstains

When blood dries, the red cells hemolyze. The direct agglutination methods that are utilized for red cell typing cannot, therefore, be used for typing dried bloodstains. Other methods must be employed, and three categories of them have been developed: (1) Detection of antibodies; (2) Inhibition procedures; and (3) Elution procedures. The antibody detection method is applicable only to the ABO system. Inhibition and elution procedures detect the presence of antigens in bloodstains, and are thus applicable to the typing of any blood group system antigens. In general, inhibition procedures were developed and used first for most of the blood group antigens. Reliable elution techniques have been developed since 1960.

The initial studies on ABO grouping in dried bloodstains were directed toward the detection of the isoantibodies. Landsteiner (1901) noted that the isoagglutinins were detectable in serum stains on linen, and these studies were extended shortly afterward (Landsteiner and Richter, 1903). The most systematic and extensive studies on the determination of the ABO group in bloodstains by detection of isoagglutinins were carried out by Lattes (1913, 1915). The technique is equivalent to "reverse grouping" of whole blood, except that bloodstain extracts are tested instead of fresh serum. These tests are still carried out, although most often now to confirm results obtained by elution technique, and they are often referred to as the "Lattes test", or "Lattes crust test". Isoagglutinin detection is of value only in ABO system typing, since it is the only blood group system having naturally occurring isoagglutinins present in serum.

Inhibition procedures are based on the principle that a bloodstain, containing a particular antigen on the red cell membranes, will reduce the strength of an antiserum containing the corresponding antibody if the two are incubated together under suitable conditions. Schütze (1921) first utilized this principle for the determination of ABO antigens in dried bloodstains. Extensive studies on the inhibition technique were carried out by Siracusa (1923), working in Lattes' laboratory (Lattes, 1923). There are different ways of doing inhibition tests. The simplest method makes use of a low titer antiserum which is completely inhibited by the quantity of corresponding bloodstain antigen with which it is incubated. The antiserum will agglutinate test cells prior to absorption, or if the stain does not contain the corresponding antigen, but will fail to agglutinate test cells after absorption in a positive test.

Part of the problem with this procedure is that the antiserum is used at a single dilution, and the test results are judged according to whether there has been complete inhibition or no inhibition. Both age and environmental factors can influence how much reactive antigen a particular quantity of dried blood contains. Thus, equivalent samples of different bloodstains could contain quite different quantities of reactive antigen, and the "all-or-none" inhibition procedure does not easily allow these variations to be taken into account. In 1931, Holzer devised an improved inhibition technique in which the antiserum is titrated before and after absorption with the bloodstained sample. This procedure enables different quantities of antigen to be detected, the degree of inhibition being related to the relative antigenic content of the sample. An even more sensitive inhibition method has been described (Hirszfeld and Amzel, 1932; Kind, 1955), although it requires a greater quantity of sample. Inhibition techniques are relatively insensitive when compared to elution, and this is a disadvantage in circumstances where a limited amount of sample is available. Another disadvantage of inhibition tests is that some kinds of bloodstained substrata show nonspecific binding of the antibodies (inhibition in the substratum control). Inhibition techniques have been described for a number of blood group antigens besides ABO, and these will be mentioned in the appropriate sections below. Elution techniques have largely supplanted inhibition for blood group antigen determination in bloodstains. Inhibition techniques remain the method of choice for Gm and Km serum group system typing, which is more fully discussed in § VIII (and they remain the method of choice for secretor body fluid ABO determinations).

Elution techniques are now preferred in most laboratories for antigen determination in bloodstains. They are based on the recovery and detection of antibodies specifically bound to blood group antigens in bloodstains (or on red cells). Samples are incubated with a relatively high concentration of antibodies under conditions favoring maximal antibody binding. Excess unbound antibody is then removed by washing, and the specifically bound antibody is recovered (eluted) using conditions which disrupt the antigen-antibody bonds. Many different procedures for eluting specifically bound antibodies have been described (Howard, 1981).

It was noted early in the studies on blood group antigen-antibody binding that the association is temperature-dependent (Landsteiner, 1902; Landsteiner and Jagic, 1903), higher temperatures favoring antibody dissociation. Most bloodstain grouping procedures have employed the heat-elution method of Landsteiner and Miller (1925).

ABO grouping in dried blood using absorption-elution was first described by Siracusa (1923), but there were technical problems with the techniques (Faraone, 1941) and many workers regarded the inhibition procedure as more reliable. In 1960, Kind described a sensitive and reliable absorption-elution procedure, applicable to dried blood smears (Kind, 1960a) and to dried blood on fabrics (Kind, 1960b). The value of this technique was quickly confirmed by many other workers, and a number of technical modifications were introduced (Schleyer, 1961; Outteridge, 1962a, 1962b, 1963; Nickolls and Pereira, 1962; Kind, 1962, 1963; Budvari, 1963; Fiori, Marigo and Benciolini, 1963). The availability of *Ulex europaeus* anti-H lectin (Wiener, Gordon and Evans, 1958) made possible the positive diagnosis of group O stains.

Elution tests may be done in test tubes, and some workers prefer this method. The tests are frequently carried out directly on a small fragment of bloodstained sample, particularly in the case of stains on textile materials. Howard and Martin (1969) described a method in which threads of bloodstained material are affixed to cellulose acetate sheets. The entire procedure is then carried out on the exposed portions of the threads. This technique simplifies the washing step, and allows relatively large numbers of samples to be processed somewhat more easily than in tubes. Bloodstain extracts can be typed by elution as well. Outteridge (1962a) used welled slides to group aqueous extracts of bloodstains, which had been redried in the wells. Kind and Cleevely (1969) described a similar technique, but applied it to dried ammoniacal extracts of bloodstains. The grouping of dried ammoniacal extracts of bloodstains for ABO can be conveniently carried out in small plastic cups, such as the 2 mL conical bottom polystyrene sample cups marketed by Scientific Products and by VWR Scientific (Gaensslen, Bremser and DeGraw, 1981).

This monograph is concerned primarily with the grouping of bloodstains for antigens of the Rh, MNSs, Kell, Duffy and Kidd blood group systems, and the Gm and Km serum group systems.

Additional background and information about other genetic marker systems and about ABO grouping in dried bloodstains may be found in the various reviews (Prokop and Uhlenbruck, 1969; Giblett, 1969; Brinkmann, 1971; Culliford, 1971; Harris and Hopkinson, 1976; Prokop and Göhler, 1976; Giblett, 1977; MPFSL, 1978; Beckman, 1978; Gaensslen and Camp, 1981; Sensabaugh, 1981; Lee, 1982; Gaensslen, 1983). Background information on the blood groups and on their typing in bloodstains may be found in Culliford (1971), Dodd (1972), Race and Sanger (1975), Issitt and Issitt (1976), Boorman, Dodd and Lincoln (1977), MPFSL (1978), Gaensslen and Camp (1981) and Gaensslen (1983).

II. Blood Group Antibodies and Agglutination

A. Blood Group Antibodies

1. Antibodies in General

Antibodies are immunoglobulins, tetrameric molecules consisting of two heavy and two light polypeptide chains. Five classes of immunoglobulins have been distinguished, based upon the nature of the heavy chains. The general structure of an immunoglobulin molecule may be written as H_2L_2, where "H" and "L" stand for the heavy and light chains, respectively. The polypeptide chains involved in immunoglobulin structure are given Greek letter designations. There are two types of light chains, κ and λ. The two light chains in a given molecule are always the same type. There are five types of heavy chains, μ, γ, α, δ and ε. The different classes of immunoglobulins, along with some of their properties, are shown in Table 1.

Table 1. Classes of Immunoglobulins

Immunoglobulin	Chain Formulae	Approximate MW	% Total Immunoglobulin in Serum
IgM	$(\mu_2\kappa_2)_5$; $(\mu_2\lambda_2)_5$	900,000	5-10
IgG	$\gamma_2\kappa_2$; $\gamma_2\lambda_2$	145,000	80
IgA	$\alpha_2\kappa_2$; $\alpha_2\lambda_2$	160,000	5-10
IgD	$\delta_2\kappa_2$; $\delta_2\lambda_2$	177,000	1
IgE	$\varepsilon_2\kappa_2$; $\varepsilon_2\lambda_2$	187,000	1

Blood group antibodies may be of the IgM, IgG or IgA classes. Those which bring about complete agglutination in saline are labeled "complete" antibodies;

those which sensitize red cells (bind the corresponding antigen on the cell surface) without bringing about agglutination are called "incomplete". Complete antibodies are often, but not always, IgM, while incomplete antibodies are often but not always IgG. Blood group antibodies of the IgA class, when they occur, may complete or incomplete. The distinction between complete and incomplete antibodies is a serological one, not an immunochemical one, but it is very useful in discussions of blood group antibodies.

2. ABO Antisera and Lectins

Commercially obtained anti-A and anti-B grouping sera contain complete antibodies, and their titer is relatively high and consistent from batch to batch, even among different manufacturers. The anti-H reagent prepared from *Ulex europaeus* seeds behaves like a complete antibody. Crude *Ulex* anti-H preparations are known to contain two lectins (Matsumoto and Osawa, 1969, 1970; Horejsi and Kocourek, 1974; Pereira *et al.*, 1978; Pereira, Gruezo and Kabat, 1979). They have been purified, and their serological and biochemical characteristics studied. So-called lectin I is inhibited by fucose, and is the active "anti-H" principle. Lectin II is not inhibited by fucose, and is relatively nonspecific with regard to its agglutination of ABO red cell types. Most laboratories use a partially purified, but still crude *Ulex* seed extract, which contains both lectins, as an anti-H reagent. Studies are currently in progress to determine whether a purified *Ulex europaeus* lectin I would be a better anti-H reagent than the partially purified crude extract in forensic serology (Blake *et al.*, 1982; studies in our own laboratory). Many laboratories prepare anti-H reagent from *Ulex* seeds using one of two basic procedures: that of Kind (1962), or that of Culliford (1971). They are similar, except that Kind's procedure contains a preliminary petroleum ether extraction step, and utilizes McIlvaine's buffer to extract the lectin rather than saline. Anti-H reagents prepared by these methods commonly have titers of 64-128 against O cells, and only somewhat lower titers against A_2 cells. They often react with A_1 and B cells as well, but have much lower titers against them. Reagents with higher titers against O cells have correspondingly higher titers against other cell types, as a rule. Those preparations with relatively high titers against O (and A_2) cells, and relatively low ones against A_1 and B cells can be diluted somewhat.

Dilution minimizes or eliminates the reactions with A_1 and B cells while retaining an O cell titer sufficiently high for use in elution tests. Optimal conditions for elution include the use of suitably high titered absorbing antisera (this matter is discussed below, § IV.A.1), and preparations of anti-H with titers of less than 32-64 against O cells are not desirable. We prefer a reagent that has a 32-64 titer against O cells, but which has very low or zero titers with A_1 and B cells. The extent to which the seeds are ground appears to have a noticeable effect on the extent to which the resulting extract will react with A_1 or B cells. Gentler grinding of the seeds, by hand rather than with mechanical or electrically-driven grinders, seems to yield a somewhat better reagent. Commercially obtained *Ulex* anti-H reagents often have low titers against O cells and/or do not show the same degree of specificity for ABO as the extracts made from seeds by hand. Table 2 shows the titers of a number of representative antisera and lectins with ABH specificity.

Table 2. Antisera and Lectins with ABH Specificity

Specificity	Source†	Titer (0.1% cells)
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-B	Ortho	512
Anti-B	Molter	512
Anti-B¶	Dade	256
Anti-B¶	Dade	512
Anti-H	seeds*	32(O); 16(A_2); 2(B); 1(A_1)
Anti-H	seeds*	128(O); 32(A_2); 8(B); 4(A_1)
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)

†See Appendix I ¶Different lots *Method of Kind (1962)

As noted above, the ABH system reagents generally have similar titers and properties from batch to batch, with the exception perhaps of commercially obtained *Ulex* anti-H, which must be evaluated for titer and specificity.

The titer and specificity of *Ulex* extracts prepared in one's own laboratory can easily be evaluated, and adjusted to some extent by varying the method of preparation. The problems associated with ABO grouping in bloodstains are not, for the most part, attributable to problems with the antisera (or the anti-H lectin). Most anti-A and anti-B sera from commercial sources are perfectly suitable for elution tests, and can of course be diluted to appropriate strengths for inhibition tests. Anti-H lectin preparations generally require more thorough evaluation, but suitable preparations can be made quite easily. The anti-H lectin behaves as a complete antibody. Blood grouping antisera for most other blood group specificities require a much more careful evaluation to determine their serological characteristics and their applicability to bloodstain grouping by elution techniques. Some contain incomplete antibodies, and their properties vary under different serological conditions. Most of the antiserum evaluation studies discussed below are concerned with the blood group systems other than ABO.

3. Other Blood Group Antibodies—Enhancement of Agglutination

In the mid-1940's, in connection with studies on the Rh system, it was found that certain antibodies could bind to their corresponding antigens on the red cell surface, without bringing about agglutination in saline media. Wiener (1944) called these "blocking antibodies". He found that red cells containing the corresponding antigen were not agglutinated by a complete antiserum of the same specificity after treatment with the "blocking" antibody. Red cells containing a blood group antigen, to which is bound corresponding "blocking" or incomplete antibody, are said to be "sensitized". In 1945, Coombs, Mourant and Race found that sensitized cells could be agglutinated by an anti-human immunoglobulin serum raised in rabbits. This finding provided a simple method for the detection of incomplete blood group antibodies, which is called the "antihuman globulin test" or "Coombs test". The rabbit anti-human globulin (AHG) serum is often called "Coombs serum". An "indirect" Coombs test involves sensitizing red cells with a serum thought to contain incomplete antibodies to the corresponding red cell antigen, washing the cells to remove excess antibody, and then treating the cells with AHG serum and reading for agglutination. In a "direct" Coombs test, red cells suspected of having been sensitized *in vivo* are tested with AHG serum directly.

The direct test is important in clinical serology. In forensic serology, the indirect test is used in testing for certain antibodies. Blood group antibodies which are Coombs reactive can be found for a number of different systems, and may be detected by the AHG test.

Some incomplete antibodies, which agglutinate red cells weakly or not at all in saline, will bring about agglutination in an albumin (or other high protein) medium. This behavior was first observed with Rh antibodies (Cameron and Diamond, 1945; Diamond and Denton, 1945), and studied further by Wiener and Hurst (1947). More recently, it has been found that bovine serum albumin preparations may vary in the quantity of polymerized albumin which they contain, and that those having a higher polymerized albumin content are more effective potentiators of agglutination by incomplete antibodies (Goldsmith, 1974; Reckel and Harris, 1978).

In 1946, Pickles observed that red cells sensitized with an incomplete Rh antibody would agglutinate if treated with the filtrate from cholera organism cultures. The active principle in the filtrate was thought to be an enzyme, and it was quickly shown that trypsin could mimic the effects of the cholera filtrate (Mortin and Pickles, 1947). Unger (1951) showed that the AHG reaction of cells sensitized with incomplete Rh antibodies was enhanced by trypsin treatment of the cells. In 1953, Stratton showed that papain treatment of red cells allowed them to be agglutinated by incomplete Rh antibodies. Papain treatment of red cells is a common procedure in many laboratories for the detection of certain incomplete antibodies. It is sometimes used in conjunction with other serological enhancement techniques as well (Cf. § IV.C.3). The papain procedure of Löw (1955) is used in our laboratory and in many others (see Boorman, Dodd and Lincoln, 1977). Mortin (1962) looked at the effects of of trypsin, papain and several other proteolytic enzymes on a number of blood group antigens. Certain enzymes destroy certain receptors, and these findings must be considered in selecting enzyme enhancement techniques for different blood groups. Chymotrypsin and the proteolytic enzymes papain, ficin and bromelin, for example, destroy Duffy receptors. Papain enhancement techniques are perhaps most commonly (though not exclusively) used with Rh antibodies. Details of different enzyme procedures are discussed by Issitt and Issitt (1976) as well.

Physicochemical studies on blood group antigen-antibody reactions (discussed in more detail below, § II.B) indicate that salt concentration is an important variable. Lower salt concentrations tend to increase antigen-antibody association, especially with incomplete antibodies (Hughes-Jones, Gardner and Telford, 1964; Hughes-Jones *et al.*, 1964), although the effect depends to some extent on the type of antibody and its avidity (Lincoln and Dodd, 1978). This finding has increased the use of media containing lower salt concentrations for the detection of certain incomplete antibodies (Löw and Messeter, 1974; Moore and Mollison, 1976; Rosenfield *et al.*, 1979; Lincoln and Dodd, 1978; McDowall, Lincoln and Dodd, 1978). Such solutions are buffers containing lower concentrations of NaCl than normal saline, and molecules such as glycine or sucrose to maintain the correct osmolarity. They are called "low ionic strength" solutions, often abbreviated "LIS" or "LISS". LISS techniques are sometimes used in conjunction with other enhancement techniques (Cf. § IV.C.3).

B. Studies on Agglutination

The mechanism of red cell agglutination by blood group antibodies is complex, and a number of studies have been carried out to try and understand it. Among other things, suitable models for the mechanism must be able to account for the enhancement of serological reactions by albumin and other high protein media, enzyme treatment of red cells, and by LISS techniques.

Antigen-antibody reactions may be regarded as occurring in two stages. The first stage, in this way of looking at the process, is the association of the antigen-binding portion of the antibody molecule with the antigenic determinant. This association alone does not give rise to any visible or detectable product. The only way of knowing that the first stage has occurred is through the occurrence of the second stage. The second stage is the visible or otherwise detectable result of antigen-antibody association, such as agglutination, precipitation, complement fixation, cell lysis, etc. In agglutination reactions, an antibody molecule must interact with receptor sites on two or more cells, thus bridging them together. A variety of factors influence the ability of the antibody to bring about the agglutination of cells containing the corresponding antigen.

These include the size of the antibody molecule relative to the cells, the number of combining sites on the antibody molecule and the distance between them, the density and relative distribution of antigenic determinants on the red cell, and other physicochemical factors which favor or allow the antigen-antibody interaction to occur. IgM molecules are some 6 to 7 times larger than IgG ones, and they have 10 potential combining sites spaced at approximately 300 Å. IgG molecules have two combining sites, spaced at about 120 Å. It is not surprising, therefore, even on the basis of size alone, that IgM antibodies are often complete saline agglutinins, where IgG antibodies frequently are not. Red cells are negatively charged particles which form stable suspensions in salt-containing aqueous media. Electrostatic repulsion between the negative surface potentials (ζ-potential) probably has a role in the stability of these suspensions. The pH, ionic strength, dielectric constant and viscosity of the medium have a role in determining the thickness of the electrical double layer surrounding the cells, and in influencing the ζ-potential, and thus ultimately in the stability of the suspension. Alterations in any of these properties which tend to destabilize a cell suspension and bring cells closer together may be expected to enhance agglutination by IgG antibodies. Many of these same variables also play roles in the interaction between antibody molecules and cell surface antigenic receptors as well. Any additive or treatment which enhances agglutination, therefore, may be doing so through an influence on antigen-antibody binding, or on the stability of the cell suspension, or both.

Pollack *et al.* (1965) conducted a number of experiments on the "second stage" red cell agglutination reaction. Their explanation of the enhancement of agglutination by incomplete antibodies emphasized the importance of the surface potential of cells. The effects of adding high molecular weight polymers to the medium, and of treating cells with proteolytic enzymes, were interpreted in this framework. The addition of high molecular weight polymeric colloids (such as albumin, ficoll, polyvinylpyrolidone and dextran) to red cell suspensions increased the dielectric constant of the medium, thereby lowering the ζ-potential and enhancing agglutination by helping to destabilize the the cell suspension, in this view. Similarly, enzyme treatment of cells brought about a significant reduction in surface charge, and thus of ζ-potential.

It was suggested that the enzymes removed ionogenic surface groups from the cell surface (perhaps sialic acid). If this explanation were right, the esterase activity of the enzymes would be more important than their proteolytic activity, and they would be mimicing the action of neuraminidase (sialidase). Other investigations indicate that the enhancement mechanisms are somewhat more complicated.

Goldsmith (1974) looked at the effect of albumin on the enhancement of agglutination of Rh+ cells by incomplete anti-D, in an effort to understand the variable effects of different lots of albumin. The different albumin preparations were found to contain quite different amounts of polymerized albumin, and optimal enhancement of agglutination was obtained with albumin solutions containing approximately 85% monomer and 15% polymer (including dimer). The alteration of the dielectric constant of the medium by albumin solutions containing different amounts of polymer was not sufficient to account for the degree of enhancement. Reckel and Harris (1978) obtained similar results and found, too, that higher polymers were more effective potentiators of agglutination than dimeric or trimeric forms. According to van Oss, Mohn and Cunningham (1978), who have reviewed much of this material fairly recently, and conducted a number of experimental studies, polymer bridging between cells is a much more important factor than dielectric constant with most of the colloidal polymers. Some of the polymers which enhance agglutination, for example, do not markedly affect the dielectric constant of the medium. In addition, certain ones such as the dextrans induce spiculation of the red cells. This alteration of cell shape increases the extent to which neighboring cells can interact. Spiculation is also induced in A and B cells by anti-A and anti-B, respectively, and this effect is thought to be important in understanding the relative ease with which these antibodies bring about agglutination in saline.

Gunson (1974) has discussed the mechanism by which enzyme treatment of red cells enhances their subsequent agglutination by incomplete antibodies. While enzyme treatment of cells does bring about a reduction in surface potential, thus allowing cells to approach one another more closely, the mechanism at work here may be more complex.

Papain, for example, is a much more effective potentiator of the agglutination of Rh+ red cells by IgG anti-D than neuraminidase, and the discrepancy cannot be explained by ζ-potential alterations alone. Other factors which could be involved, and which are thought by some workers to be more important than surface potential effects, are the removal of cell surface peptides which could hinder the approach of IgG molecules, enzyme effects on the distribution of antigenic sites on the cell surface, and enzyme effects on cell shape. Many of the enzyme enhancement studies have been carried out using IgG anti-D and Rh+ cells. In considering other blood group systems and antigens, it is important to recognize that different enzymes may have deleterious or destructive effects on various receptors (Judson and Anstee, 1977).

The interaction and binding of antibody molecules and cell surface antigens can be regarded, as noted above, as a "first stage" in agglutination. The reaction can be analyzed according to the laws of mass action and chemical equilibrium (Hughes-Jones, Gardner and Telford, 1962; Hughes-Jones, 1974, 1975). Equilibrium constants for different antibodies can be experimentally determined, and these vary from one specificity to another. They also vary among antibodies of the same specificity within the same serum, and the experimental determination yields an average value. It is reasonable to suppose that a certain minimum number of antibody molecules must be bound to cell surface antigens before agglutination can take place; that is, the ratio of antigen-antibody complex to free antigen under consideration must reach some threshold value. From the equilibrium considerations:

$$Ag + Ab \underset{k_d}{\overset{k_a}{\rightleftharpoons}} AgAb$$

$$v_f = k_a[Ag][Ab]; \quad v_r = k_d[AgAb]$$

At equilibrium, $v_f = v_r$ and $k_a[Ag][Ab] = k_d[AgAb]$

$$\frac{[AgAb]}{[Ag][Ab]} = \frac{k_a}{k_d} = K$$

where Ag denotes antigen, Ab denoted antibody, AgAb denoted antigen-antibody complex, [ ] denotes concentration, k_a is association constant, k_d is dissociation constant, v_f is the forward rate, v_r is the reverse rate, and K is the equilibrium constant.

Note that $\frac{[AgAb]}{[Ag]} = K[Ab]$, i.e., the ratio of [AgAb] to [Ag] is dependent upon the equilibrium constant as well as the free antibody concentration. Thus, antibodies with a high K value can increase the ratio more effectively at lower concentrations. An important factor that has been found to affect antigen-antibody complex formation is the ionic strength of the medium. Lowering the ionic strength of the medium from that of normal saline down to 0.03 increased the rate of reaction between anti-D and D+ cells by about 1000-fold (Hughes-Jones, Gardner and Telford, 1964), attributable to an increase in k_a. This increase should be reflected in K, and indicates that more AgAb should be formed for a given [Ag] value under low ionic strength conditions. Many antibodies besides anti-D have been shown to behave similarly when ionic strength is reduced (Hughes-Jones, 1975), and the use of LIS media has become an important enhancement technique in blood group antigen-antibody reactions (Elliott *et al.*, 1964; Jørgensen *et al.*, 1979a, 1979b; Fitzsimmons and Morel, 1979; Rosenfield *et al.*, 1979).

Agglutination is a complex phenomenon and the mechanisms of many of the serological enhancement procedures are not completely understood as yet. Many of the factors involved have been identified, however, and the information can be used to help optimize the various techniques for different applications and purposes. In summary, red cell agglutination reactions are affected in varying degrees by many factors, including: (1) the number of antigenic determinant sites on the red cell (see Table 3); (2) the distribution of the antigenic determinant sites on the cell surface; (3) the average equilibrium constant for the antibody preparation; (4) the relative concentrations of antibody and antigen (cells); (5) cell shape and spiculation; (6) the cell surface potential; (7) the dielectric constant of the medium; (8) the ionic strength of the medium; (9) the presence of polymeric colloids capable of polymer bridging; (10) extracellular colloid osmotic pressure; and (11) the hydration state of the antibody molecule and of the antigenic determinant sites. Table 3 summarizes the number of blood group antigenic determinant sites on red cells, which have been estimated primarily from radioactive iodine-labeled antibody studies. These data, from a number of different sources, were given by Hughes-Jones (1975).

Table 3. Number of Blood Group Antigenic Sites on Red Cells

	Antigen	Red Cell Phenotype	Estimated Number of Antigenic Sites Per Cell
ABO	A_1	A_1	810,000 - 1,170,000
		A_1B	460,000 - 850,000
	A_2	A_2	240,000 - 290,000
		A_2B	120,000
	B	B	610,000 - 850,000
		A_1B	310,000 - 560,000
Rh	D	R_2R_2	25,000 - 35,000
		R_1r	10,000 - 15,000
	C	Cc	37,000 - 53,000
	c	cc	70,000 - 85,000
	E	various	450 - 25,600
	e	ee	18,200 - 24,400
		Ee	13,400 & 14,500
Kell	K	KK	4,430 - 7,050
		Kk	2,100 - 5,400

III. Serological Procedures

There are many different techniques used in blood group serology, and different ways of doing many of them. Descriptions may be found in reference books such as Boorman, Dodd and Lincoln (1977), Issitt and Issitt (1976) and the MPFSL (1978) Manual. In this section, a few of the basic techniques are described along with the methods and materials we have employed and found to be useful. Additional descriptions and technical details may be found in Appendix II.

A. Titration

Blood grouping antiserums are nearly always titrated by determining the highest doubling dilution of the reagent which will give a predetermined agglutination result (1+ is common, and is what we use) under a defined set of conditions. Any change in the conditions can affect the titer obtained for a particular antiserum. In addition, it is important that different readers of agglutination in the laboratory agree on the interpretation of different degrees of agglutination.

Otherwise, different readers may obtain somewhat different results from a titration— even from the same set of tubes.

We distinguish six different degrees of agglutination, based upon low power microscopical (50X overall magnification) readings. All agglutination results are read in the same way to maintain internal consistency. The different degrees of agglutination (and approximate descriptions of what is meant by them) are as follows:

Designation	Equivalent Designation	Approximate Description
4+	c or v	complete agglutination, usually in a single large mass; virtually no free cells in field; may be distinguished visually without a microscope in small tubes, or in concavity slides
3+	++	strong agglutination; most cells are agglutinated, but a few free cells may be present; a number of masses of agglutinated cells are present rather than a single large mass; may be distinguished visually by the experienced eye
2+	+	definite masses of agglutinated cells present in the field, but along with a significant number of free unagglutinated cells
1+	±	some masses of agglutinated cells, generally smaller than in a 2+, and a large number of free cells in the field
w	w	a few agglutinates in the field, with a substantial majority of free cells
-	–	negative; no agglutination; all free cells in the field

Microscopical reading is necessary to distinguish 2+ and weaker degrees of agglutination. Titrations are usually carried out in tubes. If small test tubes (6 x 50 mm) are used, agglutination results can be read directly by viewing the bottom of the tube under the low power lens of the microscope. Some procedures require the use of larger tubes (12 x 75 mm), and the contents are then transferred to glass Boerner microtest slides (the ones we use have ten wells, each 2 mm deep and about 15 mm in diameter), in which they can be rotated if necessary, and read under the low power microscope. If tubes are to be centrifuged prior to reading, it is convenient to have a multipurpose low speed centrifuge (like the Beckman TJ-6) for the purpose.

A number of different sized test tube racks are available for this instrument. Commonly, a row of tubes representing a titration series is centrifuged by allowing the centrifuge to attain 1000 rpm before shutting it off and allowing it to decelerate to zero undisturbed. Centrifugation is not done with every kind of antiserum; in some cases, it is specifically not recommended because of the possibility of false positive agglutination results. Different antisera are titrated under different conditions, and using different techniques. Details are discussed in connection with antisera for various specific blood group antigens in the subsequent sections.

Doubling dilutions of an antiserum for titration are generally prepared by placing 1 volume of saline, or other appropriate diluent, in every tube except the first. 1 volume of neat antiserum is added to the first tube. One volume of neat antiserum is then added to the second tube, and the pipette used to mix the antiserum and diluent thoroughly. One volume of the mixture is then transferred to the third tube, and the mixing and transfer steps repeated. This process continues down the row to the last tube. The volume removed from it, after mixing, is discarded. These dilutions can be made using any convenient pipetting device and any convenient volume. Accuracy and reproducibility are important in the volume measurements, however. In addition, many of the reagents are expensive and precious, and smaller unit volumes are preferable. We have found that a spring loaded repeating pipettor, which has replaceable glass capillaries and handles relatively small volumes, is very convenient for titrations. The SMI Quikset Micropettor, for example, has a replaceable glass capillary that can easily reach the bottoms of all the different sized tubes we use (especially the 6 x 50 mm ones), and the model which can be adjusted to dispense 20, 25 or 50 µL has been found to be very useful. We often use 50 µL as one volume; when the reagent is in low supply or is very expensive, 25 or even 20 µL volumes can be used with equal ease.

B. Test Red Cells — Cell Panels

Blood grouping antisera should be titrated against red cells which are both homozygous and heterozygous for the corresponding antigen. Fresh red cells are always preferable if they are available. They are most easily obtainable from people around the laboratory. A finger or ear stick normally yields enough cells. They may be available from a blood bank or transfusion center as well.

If many different antisera for a number of different blood group system antigens are being evaluated and used, however, it is not always possible to obtain fresh red cells having all the different phenotypes required. For blood group systems other than ABO, one wants test cells with type O in the ABO system.

Several different kinds of cell panels are available commercially, and they can provide many of the different types of cells which may not be readily obtainable from individuals. These panels typically contain 10 to 12 separate vials of 3-5% cell suspensions, each from a separate, single group O donor. The cell donors have been typed for the common antigens of the Rh, MNSs, P, Lewis, Kell, Lutheran, Duffy and Kidd systems, and sometimes for other antigens, such as Xg^a, Sd^a, Yt^a and Co^b. The antigenic composition of all the cells in a particular panel is specified on a package insert. The compositions of three representative cell panels are shown in Table 4. Panel cells, like antisera, carry an expiration date, which is generally around 21 to 30 days from collection. Since each panel vial typically contains 5 to 10 mL of 3-5% cells, and many of the procedures in forensic serology are carried out with low cell concentrations, it may not be possible to consume all the panel cells before they expire. In addition, some vials will be used up more rapidly than others. Thus, to save money, some laboratories may wish to preserve some of the panel cells cryogenically in convenient quantities, and recover them as needed. Procedures are given in Appendix II for this process. Fresh cells can, of course, also be preserved in this manner, and such a procedure is helpful if certain cell donors are available only occasionally.

The titer of an antiserum is a function of the conditions used for titration, and these include the red cells that are used. Antisera may give different titer values with fresh cells, cells stored at 4°, panel cells, cells recovered from cryogenic storage, etc. It is important to be aware of these possible differences, and to be as consistent as possible in the kinds of cells used in titer determinations and as test cells for detecting an antibody in an eluate. Some examples of this behavior are shown in Table 5. It will be seen that cells retain activity better at 4° under the Ortho red cell diluent than under saline, and this effect becomes far more noticeable if longer storage periods are involved. Cells store well cryogenically at -85° under proper conditions, and we prefer this method for storing cells for longer than a few days.

Table 4. Antigenic Composition of Representative Cell Panels

	Rh Phenotype	D	C	E	c	e	C^w	M	N	S	s	P_1	Le^a	Le^b	K	k	Fy^a	Fy^b	Jk^a	Jk^b	Other
Dade Data-Cyte (Lot # DC-253)																					
1.	rhrh	-	-	-	+	+	-	+	+	-	+	-	+	-	-	+	-	-	+	+	Lu(a-b+)
2.	rhrh	-	-	-	+	+	-	-	+	-	+	+	-	+	+	+	-	+	-	+	Xg(a+)
3.	rh'rh	-	+	-	+	+	-	-	+	-	+	w	-	+	-	+	+	+	+	+	Kp(a-b+)
4.	rh"rh	-	-	+	+	+	-	+	+	+	-	+	+	-	-	+	+	+	-	+	Js(a-b+)
5.	Rh_orh	+	-	-	+	+	-	+	+	-	+	+	-	-	-	+	-	-	+	-	Xg(a-)
6.	rhrh	-	-	-	+	+	-	+	-	+	+	w	+	-	-	+	+	+	+	-	
7.	Rh_1Rh_1	+	+	-	-	+	-	-	+	-	+	+	+	-	-	+	-	+	-	+	Lu(a+b-)
8.	Rh_1Rh_1	+	+	-	-	+	-	+	+	+	+	-	-	+	+	+	+	-	+	-	
9.	$Rh_1Rh_1^W$	+	+	-	-	+	+	+	-	+	+	+	-	+	-	+	-	+	+	+	
10.	Rh_2Rh_2	+	-	+	+	-	-	+	+	-	+	+	-	+	-	+	-	-	-	+	Js(a+b+)
11.	Rh_1Rh_1	+	+	-	-	+	-	+	+	-	+	+	-	+	-	+	-	+	+	+	Kp(a+b+)
Ortho Resolve Panel A (Lot # RA 124)																					
1.	$Rh_1^WRh_1$	+	+	-	-	+	+	+	-	+	+	+	-	+	+	+	+	-	+	-	Lu(a-)
2.	Rh_1Rh_1	+	+	-	-	+	-	+	-	+	-	+	+	-	-	+	-	+	+	+	
4.	Rh_orh	+	-	-	+	+	-	+	+	+	+	+	-	+	-	+	+	-	+	+	Wr(a+)
5.	rh'rh	-	+	-	+	+	-	+	+	-	+	+	-	+	-	+	-	+	+	+	
6.	rh"rh	-	-	+	+	+	-	-	+	-	+	-	-	-	-	+	+	+	-	+	
8.	rhrh	-	-	-	+	+	-	-	+	+	+	+	+	-	-	+	+	-	+	+	
10.	rhrh	-	-	-	+	+	-	+	+	-	+	-	-	-	-	+	+	-	+	+	Co(b+)

Table 4. Continued

	Rh Phenotype	D	C	E	c	e	C^w	M	N	S	s	P_1	Le^a	Le^b	K	k	Fy^a	Fy^b	Jk^a	Jk^b	Other
Ortho Resolve Panel B (Lot # RB 508)																					
1.	rhrh	-	-	-	+	+	-	+	+	+	+	-	-	-	-	+	-	+	+	-	Sd(a+)
2.	rhrh	-	-	-	+	+	-	+	+	+	-	-	-	+	-	+	+	+	+	+	Lu(a+)
5.	rhrh	-	-	-	+	+	-	+	-	-	+	-	-	+	-	+	+	+	+	+	Lu(b+)
6.	Rh_2Rh_2	+	-	+	+	-	-	+	+	-	+	-	+	-	+	+	-	+	+	+	
7.	Rh_zRh_1	+	+	+	-	+	-	+	-	+	+	+	+	-	-	+	-	+	-	+	
9.	Rh_2rh	+	-	+	+	+	-	+	-	+	+	+	+	-	+	-	-	+	+	+	Bg(a+)
10.	Rh_orh	+	-	-	+	+	-	+	+	+	+	+	-	+	-	+	-	-	+	-	V+;He+

Table 5. Antiserum Titers With Fresh and Stored Red Cells

Antiserum	Titer (Score) With Red Cells of Condition:*			
	Fresh	4° Saline	4° Diluent	Cryogenic
Anti-A (8001)	256(82)	64(69)	256(82)	128(69)
Anti-c (8005)				
saline	128(94)	64(58)	64(58)	64(62)
papain	2000(114)	2000(100)	2000(109)	2000(104)
Anti-c (8006)				
saline	4(31)	2(18)	2(20)	2(18)
papain	2000(112)	1000(92)	1000(101)	1000(91)
Anti-e (8001)				
saline	128(70)	64(61)	128(66)	64(61)
papain	1000(100)	512(83)	512(88)	1000(89)
Anti-k (8201)¶	64(43)	32(41)	32(43)	32(41)

*Cells of type A_2, rhrh, kk three times washed in saline, then titrated at 0.1% concentration immediately, or after indicated storage. Refrigerator storage (4°) was under normal saline, or under Ortho modified Alsever's red cell diluent solution containing inosine, for one week. Cryogenic storage was in glycerol (Cf. Appendix II) at -85° for periods of weeks to many months; cells were recovered by dialysis, and thrice washed in saline prior to testing

¶By anti-human globulin technique All temperatures in °C

C. Titration Score

In addition to the titer against a particular type of cell under specified conditions, antibodies may also show different avidities for cellular antigens, and these differences may not be apparent from a comparison of the titers alone. Two antisera of the same specificity could have identical titers against a particular cell type under the same conditions, but have populations of antibodies with different avidities for the cellular antigen. The "titration score" is a simple way of looking at these avidity differences. Titration scores are determined by first assigning arbitrary numerical values to the different degrees of agglutination, and then summing up these numbers over a titration series.

A number of different scoring systems have been used, and any one of them is perfectly acceptable as long as consistency is maintained. We use the scoring system described by Issitt and Issitt (1976), slightly modified to be consistent with the different degrees of agglutination used in reading, as follows:

Degree of Agglutination	Associated Score Value
4+ or v	12
3+ or ++	10
2+ or +	8
1+ or ±	5
w or w	2
- or -	0

A score can be computed for any titration series. Antisera with the same specificity can have the same titer, but differ in titration score. Some examples of this behavior, and the titers and scores of several different antisera, are shown in Table 6.

Table 6. Titers and Scores of Some Representative Antisera

Antiserum	Cell Phenotype	Technique	Titer	Score
Anti-A #1	A_1	saline	512	92
	A_2	saline	256	88
Anti-A #2	A_1	saline	512	108
	A_2	saline	256	105
Anti-C #1	CC	papain	1000	113
	Cc	papain	512	104
Anti-C #2	CC	papain	512	104
	Cc	papain	512	102
Anti-k #1	kk	Coombs	64	58
	Kk	Coombs	64	51
Anti-k #2	kk	Coombs	64	63
	Kk	Coombs	64	58
Anti-Fy^a #1	Fy(a+b-)	Coombs	128	68
	Fy(a+b+)	Coombs	64	53
Anti-Fy^a #2	Fy(a+b-)	Coombs	128	74
	Fy(a+b+)	Coombs	64	64

Other serological procedures used in this and other studies are discussed in subsequent sections, and some are given in detail in Appendix II.

IV. Absorption-Elution — Variables and Optimization

As noted in §I, elution is now employed as the method of choice in most laboratories for the typing of antigens in dried blood. It is considerably more sensitive than inhibition techniques, and thus has the advantages of being applicable to smaller quantities of bloodstain, and of being more conservative of expensive grouping reagents.

The most complete and extensive studies of the absorption-elution process have been carried out by Lincoln (1973) and Lincoln and Dodd (1973). The absorption-elution process can be divided into a series of stages. Each of these was studied separately, with the idea of finding the most optimal conditions for the overall technique. We have gathered data on some of the variables, and the data are in agreement with the findings of Lincoln and Dodd (1973). In an optimal procedure, the maximal amount of antibody is absorbed by a given quantity of bloodstained material, the least amount of specifically bound antibody is lost during the washing procedure while all the unbound antibody must be washed away, and the maximal amount of specifically bound antibody is eluted. Finally, the best and most sensitive technique for the particular antibody is utilized to detect the eluted antibody. The following variables need to be considered: (1) Antibody concentration at the absorption stage; (2) Absorption time; (3) Antigen concentration (quantity of dried blood) in the absorption stage; (4) Washing volume, time and temperature; (5) Elution temperature; (6) Test cell concentration; and (7) The serological technique to be used for the detection of the eluted antibodies. Optimization of each of these variables is discussed below. The basic principles governing agglutination (§II.B) have to be considered in determining how to optimize each of these variables. The characteristics of the antiserum are also important, particularly with regard to choice of serological technique for detecting eluted antibodies.

In the studies on the elution process conducted by Lincoln and Dodd (1973), and in most of our studies, eluates were titrated at the end of the elution step.

The titer (and score) of an eluate provide a relative quantitative measure of the antibody recovered from the bloodstained material. This technique allows different variables in the elution procedure to be studied, and it allows comparisons to be made among different antisera and bloodstained materials. Titration of eluates is to be recommended even in routine casework grouping, because more information can be obtained about the sample, and much more confidence can be placed in many of the positive results, with only a modest amount of additional effort.

A. Absorption Stage

The absorption stage of an absorption-elution test is usually carried out using a great excess of antibody, conditions which are expected to favor the maximal formation of antigen-antibody complex. In addition to antibody concentration, the following other parameters must be considered: the rate of antigen-antibody association; the quantity of antigen (bloodstain) to be used; the ionic strength of the medium; and absorption temperature.

1. Concentration of Antibody — Titer of Antiserum

The titer of an antiserum used for absorption represents a measure of the antibody concentration. Table 7 shows the effect of changing the antibody concentration of the absorbing antiserum over a wide range, and with several different specificities. The quantity of bloodstain, and other parameters, were constant.

It can be seen from the data that antibody yield from a constant quantity of bloodstain increases as the absorbing antiserum concentration increases, but only to a certain point. The optimal titer of an absorbing antiserum is 256 - 512. Higher concentrations of antibody not only do not increase antibody yields in the eluates, but may result in antibody appearing in eluates from antigen-negative stains. The latter effect is probably caused by the inability to wash out all the excess antibody in the washing procedures that were used; this behavior is ordinarily seen only at the very high antibody concentrations. The very high titers of antiserum are not found in commercially available ABO antisera using saline technique, but can be seen with Rh antisera if enhancement techniques (like the use of papainized test cells) are employed.

Table 7. Effect of Antibody Concentration on Antibody Recovery in Eluates

	Titer of Antiserum Used for Absorption¶	Titer¶ of Eluates From Stains of Type:	
		R_2R_2	rr
A. Anti-D with Rh+ and Rh - Stains*	32000	128	32
	8000	256	2
	1000	256	2
	512	128	0
	64	32	0
	16	8	0
		R_1R_2	rr
B. 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
C. Anti-B with B and Other ABO Type Stains	256	8	0
	128	4	0
	64	2	0
	16	1	0
	4	0	0

*Data of Lincoln and Dodd (1973) ¶Anti-D titers by papain technique in absorption stage and in eluates; Anti-B by usual saline technique

Titers of absorbing antisera below 256 result in lower antibody yields in the resulting eluates. It should be realized that each increase of "one tube" in the reciprocal titers of eluates (such as those shown in Table 7) represents a doubling of the quantity of antibody recovered from the bloodstain. Thus, anti-D antibody recovery from a D+ stain was 16 times greater with an absorption titer of 512 than it is with an absorption titer of 16 (Table 7A); similarly, anti-B antibody recovery from a B stain was 8 times greater if the titer of anti-B used for absorption was increased from 16 to 256 (Table 7C).

2. Rate of Antigen-Antibody Binding — Absorption Time

The rate of antigen-antibody binding is another important factor, related to optimizing the absorption time. Results of experiments designed to test this variable are shown in Table 8. Here, all elution variables are held constant except for absorption time, and eluate titers, representing a measure of antibody recovered, are then compared.

Table 8. Effect of Absorption Time on Antibody Recovery in Eluates

A. Anti-A With Type A Stain*

Papain Titer of Anti-A Used for Absorption	Absorption Time (hrs)	Papain Titer of Eluate from 1 cm Stained Thread
1000	2	8
	16	128
256	2	4
	16	32

B. Anti-D# With R_1R_2 Stain

Absorption Time (hrs)	Papain Titer (Score) of Eluate From 1 mm^2 Stain
1	0(0)
2	0(2)
4	2(12)
6	4(15)
8	16(31)
10	32(45)
16	128(63)

C. Anti-A and Anti-B With A and B Stains and Dried Ammoniacal Extracts of A and B Stains¶

Antiserum Used for Absorption -- Titer in Saline (Papain)	Absorption Time (hrs)	Papain Titer (Score) of Eluate from 1 mm^2 With Antigen	Papain Titer (Score) of Eluate from Dried Ammoniacal Extract of 1 mm^2 Stain With Antigen
Anti-A 64 (512)	0.25	8(28)	4(18)
	1	16(36)	8(28)
	4	32(41)	32(38)
	17	64(52)	64(60)
Anti-B 512 (1000)	0.25	2(9)	2(10)
	1	4(23)	4(23)
	4	8(23)	8(33)
	17	32(41)	32(40)

*Data of Lincoln and Dodd (1973) #Papain titer 512 ¶Gaensslen, Bremser and DeGraw (1981); Absorptions carried out at 4° for ABO antisera, and 37° for Rh antisera

All the data indicate that antibody recovery is maximal at absorption times of about 16 hours. Shorter absorption times result in reduced recoveries. Most elution procedures suggest absorption periods of about 16 hours—"overnight" is common. Most workers do not titrate the eluates from these tests in everyday practice. Test cells are ordinarily added to the eluate, and agglutination is read after a suitable incubation period under appropriate conditions. If one demanded as a minimum condition for a positive result that an eluate give at least a 2+ agglutination result, then the minimum acceptable titer of an eluate under the conditions represented by the experiments in Table 8 would be 2 to 4. These titers are seen in the experimental eluates from A and B stains represented in the Table, even at relatively short absorption times. It should be noted, however, that these experiments were done with relatively fresh bloodstains, and that papain technique was used as an experimental device to enhance agglutination so that the effects and differences could be clearly observed. In practice, papain technique would normally not be used with ABO tests. The eluate titers seen in the Table would be correspondingly lower using saline technique. In addition, bloodstains of comparable quantity but older than those used in the experiments, or which had been subjected to conditions which decreased the number of reactive antigenic sites, would give lower antibody yields. In the case of Rh typing, in which there are considerably fewer antigenic receptor sites in a comparable amount of blood to begin with (Cf. Table 3) as compared with the number of ABO sites, and correspondingly less antibody is expected to be taken up (see in Table 9), optimization of antibody yield is very important. The data in Table 8B indicate that four hours absorption would have been the minimally acceptable time, under the particular conditions and with the stain used, for obtaining a positive result.

Table 9. Anti-A, Anti-D and Anti-c Antibody Uptake By a Constant Quantity of Cells from Antisera of Comparable Titer*

Antiserum	Preabsorption Papain Titer	Papain Titer After Absorption With 5 µL Red Cells
Anti-A	512	2
Anti-D	1000	256
Anti-c	512	128

*Data of Lincoln & Dodd (1973); Absorption for 1 hr at 4° for ABO and 37° for Rh antisera

ABO typing of dried ammoniacal extracts of bloodstains was originally proposed by Kind and Cleevely (1969), as noted in §I.B. Some investigators have suggested that very short absorption times (in the range of 5 to 10 min) are suitable when grouping by this technique, resulting in a very great savings of time in the overall procedure (Chisum, 1971; Dixon and Epstein, 1976). We were interested in finding out whether the rate of association between ABO antibodies and antigens was different in the case of bloodstains on cotton as against the dried ammoniacal extracts from equivalent quantities of the same stain. The results shown in Table 8C indicate that the rate of association is quite comparable in the two cases. While it appears that convincing positive results would usually be obtained from bloodstains using the ammonia extraction procedure with very short absorption times, it is also the case that maximal antibody absorption and recovery require absorption times comparable to those required for bloodstains on cloth substrata. It is possible that a stain which was fairly old, or had been exposed to influences that reduced the effective number of antigenic receptor sites, could give poor results when typed using very short absorption times, but give acceptable results if optimal absorption times had been employed. Table 10 shows the effect of absorption time on antibody recovery from stains made from diluted (1:5 with saline) blood as opposed to whole undiluted blood, using the ammoniacal extraction technique.

Table 10. Effect of Absorption Time on Antibody Recovery From Ammoniacal Extracts of Stains Made From Whole and From Diluted Blood

Sample	Antiserum (Titer)	Absorption Time (hrs)	Titer (Score) of Eluate From Ammoniacal Extract
Type A Whole Bloodstain Extract	Anti-A (256)	0.5	2(10)
		16	8(35)
Type A Diluted Bloodstain Extract	Anti-A (256)	0.5	0(4)
		16	4(18)

The whole blood stain extract gave acceptable typing results with both absorption times, although antibody recovery was four fold higher with the 16 hr absorption time. The diluted bloodstain extract, however, gave only a weak agglutination—which might easily have been regarded as less than convincing—with the shorter absorption time, while it gave a convincing A reaction when absorption was carried out for 16 hrs. Stains from diluted blood are sometimes encountered in casework situations. Blood may be diluted by other body fluids, or by rain water, for example.

Using fresh cells and continuous agitation, anti-c binding to Rh c+ cells reaches equilibrium within 30 min at 37° (Hughes-Jones, 1975), and shorter equilibration times can be seen at higher antibody concentrations. It could not be assumed, as Lincoln and Dodd (1973) pointed out, that the kinestics of binding would be the same for bloodstain antigens. Indeed, it is clear from their studies, the results of which are fully confirmed by our results, that antibody yield is substantially improved using 16 hr absorption times, other factors being equal. Ducos (1954) had observed that the titer of an anti-D, incubated with Rh+ dried blood at 37°, decreased steadily over the course of incubation time, up to a maximum of about 24 hrs.

3. Concentration of Antigen—Quantity of Bloodstain

A number of factors have to be considered in deciding what quantity of bloodstain to use for grouping by microelution techniques. The ability to group small quantities of bloodstain is very desirable in casework samples where the quantity of bloodstain available for testing is limited. If the eluate from the stain is to contain an amount of antibody sufficient for detection by the method in use, however, a certain minimum quantity of stain, representing an adequate number of active antigenic sites, must be utilized. In casework, stains which have been subjected to many different degradative influences and of amny different ages will be encountered. Selection of sample size is partly, therefore, a matter of judgment based upon experience, and the information an examiner has about the stain's history. Results may be more dependent upon sample size in testing for those antigens which are present on the red cell in smaller numbers to begin with. Finally, the effects of antigen concentration on antibody binding and recovery must be taken into account.

Lincoln and Dodd (1973) found that anti-A antibody recovery was enhanced quite markedly by using smaller quantities of bloodstain, within certain limits. Their results are shown in Table 11.

Table 11. Effect of Quantity of Type A Stain on Anti-A Antibody Recovery in Eluates*

Quantity of Packed Type A Red Cells Used to Make Stain	Papain Titer of Eluate
5 µL	8
≅2.5 µL	128
≅0.25 µL	128

*Data of Lincoln and Dodd (1973); Papain titer of anti-A used for absorption was 1000

These results indicated much poorer antibody recovery in the stain of greatest antigen concentration. Two different effects were believed to be at work here. First, the presence of excess antigen may allow the antibody molecules a greater latitude of choice in antigenic sites for binding, with the result that many bind to sites of "best fit", forming very stable complexes and making subsequent elution more difficult. Secondly, and probably of greater importance, eluted antibody may have a much greater chance of recombining with antigenic sites if there is an excess of antigen present.

In our studies designed to determine a minimally acceptable quantity of bloodstain for elution grouping with Rh antisera, we did not observe decreases in antibody recovery with larger quantities of stain, although we did not measure the quantities involved as carefully as Lincoln and Dodd (1973) had done, and the experiments were not done with ABO antisera, which might behave differently than the Rh ones. Table 12 shows the results of varying the quantity of bloodstain taken for absorption on the antibody recovery in the eluates. There appears to be no advantage in using larger pieces of stained material; in fact, they are more difficult to wash thoroughly than threads. For most applications, three 1 cm threads of stained material appears to be satisfactory for microelution typing. It is easier to keep track of three threads during washing than of only one, even though with some stains one thread represented an adequate quantity of stain for reliable antigen typing.

Table 12. Effect of Quantity of Bloodstained Sample* on Antibody Recovery in Eluates

Antiserum	Quantity of Bloodstain Used for Absorption	Titer (Score) of Eluates from Rh C+D+ Stain
Anti-D¶	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†	1 thread (1 cm)	64 (53)
	3 threads (1 cm)	128 (68)
Anti-C§	1 thread (1 cm)	16 (28)
	3 threads (1 cm)	16 (33)

*Cotton cloth, freshly prepared—not more than a few days old; ¶Papain titer 1000; eluates titrated with papain treated cells; †Titer in 0.5% albumin 512; eluates titrated by albumin technique; §Saline titer 64; eluates titrated in saline

Bloodstains may occur on almost any type of fabric or on any object or material. A number of fabrics can be used directly for elution grouping tests. The quantity required may vary from a 1 cm thread (if the material can be separated into threads or fibers) to a 1 cm^2 piece, depending upon the age, density, distribution and condition of the stain. In the case of other fabric materials, and of objects which cannot be used for grouping in tubes, the bloodstain may be transferred onto saline-wetted cotton threads. These are then carefully re-dried, and used for the typing tests. Further discussion of typing results on various substrata is presented in appropriate subsequent sections.

B. Washing Stage

The washing stage is designed to remove all traces of antibody not specifically bound to its corresponding antigenic receptor in the stain. At the same time, one wants as little specifically bound antibody as possible to be eluted and washed away. The most important variables in the washing procedure are the volume of washing fluid (saline), the time allowed for the washing fluid to remain in contact with the sample, and the temperature of the washing fluid and the environment. We carry out the absorption and washing stages of grouping in 12 x 75 mm test tubes most of the time, and the stained material occupies a small area at the bottom of the tube.

These tubes hold about 8 to 9 mL fluid. The washing procedure consists of filling the tube almost to the top with ice-cold saline, after first removing the excess absorbing antiserum with a narrow-bore capillary pipette attached to a water-driven vacuum line. The tubes are then placed in a refrigerator for 15 min. The washing fluid is the completely removed by vacuum suction pipette, and the tubes refilled with ice-cold saline and put back into the refrigerator for 15 min. This process is repeated until 6 ice-cold saline washes have been carried out. The last wash is done with ice-cold saline containing 0.5% bovine serum albumin.

When grouping ammoniacal extracts of bloodstains for ABO, the extracts are prepared in the bottoms of conical polystyrene sample cups using about six 1 mm long threads with about 100 µL ammonia solution per cup. After about 30 min, the threads are removed, and the extracts allowed to air dry completely. Absorption and washing are carried out directly on the sample residues within the sample cups (see also in § I.B). These cups hold 2 mL fluid when filled, and washing is carried out in the same way as described above for tubes, except that it is repeated 8 times.

Incomplete washing of bloodstains can cause false positive reactions in elution tests. Negative (bloodstains lacking the antigen) and substratum or cloth controls are thus always run in parallel with every test. The cloth or substratum control serves to insure that washing has been complete. If it has not, the tests must be repeated. Negative bloodstain controls should likewise be negative. Cloth or substratum controls can give positive reactions in ABO grouping tests for reasons other than incomplete washing as well.

The temperature of the saline used for washing should be 4°. In this way, the least quantity of specifically bound antibody will be lost in the washing process. While 56° is ordinarily thought of as "the temperature" at which antibody is eluted, there is no question that some antibody can be eluted at much lower temperatures. Table 13 shows the effect of the temperature on the elution of anti-A and anti-D from type A and Rh(D+) stains, respectively. While temperatures of 55°-65° are fairly optimal for the elution of antibody, with some variability in the anti-D probably caused by variations in the temperature dependence of the equilibrium constants for various antibodies in the antiserum, the thing to be noted here is that antibody can be eluted even at 22° in the case of anti-A, and at 37° in the case of anti-D.

Table 13. Effect of Temperature on Elution of Anti-A and Anti-D from Bloodstains Containing the Antigens*

Temperature (°C)	Titer of Eluates from Bloodstains Containing the Antigens			
	Anti-A		Anti-D	
	saline	papain	papain	AHG
4	2	4	0	0
22	4	32	0	0
37	4	32	2	2
45	8	128	4	2
55	16	64	16	4
60	8	64	32	16
65	4	16	64	64
70	2	16	128	16

*Data of Lincoln and Dodd (1973)

If the washing liquid is allowed to warm up, therefore, some antibody will be lost during washing, especially as this process is repeated agin and again. Accordingly, the washing fluid should be kept ice-cold, and the tubes filled with it should be kept at 4°, except during the removal and filling operations which should be carried out as quickly as possible.

C. Elution and Detection Stages

The elution step is carried out in order to dissociate the specific antigen-antibody complex, and recover the antibodies in solution so that they can be detected in agglutination reactions with fresh cells. There are any number of different methods for eluting specifically bound antibodies from cells or stains (see Howard, 1981), as noted in § I.B. Elution of antibodies from stains is most commonly carried out, however, by the heating procedure originally described by Landsteiner and Miller (1925).

1. Elution Temperature

Most workers elute at 56°, and this temperature is quite optimal for ABO antibodies, and very satisfactory for other antibodies as well. There is some variation depending upon the particular antibody and its specificity.

The variability was demonstrated in the studies of Lincoln and Dodd (1973) and is shown in Table 13. It is important to recognize that heat-eluted antibodies can be bound again to the antigen, if the antigen is available, and if the temperature decreases significantly. The eluate must, therefore, be removed quickly from contact with the bloodstain sample material before the temperature has a chance to decrease. This point was illustrated by an experiment carried out by Lincoln and Dodd (1973).

Type A cells were absorbed with an immune anti-A at 4° for 2 hrs. The cells were thoroughly washed with ice-cold saline, and divided into two aliquots. The first was eluted at 56°, and the cells then centrifuged down at 56° and the eluate removed. The second was eluted at 56°, then allowed to stand 5 min at room temperature before centrifugation and collection. The results showed that the rapidly separated eluate had a saline titer of 512 and a papain titer of 1000; the eluate which had been allowed to cool had a saline titer of 16 and a papain titer of 128.

2. Concentration of Test Cells

It has been known for a long time that the sensitivity of the agglutination reaction is inversely proportional to the concentration of the red cell suspension. This behavior provides a simple way of enhancing the sensitivity of agglutination reactions that seems to have been overlooked in designing many forensic serological procedures involving agglutination reactions. In tests designed to detect relatively small amounts of antibody by agglutination, such as in eluates, maximization of sensitivity is an important consideration. In 1941, Lund found that the use of 0.0625% cell suspensions increased the sensitivity of agglutination eight-fold, compared with 0.5-1.0% cell suspensions, with ABO antibodies. Cell suspensions of 0.007% gave agglutination reactions that were 32 times more sensitive than 1.0% suspensions.

Lincoln and Dodd (1973) studied the effect of cell concentration on the sensitivity of the agglutination reaction using a high titered anti-c reagent and papain technique. Their results are shown in Table 14A. Our studies (Table 14B) confirm their observations using an antiserum of different specificity. At lower dilutions of anti-c (higher antibody concentrations), cell suspensions of 0.5%, 0.1% and 0.05% gave the same titer, which was one to several dilutions higher than that seen with 2% cell suspensions.

Table 14. Effect of Cell Suspension Concentration on the Titer of an Antibody at Different Dilutions

A. Anti-c with Rh(c+) cells*			B. Anti-D with R_1R_1 Cells		
Dilution of Anti-c	Concentration of Cell Suspension(%)	Titer of Anti-c†	Dilution of Anti-D	Concentration of Cell Suspension (%)	Titer(Score) of Anti-D ¶
1:16	2	128	1:8	3	1000(102)
	0.5	512		1	2000(109)
	0.1	512		0.1	4000(106)
	0.05	512		0.05	4000(106)
1:64	2	32	1:32	3	64(67)
	0.5	256		1	128(67)
	0.1	256		0.1	256(71)
	0.05	256		0.05	256(76)
1:256	2	8	1:512	3	16(41)
	0.5	32		1	16(40)
	0.1	64		0.1	32(48)
	0.05	128		0.05	128(56)

*Data of Lincoln and Dodd (1973); †With papain technique; ¶With papain treated test cells and AB serum diluent

At the highest antiserum dilution, however, each successive decrease in cell suspension concentration gave a corresponding increase in the anti-c titer. A sixteen-fold increase in sensitivity is seen at the highest dilution of anti-c in going from 2% to 0.05% cell suspensions, while at the highest dilution of anti-D, an eight-fold increase is seen in going from 1% to 0.05% cells. The sensitivity of the agglutination detection reaction is a sensitive function of cell suspension concentration, particularly at comparatively low antibody concentrations, such as those in eluates. The employment of 0.05% test cell suspensions is thus to be recommended as optimal for the detection of low concentrations of antibodies eluted from bloodstained specimens.

3. Serological Technique

The serological technique selected for the detection of eluted antibodies depends primarily on the characteristics of the antibodies in the particular antiserum being used. Maximal absorption of antibody by the bloodstain antigens takes place at comparatively high absorbing antibody concentrations (see § IV.A.1).

Thus, the titer of the absorbing antiserum should be in the neighborhood of 256-512 whenever possible; in addition, it should not be *too high*, as noted above. The titer being referred to here is that determined under the same conditions that are going to be used in detecting the eluted antibodies. If papain treated cells are to be used in the detection stage, for example, then it is the papain titer of the antiserum (and at the same cell suspension concentration) that needs to be considered in determining the appropriate concentration of antiserum to be employed in the absorption stage.

One of the important steps in evaluating an antiserum for its bloodstain typing applicability is determination of its titer under different conditions. In some cases, different enhancement techniques, or a combination of them, need to be tested. In addition, it is a good practice to determine the titer with cells that are heterozygous as well as cells that are homozygous for the corresponding antigen.

Most ABO and MN antisera and lectins are of more than sufficiently high titer in saline to be applicable to bloodstains. Rh antisera vary considerably in their characteristics. Some react relatively strongly in saline, but many show optimal reactivity in high protein media, or with papainized cells. Some Rh antisera have significant AHG titers. The use of papain-treated cells with Rh antisera is a good general technique. Many of them have high papain titers and can readily be adjusted to values optimal for absorption. Lincoln and Dodd (1973) employed papain technique with many of the Rh antisera, and we have used it in most of our studies as well. Other techniques are perfectly suitable, however, provided antisera are selected and evaluated to determine their characteristics. In this way, the different serological conditions in the absorption-elution procedure can be optimized. Some Rh antibodies are chemically modified by the manufacturer so as to make them more reactive in saline or in high protein media. This information is noted on the package inserts supplied with commercial antiserum. Most Ss, Kell, Duffy and Kidd antisera are optimally reactive by the indirect AHG technique. In some cases, low ionic strength solutions (LISS media) yield enhancement of the reactions of these reagents, particularly if they are employed in the sensitization stage. LISS may increase not only the rate but also the total amount of antibody uptake.

In bloodstain grouping, LISS can be employed at the absorption stage, as a suspending medium for test cells at the detection stage, or both. Extensive studies by McDowall, Lincoln and Dodd (1978a) have shown that LISS can significantly increase the sensitivity of detection of eluted blood group antibodies. A number of Rh and Ss antibodies were included in these studies. Enhancement was apparent when absorption was carried out in LISS, and when LISS was employed as suspending medium for the test cells at the detection stage. When LISS was included in both stages, there was additional enhancement. In addition, the use of AB serum (1:10) as an eluate titration diluent gave stronger reactions than did an albumin diluent (1:100 bovine albumin in 0.15M NaCl). If LISS was used at the absorption stage, and in conjunction with AB serum as a titration diluent, there was significant enhancement compared with a technique in which no LISS was introduced and titrations were carried out in albumin diluent. A number of stains, particularly older ones, which yielded weak or unconvincing reactions without LISS, gave very satisfactory and conclusive typing results with LISS technique using several Rh antisera. LISS techniques enhanced the anti-S reactions as well, in one case yielding a 6+ reaction from a stain that had given a negative reaction by normal technique. Papain treated cells were used in conjunction with LISS in the case of the Rh antisera, but not with the anti-S, which was detected by AHG. Lincoln and Dodd (1978) have shown that LISS can enhance the papain titer of certain Rh antibodies, particularly those of low affinity (which can be selected on the basis of having low or nil AHG titers with or without LISS, but significant titers with ordinary papain technique). The LISS-papain technique was shown to be especially suitable for the detection of eluted antibodies. LISS techniques, and antisera showing LISS-enhancement, must be carefully evaluated. One of the problems with LISS is that stains which do not possess an antigen can show reactions with the corresponding antiserum under certain conditions (see Lincoln and Dodd, 1973). These problems are largely overcome by the use of the LISS described by Löw and Messeter (1974), but careful evaluations of antisera and techniques are still necessary, in addition to the incorporation of appropriate controls. It should be noted that enzyme (papain) treatment cannot be used with all blood group antigens. Enzyme treatment has a destructive effect on certain of the receptors. Papain, for example, is destructive to S and Fy^a receptors (Morton, 1962).

D. Optimization of Absorption-Elution Variables— Summary

There are a number of variables in the absorption-elution procedure as applied to blood group antigen determination in dried stains. The effects of the different variables on absorption, elution and detection can be assessed by titrating the eluates to obtain a relative estimate of antibody recovery. Adjustment of the different absorption-elution conditions for maximal antibody absorption, recovery, and detection results in the most sensitive procedure, and should give the best possible results with bloodstains. In the absorption stage, an antiserum with a titer of 256-512 should be employed if possible. Absorption should be carried out for 16 hrs to obtain maximal antibody uptake. The quantity of stain material to be used depends to some extent on the quality and age of the bloodstain and the nature of the substratum, and to some extent on the antigen being determined. There is evidence that sensitivity is decreased in some cases by the presence of an excess quantity of antigen (bloodstain), as discussed in § IV.A.3.

Washing time and volume should be adjusted to insure the complete removal of non-specifically-bound antibodies. Controls are essential to show that washing has been complete. Washing should be carried out at 4° to minimize the loss of specifically-bound antibody by unwanted elution. Optimal elution temperature is 55°-65°, although there may be slight variations with different antibodies caused by differences in the temperature dependence of the equilibrium constants of the antibodies. A 56° elution temperature is found to work well under most circumstances.

Test cell concentrations are roughly inversely proportional to sensitivity, particularly at low antibody concentrations. Test cell suspensions with concentrations of 0.05%-0.1% are to be recommended. It may be necessary to increase these concentrations somewhat for low concentrations of antibodies being detected by the AHG (Coombs) technique.

The serological technique used for the detection of eluted antibodies depends on the specificity and characteristics of the antiserum being used. A number of techniques which enhance antibody uptake and/or agglutination have been found to be helpful in increasing the sensitivity of absorption-elution, thus enabling the detection of antigens in bloodstains which might not have been detected without them.

V. MNSs System — MN Antigens and Antibodies

A. The MNSs System

Landsteiner and Levine (1927a) first described a rabbit immune anti-M, which reacted with about 80% of Caucasian and about 70% of Negro red cells. Another rabbit serum, defining the N antigen, was quickly found (Landsteiner and Levine, 1927b). These antigens constituted a new human blood group system, independent of ABO, and family studies suggested control by an allelic pair of codominant autosomal alleles *M* and *N*, giving three genotypes and phenotypes MM, MN and NN (Landsteiner and Levine, 1928a, 1928b). In 1947, Walsh and Montgomery found a new antibody in the serum of a postpartum mother in Australia. The antiserum was studied by Sanger and Race (1947), who called the antigen being detected by it "S". It was not associated with most of the then-known blood group systems, but was associated with MN. Family studies confirmed the association (Sanger *et al.*, 1948), and the finding of anti-s in the serum of a mother whose baby had hemolytic disease of the newborn (Levine *et al.*, 1951) showed that *S* was not allelic to *M* and *N*. The *Ss* locus was closely linked to that of *MN* (Sanger and Race, 1951), thus defining an MNSs blood group system. Table 15 shows the characteristics of this system.

Table 15. The MNSs System

Phenotype	Genotype(s) or Haplotype Combinations	Reactions of Red Cells With Anti-M	Anti-N	Anti-S	Anti-s
MS	MS/MS	+	-	+	-
Ms	Ms/Ms	+	-	-	+
MSs	MS/Ms	+	-	+	+
MNS	MS/NS	+	+	+	-
MNs	Ms/Ns	+	+	-	+
MNSs	MS/Ns; Ms/NS	+	+	+	+
NS	NS/NS	-	+	+	-
Ns	Ns/Ns	-	+	-	+
NSs	NS/Ns	-	+	+	+

There are a number of complexities in the MNSs system that are not indicated in Table 15.

Quite a few antigens in addition to original four are now known to belong to this system. Fuller discussion of these, and of other complexities in the MNSs system may be found in reviews (Race and Sanger, 1975; Issitt, 1981; Gaensslen, 1983).

Although the genes controlling the MN and Ss antigens are inherited together, the antisera for the MN antigens on the one hand, and for the Ss antigens on the other, tend to be quite different in their serological characteristics. MN antisera often contain complete, saline reacting antibodies, and many of the reagents are prepared in rabbits. Anti-N lectins are also known. There are certain problems associated with bloodstain MN grouping that are attributable to biochemical similarities in certain red cell sialoglycoproteins (see below). Ss antisera are human, often Coombs-reactive, and have serological characteristics more similar to Kell, Duffy and Kidd antisera than they do to MN antisera. Accordingly, MN antigens and antisera are discussed in this section; Ss antigen typing and Ss system antisera will be discussed in §VII along with the Kell Duffy and Kidd systems.

B. Development of MN Grouping of Bloodstains

The M and N antigens were first determined in dried blood by their discoverers using absorption-inhibition technique (Landsteiner and Levine, 1928a). Other earlier investigators reported successful MN grouping in dried bloodstains by inhibition techniques (Lauer, 1933; Clausen, 1933; Therkelsen, 1934; Moureau, 1935; Ponsold, 1936). Sylvia and Kirk (1961) carried out studies on MN grouping in bloodstains using anti-M serum and an anti-N lectin from *Vicia graminea*. Good results were obtained for the M antigen, but there were various problems with N.

Nickolls and Pereira (1962) first reported an elution method for MN typing in bloodstains, and a fuller description of the procedure was given by Pereira (1963). Acceptable results were obtained with anti-M, and usually with anti-N. Rabbit immune sera were used in these studies. The importance of known controls and of careful interpretation was stressed. Other workers confirmed the usefulness of elution procedures for M and N typing, and introduced various technical modifications of the procedures (Budvari, 1963; Fiori, Marigo and Benciolini, 1963; Yudina, 1972; Driesen and Keller, 1973; Schwerd, 1978). The thread technique on cellulose acetate support sheets can also be used (Howard and Martin, 1969).

The major problem in MN grouping is usually called "cross-reactivity", which refers to the fact that anti-M is usually quite specific for M antigen in its reactions, but that anti-N can react not only with N cells but also with M cells. This behavior was noticed by Landsteiner and Levine (1928b) and has been reported by many workers subsequently, including Clausen (1933), Sylvia and Kirk (1961), Pereira (1963) and Schwerd (1978). Type M stains may react, therefore, with anti-N which is then eluted and detected giving a false result. Stain typing with anti-M is not affected by this problem, and the M antigen can ordinarily be reliably detected. For a number of years it was thought that the cross-reactivity of anti-N was mainly an "antiserum problem", and that it could be overcome at least to a great extent by the careful selection and evaluation of anti-N reagents, and by the use of known control stains in every test. There is not much doubt that taking these steps improves bloodstain typing results, but it turns out that the problem is of a somewhat more fundamental nature, involving biochemical similarities in antigenic structures on the surface of the red cells. It was also believed for some time that anti-N cross-reactivity with M cells could be explained by a biochemical precursor relationship between N and M, i.e. that M antigen was made from N. The structural studies discussed below have shown that this thinking was incorrect.

C. Biochemical Studies on the MN Antigens

Within the past few years, structural studies of the glycoproteins of the red cell membrane and of the MN and associated antigenic determinants have led to a much better understanding of the biochemical basis for the MNSs blood group system. For some time, there was disagreement in the literature as to whether the difference between the M and N determinants could be accounted for by differences in attachment of sialic acid residues, or whether there was a difference in the protein structure. Much of the experimental work leading to a clarification of the structural differences, and review of this work, may be found in Fairbanks, Steck and Wallach (1971), Dahr *et al.* (1977), Blumenfeld and Adamany (1978), Dahr and Uhlenbruck (1978), Furthmayr (1978), Lisowska (1981) and Dahr (1981).

It is now clear that the structural differences between M and N (and between S and s) antigens resides in the polypeptide moieties of the sialoglycoproteins, although these findings do not rule out a role for the carbohydrate moieties in antibody-immunodeterminant association. When red cell membranes were first subjected to SDS-PAGE [polyacrylamide gel electrophoresis in gels containing sodium dodecyl sulfate], three sialoglycoprotein bands were seen, and designated PAS-1, PAS-2 and PAS-3 (the designations deriving from the periodic acid-Schiff reagent stain used to detect them in the gel). PAS-1 was found to be the MN-sialoglycoprotein (or SGP). It is now known that PAS-1 is a dimer of the MN-SGP, and PAS-2 is its monomer. PAS-3 was found to be the Ss-SGP, and the so-called "N cross reacting activity" of M cells also resides in PAS-3. Dahr denoted the cross-reacting N as 'N'. The structural studies which led to an understanding of the amino acid sequences of the MN and Ss SGPs, and of the strcutral bases for the antigenic determinant differences, were carried out primarily by Dahr and collaborators in Cologne, Lisowska and collaborators in Wroclaw, and by Marchesi, Tomita and Furthmayr at Yale in New Haven. The Yale group refer to the MN-SGP as glycophorin A, and to the Ss-SGP as glycophorin B. There has been quite a bit of work on the oligosaccharide structures, and their attachment sites to the polypeptide chains as well (see Lisowska, 1981). The SGPs are now seen as linear molecules thrust through the red cell membrane. The N-terminal portions of the chains (about 50 amino acid residues) extend outward from the outer surface of the membrane. These contain the amino acids responsible for the antigenic determinant differences, and all the oligosaccharide chains are attached in this region. There is an intra-membrane portion of the chain, and an internal segment extending into the cell. The MN-SGP is about 130 residues in overall length. Table 16 shows the sequences of the MN and Ss SGPs, and indicates the amino acid residues which determine the antigenic specificity differences. One-letter codes for the amino acids have been used here in order to fit the sequences onto the page.

The M and N antigens differ according to the residues at positions 1 and 5, while S and s differ according to a single residue at position 29. The first 26 residues of the Ss-SGP are identical to those in the N-specific SGP, and the identity of residues 1 and 5 represents the 'N' antigen.

Table 16. Structures of the N-Terminal Sequences of the MN and Ss Sialoglycoproteins

MN-SGP (Glycophorin A)	
M Specific	SSTTGVAMHTSTSSSVTKSYISSQTND THKRDTYA.....
N Specific	LSTTEVAMHTSTSSSVTKSYISSQTND THKRDTYA.....
Ss-SGP (Glycophorin B)	
S Specific	LSTTEVAMHTSTSSSVTKSYISSQTNGEMGQLVHR.....
s Specific	LSTTEVAMHTSTSSSVTKSYISSQTNGETGQLVHR.....
	homologous sequence domain | non-homologous sequence domain

Amino acids underscored indicate residues responsible for M and N, and for S and s antigenic differences; the double underscores at residues 1 and 5 in the Ss-SGP show the identity of the sequence with the type N-specific MN-SGP, and represent the 'N' receptor

In the structures published by Furthmayr (1978) for glycophorin A, residue 11 was Thr and 17 was Ser; Dahr (1981) said that the sequence had been reinvestigated and that residue 11 was Ser while 17 was Thr. Table 16 shows the structures according to the Cologne group (Dahr, 1981).

The structural determinations of the antigenic determinants provide a molecular explanation for the anti-N reaction with M cells. The 'N' antigen resides on the Ss-SGP, and is present on the cells regardless of MN type (and regardless of Ss type). But there is still more to it. Not every example of M cells shows "cross reactivity" with every anti-N serum (whether human or rabbit), nor every anti-N lectin. The cross-reactivity may be present, but it may not be, and it is not very predictable. This variability may have to do with the antigenic determinant topography on the cell surface, about which little is known, or with the still not very clear role played by the oligosaccharide structures in antibody-immunodeterminant binding, or with other factors.

D. MN Antisera— MN Typing in Bloodstains

The major problem with MN typing in stains is the possibility of anti-N reacting with M bloodstains, which would then be mistakenly typed as MN.

Anti-N sera can be tested for cross-reactivity with M cells as part of evaluating their applicability for stain typing. They can then be tested with known M stains in a further evaluative step. The difficulty is that one cannot be sure that every M stain will behave in the same way, i.e., that an unknown stain will behave toward anti-N like the known control M stains. The 'N' reactivity appears to be in part a function of the particular anti-N, but also in part a function of different M bloods. It is, therefore, almost impossible to control riogorously for the possible presence of 'N' in an M stain. For these reasons, many laboratories have ceased to carry out MN typing in their routine protocols. The detection of M antigen with anti-M does not present any great difficulty in properly controlled tests done by experienced workers, although less information is obtained from a stain if only anti-M is used.

A biochemical approach to solving this problem was described by Shaler, Hagins and Mortimer (1978). Their experiments on bloodstains were based on the work of Dahr, Uhlenbruck and Knott (1975) among others, who showed that α-chymotrypsin destroyed the blood group antigenicity of the Ss-SGP (including 'N') without affecting the reactivity of the MN-SGP. Thus, treatment of a sample with this enzyme could obliterate 'N' without affecting N. The procedure gave very promising results, although some biochemical skill was needed to adjust the conditions of the test properly, and the authors suggested that further work should probably be done before this technique was considered for use in routine casework.

In Table 17 are shown the properties of a number of representative anti-M and anti-N from among those examined in our studies. Most anti-M reagents examined had titers within one or two dilutions of one another, the rabbit immune sera tending to be higher than those of human origin. The titers against MN cells were generally one dilution lower than with homozygous cells. Anti-N reagents were somewhat more variable. Three of those shown in Table 17 reacted with M cells. The Molter anti-N from rabbits had a high enough titer that the M cell reaction could be diluted out while still retaining a substantial titer against N or MN cells. The anti-N reagents were more likely to show dosage effects with heterozygous vs homozygous cells than the anti-M ones. Reagents which show marked dosage effects can be very handy for red cell typing. MN typing of red cells requires care and attention, and an evaluation of the antisera.

Table 17. Reactivities of Representative Anti-M and Anti-N Reagents

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

Some years ago, there was some discussion in the literature about "heterozygous advantage" for the MN type. This thinking arose from an examination of some then available population frequency studies in which there were significantly more MN people than were to be expected on the basis of Hardy-Weinberg genetic equilibrium assumptions. Wiener (1962) stated flatly that he thought this notion was nonsense, and that the population data failed to fit the equilibrium model because substantial numbers of bloods had simply been mistyped. Indeed, the incorrect typing of M bloods as MN could explain the discrepancies. His own data on MN frequencies in New York City fit the genetic model well.

Antisera for MN typing have to be carefully checked, and their characteristics determined. In most cases, manufacturer's instructions specify the typing technique for which the reagent is intended (and with which it may be expected to give specific reactions). Deviations from the procedure must be carefully controlled to avoid the possibility of false reactions.

Because of the difficulty of predicting, or trying to control for, possible cross reactions with any given anti-N with any given example of type M cells or stain, bloodstain typing with anti-N—even when carefully controlled and carried out with thoroughly evaluated antisera—may not always be reliable. Although antiserum evaluation is essential for any reagent that is to be used for stain typing, the answer to this particular problem is not to be found in antiserum selection and evaluation. There does not seem to be any problem in determining M antigen in bloodstains, provided that suitable reagents, controls and procedures are used.

We have evaluated antisera first with different red cells, sometimes using several different cell typing techniques. The next step is to select those which seem to have the desired properties, and test them with "fresh" bloodstains. Those which give good results can then be used to test older and older stains. Table 18 shows the results of such an evaluation with selected anti-M and anti-N reagents.

Table 18. MN Typing in Bloodstains With Selected Antisera

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

¶Anti-M - titer (score) with cells of type: M 256 (88); MN 128 (76); N 0 (0)
*Anti-N - titer (score) with cells of type: M 2 (13); MN 128 (59); N 256 (94)

Anti-M does not react falsely with N stains. The anti-N used for illustration in Table 18 was cross-reactive with M cells, and did react with the fresh M stain. The cross-reaction disappeared as the stain aged. It should not be concluded, however, that the disappearance of cross-reactivity always occurs because stains reach a certain age. In addition, because elution techniques are sensitive, and adjusted to detect very small quantities of antibody in eluates, an anti-N could react with an M stain in an elution test even though it had not shown a reaction with M cells.

The M antigen was readily detectable in 20 week old stains of types M and MN, and can be detected in stains of more than twice that age in some cases. This result is in general agreement with the results obtained by the Aerospace group (Denault *et al.*, 1978, 1980). Truly reliable N antigen typing in bloodstains will require additional study, and perhaps the use of modified procedures.

VI. Rh System

A. The Rh Blood Group System

The antigen Rh_o (or D) was first reported in 1939 by Levine and Stetson, but it was not named. The woman in whose serum the antibody was found had been through two pregnancies, and had received a unit of blood from her husband. Landsteiner and Wiener (1940, 1941) found that rabbits immunized with Rhesus monkey red cells made an antibody which agglutinated many human cells, and was not related to ABO, MN or P. The human antigen being detected was called "Rh". In 1940, Wiener and Peters reported several cases of hemolytic transfusion reactions in which studies with the rabbit anti-Rh showed that the new Rh blood factor was the culprit. Many years later, it was realized that the antigens being detected by the Rhesus monkey cell immunized rabbit serum, and the human serum, were not the same. The human immune serum detects Rh_o or D, while the rabbit serum detects an antigen that is today called "LW" in honor of its discoverers. The "Rh" nomenclature was firmly entrenched, however, and was retained. After the finding of the first Rh antigen, it became clear that there were five common antigens that belonged to the Rh system. Two nomenclatures for Rh antigens developed because of disagreements over the mode of inheritance of Rh. There is a one-to-one correspondence between the systems, however, and typing and phenotype classification results are the same no matter which nomenclature is used. The nomenclature and mode of inheritance controversy was long and complex, and is beyond the scope of this brief discussion (see Race and Sanger, 1975; Issitt, 1979; Gaensslen, 1983).

According to the Fisher-Race model, there are six Rh genes, called *D*, *d*, *C*, *c*, *E* and *e*, located at three very closely linked loci, and thus inherited as haplotypes.

Each gene is responsible for a red cell antigen, having the same designation as the gene, except *d* which is silent. It is also common at the present time to designate genes in italic type and their corresponding antigens in roman type. There are eight haplotypes altogether, and a genotype is a combination of two haplotypes.

According to Wiener's model, there are eight genes (designated by italic type), all allelic at a single Rh locus. Each gene codes for an "agglutinogen" on the red cell surface (designated by roman type), and the agglutinogens have two or three different antigenic receptors (called "blood factors", and designated by boldface type). A genotype is the combination of any two genes. The blood factors of Wiener correspond to the antigens of Fisher and Race. The two schemes are shown in Table 19.

Table 19. Rh System Genes and Gene Products

Gene	[Gene Complex]	Agglutinogen	Blood Factors	[Antigens]	Reaction with Anti- Rh_o [D]	rh' [C]	rh" [E]	hr' [c]	hr" [e]
R^o	*cDe*	Rh_o	Rh_o,hr',hr"	c,D,e	+	-	-	+	+
R^1	*CDe*	Rh_1	Rh_o,rh',hr"	C,D,e	+	+	-	-	+
R^2	*cDE*	Rh_2	Rh_o,rh",hr"	c,D,E	+	-	+	+	-
R^z	*CDE*	Rh_z	Rh_o,rh',rh"	C,D,E	+	+	+	-	-
r	*cde*	rh	hr', hr"	c,e	-	-	-	+	+
r'	*Cde*	rh'	rh',hr"	C,e	-	+	-	-	+
r''	*cdE*	rh"	rh",hr'	c,E	-	-	+	+	-
r^y	*CdE*	rh_y	rh',rh"	C,E	-	+	+	-	-

Fisher-Race nomenclature indicated by [ ]

Rh phenotypes, their usual designations, and the genotypes responsible for them, are shown in Table 20. Designations using an upper case "R" are now often employed for "Rh+" types, i.e., those which are Rh_o (or D) positive, while those with a lower case "r" are used for "Rh-" types, i.e., those which are Rh_o (or D) negative. Several different genotypes can be responsible for the Rh+ phenotypes.

Table 20. Rh Genotypes and Phenotypes

Phenotype	Usual Designation	Genotypes	[Genotypes]	Reactions with anti- Rh$_o$ [D]	rh' [C]	rh" [E]	hr' [c]	hr" [e]
Rh$_o$	R$_o$R$_o$ (R$_o$r)	R^oR^o, R^or	*cDe/cDe, cDe/cde*	+	-	-	+	+
Rh$_1$rh	R$_1$r (R$_1$R$_o$, R$_o$r')	R^1r, R^1R^o, R^or'	*CDe/cde, CDe/cDe, cDe/Cde*	+	+	-	+	+
Rh$_1$Rh$_1$	R$_1$R$_1$ (R$_1$r')	R^1R^1, R^1r'	*CDe/CDe, CDe/Cde*	+	+	-	-	+
Rh$_2$rh	R$_2$r (R$_2$R$_o$, R$_o$r")	R^2r, R^2R^o, $R^or"$	*cDE/cde, cDE/cDe, cDe/cdE*	+	-	+	+	+
Rh$_2$Rh$_2$	R$_2$R$_2$ (R$_2$r")	R^2R^2, $R^2r"$	*cDE/cDE, cDE/cdE*	+	-	+	+	-
Rh$_1$Rh$_2$	R$_1$R$_2$ (R$_1$r", R$_2$r'	R^1R^2, $R^1r"$, R^2r'	*CDe/cDE, CDe/cdE, cDE/Cde*	+	+	+	+	+
Rh$_z$rh	R$_z$r, R$_z$R$_o$, R$_o$r^y)	R^zr, R^zR^o, R^or^y	*CDE/cde, CDE/cDe, cDe/CdE*					
Rh$_z$Rh$_1$	R$_z$R$_1$ (R$_z$r', R$_1$r^y)	R^zR^1, R^zr', R^1r^y	*CDE/CDe, CDE/Cde, CDe/CdE*	+	+	+	-	+
Rh$_z$Rh$_2$	R$_z$R$_2$ (R$_z$r", R$_2$r^y)	R^zR^2, $R^zr"$, R^2r^y	*CDE/cDE, CDE/cdE, cDE/CdE*	+	+	+	+	-
Rh$_z$Rh$_z$	R$_z$R$_z$ (R$_z$r^y)	R^zR^z, R^zr^y	*CDE/CDE, CDE/CdE*	+	+	+	-	-
rh	rr	*rr*	*cde/cde*	-	-	-	+	+
rh'rh	r'r	*r'r*	*Cde/cde*	-	+	-	+	+
rh'rh'	r'r'	*r'r'*	*Cde/Cde*	-	+	-	-	+
rh"rh	r"r	*r"r*	*cdE/cde*	-	-	+	+	+
rh"rh"	r"r"	*r"r"*	*cdE/cdE*	-	-	+	+	-
rh'rh"	r'r"	*r'r"*	*Cde/cdE*	-	+	+	+	+
rh$_y$rh	r$_y$r	r^yr	*CdE/cde*					
rh$_y$rh"	r$_y$r"	$r^yr"$	*CdE/cdE*	-	+	+	+	-
rh$_y$rh'	r$_y$r'	r^yr'	*CdE/Cde*	-	+	+	-	+
rh$_y$rh$_y$	r$_y$r$_y$	r^yr^y	*CdE/CdE*	-	+	+	-	-

Fisher-Race nomenclature indicated by []

In 1946, Callender and Race found an antiserum detecting a variant kind of C antigen, which was called C^W. The C^W gene behaves as an allele of *C* and *c* in thr Fisher-Race scheme. If C^W is included, therefore, the number of Rh types in Table 20 can be expanded. Over the years, a number of antisera have been found which appear to define additional Rh antigens. Some of them act like complexes of the basic antigens, while others have more complicated properties. Neither of the original nomenclature systems has been able to absorb all of these complexities, and Rh is obviously more involved than it once appeared to be. Further details on the Rh system may be found in reviews and specialized works (Race and Sanger, 1975; Issitt, 1979; Gaensslen, 1983).

In 1962, Rosenfield *et al.* proposed a numerical nomenclature for the Rh system. This scheme was designed to be descriptive of the types and reactions, without any prejudice one way or the other about the mode of inheritance. As new complexities became apparent, they were absorbed into the numerical system, and in some of the more recent cases the numerical designations have no counterparts in either Fisher-Race or Wiener nomenclature. By 1972, when Allen and Rosenfield reviewed the Rh system and terminology, there were 33 numbered antigens. In 1978, 35 specificities had been numbered (Gaensslen, 1983), and at the present time there are 41 (Svoboda, van West and Grumet, 1981). Mr. P.D. Issitt in Florida has apparently agreed to take on the task of keeping track of the numbers (see Rosenfield *et al.*, 1979). The numerical nomenclature scheme is shown in Table 21.

B. Development of Rh Grouping in Bloodstains

Prior to 1960, Rh antigen typing in bloodstains was carried out using inhibition techniques (Closon, 1954; Ducos, 1954; and see Gaensslen, 1983). Elution methods were applied to Rh antigen typing shortly after they had been found to be reliably applicable to ABO and MN antigens. They are much more sensitive than inhibition. Preliminary studies on the typing of D by absorption-elution were published by Nickolls and Pereira (1962). A more complete study was done by Bargagna and Pereira (1967). Various examples of both complete and incomplete antisera were tested for the detection of the five common Rh antigens as well as C^W in stains of many different phenotypes. The reactions of incomplete antisera were detected in different experiments by the use of high protein media, enzyme treated test cells, and AHG technique. The results were generally good, and the reactions with the antisera found to be specific. False positive reactions were seen with some anti-c reagents, but these could be eliminated by antiserum dilution or by employing Coombs technique.

Table 21. Rh Numerical Nomenclature

Antigen	Wiener Equivalent	Fisher-Race Equivalent	Antigen	Wiener Equivalent		Fisher-Race Equivalent
Rh 1	Rh_o	D	Rh 22	---		cis-CE
Rh 2	rh'	C	Rh 23	---		D^W(Wiel)
Rh 3	rh''	E	Rh 24	---		E^T
Rh 4	hr'	c	Rh 25	---	LW	---
Rh 5	hr''	e	Rh 26	---	Deal	"c-like"
Rh 6	hr	f; cis-ce	Rh 27	---		cis-cE
Rh 7	rh_i; hr_i	cis-Ce	Rh 28	hr^H		---
Rh 8	rh^{w1}	C^W	Rh 29	---	"total Rh"	---
Rh 9	rh^x	C^x	Rh 30	Rh_o^{Cor}	Go^a	D^{Cor}
Rh 10	hr^v	V; ce^s	Rh 31	hr^B		---
Rh 11	rh^{w2}	E^W	Rh 32	product of $\overline{R}^N$		---
Rh 12	rh^G	G	Rh 33		R^oHar	
Rh 13	Rh^A		Rh 34		Bas.	
Rh 14	Rh^B		Rh 35		FBC	(C)D(e)
Rh 15	Rh^C		Rh 36		Be^a	
Rh 16	Rh^D		Rh 37		Evans	
Rh 17	Hr_o		Rh 38	---		---
Rh 18	Hr		Rh 39	---		---
Rh 19	hr^s		Rh 40		Targett	
Rh 20		VS; e^s	Rh 41	---		---
Rh 21		C^G				

Designations in between columns have no equivalents in Fisher-Race or Wiener nomenclatures

Some incomplete anti-e reagents failed to react with stains containing the corresponding antigen. These investigators said that they had not reached a definite conclusion about whether complete or incomplete typing antisera were to be preferred. In 1968, Lincoln and Dodd reported specific and successful typing of the five common Rh antigens and C^W using carefully selected high titered antisera and papain treated test cells for the detection of eluted antibodies. Bloodstains up to 4 weeks old could be typed for all the antigens, and C, D and c could be detected in 4-6 month old bloodstains. Some difficulties were encountered with E and e in the older stains, attributable primarily to the difficulty in finding suitable antisera of these specificities. Most of the problems encountered in the earlier studies could be solved by careful selection and evaluation of typing antisera based on the principles involved in absorption-elution and application of the most appropriate serological techniques (Lincoln and Dodd, 1973; Lincoln and Dodd, 1978; McDowall, Lincoln and Dodd, 1978a; and see also § IV).

Autoanalyzer methods have been used for the typing of Rh antigens in stains by various workers. Some autoanalyzer techniques are more sensitive than some manual ones, and the increased sensitivity is desirable in attempting to overcome problems caused by the smaller numbers of antigenic sites on the red cell surface, particularly in cases like E and e in older bloodstains. In addition, autoanalyzer techniques might save examiner time in busy laboratories equipped to carry them out. Douglas and Staveley (1969) reported an autoanalyzer procedure for the Rh antigens D and E, which gave good results in artificially prepared bloodstains up to about a month old. Pereira, in Culliford (1971), gave a detailed description of autoanalyzer techniques for typing all the Rh antigens in bloodstains. Brewer, Cropp and Sharman (1976) described an autoanalyzer technique which was found to be completely satisfactory for all the Rh antigens in stains up to about a month old. The reactions of some of the antisera decreased in stains older than that. Studies have also been carried out comparing manual and autoanalyzer techniques (Lincoln, 1973; Martin, Rand and Pereira, 1975; McDowall, Lincoln and Dodd, 1978b). Manual techniques that are suitably sensitive for detecting small amounts of eluted antibody compare very favorably with autoanalyzer methods, and may actually be preferable under certain circumstances (McDowall, Lincoln and Dodd, 1978b).

Martin (1977) has described a reliable manual method for typing all the Rh antigens with carefully selected incomplete antisera.

Papain treated cells were employed for the detection of the eluted antibodies. Individual bloodstained threads were employed for testing, these being affixed to a polycarbonate support to facilitate the absorption and washing steps as originally described for ABO and MN typing (Howard and Martin, 1969; and see § I.B). The threads were cut from the supporting sheet and transferred to tubes prior to the elution and detection steps. Bargagna, Sabelli and Giacomelli (1982) have described a completely satisfactory technique for Rh typing in bloodstains using papain treated test cells in combination with LISS. With some prior evaluation, many commercially available typing sera were found to be suitable for bloodstain typing using these techniques.

C. Evaluation of Rh Antisera

1. General Procedure

Our studies were concerned with evaluating the applicability of commercially obtained antisera to bloodstain antigen typing. Most laboratories have no direct access to single donor sera, and must rely on commercial sources. Although the principles involved in evaluating antisera from these different sources are essentially the same, some commercially obtained reagents have different serological properties than freshly obtained single donor sera of the same specificity.

Antisera are generally evaluated in three stages. First, a newly obtained reagent is titrated against red cells from people who are homozygous and heterozygous for the corresponding antigen. Titrations must be carried out using all the different serological techniques that will be (or may be) used to detect antibody in eluates from bloodstains. Likewise, the serological conditions should be similar to those which will be employed in the detection stage of the elution procedure. The cell suspension concentrations, for example, may have a marked effect on the titer obtained (see § IV.C.2). Second, those antisera found to have appropriate specificity and sufficiently high titer for bloodstain antigen typing are tested in the absorption-elution procedure with fresh blood stains. Eluates are titrated to obtain a measure of antibody yield. Finally, those reagents which show appropriate specificity and which give suitably high antibody yields in the eluates from fresh bloodstains may be tested with older bloodstains of known phenotypic composition. Some antisera which detect the corresponding antigen in comparatively fresh stains will fail to do so in older ones.

It is very important to include appropriate negative control red cells and blood stains in the evaluation experiments. Anti-D reagents, for example, should be tested not only with R_1R_1 or R_1r cells and stains, but also with R_o (or R_or) or R_2 (or R_2r) cells or stains to detect any anti-C that may be present, in addition to testing them with rr cells and stains. It is good general procedure to test antisera of a given specificity with both cells and stains which are homozygous and heterozygous for the corresponding antigen, in addition to cells and stains which are negative for it. Carefully selected negative controls can be used to test for unexpected antibodies in an antiserum of a particular nominal specificity. Anti-C reagents may also contain antibodies of other specificities, and require careful evaluation (see below in § VI.C.2). If antisera are to be stored, they must be checked to see whether and to what extent reactivity has been changed by the storage conditions.

2. Some Special Considerations with Anti-D and Anti-C

There are some special problems in connection with the evaluation of anti-D and anti-C reagents. These have to do with the potential problems created by anti-G in anti-C or anti-D sera, and of the anti-C^W and/or anti-rh_i content of anti-C sera.

Since the work of Allen and Tippett (1958), it has become clear that the majority of C+ and/or D+ cells are likewise G+, and that most C- and/or D- cells are G-. Further, a significant number of anti-CD and anti-CDE sera contain anti-G (Allen and Tippett, 1958; Issitt, 1979; Issitt and Tessell, 1981). Anti-CD or anti-CDE sera are not ordinarily employed in Rh stain typing, since single specificity reagents are preferred. It is not too clear from the studies in the literature to what extent one might expect to find anti-G in anti-C or anti-D reagents, particularly the commercially obtained ones. The presence of anti-G could give rise to problems, since the majority of C+ and/or D+ cells also contain G. Accordingly, it is important to employ appropriate negative bloodstain controls in stain typing. R_2R_2 stains as negative controls for anti-C and r'r' (or r'r) stains as negative controls for anti-D would reveal any unexpected specificity in the antiserum, although one might not be able to distinguish whether anti-G was involved on this basis alone. An R_2R_2 stain showing a reaction with an anti-C, for example, would indicate that the anti-C contained an unexpected antibody, but one could not tell whether it was anti-D or anti-G. Similarly, an anti-D reaction with an r'r' (or r'r) stain would indicate anti-C or anti-G contaminants.

If R_1R_2 test cells were used, one would be unable to distinguish between them; if R_2R_2 test cells were used, however, anti-G would be implicated as the interfering antibody. We evaluated a number of different anti-D reagents with r'r cells by direct typing and with r'r stains by absorption-elution, using papain technique, and found no evidence of any reactions. Similarly, anti-C reagents were tested with R_2R_2 or R_2r cells and stains, and only one showed an unexpected antibody (see in Table 27). It appears, therefore, that unexpected antibodies were not present in most of the anti-D and anti-C reagents used in this study. This problem should be considered, however, in evaluating Rh antisera, and appropriate negative control tests performed with them.

Another consideration is the specificity of anti-C reagents. The many compexities surrounding the C antigen and anti-C reagents that have come to light as the Rh system has been more fully studied are beyond the scope of this brief discussion. Issitt (1979) has covered the subject in detail. In an assessment of eighteen different commercial anti-C typing sera, Svoboda, van West and Grumet (1981) found that all reacted strongly with C^W+c+C- red cells. In addition, ten sera failed to react with R_zR_2 cells, although the reagents reacted strongly with CCee cells. All these antisera contained anti-C^W, therefore, and ten of the eighteen contained anti-rh_i with no apparent anti-C. Anti-rh_i (anti-Ce: anti-Rh7) was described by Rosenfield and Haber (1958). Cells from people who have C and e on the same gene complex (i.e. who are cis-Ce) ordinarily contain rh_i. The anti-C content of an "anti-C" typing serum in contrast to its anti-rh_i content can be assessed by comparing the reactions of the antiserum with cis-Ce-containing cells (r'r', r'r, R_1R_1, R_1r, and some others) and cells which have C but not a cis-e (R_z and r^y types lacking R_1 and r'). Issitt and Tessell (1981) found that all six commercial anti-C reagents which they tested reacted with r^yr^y cells, although all contained substantial titers of anti-rh_i (titers and scores were higher with r'r' cells than with r^yr^y cells). They said that false results would not be expected from these reagents with cis-CE bloods. Anti-C sera which contain anti-rh_i to the exclusion of anti-C could cause problems in stain typing if an R_z- or r^y-containing stain were being tested. The cells required to assess the anti-rh_i (as against the anti-C) content of these reagents are quite rare, however, and difficult to obtain. The anti-C^W content of "anti-C" sera mentioned above can be assessed using C^W+C- cells, but these too are quite rare. A separate anti-C^W can be used in stain grouping tests to detect the C^W antigen if it is present. If one were using an anti-C which was exclusively anti-rh_i + anti-C^W in specificity, then a C antigen could be missed in an R_z or r^y stain that lacked R_1 or r'.

If a stain did contain C^W, the antigen should be detected with anti-C^W. If both anti-C and anti-C^W reacted with a stain, and the anti-C^W content of the anti-C were unknown, one might not be able to distinguish between a C^WC^W and a C^WC stain. If these complexities are kept in mind, however, and stain typing results are carefully and conservatively interpreted, few difficulties should be encountered.

3. Titrations of Commercial Rh Antisera under Different Serological Conditions

In the present study, 41 different Rh antisera were tested for reactivity with red cells under several different serological conditions. Among these were 11 anti-D, 8 anti-C, 8 anti-E, 7 anti-c, and 7 anti-e. They were obtained from a number of different commercial sources. The initial titrations provided a basis for judging the best serological conditions to be used in bloodstain typing and for possible subsequent correlations with the stain typing results. We did not have access to fresh bloods having the C^W types from which to make stains and for use as test cells, and did not, therefore, evaluate anti-C^W reagents. The results of the titrations of these reagents in media containing 0.5% albumin in saline, 1:10 dilute AB serum in saline, LISS, and with papain-treated cells are shown in Tables 22 through 26.

Table 22. Titration of Representative Commercial Anti-D

Manufacturer	Type	Cell Phenotype	Titer (Score) With Albumin	AB Serum	LISS	Papain
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)

Manufacturer	Type	Cell Phenotype	Titer (Score) With Albumin	AB Serum	LISS	Papain
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)	n.t.	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 23. Titration of Representative Commercial Anti-C

Manufacturer	Type	Cell Phenotype	Titer (Score) With Albumin	AB Serum	LISS	Papain
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	mod. 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)

Table 24. Titration of Representative Commercial Anti-E

Manufacturer	Type	Cell Phenotype	Titer (Score) With Albumin	AB Serum	LISS	Papain
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)	n.t.	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	mod. 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)	n.t.	128(72)
		R_2r	16(46)	32(48)		128(60)

Manufacturer	Type	Cell Phenotype	Titer (Score) With Albumin	AB Serum	LISS	Papain
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)

Table 25. Titration of Representative Commercial Anti-c

Manufacturer	Type	Cell Phenotype	Titer (Score) With Albumin	AB Serum	LISS	Papain
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)	n.t.	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)

Table 26. Titration of Representative Commercial Anti-e

Manufacturer	Type	Cell Phenotype	Titer (Score) With Albumin	AB Serum	LISS	Papain
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)	n.t.	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)

4. Bloodstain Typing with Commercial Rh Antisera

Following determination of titers with cells, Rh antisera were tested with bloodstains freshly prepared (one to several days old) on cotton cloth using absorption-elution procedure and papain treated test cells. R_1R_2 test cells can be used for all the antisera. In each case, bloodstains of several different Rh type bloods were used, and eluates were titrated routinely. The results of these tests are given in Table 27.

Table 27. 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

Specificity	Manufacturer/Type	Titer(Score) of Eluate from Stains of Phenotype R_1R_2	R_1R_1	R_1r	R_2r	rr
Anti-C	Ortho/saline	128(55)	n.t.	64(50)	4(10)	0
	Ortho/Novaserum	128(71)	n.t.	256(82)	0	0
	Molter/saline	64(54)	n.t.	128(63)	0	0
	Molter/high-protein	128(79)	n.t.	256(80)	0	0
	Dade/mod..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

Specificity	Manufacturer/Type	Titer(Score) of Eluate from Stains of Phenotype R_2R_2	R_1R_2	R_2r	R_1r
Anti-E	Ortho/Novaserum	128(59)	128(60)	128(54)	0
	Molter/saline	n.t.	128(62)	128(61)	0
	Molter/high-protein	128(58)	128(67)	256(75)	0

Specificity	Manufacturer/Type	Titer(Score) of Eluate from Stains of Phenotype R_2R_2	R_1R_2	R_2r	R_1r
Anti-E	Dade/saline	64(51)	128(43)	256(56)	0
	Dade/mod. IgG	n.t.	64(43)	64(38)	0
	BCA/high-protein	128(58)	128(56)	128(67)	0
	BCA/saline	n.t.	64(60)	128(58)	0
	Hyland/high-protein	n.t.	128(55)	128(68)	0
		Titer(Score) of Eluate from Stains of Phenotype 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
		Titer(Score) of Eluate from Stains of Phenotype 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

Selected examples of antisera of the five different specificities were next employed to type aging bloodstains at periodic intervals. The stains were prepared from whole blood of various phenotypes on cotton cloth, and were aged at room temperature and ordinary humidities. Papain treated test cells were employed in the detection stage, and tests were routinely carried out on 2-3 threads of about 0.5 cm lengths. The majority of the test stains were aged to 26 weeks; a few bloodstains one or more years old which were available were tested as well. The results are summarized in Table 28.

Table 28. Rh Typing of Aging Experimental Bloodstains

Specificity	Type	Papain Titer(Score) with Red Cells	Stain Phenotype	Papain Titer (Score) of Eluate from Bloodstain* Aged as Indicated ←weeks→ 1	2	4	6	8	12	16	26	←years→ 1.5	3	4	5
Anti-D	Novaserum	R_1R_2 512(86) R_1r 256(79)	R_1R_2 R_1r R_2r	256(75) 256(66) 256(64)	128(51) 64(47) 128(51)	16(36) 16(31) 16(33)		16(34) 16(31) 16(39)	16(22) 8(25) 8(23)	8(23) 4(15) 4(20)	8(23) 4(23) 8(26)	2(10)	1(5)	1(7)	1(5)
	AHG†	R_1R_1 4000(100) R_1r 4000(100)	R_1R_2 R_1r R_2r		128(53) 256(54) 512(74)	64(44) 32(45) 64(45)		16(40) 8(26) 8(33)	8(25) 16(36) 16(33)	16(34) 8(30) 8(32)	8(23) 8(25) 8(28)	2(13)	1(7)	2(10)	2(10)
	high-prot	R_1R_1 512(94) R_1r 512(89)	R_1R_2 R_1r R_2r	256(68) 128(53) 256(63)	256(56) 32(37) 128(55)	64(48) 64(49) 64(53)		16(33) 16(30) 16(33)	8(25) 8(23) 2(15)	2(10) 8(26) 2(12)	8(20) 4(20) 4(20)	4(15)	2(10)	1(7)	1(7)
	saline	R_1R_1 1000(111) R_1r 512(95)	R_1R_2 R_1r R_2r	128(55) 64(48) 128(55)	64(41) 64(40) 64(38)	32(43) 64(44) 64(48)		8(25) 8(31) 16(34)	4(20) 8(28) 8(23)	8(23) 8(23) 4(15)	4(18). 4(17) 4(18)	2(10)	1(9)	2(10)	1(5)
	saline	R_1R_1 512(89) R_1r 256(85)	R_1R_2 R_1r R_2r	64(51) 64(48) 64(53)				4(15) 2(12) 4(17)			1(5) 0 2(15)				
Anti-C	saline	R_1R_1 1000(118) R_1r 512(103)	R_1R_1 R_1R_2 R_1r	128(53) 64(46) 128(56)		32(43) 16(38) 32(45)	32(36) 16(25) 16(28)	16(36) 8(33) 8(28)	8(22) 4(26) 8(23)		8(23) 4(17) 4(18)		1(5)		1(5)
	high-prot	R_1R_1 2000(92) R_1r 1000(90)	R_1R_1 R_1R_2 R_1r	128(56) 128(56) 128(61)		32(47) 64(51) 64(57)	32(38) 32(38) 16(28)	16(45) 16(31) 8(30)	4(20) 2(12) 8(23)		8(25) 8(20) 4(20)		0(4)	1(7)	0(2)
	mod. IgG	R_1R_1 1000(107) R_1r 512(99)	R_1R_1 R_1R_2 R_1r	256(63) 64(59) 128(53)		64(46) 64(46) 64(44)	32(36) 32(30) 32(28)	16(43) 16(36) 16(43)	8(23) 8(22) 4(20)		8(26) 4(18) 4(18)		2(10)	1(7)	1(5)
	high-prot	R_1R_1 512(105) R_1r 512(95)	R_1R_1 R_1R_2 R_1r	128(63) 128(56) 128(68)		64(45) 128(46) 32(45)	32(43) 16(28) 16(31)	32(50) 8(30) 8(35)	4(20) 2(12) 8(23)		8(25) 4(20) 8(25)		2(10)	2(13)	0(2)
	saline	R_1R_1 128(86) R_1r 128(85)	R_1R_1 R_1r	128(55) 64(50)							1(5) 1(5)				

Table 28. Rh Typing of Aging Experimental Bloodstains

Specificity	Type	Papain Titer(Score) with Red Cells	Stain Phenotype	Papain Titer (Score) of Eluate from Bloodstain* Aged as Indicated											
				←weeks→								←years→			
				1	2	4	6	8	12	16	26	1.5	3	4	5
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)		1(5)	1(7)	1(5)
		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)	2(10)			
	AHG†	R_1R_1 4000(100)	R_1R_2		128(53)	64(44)		16(40)	8(25)	16(34)	8(23)		1(7)	2(10)	2(10)
		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)	2(13)			
	high-prot	R_1R_1 512(94)	R_1R_2	256(68)	256(56)	64(48)		16(33)	8(25)	2(10)	8(20)		2(10)	1(7)	1(7)
		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)	4(15)			
	saline	R_1R_1 1000(111)	R_1R_2	128(55)	64(41)	32(43)		8(25)	4(20)	8(23)	4(18)		1(9)	2(10)	1(5)
		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)	2(10)			
	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)		1(5)		1(5)
			R_1r	128(56)		32(45)	16(28)	8(28)	8(23)		4(18)				
	high-prot	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)		0(4)	1(7)	0(2)
			R_1r	128(61)		64(57)	16(28)	8(30)	8(23)		4(20)				
	mod. 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)		2(10)	1(7)	1(5)
			R_1r	128(53)		64(44)	32(28)	16(43)	4(20)		4(18)				
	high-prot	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)		2(10)	2(13)	0(2)
			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)				

Specificity	Type	Papain Titer(Score) with Red Cells		Stain Phenotype	Papain Titer(Score) of Eluate from Bloodstain* Aged as Indicated											
					←weeks→								←years→			
					1	2	4	6	8	12	16	26	1.5	3	4	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)	0(2)			
				R_1R_2	64(41)		16(25)		8(28)	2(13)		4(15)		1(9)	1(9)	0(2)
	high-prot	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)	0			
				R_1R_2	64(48)		32(47)		8(35)	4(17)		2(10)		2(10)	2(13)	1(5)
	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)	0			
				R_1R_2	8(26)		4(15)		2(13)	2(12)		2(10)		1(7)	2(10)	1(7)
	high-prot	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-prot	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)		1(7)		
		R_1R_2	512(97)	R_2r	128(63)		16(38)	16(38)	16(45)	4(15)		2(15)	0(2)			
				R_1R_2	128(51)		16(34)	8(30)	8(30)	4(15)		2(10)		0	1(5)	1(5)
	saline	rr	2000(102)	rr	128(66)		64(62)	32(51)	16(41)	8(30)		8(33)		2(12)		
		R_1R_2	512(87)	R_2r	64(56)		64(58)	32(46)	16(36)	8(28)		8(33)	2(10)			
				R_1R_2	32(55)		32(44)	16(34)	4(25)	4(15)		4(20)		1(7)	2(13)	1(5)
	high-prot	rr	2000(104)	rr	64(56)		64(67)	32(48)	16(45)			8(28)		1(5)		
		R_1R_2	2000(104)	R_2r	32(47)		64(65)	16(36)	16(43)			4(25)	2(13)			
				R_1R_2	32(51)		32(46)	32(41)	16(38)			4(23)		1(5)	0	1(5)
	high-prot	rr	512(94)	rr	256(76)		32(55)	32(48)	8(37)	4(23)		4(23)		0(2)		
		R_1R_2	256(77)	R_2r	256(75)		16(40)	16(36)	8(37)	4(18)		4(20)	1(5)			
				R_1R_2	128(68)		16(38)	8(26)	8(31)	2(12)		4(15)		0(2)	0	0
	high-prot	rr	512(106)	rr	512(92)				2(12)		0					
		R_1R_2	512(106)	R_2r	128(77)				2(10)		2(12)					
				R_1R_2	128(68)				2(10)		0					

Specificity Anti-e	Type	Papain Titer(Score) with Red Cells	Stain Phenotype	Papain Titer (Score) of Eluate from Bloodstain* Aged as Indicated											
				←weeks→								←years→			
				1	2	4	6	8	12	16	26	1.5	3	4	5
	saline	rr 512(93)	rr	128(61)		32(48)	8(23)		4(20)		2(18)		1(5)		
		R_1R_2 512(90)	R_2r	64(54)		16(31)	4(20)		4(15)		2(12)	1(5)			
			R_1R_2	64(46)		16(28)	2(12)		4(20)		4(18)		0	0	0
	Novaserum	rr 512(95)	rr	128(61)		32(45)	16(33)	4(25)	8(28)						
		R_1R_2 512(86)	R_2r	128(62)		8(25)	16(28)	4(18)	4(18)		4(18)		1(5)		
			R_1R_2	64(51)		16(28)	4(15)	2(15)	8(23)		4(18)	1(5)			
											2(10)		0(2)	0	0
	high-prot	rr 256(85)	rr	64(54)		32(44)	8(31)	16(35)	8(28)						
		R_1R_2 128(75)	R_2r	64(51)		8(31)	8(20)	4(28)	4(17)		4(21)		1(7)		
			R_1R_2	64(46)		16(38)	8(26)	8(30)	8(28)		2(15)	1(5)			
											2(13)		1(5)	0	0
	high-prot	rr 256(92)	rr	64(60)							1(7)				
		R_1R_2 256(86)	R_2r	64(59)							0				
			R_1R_2	128(70)							0				

*Same stain followed in "weeks" series; different stains tested in "years" series

†Antiserum undiluted in absorption stage; antisera adjusted to a papain titer of 256-512 (or used neat if undiluted titer was lower) in all other tests

The results in Table 28 indicate that Rh antigens can be readily detected in room-temperature aged bloodstains on cotton cloth up to 26 weeks with many of the antisera tested. In a few cases, antigens could be satisfactorily detected in bloodstains a year or more old. There is a tendency for eluate titers and scores from bloodstains made from bloods homozygous for the corresponding antigen to be slightly higher than those from bloodstains prepared from heterozygous bloods. The majority of antisera which showed relatively high papain titers with red cells, and which yielded relatively high titered eluates from fresh or week-old stains, yielded detectable antibody from 26 week old stains. With a few antisera, the antibody yield was low or nil in eluates from 26 week old bloodstains, and this behavior seemed to be more a function of the individual antiserum than of any particular specificity or type.

These results are generally in accord with other studies on the applicability of antisera (including commercial antisera) to Rh typing in bloodstains, such as the work of Lincoln and Dodd (1968), Martin (1977), McDowall, Lincoln and Dodd (1978a) and Bargagna, Sabelli and Giacomelli (1982). Martin(1977) could detect all the antigens present in six month old stains using procedures similar to those in the present studies, except that eluates were not titrated. He noticed that c and D were more readily detectable in year old bloodstains than C, E and e. These differences were not as apparent in our data, although the number of stains examined was relatively small. Bargagna, Sabelli and Giacomelli (1982) showed that D, C and c antigens were regularly detectable in stains up to six months old, the E antigen in stains up to 4 months old, and the e antigen in stains up to two months old, using papain technique. The use of LISS in conjunction with papain enabled detection of the antigens in still older stains, and the LISS enhancement was particularly noticeable in the older stains. McDowall, Lincoln and Dodd (1978a) had previously shown that the use of LISS increased sensitivity of elution tests for detecting blood group antigens in stains, and Lincoln and Dodd (1978) had shown that LISS could be productively combined with papain technique in enhancing eluted antibody detectability.

A limited number of experiments were conducted in the present studies on the detectability of Rh antigens in bloodstains in LISS and AB serum media. Papain treated cells were not used in these studies, the results of which are shown in Table 29.

Table 29. AB Serum and LISS Enhancement Effects with Rh Antisera

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

In general, AB serum and LISS media enhancement did not offer much improvement in antibody recovery in eluates as compared with papain technique, and in some cases the papain procedure gave better recoveries. With antisera that showed significantly better cell titers in AB serum than in albumin, results with stain eluates were as good as or better than those using papain technique. Similarly, eluate antibody recoveries were better in LISS with antisera which showed LISS enhancement with cells relative to albumin.

Denault *et al.* (1978 and 1980) detected C antigen in 26 week old stains, D, c and E in 13 week old stains, and e in 2 week old stains, all made on cotton cloth and kept at room temperature and normal relative humidity. Some of the antigens were not detectable as long in stains kept at higher relative humidity. These studies were carried out exclusively in saline-albumin media with little prior evaluation of the Rh antisera, and using cell concentrations somewhat higher than those in the present studies. Surprisingly, it was said that no significant improvements in detectability were observed using enzyme techniques.

Maeda *et al.* (1979 and 1980) reported that all the Rh antigens except e could be detected in stains on cotton cloth aged at room temperature up to 42 weeks. D antigen could occasionally be detected in 24 month old stains, while in 15-20 month old ones, the other three antigens were difficult to detect as a rule. The bloodstains in these studies were prepared from packed red cells, however, and not from whole blood, and it is not clear what differences this practice might cause in antigen detectability and survival studies (see also §VI.C.6).

While it does not seem possible to predict with any certainty the age of a bloodstain at which Rh antigens are no longer detectable, the data from this and other studies suggest that Rh antigens are frequently detectable in six to twelve month old stains that are in reasonably good condition, and occasionally in even older ones. The ability to detect Rh antigens in older stains is improved by careful prior evaluation and selection of antisera, and by the use of papain techniques. In some cases and with some antisera, the use of LISS or AB serum media enhancement techniques is also helpful in improving antibody detectability.

5. Detection of Antigens in Bloodstains on Different Substrata

The detection of the antigens D, C and e in relatively fresh bloodstains on a variety of different substrata was investigated because of the fact that bloodstains encountered in casework may occur on almost any material or object.

Bloodstains on some substrata can be subjected to elution tests directly, while other substrata require that the dried blood be transferred to cotton threads for the procedure. A number of the stains in the substratum study were tested both directly and after transfer to a cotton thread. Transfer is accomplished using a minimal quantity of saline, or saline-impregnated cotton threads. The threads are allowed to dry completely prior to elution testing. Papain treated R_1R_2 test cells were employed in all these studies. Elution from one or two 2 cm long cotton threads was carried out in 50 µL saline, and eluates were not titrated in these experiments. The results are shown in Table 30.

Generally, the Rh antigens for which stains were tested were detected on most of the different substrata. Denim, suede and Kodel polyester were noteworthy exceptions. With some nylon fibers, direct testing was unsatisfactory, but the antigens could be detected if the dried blood was transferred to cotton cloth. These results are generally in accord with those of Denault *et al.* (1978 and 1980), except that those workers reported considerably better success with denim substrata.

6. False Results and Stain Typing Interpretation

For a variety of different reasons having to do with the age of a bloodstain, environmental influences to which it has been subjected, the antiserum being employed, the technique used, and the serological skill and experience of the examiner, an antigen actually present in a stain may not be detected in an elution test.

Table 30. Detectability of Rh Antigens D, C and e in Bloodstains on Different Substrata

Substratum	Method[1]	Stain Phenotype	Agglutination Result in Eluate D	C	e
Wood	transfer	R_2r	+	-	+
		R_1r	+	+	+
		R_1R_2	+	+	+
		rr	-	-	+
Plastic (polyethylene)	transfer	R_2r	+	-	+
		R_1r	+	+	+
Synthetic rubber (shoe sole)	transfer	R_2r	2+	-	2+
		R_1r	2+	2+	2+
Leaf (dead, dry)	transfer	R_1r	2+	2+	2+
		R_2r	2+	-	2+
		R_1r	2+	2+	+
Denim, unwashed	direct[2]	R_2r	±	-	-
		R_1r	±	±	-
	direct[3]	R_2r	±	-	±
		R_1r	±	±	±
Denim, washed	direct[4]	R_2r	-	-	-
		R_1r	-	-	-
70% Acrylic, 30% Wool	direct	R_2r	+	-	+
		R_1r	+	+	+
65% Polyester, 35% Cotton	direct	R_2r	+	-	+
		R_1r	+	+	+
72% Rayon, 28% Cotton	direct	R_2r	3+	-	+
		R_1r	2+	2+	2+
		R_1R_2	2+	2+	+
Cotton (terry cloth) unwashed	direct	R_2r	+	-	+
		R_1r	+	+	+
Cotton (terry cloth) washed	direct	R_2r	+	-	+
		R_1r	+	+	+
Khaki	direct	R_2r	+	-	+
		R_1r	+	+	+
Polyester (Kodel)	direct	R_2r	-	-	-
		R_1r	-	-	-

Substratum	Method[1]	Stain Phenotype	Agglutination Result in Eluate D	C	e
Linen	direct	R_2r	3+	-	2+
		R_1r	3+	3+	3+
		R_1R_2	3+	2+	2+
Wool	direct	R_2r	2+	-	2+
		R_1r	2+	2+	2+
		R_1R_2	2+	2+	2+
	transfer	R_2r	2+	-	2+
		R_1r	3+	2+	2+
		R_1R_2	2+	2+	2+
Nylon (woven)	direct[5]	R_2r	+	-	±
		R_1r	+	±	±
		R_1R_2	±	±	±
Nylon, pressed (Pellon)		R_2r	+	-	+
		R_1r	2+	2+	+
		R_1R_2	2+	2+	+
Nylon carpet	direct	R_2r	-	-	-
		R_1R_2	-	-	-
	transfer	R_2r	2+	-	2+
		R_1R_2	2+	2+	2+
Nylon, woven with Scotchguard	direct	R_2r	-	-	-
		R_1r	-	-	-
		R_1R_2	-	-	-
	transfer	R_2r	+	-	+
		R_1r	+	+	+
		R_1R_2	+	+	+
Rayon	direct	R_2r	+	-	+
		R_1r	+	2+	+
		R_1R_2	2+	2+	+
Rayon, with "Zepel"	direct	R_2r	-	-	-
		R_1r	-	-	-
		R_1R_2	±	-	-
	transfer	R_2r	2+	-	+
		R_1r	2+	+	+
		R_1R_2	2+	+	+
Silk	direct	R_2r	+	-	+
		R_1r	+	+	+
		R_1R_2	+	+	+
Suede, shoe	transfer	R_2r	-	-	-
		R_1r	-	-	-
		R_1R_2	-	-	-

Substratum	Method[1]	Phenotype	Agglutination Result in Eluate D	C	e
Suede, belt	transfer	R_2r	-	-	-
		R_1r	±	-	-
		R_1R_2	±	-	-
Cotton, corduroy	direct	R_2r	+	-	+
		R_1r	+	+	+
Cotton, muslin	direct	R_2r	+	-	+
		R_1r	+	+	+
Leaf, green	transfer	R_2r	2+	-	+
		R_1r	2+	2+	2+
Cinderblock, unpainted	transfer	R_2r	3+	-	2+
		R_1R_2	2+	2+	2+
Cinderblock, latex paint	transfer	R_2r	3+	-	3+
		R_1R_2	3+	3+	3+
Teflon	transfer	R_2r	2+	-	2+
		R_1R_2	3+	2+	2+
Wax coated paper cup	transfer	R_2r	2+	-	2+
		R_1R_2	2+	2+	2+
Ceramic tile	transfer	R_2r	2+	-	2+
		R_1R_2	3+	2+	2+
Linoleum	transfer	R_2r	2+	-	2+
		R_1R_2	3+	3+	3+

[1] transfer or direct testing; [2] blue threads; [3] white threads
[4] blue or white threads; [5] 1 cm^2 pieces; 1 cm x 1 mm pieces gave identical results

These false negative results should not cause any difficulties in practice, provided it is recognized that a negative result can have two different interpretations: (1) the antigen was not present in the blood that formed the stain; or (2) the antigen was present in the blood that formed the stain, but it was not detected in the test for some reason. It is not possible to distinguish between the two possibilities by serological methods. Thus, only antigens that are detected in stains can be regarded as informative. The most informative exclusionary results are obtained when an antigen can be demonstrated in a bloodstain, but is absent in fresh blood from a suspected depositer. Likewise, inclusionary results can be informative and some discrimination in the population is achieved, even in cases where a limited number of antigens has been demonstrated in the bloodstain.

There are very few reports of false positive Rh typing results in bloodstains which could not be explained readily by such factors as incomplete washing. Absorbing antiserum that is too high titered can cause problems , and this matter was discussed in §§ IV.A.1 and IV.C.3. Some special problems with unexpected antibodies in antisera of certain nominal specificities were discussed in §VI.C.2. Incomplete washing should be apparent from substratum and/or negative control samples in properly conducted tests. No unexplained false positive results were seen in the present studies with room temperature aged bloodstains. The usual cause of such observations is incomplete washing, and the difference between incompletely washed and truly positive samples is ordinarily very evident when eluates are titrated. Denault *et al.* (1978 and 1980) said that false positive results were seen with several Rh antigens in bloodstains deposited on nylon, wool and perma-press substrata. In 1982, Maeda, Nagano and Tsuji reported some studies on antigen typing in heated bloodstains. Stains heated to 140° for 30 min lost Rh antigen activity except for c, which was destroyed by heating for 1 hr at 140°. Of greater concern, however, was their finding that bloodstains heated to 160° for 30 min or more gave false positive D, c and e results, and that those heated to 180° for 1hr gave false positive E results. The reason for this behavior is unclear, although it may be significant that the bloodstains were prepared from packed red cells and not from whole blood.

VII. Ss, Kell, Duffy and Kidd Antigens

A. The Ss Antigens

The S and s antigens are controlled by an allelic pair of genes closely linked to the *MN* locus. The relationship was discussed in §V.A. Antisera to S and s, however, are often incomplete and Coombs-reactive, similar in this respect to Kell, Duffy and Kidd antisera. The S and s are considered in this section for that reason. The Ss antigens may be considered separately from MN as the expression of a simple codominant Mendelian pair of alleles (Table 31).

Table 31. The Ss System

Genotype/Phenotype	Reaction of Red Cells With Anti-S	Anti-s
SS	+	-
Ss	+	+
ss	-	+

In U.S. populations, phenotypic distributions of SS, Ss and ss are about 10%, 42% and 47&, respectively, among Whites, and about 6%, 24% and 70%, respectively, among Blacks (Gaensslen, 1983).

B. Kell, Duffy and Kidd Blood Group Systems

1. Kell System

In 1946, Coombs, Mourant and Race reported on a number of cases of maternal-fetal incompatibility, one of which had been caused by a previously undescribed antibody formed by the mother in response to a blood group antigen inherited by the child from its father. This antigen was called "Kell" (Race, 1946). The same antigen was independently discovered by Wiener and Sonn Gordon (1947) and was initially called "Si". Wiener agreed to the name Kell once the identity between the two was established (Wiener, Brancato and Waltman, 1953). Kell was inherited, and it was predicted that an antibody to the product of its allelic partner would be found. In 1949, Levine *et al.* found the expected antibody, and called its corresponding antigen "Cellano". Kell (K) and Cellano (k) were shown to be allelic, giving rise to three genotypes and phenotypes, KK, Kk and kk.

Since these initial studies, a number of other antigens have been shown to be part of the Kell system.

The Kp^a antigen (Allen, 1956; Allen and Lewis, 1957) and the Kp^b antigen (Allen, Lewis and Fudenberg, 1958) are controlled by a second allelic pair of genes. They were originally referred to as "Penney" and "Rautenberg", respectively. Another antigen discovered by Callender and Race (1946) and called "Levay" has recently been shown to be controlled by an allele of Kp^a and Kp^b (Gavin *et al.*, 1979; Yamaguchi *et al.*, 1979); it is now called Kp^c. The antigen called Js^a was found by Giblett (1958), and more fully described by Giblett and Chase (1959). Greenwalt *et al.* (1962) and Walker *et al.* (1963) found the antigen Js^b, and the Js antigens were considered to make up a new blood group system called "Sutter". In 1965, however, Stroup *et al.* showed that the Sutter antigens belonged to the Kell complex locus. There are now more than 20 different antibodies defining antigens at the Kell locus. Following the suggestion of Allen and Rosenfield (1961), Kell antigens have been assigned numbers for some years. The established allelic loci within the Kell complex locus are: *Kk*, $Kp^aKp^bKp^c$, Js^aJs^b and *K11K17*. For most routine blood grouping tests, only antisera to K and k are employed, and these are the only antigens that have received attention in the bloodstain grouping literature. Kell is not one of the more informative systems, since more than 90% of Whites and about 98% of Blacks are *kk*.

2. Duffy System

The antigen now called Fy^a was found in 1950 (Cutbush and Mollison, 1950; Cutbush, Mollison and Parkin, 1950), and it was predicted that an antigen corresponding to the allele of Fy^a would be found. Ikin *et al.* (1951) found anti-Fy^b in a serum in Berlin, and a more detailed report was given by Blumenthal and Pettenkofer (1952). Accordingly, the system consisted of a pair of codominant alleles giving rise to three phenotypes: Fy(a+b+), Fy(a-b+) and Fy(a+b-). In 1955, Sanger, Race and Jack found that almost 70% of a small sample of New York Blacks were Fy(a-b-). This common phenotype in the Black population has been attributed to homozygosity for a silent allele, *Fy*. Several other Duffy antigens have since been found, and these have been given numerical designations: Fy3 (Albrey *et al.*, 1971); Fy4 (Behzad *et al.*, 1973); and Fy5 (Colledge, Pezzulich and Marsh, 1973). Chown, Lewis and Kaita (1965) have described an allele Fy^x the product of which reacts weakly with selected examples of anti-Fy^b. If Black people with the common Fy(a-b-) phenotype are actually homozygous for a gene which makes another Duffy antigen, no antisera defining it have as yet been found. Table 32 summarizes the Duffy system.

Table 32. Duffy System

Phenotype	Genotype(s)	Reaction of Red Cells With Anti-Fy^a	Reaction of Red Cells With Anti-Fy^b	Approximate Occurrence in U.S. Populations (%) White	Approximate Occurrence in U.S. Populations (%) Black
Fy(a+b+)	Fy^aFy^b	+	+	45	5
Fy(a-b+)	Fy^bFy^b Fy^bFy	-	+	32 ---	21
Fy(a+b-)	Fy^aFy^a Fy^aFy	+	-	22 ---	14
Fy(a-b-)	$FyFy$	-	-	rare	60

3. Kidd System

The antigen now called Jk^a was described by Allen, Diamond and Niedziela (1951), and the existence of Jk^b was predicted. The latter was found by Plaut *et al.* (1953). The major phenotypes are Jk(a+b+), Jk(a-b+) and Jk(a+b-), and they occur in approximately 50%, 22% and 28% U.S. Whites, and 36%, 8% and 56% U.S. Blacks, respectively. A pair of codominant alleles, Jk^a and Jk^b, accounts for the genetics of Kidd. A Jk(a-b-) phenotype has been described by Day, Perkins and Sams (1965), and was seen in Orientals and Asians. Antisera to Jk^a and Jk^b are somewhat more difficult to obtain than others that have been discussed, and perhaps for this reason only a few laboratories have studied Kidd antigen grouping in bloodstains.

C. Ss, Kell, Duffy and Kidd Antigen Typing in Bloodstains

Successful determination of the S antigen in bloodstains was first reported by Lincoln and Dodd (1968) using a selected anti-S and Coombs technique. The antigen could be detected in stains up to six months old. In 1975, Lincoln and Dodd showed that the s antigen could likewise be detected in stains, in this case up to 7 months old. More recently, McDowall, Lincoln and Dodd (1978a and 1978b) have shown that S could be detected in stains over a year old, that LISS was useful in enhancing the reactions of anti-S, and that the manual procedure was more satisfactory with older stains than autoanalyzer ones.

The K antigen was typed in a medicolegal case by Jones and Diamond (1955) using an inhibition technique. Lincoln and Dodd (1975) showed that K was readily determinable by elution technique in stains up to 7 months old by Coombs technique.

Two of the anti-K were significantly more reactive with the older stains than the third example. They showed further that quantities of bloodstain which gave fully satisfactory elution results did not significantly inhibit the anti-K sera. McDowall, Lincoln and Dodd (1978b) compared manual and autoanalyzer techniques for the typing of K in bloodstains. Completely satisfactory results were obtained manually, but the autoanalyzer failed to detect the anti-K in the eluates reliably. Burke and Tumosa (1978) employed the elution procedure of Lincoln and Dodd (1975) to detect K in a 4 year old bloodstain, and the results were satisfactory.

The Fy^a antigen of the Duffy system was first detected in bloodstains with an inhibition technique by Ruffié and Ducos (1957). Both Fy^a and Fy^b could be determined in stains by elution tests using Coombs technique (Lincoln and Dodd, 1975). The results were completely convincing with bloodstains of all three phenotypes. Burke and Tumosa (1978) could detect Fy^a in a 4 year old bloodstain using the elution procedure devised by Lincoln and Dodd (1975). The only report in the literature concerning the detection of Jk^a in bloodstains is that of Lincoln and Dodd (1975). They noted that both Fy^b and Jk^a were detected in quantities of bloodstain that did not give significant inhibition of the test antisera. In addition, it was said that Jk^b typing would be desirable in conjunction with Jk^a typing, but that suitable anti-Jk^b was very difficult to obtain.

D. Evaluation of Ss, Kell, Duffy and Kidd Antisera

1. General Procedure

The procedures followed in evaluating these antisera were essentially the same as those used for the Rh antisera (§VI.C.1). Antisera were titrated under various serological conditions with red cells homozygous and heterozygous for the corresponding antigens. The antisera were then used to test fresh bloodstains made from homozygous and heterozygous bloods by elution procedure, antibody yield being estimated by titration of the eluates. Finally, selected examples of the antisera were employed to follow antibody yields in eluates following application of the elution procedure to a series of aging bloodstains. Included in these tests were 4 anti-S, 5 anti-s, 5 anti-K, 3 anti-k, 4 anti-Fy^a, 2 anti-Fy^b and 4 anti-Jk^a. Suitable examples of anti-Jk^b could not be obtained.

With the exception of two anti-S reagents, all the antisera in this series of tests were Coombs reactive. Some testing was carried out in AB serum and in LISS media as well as in saline-albumin.

CONTINUED

1 OF 2

Elution tests with Coombs reactive antisera involve an additional step in the detection stage. Ordinarily, 2-3 threads of bloodstained material 0.5-0.8 cm in length are incubated with neat antiserum for 17 hrs at 37°. The titers of these antisera do not ordinarily exceed 256 even in enhancing media, and dilution is accordingly unnecessary (§IV.A.1). The threads are washed 6 times with ice-cold saline, allowing 15 min between washes. Elution is then carried out in about 50 µL of saline-albumin (or enhancing medium), and approximately 100 µL of 0.5% test cells are immediately added and the threads removed. The tubes are then incubated at 37° for 30-45 min to achieve sensitization. The contents of the tubes are next washed three times in saline and transferred to the wells of Boerner slides. A drop of appropriately diluted AHG serum is added to each well, and slides are gently rocked until being read microscopically for agglutination.

2. Anti-human Globulin Sera

Anti-human globulin sera was obtained from commercial sources along with the other grouping reagents. Since it is employed extensively in Ss, Kell, Duffy and Kidd antigen typing, it must be titrated as well, and a suitable dilution determined for use with sensitized red cells. The results of titrations of several examples of AHG sera are shown in Table 33.

Table 33. Titrations of Representative Coombs Sera

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

The first four rows of Table 33 show the effect of strong, moderate and weak sensitization of antigen-containing test cells on a single example of AHG serum. Coombs sera ordinarily have titers in the 128-256 range with approximately 0.5% suspensions of Dade sensitized cells.

For routine testing of sensitized cells for agglutination, the AHG reagents are adjusted to the dilution giving the last 4+ reaction in the titration series (column 3 in Table 33). A review of AHG sera may be found in Petz and Garraty (1978).

3. Titrations of Grouping Antisera Under Different Serological Conditions

Titration data for antisera against Ss, Kell, Duffy and Kidd system antigens are shown in Tables 34-37. Antisera were titrated in the indicated medium using Coombs technique, except for the two anti-S which were saline reacting.

Table 34. Titration of Representative Commercial Anti-S and Anti-s

Specificity	Manufacturer	Cell Phenotype	Titer(Score) With Albumin	Titer(Score) With AB Serum	Titer(Score) With LISS
Anti-S	Molter (sal)	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)	nt	nt
		Ss	16(38)		
Anti-s	Dade	ss	128(63)	nt	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)	nt
		Ss	32(51)	64(54)	

Table 35. Titration of Representative Commercial Kell Antisera

Specificity	Manufacturer	Cell Phenotype	Titer(Score) With Albumin	AB Serum	LISS
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)

Table 36. Titration of Representative Commercial Duffy Antisera

Specificity	Manufacturer	Cell Phenotype	Titer(Score) With Albumin	AB Serum	LISS
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)	nt	128(70)
		Fy(a+b+)	32(38)		128(65)

Table 37. Titration of Representative Commercial Kidd Antisera

Specificity	Manufacturer	Cell Phenotype	Titer (Score) With Albumin	AB Serum	LISS	Papain
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)	nt	128(51)	64(46)
		Jk(a+b+)	2(10)		32(41)	32(41)

4. Bloodstain Typing With Commercial Anti-Ss, Kell, Duffy and Kidd Antisera

Antisera of all the different specificities were tested with bloodstains made from both homozygous and heterozygous bloods using the absorption-elution procedure. Eluates were titrated to give a relative measure of antibody recovery for the stains. All antisera were tested with relatively fresh bloodstains, and then selected examples of each specificity were employed to follow room-temperature aging bloodstains for up to six months. Anti-Jk^b was not used to follow aging bloodstains, because of the insufficient number of different examples of it. In a few cases, tests were carried out with bloodstains a year or more old. The results of these studies are recorded in Table 38.

Table 38. Ss, Kell, Duffy and Kidd Typing of Aging Experimental Bloodstains

Specificity	Manufacturer		Titer(Score)* With Red Cells	Stain Phenotype	Titer(Score)* of Eluate from Bloodstains Aged as Indicated												
					Fresh	1	2	4	6	8	12	24	52	3	4	5	
						←---Weeks---→								←---Years§---→			
Anti-S	Ortho		SS 32(50) Ss 32(46)	SS Ss	--- ---	8(25) 8(28)	--- ---	4(18) 8(20)	4(18) 4(20)	8(26) 4(20)	4(15) 2(12)	2(10) 1(10)	--- ---	0(2) 1(7)	0	0	
Anti-S	Molter	#	SS 256(75) Ss 128(70)	SS Ss	--- ---	128(53) 64(49)	--- ---	4(18) 8(20)	8(28) 8(22)	4(17) 8(23)	8(20) 4(17)	4(15) 2(10)	--- ---	0 0	0(2)	0	
Anti-S	Molter	† #	SS 256(76) Ss 256(76)	SS	---	---	---	32(48)	---	---	---	4(12)	0				
Anti-S	Molter	¶ #	SS 256(90) Ss 256(84)	SS	---	---	---	16(36)	---	---	---	4(25)	4(17)				
Anti-S	Dade		SS 32(51) Ss 16(38)	SS Ss	--- ---	8(23) 8(20)		NFT									
Anti-S	BCA	#	SS 64(58) Ss 32(48)	SS Ss	--- ---	64(48) 64(47)	--- ---	4(15) 8(30)		NFT							
Anti-s	Ortho		ss 32(52) Ss 16(38)	ss Ss	--- ---	16(33) 8(30)	--- ---	--- ---	--- ---	1(7) 1(5)	--- ---	0 0					
Anti-s	Dade		ss 128(63) Ss 32(43)	ss Ss	--- ---	64(48) 32(40)	--- ---	16(31) 8(25)	8(25) 4(15)	8(20) 4(17)	4(17) 2(12)	1(7) 1(7)					
Anti-s	Molter		ss 64(65) Ss 64(60)	ss Ss	--- ---	16(40) 8(33)		NFT						1(5)	0(2)	0	
Anti-s	BCA		ss 128(63) Ss 32(43)	ss Ss	--- ---	64(48) 64(46)	--- ---	16(25) 4(15)	4(20) 4(23)	8(28) 4(20)	8(20) 4(15)	2(15) 1(7)		1(5)	1(7)	0(2)	
Anti-s	BCA	†	ss 128(67) Ss 64(61)	ss	---	---	---	8(33)	---	---	---	4(20)	1(7)				
Anti-s	BCA	¶	ss 128(77) Ss 64(65)	ss	---	---	---	16(43)	---	---	---	4(17)	2(12)				
Anti-s	Hyland		ss 64(58) Ss 32(51)	ss Ss	--- ---	32(43) 16(33)		NFT									
Anti-K	Ortho		KK 64(64) Kk 8(40)	Kk	64(41)		NFT										
Anti-K	Dade		KK 64(54) Kk 32(41)	Kk	64(46)	---	---	---	---	---	---	0					

Specificity	Manufacturer		Titer(Score)* With Red Cells	Stain Phenotype	Titer(Score)* of Eluate from Bloodstains Aged as Indicated											
					Fresh	1	2	4	6	8	12	24	52	3	4	5
					←----Weeks----→									←---- Years---→		
Anti-K	Molter		KK 64(51) Kk 64(51)	Kk	128(50)	16(33)	8(35)	8(30)	8(28)	8(20)	2(12)	1(2)				
Anti-K	Molter	†	KK 64(56) Kk 32(54)	Kk	---	---	32(55)	---	---	---	---	4(15)	1(5)			
Anti-K	Molter	¶	KK 64(61) Kk 64(58)	Kk	---	---	32(41)	---	---	---	---	8(25)	1(5)			
Anti-K	BCA		KK 128(59) Kk 64(54)	Kk	64(41)		NFT									
Anti-K	Pfizer		KK 256(71) Kk 128(68)	Kk	64(50)	32(38)	8(30)	8(31)	8(23)	8(20)	2(15)	2(10)				
Anti-k	Ortho		kk 64(58) Kk 32(48)	kk Kk	128(60) 32(43)	32(40) 8(28)	8(35) 8(35)	8(28) 4(27)	8(31) 4(23)	4(21) 2(10)	4(20) 4(18)	2(10) 1(7)	---	1(7)	0(2)	1(5)
Anti-k	Ortho	†	kk 64(58) Kk 64(56)	kk Kk	---	---	---	8(31) 8(31)	---	---	---	2(15) 1(9)	2(15) 1(7)			
Anti-k	Ortho	¶	kk 64(63) Kk 64(50)	kk Kk	---	---	---	8(28) 4(26)	---	---	---	4(15) 2(10)	2(10) 1(5)			
Anti-k	Dade		kk 64(60) Kk 32(48)	kk Kk	128(55) 32(40)	32(36) 8(27)	16(35) 8(28)	8(28) 4(23)	8(30) 8(28)	4(21) 2(12)		NFT				
Anti-k	Molter		kk 64(47) Kk 32(45)	kk Kk	64(55) 16(33)		NFT									
Anti-Fy^a	Ortho		a+b- 64(60) a+b+ 32(48)	a+b- a+b+	16(35) 4(25)		NFT									
Anti-Fy^a	Dade		a+b- 64(51) a+b+ 64(49)	a+b- a+b+	16(36) 16(31)	16(35) 8(28)	8(28) 8(23)	8(23) 2(10)	8(26) 2(12)	4(26) 4(18)	4(15) 2(10)	0 0	--- ---	0		
Anti-Fy^a	Dade	†	a+b- 128(63) a+b+ 64(60)	a+b-	---	---	8(28)	4(10)	---	2(12)	---	1(7)	0			
Anti-Fy^a	Dade	¶	a+b- 128(75) a+b+ 64(65)	a+b-	---	---	16(25)	8(18)	---	2(10)	---	1(7)	0			
Anti-Fy^a	Molter		a+b- 128(69) a+b+ 64(64)	a+b- a+b+	16(35) 8(29)	---	---	---	---	---	0(2) 0					

Specificity	Manufacturer		Titer(Score)* With Red Cells	Stain Phenotype	Titer(Score)* of Eluate from Bloodstains Aged as Indicated											
					Fresh	1	2	4	6	8	12	24	52	3	4	5
									←----Weeks----→					←--Years--→		
Anti-Fy^a	BCA		a+b- 64(55) a+b+ 32(47)	a+b a+b+	32(36) 8(25)	8(32) 8(25)	16(33) 16(33)	4(20) 4(20)	4(18) 4(17)	4(23) 2(13)	2(10) 2(12)	1(7) 1(7)				
Anti-Fy^a	BCA	†	a+b- 64(58) a+b+ 32(50)	a+b-	---	---	4(20)	2(15)	---	1(7)	---	0(2)	0			
Anti-Fy^a	BCA	¶	a+b- 128(68) a+b+ 64(58)	a+b-	---	---	8(31)	4(23)	---	2(20)	---	1(7)	0(2)			
Anti-Fy^b	Molter		a-b+ 64(53) a+b+ 32(45)	a-b+ a+b+	8(30) 4(20)	8(23) 8(23)	8(25) 8(23)	4(22) 2(14)	4(23) 4(15)	4(18) 2(12)	2(12) 2(12)	1(7) 1(5)		0(2)	0(2)	
Anti-Fy^b	Dade		a-b+ 32(57) a+b+ 32(38)	a-b+ a+b+	8(23) 8(26)	8(28) 8(25)	16(36) 16(30)	4(22) 4(20)	4(20) 4(20)	--- ---	--- ---	1(7) 1(5)		0(2)	0(2)	0(2)
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		0(2)	1(5)	
Anti-Jk^a	Molter	¶	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	†	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)		---	1(5)	---
Anti-Jk^a	Pfizer	∇	a+b- 64(47) a+b+ 32(41)	a+b-	---	---	---	---	---	---	8(20)	---				

*Albumin-saline medium and AHG technique unless otherwise indicated #Saline reacting; direct testing †AB serum ¶LISS ∇Papain

NFT Not further tested §Different stains used than those in the "weeks" series

The data in Table 38 indicate that the S, s, K, k, Fy^a, Fy^b and Jk^a antigens can be detected in stains up to 6 months old with selected commercial antisera. In general, those antisera which had relatively higher titers against red cells, and which gave higher antibody yields with "fresh" or week-old stains, tended to be better for the detection of the corresponding antigen in older bloodstains. Enhancement with LISS or AB serum media was more noticeable in eluate data with older stains (3 months or more). In some cases, the antigens could be detected in older stains using LISS or AB serum where they would have been undetectable in the absence of the enhancing media. Antisera which showed this behavior tended to be those which showed increased titers and scores with cells using LISS or AB serum as compared with saline-albumin. Some of the antigens could be convincingly detected in bloodstains several years old. With certain antisera, papain enhancement is significant, as can be seen in Table 37. The titers and scores of some of these antibodies in eluates from older bloodstains may then be correspondingly better by papain technique, as with the last example of anti-Jk^a in Table 38.

The results of these studies are generally in accord with those of Lincoln and Dodd (1975) and McDowall, Lincoln and Dodd (1978a). The K and s antigens could be detected in their studies in stains from 7 to 10 months old, and S antigen could be detected in a 64 week old stain. In some older stains, convincing results were obtained using LISS media which would have been negative or ambiguous in its absence. The value of including LISS at both the absorption and detection stages was established by comparing titration scores of eluates with and without LISS. In addition, AB serum diluent was shown to be valuable in enhancing the reactivity of antibodies in eluates with selected antisera. Maeda *et al.* (1979 and 1980) found that S, s, k, and Duffy antigens could be detected in all the stains they studied up to 42 weeks old. Further, all the antigens except k were detectable in all the stains up to 2 years old. The k antigen could be detected in some two-year old stains. It must be noted again, however, that the Japanese investigators prepared their bloodstains using packed red cells. Denault *et al.* (1978 and 1980) found that s antigen was detectable in stains up to 26 weeks old, regardless of substratum or humidity. The S antigen, however, was not detected in stains older than 4 weeks. No substratum or humidity effects were noticed, and they suggested that technical problems might account for the results since other workers had been able to determine S in older stains. The Fy^a, K, and Jk^a antigens were not detected in stains older than two weeks in their studies.

The data from the present studies along with those from other investigations indicate the importance of evaluation and selection of antisera for bloodstain grouping, particularly with stains that are older than about 2-3 months. Enhancement media and techniques can be very valuable in this work, with carefully selected antisera. Differences in detectability of these blood group antigens in older stains prepared from packed cells as against those made from whole blood deserve additional study.

5. Detection of the k and Fy^a Antigens in Bloodstains on Various Substrata

The Cellano and Fy^a antigens were selected arbitrarily as representative of this group of red cell antigens to be studied for detectability in relatively fresh (weeks old) bloodstains on a variety of different substrata. Eluates were not titrated in this series of experiments. Some substrata can be sampled, and subjected to direct testing in tubes. In other cases where direct testing was not possible, the dried blood can be eluted into saline and transferred to cotton threads. The threads are then dried completely, and employed for the usual microelution tests. The results of these studies are shown in Table 39.

The k antigen was detectable on most of the substrata. Reactions were negative or weak on certain denims, some synthetics, and rayon fibers treated with a waterproofing agent. Detection of Fy^a on this series of substrata was less successful. The same materials on which k was weakly detected or undetected showed weak or negative reactions for Fy^a as well. Some materials on which k reactions were satisfactory, however, did not exhibit very convincing Fy^a detectability. A number of Fy^a reactions were weaker than the corresponding k results on the same substratum material. It is possible that these results could be explained in part by a difference in the number of k and Fy^a antigenic sites on the red cells to begin with, but there is little published data on this point. Many of the substrata on which k and Fy^a reactions were weak or negative in bloodstains were the same as those on which Rh antigen detectability was poor (Table 30). Generally, an agglutination result of 1+ (i.e. +) or greater would be regarded as a convincing result, whereas ± or w results might well not be.

These substratum results are in accord with those of Denault *et al.* (1978 and 1980). They studied the S, s, Fy^a, K and Jk^a antigens, where we looked only at k and Fy^a, and they utilized a narrower range of substrata.

Table 39. Detectability of k and Fy^a in Bloodstains* on Different Substrata

Substratum	Method¶	Agglutination Result in Eluate† k	Fy^a
Plastic (polyethylene)	transfer	1+	2+
Absorbent paper	direct	3+	3+
Washed denim	direct[1,2]	-	-
Unwashed denim	direct[1]	2+	w
	direct[2]	±	-
70% acrylic, 30% wool	direct	w	w
Cotton, corduroy	direct	2+	+
Cotton, muslin	direct	2+	+
65% polyester, 35% cotton	direct	3+	+
Washed terrycloth	direct	2+	2+
Khaki	direct	+	+
Kodel polyester	direct	-	-
Linen	direct	2+	-
Wool	direct	2+	+
	transfer	2+	2+
Pressed nylon	direct	3+	+
Woven nylon	direct	w	-
Rayon	direct	3+	+
Rayon with Zepel	direct	±	-
Silk	direct	2+	+
Suede (shoe)	transfer	+	+
Suede (belt)	transfer	2+	±
Ceramic tile	transfer	3+	+
Leather	transfer	3+	+
Teflon	transfer	3+	3+
Wax cup	transfer	3+	2+
Linoleum	transfer	2+	+

*Prepared from kk, Fy(a+b-) whole blood ¶Direct= tested on piece of substratum itself; or transfer=eluted and transferred to cotton threads for testing

†Coombs technique [1]Blue threads [2]White threads

No great differences were seen in their data on the several different textile substrata. They were able to detect s and Jk^a on denim material to the same extent as on cotton. We found that k and Fy^a were difficult to type in bloodstains on denim, just as had been found in the case of the Rh antigens. No false positive reactions were seen in our studies on this series of antigens, and Denault *et al.* (1978 and 1980) likewise reported no false positive results with these antigens.

VIII. Gm and Km Antigens

A. Gm System

1. Introduction

The Gm system is made up of a complex group of inherited antigenic determinants located on the heavy (γ) chains of human serum IgG molecules. Gm (and Km) are members of the class of genetic markers in human blood called "serum group systems". They are very different from the classical blood groups, although the antigens are ordinarily typed using serological methods; they are members of the same class of genetic markers as the polymorphic serum proteins Hp, Gc, Tf and Pi, although the antigens of the Gm and Km system cannot be typed by electrophoretic or isoelectrofocusing techniques.

In 1956, Grubb found a most unusual antibody in the sera of certain rheumatoid arthritis patients. The antibody would agglutinate Rh+ cells which had been sensitized with certain incomplete anti-D. The initial studies were expanded by Grubb and Laurell (1956). It was immediately clear that the new antibody was recognizing an antigen on the IgG anti-D with which the test cells had been sensitized. An inhibition test was devised in which the antibody could be incubated with human serum to see whether its ability to agglutinate sensitized Rh+ cells in a second step could be removed. If the serum inhibited the antibody, it was inferred to possess the corresponding antigen or "factor". The first Gm antigen was called Gm^a. Milgrom *et al.* (1956) had observed an example of an anti-Gm earlier, but had not named it, nor shown that the antigen it was detecting was inherited. It soon became clear that anti-Gm antibodies were not restricted to rheumatoid arthritis patient sera, and that the antibodies obtained from such patients differed in their serological properties from those obtained from healthy donor sera.

Grubb and Laurell (1956) showed that Gm^a was an inherited characteristic. Additional Gm factors were soon found as additional sera were tested, and appropriate anti-D found. By 1965, approximately 14 distinct Gm specificities had been defined, and about 30 factors have been described altogether.

2. Gm Nomenclature—— Assignment of Gm Factors to IgG Subclasses

By the mid-1960s, it was clear that there were four immunologically distinguishable subclasses of IgG, called IgG 1 through 4. In certain neoplastic diseases, the immunoglobulins may be synthesized in excess and the IgGs isolated from the sera of people suffering from these diseases (especially multiple myeloma) are often quite homogeneous compared with those of normal sera. Relatively large amounts of IgG of a given subclass can thus be isolated from myeloma serum.

The nomenclature of IgG subclasses had been standardized by international agreement, and analysis of the isolated IgG molecules of a particular subclass for Gm factors enabled the assignment of the Gm antigens to particular subclasses. This information was taken into consideration in the international agreements on standardization of Gm factor nomenclature (World Health Organization, 1976). Gm factors were initially designated by letters, and later by numbers. Both usages are currently acceptable. We designate Gm factors by number as a rule. In addition, the recommended designation of a Gm factor indicates the IgG subclass on which the marker resides. Thus Gm(1) is the same as Gm(a), this factor occurs on IgG1, and the formal designation is G1m(1) or G1m(a). Likewise, Gm(10) is the same as Gm(b5), and the formal designation is G3m(10) or G3m(b5) because Gm(10) is found on IgG3. There are four Gm factors on IgG1, one on IgG2, and the remainder are assigned to IgG3. A list of the Gm factors and their designations is shown in Table 40.

The genetics of the Gm system antigens has been well reviewed by van Loghem (1971). Extensive background material on the Gm (and Km) systems may be found in the reviews by Prokop and Bundschuh (1963), Natvig and Kunkel (1968 and 1973), Franklin and Fudenberg (1969), Grubb (1970), Mage *et al.* (1973), Johnson, Kohn and Steinberg (1977), Stedman and Wainwright (1979), Bargagna and Domenici (1980) and Gaensslen (1983).

Table 40. Genetic Markers of the Immunoglobulins— Gm and Km

	Chain Location	Recommended Designation Alphameric	Recommended Designation Numeric	Older or Other Designation(s) Alphameric	Older or Other Designation(s) Numeric
γ Chain Markers	IgG1	G1m(a)	G1m(1)	Gm(a)	-----
		G1m(x)	G1m(2)	Gm(x)	-----
		G1m(f)	G1m(3)	Gm(b^w), (b2), (f)	Gm(3), (4)
		G1m(z)	G1m(17)	Gm(z)	Gm(17)
	IgG2	G2m(n)	G2m(23)	Gm(n)	Gm(23)
	IgG3	G3m(b0)	G3m(11)	Gm(b^β), (b0)	Gm(11)
		G3m(b1)	G3m(5)	Gm(b), (b1), (b^γ)	Gm(5), (12)
		G3m(b3)	G3m(13)	Gm(b3), (Bet)	Gm(13), (25)
		G3m(b4)	G3m(14)	Gm(b4)	Gm(14)
		G3m(b5)	G3m(10)	Gm(b^α), (b5)	Gm(10)
		G3m(c3)	G3m(6)	Gm-like, (c), (c3)	Gm(6)
		G3m(c5)	G3m(24)	Gm-like, (c), (c5)	Gm(24)
		G3m(g)	G3m(21)	Gm(g)	Gm(21)
		G3m(s)	G3m(15)	Gm(s)	Gm(15)
		G3m(t)	G3m(16)	Gm(t)	Gm(16)
		G3m(u)	G3m(26)	Gm(Pa)	-----
		G3m(v)	G3m(27)	Gm(Ray)	-----
		-----	G3m(28)	-----	Gm(28)
γ Chain Markers whose status is unclear and/or for which reagents are no longer available				Gm(r)	Gm(7)
				Gm(e)	Gm(8)
				Gm(p)	Gm(9)
				RO2, Rouen 2	Gm(18)
				RO3, Rouen 3	Gm(19)
				San Francisco 2	Gm(20)
				Gm(y)	Gm(22)

	Chain Location	Recommended Designation Alphameric	Numeric	Older or Other Designations Alphameric	Numeric
κ Chain Markers	κ	-----	Km(1)	InV, Inv(ℓ)	Inv(1)
		-----	Km(2)	Inv(a)	Inv(2)
		-----	Km(3)	Inv(b)	Inv(3)

B. Km System

In 1961, Ropartz, Lenoir and Rivat described an inherited serum protein antigen which was detected like the Gm factors, but which could be shown not to belong to the Gm system. The factor was first called "InV" or "Inv". Subsequent studies indicated that this new marker resided on the κ light chains of immunoglobulin molecules. Two additional light chain factors have also been described (Ropartz *et al.*, 1961; Ropartz, Rivat and Rousseau, 1962; Steinberg, Wilson and Lanset, 1962). These factors are now designated Km(1), Km(2) and Km(3) by international agreement. Many of the reviews cited above contain information about Km as well as about Gm.

C. Serological Methods for Gm and Km Typing

Gm and Km antigens are usually typed by serological inhibition procedures. Some have been determined by precipitation reactions, but this method is not very common. G2m(23) could only be typed by precipitin tests originally, because an IgG anti-D possessing Gm(23) was never found; the factor has also been typed serologically using test cells to which the myeloma proteins containing Gm(23) have been coupled chemically (Natvig and Kunkel, 1967). The inhibition method has been preferred in part because precipitating typing reagents are not very widely available.

Gm or Km typing by inhibition requires a pair of reagents: the anti-Gm (or anti-Km) serum, and an IgG anti-D which possesses the corresponding antigen. The anti-D member of the pair is sometimes called the "coat". Anti-Gm sera are frequently of human origin. As noted above, the first anti-Gm was seen in the serum of a rheumatoid arthritis patient. Since then, hundreds of different anti-Gm and anti-Km antibodies have been found in the sera of rheumatoid arthritis patients as well as in the sera of healthy donors. Anti-Gm sera from rheumatoid arthritis patients are called "Raggs", while those from healthy donors are called "SNaggs". Ragg sera are generally more difficult to work with, and SNaggs are the preferred reagents for Gm typing. Commercial anti-Gm sera are SNaggs.

In an inhibition test, anti-Gm sera are incubated with the serum to be tested. After some time, the test cell system, consisting of Rh+ cells sensitized with IgG anti-D containing the corresponding antigen, is added. Agglutination of the cells indicates no inhibition, and absence of the factor in the serum tested. Lack of agglutination indicates inhibition, and the presence of the factor. Gm antisera must be titrated and evaluated for their applicability to stain grouping just like any other antisera. This and other technical factors in Gm/Km typing is discussed below (§VIII.E).

D. Gm and Km Typing in Bloodstains

Methods for determining Gm factors in bloodstains were devised in the early 1960s, beginning with the studies of Planques, Ruffié and Ducos (1961). G1m(1), G1m(2) and G3m(5) could be reliably determined by these investigators in a variety of dried bloodstains on both absorbent and nonabsorbent surfaces. Both SNagg and Ragg antisera were used, and the former preferred because they gave more consistently complete inhibition results. False positive results were not observed with bloodstains, and the Gm factors in several stains 7-10 years old could be detected. Inhibition techniques have been used by all subsequent investigators for bloodstain typing. The usefulness of Gm antigens as individualizing markers in bloodstains was widely confirmed (Fünfhausen and Sagan, 1961; Fünfhausen, Sagan and Schramm, 1962; Nielsen and Henningsen, 1962; Kobiela, 1963; Sagan and Fünfhausen, 1965; Lenoir and Muller, 1966; Budyakov, 1967). In 1962, Prokop, Krämer and Rieger described a micro-inhibition procedure for Gm typing in stains. In 1967, Merli and Ronchi showed that Km(1) could be detected in dried blood. An exhaustive study of Gm typing in bloodstains was carried out by Görtz (1969). Antisera to a number of different specificities were employed, and different parameters in the inhibition test protocol were evaluated to determine optimal conditions. Some of the findings in this study were summarized by Görtz *et al.* (1970). The distribution of Gm factors varies among different major racial groups, and Blanc, Görtz and Ducos (1971) noted that the determination of Gm antigens in bloodstains could yield information about the racial origin of a bloodstain depositer.

More recently, Khalap, Pereira and Rand (1976) described a procedure applicable to bloodstained threads. Their results were generally very satisfactory. Some nonspecific inhibition was observed with woolen substrata. Khalap and Divall (1978) noted that Gm/Km antigens, and ABO antigens, could be sequentially determined from the same piece of bloodstained thread. Kipps (1979) presented a very useful methods summary for Gm and Km typing in bloodstains.

In laboratories where several different Gm antigens can be typed, it may be possible under certain circumstances to interpret negative inhibition results for certain factors. Ordinarily, one does not know whether negative results are attributable to the absence of the antigen, or to the failure to detect it. Khalap and Divall (1979) noted the value of determining Gm(5) in stains that yielded Gm(-1,-2) results, because many of the Gm(-1,-2) stains encountered in their work were from Gm(-1,-2,5) persons. Shaler (1982) carried this logic a step further, noting that the demonstration of one Gm factor on a particular IgG subclass would make possible the interpretation of a negative finding with another factor on that same subclass. Shaler (1982) discussed two cases in which Gm typing had helped to clarify the findings. Another case, in which Gm typing was used to help determine which of the occupants of two cars involved in a crash were driving the cars, was discussed by Brocteur and Moureau (1964).

Gm typing in bloodstains is valuable in part because of the apparent extraordinary stability of the antigens. Planques, Ruffie and Ducos (1961) said that Gm factors had been determined in 7 to 10 year old bloodstains. More recently, Hoste, Brocteur and Andre (1978) determined Gm(1), Gm(10) and Km(1) in bloodstains 29 to 33 years old.

A brief but thoroughly informative and readable summary of the Gm/Km antigen systems was given by Stedman and Wainwright (1979).

E. Evaluation of Commercial Anti-Gm/Anti-Km Sera

1. Summary of Reagents Examined

Nineteen different anti-Gm antisera obtained from different sources and representing seven different Gm specificities were available for these studies. In addition, three different anti-Km(1) reagents were employed. Corresponding anti-D reagents were obtained from each different supplier along with the different anti-Gm/Km sera (Appendix I).

A summary of the different specificities and their suppliers is shown in Table 41.

Table 41. Summary of Anti-Gm/Km Reagents Tested

Supplier[¶]	G1m(1)	G1m(2)	G1m(3)	G3m(5)	G3m(10)	G3m(11)	G3m(21)	Km(1)
Molter	*	*	*	-	*	-	-	*
Behring	*	*	*	*	-	-	-	*
Fresenius	*	*	*	*	-	-	*	*
Biotest	*	*	-	-	-	*	-	-

[¶]See Appendix I *Available/Tested -Not Available for Testing

2. Titration and Determination of Optimal Reagent Concentrations

Anti-Gm sera are titrated by doubling dilutions in the usual way. The optimal concentration of the corresponding anti-D to be used for sensitization of the Rh+ test cells may be determined by preparing a series of mixtures of 50% washed red cells and anti-D in different volume ratios, incubating at 37° for 45-90 min, washing the cells three times in saline, and then testing the corresponding anti-Gm/Km serum against 0.5% suspensions of the sensitized cells in albumin-saline. Determination of both the optimal sensitizing volume ratio of anti-D to red cells, and the titer of the corresponding anti-Gm/Km reagent, can be conveniently done in one operation by a two-dimensional titration set-up as indicated in Table 42.

Table 42. Two-Dimensional Titration Scheme for Determination of Optimal Anti-Gm/Km and Corresponding Anti-D Concentrations

50% Rh+ Cells:Anti-D Volume Ratio ↓	Anti-Gm/Km Dilution→ 1	2	4	8	16	32	64	128
1:1								
1:2								
1:3								
1:4								
1:5								
1:6								

The Rh+ cells:anti-D ratio yielding the highest titer and score with the corresponding anti-Gm/Km reagent is taken as the optimal sensitizing ratio. Anti-Gm/Km titers of 32-64 were commonly obtained at the optimal red cells:anti-D volume ratio with reagents examined in these studies. Typical results for the antisera tested in this way are shown in Table 43.

Table 43. Titration of Anti-Gm/Km Sera at Different Red Cells:Anti-D Ratios

Anti-Gm/Km Reagent [Manufacturer]	Titer(Score) With 30% R_1R_1 Cells:Anti-D Volume Ratio Adjusted To					
	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)	---

The anti-Gm/Km serum dilution to be used in typing was chosen as that giving the last 3+ agglutination result with test cells prepared at the optimal sensitizing volume ratio. Weaker antisera which give 3+ agglutination only when neat, or which do not give it at any dilution, are used neat for typing.

Most suppliers of these reagents recommend a particular antiserum dilution to be employed for serum typing. This recommended dilution may correspond to that determine by the titration procedure described above, and is usually within a dilution or two if it is not identical. Nevertheless, it is preferable to titrate each reagent and determine its properties under one's own testing conditions. Some adjustment in these dilutions is sometimes required for application to bloodstain typing. Table 44 shows some representative examples of the determination of optimal anti-Gm/Km dilution.

Table 44. 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

The data presented thus far indicate that the optimal volume ratio of sensitizing anti-D to cells as well as the titer of different examples of anti-Gm/Km are variable. These properties are dependent to some degree on the actual procedure employed in the sensitization and agglutination stages of the tests. Such factors as the reactivity of the cells used for sensitization, time of sensitization, suspending medium used for sensitized cells, test cell concentration, time and temperature of incubation with anti-Gm/Km serum, and the details of the agglutination detection method as such, may all have some effect on the results obtained.

Cell reactivity is affected primarily by storage (Table 5). Likewise, storage may alter the properties of the antisera (Appendix III). In general, the same groups of cells, reagents and procedures should be used for the evaluation of reagents and for typing. Detailed techniques will vary from one laboratory to another. For the Gm/Km reagents, the general procedures followed in our laboratory consist of sensitization at predetermined optimal volume ratios of 50% R_1R_1 cell suspensions and anti-D at 37° for 45 min, followed by washing three times in saline, and resuspension of the sensitized cells in saline containing 0.5% albumin (saline-albumin). The sensitized cells are next tested with an anti-human globulin reagent (see § VII.D.2) to insure that sensitization has been achieved. Agglutination tests with anti-Gm/Km reagents are carried out by incubating equal volumes of anti-Gm/Km (diluted in saline-albumin as necessary) and 0.5% test cells for about an hour at 4°. The mixtures are then transferred to Boerner slides and placed on a rotator for about 20 min after which agglutination is read microscopically.

Rh+ cells may be sensitized for periods longer than 45 min, but little improvement in the degree of antibody binding appears to be gained by doing so. Table 45 shows the results of a time course of sensitization experiment.

Table 45. 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)

Since there are a number of different types of Rh+ cells (§ VI.A; Table 20), some attention has been given to which of them is best employed for test cells in Gm/Km procedures. Giblett (1969) correctly noted that cells with the greatest number of D binding sites were to be preferred.

Most workers have tended to recommend homozygous R_1R_1 or R_2R_2 cells, some tending to prefer the former. Table 46 shows titration results with several anti-Gm sera employing different types of Rh+ cells for sensitization with the corresponding anti-D.

Table 46. Effect of Rh Cell Phenotype on Anti-Gm Titers

Anti-G1m(1)	Titer(Score) With Sensitized Rh+ Cells of Indicated Type		
	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)

Differences were not very significant among the three types tested. Most of the tests carried out in the present studies have utilized R_1R_1 test cells. The Rh+ cells used in these procedures should obviously be of ABO group O.

Most of the anti-Gm/Km sera were tested using corresponding anti-D from the same supplier. The anti-Gm/Km and anti-D can, of course be interchanged, but the behavior of the anti-Gm/Km serum depends to some extent on that of the corresponding anti-D. Thus, evaluation is made easier by doing the tests on a particular reagent pair, and then using it consistently in subsequent typing tests. Table 47 shows the titration results of two anti-Gm(1) with test cells sensitized with two different anti-D/Gm(1) sera.

Table 47. Effect of Different Anti-D/Gm(1) on Different Anti-G1m(1) Serum Titers

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

3. Reagent Stability on Storage

There are obvious advantages to being able to store blood grouping reagents over extended periods of time, particularly if they are not expected to be used up quickly in routine tests. If reagents can be stored with little or no loss of activity, it is more economical to acquire larger quantities initially, conduct necessary evaluations, and then store them in convenient quantities. Accordingly, limited studies were carried out to test the retention of activity of Gm/Km antisera after being stored frozen at -85°, and thawed once. Since multiple freeze-thaw cycles are known to have adverse effects on a number of protein containing reagents (some experimental support for this is given in Appendix III), our practice has been to transfer reagents into small tubes in convenient quantities, and thaw the individual tubes as needed. Other studies on the stability of blood grouping reagents (Appendix III) indicate that most of them retain activity well at -85° for extended periods of time. The anti-D members of the Gm/Km typing reagent pair are not expected to be different in this respect from any other anti-D. Table 48 shows the results of tests done to determine the stability of the anti-Gm sera.

Table 48. 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)

The anti-Gm retain serological activity well through one free-thaw cycle, and are thus good candidates for cryogenic storage. The reagents tested in Table 48 were supplied initially as liquid antisera. A few examples of lyophilized anti-Gm reagents were obtained from Behring. Their activity was comparable to that of other anti-Gm reagents upon reconstitution from the dried state. We noticed, however, that these antisera lost activity fairly quickly at 4° following reconstitution. In one case, for example, an anti-G1m(1) with a titer of 64 (score 65) upon reconstitution had a titer of 16 (score 38) after 14 days at 4°.

Whether this behavior was peculiar to these reagents, or to some particular lot of them, is not clear from our limited studies. Loss of activity was even more dramatic if the lyophilized reagents were reconstituted, frozen at -85°, and then placed at 4° after one thaw. Stability of reagents should be checked under the conditions in one's own laboratory.

4. Gm/Km Typing in Bloodstains

A number of different bloodstains from individuals of previously determined Gm/Km type, deposited on cotton cloth swatches, were examined at various times following deposition of the blood using available Gm/Km reagents. All the stains were aged at room temperature. A limited number of stains a year or more old were tested. Bloodstains on cotton cloth can be tested directly by using threads for the inhibition test. Alternatively, extracts of bloodstains can be prepared, and the extracts employed in inhibition tests. The tests reported here were carried out directly on bloodstained threads. Three 1 cm threads were used routinely; occasionally 1 cm^2 portions of bloodstained material were employed. Representative results are shown in Table 49.

No great differences were noted among antisera of the same specificities, provided that they had been titrated in advance and used at proper dilutions. A dilution representing the last 3+ agglutination in a titration series, given optimally sensitized test cells, ordinarily works well. Reagents that give a 3+ only when neat, or do not give 3+ at any dilution, are used neat for typing. Positive and negative control bloodstains should be employed in each test, and the positive control stain should give complete inhibition. The control stains should be similar in age to the questioned stain. If a positive control stain gives weak inhibition, it may be possible to obtain more clear cut results with a higher antiserum dilution, with more sample, or both. In doubtful cases, the antiserum can always be fully titrated before and after incubation with the stains to reveal the extent of inhibition. A substratum control should be included in tests with questioned stains since these are inhibition procedures. In serum typing, a saline control is always included to be sure the serum being tested is negative for any anti-Gm/Km activity (Appendix II). Such a saline control should be employed in bloodstain tests as well; it is prepared by substituting saline for antiserum in the inhibition stage of the test.

Table 49. Gm/Km Typing Results in Experimental Bloodstains

Stain Phenotype	Gm/Km Typing Results[¶] in Bloodstains[*] Aged as Indicated			
	Fresh	←-------weeks-------→ 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)	

Stain Phenotype	12	26 ←-weeks-→	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)	

Stain Phenotype	1 ←---years---→	2 - 5
1,2,-3;-10		(-1,-2,-3;-10)
-1,-2,3:5,10,11,21	(-1,-2,-3;-5,-10,-11,-21)	

[¶]Results shown for specificities actually tested; the prefix "Gm" has been omitted. In many cases, a number of different stains were tested, and different examples of antisera of the same specificity were used

[*]Cotton cloth

All the Gm/Km factors present could be detected readily in bloodstains aged up to 6 months, and in all but one of the stain aged up to 11 months. In one stain 39 weeks old, the IgG1 markers were detected but the IgG3 ones were not. In another stain, all the markers present could be detected at 11 months, but not at 1 year. The ability to detect the markers by the inhibition test is a function of a number of variables, including the amount of serum in the stain to begin with, the amount of bloodstain used in the test, the test procedure, and the reagents. It has been reported (see in § VIII.D) that certain Gm factors could be detected in dried blood as old as 30 years. The most important parameter determining whether the factors present are detected in solder stains is probably the quantity of sample taken for the test. Indeed, the serum content of any bloodstain is probably the most important variable determining whether immunoglobulin markers can be detected in the quantity of stain tested. Planques, Ruffié and Ducos (1961) detected IgG1 markers in bloodstains 7 and 10 years old using 20 mg dried blood powder. Görtz (1969) was able to detect several factors in 10-15 year old dried blood in some cases. He used a fairly large quantity of dried blood for extraction, and then tested the extracts. In one case where the test failed to detect a factor known to be present, he could show that the extract contained comparatively little serum protein (and hemoglobin). Hoste, Brocteur and Andre (1978) detected Gm factors in 30 year old dried blood by extracting 250 mg dried blood powder and testing the extract. The sample used for testing of a given factor contained an extract of about 50 mg dried blood.

In this context, a limited number of experiments were carried out to determine the maximal serum dilution at which Gm factors known to be present could be detected. The results are shown in Table 50.

Table 50. Detection of Gm Factors in Serum Dilutions

Gm Factor Tested	Gm Phenotype of Serum	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	++	++	++	++	++	++

These results indicate that IgG1 markers can usually be detected in 1:1000 dilutions of serum, while 1:100 dilutions are usually the greatest in which IgG3 markers can be detected. Serum contains an average of about 12 mg/mL (range 8 to 17) IgG, of which about 70% is IgG1 and 10% is IgG3. If a drop of serum is taken to be 50 µL, it would then contain about 600 µg IgG, of which about 420 µg is IgG1 and about 60 µg is IgG3. The serum dilution experiment indicates that something on the order of 0.4-0.6 µg IgG is needed to detect the Gm factors present in fresh material. More sample may be required once the sample has dried, and perhaps still more is needed as the specimen ages. Our studies on Gm typing in bloodstains routinely utilized three 1 cm bloodstained threads, a sample probably containing on the order of 50-100 µg serum, based on the assumptions that whole blood has a specific gravity of about 1.02, that it is about 80% water, and that about half the dried residue is serum, and further on some assumptions about the area occupied by a drop of blood, and the fraction of the material taken when three threads are removed. By contrast, in reports in which dried blood many years old has been grouped for Gm antigens, much larger quantities of sample (from 400 to 1000 times as much) have been employed. Since serum contains approximately seven fold more IgG1 than IgG3, more sample would presumably be required to detect IgG3 markers than IgG1 factors, all other things being equal. It thus appears that the Gm antigens exhibit considerable intrinsic stability, but that sample quantities must be increased if the factors are to be detected in older stains. For workers who utilize extracts for Gm typing, the well known resistance of serum proteins in older stains to extraction into saline or buffer should be kept in mind.

In the aging studies on Gm antigens, we noticed that the factors present seemed to be detectable longer in stains made from drawn whole blood than in stains made directly on cloth from finger sticks. We commonly collect known bloodstains by finger stick, and it seemed possible that these stains might actually contain less serum than comparable stains made from drawn whole blood. Accordingly, three pairs of bloodstains were examined for serum content. Each pair came from a different individual. One member of the pair was obtained by finger stick, the other by application of drawn whole blood to the cloth. A radial immunodiffusion (RID) system was arranged using a high titered anti-human albumin (albumin to serve as a serum marker), and the system was calibrated using commercially available serum calibrators.

Comparable quantities of each member of the stain pair were then extracted for a week at 4°, and duplicate samples were taken for application to the calibrated RID plates. Duplicate results were averaged, and expressed in terms of µg albumin per mL extract. Results are shown in Table 51.

Table 51. Estimation of Albumin Concentration in Bloodstains from Finger Stick and Made from Drawn Whole Blood

Stain	Stain Age (m)	Fingerstick (F) or Venipuncture (V)	µg Albumin/ mL Extract
#1	14	F	20
		V	30
#2	12	F	92.5
		V	187.5
#3	10	F	170
		V	340

The results indicate that bloodstains obtained by finger stick routinely contain less serum, as estimated by albumin content, than comparable stains from the same person prepared from drawn whole blood. If older stains on cloth are wanted for Gm typing standards, therefore it would be best to prepare them from drawn whole blood. This finding may incidentally have implications for the preparation of stain standards for other serum protein markers, such as Hp, Gc and Tf, particularly if older stain standards are required.

5. Gm/Km Typing in Bloodstains on Various Substrata

A series of relatively fresh bloodstains on a variety of different substrata were tested for the detectability of Gm/Km antigens known to be present in the blood. Substrata which could be divided into threads were examined directly. Some bloodstains which formed dried crusts on certain substrata were examined directly as crusts. Finally, dried blood on some of the substrata were dissolved in saline and transferred to cotton threads for typing. None of the substrata tested showed nonspecific binding of the anti-Gm sera. Results are shown in Table 52.

Table 52. Gm/Km Typing in Bloodstains on Various Substrata

Substratum	Method*	Gm/Km Phenotype¶ of Bloodstain	Gm/Km Factors¶ Detected
Corduroy (cotton)	direct	2	2
Muslin	direct	2	2
Knit (polyester)	direct	2	2
Polyester-cotton	direct	2	2
Denim	direct	2	2
Kodel polyester	direct	2,3;10 Km 1	2,3;10 Km 1
Polyethylene	transfer	2	2
Pellon (nylon)	direct	2,3;10 Km 1	2,3;10 Km 1
Wool	direct	2;10	2;10
Linen	direct	2;10	2;10
Nylon (close weave)	direct	2;10	2;10
Glass	transfer	2;10	2;10
Glass	crust	2;10	-2;-10
Ceramic tile	transfer	2;10	2;10
Ceramic tile	crust	2;10	-2;-10
Linoleum	transfer	2;10	2;10
Linoleum	crust	2;10	-2;-10
Wax cup	transfer	2;10	2;10
Wax cup	crust	2;10	-2;-10
Nylon carpet	direct	2;10	2;10
Teflon	transfer	1;10	1;10
Rayon 100%	direct	1;10	1;10
Rayon-cotton (77:38)	direct	1;10	1;10
Wood	direct	2;10	2;10
Wood	transfer	2;10	2;10
Rayon with Zepel	direct	1;10	1;10
Nylon with Scotchguard	direct	1;10	1;10
Silk-cotton (40:60)	direct	1;10	1;10
Terry cloth (new)	direct	1;10	1;10
Terry cloth (washed)	direct	1;10	1;10

*Direct on threads; direct on crust; or transfer to cotton threads

¶Only factors tested for are shown; prefix "Gm" is omitted

While these studies are by no means exhaustive, the results indicate that Gm/Km factors are detectable in relatively fresh (up to 2 months) bloodstains on a wide variety of substrata. Failure to detect the Gm factors in the blood crusts was probably the result of using sample quantities that were too small.

The Gm/Km antigens are valuable genetic markers in bloodstains, and the principal drawback to their incorporation into laboratory routines on a wider basis is that commercial sources of reagents are not readily available in this country. This situation may already be showing signs of improvement. With monclonal antibody technology advancing rapidly, selections of reagents of many specificities may soon be available and affordable.

References

Albrey, J.A., E.E.R. Vincent, J. Hutchinson, W.L. Marsh, F.H. Allen, Jr., J. Gavin and R. Sanger. 1971. A new antibody, anti-Fy3, in the Duffy blood group system. *Vox Sang.* 20: 29-35

Allen, F.H.,Jr. 1956. A new antigen in the Kell blood group system, *in:* Proc. 6th Congr. Int. Soc. Blood Transf., Boston, Sept., 1956, *Bibliotheca Haematol.*, Fasc. 7, 106-109, S. Karger, Basel & New York (1958)

Allen, F.H., Jr., L.K. Diamond and B. Niedziela. 1951. A new blood-group antigen. *Nature* 167: 482

Allen, F.H., Jr. and S.J. Lewis. 1957. Kp^a (Penney), a new antigen in the Kell blood group system. *Vox Sang.* 2: 81-87

Allen, F.H., Jr., S.J. Lewis and H. Fudenberg. 1958. Studies of anti-Kp^b, a new antibody in the Kell blood group system. *Vox Sang.* 3: 1-13

Allen, F.H., Jr. and R.E. Rosenfield. 1961. Notation for the Kell blood group system. *Transfusion* 1: 305-307

Allen, F.H., Jr. and R.E. Rosenfield. 1972. Review of Rh serology. Eight new antigens in nine years. *Haematologia* 6: 113-120

Allen, F.H., Jr. and P.A. Tippett. 1958. A new Rh blood type which reveals the Rh antigen G. *Vox Sang.* 3: 321-330

Bargagna, M. and R. Domenici. 1980. *Genetic Markers of Human Immunoglobulins in Forensic Medicine*, Biotest-Serum-Institut GmbH, Frankfurt am Main

Bargagna, M. and M. Pereira. 1967. A study of absorption-elution as a method of identification of Rhesus antigens in dried bloodstains. *J. Forensic Sci. Soc.* 7: 123-130

Bargagna, M., M. Sabelli and C. Giacomelli. 1982. The detection of Rh antigens (D,C,c,E,e) on bloodstains by a micro-elution technique using low ionic strength solution (LISS) and papain-treated red cells. *Forensic Sci. Int.* 19: 197-203

Beckman, G. 1978. Enzyme polymorphism, *in:* D.J.H. Brock and O. Mayo (eds) *The Biochemical Genetics of Man*, 2d ed., 185-269, Academic Press, New York, London, San Francisco

Behzad, O., C.L. Lee, J. Gavin and W.L. Marsh. 1973. A new anti-erythrocyte antibody in the Duffy system: anti-Fy4. *Vox Sang.* 24: 337-342

Blake, E.T., M. Gibbons, J. Bashinski and G.F. Sensabaugh. 1982. Purification and serological studies on two lectin specificities from *Ulex europaeus* seeds: a preliminary report. Paper presented at 58th Semi-annual Meeting, Calif. Assn. of Criminalists, *J. Forensic Sci. Soc.* 22: 317 [Abstr]

Blanc, M., R. Görtz and J. Ducos. 1971. The value of Gm typing for determining the racial origin of blood stains. *J. Forensic Sci.* 16: 176-182

Blumenfeld, O.M. and A.M. Adamany. 1978. Structural polymorphism within the amino-terminal region of MM, NN and MN glycoproteins (glycophorins) of the human erythrocyte membrane. *Proc. Nat. Acad. Sci. USA* 75: 2727-2731

Blumenthal, G. and H.J. Pettenkofer. 1957. Über das neuentdecke Anti-$Duffy^b$ (Fy^b). *Z. Immunitaetsforsch. Exp. Ther.* 109: 267-273

Boorman, K.E., B.E. Dodd and P.J. Lincoln. 1977. *Blood Group Serology*, 5th ed., Churchill Livingstone, Edinburgh, London and New York

Brewer, C.A., P.L. Cropp and L.E. Sharman. 1976. A low ionic strength, hemagglutinating Autoanalyzer for Rhesus typing of dried bloodstains. *J. Forensic Sci.* 21: 811-815

Brinkmann, B. 1971. Erythrocytäre Enzympolymorphismen in der forensischen Serologie. *Z. Rechtsmed.* 69: 83-117

Brocteur, J. and P. Moureau. 1964. Un cas d'application pratique des groupes plasmatiques Gm à l'identification de taches de sang. *Ann. Med. Leg.* 44: 315-321

Budvári, R. 1963. Über eine neue Methode Blutgruppenuntersuchungen in Blutflecken mittels Agglutininelution. *Dtsch. Z. Gesamte Gerichtl. Med.* 54: 24-26

Budyakov, O.S. 1967. O vozmozhnosti primeneniya v sudebno-meditsinskoy ekspertize syvorotochnykh γ-globulinovykh faktorov Gm. *Sud. Med. Ekspert.* (Moscow) 10 (1): 41-45

Burke, R. and C.S. Tumosa. 1978. The persistence of K and Fy^a antigens in bloodstains. *Forensic Serol. News* 4(3)

Callender, S.T. and R.R. Race. 1946. A serological and genetical study of multiple antibodies formed in response to blood transfusions by a patient with lupus erythematosus diffusus. *Ann. Eugenics* 13: 102-117

Cameron, J.W. and L.K. Diamond. 1945. Chemical, clinical and immunological studies on the products of human plasma fractionation. XXIX. Serum albumin as a diluent for Rh typing reagents. *J. Clin. Invest.* 24: 793-801

Chisum, W.J. 1971. A rapid method for grouping dried bloodstains. *J. Forensic Sci. Soc.* 11: 205-206

Chown, B., M. Lewis and H. Kaita. 1965. The Duffy blood group system in Caucasians: evidence for a new allele. *Am. J. Hum. Genet.* 17: 384-389

Clausen, Johs. 1933. Über die serologischen Eigenschaften M und N und ihre Bedeutung in der Gerichtsmedizin. *Z. Rassenphysiol.* 6: 49-65

Closon, J. 1949. L'antigène Rh dans le sang desséché. *Acta Med. Leg. Social.* 2: 227-230

Colledge, K.I., M. Pezzulich and W.L. Marsh. 1973. Anti-Fy5, an antibody disclosing a probable association between the Rhesus and Duffy blood group genes. *Vox Sang.* 24: 193-199

Coombs, R.R.A., A.E. Mourant and R.R. Race. 1945. A new test for the detection of weak and "incomplete" Rh agglutinins. *Br. J. Exp. Pathol.* 26: 255-266

Coombs, R.R.A., A.E. Mourant and R.R. Race. 1946. In-vivo isosensitisation of red cells in babies with haemolytic disease. *Lancet* 1: 264-266

Culliford, B.J. 1971. *The Examination and Typing of Bloodstains in the Crime Laboratory*, U.S. Government Printing Office, Washington, D.C.

Cutbush, M. and P.L. Mollison. 1950. The Duffy blood group system. *Heredity* 4: 383-389

Cutbush, M., P.L. Mollison and D.M. Parkin. 1950. A new human blood group. *Nature* 165: 188-189

Dahr, W. 1981. Serology, genetics and chemistry of the MNSs blood group system. *Rev. Fr. Transf. Immunohematol.* 24: 85-95

Dahr, W. and G. Uhlenbruck. 1978. Structural properties of the human MN blood group antigen receptor sites. *Hoppe-Seyler's Z. Physiol. Chem.* 359: 835-843

Dahr, W., G. Uhlenbruck, E. Janssen and R. Schmalisch. 1977. Different N-terminal amino acids in the MN-glycoproteins from MM and NN erythrocytes. *Hum. Genet.* 35: 335-343

Dahr, W., G. Uhlenbruck and H. Knott. 1975. Immunochemical aspects of the MNSs-blood group system. *J. Immunogenet.* 2: 87-100

Day, D., H.A. Perkins and B. Sams. 1965. The minus-minus phenotype in the Kidd system. *Transfusion* 5: 315-319

Denault, G.C., H.H. Takimoto, Q.Y. Kwan and A. Pallos. 1978. *Detectability of Selected Genetic Markers in Dried Blood Upon Aging*, Nat. Inst. Law Enforcement Crim. Just., Law Enforcement Assistance Administration, U.S. Dept. of Justice, Washington, D.C.

Denault, G.C., H.H. Takimoto, Q.Y. Kwan and A. Pallos. 1980. Detectability of selected genetic markers in dried blood on aging. *J. Forensic Sci.* 25: 479-498

Diamond, L.K. and R.L. Denton. 1945. Rh agglutination in various media with particular reference to the value of albumin. *J. Lab. Clin. Med.* 30: 821-830

Dixon, T. and B. Epstein. 1976. Absorption elution by ammonia extraction. *Forensic Serol. News* 2 (2)

Dodd, B.E. 1972. Some recent advances in forensic serology. *Med. Sci. Law* 12: 195-199

Douglas, R. and J.M. Staveley. 1969. Rh and Kell typings of dried bloodstains. *J. Forensic Sci.* 14: 255-262

Driesen, H.H. and M. Keller. 1973. Beitrag zur Erfassung der Antigene M und N in angetrockneten Blutspuren. *Arch. Kriminol.* 151: 180-183

Ducos, J. 1954. Etude de l'absorption des anticorps anti-Rhesus par le sang sec. (Application au groupage des taches de sang). Thesis, Doctor of Medicine, Imprimerie Cleder, Toulouse

Elliot, M., E. Bossom, M.E. Dupuy and S.P. Masouredis. 1964. Effect of ionic strength on the serologic behavior of red cell isoantibodies. *Vox Sang.* 9: 396-414

Fairbanks, G., T.L. Steck and D.F.H. Wallach. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry* 10: 2606-2617

Faraone, G. 1941. Sulle cause della restituzione aspecifica delle isoagglutinine nella dimostrazione della sostanza isoagglutinabile delle macchie di sangue. *Zacchia* 5 (2 ser.): 1-20

Fiori, A., M. Marigo and P. Benciolini. 1963. Modified absorption-elution method of Siracusa for ABO and MN grouping of bloodstains. *J. Forensic Sci.* 8: 419-455 and 535-567

Fitzsimmons, J.A. and P.A. Morel. 1979. The effects of red blood cell suspending media on hemagglutination and the antiglobulin test. *Transfusion* 19: 81-85

Franklin, E.C. and H.H. Fudenberg. 1969. Genetic polymorphism of human γ-globulins. The Gm and Inv factors. *Rheumatology* 2: 62-112

Fünfhausen, G. and Z. Sagan. 1961. Über die Möglichkeitdes Nachweises der Gruppeneigenschaft Gm in Blutspuren. *Dtsch. Gesundheitswes.* 16: 2468-2469

Fünfhausen, G., Z. Sagan and H. Schramm. 1962. Über den Nachweis von Gm in Blutspuren. *Dtsch. Z. Gesamte Gerichtl. Med.* 53: 18-27

Furthmayr, H. 1978. Structural comparison of glycophorins and immunochemical analysis of genetic variants. *Nature* 271: 519-524

Gaensslen, R.E. 1983. *Sourcebook in Forensic Serology, Immunology and Biochemistry*, U.S. Government Printing Office, Washington, D.C.

Gaensslen, R.E., J.K. Bremser and S.S. DeGraw. 1981. Studies on antibody elution from ammoniacal extracts of bloodstains. Paper delivered at Annual Meeting, Northeastern Assn. Forensic Sci., Allentown, PA

Gaensslen, R.E. and F.R. Camp, Jr. 1981. Forensic serology: Analysis of bloodstains and body fluid stains, *in:* C.H. Wecht (ed.) *Forensic Sciences*, v. 2, Chpt. 29, Matthew Bender, New York

Gavin, J., G.L. Daniels, H. Yamaguchi, Y. Okubo and T. Seno. 1979. The red cell antigen once called Levay is the antigen Kp^c of the Kell system. *Vox Sang.* 36: 31-33

Giblett, E.R. 1958. Js, a 'new' blood group antigen found in Negroes. *Nature* 181: 1221-1222

Giblett, E.R. 1969. *Genetic Markers in Human Blood*, Blackwell Scientific Publs., Oxford and Edinburgh

Giblett, E.R. 1977. Genetic polymorphisms in human blood. *Ann. Rev. Genet.* 11: 13-28

Giblett, E.R. and J. Chase. 1959. Js^a, a 'new' red-cell antigen found in Negroes; evidence for an eleventh blood group system. *Br. J. Haematol.* 5: 319-326

Goldsmith, K.L.G. 1974. The effect of bovine serum albumin on red cell agglutination, *in: Enhancement of Serological Reactions*, One-day symposium held at London Hospital Medical College, Nov., 1974, Biotest-Folex, Ltd., Birmingham

Görtz, R. 1969. *La Recherche des Antigenès Gm dans les Taches de Sang pour leur Identification Biologique et Médico-légale*, Hermann, Paris

Görtz, R., M. Blanc, J.F. DeBoissezon and J. Ducos. 1970. Les causes d'erreur au cours de l'identification des facteurs Gm dans les taches de sang sec. *Arch. Belg. Med. Soc. Hyg. Med. Trav. Med. Leg.* 28: 465-472

Greenwalt, T.J., R.H. Walter, C.I. Argall, E.A. Steane, R.T. Can and T.T. Sasaki. 1962. Js^b of the Sutter blood group system, *in:* Proc. 9th Congr. Int. Soc. Blood Transf., Mexico City, 1962, *Bibliotheca Haematol.*, Fasc. 19, 235-237, S. Karger, Basel and New York (1964)

Grubb, R. 1956. Agglutination of erythrocytes coated with 'incomplete' anti-Rh by certain rheumatoid arthritis sera and some other sera. The existence of human serum groups. *Acta Pathol. Microbiol. Scand.* 39: 195-197

Grubb, R. 1970. *The Genetic Markers of Human Immunoglobulins*, Springer-Verlag, New York and Berlin

Grubb, R. and A.-B. Laurell. 1956. Hereditary serological human serum groups. *Acta Pathol. Microbiol. Scand.* 39: 390-398

Gunson, H.H. 1974. The use of enzymes in blood group serology, *in: Enhancement of Serological Reactions*, One-day Symposium at London Hospital Medical College, Nov., 1974, Biotest-Folex, Ltd., Birmingham

Harris, H. and D.A. Hopkinson. 1976. *Handbook of Enzyme Electrophoresis in Human Genetics*, North Holland, Amsterdam and Oxford, and American Elsevier, New York

Hirszfeld, L. and R. Amzel. 1932. Beitrag zur gerichtlich-medizinischen Verwertung der Blutgruppen. *Dtsch. Z. Gesamte Gerichtl. Med.* 19: 133-148

Holzer, F.J. 1931. Ein einfaches Verfahren zur Gruppenbestimmung an vertrocknetem Blut durch Agglutininbindung. *Dtsch. Z. Gesamte Gerichtl. Med.* 16: 445-458

Hořejšf, V. and J. Kocourek. 1974. Studies on phytagglutinins. XVII. Some properties of the anti-H specific phytohemagglutinin of the furze seeds *(Ulex europaeus* L.). *Biochim. Biophys. Acta* 336: 329-337

Hoste, B., J. Brocteur and A. Andre. 1978. Indefinite storage of dried Gm and Km (Inv) antigens: (Examination of bloodstains 33 years old). *Forensic Sci.* 11: 109-113

Howard, H.D. and P.D. Martin. 1969. An improved method for ABO and MN grouping of dried bloodstains using cellulose acetate sheets. *J. Forensic Sci. Soc.* 9: 28-30

Howard, P.L. 1981. Principles of antibody elution. *Transfusion* 21: 477-482

Hughes-Jones, N.C. 1974. Factors affecting the enhancement of serological reactions, *in: Enhancement of Serological Reactions*, One-day Symposium at London Hospital Medical College, Nov., 1974, Biotest-Folex Ltd., Birmingham

Hughes-Jones, N.C. 1975. Red-cell antigens, antibodies and their interactions. *Clin. Haematol.* 4: 29-43

Hughes-Jones, N.C., B. Gardner and R. Telford. 1962. The kinetics of the reaction between blood-group antibody anti-c and erythrocytes. *Biochem. J.* 85: 466-474

Hughes-Jones, N.C., B. Gardner and R. Telford. 1964. The effect of pH and ionic strength on the reaction between anti-D and erythrocytes. *Immunology* 7: 72-81

Hughes-Jones, N.C., M.J. Polley, R. Telford, B. Gardner and G. Kleinschmidt. 1964. Optimal conditions for detecting blood group antibodies by the antiglobulin test. *Vox Sang.* 9: 385-395

Ikin, E.W., A.E. Mourant, H.J. Pettenkofer and G. Blumenthal. 1951. Discovery of the expected haemagglutinin anti-Fy^b. *Nature* 168: 1077-1078

Issitt, P.D. 1979. *Serology and Genetics of the Rhesus Blood Group System*, Montgomery Scientific Publs., Cincinnati

Issitt, P.D. 1981. *The MN Blood Group System*, Montgomery Scientific Publs., Concinnati

Issitt, P.D. and C.H. Issitt. 1976. *Applied Blood Group Serology*, 2d ed., Spectra Biologicals, Divn. of Becton-Dickinson, Oxnard, CA

Issitt, P.D. and J.A. Tessel. 1981. On the incidence of antibodies to the Rh antigens G, rh_i (Ce), C and C^G in sera containing anti-CD or anti-C. *Transfusion* 21: 412-418

Johnson, W.E., P.H. Cohn and A.G. Steinberg. 1977. Population genetics of the human allotypes Gm, Inv and A2m. An analytical review. *Clin. Immunol. Immunopathol.* 7: 97-113

Jones, A.R. and L.K. Diamond. 1955. Identification of the Kell factor in dried blood stains. A case report. *J. Forensic Med.* 2: 243-246

Jørgensen, J., M. Nielsen, C.B. Nielsen and J. Nørmark. 1979a. The influence of ionic strength, albumin, and incubation time on the sensitivity of the indirect Coombs test. *Vox Sang.* 36: 186-191

Jørgensen, J., M. Nielsen, J. Laugesen and S. Stoltenberg. 1979b. The influence of ionic strength and incubation time on the sensitivity of methods using papainized red cells. *Vox Sang.* 37: 111-115

Judson, P.A. and D.J. Anstee. 1977. Comparative effect of trypsin and chymotrypsin on blood group antigens. *Med. Lab. Sci.* 34: 1-6

Khalap, S. and G.B. Divall. 1978. The sequential determination of Gm/Km and ABO blood groups from the same piece of bloodstained thread. *Forensic Sci.* 11: 41-45

Khalap, S. and G.B. Divall. 1979. Gm(5) grouping of dried bloodstains. *Med. Sci. Law* 19: 86-88

Khalap, S., M. Pereira and S. Rand. 1976. Gm and Inv grouping of bloodstains. *Med. Sci. Law* 16: 40-43

Kind, S. 1955. A modified absorption technique of determining the ABO group of bloodstains. *Vox Sang.* 5 [Old Ser.]: 15-19

Kind. S.S. 1960a. Absorption-elution grouping of dried blood smears. *Nature* 185: 397-398

Kind, S.S. 1960b. Absorption-elution grouping of dried blood-stains on fabrics. *Nature* 187: 789-790

Kind, S.S. 1962. The ABO grouping of blood stains. *J. Crim. Law, Criminol. Police Sci.* 53: 367-374

Kind, S.S. 1963. Some properties of ABH antigens considered in relation to bloodstain grouping. *J. Forensic Med.* 10: 51-56

Kind, S.S. and R.M. Cleevely. 1969. The use of ammoniacal bloodstain extracts in ABO groupings. *J. Forensic Sci. Soc.* 9: 131-134

Kipps, A.E. 1979. Gm and Km typing in forensic science--a methods monograph. *J. Forensic Sci. Soc.* 19: 27-47

Kobiela, J. 1963. Investigation of blood-stains in practice, *in: Forensic Immunology, Medicine, Pathology and Toxicology*, Rept. 3rd Int. Meeting, Int. Congr. Ser. No. 80, Excerpta Medica Foundation, Amsterdam

Landsteiner, K. 1901. Über Agglutinationserscheinungen normalen menschlichen Blutes. *Wien. Klin. Wochenschr.* 14(46): 1132-1134

Landsteiner, K. 1902. Ueber Serumagglutinine. *Muench. Med. Wochenschr.* 49(46): 1905-1908

Landsteiner, K. and N. Jagic. 1903. Ueber die Verbindungen und die Entstehung von Immunkörpern. *Muench. Med. Wochenschr.* 50(18): 764-768

Landsteiner, K. and P. Levine. 1927a. A new agglutinable factor differentiating individual human bloods. *Proc. Soc. Exp. Biol. Med.* 24: 600-602

Landsteiner, K. and P. Levine. 1927b. Further observations on individual differences of human blood. *Proc. Soc. Exp. Biol. Med.* 24: 941-942

Landsteiner, K. and P. Levine. 1928a. On individual differences in human blood. *J. Exp. Med.* 47: 757-775

Landsteiner, K. and P. Levine. 1928b. On the inheritance of agglutinogens of human blood demonstrable by immune agglutinins. *J. Exp. Med.* 48: 731-749

Landsteiner, K. and C.P. Miller. 1925. Serological studies on the blood of the primates. II. The blood groups in anthropoid apes. *J. Exp. Med.* 42: 853-862

Landsteiner, K. and M. Richter. 1903. Ueber die Verwerthbarkheit individueller Blutdifferenzen für die forensische Praxis. *Z. Medizinalbeamte* 16(3): 85-89

Landsteiner, K. and A.S. Wiener. 1940. An agglutinable factor in human blood recognized by immune sera for Rhesus blood. *Proc. Soc. Exp. Biol. Med.* 43: 223

Landsteiner, K. and A.S. Wiener. 1941. Studies on an agglutinogen (Rh) in human blood reacting with anti-Rhesus sera and with human isoantibodies. *J. Exp. Med.* 74: 309-320

Lattes, L. 1913. Sull'applicazione pratica della prova di agglutinazione per la diagnosi specifica ed individuale di sangue umano. *Arch. Antropol. Crim. Psichiatr. Med. Leg.* 34 (4 ser. 5): 310-325

Lattes, L. 1915. L'individualita del sangue umano e la sua dimostrazione medico-legale. *Arch. Antropol. Crim. Psichiatr. Med. Leg.* 36 (4 ser. 7): 422-447 and 538-554

Lattes, L. 1923. Le diagnostic individuel des taches de sang. *Ann. Med. Leg. Criminol. Police Scientif.* 3: 213-226

Lauer, A. 1933. Zur Technik der Blutfleckdiagnose nach M und N. *Dtsch. Z. Gesamte Gerichtl. Med.* 22: 86-94

Lee, H.C. 1982. Identification and grouping of bloodstains, *in:* R. Saferstein (ed.) *Forensic Science Handbook*, 267-337, Prentice-Hall, Englewood Cliffs, NJ

Lenoir, L. and P.H. Muller. 1966. Determination des groupes Gm sur taches de sang. *Ann. Med. Leg.* 46: 191-193

Levine, P., M. Becker, M. Wigod and R. Ponder. 1949. A new human hereditary blood property (Cellano) present in 99.8% of all bloods. *Science* 109: 464-466

Levine, P., A.B. Kuhmichel, M. Wigod and E. Koch. 1951. A new blood factor, s, allelis to S. *Proc. Soc. Exp. Biol. Med.* 78: 218-220

Levine, P. and R.E. Stetson. 1939. An unusual case of intragroup agglutination. *J. Am. Med. Assoc.* 113: 126-127 and see *Vox Sang.* 38: 297-300 (1980)

Lincoln, P.J. 1973. A study of elution of blood group antibodies from red cells and its useful application to forensic serology. *Lond. Hosp. Gaz., Clin. Scientif. Suppl.*, June, 1973

Lincoln, P.J. and B.E. Dodd. 1968. The detection of the Rh antigens C, C^w, c, D, E and e and the antigen S of the MNSs system in bloodstains. *Med. Sci. Law* 8: 288-295

Lincoln, P.J. and B.E. Dodd. 1973. An evaluation of factors affecting the elution of antibodies from bloodstains. *J. Forensic Sci. Soc.* 13: 37-45

Lincoln, P.J. and B.E. Dodd. 1975. The application of a micro-elution technique using anti-human globulin for the detection of the S, s, K. Fy^a and Jk^a antigens in stains. *Med. Sci. Law* 15: 94-101

Lincoln, P.J. and B.E. Dodd. 1978. The use of low ionic strength solution (LISS) in elution experiments and in combination with papain-treated cells for the titration of various antibodies, including eluted antibodies. *Vox Sang.* 34: 221-226

Lisowska, E. 1981. Biochemistry of M and N blood group specificities. *Rev. Fr. Transf. Immunohematol.* 24: 75-84

Löw, B. 1955. A practical method using papain and incomplete Rh-antibodies in routine Rh blood-grouping. *Vox Sang.* 5[O.S.]: 94-98

Löw, B. and L. Messeter. 1974. Antiglobulin test in low ionic strength salt solution for rapid antibody screening and cross matching. *Vox Sang.* 26: 53-61

Lund, H. 1941. Relation of the concentration of red blood cells to the sensitivity of the iosagglutination reaction. *Arch. Pathol.* 31: 458-466

Maeda, H., T. Nagano and T. Tsuji. 1982. Serological thermostabilities of blood group antigens on human red cells. Paper delivered at XII Congr. Int. Acad. Forensic Soc. Med., Vienna

Maeda, H., K. Nishi, S. Okazaki, T. Tsuji, N. Tanaka and T. Nagano. 1979. Activity changes of blood group antigens in dried bloodstains on standing. *J. Wakayama Med. Soc.* 30: 211-218

Maeda, H. N. Tanaka, T. Azuma and T. Nagano. 1980. Activity changes of blood group antigens in dried bloodstains on standing (II). *J. Juzen Med. Soc.* 89: 564-570

Mage, R., R. Lieberman, M. Potter and W.D. Terry. 1973. Immunoglobulin allotypes, *in:* M. Sela (ed.) *The Antigens*, v. I, 299-376, Academic Press, New York & London

Martin, P.D. 1977. A manual method for the detection of Rh antigens in dried bloodstains. *J. Forensic Sci. Soc.* 17: 139-142

Martin, P.D., S.P. Rand and M. Pereira. 1975. Comparison and evaluation of methods of detection of Rh antigens in dried bloodstains. *Forensic Sci.* 5: 150-151

Matsumoto, I. and T. Osawa. 1969. Purification and characterization of an anti-H(O) phyto-hemagglutinin of *Ulex europaeus*. *Biochim. Biophys. Acta* 194: 180-189

Matsumoto, I. and T. Osawa. 1970. Purification and characterization of a *Cystisus*-type anti-H(O) phytohemagglutinin from *Ulex europaeus* seeds. *Arch. Biochem. Biophys.* 140: 484-491

Matsumoto, I. and T. Osawa. 1972. The specific purification of various carbohydrate binding hemagglutinins. *Biochem. Biophys. Res. Comm.* 46: 1810-1815

McDowall, M.J., P.J. Lincoln and B.E. Dodd. 1978a. Increased sensitivity of tests for the detection of blood group antigens in stains using a low ionic strength medium. *Med. Sci. Law* 18: 16-23

McDowall, M.J., P.J. Lincoln and B.E. Dodd. 1978b. Observations on the use of an autoanalyzerand a manual technique for the detection of the red cell antigens C, D, E, c, K and S in bloodstains. *Forensic Sci.* 11: 155-164

Merli, S. and G.V. Ronchi. 1967. La determinazione su macchie di sangue dei fattori del sistema Inv. Indagini preliminari sull'Inv(1). *Zacchia* 42 (3 ser. 3): 249-255

Metropolitan Police Forensic Science Laboratory. 1978. *Biology Methods Manual*, Metropolitan Police, Commissioner of Police of the Metropolis, London

Milgrom, F., S. Dubiski and G. Woznizko. 1956. Human sera with 'anti-antibody'. *Vox Sang.* 1: 172-183

Moore, H.C. and P.L. Mollison. 1976. Use of a low-ionic-strength medium in manual tests for antibody detection. *Transfusion* 16: 291-296

Morton, J.A. 1962. Some observations on the action of blood-group antibodies on red cells treated with proteolytic enzymes. *Br. J. Haematol.* 8: 134-148

Morton, J.A. and M.M. Pickles. 1947. Use of trypsin in the detection of incomplete anti-Rh antibodies. *Nature* 159: 779-780

Moureau, P. 1935. Contribution à l'étude des facteurs d'individualisation du sang humain et leurs applications en médecine légale. 1. Les groupes d'isoagglutination de Landsteiner; 2. Les groupes M et N d'hémo-agglutination de Landsteiner et Levine; 3. Applications pratiques des propriétés individuelles du sang. *Rev. Belge Sci. Med.* 7: 177-233; 540-588; 589-629

MPFSL. 1978. *Biology Methods Manual* [see at Metropolitan Police.....]

Natvig, J.B. and H.G. Kunkel. 1967. Detection of genetic antigens utilizing gamma globulins coupled to red blood cells. *Nature* 215: 68-69

Natvig, J.B. and H.G. Kunkel. 1968. The genetic markers of human immunoglobulins. The Gm and Inv systems. *Ser. Haematol.* 1: 66-96

Natvig, J.B. and H.G. Kunkel. 1973. Human immunoglobulins: classes, subclasses, genetic variants and idiotypes. *Adv. Immunol.* 16: 1-60

Nickolls, L.C. and M. Pereira. 1962. A study of modern methods of grouping dried blood stains. *Med. Sci. Law* 2: 172-179

Nielsen, J.C. and K. Henningsen. 1962. Experimental studies on the determination of the Gm groups in bloodstains. *Med. Sci. Law* 3: 49-58

Outteridge, R.A. 1962a. Absorption-elution grouping of bloodstains. Modification and development. *Nature* 194: 385-386

Outteridge, R.A. 1962b. Absorption-elution method of grouping blood-stains. *Nature* 195: 818-819

Outteridge, R.A. 1963. Bloodstain grouping--elution vs. inhibition. *J. Forensic Sci. Soc.* 4: 87-90

Petz, L.D. and G. Garratty. 1978. Antiglobulin sera---past, present and future. *Transfusion* 18: 257-268

Pereira, M. 1963. The identification of MN groups in dried bloodstains. *Med. Sci. Law* 3: 268-271

Pereira, M.E.A., F. Gruezo and E.A. Kabat. 1979. Purification and characterization of lectin II from *Ulex europaeus* seeds and an immunochemical study of its combining site. *Arch. Biochem. Biophys.* 194: 511-525

Pereira, M.E.A., E.C. Kisailus, F. Gruezo and E.A. Kabat. 1978. Immunochemical studies on the combining site of the blood group H-specific lectin 1 from *Ulex europaeus* seeds. *Arch. Biochem. Biophys.* 185: 108-115

Pickles, M.M. 1946. Effect of cholera filtrate on Rh cells as demonstrated by incomplete Rh antibodies. *Nature* 158: 880

Planques, J., J. Ruffié and J. Ducos. 1961. Le système Gm en médecine légale. *Toulouse Med.* 62: 685-699

Plaut, G., E.W. Ikin, A.E. Mourant, R. Sanger and R.R. Race. 1953. A new blood-group antibody, anti-Jk^b. *Nature* 171: 431

Pollack, W., H.J. Hager, R. Reckel, D.A. Toren and H.O. Singher. 1965. A study of the forces involved in the second stage of hemagglutination. *Transfusion* 5: 158-183

Ponsold, A. 1936. Ein Absorptionsverfahren ohne Titerreduktionsbestimmung zum Nachweis der Blutimmunfaktoren M und N. *Dtsch. Z. Gesamte Gerichtl. Med.* 26: 303-310

Prokop, O. and G. Bundschuh. 1963. *Die Technik und die Bedeutung der Haptoglobine und Gm-Gruppen im Klinik und Gerichtsmedizin*, Walter deGruyter, Berlin

Prokop, O. and W. Göhler. 1976. *Die menschlichen Blutgruppen*, 4th Aufl., Gustav Fischer Verlag, Stuttgart & New York

Prokop, O., K. Krämer and A. Rieger. 1962. Ein Mikroverfahren zum Nachweis der Gm-Gruppen in Blutspuren. *Z. Aerztl. Fortbild.* 56(14): 772-775

Prokop, O. and G. Uhlenbruck. 1969. *Human Blood and Serum Groups*, Wiley Interscience, New York

Race, R.R. 1946. A summary of present knowledge of human blood-groups, with special reference to serological incompatibility as a cause of congenital disease. *Br. Med. Bull.* 4: 188-193

Race, R.R. and R. Sanger. 1975. *Blood Groups in Man*, 6th ed., Blackwell Scientific Publ., Oxford & London

Reckel, R.P. and J. Harris. 1978. The unique characteristics of covalently polymerized bovine serum albumin solutions when used as antibody detection media. *Transfusion* 18: 397-406

Ropartz, C., J. Lenoir and L. Rivat. 1961. A new inheritable property of human sera: the Inv factor. *Nature* 189: 586

Ropartz, C., L. Rivat and P.Y. Rousseau. 1962. Deux nouveaux facteurs dans les systemes hereditaires de gamma-globuline: Le Gm(e) et l'Inv (1), *in:* Proc. 9th Congr. Int. Soc. Blood Transf., Mexico City, *Bibliotheca Haematol.*, Fasc. 19, 455-458 (1964)

Ropartz, C., P.Y. Rousseau, L. Rivat and J. Lenoir. 1961. Etude génétique du facteur sérique Inv fréquence dans certaines populations. *Rev. Fr. Etud. Clin. Biol.* 6: 374-377

Rosenfield, R.E., F.H. Allen, Jr., S.N. Swisher and S. Kochwa. 1962. A review of Rh serology and presentation of a new terminology. *Transfusion* 2: 287-312

Rosenfield, R.E., F.H. Allen, S.N. Swisher and S. Kochwa. 1979. Rh nomenclature. *Transfusion* 19: 487

Rosenfield, R.E. and G.V. Haber. 1958. An Rh blood factor, rh_i (Ce), and its relationship to hr (ce). *Am. J. Hum. Genet.* 10: 474-480

Rosenfield, R.E., S.H. Shaikh, F. Innella, Z. Kaczera and S. Kochwa. 1979. Augmentation of hemagglutination by low ionic conditions. *Transfusion* 19: 499-510

Ruffié, J. and J. Ducos. 1957. Absorption des anticorps anti-Fy^a par le sang desséché. *C.R. Soc. Biol.* 151: 393-395

Sagan, Z. and G. Fünfhausen. 1965. Gm group characteristics in blood stains. *Arch. Immunol. Ther. Exp.* 13: 149-156

Sanger, R. and R.R. Race. 1947. Subdivisions of the MN blood groups in man. *Nature* 160: 505

Sanger, R. and R.R. Race. 1951. The MNSs blood group system. *Am. J. Hum, Genet.* 3: 332-343

Sanger, R., R.R. Race and J. Jack. 1955. The Duffy blood groups of New York Negroes; the phenotype Fy(a-b-). *Br. J. Haematol.* 1: 370-374

Sanger, R., R.R. Race, R.J. Walsh and C. Montgomery. 1948. An antibody which subdivides the human MN blood groups. *Heredity* 2: 131-139

Schleyer, F. 1961. Weitere Untersuchungen über den optimalen ABO-Gruppennachweis in Blutflecken auf Textilien. *Arch. Kriminol.* 127: 161-166

Schütze, H. 1921. Haemagglutination and its medico-legal bearing, with observations on the theory of isoagglutinins. *Br. J. Exp. Pathol.* 2: 26-33

Schwerd, W. 1978. Zum Beweiswert der MN-Differenzierung aus Blutspuren. *Z. Rechtsmed.* 80: 293-298

Sensabaugh, G.F. 1981. Uses of polymorphic red cell enzymes in forensic science. *Clin. Haematol.* **10**: 185-207

Shaler, R.C. 1982. Interpretation of Gm testing results: two case histories. *J. Forensic Sci.* 27: 231-235

Shaler, R.C., A.M. Hagins and C.E. Mortimer. 1978. MN determination in blood-stains--selective destruction of cross-reacting activity. *J. Forensic Sci.* 23: 570-576

Siracusa, V. 1923. La sostanza isoagglutinabile del sangue. *Arch. Antropol. Crim. Psichiatr. Med. Leg.* 43(4 ser. 14): 362-384

Stedman, R. and P. Wainwright. 1979. Gm and Km polymorphism in man, *in:* D. Tills (ed.) *Biotest Bulletin 4*, Biotest-Serum-Institut, Frankfurt a.M.

Steinberg, A.G., J. Wilson and S. Lanset. 1962. A new human gamma globulin factor determined by an allele at the Inv locus. *Vox Sang.* 7: 151-156

Stratton, F. 1953. A slide test for the detection of Rh-antibodies using papain treated red cells. *Vox Sang.* 3[O.S.]: 43-50

Stroup, M., M. MacIlroy, R. Walker and J.V. Aydelotte. 1965. Evidence that Sutter belongs to the Kell blood group system. *Transfusion* 5: 309-314

Svoboda, R.K., B. Van West and F.C. Grumet. 1981. Anti-Rh41, a new Rh antibody found in association with an abnormal expression of chromosome 1 genetic markers. *Transfusion* 21: 150-156

Sylvia, F.R. and P.L. Kirk. 1961. Specificity in blood grouping with plant lectins and the application of N-lectin to dry blood stains. *J. Forensic Med.* 8: 172-181

Therkelsen, F. 1934. Die gerichtsmedizinische Fleckenuntersuchung durch Typenbestimmung. *Dtsch. Z. Gesamte Gerichtl. Med.* 23: 29-34 and 35-39

Unger; L.J. 1951. A method for detecting Rh_o antibodies in extremely low titer. *J. Lab. Clin. Med.* 37: 825-827

van Loghem, E. 1971. Formal genetics of the immunoglobulin systems. *Ann. N.Y. Acad. Sci.* 190: 136-149

van Oss, C.J., J.F. Mohn and R.K. Cunningham. 1978. Influence of various physicochemical factors on hemagglutination. *Vox Sang.* 34: 351-361

von Decastello, A. and A. Sturli. 1902. Über die Isoagglutinine im Serum gesunder und kranker Menschen. *Muench. Med. Wochenschr.* 49(26): 1090-1095

Walker, R.H., C.I. Argall, E.A. Steane, T.T. Sasaki and T.J. Greenwalt. 1963. Anti-Js^b, the expected antithetical antibody of the Sutter blood group system. *Nature* 197: 295-296

Walsh, R.J. and C.M. Montgomery. 1947. A new human iso-agglutinin subdividing the MN blood groups. *Nature* 160: 504-505

Wiener, A.S. 1944. A new test (blocking test) for Rh sensitization. *Proc. Soc. Exp. Biol. Med.* 56: 173-174

Wiener, A.S. 1962. Blood groups and disease. A critical review. *Lancet* 1: 813-816

Wiener, A.S., G.J. Brancato and R. Waltman. 1953. Observations on isosensitization to the Kell factor. *J. Lab. Clin. Med.* 42: 570-580

Wiener, A.S., E.B. Gordon and A. Evans. 1958. The value of anti-H reagents (Ulex europaeus) for grouping dried blood stains. *J. Forensic Sci.* 3: 493-502

Wiener, A.S. and J.G. Hurst. 1947. A new sensitive test (direct test) for Rh blocking antibodies. *Exp. Med. Surg.* 5: 285-298

Wiener, A.S. and H.R. Peters. 1940. Hemolytic reactions following transfusions of blood of the homologous group, with three cases in which the same agglutinogen was responsible. *Ann. Int. Med.* 13: 2306-2322

Wiener, A.S. and E.B. Sonn Gordon. 1947. Réaction transfusionelle hémolytique intra-groupe due a un hémagglutinogene jusqu'ici non décrit. *Rev. Hematol.* 2: 1-10

World Health Organization [Meeting on Human Immunoglobulin Allotypic Markers]. 1976. Review of the notation for the allotypic and related markers of human immunoglobulins. *J. Immunogenet.* 3: 357-362

Yamaguchi, H., Y. Okubo, T. Seno, K. Matsushita and G.L. Daniels. 1979. A 'new' allele, Kp^c, at the Kell complex locus. *Vox Sang.* 36: 29-30

Yudina, G.S. 1972. Reaktsiya absorbtsii-elyutsii primenitel'no k vyyavleniyu antigenov M i N v pyatnakh krovi. *Sud. Med. Ekspert. (Moscow)* 15(3): 37-40

Appendix I. Manufacturers/Suppliers of Antisera*

[Ortho]
Ortho Diagnostics, Inc.
Raritan, NJ 08869

[Dade]
American Dade, Division of
American Hospital Supply Corp.
P. O. Box 520672
Miami, FL 33152
Orders placed through regional offices of American Scientific Products Co.

[BCA]¶
Biological Corporation of America
1230 Wilson Drive
West Chester, PA 19380

[Hyland; Travenol-Hyland]¶
Accugenics
P. O. Box 7300
Costa Mesa, CA 92626

[IVRS]
In Vitro Research Sources, Inc.
224 East Broadway
Bel Air, MD 21014

[Behring]
Calbiochem-Behring Corp.
10933 North Torrey Pines Road
La Jolla, CA 92037

Routes 202-206 North
Building J-O
Somerville, NJ 08876
(Eastern U.S.A.)

distributor for products of
Behring Diagnostics
Hoechst AG
Postfach 80 03 20
6230 Frankfurt a.M. 80
BRD (West Germany)

[Molter]
Dr. Molter GmbH
Postfach 10 40 49
6900 Heidelberg 1
BRD (West Germany)

Industriestrasse 55-61
6901 Bammental bei Heidelberg
BRD (West Germany)

[Fresenius]
Dr. E. Fresenius
Chem.-Pharm. Industrie KG
Bad Homburg v.d.H.

6370 Oberursel/Ts.1
Postfach 1809
Borkenberg 14
BRD (West Germany)

[Biotest]
Folex-Biotest-Schleussner, Inc.
6 Daniel Road East
Fairfield, NJ 07006

distributor for products of
Biotest-Serum-Institut GmbH
Landsteinerstrasse 5
Postfach 40 11 08
6072 Dreieich
BRD (West Germany)

[Pfizer]
Pfizer Diagnostics Division
16700 Red Hill Ave.
Irvine, CA 92705

230 Brighton Rd.
Clifton, NJ 07012

Diagnostics Division has since been sold, with different products segments going to different companies.

Blood banking and blood products currently handled by Immucor, Inc., 3130 Gateway Dr., Norcross, GA 30071

*Brief descriptive names of different suppliers used in the text indicated in the square brackets [ ]

¶ Products of BCA and Accugenics currently handled by BCA-Accugenics, One Technology Court, Malverne, PA 19355

Appendix II. Selected Methods and Procedures

Solutions and Reagents

1. Saline (Normal Saline; Physiological Saline)

   0.85% NaCl in distilled water

   Dissolve 8.5 g NaCl in 1 L distilled water

2. PBS (Phosphate Buffered Saline)

   0.85% NaCl in 50 mM sodium phosphate buffer, pH 7

   (a) 50 mM NaH_2PO_4

   (b) 50 mM Na_2HPO_4

   (c) Add the Na_2HPO_4 solution to the NaH_2PO_4 solution until the pH is 7; this procedure is most easily done with a pH meter and a magnetic stirrer

   (d) Dissolve 8.5 g NaCl in each liter of the 50 mM phosphate buffer, pH 7

3. Saline-Albumin

   0.85% NaCl containing 0.5% (v/v) bovine serum albumin

   Bovine serum albumin is commercially available as 22% or 30% solutions; these stocks are diluted with saline to a final albumin concentration of 0.5%

4. Löw's Papain (Papain)

   (a) 0.067M KH_2PO_4

   (b) 0.067M K_2HPO_4

   (c) Add the K_2HPO_4 solution to the KH_2PO_4 solution until the pH is 5.4; this procedure is conveniently done with a pH meter and a magnetic stirrer

   (d) Dissolve 8.5 g NaCl per liter of 0.067M phosphate buffer, pH 5.4, thus making a phosphate buffered saline, pH 5.4

   (e) 1M cysteine HCl, adjusted to pH 7 with NaOH solution; 10 mL of this solution is required

   (f) Grind 2 g papain powder with 100 mL PBS, pH 5.4

   (g) Filter to clarity

   (h) Add 10 mL neutralized cysteine HCl to each 100 mL clarified papain solution; add PBS, pH 5.4 to make 200 mL final volume

   (i) Incubate at 37° for 1 hour

   This product is stable frozen for many months, and can be stored in small, convenient quantities in a conventional or cryogenic freezer

5. Saline - AB Serum ("AB Serum"; "Dilute AB Serum")

   AB Serum is diluted 1 in 10 with saline; AB Serum can be obtained commercially

6. LISS (Low Ionic Strength Solution)

   The preferred solution is that of Löw and Messeter (1974)

   (a) 18.02 g glycine in 800 mL distilled water; adjust pH to 6.7 with 1M NaOH

   (b) 0.17M NaCl

   (c) 0.15M NaH_2PO_4 and 0.15M Na_2HPO_4; add the latter to the former until the pH is 6.7 (conveniently done with a pH meter and magnetic stirrer)

   (d) To the 800 mL glycine-NaOH, pH 6.7, solution are added 180 mL of 0.17M NaCl and 20 mL phosphate buffer, pH 6.7

   Solutions readily grow microorganisms upon storage; they should therefore be stored in small convenient volumes in a deep freeze, or at 4° following autoclaving

   [Several examples of commercially available LISS have been found to be suitable if they are evaluated in advance with known samples using the same procedures that are to be employed for unknown sample typing]

7. Glycerol "Laying-Down Solution" for Red Cells (LDS)

   (a) 3.25% (w/v) tripotassium citrate·H_2O, 0.47% (w/v) KH_2PO_4 (anhydrous), 0.60% (w/v) K_2HPO_4 (anhydrous) in distilled water

   (b) 40% glycerol (v/v) in the above phosphate buffered potassium citrate solution

   This solution may be employed to store red blood cells at cryogenic temperatures (-85°C or lower) for indefinite lengths of time

Procedures

1. Titrations

   Blood grouping reagents are all normally titrated by carrying out doubling dilutions of the antiserum. The dilutions can be conveniently made in tubes by adding one volume of saline (or other diluent) to every tube in the row except the first; one volume of "neat" antiserum is then added to the first ("neat" or "1") tube. One volume of antiserum is then added to the second ("1:2") tube, and the contents thoroughly mixed. One volume of this mixture is transferred to the third tube, the contents mixed, and a volume of this mixture transferred to the next tube. This procedure is continued down the row to the end. The remaining volume removed from the last tube is discarded.

   Any convenient quantity may be defined as "one volume". A drop from a particular type of pipette may be taken as one volume. We prefer to use calibrated, spring-loaded repeating capillary pipettors ("Micropettors") for titration. Commercial micropettors are available to dispense any number of different volumes repeatedly. 50 µL is a convenient "volume" for most work; if reagents or sample is very precious, it may be preferable to use 25 or even 20 µL volumes.

   Following the preparation of the doubling dilution series, a volume of test red blood cells is added to each tube, and the contents mixed. The red cells may be adjusted to various concentrations, but the value should be kept consistent for all related work.

The tubes are next incubated under conditions optimal for the antibody being tested, usually room temperature or 37°, for periods varying from 10-15 min to more than an hour depending upon the system. With many antibodies, the tubes may next be centrifuged briefly to pack the cells. Contents of the tube are then transferred to microscope slides, Boerner slides, tiles, or some other convenient reading device. If larger volumes of reagents and more concentrated cell suspensions are being used, agglutinations may be read macroscopically. Most of the work in our laboratory involves small volumes and thinner cell suspensions, and we prefer microscopical reading. Even under these conditions, 4+ and 3+ agglutinations are evident to the experienced eye.

The most important feature of titrations is that they be carried out under the same conditions as the tests for which the antisera being evaluated are intended.

2. Cell Suspension Concentration

Red cells are washed by successive centrifugation and removal of the saline or other diluent almost completely. Packed red cells following centrifugation are taken to be a 100% cell suspension. Various % cell suspensions are prepared by making v/v dilutions of packed cells in saline or other diluent. One volume of packed cells in 99 volumes of saline, for example, would give after mixing thoroughly a 1% cell suspension. Most procedures require cell suspensions varying from 0.05% to 0.5%.

3. Washing/Handling of Red Blood Cells

Red cells are washed three times in saline before use in our laboratory regardless of the conditions under which they have been stored. Red cell washing is conveniently carried out with a clinical centrifuge, and a water-driven vacuum aspirator, although there are other ways of doing it that are equally good.

Red cells should be handled gently as a general rule; tubes in which red cells are being resuspended should be mixed gently.

As a matter of laboratory safety, it is good practice to treat all blood and blood products as being capable of transmitting hepatitis, and other blood borne viruses. Contact with blood should be avoided as much as possible.

4. Direct Agglutination Tests

Direct agglutination tests can be carried out with so-called "complete" antibodies, generally ABO, MN, Lewis and some S ones. Direct tests can usually also be carried out with Rh antisera under some serological conditions, especially with papain treated cells.

Direct agglutination tests consist of adding a volume of antiserum and a volume of appropriately diluted cell suspension together, mixing, incubating, often centrifuging quickly, and then reading. Occasionally, two volumes of antiserum for each volume of cells is employed for certain systems.

These tests are done in tubes if the antiserum is being titrated as a rule. They may also be done in Boerner slides, on microscope slides, etc., however, depending on the test actually being done.

5. Anti-human Globulin Tests ("AHG Tests", "Indirect Coombs Tests")

AHG tests are employed with antisera that do not directly agglutinate red cells containing the corresponding antigen in saline. The "direct" Coombs test is very important clinically, but has little application in forensic blood grouping as such. The "indirect" Coombs test is used with any antibody that will not bring about agglutination in saline, and which is not amenable to other kinds of enhancement procedures. LISS is often used in conjunction with indirect Coombs tests. Most anti-s, anti-Duffy, anti-Kidd, and some anti-Rh and anti-S sera are principally AHG reactive.

The test is carried out in three stages: sensitization; washing to remove all traces of the antiserum; and addition of the AHG to bring about agglutination.

Sensitization of red cells is achieved by mixing equal volumes of antiserum and red cells (about 3-5% suspension), and incubating at 37° for 45-90 min. The cells are then washed three times in saline to remove all traces of the sensitizing antiserum. One volume of AHG serum is then added to one volume of sensitized cells (about 0.5% suspension) in a tube or on a tile or slide, and these mixtures are incubated for 15-30 min as a rule. Agglutination is then read.

AHG sera may be titrated, and used at dilutions other than "neat". This matter was discussed in § VII.D.2.

6. Papain Treatment of Red Cells

Papain treated red cells are used most commonly in forensic serology for Rh typing. They may be used occasionally in the typing of other blood group antigens as well.

One volume of Löw's papain is added to one volume of a 50% suspension of washed red blood cells. The mixture is incubated for 15 min at 37° (the timing should be precise). The cells are then washed three times in saline, and are ready for use.

Papainized red cells are stable for a matter of hours, and must be made up fresh each day.

7. Glycerol Freezing of Red Blood Cells

Red blood cells may be frozen in glycerol solutions at -85° or lower, and recovered as intact cells. They are stable in the frozen state for years under these conditions. There is always some loss of cells by hemolysis upon recovery.

An equal volume of washed, packed red cells and "laying down solution" (LDS) [see above] are slowly mixed, the LDS being added slowly to the cells with gentle mixing. The mixtures are then frozen at -85° or lower.

The cells may be recovered from the frozen state most readily by dialysis against phosphate buffered saline for at least two hours with two changes of PBS. The recovered cells are then washed three times in saline, and are ready for use.

Alternatively, cells may be recovered by successively washing them in 16%, 8%, 4% and 2% glycerol made up in phosphate-citrate buffer [3.25% tripotassium citrate monohydrate, 0.47% KH_2PO_4 (anhyd) and 0.60% K_2HPO_4 (anhyd)], and finally washing in saline.

8. Absorption-Elution Procedure

The elution procedure for blood group antigens other than ABO is carried out in test tubes. For ABO, the procedure may be done in tubes, or with threads affixed to inert backings (Howard and Martin, 1969). Ammoniacal extracts of bloodstains may be tested in tubes, in welled slides, or in conical polyethylene sample cups (see § I.B); such extracts can be grouped only for ABH antigens.

With minor variations depending upon the nature of the sample, the quantity available, the substratum, and the antigen being typed, the elution procedure followed in our laboratory is as follows:

(a) Three 1 cm threads of bloodstained sample (or as much as 1 cm^2) are placed in tubes (12 x 75 mm); a separate tube is prepared for each antigen that is to be tested for

(b) Cloth or other suitable substratum controls, and positive and negative bloodstain controls are prepared in the same manner

(c) Antiserum is added to the tube in sufficient quantity to cover the threads completely; the antiserum should have a titer of 256-512 under the serological conditions that will be used for detection insofar as is possible

(d) Tests for ABH or MN antigens are incubated at 4°, and tests for Rh, Ss, Kell, Duffy and Kidd antigens are incubated at 37°, for 17 hours

(e) The excess antiserum is removed from the tube, and the samples are washed six times (in 12 x 75 mm tubes) with ice cold saline, allowing 12-15 min for each wash

(f) The last wash is performed in saline-albumin, and all traces of wash fluid are removed following the last wash

(g) Saline sufficient to cover the threads (at least enough to be able recover one volume) is added, and the tubes are incubated at 56° for 15-30 min

(h) Eluates are removed quickly to a Boerner slide and appropriate test cells can then be added. If the eluates are to be titrated, there must be sufficient eluate available to remove two volumes, and in this case, the eluate would be placed in tubes in a pre-prepared titration row. Eluates are also removed to tubes if the antibody requires detection by AHG.

(i) For systems in which agglutination can proceed directly once the eluted antibody and test cells are mixed together, the tubes or Boerner slides are incubated at the appropriate temperature (20° or 37°), and agglutination is read microscopically

(j) For eluates containing antibodies that can be detected without an AHG test, the test cell concentration is 0.05-0.1%

(k) For eluates containing Coombs reactive antibodies, the eluates are placed in 12 x 75 mm tubes, and 0.5% test cells are added (one volume of cells per volume of eluate). If the eluate is to be titrated, two volumes of it must be recoverable, and these are added to a pre-prepared titration row. The eluates and cells are incubated at 37° for 45-60 min. The cells are then washed three times with saline, transferred to a Boerner slide well and a volume of appropriately diluted AHG is added. After a 15-30 min incubation on the rotator, the agglutinations are read microscopically.

9. Gm/Km Typing Procedure for Serum

(a) Three sample tubes are employed. The first two should receive one volume of 1:10 diluted serum, the third should receive one volume of 1:20 diluted serum

(b) Two positive control tubes are set up, containing 1:10 and 1:20 dilute antigen-positive serum; two negative controls are similarly set up

(c) A volume of appropriately dilute anti-Gm/Km serum (see § VIII.E.2) is added to the control tubes, and to the 1:20 serum sample tube and one of the 1:10 serum sample tubes. To the remaining 1:10 serum sample tube is added a volume of saline (saline control).

(d) The tubes are incubated at 4° for 17 hours

(e) Sensitized test cells are prepared by incubating group O, R_1R_1 red cells with an optimal volume ratio of anti-D possessing the factor being tested (see § VIII.E.2). Sensitization is checked by an AHG test.

(f) One volume of sensitized test cells (0.5%) is added to every inhibition tube, the contents mixed, and the contents of each tube then transferred to a separate well in a Boerner slide. The Boerner slides are placed on a rotator for 15-20 min (until the negative serum controls show definitive agglutination). All the wells can then be read for agglutination microscopically.

(g) Controls should be read first. Positive serum controls should be completely inhibited (negative agglutination), and negative serum controls should show definitive agglutination. If there is agglutination in the saline control, the tests cannot be interpreted; this result indicates some kind of anti-Gm/Km activity in the serum being tested

(h) In a positive test, both the 1:10 and 1:20 serum sample test tubes should show complete inhibition. If there is inhibition in the 1:10 but not in the 1:20, the test may be repeated using slightly more dilute anti-Gm serum; alternatively each test may be completely titrated out.

10. Gm/Km Typing Procedure for Stains

(a) For most relatively fresh bloodstains, three 1 cm threads is sufficient sample; more sample may be taken for older or thinner stains; a cloth or substratum control must be set up; and a sample should be prepared for use as a saline control. Positive and negative known stain controls are required as well.

(b) Anti-Gm/Km sufficient to cover the threads (but at least one volume; two volumes will be needed if the tests are to be titrated out) is added to all the tubes except the "saline control" which receives an equivalent volume of saline. Tubes are incubated 17 hrs at 4°.

(c) Sensitized test red cells are prepared exactly as described for serum typing

(d) Cloth samples are removed from each tube carefully, allowing the adhering liquid to run back into the tube as much as possible.

(e) A volume of sensitized test cells is added for each volume of antiserum present in the tube, and the tests are then handled exactly as described above for serum testing; in questionable cases, each tube may be titrated out, as noted above in testing serum.

Appendix III. Blood Grouping Reagent Stability and Storage

It is well known that biological materials often lose activity upon storage or aging, and blood grouping reagents are no exception to this general rule of thumb. With antisera (or red cells) that are not used up fairly quickly in routine tests and/or are not routinely re-supplied by standing orders, it would be desirable to be able store them under conditions that give the best retention of activity.

Long term storage of red cells is possible if liquid nitrogen or a mechanical freezer that pulls down to -85° or lower is available. Procedures are given in Appendix II, and some data on the activity of red cell antigens in red cells stored under various conditions is presented in § III.B.

Limited experiments were carried out to determine the stability of antisera under several different storage conditions. An anti-B serum, an anti-c serum and an incomplete anti-k serum were included in these studies. Some data was also gathered on several precipitating antisera. The results are shown in the accompanying table (page 126). Some discussion of the storage and stability of anti-Gm/Km sera was presented in § VIII.E.3.

Most of our antisera are stored at -85° in small quantities, so that only one thawing is ever required. Precipitating antisera in general seem to lose more activity (as estimated by titration) than agglutinating (blood grouping) antisera. The data in the table show this effect. In addition, it has been noticed that monospecific precipitins seem to lose more activity upon freezing and thawing than polyspecific ones.

Both glycerol and DMSO were used in the experiments to see whether their presence would give greater retention of activity upon freezing and thawing. Neither of them had any effect that could be measured, and there seems to be no reason for including either of them.

The data indicate that conventional freezing of antisera was as effective in preserving activity as was -85° for the most part. The data indicate too that it is better to thaw the reagents only once.

Retention of Activity of Various Antisera Under Various Storage Conditions

Conditions	Titer(Score) of Antiserum with Red Cells* or Human Serum¶					
	Anti-B[1]	Anti-c[2]	Anti-k[3]	Anti-IgG[4]	Anti-human Serum[5]	Anti-IgM, IgG, IgA[6]
Fresh	512(92)	1000(97)	64(51)	1600	5000	2000
-15° 1X thaw	256(87)	512(88)	32(44)	1000	4000	500
-15° 2X thaw	256(75)	256(74)	8(30)	100	4000	100
-85° 1X thaw	256(87)	512(88)	16(39)	1000	4000	500
-85° 2X thaw	256(75)	256(76)	8(28)	500	4000	500
-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)	---	---	---
4° 1 week	---	---	---	---	4000	1000
-15°, then 4° 1 wk	256(82)	---	---	500	4000	1000
-85°, then 4° 1 wk	256(82)	---	---	500	4000	500

*B cells with anti-B; rr cells with anti-c; kk cells with anti-k

¶Ouchterlony technique in 1% agarose in 50 mM phosphate buffer, pH 6.8; titer is highest dilution of human serum still giving visible precipitin reaction

[1]saline [2]papain technique [3]AHG technique [4]Dako [5]Miles-Yeda (lyophilized) [6]Miles-Yeda

END