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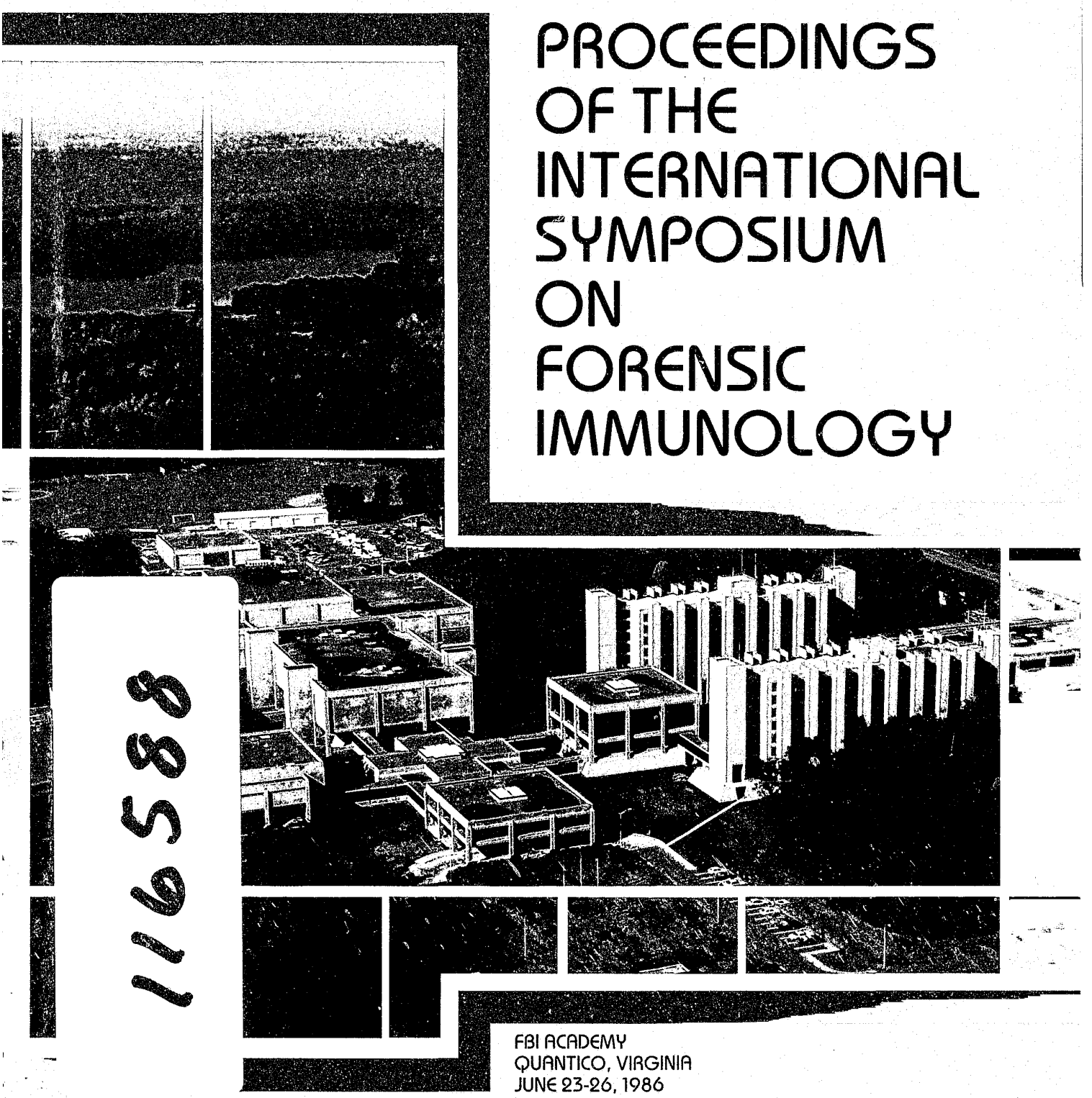
PROCEEDINGS OF THE INTERNATIONAL SYMPOSIUM ON FORENSIC IMMUNOLOGY



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JUNE 23-26, 1986

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FOREWORD

On June 23–26, 1986, the FBI Laboratory hosted an "International Symposium on Forensic Immunology." The symposium was held at the Forensic Science Research and Training Center, Quantico, Virginia, and there were 175 scientists in attendance from industry, university and government laboratories in the United States, Canada, the Federal Republic of Germany, France, Italy, Japan and the United Kingdom.

Plenary sessions were held on topics such as the immunologic basis for species differentiation, the biochemical genetics of the ABO and Lewis cellular and soluble antigens, inhibition-elution and mixed agglutination methods, ELISA methodology, quality control of immunology reagents and laboratory safety. In addition, there were short oral and poster presentations and panel discussions on relevant topics.

The symposium provided an exchange of ideas which, it is hoped, will generate future research and strengthen the scientific merit of the forensic applications of immunology. Consequently, I believe the objectives of this symposium were met by gathering together respected scientists to discuss immunology and forensic applications.

On behalf of the FBI, I would like to thank all those who participated in the symposium.

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INHIBITION, ELUTION AND MIXED AGGLUTINATION

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The forensic serologist dealing with the problem of the detection of antigens in stains of blood, body fluids and other materials will not be able to use the normal agglutination techniques for grouping fresh blood samples but can use the techniques of inhibition, elution and mixed agglutination. These methods were originally developed by blood group serologists and have been adapted by forensic serologists to the special problems encountered in their field. The general problem is one of antigenic material, such as dried red cells, which cannot be agglutinated by corresponding antibodies in the same way as normal red cells from a fresh blood sample. It has been suggested that the three processes of inhibition, elution and mixed agglutination could be compared with using a mop to clean up a puddle. By inhibition, we examine the puddle to see what has been taken up, by mixed agglutination, we look at the mop itself and the elution is equivalent to squeezing out the mop.

INHIBITION

The technique of inhibition is the first to be used when the antigen is presented in a form that will not allow its reaction with the corresponding antibody to be directly observed by agglutination. The principle of the inhibition technique is shown in Figure 1. The specific antibody is added to the test material, saliva samples being the example shown. If the corresponding antigen is present, it will combine with the antibody. Subsequent testing with fresh red cells containing the corresponding antigen will demonstrate if a measurable amount of the antibody has combined with the test material by showing a reduction of the activity of the antiserum. One can envisage that to obtain a high degree of sensitivity, the concentration of antibody must be low so that even small amounts of antigen will cause detectable reduction in the activity of the antibody.

The technique may be performed in various ways depending on the quantity of antigen in the test material. Since the technique is particularly useful in the detection of the ABO substances in body fluids, such as saliva and semen, it is convenient to use such fluids as examples for describing inhibition.

If the stain material has a high concentration of antigen, then an extract from this can be diluted to give a number of concentrations, each of which can be tested against a suitably diluted antiserum. After incubation of the antiserum and the various concentrations of stain extract, each mixture is tested with indicator red cells of the appropriate group to detect any remaining antibody. As can be seen from the results shown in Table 1a, a number of dilutions of the extract may have sufficient concentration of antigen to cause complete inhibition of the antibody so that no agglutination of the red cells occurs. Such a technique allows some assessment of the quantity of antigen in the stain under examination.

Far greater sensitivity can be achieved if the stain extract is tested with a number of dilutions of the antiserum, made, for instance, by titrating the antiserum with doubling dilutions. An equal quantity of the neat extract from the stain is added to each dilution of

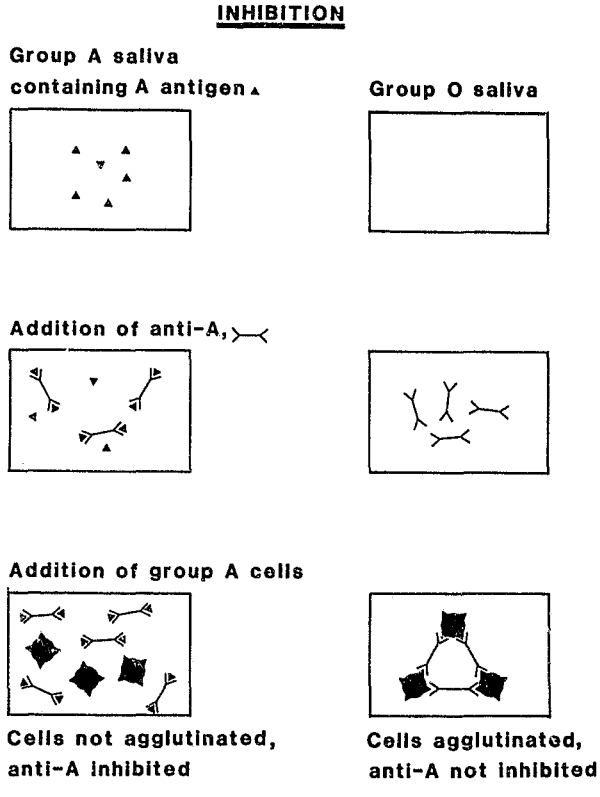

Figure 1. Detection of blood group antigens by the inhibition technique.

Table 1. EXAMPLE RESULTS FROM INHIBITION TESTS USING ANTI-A AND SALIVA STAINS FROM INDIVIDUALS OF GROUP O AND GROUP A

a) *Doubling dilutions of stain extract each tested with an equal volume of anti-A*

	N	½	¼	⅛	1/16	1/32	1/64	1/128	1/256	1/512
Group A	–	–	–	–	–	–	–	W	+	V
Group O	V	V	V	V	V	V	V	V	V	V
PBS control	V	V	V	V	V	V	V	V	V	V

b) *Doubling dilutions of anti-A each tested with an equal volume of neat extract from stain*

	N	½	¼	⅛	1/16	1/32	1/64
Group A	V	+	W	–	–	–	–
Group O	V	V	++	+	(+)	W	–
PBS control	V	V	++	+	(+)	W	–

c) *Equal volume of anti-A and neat extract incubated and any remaining antibody activity measured by titration*

	½	¼	⅛	1/16	1/32
anti-A+ Group A stain extract	++	W	–	–	–
anti-A+ Group O stain extract	V	V	++	+	W
anti-A+ PBS control	V	V	++	+	W

the antiserum. After incubation, the appropriate red cells are added to each tube to test for antibody activity. Such a test system allows the neat stain extract the opportunity to react with a number of concentrations of the antibody. As can be seen from the results in Table 1b, such a test system may be sensitive enough to detect antigenic activity that is not sufficient to completely inhibit all of the activity in the normally selected concentration of antiserum and therefore not produce a positive reaction in the alternative technique described above as Table 1a.

A third variation of the inhibition test, which provides a sensitivity somewhere between these two methods, involves incubation of equal volumes of the selected antiserum and stain extract. Subsequently, this mixture is titrated by making a series of doubling dilutions and its activity compared with the antiserum after incubation with the same quantity of control material. Results from such a test are shown in Table 1c. In this example, the extract from the A saliva stain contained sufficient A activity to produce a significant inhibition of the anti-A, but not complete inhibition. If such a test is performed as an initial test on an unknown stain and complete inhibition of the antiserum is obtained, then method 1a can be subsequently used to make a measurement of the amount of antigen activity in the extract. Alternatively, if no significant inhibition is detected by this technique, then it might be appropriate to proceed with the more sensitive technique 1b, which can detect very small amounts of antigen.

The sensitivity of each of these variations of inhibition test will depend, among other things, on the concentration of antibody used in the test. For example, in method 1a, where a number of dilutions of the stain extract are tested against the same concentration of antibody, total inhibition of the antibody activity by a number of concentrations of the extract will occur only if a very low concentration of antibody is used. In any inhibition test, the test antigen must be able to combine with a large proportion of the antibody molecules offered to it, if there is to be a resultant detectable reduction in the antibody activity. It is essential, therefore, that a suitable dilution of the antibody be selected by titration and that the selected concentration be carefully evaluated through the use of known test materials. In some laboratories, the highest dilution of the antiserum which produced a certain agglutination reaction, such as the maximum agglutination, is selected for use. This criterion may not always be the most successful one for selection. The results of titration of two examples of potential inhibition anti-A reagents are shown in Table 2. Adopting the procedure of selecting the highest dilution that produces the maximum agglutination reaction would result in the same dilution, 1 in 4 being selected for inhibition for both these reagents. However, serum 1 at this dilution has a titer of 1 in 4, whereas serum 2 has a titer of 1 in 16 and is likely to have a higher concentration of antibody than serum 1. This means that, used at this dilution, serum 1 may be completely inhibited by less antigen than is needed to produce the same inhibition of serum 2. Consequently, serum 1 may be more sensitive than serum 2 in the inhibition test and prove to be a more satisfactory reagent if the concentration selected for use is determined in this way.

When ABO antibodies are considered for use in inhibition tests, it is important to remember that qualitative as well as quantitative features are likely to

Table 2. RESULTS FROM TITRATION OF TWO ANTI-A REAGENTS THAT ARE FOR POSSIBLE USE IN INHIBITION TESTS

	N	½	¼	⅛	1/16	1/32	1/64	1/128
anti-A (1)	V	V	V	+	W	–	–	–
anti-A (2)	V	V	V	++	++	+	(+)	–

be significant and the final selection should be made with the aid of results obtained in pilot experiments. Even where concentrations of antibodies giving the same titer have been selected, these may vary in their suitability. One possibly important factor is whether the antibodies are IgG or IgM, since it is well known that IgG ABO antibodies are less well inhibited by soluble blood group substances than are IgM antibodies. Polley *et al.* (1963) estimated that IgG anti-A required 20 times more purified A substance IgM anti-A to effect a comparable reduction in titer. In fact, this characteristic was used in clinical work to distinguish between IgG and IgM antibodies (Witebsky test; Witebsky 1948). This means that when ABO antibodies are selected, it is as well to select serum from an individual who has not been immunized, since immunized individuals are more likely to have IgG antibodies in their serum. Moreover, an antiserum that has been selected as suitable for typing of fresh red cells by normal agglutination tests may not necessarily be ideal for inhibition. Even if the antiserum is diluted to a low concentration, the proportion of IgG antibodies may be too high for good inhibition to be achieved because such reagents produced for grouping purposes are usually prepared from selected immunized donors. Therefore, even when an apparently suitable concentration of antibody has been selected, some antisera may be found to be more suitable than others and hence the need for pilot experiments to make the final selection.

Although the inhibition test was originally used for the detection of antigens not only of the ABO system but also of the MN, Rh and Kell antigens in bloodstains, the amount of stain material required to show unequivocal inhibition of the corresponding antibody is too large to make the test useful for widespread application in casework. Since the development of an elution technique with superior sensitivity for the detection of a wide range of red cell antigens in bloodstains, as illustrated by Lincoln and Dodd (1975), the use of inhibition has declined. At the present time, the elution technique is used mainly for the detection of ABO and Lewis antigens in body fluids other than blood, but it has also been found to be the method of choice for the detection of the Gm and Km factors in bloodstains.

ELUTION

The reaction between antigen and its corresponding antibody can be reduced in a number of ways so that the two are dissociated, that is, antibody is eluted from antigen. Many such procedures have been reported and continue to appear in the literature, but the only method routinely applied to the detection of antigens in stains has been the Landsteiner technique of heat elution. The original Landsteiner technique was considerably modified by forensic serologists, but the principle remains the same and is summarized in Figure 2. The antigens of the stain material are allowed to specifically adsorb antibody, unbound antibody is removed by washing and then any specifically bound antibody is dissociated from the stain antigens by raising the temperature to 55° C or 60° C. Antibody that has been eluted can be detected by indicator cells carrying the appropriate antigen. A number of criteria can help provide optimum conditions throughout the test so that the maximum sensitivity will be obtained. Failure to provide all the optimum conditions at each stage of the test will not usually result in failure, but each parameter makes its contribution towards optimum sensitivity.

Consideration of the nature of the antigen antibody reaction is essential in understanding the influence of Dr. Steane. (A concise review of this topic is provided by Moore 1982.) It is clear that goodness of fit between antigen and antibody, distribution of antigenic determinants, relative concentrations of antigen and antibody, ionic strength of the reaction medium and temperature and incubation time all have their influence on the combination of antigen and antibody. Thus, when the technique of elution is considered, these factors must be borne in mind during each stage of the test of adsorption elution and detection of the eluted antibody.

Adsorption

At this stage, adsorption of antibody by the antigens in the stain and the relative concentrations of antigen and antibody are important. When an antiserum is selected for use in this test, the sensitivity can be increased by increasing the concentration of antibody, but it is possible to use a concentration that is too high (Lincoln and Dodd 1973; Gaensslen *et al.* 1985). Apart from being an unnecessary waste of reagent, this may lead to failure because of the difficulty of washing away all traces of uncombined antibody. If this occurs, positive reactions will be obtained even where the antigen under investigation is

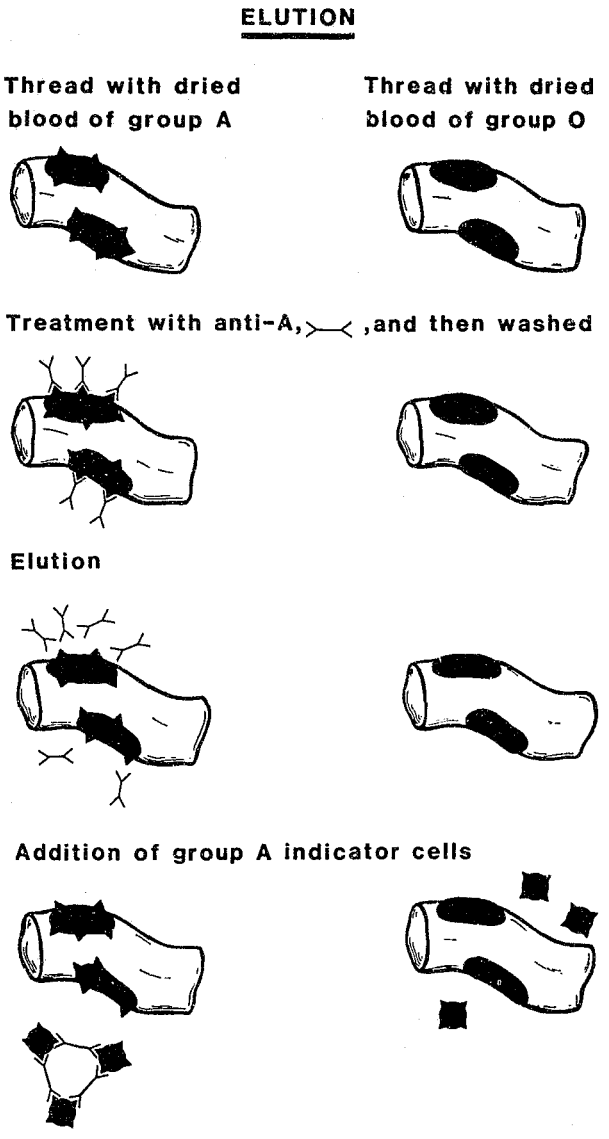

Figure 2. Detection of blood group antigens by the elution technique.

not present in the stain. Results obtained from an experiment using a potent example of a monoclonal anti-D reagent illustrate this point and are shown in Table 3. The higher concentrations of this reagent do not produce satisfactory results because anti-D is seen to be recovered not only from the D positive bloodstains but also from the D negative stains. However, further dilution produces improvement, and at a dilution of 1 in 64, the positive reactions are retained with the D positive stains and negative reactions are achieved with the D negatives. Thus, correct D typing of bloodstains can be produced by a careful selection of the appropriate concentration of this reagent.

Table 3. RESULTS FROM ELUTION TESTS TO DETECT THE D ANTIGEN IN BLOODSTAINS USING VARIOUS CONCENTRATIONS OF A MONOCLONAL ANTI-D[a]

D type of stain	Age of stain	Dilution of anti-D 1/8	1/16	1/32	1/64
D+	1 week	4+	2+	4+	4+
D+	1 week	4+	4+	4+	4+
D–	1 week	2+	–	1+	–
D–	1 week	2+	–	–	–
D+	4 months	4+	4+	4+	4+
D+	4 months	4+	4+	4+	4+
D+	4 months	4+	4+	4+	4+
D–	4 months	2+	2+	1+	–

[a]Anti-D UCH D4 was kindly supplied by Dr. P. Crawford of University College Hospital, London

It is important to remember that this anti-D reagent has been standardized for D typing of fresh red cells by normal agglutination procedures, for example, albumin, papain and ahg technique, and with these techniques, even the undiluted reagent produces specific reactions with no agglutination of the D negative cells. These results illustrate how a reagent must be restandardized when it is to be used in a technique for which it was not originally intended. This applies to all blood grouping antisera. Such reagent will have been standardized to produce the specificity claimed, but this can be assumed to be true only for the techniques specified for their use, and these techniques are most unlikely to include elution.

The concentration of antigen used during adsorption can affect the success of the test. Usually the forensic serologist is plagued with the problem of insufficient stain material and the problem of identifying antigens when their quantity is insufficient to allow detection. However, there are instances, particularly when one considers the ABO system, when the concentration of antigen may be too high and lead to loss of sensitivity or even apparent failure of the test (Lincoln and Dodd 1973; Gaensslen *et al.* 1985). Examples of this are seen when testing body fluids, such as saliva and semen, for ABO antigens, and the problem is illustrated by the results shown in Table 4. The test material originated from a group A secretor stain. The higher concentrations of antigen produce only weak reactions or even no reaction with the anti-A and anti-H. It can be seen that use of more than the optimal amount of antigen in the test can result in failure to detect the antigen or at least in reduced activity.

Both the length of the incubation period and the temperature used have been shown to affect the final yield of antibody and hence, the sensitivity of the test.

Table 4. RESULTS FROM ABO GROUPING OF EXTRACTS FROM A GROUP A SECRETOR STAIN BY ELUTION

Dilution of extract	anti-A	anti-B	anti-H
Neat	–	–	–
1/5	1+	–	2+
1/10	2+	–	2+
1/20	3+	–	3+
1/40	4+	–	1+

There appears to be little doubt that antibody will be taken up by the antigens in a stain when the adsorption stage is allowed to continue for only the normal time used for fresh blood samples, for example, 2 hours. However, it appears that there will be a greater chance that more antibody will become bound if the incubation is extended, and about 16 hours (overnight) has been shown to be both successful and convenient (Lincoln and Dodd 1975; Gaensslen *et al.* 1985).

The optimum temperature used for the adsorption will depend on the specificity of the antibody. The ABO antibodies tend to show maximum activity at 4° C, and such a temperature is selected for adsorption. So-called warm reacting antibodies will react optimally at 37° C, and this temperature should be selected for the adsorption, even though the incubation is overnight. Having achieved the maximum uptake of antibody by the antigens of the stain, the object is to keep it there until it is dissociated and recovered in the eluate. Minimum elution will occur at 4° C, whether the antibodies are cold reacting such as ABO antibodies or warm reacting antibodies such as those of Rh specificity. Sufficient ice-cold saline must be used in the washing to remove all traces of nonspecifically bound antibody that could be detected by the subsequent sensitive test procedure with indicator cells. As well as the quantity of saline, the time span appears to be an important contribution to efficiency of this washing and in our experience, a 2 hour wash seems to be satisfactory.

Dissociation or Elution of Antibody

As already mentioned, a wide variety of elution procedures is available, but there is evidence that no one technique produces maximum recovery of all antibodies (Judd 1982). The method chosen by forensic serologists is elution by heat, based on the original Landsteiner technique. The method does not appear to be particularly efficient as far as antibody yield is concerned when compared with other techniques for eluting from fresh red cells. However, the method can be conveniently applied to the forensic situation that can involve large numbers of bloodstains, and the success that it achieves appears to have deterred serologists from searching for alternative methods. Dissociation or elution of antibody from antigen takes place over a wide temperature range, and indeed different antibodies may have different optima. An important factor is the combining capacity or K value of the antibody molecules. Those with a low combining capacity will be eluted at a lower temperature than those with a higher K value, and depending on the composition of the antibody molecules in a particular antiserum, the optimum temperature for elution may be at 55° C or at 60° C (Lincoln and Dodd 1973).

Moreover, IgM antibodies may be more heat labile than are IgG antibodies, so that for the ABO system, where the antibodies may be largely IgM, a higher temperature may not produce the optimal results. Ideally, the optimal temperature should be determined for each antibody as part of the assessment program for selection of suitable reagents.

The Landsteiner elution procedure, as developed for elution from fresh red cells, involves an elution period of about 7 minutes, this time being determined to a large extent by the fact that the red cells are hemolyzed if subjected to longer periods at this temperature. It has been found that dissociation is by no means complete in this period, and extension of the elution time can improve the quantity of antibody recovered (Dodd *et al.* 1967; Lincoln and Dodd 1975). In our laboratory, we routinely use a period of 15 minutes at 55° C when using ABO antibodies, and generally this period is extended to 30 minutes at 60° C for antibodies of all other systems.

At the end of the elution period, the standard serologic procedure would be to separate the eluted antibody from the antigen as quickly as possible. However, experiments have shown that there is an advantage in not separating the eluate from the stain and that the indicator cells should be added to the eluate without first removing the stain material (Lincoln and Dodd 1975). The stronger agglutination that is apparent if this procedure is followed appears to be because the indicator cells continue to acquire antibody both by competing successfully for any antibody remaining in dynamic equilibrium with the antigen of the stain and by combining with antibody that continues to elute from the stain during this incubation with the indicator cells.

Detection of Eluted Antibody

The amount of antibody eluted is limited by the very small quantity of antigen present on the stain used in the test. Its concentration in the eluate will be

low, and maximum sensitivity will be needed for its detection. The marked effect of reducing the red cell concentration when detecting low concentrations of antibody is illustrated by the results shown in Table 5. As has been demonstrated previously (Lincoln and Dodd 1975; Gaensslen *et al.* 1985), greater enhancement of sensitivity is often more marked with lower concentrations of antibody.

Experience has shown that papain treated cells provide a more sensitive technique for the detection of eluted antibodies of some systems, particularly Rh antibodies (Lincoln and Dodd 1978). In practice, we found that an 0.5% suspension of cells is very suitable as indicator cells for ABO grouping and that a 0.1% suspension of papain treated cells is very satisfactory for the detection of Rh antibodies. In a test for antigens in stains whose corresponding antibodies react preferentially by the antiglobulin technique (for example, S, s, K, Fy^a or Jk^a), a drop of 1% concentration of the indicator cells is used.

The appropriate detection method for the antibody used in the test, be it, for instance, saline, enzyme treated cells or antiglobulin test, is important. We know that some antibodies, such as those of the Kell and Duffy systems, may not be detectable by any technique other than ahg, but papain may be better than ahg for most Rh antibodies. Moreover, some antibodies show quite marked variation in activity depending on whether cells used for testing are homozygous or heterozygous for the gene concerned, and it is always preferable to use cells that are homozygous.

The Use of Low Ionic Strength Solution

When red cells in normal saline suspension are added to a serum containing corresponding antibody, opposing charges on the red cells and antibody molecules have a tendency to hold the two apart. If the ionic constitution of the medium is changed, some of the opposing charges may be negated so that antigen and antibody can get closer together, and they may have an increased chance of combining with each other. Early workers found it difficult to find a suitable ionic strength that would increase the reaction between antigen and antibody but at the same time not allow nonspecific binding to occur. Also, the early work was performed with the object of increasing the rate of reaction so that agglutination would occur more quickly and faster testing could be achieved in clinical emergencies concerning blood transfusions. For the forensic serologist, speed of reaction is not important, but the use of low ionic strength solution (LISS) with a normal incubation period appears to increase the amount of antibody detectable in eluates prepared from stains and thus the sensitivity of the test system could be improved. More recently, Merry *et al.* (1984) provided additional evidence of the increased uptake of antibody in the presence of LISS.

The LISS can be incorporated both during the reaction between antigen and antibody at the adsorption stage and also during the detection of the eluted antibodies with fresh indicator cells. We have reported the results of trials that were undertaken to investigate the use of LISS in the elution technique and shown how LISS could improve sensitivity when included at both the adsorption stage and during detection of the eluted antibodies (McDowall *et al.* 1978).

Results comparing tests with and without the use of LISS (Table 6) demonstrate how, in many instances, improvement in sensitivity can be obtained with antibodies of many different specificities. A wide variation in the effect can be observed. In some instances, little or no increase in sensitivity is discernable, but often there is some increase and there are some examples where the use of LISS would result in

Table 5. RESULTS OBTAINED FROM USING VARIOUS RED CELL CONCENTRATIONS FOR THE TITRATION OF ANTI-D

	Percent concentration of cells	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256
Anti-D	2	++	++	++	+	+	–	–		
1 in 100	1	C	V	V	++	++	+	–	–	
	0.5	C	C	V	V	++	+	W	–	–
	0.1	C	C	C	C	V	++	+	+	–
	0.05	C	C	C	C	V	V	++	+	–
Anti-D	2	++	+	–	–					
1 in 500	1	++	++	+	(+)	–	–			
	0.5	V	++	++	+	W	–			
	0.1	C	V	V	++	+	W	–		
	0.05	C	V	V	++	+	+	–		

Table 6. RESULTS OF TYPING BY ELUTION FOR VARIOUS ANTIGENS SHOWING THE ENHANCEMENT OBTAINED USING LISS

Antiserum	Phenotype of stain	Age stain in weeks	Antibody activity in eluate	
			Normal	LISS
Anti-D	CcDEe	8	++	++++
Anti-D	ccDEe	32	+	++++
Anti-D	ccddee	32	–	–
Anti-C	CcDEe	1	++	++++
Anti-c	ccddee	2	++++	++++
Anti-c	ccDEe	24	(+)	++++
Anti-E	CcDEe	1	++++	++++
Anti-E	CcDEe	20	+	++++
Anti-S(1)	Ss	2	+++	++++
Anti-S(1)	Ss	36	++	++++
Anti-S(2)	Ss	1	–	++
Anti-S(2)	SS	1	–	++++
Anti-Fy^a	Fya+b+	20	+++	+++
Anti-Fy^a	Fya+b+	32	++	++
Anti-Fy^b	Fya–b+	20	(+)	++
Anti-Fy^b	Fya+b+	32	(+)	+
Anti-Jk^a	Jk a+b–	12	++	++
Anti-Jk^a	Jk a+b+	32	–	+
Anti-Jk^b	Jk a–b+	4	+	++
Anti-Jk^b	Jk a+b+	32	–	++

the detection of an antigen that would remain undetected by the corresponding test without LISS.

No instances of reduced sensitivity have been found. In our laboratory, we regularly use LISS in our elution test system for all red cell antigen systems except for ABO and Kell, since there seems to be little evidence for any improvement in detection of antibodies of these systems.

MIXED AGGLUTINATION

The application of the mixed cell agglutination principle to the detection of antigens in stains should be mentioned. Although it was greeted with acclaim when it was first described for this purpose by Coombs and Dodd (1961), it was found to have only limited application and has been replaced by elution.

Mixed agglutination was developed as a technique for detecting antigen on cells that are unsuited for direct agglutination techniques, for example, buccal cells. The principle of the technique is outlined in Figure 3, using a bloodstain as an example. The stain material is mixed with the appropriate antisera, and subsequently, all antibody that has not become specifically attached to the antigens in the stain is washed away. If fresh red cells containing the antigen corresponding to the antibody used in the test are then added to the threads, these cells become bound to the stain material by the antibody molecules that are attached to the antigens on the stain but that have further antigen receptors available to combine with the antigens of the indicator cells. Often the indicator cells align themselves to cover the surface of the stain material, but sometimes they compete more successfully with the stain antigens for the available antibody molecules, and then agglutinated cells are seen which are not attached to the stain material.

The technique is very elegant, but it was found that relatively few antisera were suitable and it did not appear to work satisfactorily for antigens of systems other than ABO. It remains as a research technique and does have the advantage that the attachment of the indicator cells to the material under test can be useful because it locates the precise area of antigenic activity in sections of organs.

SELECTION OF ANTISERA

Selection of suitable antisera has been mentioned in various contexts, but it is of primary importance and warrants further comment. Each of the techniques mentioned as being applicable to the detection of red cell antigens in stain material requires certain antibodies and/or conditions if maximum sensitivity is to be achieved. A single antiserum may be found to produce results when used by all three techniques, but it is unlikely to provide the maximum sensitivity by them all.

The object of the inhibition test is to allow all or at least a very high proportion of the antibody in the

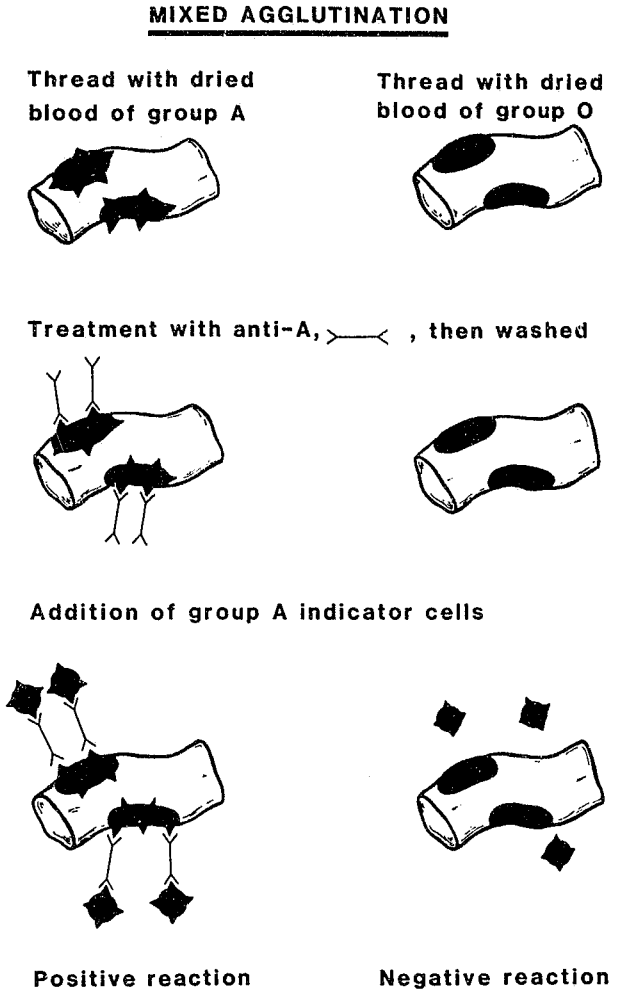

Figure 3. Detection of blood group antigens by the mixed agglutination technique.

test serum to be adsorbed by the antigen in the stain, so that there is maximum reduction in antibody activity in the serum. Thus, a high proportion of antigen relative to antibody is needed. In contrast, in the elution test the object is to achieve maximum number of antibody molecules on the antigens in the stain, and this is more likely to be obtained using an excess of antibody. For the elution technique, the antibodies need to have a reasonable affinity for the stain antigen such that a high proportion combine with the stain antigens and remain attached during the washing, but they must not be of such a high affinity that they are not recovered well during elution.

Bearing in mind these factors and also the importance of the relative concentrations of antigen and antibody as discussed earlier, it is not surprising that careful selection by experiment is an essential prerequisite to successful stain grouping. Moreover, the success achieved with a particular antiserum may vary according to the age of the stain material under test. This can be noticeable when dealing with the detection of ABO antigens in body fluids such as saliva and semen, where the prozone effect mentioned earlier as a feature of the elution technique can become apparent on very fresh stain material but nonexistent on older stains. Also experience has shown that whereas it may be relatively easy to select antisera suitable for detection of antigens in stain material that is just a week or two old, far fewer reagents may be suitable when stains are older, and it may be necessary to use higher concentrations of antibodies where the stain material is of reduced quality or quantity.

Some of these points are well illustrated by results that have been performed in our laboratory. In the results shown in Table 7, a number of monoclonal anti-A reagents have been used for typing bloodstains together with a routinely used polyclonal anti-A of human origin. Also included are results from tests using these monoclonal anti-B reagents. It was found that very little difference in activity was seen in a comparison of the results obtained with tests on stains of group A_1 and A_2 which were not more than 1 month old. However, reducing the available antigen by using A_1 and A_2 stains that were 3 years old demonstrated some variation between the monoclonal reagents and the polyclonal routine antiserum in regard to ability to detect the A antigen. This variation in the suitability of the monoclonal reagents became even more marked when stains of group A_2B were investigated, and some of the monoclonal reagents tested failed to detect the A antigen in such stains.

The monoclonal reagents used in this investigation all produced titration values against fresh red cells which were at least equivalent to those of the routinely used polyclonal reagent. The three monoclonal anti-B reagents all produced results that were as good as those produced by the polyclonal anti-B serum as far as the extent of testing in this trial was concerned.

The variability in the suitability of the monoclonal reagents used in this trial is interesting. As mentioned earlier, it has been postulated that one of the features of an antibody which will make it suitable for the elution technique is that it should have the necessary affinity for the corresponding antigen to combine and remain attached through the washing process and yet be recovered well at elution. A polyclonal serum is likely to have a heterogeneous selection of antibody molecules with various affinities, and at least some are possibly well suited for the elution technique, but the monoclonal reagent, by definition having only one population of antibody

Table 7. RESULTS OBTAINED FROM AN ASSESSMENT OF USE OF MONOCLONAL REAGENTS FOR THE DETECTION OF A AND B ANTIGENS IN BLOODSTAINS BY ELUTION

ABO type of stain	Age	Anti-A reagents				Anti-B reagents		
		poly	monoclonal			monoclonal		
		1	1	2	3	1	2	3
A_1	fresh	3+	3+	3+	3+	–	–	–
	3 yrs	3+	3+	3+	3+	–	–	–
A_2	fresh	3+	3+	3+	3+	–	–	–
	3 yrs	3+	–	2+	2+	–	–	–
A_2B	fresh	3+	1+	2+	3+	3+	3+	3+
	3 yrs	3+	–	–	2+	3+	3+	3+
B	fresh	–	–	–	–	3+	3+	3+
	3 yrs	–	–	–	–	3+	3+	3+
O	fresh	–	–	–	–	–	–	–
	3 yrs	–	–	–	–	–	–	–

molecules, is prone to the risk of being composed entirely of molecules that may be less than ideal for elution.

Our experiments have provided some evidence that older stains may need antibodies at a higher concentration than do fresh stains and moreover, that the introduction of LISS into the test system may show more advantage when older stains are tested and may even result in antisera being suitable for typing bloodstains that otherwise appeared unsatisfactory. When antisera are selected for routine stain grouping, it is therefore important to include stains of various ages to obtain a true assessment of the full potential of the antisera under investigation.

The final and perhaps most important point concerning the selection of suitable antisera is that the forensic serologist has developed techniques for a specific task and the reagents normally available may not necessarily maintain their specificity when used by these specialized techniques. It is essential therefore that the forensic serologist must perform his own assessment and standardization program of reagents if he is to produce successful and reliable results.

CONCLUSIONS

Through the use of the techniques of inhibition and elution, it is possible for the forensic serologist to obtain successful grouping for a wide range of red cell antigens that are present in stains of blood and other body fluids. To achieve success, great care must used in the selection of suitable antisera. Attention to the detailed performance of the techniques can achieve considerable increases in sensitivity which sometimes could mean the difference between success and failure in the detection of a particular antigen in the material under investigation.

ACKNOWLEDGMENT

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DISCUSSION

Souhrada: Do you recommend using low ionic strength solution (LISS) with monoclonal anti-D?

Lincoln: Yes, with that monoclonal anti-D I was using LISS. We routinely use LISS for all antibodies, except for ABO. This anti-D is a very potent antibody.

Question: Regarding a comment made by Dr. Steane concerning the antigenic binding qualities of IgG versus IgM, you indicated that IgG was weaker and Dr. Steane said it was more concentrated, but your comments concerned body fluid antigens. Could you explain the differences?

Lincoln: I think it is true that when you are using soluble substances, IgG is not inhibited as well as the IgM antibodies.

Steane: Immunoglobulin is a much better agglutinator than IgG. Therefore, although it is true that the antibody is more difficult to combine, or combines more strongly, the amount of antibody left may be much better than with IgM, but it does not agglutinate as well because IgG is not a good agglutinator.

Question: One practical problem with many immunologic techniques is the presence of a prozone of negative reactions. You seem to also have some of these prozone phenomena in stains. What is the theoretical approach to understanding this prozone in the kind of experiments you do and what are the practical solutions? Do we have to go through the whole set of dilutions, or is there any way to get around this problem?

Lincoln: The danger of using the elution technique by itself is that you can see a prozone and get a negative reaction when, in fact, the antigen is present. You overcome this if you use the inhibition technique in parallel and do not use the elution by itself. In the inhibition technique, you do not see the prozone. We can get around it from the practical point of view, but I am sure others will be discussing this.

Schanfield: The difference between IgM agglutination and IgG agglutination involves major orders of magnitude. Pure IgG anti-A needs in the neighborhood of 20,000–40,000 molecules per cell for agglutination, whereas IgM involves only hundreds of molecules per cell for agglutination. It takes much less antigen to neutralize 400–500 molecules than it does to neutralize 20,000–40,000 molecules.

Question: Do you find the same principles exist when selecting a lectin for your elution work with the O antigen or H antigen?

Lincoln: I assume that the same criteria exist. You must select the right dilutions and the right concentrations and so on. Generally we select procedures using the same criteria, but I think there may be different problems with anti-H reagents.

Question: Would you say generally that in selecting a lectin for elution that the higher concentration of the lectin, the better off you are for sensitivity in your elution assay for the H antigen?

Lincoln: I find with anti-H that sometimes you can run into trouble because the anti-H is not strong enough. However, you can get to a point where it is too concentrated, and then you have trouble getting rid of some of the unbound antibody.

Question: Is that due to the washing procedure?

Lincoln: I think it is. I do not have good scientific evidence, but I suspect that most of the time our problem is one of physical inability to wash away the uncombined antibody. You see this particularly if you use a larger quantity of material or thicker material.

Question: In the O row, if you are to use your control standards then you are using an A. If you are

using all the controls, A, B, AB and O, you could expect to see some H in all those.

Lincoln: Yes, but you can get to a point where after years of experience, you can describe whether your stain is A, B or O according to the reaction you get with the anti-H.

Question: But your assay should be sensitive enough to pick up those rows.

Lincoln: I think so. I like to be able to equate the ABO status of the bloodstain with the H reaction obtained.

IMMUNOLOGIC BASIS FOR SPECIES DIFFERENTIATION

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Proteins are the most abundant and diverse class of antigens to which the immune system can respond. These antigens include toxins, allergens, products of infectious organisms and transplantation antigens, as well as proteins, such as the blood and tissue proteins, both soluble and cell surface, secreted or nonsecreted, which forensic scientists find of particular interest.

The ultimate goal of using immunologic methods in forensic medicine is not only to detect specific proteins or identify the species of origin of a given forensic sample but in many cases to identify the individual from whom that specimen was derived. The ability to do so rests on the assumption that the immunologic reagents used do indeed have the ability to specifically distinguish between similar, yet antigenically very complex, proteins from closely related species or individuals.

This goal has been elusive, and only recently with the advent of monoclonal antibody (MCA) and recombinant DNA technologies have we been able to better understand the structural and genetic basis for immunogenicity and antigenicity and to produce reagents with the specificity required. The discussion presented below is designed to give the forensic scientist a better understanding of antigen structure and immunologic crossreactivity.

ANTIGEN STRUCTURE

Immunogenicity is defined as the ability to induce an immune response, whereas antigenicity merely implies the ability to be recognized by the product of that immune response, for example, antibody. Antibodies formed in response to a native protein often do not react with the denatured form (Landsteiner 1936; Brown *et al.* 1959), but antibodies can be raised against peptides of undefined conformation (Shinka *et al.* 1967; Gerwing and Rhompson 1968; Young and Leung 1970; Sela 1969; Sela *et al.* 1967), for example, synthetic vaccines. Antigenic sites (also known as antigenic determinants or epitopes) are of one of two types: assembled and segmented (Benjamin *et al.* 1984) (Figure 1 and Table 1). Although assembled antigenic sites appear to be more numerous, both sites are topographic in that they are composed of structures on the protein surface. However, it is quite clear that antibody exerts high affinity binding to segmental sites only when those sites are in a preferred conformation. Therefore, all determinants are now thought to be conformational.

There are two views of what constitutes an antigenic site. One view maintains that antigens possess a very limited number of sites that are immunogenic irrespective of the individual or species responding to that antigen (Atassi 1975, 1981; Twining *et al.* 1981), suggesting that the chemical and/or physical properties of certain parts of protein molecules make them intrinsically more immunogenic. The opposing view (Benjamin *et al.* 1984) states that most, if not all, of the accessible surface of a protein molecule is potentially immunogenic, that one can define which sites are immunogenic only with respect to a particular individual and that the total antigenic structure of a protein is the sum of all sites recognized by a large variety of individuals and species. The vast majority of evidence, which recently has been reviewed (Benjamin *et al.* 1984), supports the following two concepts:

1. The surface of a protein is essentially a continuum of potential antigenic sites.

2. The structural differences between the antigen and self-protein as well as the host's immunologic regulatory mechanisms are the important factors influencing the outcome of the immune response.

The first concept, that is, that of a continuum of antigenic sites, is supported by studies of antibody responses to, among others, hen egg white lysozyme (Underwood 1982) and by our own studies on serum albumins (Benjamin *et al.* 1983a, 1983b, 1985). Using a panel of 60 MCAs, we were able to define a minimum of 35 antigenic sites on bovine serum albumin (BSA). An extensive study of competition between pairs of MCAs for binding simultaneously to BSA has suggested complex patterns of overlap which

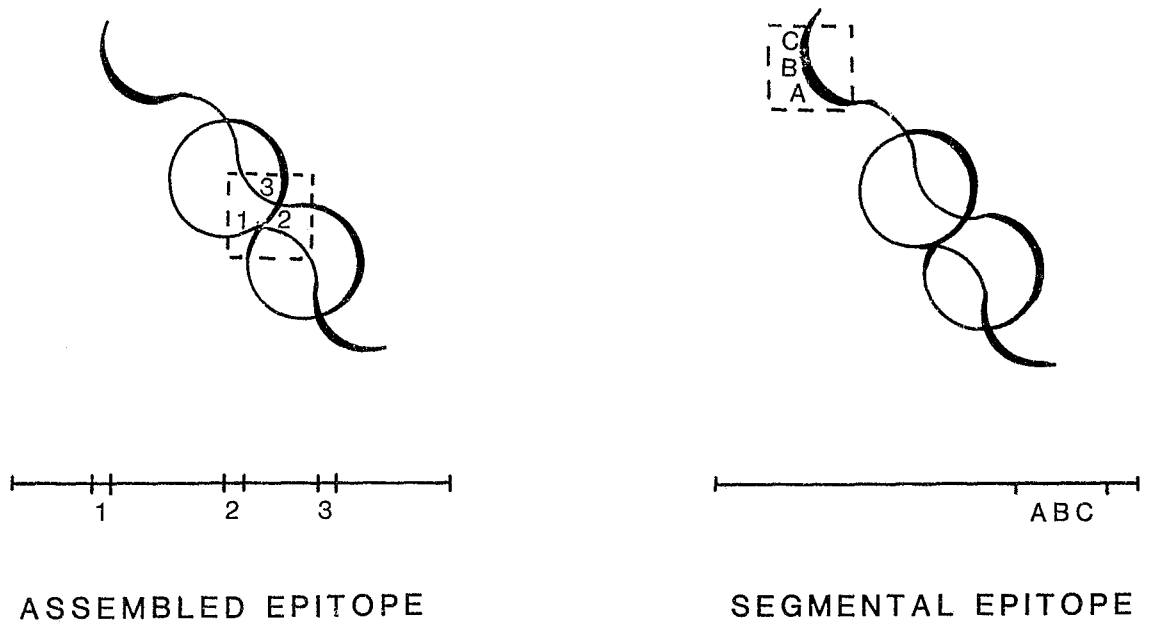

Figure 1. Diagrammatic representation of two different types of antigenic determinants. An assembled epitope is composed of amino acid side chains (Nos. 1, 2 and 3) which are well separated in the linear sequence of the protein but which are brought together during folding of the protein. A segmental epitope is composed of amino acid side chains (A, B and C) which are contiguous both in the linear sequence of the protein and on the surface of the protein in its folded form.

Table 1. TYPES OF ANTIGENIC DETERMINANTS

Segmental – exists wholly within a continuous segment of the amino acid sequence
Assembled – consists of amino acids far apart in the primary sequence which are brought together on the surface of the antigen as it folds into its unique three-dimensional shape

are consistent with the entire surface of BSA being immunogenic. For example, numerous cases of three antibodies, called A, B and C, interacting in a pattern such that A competes with B, B competes with C but A does not compete with C, have been found.

The second concept is derived from a large number of studies on the antigenicity of slowly evolving proteins such as cytochrome c (Jermerson and Margoliash 1979; Urbanski and Margoliash 1977a, 1977b), from studies on the effect of evolutionary substitutions in the primary sequence of proteins on immunologic crossreactivity (Prager and Wilson 1971; Wilson *et al.* 1977; Champion *et al.* 1975; Berzofsky *et al.* 1979) and from studies on the effect of specific regulatory cells on antibody responses (Berzofsky *et al.* 1979; Riley *et al.* 1982; Katz *et al.* 1982; Kapp *et al.* 1979; Schwartz *et al.* 1978). Cytochrome c, in contrast to serum albumin, is an example of a slowly evolving protein (Table 2) with few sequence differences between each cytochrome c and that of the responding individual. The rabbit antibody response

Table 2. EVOLUTIONARY VARIANCE IN AMINO ACID SEQUENCE

Protein	Difference[a]
Cytochrome c	9.6
Serum albumin	27.5

[a]Mean number of amino acid differences per 100 residues

to pigeon cytochrome c was found to be directed against four sites on the pigeon cytochrome (Benjamin *et al.* 1984). These four sites contained all seven sequence differences between the rabbit and pigeon cytochrome c.

In other studies (Prager and Wilson 1971; Wilson *et al.* 1977; Prager *et al.* 1978), rabbits were immunized with a particular protein from one organism and the antisera tested for reactivity with evolutionary variants of that protein. The results showed a high degree of correlation between immunologic distance (calculated from the immunologic crossreactivity observed) and the number of amino acid differences

between each pair of proteins tested (Table 3). From such data, one can estimate that most, if not all, of the differences in surface amino acids between the antigen and the self-protein of the responding host can be immunologically detected.

Structurally, each antigenic site on the surface of a protein antigen is composed of a number of side chains of amino acids. To date, the exact boundaries of a single antigenic site have not been defined. However, based on what is known of the surface topography of proteins and the structure of the antibody combining site, we can estimate that the antigenic site consists of six to eight amino acids. Within this six to eight amino acid surface configuration, a difference of a single amino acid, between antigen and a similar protein in the responding host, may be sufficient to render that region immunogenic. Although there may be only a single amino acid difference with any given site, X-ray crystallographic structural analyses on antigen antibody complexes clearly show that adjacent amino acids, identical to those in the same region of the responding host's protein, are involved in antibody binding to the site (Amit *et al.* 1986).

For those sites that have been studied in detail, we can see that no single amino acid is predominant. Indeed, aliphatic, charged, neutral and hydrophobic amino acids can all be found within one or more sites. Similarly, no particular physical feature of the surface of a protein antigen dictates antigenicity. Therefore, an antigenic site can only be defined with reference to a single antibody molecule, that is, an MCA. It is a specific configuration of amino acid side chains on the surface of the protein antigen which possesses a configuration complementary to the antibody combining site.

CROSSREACTIVITY

As a general rule, antibodies made to one protein antigen will also react (crossreact) with a wide variety of similar proteins from other species. For example, 15% of the antibody in a polyclonal antiserum made to BSA will react with human serum albumin. The molecular basis for this crossreaction is not clearly understood but is believed to be due to the presence of a configuration of amino acid side chains, on another protein molecule, which has a sufficient overall similarity to the specific configuration on the original antigen that induced antibody formation. Figure 2 shows three protein antigens, each with a single antigenic site. The configuration of each site is such that each is similar, for example, proteins A and C share three of four amino acids, whereas protein A shares two of four amino acids with each of proteins B and C. Therefore, the efficiency of reaction of an antibody made to protein B can be predicted to be in the order B>C>A, whereas one might expect an antibody made to protein A to react equally well with proteins B and C. However, the exact effect of any given substitution cannot be predicted.

Berzofsky *et al.* (1982) have shown that one antigenic site, defined by a single MCA, on the surface of sperm whale myoglobin involves the amino acids glutamic acid, alanine and lysine at positions 83, 144 and 145, respectively. These three amino acids are brought together on the surface of the antigen by its specific folding after synthesis, that is, it is an assembled site. Although these amino acids constitute only a part of the site, these studies provided considerable information on crossreactivity in addition to localizing the site on the surface of myoglobin. The data in Table 4 summarize their results and show that a change of glutamic acid to aspartic acid at position 83 in killer whale myoglobin had a 24-fold effect on binding of antibody, whereas a single amino acid change from alanine to glutamic acid at position 144 in beef myoglobin essentially eliminated all reactivity. Thus, with respect to this MCA, only killer whale myoglobin is a crossreacting antigen.

As time passes since two species diverge from a common ancestor, mutations occurring in the structural genes encoding similar proteins result in amino acid differences between the proteins produced by two species. As stated above, the degree of immunologic similarity between any two such proteins is directly related to the evolutionary distance between the two species. Different proteins evolve at different rates. The structural basis for this is that for slowly evolving proteins, changes in amino acid sequence have a greater effect on function and are less well tolerated. The examples given in Table 2 then indicate that, on the average, changes in serum albumins have a lesser effect on critical function than do changes in cytochrome c.

Some of these evolutionary changes may influence antigenicity and crossreactivity through long-range effects. However, for most proteins, these evolutionary changes have only local effects on structure

Table 3. CORRELATION BETWEEN AMINO ACID SEQUENCE IDENTITY AND IMMUNOLOGIC CROSSREACTIVITY[a]

Protein	Correlation Coefficient
Lysozyme c	0.95
Ribonuclease	0.92
Cytochrome c	0.87

[a]Adapted from Benjamin *et al.* (1984) and based on data from Berzofsky *et al.* (1982)

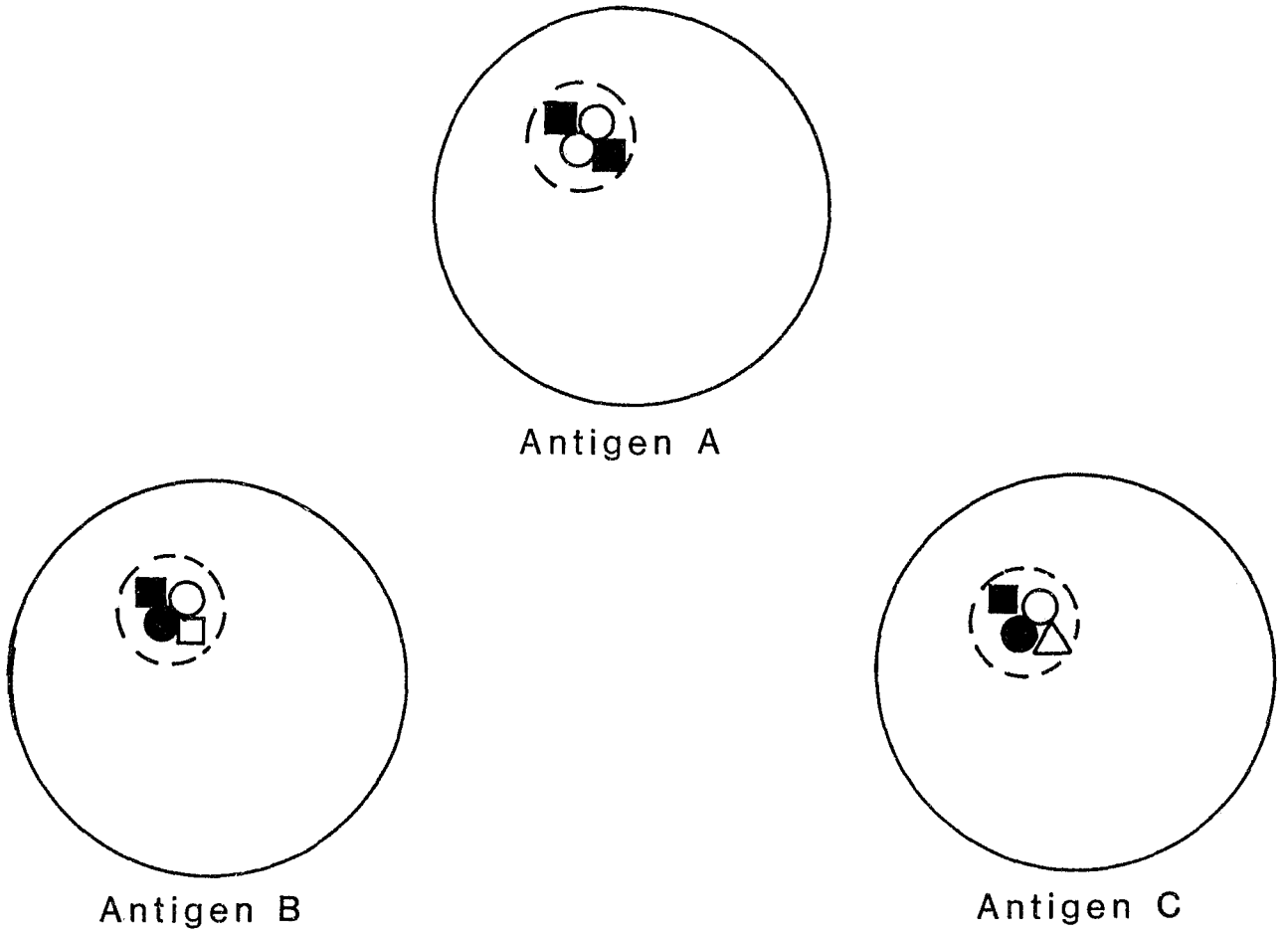

Figure 2. Diagrammatic representation of three protein molecules possessing crossreacting antigenic sites. Only one site is shown per protein molecule. The antigenic site on each protein is similar, yet not identical, to the site on the other proteins.

Table 4. INHIBITION OF MONOCLONAL ANTIBODY BY MYOGLOBIN VARIANTS[a]

	Amino Acid			
Inhibitor myoglobin	83	144	145	Myoglobin required for 50 percent inhibition
Sperm whale	Glu	Ala	Lys	10 nM
Killer whale	Asp	Ala	Lys	240 nM
Bovine	Glu	Glu	Lys	>40,000 nM

[a]Adapted from Berzofsky *et al.* (1982)

and function. Indeed, substitutions that markedly affect the binding of one MCA have little effect on the binding of a second MCA to an adjacent site (Berzofsky *et al.* 1982; Smith–Gill *et al.* 1982). Any long-range hypothesis would have predicted otherwise.

SPECIES DIFFERENTIATION USING MONOCLONAL ANTIBODIES

Since the turn of the century, forensic scientists have been using polyclonal antisera to distinguish between species (Uhlenhuth 1901a, 1901b; Wasserman and Schutze 1901). The reliability of the results depends on high quality, carefully tested antisera that have been exhaustively adsorbed to eliminate cross-reactivity with proteins from other species (Gaensslen 1983). This crossreactivity of the polyclonal antisera is the major limitation of current tests for species of origin. Thus, the phylogenetic relationships between species impose intrinsic limits on the ability to differentiate related blood or tissue specimens. As noted by Sensabaugh (1976), forensic immunologic analyses

would be far more satisfactory if monospecific antisera to rapidly evolving blood marker proteins were used, thereby taking advantage of greater differences in the structure of similar proteins, even in very closely related species.

Obviously, proteins such as cytochrome c are not good candidates for forensic testing. Serum albumin, on the other hand, is a rapidly changing protein (see Table 2) that has proved amenable to complete immunochemical analysis with both polyclonal and monoclonal antibodies (Benjamin *et al.* 1983a, 1983b, 1985). Albumins from thousands of pairs of vertebrate species have been compared immunochemically, and the results have been used to measure evolutionary distances (Wilson *et al.* 1977).

A large body of literature, spanning three decades, has shown albumin to be a highly complex antigen with multiple antigenic sites (Benjamin *et al.* 1984). Our own studies on the antigenic structure of BSA (Benjamin *et al.* 1983a, 1983b, 1985) showed that species-specific MCAs could be produced and that a panel of crossreactive antibodies could be assembled for use in identifying a number of other species.

These studies suggested that species-specific MCAs might be produced to human albumin and would be of value in determining the human origin of blood, tissue and other body fluids. We have been successful in producing an MCA that appears to react with a human-specific epitope on serum albumin.

Overall, the MCAs produced to serum albumin were found to be of one of three general types (Table 5). Type A is highly specific for human albumin, type B crossreacts extensively with primate albumins with little or no crossreactivity with albumins from other species and type C shows variable reactivity with albumins from a variety of species. The species tested are shown in Table 6. Two MCAs were selected for further study: human serum albumin (HSA)-1 is of type A and highly human specific, whereas HSA-2 is of type B. These two MCAs were used in a double antibody trapping enzyme linked immunosorbent assay (ELISA) to detect and quantitate human albumin in blood, serum and a variety of other human body fluids and tissue extracts.

First of all, the sensitivity of the assay was determined by using dilutions of purified human albumin and human serum. As shown in Figure 3, the assay could specifically detect as little as 30 ng/ml purified albumin, or expressed in another way, the albumin present in a $1{:}10^6$ dilution of human serum (equivalent to 1.0 nl or less of serum) within a few minutes after addition of substrate to the ELISA. Longer incubation times permit greater sensitivity without loss of specificity. The assay can be made

Table 5. CLASSES OF MONOCLONAL ANTI-ALBUMIN ANTIBODIES

	H	C	G	O	D	Ca	B	S	De	Ho[a]
Type A	+	–	–	–	–	–	–	–	–	–
Type B	+	+	+	+	–	–	–	–	–	–
Type C	+	–	+	–	–	+	+	+	–	+

[a]H–human, C–chimpanzee, G–gorilla, O–orangutan, D–dog, Ca–cat, B–bovine, S–sheep, De–deer, Ho–horse

Table 6. SPECIFICITY OF HSA-1 MONOCLONAL ANTIBODY[a]

Human	Chimpanzee	Gorilla
Orangutan	Dog	Cat
Hamster	Cow	Sheep
Pig	Rat	Raccoon
Goat	Rabbit	Antelope
Mule deer	Elk	Horse
WT deer	Guinea pig	Turkey

[a]Serum samples from the listed species were tested. Only human was reactive at any concentration.

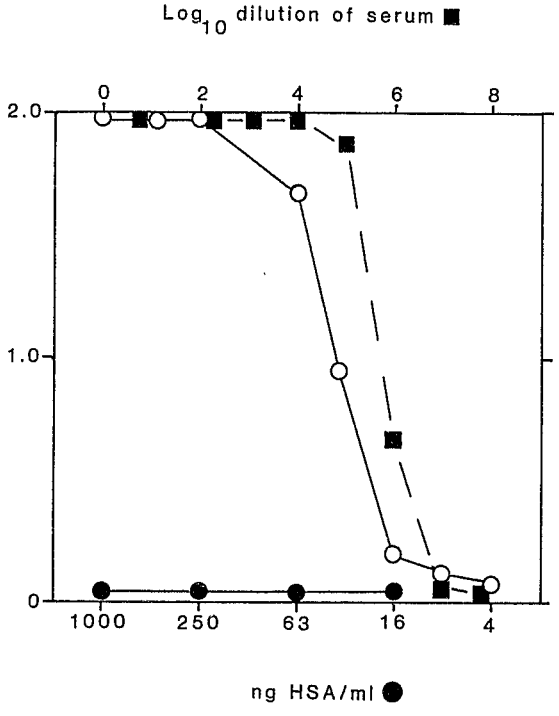

Figure 3. Sensitivity of antigen trapping assay (ATA) employing HSA-1 and HSA-2 monoclonal antibodies. The HSA-2 antibody (type B) was coated on the plate, and then human albumin or serum was diluted as indicated and added to the wells. Biotin-conjugated HSA-1 served as the specific probe. Optical density at 410 nm was read after 15 minutes.

more or less sensitive by varying any one of a number of parameters, for example, the amount of antibody coated on the ELISA plate or the reaction times with the various reagents.

Albumin was easily detected in a variety of body fluids and tissue extracts including seminal fluid, milk, urine, saliva, vaginal secretions and extracts of liver, kidney and pancreas. The concentrations of albumin in each of these fluids, relative to serum, was such that assay conditions could be easily adjusted to readily distinguish between blood samples and other body fluids.

Every specimen from a large panel of human serum samples from various races and sexes reacted with the HSA-1 monoclonal antibody. No human serum has been tested that did not react, indicating the absence of genetic variation in the antigenic site detected by the HSA-1 antibody.

The HSA-1 MCA thus fulfills two essential criteria for an immunologic probe for forensic use in the identification of species of origin: species specificity and intraspecies conservation of the antigenic site. The unequivocal assignment of the species of origin to unknown bloodstains, body fluids and/or tissue fragments is critical to forensic medicine. South African forensic scientists in particular have a serious problem in their practice differentiating human and primate blood (Taylor 1952).

High titer anti-human globulin polyclonal antiserum in double diffusion assays has been the method generally employed to identify human blood in forensic specimens (Sivram *et al.* 1975). However, given the number of antigenic sites on complex proteins, it is not surprising that it has been difficult to produce a truly species-specific polyclonal antiserum. It is also not surprising that, given the large number of structural variations between proteins from related species, species-specific surface structures exist on proteins which may be immunologically detected with MCAs. Species-specific immunoreagents, such as HSA-1, may offer the forensic community the opportunity to standardize identification of human blood and tissue, allowing direct comparison of results from different laboratories without questioning the specificity of the antibody, thus, giving an added measure of certainty to the testimony of the forensic specialist.

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DISCUSSION

Question: Have you ever seen polymorphisms within human serum albumin (HSA)?

Benjamin: With HSA we have not seen any polymorphisms, but I do not think we have looked at the right individuals for that. There is electrophoretic polymorphism. As far as I know, we have not looked at serum samples from any of those individuals that might be polymorphic.

Question: Would it be possible for a polymorphic individual to fail to react with your monoclonal antiserum?

Benjamin: Yes.

Fletcher: Have you actually used this on bloodstain extracts?

Benjamin: Yes, with artificial ones that we generated ourselves, as well as a large panel of material that the FBI has provided, and we are waiting for decoding of those results now.

Question: How old were the bloodstains?

Benjamin: As old as 6 months.

Question: We performed a large study using species specific onto human IgG, and we found that we were unable to get the material out of bloodstains older than 3 years.

Benjamin: I think that may be one of the advantages that our albumin also offers. It is a very tough protein. As evidence for this, a group at the University of California at Berkeley did a study on tissue taken from a 200-year-old bone of a Tasmanian wolf from the Australian Museum. The group was able to use an anti-albumin to definitively place the sample in the evolutionary tree. They also looked at a tissue sample taken from the woolly mammoth harvested out of the ice in the Soviet Union and were able to determine its relationship to both the African and Indian elephants using anti-albumin.

Question: I am not arguing with your definition of species specificity in the practical sense, but in the abstract sense, I think it ought to be clear that what you define as a human specific type A, could be a type C, where a determinate is on a species that you have not yet looked at. So, your specificity really is operationally defined. I think there are some examples of very distant species that share an antigenic determinate that would be entirely unexpected based on evolutionary consideration.

Benjamin: You are correct. That monoclonal really is operationally defined with respect to the species we have tested so far. It is very possible with this or any monoclonal that you may find reactivity with *E. coli* or some unknown distantly related organism.

THE BIOCHEMICAL GENETICS OF ABH AND LEWIS CELLULAR ANTIGENS

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BIOCHEMISTRY

Anti-ABH and Anti-Lewis Reagents

The discovery of the ABO and Lewis (Le) systems and most of the related pioneering work have used polyclonal human antisera. Now, monoclonal antibodies (CHEMBIOMED, Ltd., University of Alberta, Edmonton, Canada; CELLTECH, Slough Berkshire, United Kingdom; CNTS, Paris, France) and lectins (E-Y, San Mateo, California, United States; VECTOR, Burlingame, California, United States) are beginning to be commercially available. The use of these reagents has shown that, for each main antigenic specificity of the ABO or Le systems, there are many reagents with differing properties.

Each antibody or lectin may recognize different areas of the same epitope or of different epitopes with the same main blood group specificity. Differences in the recognition sites of anti-A, (Gooi *et al.* 1985), anti-A and anti-B (Chen and Kabat 1985; Mollicone *et al.* 1986a) and anti-Le (Young *et al.* 1983; Francois *et al.* 1986) reagents have already been documented. Therefore, to compare the results obtained with different reagents, the chemical specificity of each binding site must be determined. In other words, it is not enough to compare the hemagglutinating activities of different reagents, since antibodies with similar agglutination titers for red cells may have different specificities in other tissues.

The binding sites of some lectins (Hindsgaul *et al.* 1985; Spohr *et al.* 1985a), monoclonal antibodies (Lemieux *et al.* 1985; Spohr *et al.* 1985b) and purified polyclonal antibodies (Lemieux *et al.* 1981) were determined with the series of blood group-related synthetic oligosaccharides prepared by the team of Professor Lemieux (1978). These oligosaccharides, originally prepared as haptens with an aliphatic spacer ($R=(CH_2)_8COOCH_3$), can be covalently linked to proteins to make artificial antigens or to a solid support to make immunoadsorbents.

The artificial antigens, made by coupling 20 molecules of synthetic hapten to a molecule of bovine serum albumin (Hp_20-BSA), can be used to quantitate ABH and Le antigens in biologic fluids by radioimmunoassay (RIA) (Le Pendu *et al.* 1982, 1983a, 1983b) or enzyme linked immunosorbent assay (ELISA). The same Hp_20-BSA antigens can be used for immunization (Lemieux *et al.* 1977). However, the antibodies obtained with this type of antigen react better with the artificial molecules used as immunogens than with the corresponding natural antigens. Consequently, they cannot be used in competitive RIA or ELISA tests.

The immunoadsorbents, available under the trade name of Synsorbs (CHEMBIOMED, Ltd.), can be used to purify the anti-ABH or anti-Le reagents (Lemieux *et al.* 1981) or to remove natural anti-A or anti-B activity from antisera containing other previous antibodies. This technique has been used successfully to obtain human specific anti-human leukocyte antigen reagents (Bernoco *et al.* 1985).

Chemical Structures and Glycosyltranferases

Each of the main ABH and Le structures can be built on different backbone oligosaccharide chains. When only the terminal disaccharide of the backbone chains is considered, at least four types of blood group-related precursor chains are found in humans:

Type 1	βGal (1-3) βGlc*N*Ac-R
Type 2	βGal (1-4) βGlc*N*Ac-R
Type 3	βGal (1-3) αGal*N*Ac-R
Type 4	βGal (1-3) βGal*N*Ac-R

Gal=Galactose, Glc*N*Ac=*N*-Acetylglucosamine, Gal*N*Ac=*N*-acetylgalactosamine

Each of these four precursors can acquire the H antigenic specificity by addition of a fucose residue in an α(1-2) linkage on the terminal galactose through the action of specific glycosyltransferases. Two enzymes can effect this glycosylation: the H and the secretor (Se) fucosyltransferases (Le Pendu *et al.* 1986a, 1986b).

Another fucose residue can be added to the subterminal Glc*N*Ac residue. The transfer of this last fucose can also be effected by two different enzymes: the Le and the X fucosyltransferases (Watkins 1980).

The Se and Le enzymes can use type 1 and type 2 substrates, whereas the H and X enzymes preferentially use type 2 substrates. It is not known yet if the same fucosyltransferases can use type 3 and type 4 precursor chains as acceptors or if type 3 or type 4 antigens are made by other, not yet characterized, fucosyltransferases.

The Le^b antigen, found in Le(a–b+) secretor individuals, results from the combined action of both Le and Se enzymes on type 1 chains. The same enzymes can produce the Y antigen on type 2 chains (Table 1). The type 1 antigens (Le^a, Le^b, Le^c and Le^d) are produced mainly by the combined action of the products of the *Le* and *Se* genes. The type 2 antigens (X, Y, I and H) can be made by the combined action of the products of the *Le* and *Se* genes, as shown in Table 1 but can also result from the interaction of the X and H enzymes.

The A and B antigens are obtained by addition of an *N*-acetylgalactosamine residue and a galactose residue, respectively, to the H antigens. The transfer of these terminal sugars is catalyzed by the specific glycosyltransferases (Watkins 1980). Individuals who are H deficient also lack A and B antigens, since the A and B enzymes use the H antigen as acceptor substrate.

GENETICS

The ABO locus has been mapped to chromosome 9 (Ferguson *et al.* 1976). The A and B codominant alleles at this locus code for the A and B enzymes. The O allele codes for a protein that is antigenically related to the A and B transferases but devoid of any known enzymatic activity (Yoshida 1982).

The loci for three of the fucosyltransferases (H, Se and Le) have been mapped to chromosome 19 (Westerveld and Naylor 1984). The *H* and *Se* are distinct but very closely linked loci (Oriol *et al.* 1981a). The *H* gene codes for the α-2-fucosyltransferase that preferentially uses type 2 acceptor substrate, and the *Se* gene codes for the α-2-fucosyltransferase that uses both type 1 and type 2 acceptor substrates.

The *Le* locus that codes for the α-3 or α-4-fucosyltransferase that uses type 1 and type 2 substrates is also on chromosome 19 but is probably distant from the *Se* and *H* loci, since a large family study failed to show any linkage between *Le* and *Se* or *H* (Oriol *et al.* 1984a). Each of the *H*, *Se* and *Le* loci has a dominant active allele and a recessive silent allele. The deficient or negative phenotype for each locus results from the inheritance in double dose of the corresponding silent allele.

The mode of inheritance of the *X* gene is not known as yet, but four individuals lacking the X enzyme (α-3-fucosyltransferase using type 2 acceptors) in serum have been reported, and they have some common traits: they are of African origin and have the red cell phenotype Le (a-b-). The second observation suggests that the *X* gene could be closely linked to the *Le* gene in chromosome 19 analogous to the *H* and *Se* genes (Oriol *et al.* 1986); however, as yet there is no direct evidence for this assumption.

The H deficient phenotype (Bombay) is also very rare—the frequency of individuals lacking the H

Table 1. ANTIGENIC DETERMINANTS MADE BY THE FUCOSYLTRANSFERASES UNDER CONTROL OF THE *SE* AND *LE* GENES[a]

Phenotype			Antigens found in tissues		
ABH in saliva	Lewis on erythrocytes	Percent	Chemical structures[b]	Type 1	Type 2
Nonsecretor	Le(a+b–)	20	βGal→βGlcNAc-R ↑ αFuc	Le^a	X
Secretor	Le(a–b+)	69	βGal→βGlcNAc-R ↑ ↑ αFuc αFuc	Le^b	Y
Nonsecretor	Le(a–b–)	1	βGal→βGlcNAc-R	Le^c	I
Secretor	Le(a–b–)	9	βGal→βGlcNAc-R ↑ αFuc	Le^d	H

[a]Lewis positive nonsecretors have the enzyme that produces the Le^a and X antigens, Lewis negative secretors have the enzyme that makes the Le^d and H antigens, Lewis positive secretors have both enzymes and Lewis negative nonsecretors have neither.
[b]The four chemical structures shown can be of type 1 (Le^a, Le^b, Le^c, Le^d) if the βGal–βGlcNAc linkage is 1–3 or of type 2 (X,Y,I,H) if the same linkage is 1–4. In type 1 structures the αFuc–βGlcNAc linkage is 1–4 and in type 2 structures it is 1–3. The αFuc–βGal linkage is 1–2 in both type 1 and type 2 structures.

enzyme in serum and red cells has been estimated to be about 1 x 10^{-4} in India where this phenotype was discovered (Bhatia 1977), but it is about 10 times more frequent in Reunion Island (Gerard *et al.* 1982).

Ten percent of caucasians are Le negative, but this phenotype can occur in up to 40% of blacks. Finally, about 20% of the overall population are nonsecretors of ABH in saliva, with small variations in different ethnic groups (Table 1).

IMMUNOFLUORESCENCE

Immunochemical location of the ABH and Le cellular antigens has been greatly improved by the use of the synthetic immunoadsorbents (Synsorbs™, CHEMBIOMED, Ltd.) to select and purify the corresponding reagents. In this way, reagents giving a completely negative background can be obtained. With such reagents, it is necessary to use a counterstain to identify the negative cells or tissues. Nuclei are the most appropriate structures for this counterstain because they are easily labeled and they should not interfere with the fluorescent staining of ABH or Le antigens, since they do not express these antigens.

The hematoxylin blue staining of nuclei can be combined with fluorescein isothiocyanate (FITC) labeling of specific cellular antigens (Weinstein and Lechago 1977). However, the need for duplicate, fluorescent and normal illuminations, which is required for this technique, significantly reduces the intensity of the specific green fluorescence. This difficulty can be overcome by using a fluorescent counterstain of nuclei which does not interfere with the specific cellular florescence. Oxidized *p*-phenylenediamine in glycerol, used as a mounting medium, fulfills this requirement. It gives a brown-yellow fluorescence of nuclei and reduces the fading of FITC. Therefore, it gives a relative increase in the intensity of the green fluorescence of FITC. Furthermore, this mounting medium gives a bright yellow-orange fluorescence of amyloid deposits (Oriol and Mancilla-Jimenez 1983). Consequently, four structures can be identified, each one stained a different color, in a single immunofluorescent analysis: the nuclei (yellow), amyloid deposits (orange) and two antigens one labeled with a FITC conjugate (green) an another labeled with a tetramethylrhodamine isothiocyanate (TRITC) conjugate (red). The green, yellow and orange fluorescent stains are observed with the conventional filterset for fluorescein (I-2, LEITZ) and the red and orange stains with the conventional filterset for rhodamine (M, LEITZ). Double photographic exposure of the same field, with the two filter blocks are made. For this superimposition of the red and green fluorescent emissions, the automatic advance of the film, after the first photographic exposure, has to be blocked and the optical paths of the two filter blocks should be well aligned. The alignment of the standard fluorescence microscopes are not sufficiently perfect for this type of double exposure. A special request should be made to the manufacturer when the microscope is ordered, or the whole illuminator block may have to be sent back to the factory for realignment of the red and green optical paths.

Phylogeny of the ABH Antigens

The occurrence of ABH was studied in different animal species, with polychromatic immunofluorescence in conventional formalin-fixed paraffin embedded tissues. Snakes and frogs did not express the conventional ABH antigens, although the mucins of their exocrine secretions were stained by other carbohydrate specific reagents. On the other hand, all mammals tested had some structures that were positive with anti-ABH reagents.

The oligosaccharide structures of the ABO system are thought of as the main erythrocyte antigens. This concept is correct for the human species. However, studies of other animals showed that the ABH antigens appear first as tissue antigens. They are found in ectodermal and endodermal epithelial cells of lower mammals (Mollicone *et al.* 1985a) whose red cells are completely devoid of ABH antigens. Furthermore, even in baboons in whom ABH antigens are expressed on vascular endothelial cells (Oriol *et al.* 1984b), the red cells are negative for ABH. In fact, erythrocytes are the most recent cells to acquire the ABH antigens in the phylogenetic evolutionary tree (Figure 1). Only man and some anthropoid apes, the orangutan and the gorilla, express ABH antigens on red cells (Socha and Ruffie 1983). The distribution of ABH blood group substances in other human tissues was first studied by immunofluorescence more than 30 years ago (Glynn and Holborrow 1959; Szulman 1960, 1962). Most of this early work is still valid, but the discovery of the existence of the subtypes of ABH oligosaccharides and the multiple interactions in the products of the different gene loci has led to a more precise definition of the human tissue ABH antigens.

Type 1 and Type 2 ABH Antigens on Circulating Human Cells

The main ABH antigens of red cells and platelets are of type 2 and are independent of *Se* and *Le* genes. These antigens are synthesized by erythroblasts and megakaryocytes, respectively, and they are strongly

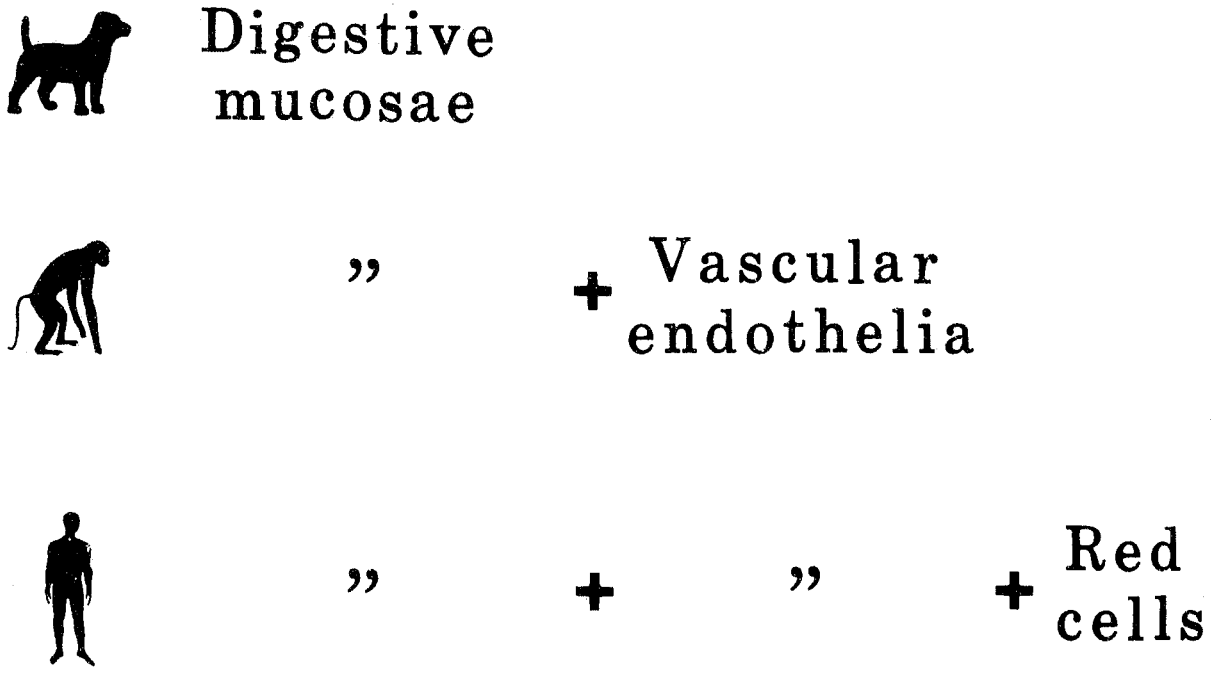

Figure 1. Appearance of cellular ABH antigens in different animal species. The most recent structure to acquire ABH antigens, through evolution, is the erythrocyte.

bound onto the cell membranes. They can be detected by immunofluorescence on paraffin-embedded tissues, and they always reflect the ABO phenotype of the cell or tissue donor.

In addition, small amounts of ABH and Le antigens, under control of *Se* and *Le* genes, are adsorbed into the red cell membranes from the pool of circulating glycosphingolipids (Marcus and Cass 1969). These antigens are not detected by the conventional immunohistochemical techniques and can be changed *in vitro* by incubation of red cells in plasma of a different donor or *in vivo* by transfusion or bone marrow transplantation (Oriol *et al.* 1981b). In these cases, the donated erythrocytes acquire the recipient's ABH and Le glycosphingolipids. These glycosphingolipids are probably synthesized by the same cells that make the glycoproteins of the exocrine secretions, but instead of being secreted outwards, the glycosphingolipids are shed into the plasma compartment.

In addition to these medically induced chimeras, there are naturally occurring genetic chimeras (Watkins 1980). Two types of this rather infrequent condition have been described: twin chimeras and dispermic or tetragametic chimeras. Twin chimeras result from placental cross circulation in dizygotic twins. In this case, the cells from the different zygotic lineage are confined to the hemopoietic tissue. Two red cell populations, carrying different ABO markers can be found, although sometimes only a lack of the anti-A or anti-B agglutinin is observed. This result would be expected on the basis of their apparent ABO red cell group. Dispermic or tetragametic chimeras are believed to result from fertilization by two sperm of two maternal nuclei and subsequent fusion of the two zygotes. A double cell population can be observed in erythrocytes and in other tissues giving gonadal abnormalities, patchy skin color or eyes of different color. Subsequent cytogenetic, serologic and glycosyltransferase studies of the propositus and other members of the family are needed to confirm this condition. Although natural chimeras are exceptional, this possibility has to be considered whenever a dual population of circulating cells or a discrepancy between red cell and secreted ABH or Le antigens is found.

The T and B lymphocytes do not synthesize their own ABH and Le antigens; they only have the ABH and Le antigens glycosphingolipids adsorbed from the plasma. These antigens can be detected by lymphocytotoxicity (Oriol *et al.* 1980a) or by agglutination and are under control of the *Se* and *Le* genes, as are the corresponding ABH and Le glycosphingolipids of red cells. As in the case of their red cell counterparts, these antigens are washed off by the different organic

solvents that are used for conventional histology and thus cannot be detected by immunohistochemistry.

Type 1 and Type 2 ABH Antigens in Other Tissues

Epidermis: The human body is covered by a continuous sheet of two to four cell layers of ABH positive cells. These cells are located in the most external layers of the stratum spinosum and the stratum granulosum, next to the stratum corneum (Figure 2a). The epidermal ABH antigens are present in almost everyone irrespective of the salivary secretor status of the individual. The only negative skin sample, found to date, belonged to an H deficient individual (*h/h*) from Reunion Island (Le Pendu *et al.* 1986a, 1986b). These observations demonstrated that the epidermal ABH antigens are independent of the *Se* gene and are under control of the *H* gene.

The epidermis of the fingertips, palms and soles has an expression of ABH antigens different from the rest of the body. This particularly thick area of the epidermis shows a clear dissociation between the expression of H and A or B antigens. The expression of H antigen is increased, and it is detected in the membranes and the cytoplasm of all the cell layers of the epidermis, from the stratum germinativum to the stratum corneum, the only exception being the basement membrane that remains negative (Figure 2b). In contrast, the expression of A and B antigens is diminished, and only a few scattered cells in the most external cell layer of the stratum granulosum remain positive (Figure 2c).

A dissociation between the expression of H and A or B was also reported in the oral mucosa (Dabelsteen *et al.* 1982). In this mucosa, A or B antigens were found in the upper cell layers, H in an intermediate region and the nonfucosylated precursor of H in the deepest layers. These observations were interpreted as a sequential appearance of more complex oligosaccharides after the cell maturation that takes place during the migration of cells from the germinal layer to the surface of the mucosa in adults. Similar, although more complex, changes in the expression of blood group carbohydrates were later described in fetal oral mucosa (Vedtofte *et al.* 1984). Anti-Lewis reagents are negative on the epithelial cells of the epidermis, except for the inner cell layer of the sweat ducts.

Sweat glands: The sweat ducts express ABH antigens irrespective of the secretor status of the individual and Le antigens only in Le positive individuals. The sweat ducts of the aforementioned H deficient subject (Le Pendu *et al.* 1986a, 1986b) were negative with anti-H and positive with anti-Le. These observations suggest that the ABH antigens expressed on sweat ducts are produced by the combined action of the products of the *H* and *Le* genes.

The sweat ducts of Le^b individuals are positive with anti-Le^b (Figure 2d). Therefore, we have to conclude that the H enzyme can make H type I structures that are transformed into Le^b by the Le enzyme, since the anti-Le^b reagent RO-23 does not crossreact with the type 2 Y structure (Francois *et al.* 1986). Lewis antigens are almost absent from the acinar cells of the coiled sweat glands (Figure 2d). On the contrary, this portion of the sweat gland expresses large amounts of ABH antigens (Figure 2e) which are under the control of the *Se* gene.

In conclusion, the expression of ABH and Le antigens in the skin has three different genetic controls: The epidermis has ABH antigens under the control of the *H* gene; the sweat ducts have ABH and Le antigens under the control of the *H* and *Le* genes and the coiled portion of the sweat glands secrete ABH antigens under the control of the *Se* gene. It is evident that sweat itself must contain a rather complex mixture of ABH antigens under different genetic controls, which may explain the difficulties found in defining the genetic control of the ABH antigens present in sweat. In addition to these epithelial ABH antigens, the vascular endothelium of the dermis has ABH antigens under the control of the *H* gene as in all the other tissues of the human body.

Kidney: Vascular endothelium of glomeruli, intertubular capillaries and larger blood vessels have ABH antigens (Figure 2f, green) under the control of the *H* gene and independent of the *Se* gene. Some distal convoluted tubules also express Le antigens under the control of the *Le* gene (Oriol *et al.* 1980b).

The glomerular epithelial cells or podocytes have no ABH antigens, but they are very rich in neuraminic acid. After neuraminidase treatment, they can be easily stained with *Arachis hypogaea* (peanut agglutinin) or *Helix pomatia* lectins (Figure 2f, g, red). These lectins also stain the apical pole of the epithelial cells of the ascending limb of the Henle loop, the distal convoluted tubules including the macula densa (Figure 2f, g, red) and the collecting tubules (Figure 2i, red). Unlike the podocytes, the tubular cells can be labeled with these lectins by direct staining without any prior enzymatic treatment (Figure 2h, i, red).

Figure 2g illustrates, in bright yellow-orange, the fluorescence of amyloid deposits in glomeruli of a patient with secondary amyloidosis. The green fluorescence seen in this photograph was obtained with anti-X (SSEA-1 or Le^x), the type 2 isomer of Le^a. This antigen is present in all proximal convoluted tubules (Fox *et al.* 1983) and in the thin descending limb of

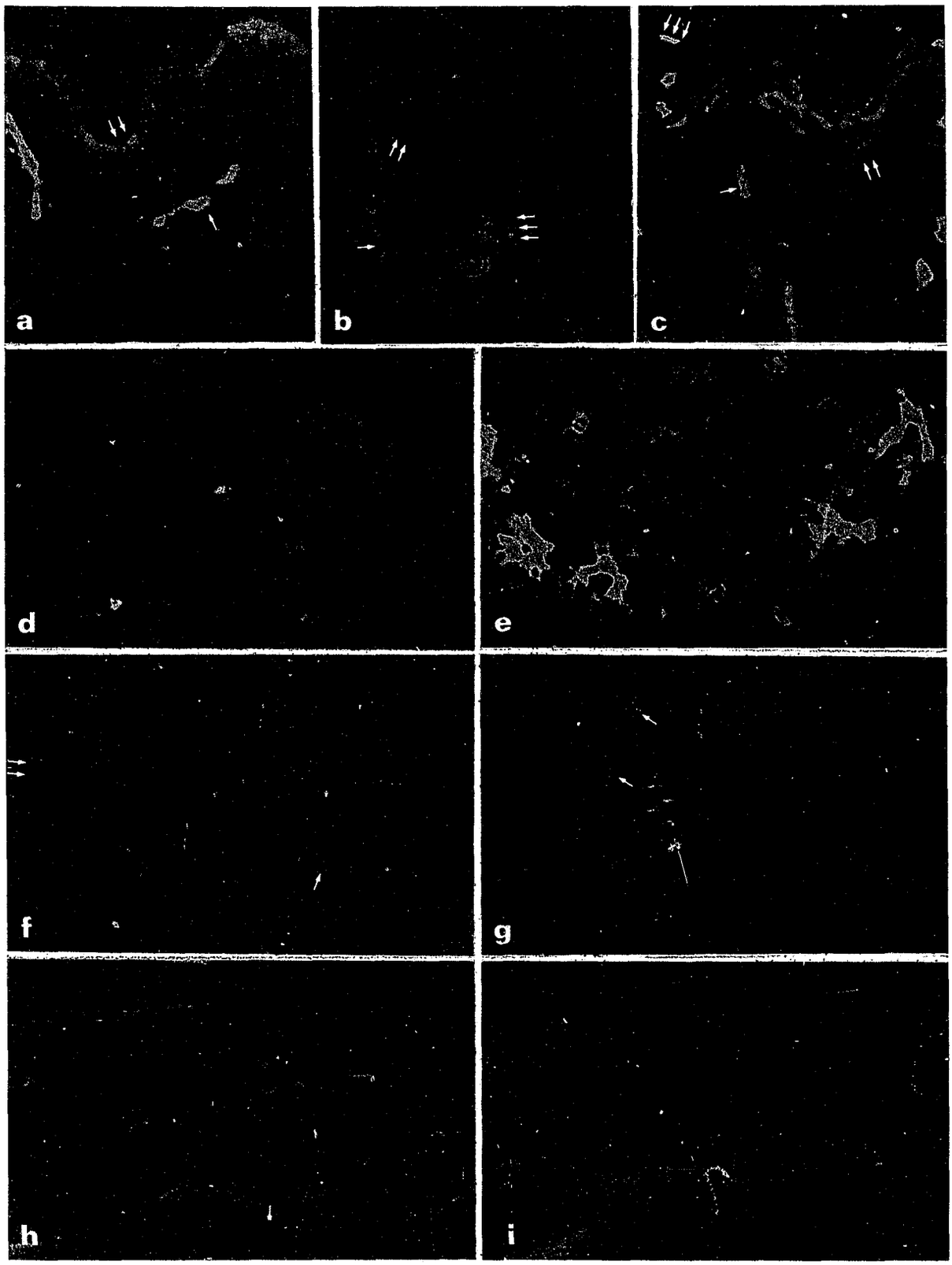
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Figure 2. Polychromatic immunofluorescence staining of formalin-fixed, paraffin embedded human tissues with FITC (green) and TRITC (red) labeled reagents. Stained preparations were mounted with one drop of *p*-phenylenediamine (1 mg/ml in pH 8 buffered glycerol).

Figure 2a. Abdominal skin of an O Le^b individual stained with anti-H (green). Vascular endothelium of capillaries (arrow) and a few upper cell layers of the stratum spinosum and granulosum (double arrows) are positive. ×250.

Figure 2b. Sole of the feet of a B Le^e individual stained with anti-H (green). Vascular endothelium of capillaries (arrow) and epithelial cells of all cell layers of the epidermis are positive. The basement membranes (double arrow) are negative. Ducts of sweat glands (triple arrows) are positive. ×250.

Figure 2c. Same tissue sample as figure 2b stained with anti-B (green). Most of the deep epidermal cells are negative. Only a few scattered cells in the surface of the stratum granulosum are positive (double arrows). Vascular endothelium (arrow) and sweat ducts (triple arrows) are positive. ×250.

Figure 2d. Sweat gland in the dermis of an O Le^b individual stained with anti-Le^b (green). The inner cell layer of epithelial cells of the ducts is positive. Acinar cells on the coiled portion of the gland are negative. ×250.

Figure 2e. Same tissue sample as figure 2d stained with anti-H (green). Both ductal and acinar cells are positive. ×250.

Figure 2f. Cortex of the kidney of an A2 Le^a normal donor treated with neuraminidase (*Vibrio cholera*) and stained with anti-A (green) and *Helix pomatia* (red). Capillaries and some distal tubules (arrow) are positive with anti-A. Podocytes and the apical areas of epithelial cells of ascending Henle loops (double arrow) and distal convoluted tubules are positive with the *H. pomatia* lectin. Proximal convoluted tubules are negative with both reagents. ×100.

Figure 2g. Neuraminidase treated kidney of an AB Le^b patient with amyloidosis. Podocytes and distal convoluted tubules are stained red with the *Helix pomatia* lectin. Proximal convoluted tubules are stained green with anti-X. Amyloid deposits are stained in bright yellow-orange with *p*-phenylenediamine. ×250.

Figure 2h. Medulla of a normal kidney of a blood group O Le^b donor stained with anti-X (red) and anti-uromucoid (green). The descending limbs of the Henle loops are positive with anti-X and the ascending limbs of the same loops are positive with anti-uromucoid. The collecting ducts (arrow) are negative. ×250.

Figure 2i. Same tissue sample as figure 2h stained with anti-uromucoid (green) and *Helix pomatia* (red). The cytoplasm of distal convoluted tubules is stained with anti-uromucoid. The apical zone of the epithelial cells of both distal convoluted and collecting tubules is stained with *H. pomatia.* The bright yellow fluorescence of the inner border of distal convoluted tubules is due to the superimposition of both red and green florescence stains in this zone. Collecting ducts are only stained with *H. pomatia.* ×400.

the Henle Loop (Figure 2h, red). The X antigen is also present in circulating leukocytes (Figure 2g, arrows, green). Finally, antibodies against uromucoid or Tamm-Horsfall urinary mucoprotein stain specifically the cytoplasm of the ascending limb of the Henle loop (Figure 2h, green), and all the distal convoluted tubules but are negative on collecting ducts (Figure 2i). Consequently, with a judicious choice of reagents, all the different segments of the nephron can be easily and selectively stained.

Liver: Type 1 and type 2 ABH antigens are expressed in different cells. Type 2 ABH antigens, independent of *Se* and *Le* genes, are found on vascular endothelium and red cells (Figure 3a, green). Type 1 ABH and Le antigens, under the control of the *Se* and *Le* genes, are synthesized by the epithelial cells of the biliary ducts (Figure 3b, green) and the gallbladder. Therefore, the biliary ABH antigens are only found in ABH secretors, and they are particularly abundant in Le negative secretors. Lewis positive secretors have most of their ABH epitopes as the ALe^b, BLe^b or Le^b and are not detected with the usual anti-A or anti-B reagents, which react poorly with the difucosylated structures. The biliary cells of Le positive ABH nonsecretor individuals express mainly the Le^a antigen, which cannot be transformed into A or B epitopes by the corresponding A or B glycosyltransferases.

Normal hepatocytes do not express ABH antigens. However, membrane and cytoplasmic H antigen has been found in some hepatocytes in areas close to the portal tracts in patients with hepatocarcinomas or cirrhosis (Oriol 1983).

Figures 3a and 3b also illustrate two types of amyloid deposits. Vascular deposits in a patient with systemic amyloidosis are stained red (Figure 3a). Interstitial amyloid deposits in the conjunctive tissue of the portal tract of a patient with a primary hepatocarcinoma are stained yellow (Figure 3b). For any given patient, the deposits in different organs have the same staining properties. However, from one

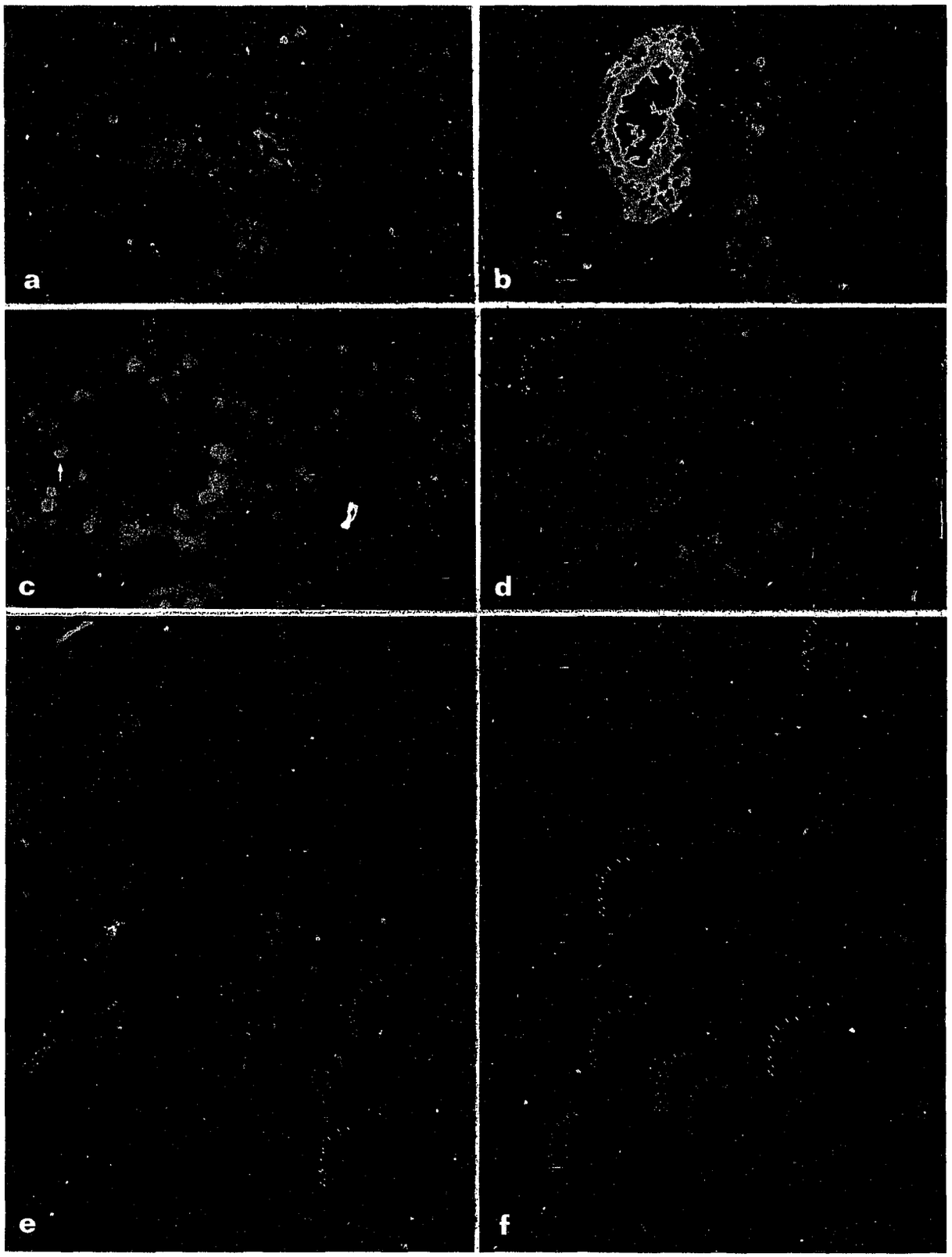
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Figure 3. ABH antigens.

Figure 3a. Liver of an O Le^b patient with systemic amyloidosis, stained with anti H (green). Vascular endothelium and red cells are positive. The bright orange-red fluorescence corresponds to the perivascular amyloid deposits (arrow). ×250.

Figure 3b. Liver of an O Le^b patient with a primary hepatocarcinoma. The section is stained with anti-Le^b (green) Epithelial cells of biliary ducts are positive with the anti Le^b. The bright orange fluorescence spots are amyloid deposits in the border of the portal tract. Hepatocytes are negative in both Figures 3a and 3b ×250.

Figure 3c. Primary sensory neurons of the posterior root ganglia of a B Le^b individual stained with anti-B (green). The Golgi apparatus (arrow), the cell membrane and the cytoplasm are strongly positive. ×1000.

Figure 3d. Brunner's glands of a blood group A Le^a donor stained with anti-A (green) and the *Ulex europaeus* lectin 1 (red). The superimposition of the green and red fluorescent stains shows the "Harlequin" pattern with alternate and complementary positive and negative cells and glands. About half of the cells secrete A antigen and the other half secrete Y antigen which is recognized by the *U. europaeus* lectin 1. ×100.

Figure 3e. Pyloric mucosa of an A Le^b individual stained with anti-Le^b (green) and the lectin 1 of *Ulex europaeus* (red). Most mucous cells from the proliferative compartment up to the surface epithelium are positive with both reagents, whereas the deep gastric glands secrete only the type 2 disfucosylated structure (Y) recognized by the lectin 1 of *U. europaeus*. ×130.

Figure 3f. Duodenal villi of an A Le^d subject stained with anti-A type 3 (green) and the lectin 1 of *Ulex europaeus*. Only the Golgi cysternae are positive with anti-A type 3, whereas the secreted mucus, the goblet cells and the Brunner's glands (lower right angle) are stained with the *U. europaeus* lectin 1. ×300.

patient to another, the color may vary from bright yellow to red, and the different color stains have not yet been correlated with any particular form of amyloidosis.

Nervous system: Tissue from the nervous system has been classically thought to be devoid of ABH antigens. However, some primary sensory neurons express ABH antigens independent of the control of the *Se* and *Le* genes (Mollicone *et al.* 1986a). The pseudo-unipolar neurons of the posterior root ganglia (Figure 3c) have ABH antigens in the Golgi cisternae, the membrane and the cytoplasm. This positive reaction extends centrally to the final synapsis in the substantia gelatinosa of the posterior horn of the spinal cord and peripherally to the sensory receptors.

Neurons of sympathic and parasympathic ganglia (Oriol *et al.* 1984b) and the neurons of the mesencephalic nucleus of the trigeminal nerve (Mollicone *et al.* 1986a) are also positive. Some of the primary sensory neurons of the cranial nerves also synthesize ABH antigens, that is, olfactory, auditory (Mollicone *et al.* 1985a) and taste receptors. All these sensory cells, expressing ABH antigens, are derived from ectodermal placodes or from the neural crest. By contrast, sensory cells derived from the central nervous system, for example, the optical receptors of the retina, have no ABH antigens at all. It is interesting to note that the epithelial and endothelial cells of the cornea, which have no sensory function but are derived from the ectoderm directly overlying the optic vesicle, do express ABH antigens (Salisbury and Gebhardt 1981). The rest of the central nervous system and all the connecting and motor neurons of the peripheral nervous system are devoid of ABH antigens.

Digestive mucosa: All the epithelial cells from the oral to the anal mucosa express some sort of blood group-related oligosaccharide antigens. Most of them are ABH and Le antigens under the control of the *Se* and *Le* genes. However, some exceptions to this general rule have been observed.

The mucosa of the gastroduodenal junction was the first portion of the digestive tract that was carefully studied for the presence of ABH antigens using immunofluorescence (Glynn *et al.* 1957). The Brunner's glands and the deep gastric glands express AY, BY or Y antigens, according to the respective ABO blood group of the individual. These antigens are independent of the *Se* and *Le* genes. A and B individuals give a patchy pattern (Figure 3d) when stained with anti-A or anti-B (green) and anti-Y (red). This pattern is called "Harlequin" because it resembles the multicolored costume of this character from the Italian theater (Mollicone *et al.* 1986b). By contrast, blood group O individuals give a uniform staining in the same areas with anti-Y. These observations demonstrate that the Harlequin pattern is due to a selective activation of the genes controlling the A and B glycosyltransferases in about half of the mucous cells, whereas the other half continue to produce only the Y precursor chain.

The surface epithelium of the duodenum and the pylorus express both type 1 and type 2 antigens under the control of *Se* and *Le* genes (Mollicone *et al.* 1985b). Figure 3e shows the double staining that is

obtained with anti-Le^b (type 1 green) and anti-Y (type 2 red) at the surface of the pyloric mucosa, in contrast with the single red stain given by the anti-Y in the deep gastric glands. In addition to these difucosylated ABH antigens, both pyloric surface epithelium and the duodenal mucosa also express monofucosylated type 1 and type 2 blood group antigens that are not found in the deep gastric or Brunner's glands. This rather complex pattern of expression of ABH antigens can be interpreted using a simple model that assumes the existence of two main differentiation pathways (Mollicone *et al.* 1985b). Starting from the proliferative compartment, the epithelial cells can migrate in two opposite directions. According to this model, the differentiation of the cells, migrating towards the surface, results in the derepression of the fucosyltransferases that are controlled by the *Se* and *Le* genes and the synthesis of blood group antigens of type 1 and type 2 under the control of the *Se* and *Le* genes. On the contrary, the differentiation of the cells migrating down towards the deeper areas of the glands would derepress the synthesis of the fucosyltransferases under the control of the *H* and *X* genes and produce only type 2 blood group antigens independent of *Se* and *Le* genes.

The remainder of the small intestine expresses ABH and Le antigens under the control of *Se* and *Le* genes as does the surface of the pyloric and duodenal mucosa. The cecum and the ascending and transverse portions of the colon express these antigens under similar genetic controls. In the mucosa of the descending and sigmoideum colon, there is a progressive diminution of the expression of ABH and Le^b antigens. These antigens are practically absent from the normal rectal mucosa (Wiley *et al.* 1981). However, the Le^a antigen persists throughout all the colonic mucosa, and other Le-related antigens (of as yet unidentified structure) appear in this area (Macartney *et al.* 1986).

Pancreas: The ABH and Le antigens have also been found in acinar cells and epithelial cells of secretory ducts of the exocrine pancreas (Rouger *et al.* 1981).

Type 3 and Type 4 ABH Antigens

These structures were described in red cells (Takasaki *et al.* 1978) and saliva (Donald 1981). Since then, they have been found in all cells expressing ABH antigens, but they seem to be restricted to the area of the Golgi apparatus (Figure 3f, green fluorescence). This observation suggests that the type 3 and 5 structures might be masked by the elongation of the oligosaccharide chains by addition of other sugar units in the Golgi cisternae (Le Pendu *et al.* 1986b). The expression of type 3 and 4 H antigens is independent of the *Se* gene (Clausen *et al.* 1986). The A type 3 (Clausen *et al.* 1985) and the A type 4 (Clausen *et al.* 1984) antigens are not found in erythrocytes of A2 individuals, and they seem to contribute to the A1-A2 difference that is independent of the expression of type 1 and type 2 A structures (reviewed in Oriol *et al.* 1986).

Finally, an A type 4 heptaglycosylceramide has been isolated from human kidneys and represents the major blood group-related glycolipid structure of this organ (Breimer and Joval 1985; Breimer and Samuelsson 1986).

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DISCUSSION

Tomaso: Are you saying there are no AB or H receptor sites on red blood cells other than human red blood cells?

Oriol: The ABH receptor sites are on red blood cells of humans, gorillas and orangutans, but these ABH receptor sites do not appear on red blood cells of baboons.

Tomaso: For example, there is an antigen in dogs called Tr which is closely related.

Oriol: The antigens are in the animals. The red blood cell has antigens coming from the ectoderm and entoderm. The intrinsic structures present on the red blood cells are not present.

Tomaso: It is fairly easily detected by adsorption-elution techniques.

Oriol: Yes, in the same way that lymphocytes or red blood cells are detected. These kinds of ABH coming from an exocrine secretion and going through the plasma, as sphingolipids do, are present in all mammals.

Konzak: Can you recommend any review articles on this topic?

Oriol: Vox Sanguinis, in the latter part of 1986, has an editorial on this subject.

Schanfield: If one used a monoclonal antibody or any antibody to the Le^c structure, would there be a confirming test for a negative Le^a negative, Le^b negative saliva, that in fact you have adequate salivary material in your stain?

Oriol: Yes, that is a good point. You must consider that each molecule, when under control of Le secretors, is the result of the interaction of all the genes. When the gene is absent and you get a Le negative, the structure is there and you get the precursor, so the antibody is specific for the precursor. You should get a positive reaction for the molecule that is negative.

Schanfield: Have you quantitated in nonsecretor saliva approximately the same levels of the precursor structure?

Oriol: That is a problem because Le^c is a tricky structure. I think there are only a few antibodies in the world that react with this structure. We have one that is polyclonal and another one that is monoclonal. This structure contains the type 1 naked precursor, plus a type 2 X, because you can be negative for Le in secretor but positive for X. Therefore, it is an antibody that reacts with both, with the X that is a polymorphic system. We have found four people who are X negative. Now we can define these in serum and in tissues with specific antibodies. Be careful where you obtain anti-C because it is one of the most common monoclonals. The SSEA1 that was described by the Wistar Institute is an anti-X, and there are many antibodies raised against tumor cells, which have the X specificity. Now X is present from the proximal convoluted tubule of the kidney, from the polymorphonuclears, of all sorts of leukocytes. There are X positive people and X negative people. It is a new polymorphism that can be added to the battery of tests that we have.

Steane: There has been a discussion for several years as to the relative quantity of intrinsic versus extrinsic ABH substance in the red blood cell. Now you have these nicely adsorbed antibodies. Can you tell us the answer to this?

Oriol: I will say that more than 90% are intrinsic structure made by the erythroblast and will stay forever in the membrane. The percentage of the adsorbed molecule is about 5%–10%, so the ABH adsorbed is a very small amount.

Errera: Are the Le X and Le Y structures on type 2 chains under the genetic control of the *X* gene or the dominant *Le* gene?

Oriol: You can have both. Sometimes two enzymes can make the same final structure and sometimes the same enzymes can make two different structures.

Errera: Liquid blood is phenotyped Le^a negative or Le^b negative. Based on the serum that you discussed, are you saying the possibility exists to determine these Le X and Le Y structures adsorbed to red blood cells and infer secretor status from that?

Oriol: It is possible, but the amount of Le X and Le Y adsorbed is going to be very small, and you need very potent reagents to do that. This is not routine work. There will be a way to determine secretor status because you will get more anti-Y in secretors than in nonsecretors.

Errera: Are you aware of any situations in which Bombay secretors will produce A- and B-like structures in semen and saliva?

Oriol: Yes. The H deficiency is very rare worldwide, but there are a few places in the world with a fairly high incidence of H deficient individuals, such as Reunion Island in the Indian Ocean. There 1 person in 100 is H deficient.

Sensabaugh: Have you looked at reproductive tract tissues, for example, the vaginal and seminal tract?

Oriol: The study is not yet complete. ABH is not found in the spermatozoa in the testes. The spermatozoa do have AB and H that probably is adsorbed from the secretions later in the seminal tract. Epithelial cells of the seminal duct secrete AB and H, which are found in semen.

BIOCHEMICAL GENETICS AND METHODOLOGY FOR THE FORENSIC ANALYSIS OF HLA ANTIGENS

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Just as the problems resulting from transfusions of unmatched blood stimulated the recognition of the ABO system, so the rejection of unmatched skin grafts stimulated the discovery of the human leukocyte antigen (HLA) system (Cohen and Collins 1977). Nobel prize winner Jean Dausset described the first HLA antigen in 1958 (Dausset 1958), and 103 factors are now assigned to 7 closely linked regions called the A, B, C, D, DR, DQ and DP loci within the major histocompatibility complex on chromosome 6 (Table 1). An official World Health Organization Committee on HLA nomenclature numbered each internationally recognized antigen, gave a provisional W number to the less well-defined antigens and described subspecificities by including the bracketed major specificity. Since each individual has two antigens at each locus, the potential polymorphism of the system is 7–8 million phenotypes.

The relevance of this extreme HLA polymorphism has been applied to many clinical problems, for example, its role in the immune system (Green 1974). The association of many established diseases with HLA phenotypes (Grumet 1977) and the association of tissue typing with successful organ transplantation (Terasaki *et al.* 1978) are of particular value in paternity investigations (Mayr 1974). The system fulfills the basic requirements for such application because the mode of inheritance is well established, the testing techniques are reproducible and the markers remain constant throughout life.

The chance of exclusion in false accusations of paternity by testing for the gene products of the HLA–A, –B and –C loci alone is 96%. If red cell antigens such as ABO, MNSs, Rh, K, P, Fy, Jk, Lu, Xg and Se, polymorphic enzymes such as erythrocyte acid phosphatase (EAP), AK, ADA, phosphoglucomutase (PGM), GPT, EsD and GLO and serum factors such as haptoglobin (Hp), group specific component (Gc), gamma marker (Gm), kappa marker (Km) and Bf are all also determined, the chance of exclusion rises 3%– 99.9% (Mayr *et al.* 1979). So in practical terms, the scarcity and cost of reliable HLA antisera are balanced by the efficiency of the system for clarifying problems of disputed paternity.

BIOCHEMISTRY AND SEROLOGY

Antigens

The HLA antigens are found on the cell membrane of all nucleated cells as well as in soluble form in body fluids (Berah *et al.* 1970). Erythrocytes that do not carry HLA antigens number 4,500,000–6,000,000/ml of whole blood in comparison with platelets that number 200,000–400,000 and with leukocytes that include granulocytes, monocytes and lymphocytes and number 5,000– 20,000/ml of whole blood, about 1/20 the concentration of red cells.

The HLA–A, –B and –C antigens are glycoproteins comprising two polypeptide chains: a heavy chain and a light chain (Figure 1). The light chain is β-2-microglobulin ($\beta 2\mu$), a 12,000 molecular weight protein homologous to an immunoglobulin domain. It is coded for by a gene on chromosome 15 and is not polymorphic (Zeuthen *et al.* 1977).

The C terminal portion of the molecule extends through the cell membrane such that a hydrophobic sequence is surrounded by hydrophilic regions (Strominger *et al.* 1977). Papain cleavage releases the antigen from the membrane to produce a soluble serologically active molecule. There is evidence that the antigens are in fact constantly released from the membrane and resynthesized (Appella *et al.* 1977). The serologically detectable HLA determinants are carried on the heavy chain that has a molecular weight of about 44,000 and is coded for by genes on chromosome 6. The chains are not covalently bound, and the serologic activity does not depend on their association. Determination of the sequence of the 22 *N*-terminal amino acids has demonstrated striking similarity among antigens A1, A2, B7, B8, B12, B13 and B14. Heavy chains with A2 or B7, B14 specificities differ at only one position, 9.

There seems to be a structural difference between the antigens of the A and B loci, since crossreacting HLA antibodies directed against several A or against several B antigens have been found but not directed against antigens of both series at the same time (Shreffler *et al.* 1971). The basic structure of HLA–DR antigens differs significantly from those of

Table 1. LOCI ON REGIONS OF THE HUMAN LEUKOCYTE (WITH THE KIND PERMISSION OF DR. W. MAYR, WEIN)

HLA-A	HLA-B	HLA-B	HLA-C
A1	B5	Bw48	Cw1
A2	B7	B49 (21)	Cw2
A3	B8	Bw50 (21)	Cw3
A9	B12	B51 (5)	Cw4
A10	B13	Bw52 (5)	Cw5
A11	B14	Bw53	Cw6
Aw19	B15	Bw54 (w22)	Cw7
A23 (9)	B16	Bw55 (w22)	Cw8
A24 (9)	B17	Bw56 (w22)	
A25 (10)	B18	Bw57 (17)	
A26 (10)	B21	Bw58 (17)	
A28	Bw22	Bw59	
A29 (w19)	B27	Bw60 (40)	
A30 (w19)	B35	Bw61 (40)	
A31 (w19)	B37	Bw62 (15)	
A32 (w19)	B38 (16)	Bw63 (15)	
Aw33 (w19)	B39 (16)	Bw64 (14)	
Aw34 (10)	B40	Bw65 (14)	
Aw36	Bw41	Bw67	
Aw43	Bw42	Bw70	
Aw66 (10)	B44 (12)	Bw71 (w70)	
Aw68 (28)	B45 (12)	Bw72 (w70)	
Aw69 (28)	Bw46	Bw73	
	Bw47		
	Bw4, Bw6 } broad specific antigens		

HLA-D	HLA-DR	HLA-DQ	HLA-DP
Dw1	DR1	DQw1	DPw1
Dw2	DR2	DQw2	DPw2
Dw3	DR3	DQw3	DPw3
Dw4	DR4		DPw4
Dw5	DR5		DPw5
Dw6	Drw6		DPw6
Dw7	DR7		
Dw8	DRw8		
Dw9	DRw9		
Dw10	DRw10		
Dw11 (w7)	DRw11 (5)		
Dw12	DRw12 (5)		
Dw13	DRw13 (w6)		
Dw14	DRw14 (w6)		
Dw15	DRw52		
Dw16	DRw53		
Dw17 (w7)			
Dw18 (w6)			
Dw19 (w6)			

the HLA–A, –B and –C antigens. The HLA–DR molecule is also composed of two chains. They are covalently bound and are of similar molecular weight: 35,000 and 28,000 (Figure 2).

Genetics

The observed recombination frequency between the HLA–A and HLA–B loci is 0.8% (Waltz and Rose 1977). Certain combinations of A and B alleles occur at much higher frequencies, whereas other combinations occur at much lower frequencies than would be expected. This unique phenomenon is called linkage disequilibrium (Table 2). It was apparent at the Histocompatibility Workshop in 1975 (Kissmeyer–Nielsen 1975) that some of the allele frequencies varied quite considerably depending on which specific population was tested. For example, A1 and

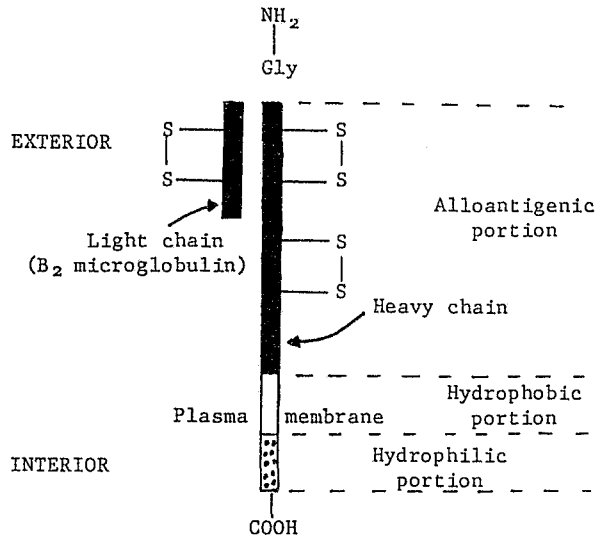

Figure 1. Diagram of an HLA-A, B or C Antigen. Modified from Strominger *et al.* 1977.

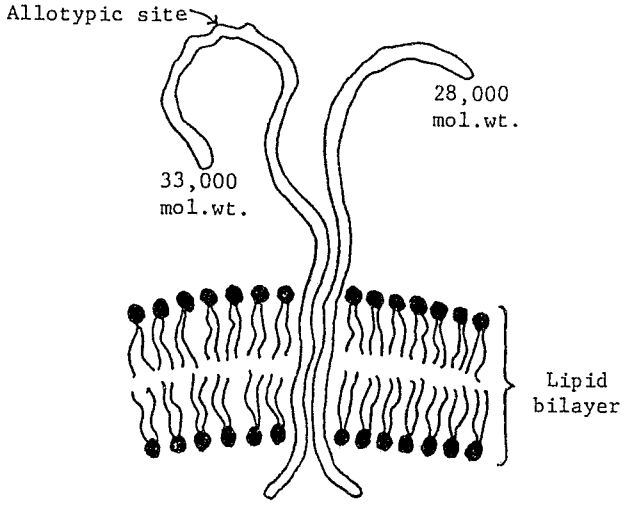

Figure 2. Diagram of an HLA-DR Antigen. Modified from Barnstable 1978.

Table 2. LINKAGE DISEQUILIBRIUM IN THE HLA SYSTEM AS ILLUSTRATED BY EXPECTED AND OBSERVED HAPLOTYPE FREQUENCIES

	Expected	Observed
A1B7	0.0196	0.0072
A1B8	0.0160	0.0815
A3B8	0.0153	0.0016

A3 are characteristic of caucasians and rarely found in orientals or blacks.

The HLA–A9 is among the most common antigens at the A locus. It has been subdivided into two specificities, W23 and W24, but broad antisera that react with both specificities are available. The A9 occurs with a frequency of about 20% in the European population, although international frequencies vary from 8.6% in a Scottish population to 97.8% in the New Guinea Highlands. The W23 ranges in frequency from not detected in Lapps and Pakistanis to 21% in the A9 West Indian population, with W24 accounting for the remaining 80%–85%. The A28 is identified 10 times as often in blacks as in caucasians or orientals. Similarly, at the B locus, B5 is characteristic of orientals, B12 of caucasians and B17 of blacks, although the differences in frequencies are not pronounced (Table 3).

Crossreactions

Each antigen of the HLA–A, –B and –C loci can be defined. However, certain HLA antigens seem to be closely related serologically as a result of striking similarities in their molecular structure. Consequently, some antigens are recognized to varying degrees by antisera against other antigens at the same locus. These crossreactions have been carefully documented by numerous laboratories and are a well–established phenomenon in HLA typing. Attempts to remove the crossreactivity from an antiserum by adsorption with a cell carrying the crossreactive antigen result in a substantial loss in antibody titer.

Professor Mayr has classified the crossreactions into three categories according to the frequency of their occurrence: rare, frequent or very frequent (Figures 3 and 4). Although the crossreactions can be anticipated, they cannot be predicted as they do not necessarily occur. Each antiserum may differ in its particular antigen reaction patterns so the results with at least two antisera should be compared for each antigen specificity to be typed.

Table 3. HLA ALLELE FREQUENCIES IN MAJOR RACES

	Caucasians	Orientals	Blacks
A1	0.14	0.02	0.05
A3	0.14	0.02	0.05
Aw24	0.08	0.23	0.02
Aw30	0.02	0.02	0.20
B5	0.06	0.19	0.04
B12	0.12	0.08	0.09
B17	0.04	0.02	0.18

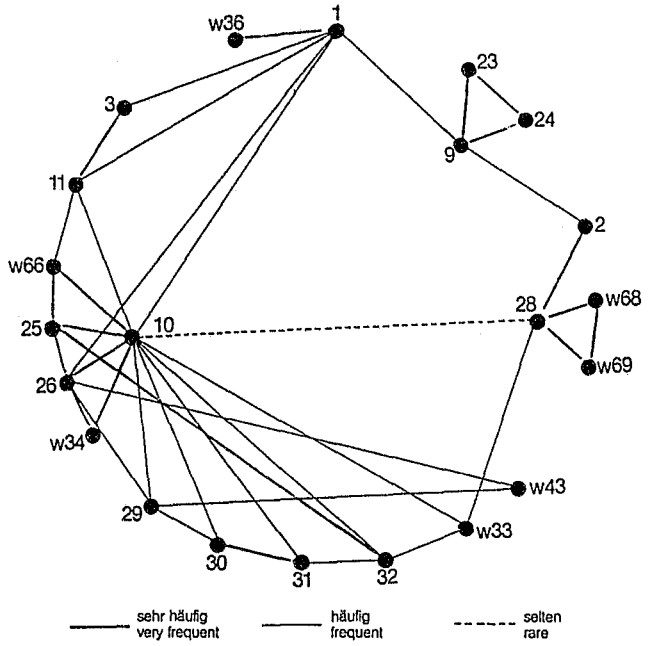

Figure 3. HLA-A Crossreactions.

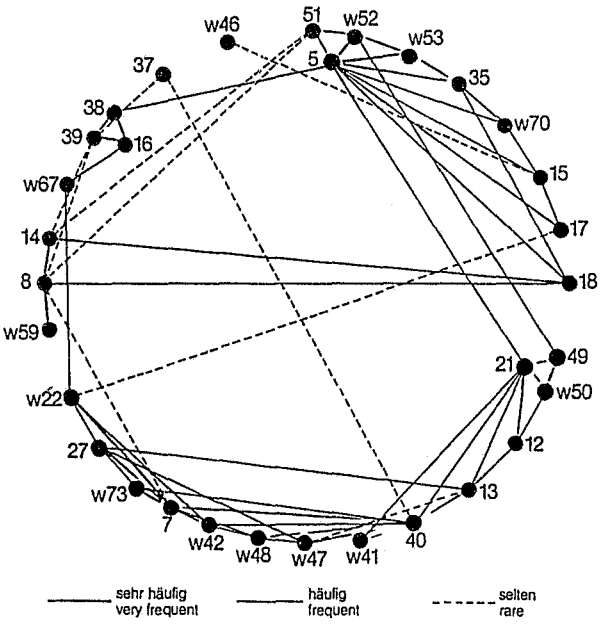

Figure 4. HLA-B Crossreactions.

The Microlymphocytotoxicity Test

An HLA antigen is carried on the surface of a lymphocyte. When its specific antibody is added, an antigen antibody complex is focused on the cell surface. If complement is then added, the complement is fixed, the cascade reaction is activated and the cell membrane is ruptured. This cell can no longer exclude a vital dye.

Terasaki designed a micro technique (Terasaki and McClelland 1964). One microliter of freshly prepared lymphocytes is applied through liquid paraffin to each well of a tray containing 1 μl each of different antisera and left at room temperature for 30 minutes. Complement is added next and left for 60 minutes, then eosin is added, and after 5 minutes, the reaction is fixed by the addition of formalin. All reagents are applied using 50-μl Hamilton microsyringes and multiple dispensers. The entire tray is then flooded with light paraffin oil, covered with microscope cover glasses and read under phase contract microscopy 100X magnification. The percentage of stained or dead cells is estimated (Figure 5).

A population of live lymphocytes is shown in Figure 6, and a mixed population with approximately 60% dead lymphocytes is shown in Figure 7. To establish a control panel, 33 donors were typed using Terasaki trays. These trays contained antisera against 15 A antigens, 21 B antigens and 3 C antigens. The results are shown in Table 3.

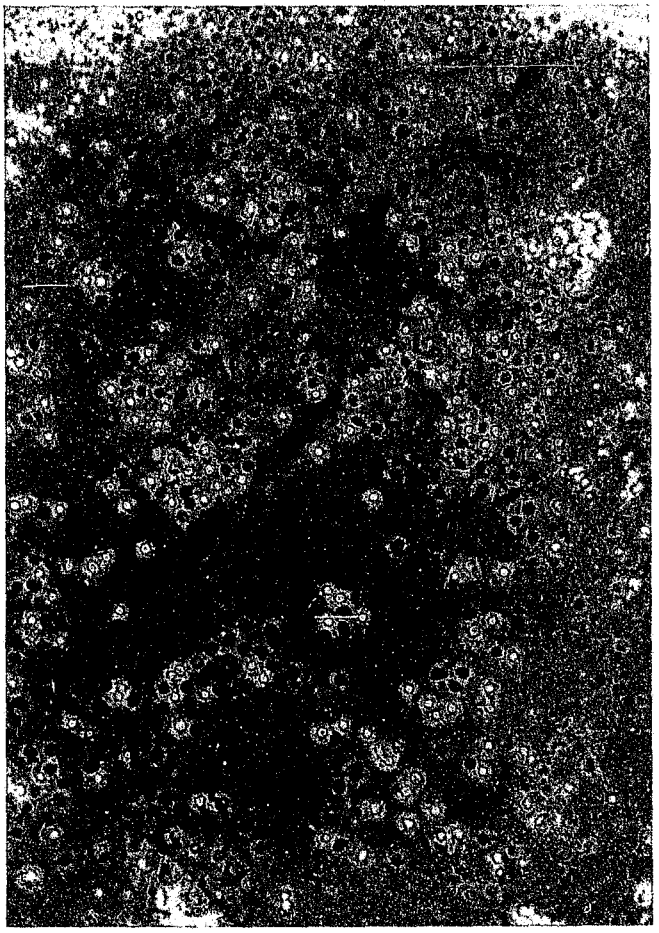

Figure 5. A mixed population of live and dead lymphocytes. Magnification $\times 100$.

Antiserum

The HLA antisera should be obtained from a laboratory that specializes in HLA typing, such as a blood donor center or a tissue typing laboratory for organ transplantation at a major hospital. The antisera should be purchased neat and then titrated in small steps to establish the most sensitive dilution, at best 1:8. They should be stored in small aliquots frozen at –20° C and preferably not refrozen because the titer decreases with repeated freezing and thawing. Antisera that are monospecific with fresh blood are not always monospecific in dried stain tests.

Complement

Rabbit serum is the preferred source of complement and should be designated for use in HLA typing. As it is very labile, it should be stored lyophilized and reconstituted over ice immediately before being used. Any excess must be discarded. As the titer of an antiserum may change when the complement pool is changed, large quantities should be purchased at one time.

Lymphocytes

Target lymphocytes must be used within 24 hours of collection. All four HLA–A and –B specificities must be known. The donor must be in good health because certain viral infections can reduce the number of available HLA antigenic sites by 50% (Ferrara 1977).

Lymphocytes are purified by isopycnic centrifugation. About 7 ml of blood taken by venipuncture into heparin and diluted in saline is layered over Ficoll–Hypaque and centrifuged for 20 minutes at 400 gat the interface. The resulting band of lymphocytes is carefully removed and washed three times. The first wash is slow, 100 rpm, to leave the platelets in suspension as they are rich in HLA antigens.

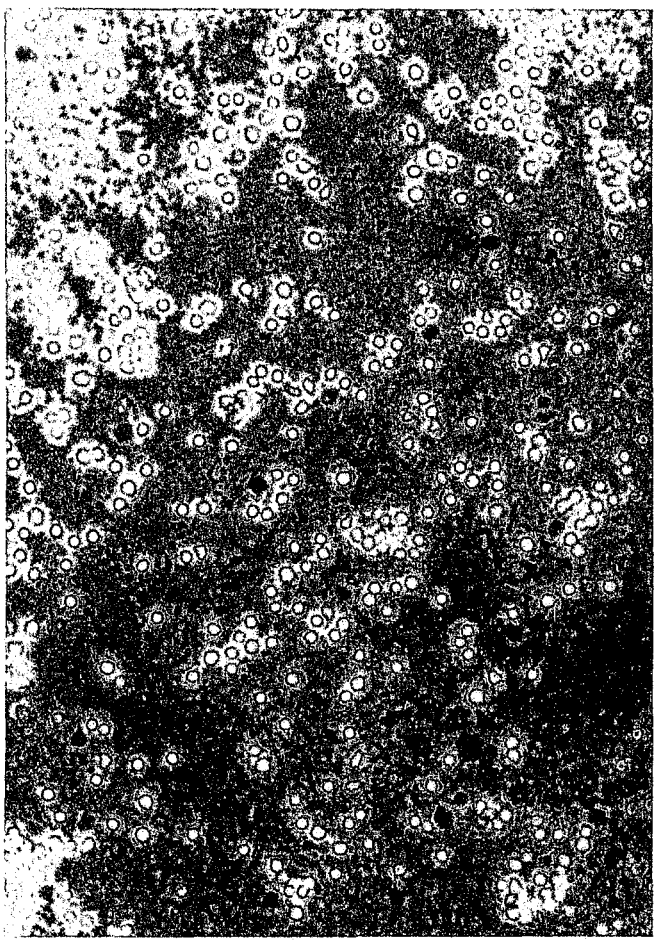

Figure 6. Live lymphocytes. Magnification ×100.

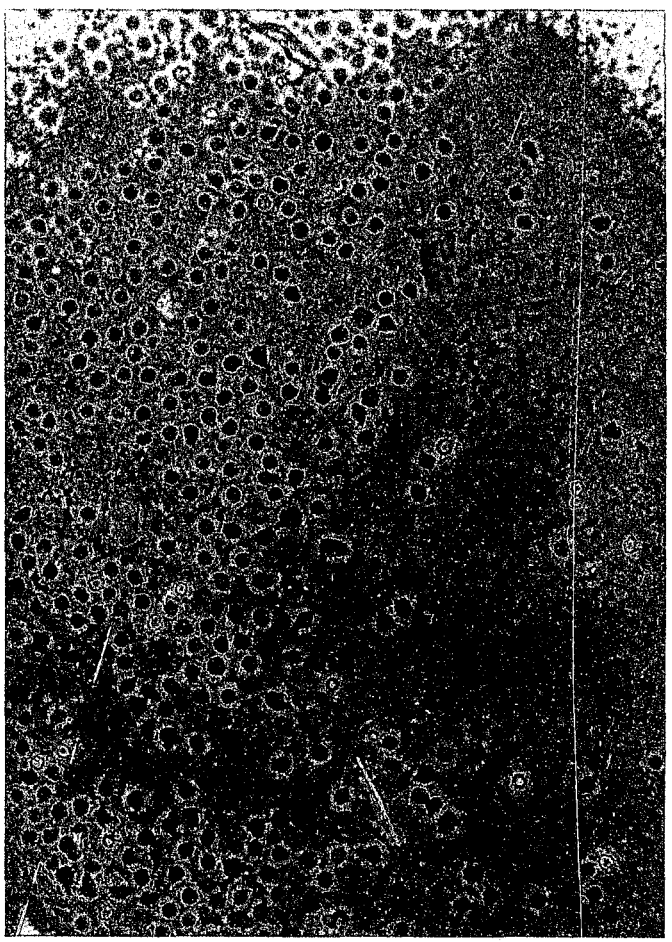

Figure 7. Dead lymphocytes stained with eosin. Magnification ×100.

Unlike red cells for ABO typing, lymphocytes from different sources, regardless of their phenotype, are not interchangeable as target cells unless they have been individually pretitered against that antiserum because the titer of the antiserum changes with different cells. One cannot accurately predict the effects of different doses between homozygous and heterozygous forms of antigen in different target cell donors as the known synergistic effect of certain antigen combinations may act to boost or to suppress the activity of the antiserum.

Washed lymphocytes have been suspended in AB serum or Hank's buffered saline solution (Dick and Crichton 1972), McCoy's modified medium containing fetal calf serum (Newall 1979, 1981) or barbital buffer (Hodge *et al.* 1980; Nelson *et al.* 1983). The working concentration as established in a hemocytometer has varied from 1,000/μl to 2,500/μl in various publications. The viability is established with vital dye. In one laboratory, lymphocytes were stored frozen and then thawed in a programmed liquid nitrogen freezer (Hodge *et al.* 1980) for use as target cells.

The standard tissue typing method of interpreting liquid blood HLA typing tests for the A, B and C loci antigens is to briefly examine each well under phase contrast 100X magnification with an inverted microscope and estimate the percentage of dead lymphocytes. Scores of 1–8 are assigned (Terasaki and McClelland 1964).

1 = 0%–20% cell lysis – negative reaction
2 = 20%–40% cell lysis – doubtful negative
4 = 40%–60% cell lysis – doubtful positive
6 = 60%–80% cell lysis – positive
8 = 80%–100% cell lysis – strong positive reaction

We (Newall 1979) read according to three simple categories:

0%–45% cell lysis = inhibition = positive reaction
45%–55% cell lysis = uninterpretable reaction
55%–100% cell lysis = no inhibition = negative reaction

HLA TYPING OF DRIED BLOODSTAINS

Stability

Stastny (1974) demonstrated specific HLA activity in mummified pre–Columbian tissue, but treatment at pH3 or boiling destroys antigenic activity (Heinrich *et al.* 1974). Specificity has been demonstrated after incubation at 50° C in distilled water, in high and low ionic strength salt solution and after treatment with trypsin.

Only the serologically detectable antigens of the A, B and C loci have been studied in dried stains, since antigens of the D loci are detected by mixed lymphocyte culture, a technique requiring live intact lymphocytes not found in dried stains. Since the accepted technique in tissue typing laboratories is complement–mediated microlymphocytotoxicity, most bloodstain studies investigated inhibition of the toxicity by soluble HLA antigen. Although seven laboratory groups have published successful results with HLA grouping of dried bloodstains, a unanimously accepted technique has not yet been adopted because each group introduced their own modifications. Probably the most significant difference was that each group used different antisera. Despite the modifications, most of the studies achieved accuracy rates of 90%–100%.

The two major problems anticipated were the ability to demonstrate adequate sensitivity and absolute specificity. Since there are only 1/20th as many lymphocytes and platelets as erythrocytes per unit volume of whole blood, the potential sensitivity of the procedure was in question. Crossreactions had been described in some detail, so demonstration of the specificity of the test was very important.

Preparative Extraction

Rittner and Waiyawuth (1974) published a report of the first attempt to identify HLA antigens in dried bloodstains. They hypothesized that the antigens in dried form required activation, so they extracted the stains in saline and incubated the extracts at 37° C. They then compared the inhibiting activity of four quantities of extract (0.5, 1.0, 1.5 and 2.0 μl), each with 1 μl of titrated antiserum in the lymphocytotoxicity test. They considered the modification of adding complement and target lymphocytes at once but rejected it as it decreased the sensitivity. Two monospecific antisera, anti–A1 and anti–B7, and four multi–specific antisera, pretitrated to establish the 95% cytotoxicity point, were tested with bloodstains from 18 donors. The investigators concluded that, with extracts of 1.0 μl, inhibition of more than 70%, although not often observed, could always be attributed to a specific reaction. However, they found that the high hemoglobin levels in the 1.5 and 2.0 μl extracts obscured the target cells, whereas with smaller quantities, the extracts often contained insufficient quantities of HLA antigens to cause significant inhibition of the antiserum. They reported that dried stains of whole blood had higher quantities of HLA antigen than did

washed red cells, crude hemoglobin, pure lymphocytes or fresh serum.

Simultaneous Adsorption–Extraction

Takasugi and Akira under the direction of Terasaki (Takasugi *et al.* 1974) were the first to investigate the persistence of HLA antigens in dried blood clots using a direct adsorption technique rather than preparative extraction. As their aim was merely to establish the fact that specific HLA activity could be demonstrated, they did not attempt to develop a technique for routine practical application. Four to five increasing quantities of clot from 2 to 10 mg were required, each tested against two dilutions of antiserum, a rather formidable approach to casework for a polymorphic system of so many antigens. Four specificities at the A locus, 1, 2, 3 and 9 and B7 were studied in 33 dried blood samples.

Profound crossreactions were encountered in which dried stains lacking an antigen inhibited antiserum against that particular antigen to a greater degree than did stains containing the antigen. Several blood samples adsorbed all of the antisera regardless of the labeled specificity of the antisera and the known phenotype of the sample. However, the investigators accomplished their aim and demonstrated quite conclusively that when specific HLA antigen activity was present, it could be retained in dried blood for at least 1 month.

Newall (1979) found that using Rittner and Waiyawuth's (1974) preparative extraction and activation technique to test for HLA–A2, 70% accuracy at best could be achieved. More important, it was impossible to differentiate between the true and false positives or the true and false negatives.

The literature suggested that HLA antisera were extremely labile, could be titrated only on cold plates, could be thawed for very short periods of time and quickly lost their cytotoxic properties. Yet an extractive procedure that protected the antiserum did not work well. The A2 antigen was simultaneously extracted and adsorbed from a bloodstain in pretitrated antiserum during an overnight incubation at 4° C. Adsorbed antiserum was placed in the well and covered with mineral oil; the plates were then treated in exactly the same sequence as previously described for liquid blood grouping using appropriate target lymphocytes as established for each antiserum.

Positive identification of an antigen required 50% inhibition. Ten A2 stains (donors 5–10), 2–13 days old, were tested for their ability to specifically adsorb anti–A2. All stains caused at least 64% inhibition of the antiserum (Table 4). Five 2– to 4–day–old stains (nos. 7–16) lacking A2 but containing A9, a crossreactive antigen, inhibited the antiserum from 8% to 35%, and one stain (donor 4) lacking A2, A9 and A28 caused 8% inhibition of the antiserum.

As these results were acceptable, the series was expanded by testing 13–day–old stains from nine A2 donors with two additional anti–A2 antisera. The A2 was identified in 22 of the 26 stains (Table 4). Four stains (9, 14, 21 and 31) did cause less than 50% inhibition, but none of the stains caused less than 50% inhibition of more than one of the three antisera.

The results with crossreacting antigens, represented by donors 3–11 in Table 4, supported the earlier findings. Ten of the 11 stains caused less than 40% inhibition and would be correctly assessed as not containing A2. The one stain (donor 16) which caused 60% inhibition of serum 3849 caused only 13% inhibition of serum 4631. With this one exception, there was no overlap above 50% of the degree of inhibition caused by a stain containing a crossreacting antigen or by a stain containing the antigen against which the serum was directed. As seen with the positive A2 stains, a confirmatory test with a second antiserum of the same specificity would avoid interpretive errors. When negative stains from donors 4 and 30 were tested, four stains caused less than 27% inhibition.

Next, 70–day–old stains from eight A2 donors were tested with the same three anti–2 antisera. Eighteen of the 23 stains caused at least 50% inhibition and were interpreted as clearly positive (Table 5). Stains from two donors (nos. 9 and 14) accounted for four of the five results that were not clearly positive. Unfortunately, unlike the inhibition observed with fresher stains, the comparison of results from these stains with any two of the three antisera would not be sufficient to rule a stain unreadable but rather could falsely confirm a lack of specific inhibition. The interpretation of negative results should be limited to stains less than 10 weeks old with this technique.

The degree of inhibition of anti–2 antisera by crossreactive antigens at 70 days was very similar to that observed in younger stains. Five stains from donors 3 and 19 caused 40% inhibition or less.

An antigen from the B locus, B5, was then tested. Three 2–day–old B5 stains were tested with one anti–B5 serum that was inhibited 67%–71%. To test the activity of antigens known to be within the crossreactive B5 group, stains from donors 5, 6 and 14 with phenotypes B15, B17 and B18 were used. The observed inhibition was only 4%–27%. Two stains from donors 16 and 13 lacking B5 or other crossreactive antigens caused 0% and 7% inhibition, respectively, of the antiserum (Table 6).

Table 4. INHIBITION OF ANTI-HLA-A2 ANTISERA BY 13-DAY-OLD BLOODSTAINS; ONE-STAGE EXTRACTION-ADSORPTION TEST

Donor #	HLA-A Phenotype	Percent Inhibition		
		Serum # 3849	Serum # 4631	Serum # 6080
5	1,2	87	73	80
2	2,9	84	91	93
9	2,W30	64	77	47
12	2,28	91	91	91
13	1,2	73	73	66
14	2,29	47	53	66
21	2,9(24)	NR	24	80
29	2	80	77	87
31	2,W31	47	91	80
3	9(24),10	31	0	0
16	9(24),28	60	13	NR
19	9,28	11	13	24
11	3,9(23)	37	40	11
4	1,10(25)	27	4	4
30	1,3	0	NR	0

Table 5. INHIBITION OF ANTI-A2 ANTISERA BY 70-DAY-OLD BLOODSTAINS; ONE-STAGE EXTRACTION-ADSORPTION TEST

Donor #	HLA-A Phenotype	Percent Inhibition		
		Serum # 3849	Serum # 4631	Serum # 6080
5	1,2	83	87	80
2	2,9	87	93	93
9	2,W30	20	60	40
13	1,2	87	53	53
14	2,29	20	40	77
21	2,9(24)	80	33	67
29	2	93	NR	73
31	2,W31	67	87	87
3	9(24),10	24	40	NR
19	9,28	0	0	27

Table 6. INHIBITION OF ANTI-HLA-B5 ANTISERUM BY 2-DAY-OLD BLOODSTAINS

Donor #	HLA-B Phenotype	Percent Inhibition
3	5,W40	71
4	5,18	67
2	5,12	67
14	12,W15	27
5	7,W17	4
6	18,27	4
16	27,W40	0
13	8,27	7

Then, 18–day–old stains from six B5 donors were tested with a second anti–B5 serum. All stains caused at least 60% inhibition (Table 7). When four stains (nos. 10–14) containing crossreactive antigens were included, the maximum inhibition observed was 40% and the minimum was 27%. Although this value was higher than that observed with A2 crossreactions, it is at least 20% lower than the inhibition observed when stains known to contain B5 itself are tested. Stains from donors 12 and 16, neither of which contained B5 or known crossreactive antigens, caused 33% and 27% inhibition, respectively, values again higher than

Table 7. INHIBITION OF ANTI-HLA-B5 ANTISERUM BY 18-DAY-OLD BLOODSTAINS; ONE-STAGE EXTRACTION-ADSORPTION TEST

Donor #	HLA-B Phenotype	Percent Inhibition
2	5,12	60
3	5,W40	73
4	5,18	80
19	5,W22.1	70
27	5,12	67
30	5.1,8	77
14	12,W15	27
5	7,W17	40
11	18	NR
8	18,W22	40
10	7,W35	40
12	12,W40	33
16	27,W40	27

those previously found (Table 7). This sensitivity may be characteristic of this particular antiserum rather than of the B5 antigenic site, since it was not observed with all anti–B5 antisera.

Seventy–eight–day–old stains from seven phenotype B5 donors were tested next. The results confirmed the earlier observation that HLA antigen is usually readily detectable in older stains, since six of the seven stains caused at least 50% inhibition (Table 8). The stain from donor 30, which at 47% was classified as a crossreactive or weakly positive reaction, was actually BW51, a recently described split of B5, and serum 4958, although sensitive to this antigen in an 18–day–old stain, was perhaps not as sensitive to the 78–day–old antigen.

The sensitivity of the technique was reasonable. The quantity of bloodstained material required for the determination of one antigen was approximately the same as that required for a PGM typing. The stability of both antigen and antiserum as demonstrated in this study was acceptable.

In summary, 113 dried bloodstains, 2–78 days old of known phenotype, were tested for the presence of HLA–A2 and HLA–B5 antigens using a one stage extraction and adsorption technique and the two stage microlymphocytotoxicity test. An overall total of 87% of the stains containing the specificity for which they were being tested were positive, and 97% of the stains lacking the specificity were negative, but when adjusted, the values were 93% correctly positive and 100% correctly negative.

An attempt to identify A9 was made. Bloodstains from 33 donors of known HLA phenotype were aged for 2 weeks at room temperature, and 113 tests were

Table 8. INHIBITION OF ANTI-HLA-B5 ANTISERUM BY 78-DAY-OLD BLOODSTAINS; ONE-STAGE EXTRACTION-ADSORPTION TEST

Donor #	HLA-B Phenotype	Percent Inhibition
2	5,12	60
3	5,W40	80
4	5,18	60
19	5,W22.1	64
27	5,12	73
30	5.1,8	47
32	5,W35	50

conducted for specific reaction with two anti–HLA–A9 antisera. Contrary to findings in previous publications, both antisera were sensitive to A9 in stains from each member of the positive control panel. Since there was no advantage or necessity to confirm a stain's reactivity with a second antiserum, all results were combined (Table 9). As can be seen, the accuracy of the readable results was 97.5% for the 52 stains containing A9 and 100% for the 61 stains containing only antigens other than A9. Although the percentage of results that could not be conclusively interpreted was higher than would be desired, many were a result of failure in technique, and it is hoped that the success rate can be improved. The single false negative result could not be repeated when stains from that individual were reexamined. Crossreactions reported with A9 are A1, A2 and, to a lesser extent, A28. Forty–three stains of blood containing antigens A1, A2 or A28 were tested but had no more effect on either antiserum, within described interpretation criteria, than stains lacking A9.

Aged Bloodstains

Although HLA–A9 was correctly identified in a few stains 3 months old, in 14 stains aged 15 months, only 2 of the known positives could be correctly grouped. No false positive results were observed in either known negative or crossreactive stains (Table 10).

Blood Samples from Casework

Liquid blood samples submitted to this laboratory for comparison purposes are collected in a variety of anticoagulants and may be in transit for several days during which they are often subjected to a variety of temperatures. The lymphocytes are not viable, and often even the red cells are lysed when the samples arrive. Since identification of HLA antigens

Table 9. IDENTIFICATION OF HLA-A9 IN 2-WEEK-OLD BLOODSTAINS

HLA-A9 Group of Bloodstain	Number of Stains	Positive	False Positive	False Negative	Negative	Not Readable
Positive	52	40		1		11
Negative	18		0		16	2
Cross-reactive[a]	43		0		35	8
Total	113	40	0	1	51	21

[a]HLA-A1, A2 or A28

Table 10. IDENTIFICATION OF HLA-A9 IN AGED BLOODSTAINS

HLA-A9 Group of Bloodstain	Age (months)	Number of Stains	Positive	False Positive	False Negative	Negative	Not Readable
Positive	3	2	2				
Positive	15	14	2		9		3
Negative	15	4			2	2	
Cross-reactive[a]	15	4			4		
Total		24	4	0	9	6	5

[a]HLA-A1, A2 or A28

in a bloodstain is of no relevance without known comparison grouping, the detectability of HLA–A9 in these comparison blood samples from casework was examined.

Twenty–four stains made from 12 liquid comparison bloods were assessed. Eight of the stains (33%) significantly inhibited the antisera, and 12 stains (50%) did not. Four of the results were not readable. Although the actual group of the donors was unknown, it was interesting to note that both the degree and frequency of inhibition were similar to results observed in the control panel of known phenotypes (Newall 1981).

The most extensive study was that by Lotterle (1981), who also changed the technique by almost tripling the quantity of bloodstain to which the antiserum was added and tested each stain with six antisera against each antigen. He used fresh lymphocytes only when he found that deep frozen target cells had cell membrane alterations, resulting in changed specificity. He studied four antigens at the A locus (1, 2, 3 and 9) and two at the B locus (7 and 8). He tested 515 stains and reported an unadjusted accuracy rate of 96% for stains 11–30 days old and an overall accuracy rate of 91% with stains up to 100 days old. The greatest source of error was failure to detect, rather than false detection of a crossreactive antigen.

Lotterle assessed each reaction as either positive inhibition or negative inhibition, since the intermediate "indeterminate" range did not significantly improve the accuracy of his results. As no basic data were included, it is difficult to interpret the specific source of the false positive reactions and to determine whether a qualifying statement including crossreactive antigens in recording the results would be helpful in eliminating these false positives.

False positives must either be conclusively excluded or explained by qualifying statements, but false negatives are a common occurrence in bloodstain grouping when aging or partially degraded stains are being tested. Martin (1981) assessed a commercial source of antisera, Biotest. He found his reagents very labile, quoting a decreased lymphocytotoxicity of 25% on overnight standing at 4° C. From his test of HLA–A1 and A2, he concluded that the problems encountered in obtaining active antisera and viable target lymphocytes and producing consistent results in an exacting technique were so great that further exploration of the method was unjustified.

Frozen Lymphocytes

The British team of Hodge *et al.* (1980) also tried simultaneous adsorption–extraction of the bloodstain in antiserum. The modifications they adopted were as follows:

1. Dilution of the antisera and suspension of the target lymphocytes in Complement Fixation Test Buffer (a barbitone buffer).
2. Inclusion of antibiotic in the lymphocyte suspension.
3. Use of frozen lymphocytes as target cells.
4. Assessment of morphologic changes in the lymphocyte cell membrane rather than dye exclusion to score the cytotoxicity reaction.
5. Use of mean inhibition to measure results rather than best assessment.

They studied HLA–A1 first, since A1 crossreacts with A3, A9, A10, A11 and A26. They found significant inhibition with stains containing A3, A26 and particularly A11. Sixty stains from 40 donors were tested against 3 antisera and gave 80%–90% overall accuracy. The stains were up to 230 days old, the oldest stains yet tested. In fact, the incidence of false negative results increased rather significantly after 100 days from 7.7% in the 21– to 100–day series to 17.8% in the 100–days–plus series and with a second antiserum from 5.9% to 42.9% in the 100–days–plus age group. To solve the problem of typing whole blood control samples in which the lymphocytes are no longer viable, they successfully

grouped heparinized whole blood using the inhibition technique.

A second report by the same group (Hodge *et al.* 1981) expanded their series to include A2, B8 and CW3. Failure to identify an A2 stain accounted for 80% of the inaccuracies in an overall accuracy rate of 90% in the identification of A2 stains up to 79 days old using three antisera. They had no success with B8, since the antiserum failed to recognize the antigen in stains known to be positive. With CW3 in stains up to 39 days old, the authors achieved 87% accuracy. All the errors were failure to detect the antigen rather than inaccurate identification of an absent antigen. Crossreactions at the C locus were not described.

It is tempting to speculate that their relatively low rate of success can be accounted for by some specific technical modifications as well as by their choice of the crossreactive antigen A1. Freezing a single batch of target lymphocytes solves the problem of their availability in the application of HLA typing in a forensic laboratory. The variability of lymphocytes from bleed to bleed in one donor is eliminated as is the necessity not only to repeatedly bleed by venipuncture a single donor but also to always have him available within hours of completing the test. However, perhaps frozen lymphocytes are more susceptible to lysis by a partially adsorbed antibody molecule such as might result from its reaction with a crossreactive rather than a specific antigen. Also, the programmable freezing mechanism required to freeze and thaw lymphocytes, yet maintain viability, is standard equipment in few laboratories.

Singular Reagent Application

Nelson and coworkers (Nelson *et al.* 1983) presented an extremely optimistic report on the feasibility of HLA typing dried bloodstains. They included six specificities (A2, A3, A1O, B7, B8 and B14) in their studies but reported results mainly with A3 and B8. Like Hodge *et al.* (1980) and Martin (1981), they suspended the lymphocytes in barbital buffer. They also used the Terasaki technique of adding successive dilutions of antiserum to a constant bloodstain sample, as they found that comparison of results with three dilutions produced clearer and more dramatic results than a calculation of percent inhibition with one quantity of pretitrated antiserum.

Their technique was very different from that used by others. The cytotoxicity test was not performed under mineral oil but was noncumulative, similar to an enzyme linked immunosorbent assay (ELISA). After each incubation step, excess reagents such as antiserum or complement were removed by flicking the plate, and the remaining target lymphocytes were washed with barbital buffer. Their results were quite startling. They observed absolutely no crossreactions and attributed this success to the quality of their antiserum.

Their aging studies for A3 which showed differences with stains older than 19 days may be a reflection of the short 3–hour adsorption–extraction time they used. Their criteria for interpretation may have been less rigid than others. For example, in recording the results for an undiluted anti–B8 antiserum, 15%–20% cell lysis was interpreted as positive inhibition, whereas 20%–30% cell lysis was interpreted as negative inhibition. Nevertheless, they presented a technique and results that, at least for HLA–A3, were absolutely accurate for both positive and negative stains. Perhaps antisera with similar performance characteristics will be described in the near future for the other HLA–A and –B locus antigens.

Monoclonal Antibodies

Hodge *et al.* (1981) published the only investigation to date of dried bloodstains using monoclonal antibodies (MCAs). Their technique required incubation of dilutions of monoclonal anti–HLA–A2 antibodies with bloodstains followed by incubation with radioactive labeled antiglobulin. They found the sensitivity of the method was such that 25 μl of whole blood was required to distinguish between an HLA–A2 positive and an HLA–A2 negative sample. Even a 20 cm bloodstained fiber yielded an insufficient quantity of HLA antigen for differentiation between a positive and negative stain.

ELISA

The most recent development with dried material involved the successful demonstration of HLA–B14 on 4–week–old dried lymphocytes using ELISA. The results published by Bishara *et al.* (1983), although promising, leave several questions unanswered regarding its direct application to bloodstains, as the authors started with purified lymphocytes aged in microplates rather than with whole blood. No mention is made of crossreactions, although B14 very often crossreacts with B8 and often with B18. Nevertheless, the work offers potential, since the increased sensitivity of ELISA and possibility for automation are great advantages over the time–consuming technique of microlymphocytotoxicity, particularly for dried stain analysis.

Isoelectric Focusing

Recent HLA work by Yang *et al.* (1984) compared the isoelectric focusing (IEF) patterns of HLA–A locus and –B locus antigens after sequential immunoprecipitation with three MCAs with very general specificities. The technique identified HLA heterogeneity and the polymorphisms detected by IEF can identify functionally important differences between HLA antigens. It is not known whether this approach could be successfully applied to dried bloodstain testing.

HLA TYPING OF DRIED STAINS OF OTHER BODY FLUIDS

As well as being found on the membrane of all nucleated cells, active HLA antigens have been identified in soluble form in many body fluids. Antigens of the A and B loci have been grouped by the inhibition of cytotoxicity technique in serum (Pellegrino *et al.* 1974), urine (Vincent *et al.* 1976) and seminal plasma (Singal and Berry 1972).

Semen

Some controversy is evident in the literature regarding the exact location of the HLA antigens in semen. Techniques such as dye exclusion, cytotoxic assay with alloantisera (Fellous and Dausset 1970), direct and indirect labeling, haploid testing by inhibition of motility assay (Arnaiz–Villena and Festenstein 1976) and two color–fluorescence (Halim *et al.* 1982) have demonstrated not only single HLA antigens but HLA haplotypes on spermatozoa. However, independent studies did not duplicate the earlier findings (Law and Bodmer 1978). To a much lesser degree, this controversy has been carried into forensic studies, although for practical purposes, it does not matter whether the HLA activity was originally present in soluble form in seminal plasma or on the spermatozoa. However, a system as complex to implement as the HLA system would be of much greater general value if it could be applied to secretion stains as well as to bloodstains.

Three studies have attempted to group semen stains. Newall (1981) and Nelson *et al.* (1983) used the same basic technique, inhibition of lymphocytotoxicity, as they used to type bloodstains. The Newall study failed to detect HLA–A9 in a limited study of seminal stains from 12 donors, as A9 could be conclusively identified in only one of four known stains (Table 11). No false positive reactions were encountered. On the other hand, Nelson *et al.* studying HLA–A2 and –A10 reported no difficulty in demonstrating these antigens in seminal stains, although they published actual inhibition levels with only one A2 stain. Regueiro and Arnaiz–Villena (1981) reported successful results with a radioactive microcompetition assay as well as with a macroadsorption test using MCAs. They studied A3, A11, A25, AW32 and B7, and B14 and BW35. In the radioactive technique, extracts of seminal stains were concentrated up to five times with Minicon B volume reducers and then tested as inhibitors against HLA antisera. The detection system was target lymphocytes labeled with chromium–51. At the endpoint of the microlymphocytotoxicity test, 15 μl of supernatant was removed and counted in a gamma counter. The cytotoxic index was defined as:

$$\frac{{}^{51}\text{Cr activity in test} - {}^{51}\text{Cr activity in negative control}}{{}^{51}\text{Cr activity in positive control} - {}^{51}\text{Cr activity in negative control}} \times 100$$

The range of cytotoxicity of the alloantisera after adsorption with concentrated semen extract eluates was variable. For example, for A11, 68% residual cytotoxicity was interpreted as positive inhibition and

Table 11. IDENTIFICATION OF HLA-A9 IN STAINS OF BODY SECRETIONS

Secretion	HLA-A9 Group of Stains	Number of Stains	Positive	False Positive	False Negative	Negative	Not Readable
Semen	Positive	4	1		3		
	Negative	2		0		2	
	Crossreactive[a]	6		0		4	2
Vaginal Secretion	Positive	2	0		2		
	Crossreactive[a]	2		1		0	1
Urine	Positive	2	0		1		1
Perspiration	Positive	2	0		2		
Total		20	1	1	8	6	4

[a]HLA-A1, A2 or A28

93% residual cytotoxicity was interpreted as negative inhibition, whereas for BW35, 3% residual cytotoxicity was interpreted as positive inhibition and 17% residual cytotoxicity was interpreted as negative inhibition.

When semen eluates were tested with MCAs in a macroadsorption test, the results were very clear. For example, 0% residual cytotoxicity was interpreted as positive inhibition and 98% residual cytotoxicity as negative inhibition for HLA–A25. Clearly, more data must be accumulated before a reliable technique for seminal stain HLA grouping can be adopted.

Saliva

As with seminal stains, the literature is controversial regarding the reliability of grouping saliva stains. Two brief studies have been published. Newall (1981) reported that HLA–A9 could not be detected in 12 saliva stains from A9 donors. In addition, positive results were obtained with two saliva stains known to contain only A2. Failure to detect antigens known to be present represented 36% of the results, and 11% of the results were recorded as not interpretable in tests on 22 saliva stains (Table 12). On the other hand, Nelson *et al.* (1983) reported the accurate grouping of an A2 saliva stain, although results with stains carrying crossreactive antigens or lacking the antigen were not reported.

Table 12. IDENTIFICATION OF HLA-A9 IN SALIVA STAINS

Group of Stains	Age	Number	Positive	False Positive	False Negative	Negative	Not Readable
Positive	0-2.5 wk	8	0		5		3
Negative		4		0		2	2
Cross-reactive[a]		2		0		1	1
Positive	3-5 mo	4	0		3		1
Cross-reactive[a]		2		2		0	
Negative	8 mo	2				0	2
Total		22	0	2	8	3	9

[a]HLA-A1, A2 or A28

Urine, Perspiration and Vaginal Secretion

If the HLA antigens present on all nucleated cells and in soluble form in serum and urine are also present in other fluids often found as background contaminants in forensic case material, such as perspiration or urine, care similar to that exercised in the ABO system must be taken to interpret accurately the results of mixtures of fluids and antigens from different sources. Newall (1981) tested two stains each of vaginal secretion, urine and perspiration, donated by known A9 individuals. In no stain could A9 be detected. Mixtures of body fluids have not yet been evaluated.

CONCLUSIONS

A new blood grouping system must have many favorable attributes to qualify for routine application in a forensic science laboratory. Most important is the potential of the system to discriminate between individuals. However, markers detectable in both blood and semen are of greater general forensic value than those isolated to one body fluid. A new technique should be reasonably easy to learn, and high quality reagents should be readily accessible. Specificity, sensitivity and stability sufficient to satisfy the practical limits of casework demands must be demonstrated. It is absolutely essential that the accuracy of the positive results be dependable.

As has been discussed, HLA–A and –B antigens qualify in many respects for routine use in forensic science laboratories. Research continues to eliminate the remaining variables preceding the application to casework of this demanding but exceptionally valuable system.

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DISCUSSION

Question: Does freezing the bloodstains change or decrease the soluble human leukocyte antigens (HLAs)?

Newall: No, I do not think freezing HLA in a soluble form in a bloodstain would have as much effect on the entire test as freezing the cell membrane of the target lymphocyte that is being used as the target cell.

Lincoln: A solution to the problem of maintaining a constant supply of target cells is freezing known lymphocytes.

Newall: Exactly.

Schanfield: When you said specificity changed on frozen cells in the reported articles, does that mean there was an increase in apparent crossreactive groups or that there were actual changes? Cells are frozen with dimethyl sulfoxide that chews up the membrane making the cells more sensitive to lysis after freezing than the cells were before freezing, resulting in an increase in crossreactions.

Newall: This is what Lotterle was suggesting. I have not worked with frozen cells myself, but he suggested that the number of crossreactions you would observe would go up substantially if you worked with frozen target lymphocytes. Terasaki groups frozen lymphocytes frequently and on a routine basis. I cannot give you a definitive answer.

Oriol: The plague of HLA testing is the quality of antisera. What is operationally nonspecific under one test system often may not be mono-specific or operationally mono-specific under another test system. My original training was done on frozen cells, which results in more mono-specific antisera than when fresh samples are used. Also, the problem with HLA in the United States, which is not as much of a problem in Canada, England or West Germany, is that most of the antisera comes from white individuals and HLA antigen reactivity with black individuals is often different.

I have not worked with HLA, but I have worked in Terasaki's laboratory for several years and he has over 100 permanently frozen cells. Terasaki accepted an error of approximately 5%. Use of frozen cells results in more dead cells, increases the sensitivity to certain specificities and increases the probability of crossreaction, but you can compensate for these problems by multiplying by 5 or 10 the number of cells in the final tests. You can figure out the optimal conditions, and the results will be much better if you use 120 frozen cells rather than 3 fresh donors.

THE BIOCHEMICAL GENETICS AND METHODOLOGY FOR THE ANALYSIS OF THE Gm AND Km ANTIGENS

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The Metropolitan Police Forensic Science Laboratory has routinely used the Gm system, and to a lesser extent the Km system, for the characterization of bloodstains for about the last 12 years. Both systems have proved to be very reliable for this purpose, but, unlike the situation for other genetic markers such as erythrocyte acid phosphatase (EAP), phosphoglucomutase (PGM) and haptoglobin, the gamma marker (Gm) and kappa marker (Km) systems have not been used to such a very great extent by other forensic science laboratories. This discussion introduces the Gm and Km systems and will explain them, how they can be detected in bloodstains and how they compare with other genetic markers for the purpose of bloodstain identification.

BASIC NOMENCLATURE

The Gm and Km systems are a series of inherited antigenic determinants present on human immunoglobulin molecules (Grubb and Laurell 1956a, 1956b; Grubb 1970; Natvig and Kunkel 1968; Ropartz *et al.* 1961). These antigens vary between different individuals and are known as allotypes or allotypic markers. More than 20 antigens in the Gm system and 3 in the Km system have now been described. Before 1976, each antigen or factor was designated by a letter, but as recommended by the World Health Organization, they are now identified by numbers.

The large number of antigens in the Gm system indicates that if it were possible to detect all of them in dried bloodstains, the system would be more useful than any other genetic polymorphism currently used for bloodstain identification. In practice, it has not been possible to realize this potential on a routine basis, and our experience is limited to the detection of Gm(1), Gm(2), Gm(3), Gm(5), Gm(10) and Km(1).

A particular antigen may be present or absent in an individual's serum, and the person is said to be positive or negative for that factor. For example, the presence of Gm(1) is indicated by "Gm(1)" (note: there is no "+" sign) and its absence as "Gm(–1)." When more than one Gm antigen is considered, these are written together in parentheses after the Gm prefix. For example, Gm(1,2) indicates the presence of both Gm(1) and Gm(2), Gm(1,–2) indicates the presence of Gm(1) but the absence of Gm(2) and Gm(–1,–2) indicates the absence of both factors. The principle is extended to three or more factors. Note, however, that antigens on the same subclass of IgG (see later) are separated by commas, for example, Gm(–1,–2,3), whereas antigens on different subclasses are separated by a semicolon, for example, Gm(–1,–2;5). The Gm and Km systems are independently inherited and are written separately, for example, Gm(1,–2), Km(1).

As we have seen, the Gm and Km antigens are present on immunoglobulin molecules, and in order to understand the biochemistry and genetics of these two blood group systems, it is necessary to consider some aspects of immunoglobulin structure. The information presented here has been drawn from reviews and original articles (Edelman 1971; Fleischmann *et al.* 1963; Koshland 1967; Natvig and Kunkel 1973; Steward 1984; Tomasi 1965).

CLASSES OF IMMUNOGLOBULIN

The five classes of immunoglobulin are designated IgG, IgA, IgM, IgD and IgE. In normal individuals, IgG comprises about 80% of the total serum immunoglobulin (Table 1). Molecules of each class are composed of two types of polypeptide chain (Edelman and Poulik 1961), which are known as light (L) chains with a molecular weight of about 23,000 and heavy (H) chains with a molecular weight of about 50,000.

The two types of light chain are termed kappa (κ) and lambda (λ). Both types are found in each class of immunoglobulin but not in the same molecule. In humans, about 65% of immunoglobulins have κ chains, and the rest have λ chains. The Km markers (formerly Inv) are located on κ chains, so these factors are present in all five classes of immunoglobulin. The heavy chains are specific to each class of immunoglobulin. For the five classes IgG, IgA, IgM, IgD and IgE, the heavy chains are designated gamma (γ), alpha (α), mu (μ), delta (δ) and epsilon (ϵ), respectively (Table 1). The Gm markers are located on the γ chains and are present, therefore, only on IgG molecules.

Table 1. CLASSES OF IMMUNOGLOBULIN IN NORMAL HUMAN SERUM

Class	Relative abundance in normal serum	Heavy Chain
IgG	80%	Gamma γ
IgA	13%	Alpha α
IgM	6%	Mu μ
IgD	1%	Delta δ
IgE	low	Epsilon ϵ

Table 2. SUBCLASSES OF HUMAN IMMUNOGLOBULIN G

Subclass	Relative Abundance in normal serum	Heavy chain
IgG1	65%	Gamma 1 γ1
IgG2	23%	Gamma 2 γ2
IgG3	8%	Gamma 3 γ3
IgG4	4%	Gamma 4 γ4

Subclasses of IgG

Immunoglobulin G itself can be divided into four subclasses: IgG1, IgG2, IgG3 and IgG4. The relative abundance of each subclass in normal human serum is 65%, 23%, 8% and 4%, respectively. The heavy chains are specific to each subclass and are designated as γ1, γ2, γ3 and γ4 (Table 2). Each Gm antigen is found on only one type of γ chain. For example, Gm(1), (2) and (3) are located on γ1 chains, Gm(23) is located on γ2 chains and Gm(5), (10) and (13) are found on γ3 chains. This association is sometimes indicated by writing the prefix G1m, G2m, and so on, before the appropriate antigen. Thus, Gm(1) would be written as G1m(1), Gm(2) as G1m(2) and Gm(5) as G3m(5). Since this can be confusing, we prefer to use the shorter notation. It will be noticed that because the Gm antigens are subclass specific and because of the relative abundance of the different subclasses, the Gm factors will not necessarily be found in equimolar concentrations. For example, in the serum of a person who is homozygous positive for Gm(1) and Gm(5), the molar concentration of Gm(1) would be about eight times as great as that of Gm(5). The different concentration of each antigen is one of the factors that affects the ease with which the antigen can be detected in bloodstains.

Structure of IgG

IgG molecules consist of two light chains (κ or λ) and two heavy chains (γ) joined together by covalent disulfide bonds and other noncovalent forces (Edelman *et al.* 1969). The light chains comprise 214 amino acid residues and have a molecular weight of about 23,000. When their amino acid sequence is considered, two distinct regions can be recognized. First, there is the variable or V_L region that extends from residue 1 to 108, and second, there is the constant or C_L region that extends from residue 109 to 214. The amino acid sequence of the V_L region is extremely variable from one molecule to the next and constitutes part of the antigen combining site of the intact immunoglobulin (antibody) molecule. In contrast, the amino acid sequence of the C_L region remains relatively constant. An exception to this is at position 191 on κ chains where the Km(1) antigen is associated with leucine and Km(–1) or Km(3) is associated with a valine residue.

The heavy chains of IgG comprise 446 amino acids, and have an average molecular weight of about 50,000; therefore, they are about twice the size of the light chains. Heavy chains, like light chains, consist of a region in which the amino acid sequence is extremely variable (V_H) and a region in which the amino acid sequence is relatively constant (C_H). The V_H region extends from position 1 to 120 and is about the same length as the V_L region of the light chain. In the intact immunoglobulin molecule, each V_H region is in juxtaposition with a V_L region, and in combination, the two form the antigen combining site of the immunoglobulin (antibody) molecule. The C_H region extends from residue 121 to 446 and is about three times as long as the constant region of the light chain. Within the C_H region itself, there are three parts of the polypeptide chain whose amino acid sequences are very similar to each other and also to the constant region of the light chain (Wailmart and Urbain 1976). These three areas are referred to as C_H1, C_H2 and C_H3 and, along with the C_L region of the light chain, are folded into compact, discrete domains, each of which is stabilized by an intrachain disulfide bond (Beale and Feinstein 1976). A diagrammatic representation of these structural features of IgG is shown in Figure 1.

LOCATION AND NATURE OF THE Gm ANTIGENS

The Gm factors are associated with the constant region of the heavy chain, and in most instances, it is known on which domain a particular antigen is located (Natvig and Turner 1971). For example, Gm(1) and Gm(2) are found on the C_H3 domain of IgG1, Gm(3) is found on C_H1 of IgG1, Gm(5) is found on C_H2 of IgG3 and Gm(10) is found on C_H3 of IgG3. Furthermore, the amino acid sequences associated with some factors are known. For example, the

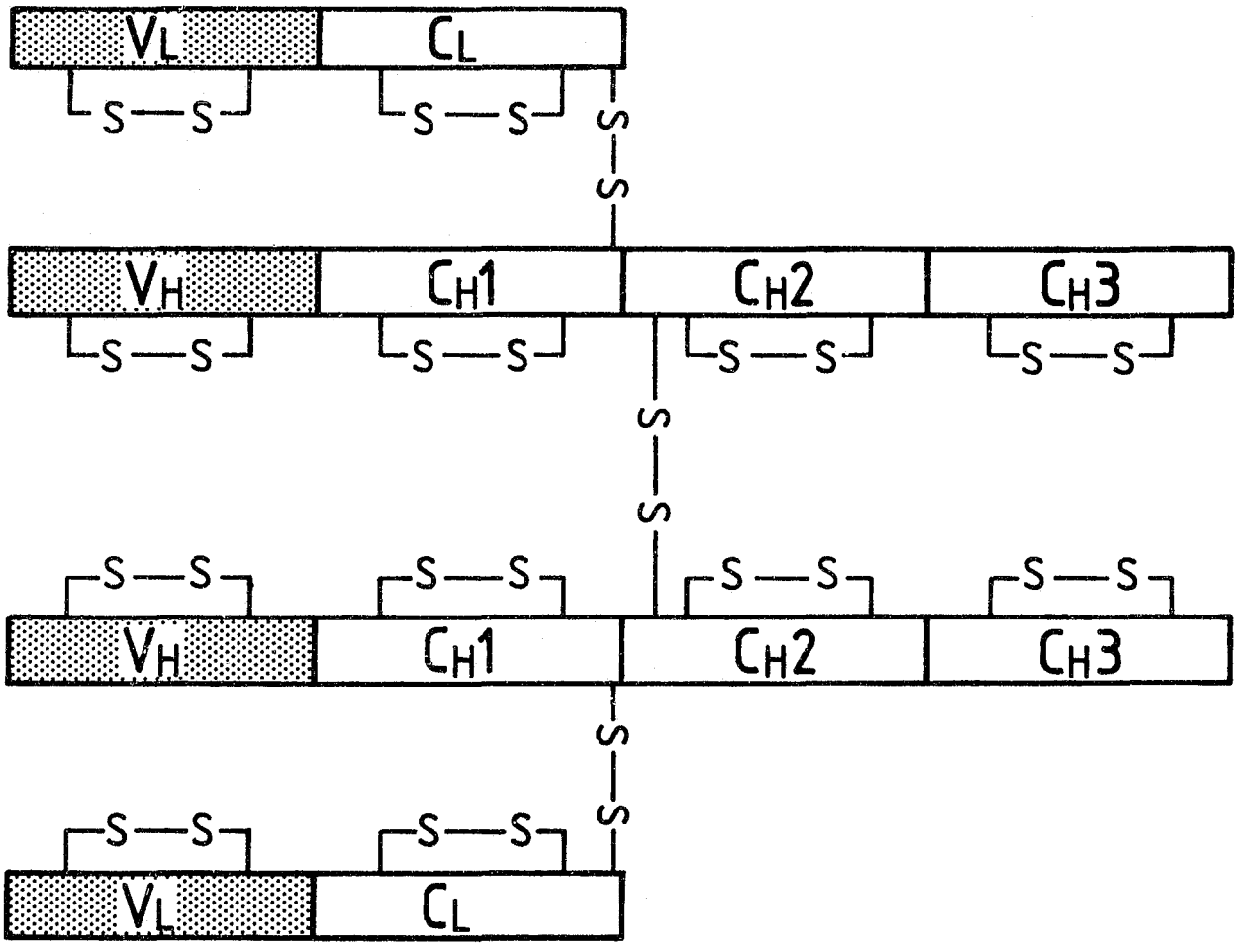

Figure 1. A diagrammatic representation of the four chain domain structure of IgG.

sequences associated with Gm(1) and Gm(–1) are as follows:

Residue No.	355	356	357	358	359	360
Gm(1)	Arg	Asp	Glu	Leu	Thr	Lys
Gm(-1)	Arg	Glu	Glu	Met	Thr	Lys

It can be seen that the Gm(1) peptide differs from that of Gm(–1) at residue 356 where aspartate is substituted for glutamic acid and at residue 358 where leucine is substituted for methionine. The Gm(1) sequence is found only in $\gamma 1$ chains, whereas the Gm(–1) sequence occurs in some $\gamma 1$ chains and all $\gamma 2$ and $\gamma 3$ chains. Thus, everyone possesses the Gm(–1) sequence; for this reason, an anti-Gm(–1) has not been found in human sera and, even if such an antiserum was available, it would be of no diagnostic value.

GENETICS OF THE Gm AND Km SYSTEMS

The genes that control the production of the κ and γ chains are not linked, so the Gm and Km antigens are inherited as independent blood group systems. The Gm factors, however, are not inherited independently of each other because the structural genes that control the constant regions of the $\gamma 1$, $\gamma 2$, $\gamma 3$ and $\gamma 4$ heavy chains are closely linked on the same chromosome. The Gm antigens are, therefore, inherited as sets or haplotypes. For example, an individual who types as Gm(1,2;5) with the genotype $Gm^{1,-2;5}/Gm^{1,2;-5}$ will pass on the gene complex $Gm^{1,-2;5}$ or $Gm^{1,2;-5}$ to his offspring.

The population frequencies for the different Km and Gm phenotypes vary enormously between different ethnic groups (Johnson *et al.* 1977), and this variation, of course, affects the usefulness of these

systems for the identification of bloodstains. The point is well illustrated in Table 3, which gives the phenotype frequencies of Gm(1), Gm(2) and Km(1) for the white and black populations of southeast England.

DETECTION OF THE Gm AND Km ANTIGENS IN DRIED STAINS

The Gm and Km factors can be readily detected in stain extracts by hemagglutination inhibition (Khalap *et al.* 1976; Khalap and Divall 1979a, 1979b; Kipps 1979; Tahir and Brown 1984). Several workers have also described methods based on the enzyme linked immunosorbent assay (ELISA) principle (Fletcher *et al.* 1983; Newall 1985). Such assays are more sensitive than the inhibition test and lend themselves to automation and precise quantitation. Despite these advantages, the ELISA methods have not been used as yet for the routine detection of the Gm/Km antigens in operational casework.

In the hemagglutination inhibition assay, a particular antigen is detected by the ability of a stain extract to inhibit the agglutination of indicator cells by the corresponding Gm or Km antiserum. A test system therefore consists of the test sample, selected anti-Gm or Km antisera and indicator cells.

The Test Sample

Serum samples are usually prepared as a 1:20 dilution with saline, although some workers prefer to test a higher dilution of between 1:50 and 1:100. Others test each serum sample at a range of dilutions, for example, 1:20, 1:40 and 1:80. For dried bloodstains, about 5 mm^2 or its equivalent is extracted in 12 drops (400 μl) of saline for 3–4 hours at room temperature or at 37° C. The higher temperature and longer periods of extraction are advantageous when dealing with old stains. After the extraction period, it is common practice to centrifuge the extracts to remove any insoluble debris. Tests are then made on the neat extract or on a dilution series (neat, 1:2 and 1:4) prepared from it. We prefer the latter because it allows, in a semiquantitative manner, the relative amounts of each antigen to be assessed and also, to a large extent, duplicates the test. Tris–hydrochloric acid buffered saline (pH 7.5) can be used as the extractant and diluent but is not essential for bloodstains. It is worth noting that in many instances, the piece of substrate material that remains after extraction can be used for ABO typing (Khalap and Divall 1978).

A substrate control must always be tested at the same time as the stain itself. The control consists of a piece of unstained substrate, taken from an area immediately adjacent to the bloodstain, which is extracted and tested in a manner identical to that used for the bloodstain.

Table 3. Gm AND Km FREQUENCIES FOR THE WHITE AND BLACK POPULATIONS OF SOUTH EAST ENGLAND

Phenotype	Frequency %	
	Whites	Blacks
Gm(1,2)	21.9	4.6
Gm(1,-2)	34.1	93.0
Gm(-1,-2)	44.0	2.4
Km(1)	17.7	53.7
Km(-1)	82.3	46.3

Antisera

The availability of good reagents is always a critical factor when deciding whether to run a particular blood group system on a regular basis. Fortunately, anti-Gm and anti-Km sera are now available from a number of commercial companies and blood transfusion centers around the world (Kipps 1979). The antisera used for Gm and Km testing are of human origin and are of two types (Divall and Khalap 1976). The first type is the anti-gamma globulins (rheumatoid factors) with Gm or Km specificity commonly found in the sera of patients with rheumatoid arthritis. These antisera are called Raggs, or rheumatoid agglutinators, and although they generally lack specificity for one marker, they have been useful for the elucidation of the Gm system. Second, there are the Gm/Km antibodies occasionally found in the sera of clinically healthy individuals. These antisera are called SNaggs, or serum normal agglutinators and arise from immunization of a person with genetically incompatible gamma globulin either by injection, transfusion or placental transfer (Ropartz *et al.* 1960). They are usually monospecific for one Gm or Km determinant and are the preferred reagents for routine Gm/Km typing.

Prior to routine use, each antiserum must be checked for specificity by testing it in the assay that is to be used against a number of samples known to be positive or negative for the antigen in question. The next stage, as with any other inhibition assay, is to titrate the antiserum so that it can be used at the optimal dilution in the test system by preparing a dilution series of the antiserum and testing each dilution against an aliquot of the indicator cells. A typical titration for an anti-Gm(1) serum is shown in

Table 4. In this instance, the antiserum would be diluted about 1:20 for use in the inhibition test. If it was used at a higher concentration, more antigen would be required to inhibit the antiserum and the test would lack sensitivity. If it was used at a dilution of much greater than 1:25, the indicator cells would not be fully agglutinated when a negative sample is tested. Consequently, the results would be difficult to read, and a false positive result could be obtained.

Indicator Cells

The final requirement for the test system is the indicator cells, which are human group O rhesus positive (D) cells that are sensitized with an anti–D that is itself positive for one or more particular Gm or Km factor. Invariably, the anti–D will be composed of one IgG subclass, so when in a test for Gm factors found on different IgG subclasses, for example, Gm(1) and Gm(5), it is necessary to prepare two lots of indicator cells. The sensitivity of the inhibition test is also influenced by the relative proportion of red cells to anti–D used to prepare the indicator cells. The best ratios to use are determined empirically at the reagent testing stage. Indicator cells must be prepared before each set of assays. Since this stage is time consuming, it would be advantageous to use indicator cells that could be stored over a period of time. To this end, we have attempted to use the chromium chloride and tannic acid methods for coating purified gamma globulin onto red blood cells but have found that cells prepared by the anti–D sensitization method always gave better results in the inhibition test.

Performing the Test

The inhibition test is performed by incubating aliquots of the test sample and suitably diluted antiserum. Stain extracts are incubated overnight at 4° C but the period for serum can be reduced to 4 hours because of the greater concentration of immunoglobulin in the sample. An aliquot of indicator cells then is added to each sample. The samples are left at room temperature for 60–75 minutes. All these stages are performed in tubes, which are then centrifuged at 1000 rpm for 1 minute. The cell button then is carefully removed with a Pasteur pipet and spotted into a drop of saline on a microscope slide. The agglutination of the indicator cells then is assessed by direct (microscopical) observation. The tests can be performed in tubes or microtiter plates (Davie 1979). The latter are said to result in a more sensitive assay and a more economical use of reagents. We prefer the tube method, partly through tradition, but mainly because the agglutination of the indicator cells is assessed by direct observation, whereas with the microtiter plates, the agglutination is assessed indirectly by reading either the sedimentation pattern of the cells or the pattern caused by tilting the plate making the cells streak to varying degrees.

Table 4. A TYPICAL TITRATION FOR ANTI-Gm(1)

	Dilution of antiserum							
	½	¼	⅛	1/16	1/32	1/64	1/128	1/256
Agglutination score	4	4	4	4	3	2	1	–

Interpretation of the Results

The results are relatively easy to interpret. No agglutination of the indicator cells means the antiserum has been inhibited and so demonstrates the presence of the particular antigen. Alternatively, agglutination of the indicator cells means the antiserum has not been inhibited and so demonstrates the absence of the antigen. Some typical results are shown in Table 5.

Inhibitor Control

The inhibitor control is one of the important steps to perform when testing for the Gm and Km antigens. This procedure tests an aliquot of the stain extract or serum sample for its ability to agglutinate the indicator cells in the absence of any anti–Gm or anti–Km antiserum. This can happen since, as previously explained, the serum in some individuals contains antibodies to foreign immunoglobulin and can therefore agglutinate red blood cells that are sensitized with the appropriate foreign immunoglobulin. Alternatively, a person's serum may contain antibodies directed against a red cell membrane antigen that is present on the erythrocytes used to prepare the indicator cells. In practice, the inhibitor control is rarely positive, but it can be a source of misinterpretation. A series of inhibition results shown in Table 6 is an example. At a serum dilution of 1:20, the indicator cells are agglutinated, that is, there appears to be no inhibition of either the anti–Gm(1) or the anti–Gm(2) and the sample is apparently Gm(–1,–2). However, at a dilution of 1:20, the serum can agglutinate the indicator cells by itself, so the inhibition results are inconclusive. When a dilution series of the serum sample is tested, the agglutinating ability is diluted out and the true phenotype of Gm(1,–2) becomes manifest at 1:80

Table 5a. TYPICAL RESULTS OBTAINED FROM A HEMAGGLUTINATION INHIBITION ASSAY FOR Gm(1) AND Gm(2)

Dilution of extract	Agglutination score with: Anti-Gm(1)	Anti-Gm(2)
Bloodstain:		
N	–	–
½	–	–
¼	–	1+
Substrate control:		
N	4	4
½	4	4
¼	4	4
Result: Gm(1,2)		

Table 5b. TYPICAL RESULTS OBTAINED FROM A HEMAGGLUTINATION INHIBITION ASSAY FOR Gm(1) and Gm(2)

Dilution of extract	Agglutination score with Anti-Gm(1)	Anti-Gm(2)
Bloodstain:		
N	–	3+
½	–	4
¼	–	4
Substrate control:		
N	3	4
½	4	4
¼	4	4
Result: Gm(1,-2)		

Table 5c. TYPICAL RESULTS OBTAINED FROM A HEMAGGLUTINATION INHIBITION ASSAY FOR Gm(1) and Gm(2)

Dilution of extract	Agglutination score with Anti-Gm(1)	Anti-Gm(2)
Bloodstain:		
N	–	+
½	1	1
¼	1+	2
Substrate control:		
N	1	1+
½	1+	2+
¼	2+	3
Result: Inconclusive		

dilution. This effect is another good reason for testing a dilution series of either the serum or stain extract.

The Problem of Negative Results

A recurrent problem with the inhibition assay is the interpretation of negative results. For example, when a sample fails to inhibit anti–Gm(1) and anti–Gm(2), does this represent a true Gm(–1,–2) result or was there insufficient immunoglobulin to inhibit each antiserum? Several approaches have been used to solve this particular problem.

Table 6. HEMAGGLUTINATION INHIBITION RESULTS OBTAINED FROM A SERUM SAMPLE SHOWING INHIBITOR CONTROL REACTIONS

Dilution of serum (1 in)	Agglutination score with: Anti-Gm(1)	Anti-Gm(2)	Saline
10	4	3+	4
20	3	3+	4
40	1	4	2+
80	–	4	+
160	–	4	–
320	–	4	–
640	2	4	–
1280	3	4	–
2560	4	4	–

The first of these approaches, and the most successful, is to use another Gm or Km antigen as a positive marker. In order to do this, the marker antigen must fulfill two criteria. First, it must be present when the other antigen(s) for which we are testing is absent. Second, the ability to detect the positive marker must be equal to or preferably less than that for the other antigens. In this way, we have used Gm(3), Gm(5) and Gm(10) as positive markers for Gm(–1,–2), and Tahir has used Gm(11) (Khalap and Divall 1979a; Tahir 1984); Km(3) can be used as a positive marker for Km(–1). These points are further explained by reference to Table 7. Here, a dilution series of a Gm(1,2,3) serum sample was tested against anti–Gm(1), anti–Gm(2) and anti–Gm(3). At a dilution of 1:8, all three antigens were detected, whereas when the serum sample was diluted 1:32, only Gm(1) and Gm(2) were detected. These results confirm that in this instance, the Gm(3) assay is less sensitive than are the Gm(1) and Gm(2) assays. From the results, it can be concluded that when a sample inhibits anti–Gm(3) but gives no inhibition of anti–Gm(1) or anti–Gm(2), then it is a true Gm(–1,–2). It is important to note that if no inhibition of all three antisera is observed, the result is inconclusive.

Another important aspect of the use of positive markers must now be described. It can be seen from the results shown in Table 7 that the sensitivities of the Gm(1) and Gm(2) assays are about the same. This is generally the case and is the reason that Gm(1) can be used as a positive marker for Gm(–2). (Note: Gm(2) can also be used as a positive marker for Gm(–1), but the Gm(–1,2) phenotype is very rare.) However, when the Gm(1) assay is more sensitive than the Gm(2) assay, then Gm(1) cannot be used as a positive marker for Gm(–2), and if this is not recognized, it can be a

Table 7. HEMAGGLUTINATION INHIBITION RESULTS OBTAINED A Gm(1,2,3) SERUM SAMPLE SHOWING THE LOWER SENSITIVITY OF THE Gm(3) ASSAY

Dilution of serum (1 in)	Agglutination score with: Anti-Gm(1)	Anti-Gm(2)	Anti-Gm(3)
4	–	–	–
8	–	–	–
16	–	–	1
32	–	–	3
64	+	–	3+
128	2	3	4
256	3+	3+	4

Table 8. HEMAGGLUTINATION INHIBITION RESULTS OBTAINED FROM A Gm(1,2) SERUM SAMPLE SHOWING AN IMBALANCE IN THE SENSITIVITIES OF DETECTING Gm(1) AND Gm(2)

Dilution of serum (1 in)	Agglutination score with: Anti-Gm(1)	Anti-Gm(2)
4	–	–
8	–	–
16	–	–
32	-	1+
64	+	3
128	1	3+
256	3	3+

source of mistyping. For example, Table 8 shows the results of testing a Gm(1,2) sample against a particular anti–Gm(1) and anti–Gm(2). At a dilution of 1:16, the serum sample inhibits both antisera and types correctly as Gm(1,2), whereas at a dilution of 1:64, only anti–Gm(1) is inhibited and the sample now types incorrectly as Gm(1,–2). With these antisera, it is apparent that the Gm(1) assay is more sensitive than the Gm(2) assay, and consequently Gm(1) cannot be used as a positive marker for confirming an apparent Gm(–2) result. It is always necessary to check for such imbalance and, if it is observed, to dilute the antisera so that the sensitivities are the same.

A second approach to the problem of interpreting negative results in the inhibition test has been to measure the total protein or immunoglobulin level in a stain extract. For example, in an attempt to find a way of confirming an apparent Km(–1) result, we measured the total immunoglobulin level in 200 Km(1) bloodstains using radial immunodiffusion and related this to the degree of inhibition of anti–Km(1). The results indicated that if an extract contained more than 60 μg/ml of total immunoglobulin but failed to inhibit the anti–Km(1), then it could be considered to be a true Km(–1) (Divall 1981).

USE OF THE Gm AND Km SYSTEMS AS GENETIC MARKERS IN STAIN ANALYSIS

Discriminating Power

As previously mentioned, Gm and Km phenotype frequencies vary widely between different racial groups. Consequently, the usefulness of the Gm and Km markers for bloodstain identification will vary according to the population under consideration (Blanc *et al.* 1971). For example, the discriminating power of Gm(1) and (2) for the white population of southeast England is 0.64; this value compares favorably with those of other genetic markers used for stain analysis (Table 9). For the black population of southeast England, however, the discriminating power of Gm(1) and (2) is only 0.14, since 93% of this population are Gm(1,–2). In contrast, Km(1) has a poor discriminating power (0.29) for the white population but is better for the black population (0.50) because of the more even distribution of the Km(1) and Km(–1) phenotypes (see Table 3).

Sensitivity

The hemagglutination inhibition test is relatively sensitive and, as a rough guide, Gm(1), (2) and (3) can be readily detected in about 4 mm^2 of bloodstained material. In comparison with the other genetic markers used for stain analysis, the Gm assay used to be one of the most sensitive. However, the introduction of isoelectric focusing on ultra–thin polyacrylamide gels for the typing of group specific components (Gc), EAP and PGM has made the detection of these markers more sensitive than that for Gm by hemagglutination inhibition.

Longevity

The Gm and Km factors are very stable in dried stains and in this respect do better than any other genetic marker except for ABO. We have readily detected Gm factors in bloodstains that are many months old, and some workers have been able to detect Gm(1), Gm(10) and Km(1) in bloodstains that were 29–33 years old (Hoste *et al.* 1978).

Ease of Analysis

The hemagglutination inhibition assay is not difficult to perform but does require practice to learn

Table 9. DISCRIMINATION POWER OF SOME BLOOD GROUP SYSTEMS FOR THE WHITE AND BLACK POPULATIONS OF SOUTH EAST ENGLAND

Blood group system	Discriminating power	
	Whites	Blacks
ABO	0.61	0.64
AK (adenylate kinase)	0.14	0.04
EAP (erythrocyte acid phosphatase)	0.65	0.53
EsD (esterase D)	0.36	0.29
Gm(1) and (2)	0.64	0.14
Hp (haptoglobin)	0.61	0.70
Km	0.29	0.50
PGM (phosphoglucomutase)	0.75	0.72

and continued experience to maintain a satisfactory level of proficiency. The tests take a relatively long time to complete, since an overnight incubation period is required when bloodstains are being tested, a disadvantage if the analysis is urgent.

Some workers see the testing procedure as relatively complicated, especially in view of the need to prepare fresh indicator cells and to test and titrate the antisera and in view of the number of controls that also have to be tested. This objection, however is generally based on lack of familiarity or experience.

Reagent Availability and Cost

The difficulty in obtaining the necessary reagents probably has been the main reason Gm and Km testing has not been adopted by many forensic science laboratories. Fortunately, good reagents are now available from several commercial companies (Kipps 1979) and blood transfusion centers, although their cost and quality can vary.

Detection in Other Body Fluids

The ability to detect a genetic marker in body fluids other than blood is always an advantage. Theoretically, the Gm and Km factors could be detected in any body fluid that contains immunoglobulins of the correct class and subclass. In practice, the levels of immunoglobulin in those body fluids of interest to the forensic scientist are very low, and these low levels probably account for the lack of success by earlier workers (Nielsen and Henningsen 1963). For example, the immunoglobulin level in semen is about 50 times lower than in blood (Blake and Sensabaugh 1978), and in saliva, it is about 1,000 times lower (Waissbluth and Langman 1971). Despite these difficulties, several workers have now reported the successful detection of some Gm and Km antigens in semen and saliva (Khalap and Divall 1979b; Davie and Kipps 1976).

We have found that Gm(1), Gm(2) and Gm(10) can be reliably detected in seminal stains with a slightly modified hemagglutination inhibition test. Antisera must be carefully selected on the basis of titer, specificity and suitability in the test system. For example, because the immunoglobulin level in semen is so low compared with that in blood, care must be taken not to mask inhibition with excess antiserum. For this reason, it is necessary to titrate the antiserum at much closer dilutions than is necessary for Gm grouping of bloodstains. Furthermore, we have observed nonspecific inhibition when using some batches of antiserum, whereas others were difficult to inhibit. Such antisera were not suitable for the Gm typing of seminal stains but were found to be suitable for bloodstain grouping. Thus, although suitable reagents do exist, they are difficult to find, and this is a major limiting factor in attempting to group seminal stains on a routine basis.

Mixtures of semen and vaginal secretions present further problems for Gm typing. First, it is necessary to extract stains into Tris–hydrochloric acid buffered saline, rather than just saline, to prevent the lysis of the indicator cells. Second, the Gm antigens can be detected in vaginal secretions alone so when semen/vaginal secretion mixtures are typed, the results must be interpreted in the light of a contribution from both components. Our experience when examining semen–stained vaginal swabs is that we generally observe no inhibition of the antisera or obtain the Gm type of the female. In either case, no information is obtained about the Gm type of the semen. Thus, although the testing procedure is valid, it is not particularly cost effective.

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DISCUSSION

Konzak: Could you go over again the nature of the positive control and the balancing of the inhibition of the antisera?

Divall: When you do a serial dilution of a sample that is Gm(1) positive, Gm(2) positive, then you see that we are detecting the Gm(1) factor and the Gm(2) factor to about the same level. So if we get a sample that is Gm(1) positive, but apparently Gm(2) negative, we can assume that it is a true negative because we are able to detect those two factors to the same extent. In this case, our ability to detect Gm(2) is less than our ability to detect Gm(1). If you have a bloodstain extract equivalent to this dilution of 1:64, then one would falsely conclude that this sample was a Gm(1) positive, Gm(2) negative, because with these two antisera the relative sensitivity of the assay is different.

Schanfield: My preference is to use Gm(3) because it has an allelic relationship. The reason you type for B or G, (21), (5) or whatever is that it provides a control for your (2) and (3) typing which may or may not be less sensitive because if there is enough material to detect, especially (21) that is the lower concentration, then you can be fairly sure that the sample is a true Gm(1) positive, Gm(2) negative.

In our laboratory we do not routinely titrate bloodstains. We type for lots of markers and lots of different subclasses, so the relative concentration of the subclasses tends to be its own internal control. The immunoassay sequence of the allotypes, as far as we can tell, is determined by at least two amino acids, with the exception of X. All of them appear to be assembled antigens and when you de-assemble the molecules, you largely lose antigenicity. So even though Km(1) has traditionally been reported as a substitution at 191, it actually is the interaction of 156 and 191.

In some jurisdictions in the United States where the population is largely nonwhite, typing for (1), (2) and (3) unfortunately gives a very poor discriminating power. So it is necessary to go to the Gm(3) markers to be able to really identify the population. In the black population, with the reagents we have available, if we use Gm and Km we can generate about 96 phenotypes, of which the most common one is only about 8%. So it is possible to get very high levels of discrimination. I would point out, if you use (1), (2), (3), (10) or (11) and (21) in the white population, you get five phenotypes and your discriminatory power does go up to some extent.

Divall: That is why I put up the discriminatory power for the caucasian population. The discriminatory power for any blood group system depends on the frequency distribution in the population under consideration. You have these reagents available, but a very important consideration for most routine laboratories is to have all those reagents available and in sufficient amounts not just to deal with one or two bloodstains a week but with the vast number of bloodstains that a forensic science laboratory would be getting.

The concentration of immunoglobulins in blood is 1,200 mg/100 ml, in semen, 20 to 30 mg/100 ml and in vaginal fluids, 10 to 15 mg/100 ml. The concentration of immunoglobulins is very low in saliva and sweat. The Km(1) marker is higher because the IgA is being secreted. The Km is detectable in saliva, but Gm is not detectable in saliva.

Grimm: We do Gm(1), Gm(2), Gm(3) and Gm(10) routinely. We measure the total protein content in each dilution step and also in the stains that we have extracted, and we compare the minimum protein concentration at which we got inhibition with the appropriate antiserum and see if the protein content of the bloodstains extract is higher or lower than this minimum concentration. This approach makes the Gm typing much more reliable than the approaches in which one marker is a marker for the reliability of another marker.

Divall: In this situation, we only use Gm(3) as a positive marker. When we get Gm(1) negative, Gm(2) negative and Gm(3) positive, we only use that to confirm the negative result. Then we do not report Gm(3) negative. In regard to the protein concentration, that is exactly what we were trying to do, but as opposed to measuring protein. I go one step further and measure the total immunoglobulin level.

THE BIOCHEMISTRY, GENETICS AND APPLICATIONS OF THE ABO AND LEWIS SOLUBLE ANTIGENS

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GENETICS AND BIOCHEMISTRY

The genetics and biochemistry of the ABO and Lewis (Le) systems have been the subject of several reviews (Watkins 1980; Hakomori 1981; Kabat 1982; Gaensslen 1983; Fiori 1985; Oriol *et al.* 1986). The two systems are inextricably linked, but provided the interrelationships are thoroughly understood, the forensic scientist should be able to make reasonable predictions about the phenotype of the individual from whom the secretion or secretion stain originated. The biosynthetic pathways that lead to the overall picture, as shown in Figures 1 and 2, can be examined simply in distinct sequences.

First, the production of the ABH and Le factors is controlled by genes at a minimum of five distinct genetic loci: secretor, ABO, Le, X and H. The factors are coded for indirectly, since the DNA codes for proteins, in this particular case, a number of transferase enzymes, and these in turn add sugars to existing

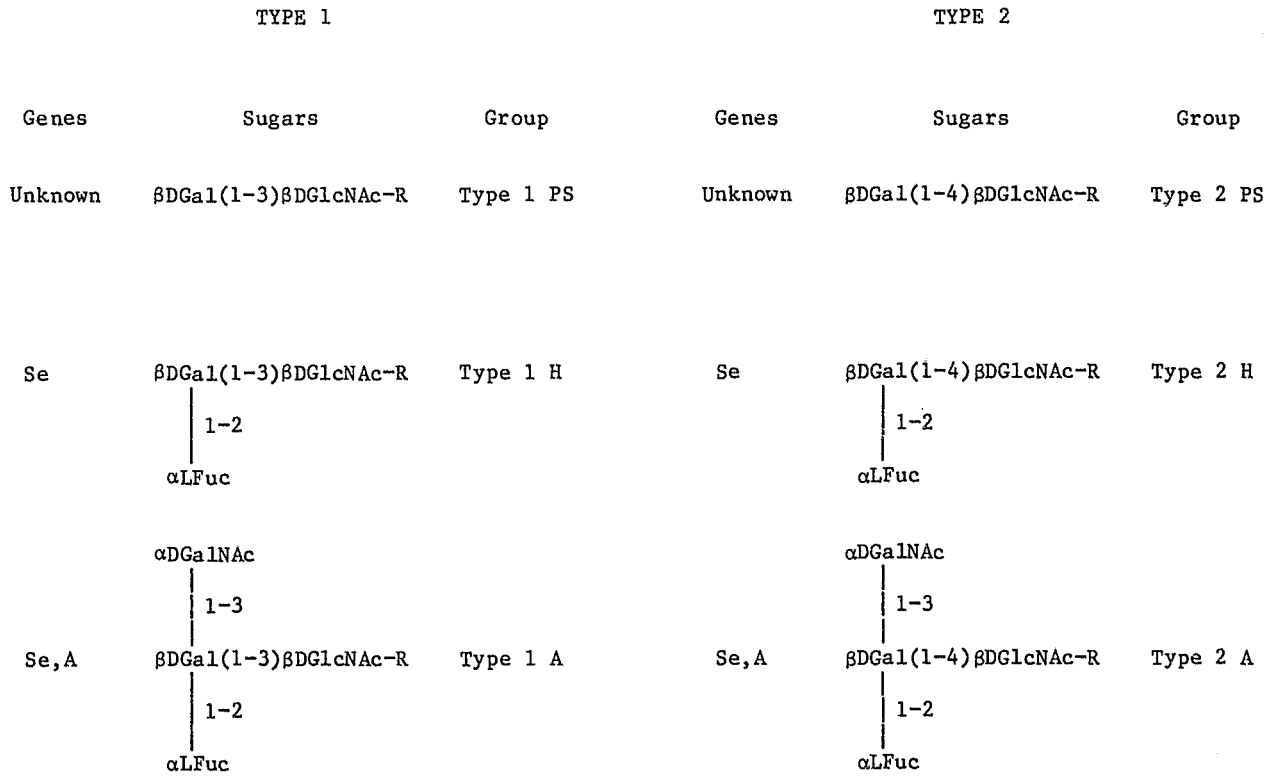

Figure 1. The sequential development of carbohydrate chains in ABH secretions. In the presence of the dominant secretor gene, *Se*, the ubiquitous type 1 and type 2 precursor substances (PS) are converted to type 1 H and type 2 H respectively. For individuals with an *A* gene (shown) and/or a *B* gene (not shown) the H substances are further glycosylated to form A and/or B active chains by the addition of *N*-acetyl-D-galactosamine (Gal*N*Ac) and D-galactose respectively in an α1-3 linkage to the terminal galactose (Gal). The *O* gene produces an enzyme with no detectable product (Yoshida, 1980) and the H chains remain unchanged. In the absence of the dominant Secretor gene, *Se*, the Production of A and/or B chains is not possible since their transferases only act on type 1 and type 2 H substances.

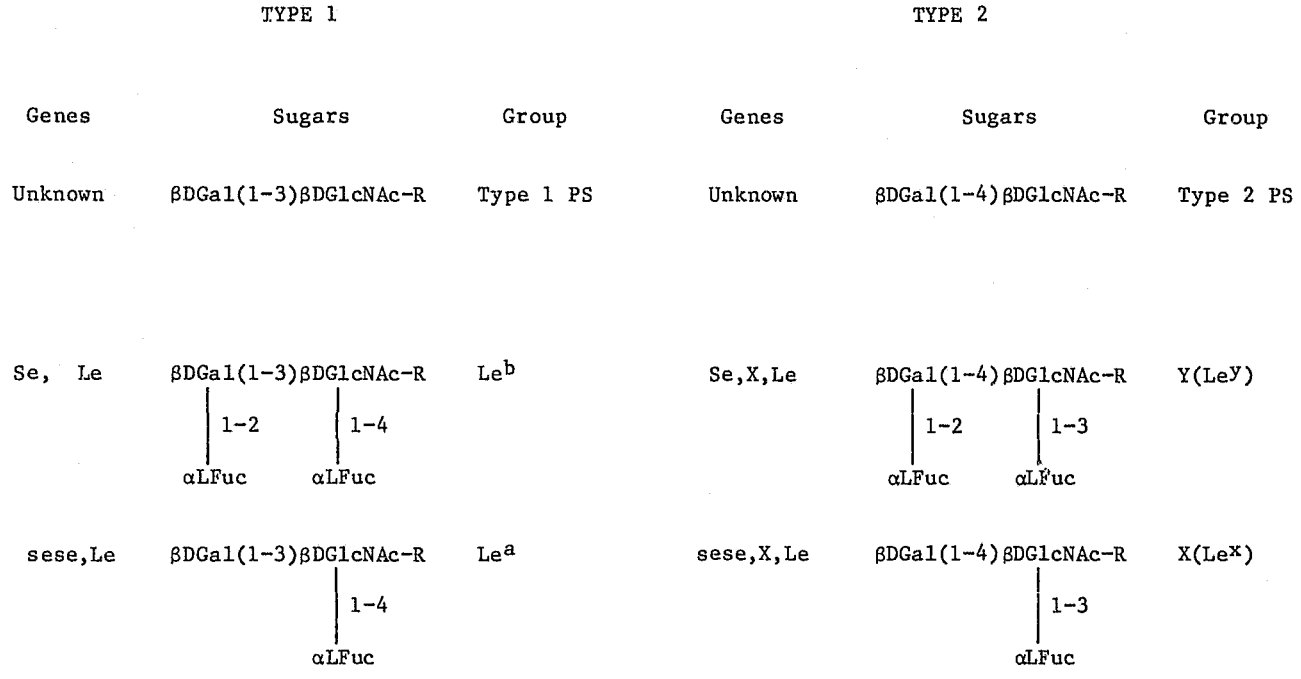

Figure 2. The sequential development of carbohydrate chains in Lewis and related secretions. In the presence of the dominant secretor gene, *Se*, type 1 and type 2 precursor substances (PS) are α1–2 fucosylated on the terminal galactose (Gal). The α3–fucosyltransferase of the high frequency *X* gene attaches a further fucose (Fuc) in an α1–3 linkage to the subterminal *N*–acetyl–D–glucosamine (Glc*N*Ac) of type 2 chains to give Y(Le^y) activity. Where the Lewis gene, *Le*, is also present its α3/4 fucosyltransferase couples a second fucose in an α1–4 linkage to type 1 chains giving Le^b and in an α1–3 linkage to type 2 chains giving further Y(Le^y). In the absence of the secretor gene, *Se*, the terminal galactose remains unfucosylated, and the *X* gene gives X(Le^x) on the type 2 structure and the *Le* gene gives Le^a on type 1 structures and further X(Le^x) on type 2 precursor substances.

molecules to form the antigens of interest. Thus, the forensic tests are performed on secondary gene products. More precisely, the transferase enzymes attach terminal sugars onto carbohydrate precursor substances in blood and secretions. These precursor substances are themselves attached to glycolipids and glycoproteins by carbohydrate chains of various lengths and degrees of branching, represented here by R. The precursor substances are present in all individuals and are of two types that are determined by the linkage between the terminal and subterminal sugars. In type 1 precursor substance, the terminal D–galactose (Gal) is linked to the subterminal *N*–acetyl–glucosamine (Glc*N*Ac) in a 1 to 3 linkage. In type 2 precursor substance, the terminal Gal is linked to the Glc*N*Ac in a 1 to 4 linkage. The precursor transferases are coded for by genes at unknown genetic loci.

For human secretions such as semen, saliva, vaginal fluid and milk, the dominant secretor gene, *Se*, produces a 2–fucosyltransferase attaching L–fucose to the terminal galactose of mainly type 1 but also type 2 precursor substances (Oriol *et al.* 1986). The chains now have type 1 and type 2 H activities. Since in secretions both type 1 H and type 2 H act as precursors for A and B, the presence of *Se* allows the production of A and/or B factors under the control of the *A* and *B* genes.

The group A transferase enzyme attaches *N*–acetyl–galactosamine (Gal*N*Ac) to the terminal galactose of both types of H, producing type 1 A and type 2 A. The group B transferase enzyme attaches Gal in a 1 to 3 linkage to the terminal galactose of both types of H, producing type 1 B and type 2 B.

In the absence of the *A* and *B* genes, the *O* gene has no detectable product, and the H substances remain unconverted. In the absence of the dominant *Se* gene, the production of H and subsequently A and/or B is not possible in secretions. The recessive nonsecretor gene has no active product, and the precursor chains are not converted to H.

If we now examine the role of the *Le* gene in secretions, the dominant *Le* gene produces a 3/4–fucosyltransferase that attaches an L–fucose in a 1 to 4 linkage to type 1 precursor chains. This confers Le^a activity on these chains. The type 2 chain cannot accept the L–fucose in the 1 to 4 coupling, since the carbon 4 of the Glc*N*Ac is already occupied. However,

there is a high frequency *X* gene that codes for a 3–fucosyltransferase. This couples an L–fucose in a 1 to 3 linkage to type 2 precursor chains (Watkins 1980). In addition, the dominant *Le* gene transferase can also use type 2 chains as an acceptor for a 1 to 3 linked L–fucose (Prieels *et al.* 1981; Johnson *et al.* 1981). The type 2 chain now has Le^x(X) specificity. Thus, the Le^x(X) factor is essentially the type 2 analog of Le^a, and because the *X* gene is of high frequency, it is present in the secretions of the vast majority of individuals.

When both the dominant *Se* and *Le* genes are present, the type 1 precursor chains are fucosylated on the terminal galactose in the 1–2 position to produce type 1 H and also fucosylated on the subterminal Glc*N*Ac in the 1 to 4 linkage. This difucosylated antigen has Le^b specificity. For type 2 chains, the terminal Gal is again fucosylated, and fucosylation of the subterminal Glc*N*Ac, this time in a 1 to 3 linkage, is carried out by the transferases of the *X* and *Le* genes to form Le^y(Y). Thus, just as Le^x(X) is the type 2 analog of Le^a, Le^y(Y) is the type 2 analog of Le^b.

In the absence of the dominant *Se* and *Le* genes, the recessive alleles, se and le, do not produce any known active enzyme; the individual then types as Le(a–b–) and is, of course, a nonsecretor of ABH substances. The body fluids are now strongly positive for type 1 precursor substance, which has been called rather confusingly Le^c. The ubiquitous *X* gene, however, does convert the type 2 chains that are present, and so Le^x(X) activity can still be detected in the secretions.

If the dominant *Se* gene is present in the absence of the dominant *Le* gene, then the individual still types as Le(a–b–) but will be a secretor of ABH activity. Thus, the type 1 precursor substances are converted, and the individual may be strongly positive for type 1 H, which has been referred to again rather confusingly as Le^d. In these individuals, the *X* gene will convert type 2 H chains to Le^y(Y).

The confusing nomenclature for type 1 precursor substance and for type 1 H as Le^c and Le^d, respectively, seems to have arisen when polyclonal antibodies to these blood group substances were presumed to be acting against type 2 analogs of Le^a and Le^b, respectively (Gunson and Latham 1972). [For an excellent explanation of how the confusion arose, see Lodge (1982).] However, as we have seen, Le^c and Le^d are not products of the *Le* gene transferase, and the type 2 analogs of Le^a and Le^b have been established as Le^x(X) and Le^y(Y). We have also seen that Le^x(X) and Le^y(Y) are not totally products of the *Le* gene but of a combination of the *X*, *Le* and *Se* genes in secretions. They are perhaps more conveniently referred to as just X or Y. Of course, it could also be said that Group O is also a misnomer because H type 1 substance, the recognized character of group O individuals in secretions, is not a product of the *O* gene but of the dominant *Se* gene.

For simplicity, the action of each transferase enzyme has been considered independently. In reality, of course, the transferases will act sometimes sequentially on the available substrates and sometimes in competition for the substrates. Thus, the production of the antigens may be affected by this interplay between the transferase enzymes. However, predictions of whether antigens will be detected by an examination of the biochemical pathways alone should be tempered with caution. Technique and reagents, particularly the antisera used, may play an overriding role. For example, both Dorrill *et al.* (1979) using autoanalyzer technology and Baechtel (1985) using manual inhibition noted a lack of correlation between A and H levels and B and H levels in the semen of an A and B secretor, respectively, when it might be expected that, in the presence of large amounts of A or B, H substance would be depleted. However, it was interesting that in Baechtel's study, 75% of group A individuals were found to have A:H ratios of 1, which corresponds approximately to the expected A^1:A^2 proportions. If the effects of such practical constraints are borne in mind, it may be useful to examine the predictions that can be made from the genetics and biochemistry of the two systems.

It should be noted that this paper follows the interpretation of the dominant *Se* gene as a structural gene, as reviewed by Oriol *et al.* (1981), rather than as a regulatory gene acting on the *H* gene as reviewed by Watkins (1980). Indeed, Betteridge and Watkins (1985) now concede that any regulatory model for the function of the *Se* gene must take into account not only some expression of an a–2–fucosyltransferase in the absence of the dominant *Se* gene but also the production of a variant enzyme in the presence of this gene. In the absence of any direct evidence for this proposal, we have preferred the more simple explanation.

Finally, as a general observation, the genetics and biochemistry of the two systems continue to increase in complexity. This complexity provides the forensic scientist with a daunting array of new ABH and Le structures, an example of which is the type 3 A chain of group A and AB individuals as reported by Clausen *et al.* (1985). This structure is based on elongated or repetitive type 2 A chains (Figure 3). However, the production of monoclonal antibodies (MCAs) to these structures (Furukawa *et al.* 1985) does provide new exciting possibilities for the subgrouping of the ABH

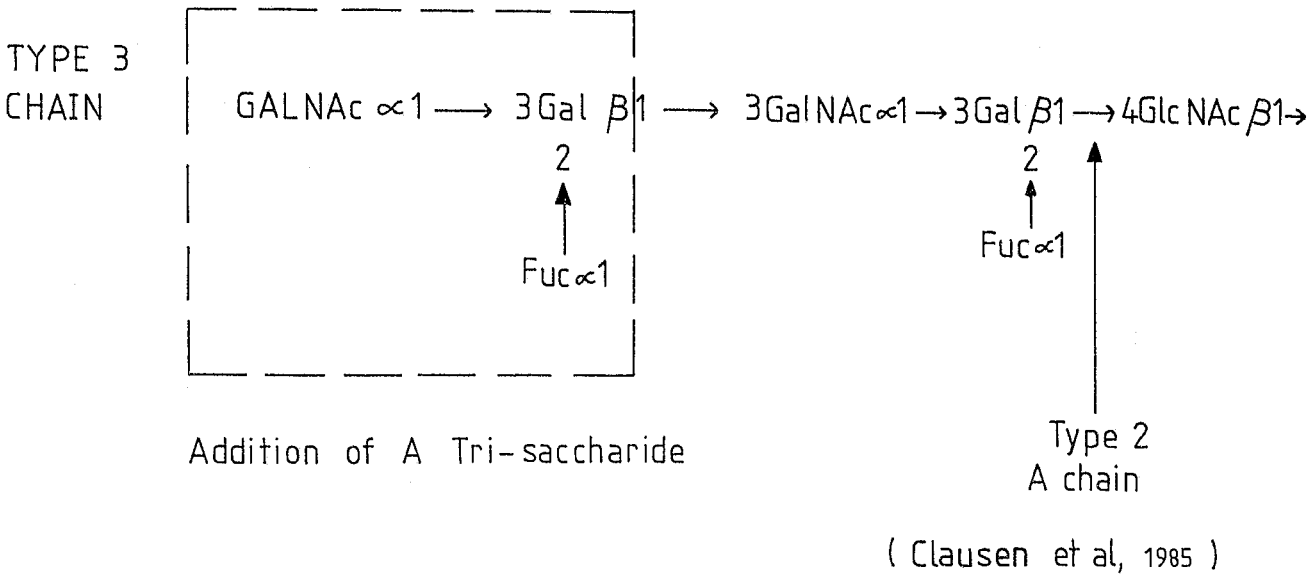

Figure 3. The repetitive type 3 A chain from group A and B individuals as described by Clausen *et al.* (1985). An A active trisaccharide is added to an existing type 2 A chain.

determinants. The forensic scientist should also be aware that the ABO and Le structures may become sialylated (Hannisch *et al.* 1985).

DEDUCTIONS BASED ON GENETICS AND BIOCHEMISTRY

The Prediction of Secretor/Nonsecretor Status

For Le positive individuals, the familiar relationship has now been established between the ABO and Le^a and Le^b factors. However, according to Chester (1971) and Watkins (1980), it may be that the preferred pathway of production for Le^b antigen is in fact from type 1 H to the difucosylated Le^b and not via the Le^a structure. Thus, in secretions at least, monofucosylated Le^a structures should be detected in Le^b positive individuals. In practice, several authors, for example, Piner and Sanger (1980) and Bassler (1986), have reported the detection of Le^a in Le^b positive individuals. Thus, including Le negative individuals, possible Le phenotypes emerge in secretions (Figure 4). Therefore, Le^a cannot be considered indicative of a nonsecretor because it is commonly found in secretors. The only diagnostic feature of the nonsecretor stain is then the absence of Le^b in the presence of Le^a.

In practice, Le grouping is generally carried out when no ABH activity has been detected and when its objective is the prediction of secretor status. The efficacy of the test will lie in the relative stabilities of the ABH and Le factors and our ability to detect them. Stains that have failed to ABH type (Figure 5) may have done so not because the stain has originated from a nonsecretor, but because the ABH blood group substances have deteriorated. If Le^b should deteriorate more rapidly than Le^a, then in precisely the situation where Le grouping is most likely to be used, it is also most likely to give a false Le(a+b–) result, confirming the erroneous prediction that the stain originated from a nonsecretor. Table 1 lists a series of results from U. K. laboratories in 1981. These were obtained after grouping by inhibition duplicate sets of experimentally prepared stains. Clearly, the detection of Le^a is susceptible to failure. However, the Le^b antigen is readily and reliably detected. A limited followup study at one laboratory demonstrated that it was also possible to preferentially fail to detect the Le^b antigen. These results indicate that the test system may be working close to the limits of its sensitivity and that it may not be the deterioration of the antigen that is the problem but, in fact, the ability to detect the Le^a and Le^b factors. Subsequent work at the Central Research Establishment (CRE) confirmed the very occasional preferential loss of Le^b in a secretor individual and identified the problem as a lack of balance between the Le^a and Le^b reactions, that is, the ability to detect Le^a and Le^b varied independently. At the Metropolitan Police Forensic Science Laboratory, De Soyza (1984) also encountered this problem, a saliva stain from a known Le(a–b+) secretor individual typed erratically,

	Lewis Antigens Detected in Secretions	Other Antigens Predicted	
		Present	Absent
Lewis Positve Individuals			
Non-Secretor	a+b-	T1 and T2 PS	ABH
Secretor	a-b+	T1 and T2 PS ABH	
Secretor	a+b+	T1 and T2 PS ABH	
Lewis Negative Individuals			
Non-Secretor Secretor	a-b-	No predictions can be made.	

Figure 4. The relationship of Lewis antigens detected in secretions and the prediction of ABH secretor/non-secretor status. Lewis a may be present in both secretors and non-secretors for Lewis positive individuals. No predictions can be made for Lewis negative individuals.

sometimes as a Le(a+b–). This led to a revision of the routine test method. Essentially to balance antigen detection, it was necessary to use the Le^a antisera at the last but one dilution giving complete agglutination in contrast to Le^b, which is used at the ultimate dilution giving complete agglutination (De Soyza, personal communication).

An Alternative to Lewis A and B

The presence of X and Y factors in secretions is of great interest because they provide the forensic scientist with a potential alternative to Le^a and Le^b. As we have seen X and Y are essentially the type 2 analogs of Le^a and Le^b, respectively. Furthermore, the production of these analogs is partly independent of the *Le* gene and, therefore, these factors will be present in Le(a–b–) individuals, X in the absence of Y being diagnostic of an Le(a–b–) nonsecretor individual and Y being diagnostic of an Le(a–b–) secretor. Thus, the overall picture of prediction of secretor status could become as shown in Figure 6. From Figure 6, it can be seen that, like Le^a, the X antigen will be detected in the secretions of secretors. The problem, therefore, remains that no one antigen is exclusively found in nonsecretors. Although proven MCAs to these factors are not commercially available, the potential application of Le X and Y is being explored at the CRE.

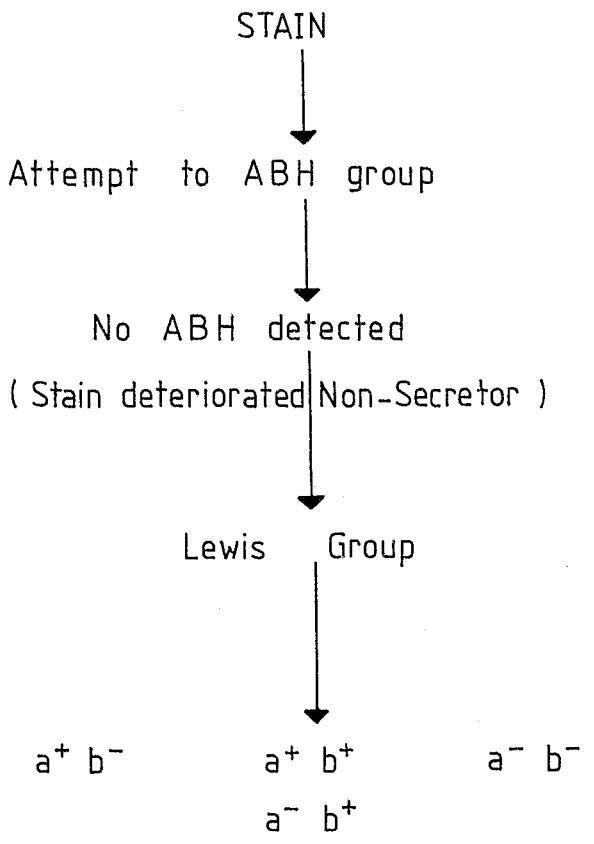

Figure 5. The role of Lewis grouping: The relative stabilities of ABH and Lewis blood group substances, particularly Lewis a and b, plays a crucial role in the efficacy of the test.

Table 1. REPLICATE SETS OF SEMEN STAINS TESTED FOR LEWIS ACTIVITY BY SIX LABORATORIES[a]

LABORATORY	SAMPLE 1 LEWIS		SAMPLE 2 LEWIS		SAMPLE 3 LEWIS		SAMPLE 4 LEWIS		SAMPLE 5 LEWIS		SAMPLE 6 LEWIS	
	a	b	a	b	a	b	a	b	a	b	a	b
DONOR GROUPS	+	–	–	+	–	+	+	–	–	+	–	+
A	+	–	+	+	NR	+	+	NR	+	+	NR	NR
B	+	–		+	–	+	+	–		+	Inc	Inc
C	+	–	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
D	+	–	–	+	–	+	+	–	–	+	–	+
E												
F												
G	+	–	+	+	+	+	+	–	+	+	NR/+	NR/+
H	+	–	+	+	+	+	+	–	+	+	–	+
I												

[a]Note the susceptibility of Lewis a detection to failure (sample 3 in particular). NR = No Result. Inc = Inconclusive. Nine laboratories surveyed (A–I) Six returned results

	Lewis Antigens Detected in Secretions	Other Antigens Predicted	
Lewis Positive Individuals		Present	Absent
Non-Secretor	Lewis a, X	T1 and T2 PS	ABH
Secretor	Lewis a- (a+),b+ X and Y	T1 and T2 PS ABH	
Lewis Negative Individuals			
Non-Secretor	X	T1 and T2 PS	ABH
Secretor	X and Y	T1 and T2 PS ABH	

Figure 6. Prediction of secretor status including the X and Y antigens. The presence of X and Y may provide a means of predicting secretor status in Lewis negative as well as Lewis positive individuals.

The Specific Detection of Type 1 and Type 2 Blood Group Substances

In recent years, there have been reports of antibodies to various ABH and Le factors, including reagents with the ability to identify exclusively type 1 or type 2 blood group substance (for example, Abe *et al.* 1984; Sakamoto *et al.* 1984). The findings in these reports may provide the ability to distinguish between blood group activity from red cells and secretions in mixtures of stains. However, examination of the genetics and biochemistry of the two systems predicts the presence of both type 1 and type 2 chains in secretions. Therefore, the presence of traces of type 2 blood group substance in stains of secretions is expected. Thus, specific identification of blood group substances originated from red cells may have to rely on the identification of the carrier molecule as well as specific carbohydrate moiety. Alternatively, a quantitative approach might have to be adopted.

LEVELS OF BLOOD GROUP SUBSTANCE IN SEMEN AND SALIVA

Levels of expression of the various ABH and Le factors vary in the secretions. Concern about this variation, together with the consequential effect that this could have on the operational scientist's ability to group stains, led to the CRE's investigating the levels of blood group substances in both semen and saliva in the early 1970's. The work followed on from the studies of Sturgeon and McQuiston (1966). The method of choice was adsorption–inhibition using an autoanalyzer. Davie and Dorrill (personal communication) first derived units of activity for A, B and H substance by pooling approximately 2 liters of boiled human saliva from a large and approximately equal number of A, B and O secretor donors. The pooled saliva was then given arbitrary units per milliliter for A, B and H activity based on the reciprocal of the end point titer achieved by manual inhibition. Their results (Dorrill *et al.* 1979) demonstrated that A, B and H levels in both semen and saliva varied enormously, particularly between individuals (Table 2), but also to a lesser extent within some individuals (Table 3). The levels of blood group substance were found to be generally higher in semen than in saliva (Table 4). In particular, the mean levels of A and B blood group substances in semen were approximately twice that found in saliva. However, the difference was much more dramatic for H, there being approximately 20 times more H in semen than in saliva, and this was reflected by A:H and B:H ratios of generally much less than 1.

The variation within individuals led to the recommendation that considerable caution should be exercised when a common origin is inferred for semen stains from different scenes of crime. Indeed, it was possible that, with a limited number of individuals, the amounts of blood group substance would be adequate for successful typing by both adsorption–elution and adsorption–inhibition on one occasion, but on a subsequent day, positive results might only be obtained by adsorption–elution.

A followup study to these findings by Shaw *et al.* (1981) related the levels of blood group substance, as defined by the autoanalyzer, to the chances of successful grouping by manual methods, as performed by two separate laboratories. In this study, 42 samples of either saliva or semen were selected so that some, by virtue of their levels of blood group substance(s) as determined by the autoanalyzer on liquid samples, might be difficult to group by manual methods from stains. The results demonstrated that there was a reduced ability of the manual techniques to detect H, in comparison with A and B, confirming the earlier work of Pereira and Martin (1976). It was projected that this failure to detect H by adsorption–inhibition, in particular, would invalidate a large proportion of successful determinations by adsorption–elution and up to 50% of the group O saliva stain results. Thus, there appears to be a marked difference in the ability of manual methods and the autoanalyzer to detect H substance. Dorrill *et al.* (1979) had found relative levels of blood group substance, particularly in semen, that supported the prediction that, in a small number of individuals, the low levels of A or B substance might not be detected by manual methods, whereas

Table 3. LEVELS OF BLOOD GROUP SUBSTANCE RANGES WITHIN AN INDIVIDUAL (Davie *et al.* pers. comm. 1979

BGS	BODY FLUID	RANGE	MEAN	N
B	SALIVA	130–3,750	1,690	66
B	SEMEN	150–13,500	3,700	13
H	SALIVA	125–770	340	66
H	SEMEN	9,250–52,000	22,800	13

Table 2. LEVELS OF BLOOD GROUP SUBSTANCES RANGES BETWEEN INDIVIDUALS FOR SALIVA AND SEMEN (Dorrill *et al.* 1979

BGS	RANGE	
	SALIVA	SEMEN
A	150–9,250	20–28,500
H	100–7,200	30–48,750
B	133–3,750	150–13,500
H	125–770	125–770
H	40–4,300	70–82,000

Table 4. THE MEAN LEVELS OF BLOOD GROUP SUBSTANCE COMPARED FOR SEMEN AND SALIVA (Dorrill *et al.* 1979

BGS	MEAN	
	SALIVA	SEMEN
A	1,360	4,300
H	930	21,800
B	1,690	3,200
H	340	22,800
H	1,260	22,000

the relatively high coincidental level of H would be. Simply, the secretion might type as group O unless both adsorption–elution and adsorption–inhibition were used in parallel. Although Shaw *et al.* (1981) found that low levels of both A and B substance were generally detected, one laboratory failed to detect A and B blood group substance in one A and one B stain, respectively, such that, by adsorption–inhibition alone, the stains were close to being reported as group O (Figure 7).

Baechtel (1985), using a very sensitive manual inhibition technique, confirmed the findings of Shaw *et al.* (1981) and concluded that the major cause of disparity between the two methods for the assay of H was that the autoanalyzer assays for semen were based on a saliva standard. However, he also found that the level of H blood group substance in semen was generally much higher than in saliva, and therefore, it is probable that it is not reference to an inappropriate standard which causes the conflict but a genuine difference in the relative sensitivities of the two techniques to detect A, B and H blood group substances. As to why the autoanalyzer should be more sensitive to H than are conventional manual methods is open to conjecture. Apart from the obvious differences between the two techniques, there are the added complications that the autoanalyzer method at CRE uses enzyme treated red cells and crude extracts of *Ulex europeaus* (Boorman *et al.* 1977). Baechtel used a commercial preparation.

Clearly, it is very difficult to generalize about the potential of the performance of one technique based on observations that were made using a second and different technique. However, some generalizations are possible, and the observations of Dorrill *et al.* (1979) provided the valuable quantitative evidence for low level secretors. These observations clearly demonstrated that the Le grouping of ABH negative casework stains, as recommended by Pereira and Martin (1976), was essential if the nonsecretor phenotype was to be unequivocally demonstrated. Furthermore, large uncorrelated variations in A:H and B:H ratios were observed between individuals, a finding that could not be predicted from the known biochemical pathways. Indeed, these observations firmly established, at least in the United Kingdom, the testing of all casework

	ABSORPTION INHIBITION		ABSORPTION ELUTION		ASSAY LEVELS			TYPE	BLOOD GROUPS ABO	BLOOD GROUPS LEWIS	
DILUTION	Lab X	Lab Y	Lab X	Lab Y	A	B	H			a	b
	A B H	A B H	A B H	A B H							
N	+ 4 0	0 4 0	+ 0 0	4 0 4	490	0	16750	SEMEN	A	−	+
1/5	3 4 1	0 4 0	+ 0 0	4 0 4							
1/10	3 4 3	0 4 0	3 0 3	3 0 3							
1/20	3 4 3	0 4 2	3 0 3	3 0 3							
1/40			3 0 4	3 0 3							
N	3 + 0	4 0 0	0 0 0	0 2 0	0	223	16500	SEMEN	B	−	+
1/5	3 2 0	3 0 1	0 3 0	0 2 2							
1/10	4 3 3	3 0 2	0 4 0	0 1 2							
1/20	4 3 4	4 0 3	0 4 0	0 2 1							
1/40			0 3 0	0 2 1							

Figure 7. Detection of H preferentially, in secretors with low levels of A and B blood group substance, by manual inhibition (Shaw *et al.*, 1981).

material by adsorption–elution and adsorption–inhibition in parallel. The more sensitive adsorption–elution was used to ensure the detection of either low level secretors or aberrant secretors as defined by Clarke *et al.* (1960) and illustrated by Smerling (1972) using mixed agglutination and inhibition in parallel. Adsorption–elution, however, does provide its own problems such as the detection of the so–called acquired antigens (Davies *et al.* 1984) and the detection of blood group substance in the secretions of Le(a+b–) individuals. The latter will depend largely on the particular method of adsorption–elution that is used.

Before the techniques currently used by the operational laboratories in the United Kingdom are documented, the disparate distribution of blood group substances across a stain should be discussed. Pereira *et al.* (1969) noted that ABH blood group substance tended to be concentrated towards the center of a stain. Rutter (1981), using autoanalyzer technology, quantitated the distribution of blood group substance and found that ABH and Le blood group substance were not necessarily coincidentally distributed. He confirmed that ABH tended to be concentrated in the center of the stain but that Le substance showed no equivalent distribution. Similarly, sequential semen-free vaginal swabs (Martin 1983) and semen-contaminated vaginal swabs can give very different levels of blood group substances and, by complete analogy with the observations made on stains, it is possible to obtain large variations in ABH and Le activity from different areas of the same vaginal swab (Divall, personal communication). Extraction procedures must, therefore, be modified to take such variations into account.

METHOD, INTERPRETATION AND REAGENTS

Method

Figure 8 lists the adsorption–elution techniques and, in particular, the dilutions used for stain extracts in the U. K. laboratories. All of the laboratories have derived methods from the Howard and Martin (1969) technique for bloodstains with the important addition of an antigen dilution series. Two of the laboratories continue to use dimpled welled sheets and threads. The remaining laboratories use a more sensitive drying down technique (Figure 8) which is more akin to earlier extractive methods, as reviewed by Kind and

DILUTION OF STAIN EXTRACT	LABORATORY						
	ALDER	BIRM	CHEP	CHORL	HUNT	MPL	WETH
Method	1	1	2	2	2	2	2
N	N	N	N	N		N	N
1/2			1/2			1/2	
1/5	1/5	1/5	1/5	1/5		1/5	
1/10	1/10	1/10	1/10	1/10	1/10	1/10	1/10
1/20	1/20	1/20	1/20			1/20	
1/40			1/40			1/40	
1/50		1/50		1/50			1/50
1/100		1/100		1/100	1/100		1/100
1/500							1/500
1/1000					1/1000		
1/10,000					1/10,000		

Figure 8. Dilutions used in adsorption-elution tests and minimum elution levels required to give an acceptable result. Method 1 = Howard & Martin with threads-dimpled plates. Method 2 = extractive method, no threads.

Cleevely (1969). The latter technique tends to detect traces of blood group substance in the secretions of Le(a+b–) nonsecretor individuals. This finding is in accord with the finding of a type 2–specific a–2–fucosyltransferase in the salivary gland of nonsecretors (Betteridge and Watkins 1985). Agglutination of indicator cells in the 1:10 to 1:20 dilutions of the original extract is considered to be a reliable indicator of the presence of antigen for most laboratories.

The adsorption–inhibition methods in use throughout the U. K. laboratories are more varied (Figure 9), ranging from the classical tube technique through to the microtiter spin and streak technique of Davie (1979, 1980), but again the presence of significant amounts of antigen centers around, in this instance, inhibition of agglutination by the 1:10 dilution.

Manual methods are difficult to standardize, and therefore, uniformity of technique *per se* among laboratories has not been considered necessary. What is required is a uniformly high standard. Thus, the performance of these techniques is measured by a Quality Assurance Program. The figures for saliva stain and semen stain grouping for 1984 are given in Table 5. In 1985, a total of 386 ABO/Le tests were carried out with only 1 failure to detect Le^b. It is

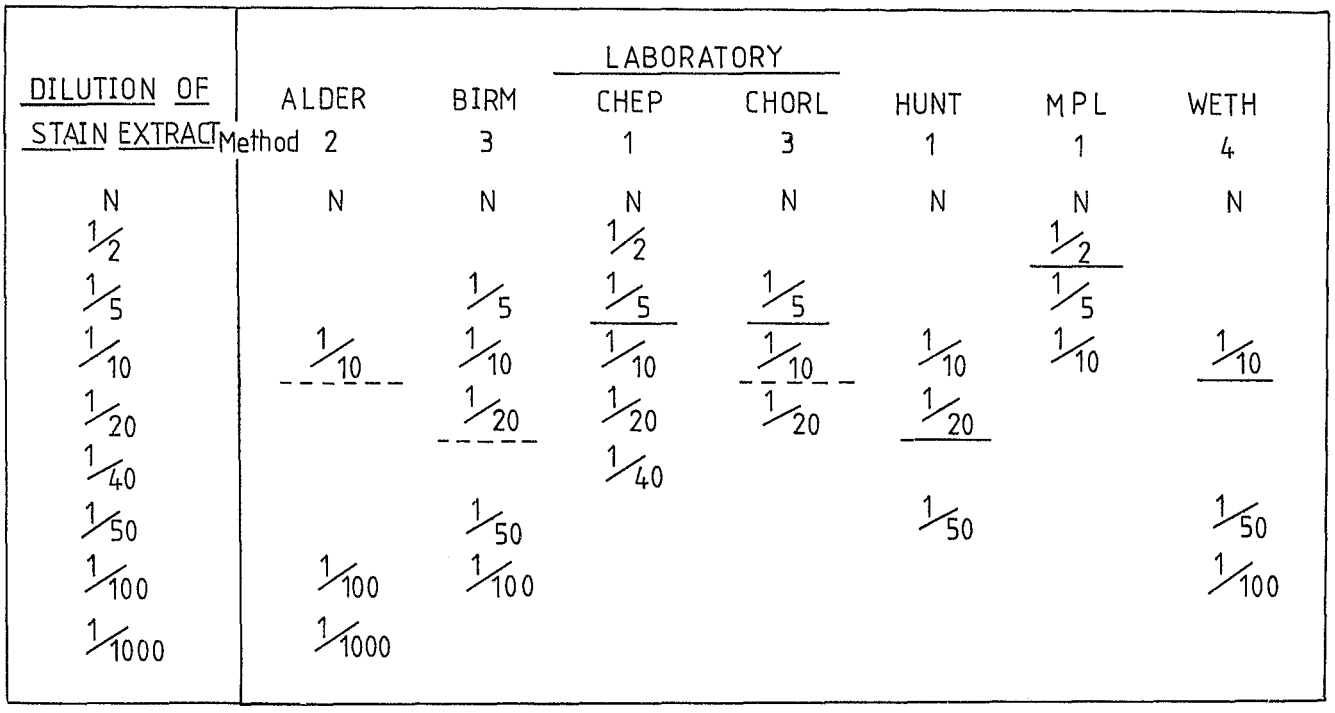

Figure 9. Dilutions used in adsorption-inhibition tests and minimum inhibition levels required to give an acceptable result. Method 1 = tube. Method 2 = dimpled cellulose acetate. Method 3 = microtiter plate. Method 4 = WHO plate.

Table 5. RESULTS OF BODY FLUID GROUPING TESTS—HOFSS INTERNAL QA TRIAL PROGRAMME 1984

Material Tested	Group System	Total Tests	INCORRECT RESULTS TESTS	%	INCONCLUSIVE RESULTS TESTS	%
Saliva (liquid + stains)	A B O	112	0	—	6	5%
	Lewis	103	0	—	6	6%
Semen (stains)	A B O	67	0	—	12	18%
	Lewis	45	0	—	15	33%
	PGM (SE)	38	0	—	3	8%
	PGM (IEF)	67	0	—	7	10%

essential that laboratories do conduct rigorous testing of their ability to detect the ABO and Le soluble antigens in body fluid stains.

Interpretation

If the antigens are detected, then the forensic scientist has the sometimes onerous task of interpretation. The task is often most difficult when dealing with vaginal swabs. Significant amounts of ABH blood group substance can be produced by the vagina (Masis 1967; Hossaini *et al.* 1981; Martin 1983). Vaginal blood group substance could, therefore, mask the seminal contribution. Through the use the population statistics reported by Rothwell (1985) in Table 6, the maximum percentage of cases in which foreign antigen can be theoretically detected on vaginal swabs is 38%. (In deriving that figure, the assumption has been made that significant levels of blood group substance are not secreted by an Le(a+b–) woman in her vaginal fluid.) From the table, it can be seen that the value of the test as a means of identifying a suspect is limited; in fact, the most valuable use of the technique can be at the investigative stage as a means of excluding suspects. However, some forms of potential exclusion present the forensic scientist with the most difficult interpretative problems. For example, can a group A suspect be safely excluded when only H is detected on a semen stained swab from a group O woman? This difficult situation has been the subject of debate both in the United Kingdom and in the United States (Inman 1985). Of course, the resolution of this problem lies in the ability to correlate blood group substance levels with levels of semen markers. If it can be confidently predicted that sufficient semen is present for a successful ABH test, then, in this particular example, group A suspects can be confidently excluded.

The ability to produce convincing correlation studies between ABH, Le and semen markers relies on quantitation. The currently used semi–quantitative methods for ABH grouping are not ideal for precise studies of this type. Consequently, the CRE has continued to use autoanalyzer technology to investigate this problem as well as the potentially objective and sensitive enzyme linked immunosorbent assay (ELISA). For secretions, autoanalyzers have the disadvantage of using red cells as indicators of blood group activity. As such, they might not be able to take advantage of specific monoclonal reagents for type 1 blood group substance; simply, the indicator red cells may not be agglutinated by antibodies specific for type 1 chains (Abe *et al.* 1984).

Reagents

The supply of MCAs has become widespread, and these antibodies do offer, potentially, a uniform supply of high quality antisera, for example, the monoclonals examined by Gaensslen *et al.* (1984). Their performance, however, is sometimes unpredictable. Unexpected results may be obtained of which the operational scientist should be aware before the reagents are adopted for routine casework investigation, whatever the method used. This point can be illustrated by reference to some commercially available Le monoclonal antisera. Some results have been obtained with a four stage ELISA. Since 1978, when the potential of ELISA in forensic science was reported to the International Association of Forensic Science by Werrett and Dolton (1978), the CRE has concentrated on this type of assay.

The four stages of ELISA are immobilization of the antigen in a microtiter plate well, addition of MCA, addition of conjugate and incubation with

Table 6. THE MAXIMUM PERCENTAGE OF CASES IN WHICH FOREIGN ANTIGEN CAN BE THEORETICALLY DETECTED[a]

Frequencies (After Rothwell, 1985)					
ABO			Lewis		
A	38.3		$a^+ b^-$		24.4
B	10.8		$a^- b^+$		67.5
O	47.6		$a^- b^-$		8.0
AB	3.2				
Possible combinations and inferred seminal group					
FEMALE'S GROUP	Non–Sec	O	A	B	AB
ANTIGENS DETECTED	HA B A,B	A B A,B	A B A,B	A B A,B	A B A,B
INFERRED SEMINAL PHENOTYPE	O A B AB	A B AB	— B B AB	A—A AB	———

[a]Theoretical maximum for detecting foreign antigen = approx 38%

substrate, in this case *p*–nitrophenol phosphate (Figure 10). Chembiomed™ MCAs to Le^a and Le^b, together with antisera supplied by Lund University Hospital, Sweden, were examined for their ability to identify Le^a and Le^b factors in liquid saliva, semen and stains made from both fluids. For liquid saliva, the appropriate Le activity was detected up to a dilution of 1:100,000 (Figure 11); for saliva stains, this activity was reduced 100–fold, but the relative amounts of Le^a and Le^b were comparable (Figure 12). However, for semen, with the same antisera and a modified three stage ELISA in the MCA and with conjugate added simultaneously, Le^a, even in Le^a individuals, was barely detectable. The Le^b on the other hand was detected to a dilution of 1:300,000 in semen stains that had originated from Le(a+b–) individuals, and, with an MCA supplied by the Wistar Institute (Philadelphia, Pennsylvania) X activity was shown to a similar dilution.

Recently, Uhlenbruck *et al.* (1984) reported the presence of sialylated Le^a antigen in seminal plasma. Furthermore, the treatment of sialylated Le^a antigen with neuraminidase was found to destroy not only sialylated Le^a activity in saliva (Brockhaus *et al.* 1985), but also to enhance levels of Le^a on erythrocytes (Picard *et al.* 1985). If, in semen, the majority of Lea activity is presented in a sialylated form and if the Chembiomed™ and Lund MCAs are specific to the native Le^a, then treatment of semen stain extracts with neuraminidase should facilitate the assay of Le^a.

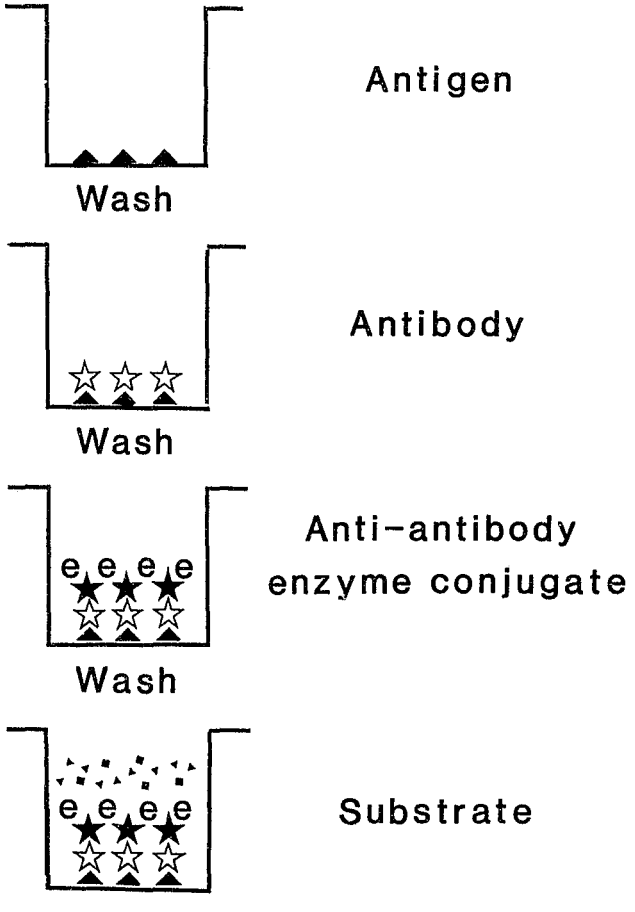

Figure 10. The four stage ELISA technique.

Initially for convenience, approximately 3.0 units of neuraminidase, prepared from *Vibrio cholera* as supplied by BDH Ltd., was added to the semen stain extracts at the immobilization stage, which was carried out at 37° C for 150 minutes at pH 7.4 (even though it was realized that this would compromise the optimum pH for neuraminidase activity). After washing, the tests were treated with MCA/conjugate mix before a final washing step and addition of substrate. The results were dramatic: the Le^a activity in both secretor and nonsecretor semen was fully visualized to a dilution of 1:24,000 of the original semen proteins by the Chembiomed™ anti–Le^a, in particular (Figure 13).

In a further investigation, the amount of neuraminidase was optimized at 0.5 units per test well. A double comparison was made between neuraminidase treatment at the immobilization stage and at the antibody/conjugate stage with the antibody/conjugate mix incubated at either 20° C or 37° C. These tests showed that incubation of the monoclonal anti–Le/conjugate stage at 20° C rather than at 37° C was superior for detection of Le^a and Le^b, even when neuraminidase treatment was being applied simultaneously. The Le^b activity was shown to at least a dilution of 1:300,000 of the original semen proteins with or without neuraminidase treatment and Le^a to a similar dilution after neuraminidase treatment either at the immobilization or MCA/conjugate stage. Clearly, the anti–Le^a antibody is extremely specific to the unsialylated form of the Le^a structure (Figure 14).

Somewhat paradoxically, however, these same Le^a and Le^b MCAs from Chembiomed™ have been found to react with two Le(a–b–) nonsecretor saliva and five Le(a–b–) secretor saliva, respectively. One can only speculate as to the crossreacting molecule in each case. It could be the type 2 analogs X and Y, respectively, or in the case of the anti–Le^b, it could depend on the precise specificity of the antibody, that is, whether it is predominantly of type Le^bH or Le^bL, the former giving crossreactions with type 1 H in the secretions of Le(a–b–) secretors (Figure 15).

Both of these Chembiomed™ preparations are very useful and powerful reagents. At CRE, they have been used to type extracts from experimentally prepared 2 μl semen stains, equivalent to a 1:1,300

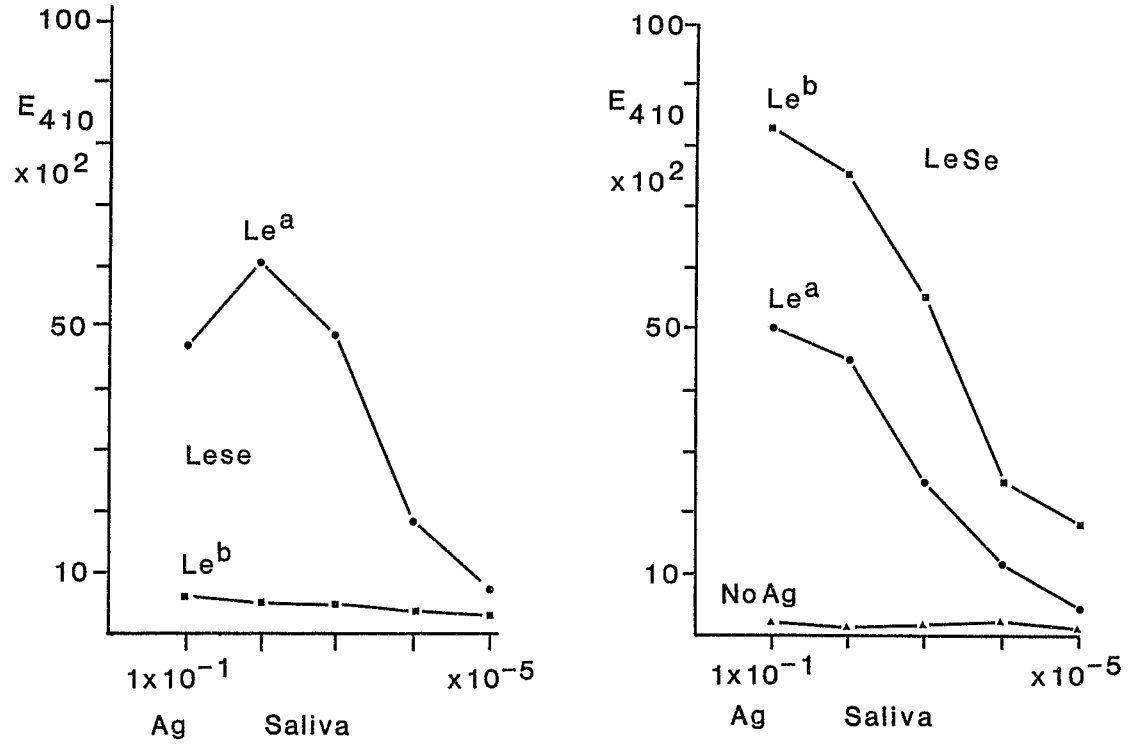

Figure 11. Detection of Lewis a and b antigens by ELISA in a Le(a–b+) individuals (LeSe) and a Le(a+b–) individual's (Lese) liquid saliva.

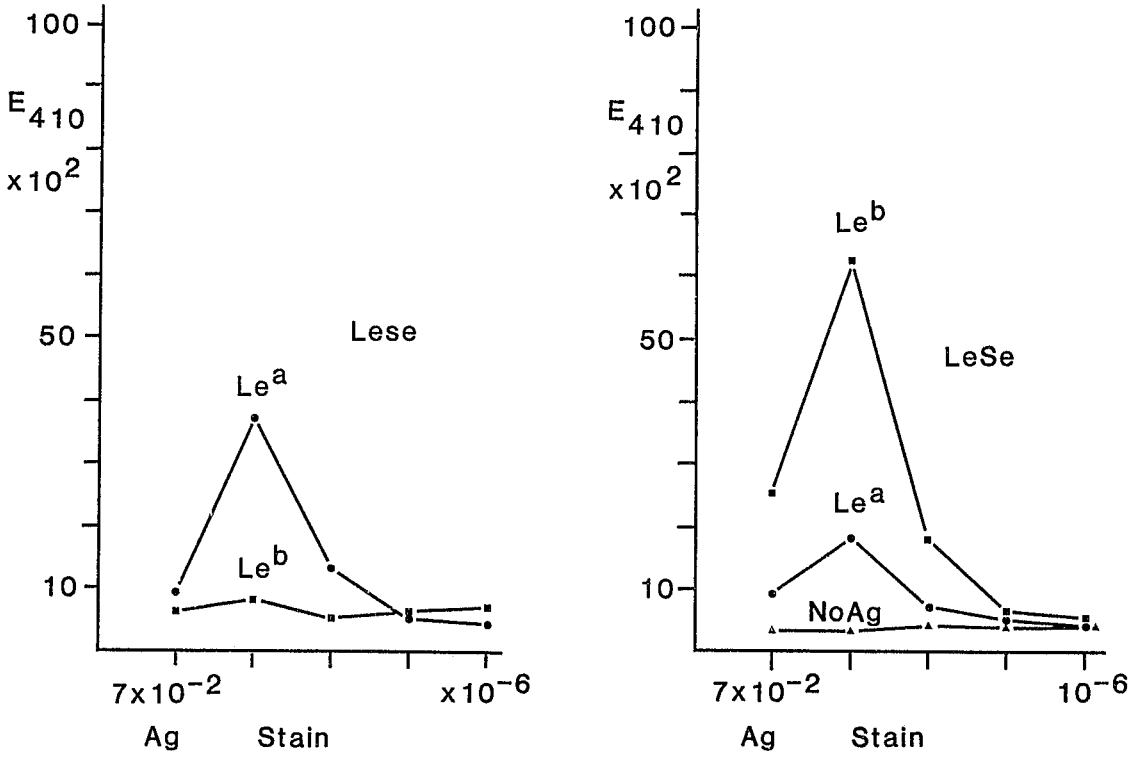

Figure 12. Detection of Lewis a and b antigens by ELISA in a Le(a–b+) individual's (Lese) and a Le(a+b–) individual's (Lese) saliva stain extracts.

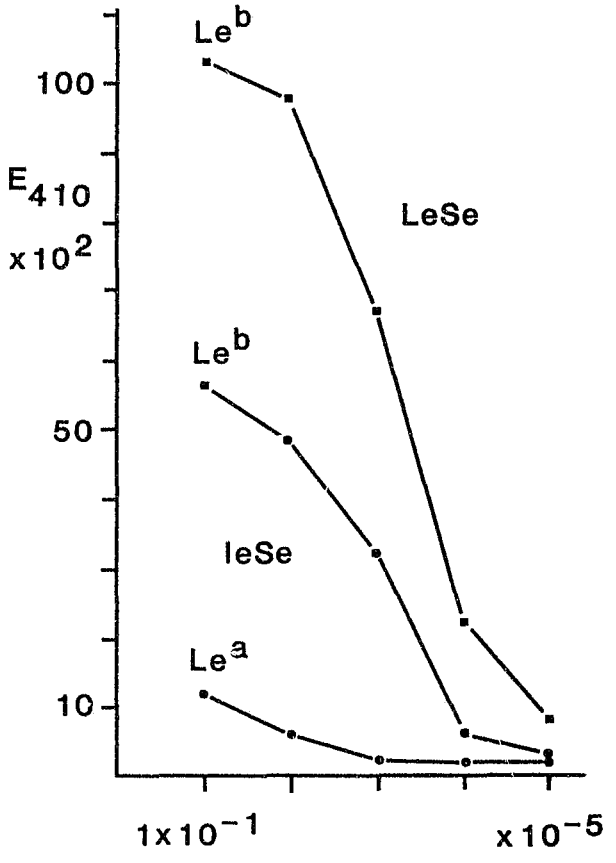

Figure 13. Detection of Le^a and Le^b activity by ELISA in a Le(a–b–) secretor (leSe) liquid saliva compared with Le^b activity in a Le(a–b+) (LeSe) secretor liquid saliva.

dilution of original semen. However, their peculiarities serve as a timely reminder that the specificity of all monoclonals cannot be taken for granted. Furthermore, by virtue of their specificities, they may reveal in conjunction with the ELISA technique, idiosyncracies in the biochemistry of the ABO and Le systems which are not found using polyclonal antisera by more traditional techniques.

SUMMARY

The genetics and biochemistry of the ABO and Le systems in secretions were described with reference to the sometimes confusing nomenclature. A tape slide presentation on this subject is being produced. The prediction of secretor status by Le typing was explored together with the value of X and Y determination. The presence of type 1 and type 2 H in secretions was explained and the significance of the presence of type 2 considered. The levels of ABH blood group substances in secretions vary both between and within individuals. Technique also plays an important role. Quantitative assays of blood group substances have firmly established the need to perform adsorption–elution and adsorption–inhibition in parallel, together with the need to Le type stains giving negative ABH results.

The current methods in use in the United Kingdom were documented. For stains, significant antigen detection focuses around the 1:10 dilution level. The onerous task of interpretation was examined with particular reference to the exclusion of individuals.

Monoclonal antibodies and ELISA are recognized as the potential reagents and technique of the future, but the performance of the reagents must not be taken for granted. An example of both extreme specificity and apparent crossreactivity was given for monoclonal anti–Le reagents.

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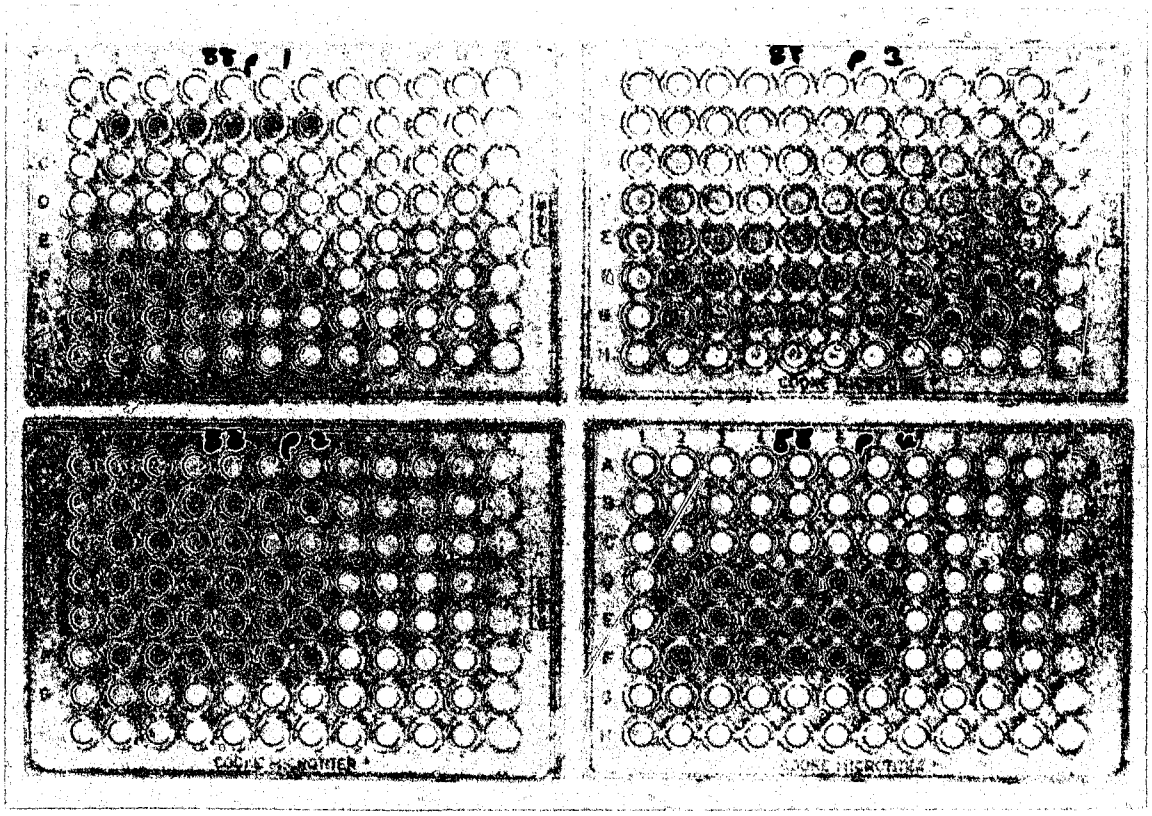

Figure 14. A comparison of Le^a, Le^b and X (Le^x) activities detected by ELISA in extracts of 3m. old semen stains with and without neuraminidase treatment.

(a) All the outside wells of each microtiter plate are "pNPP only" controls except "plastic only" dry wells in column 12.

(b) Neuraminidase solution was added to all wells except outside wells at the immobilization stage in Plates 1 (0 Le a+b–) and 2 (0 Le a–b+)

(c) No neuraminidase was added to Plates 3 (0 Le a+b–) and 4 (0 Le a–b+)

(d) Rows B–G contained antisera to Le^a CB, Le^aL Le^b CB, Le^bL, X (Le^x) W and a no MCA control.

CB=Chembiomed. L=Lund, W=Wistar. MCA=Monoclonal antibody

(e) Each 20 microliter stain was extracted in 600 microliters of PBS pH 7.4, giving a 1/30 dilution of the original semen proteins

(f) Columns 2–11 contained three dilutions of semen stain extract, a cloth control extract and a no antigen (diluent) control, each test in duplicate as follows;

		Semen stain extract		Cl. Con.	No antigen
Cols.	2 & 3	4 & 5	6 & 7	8 & 9	10 & 11
	1/8	1/80	1/800	1/8	Diluent
	(1/240)	(1/2,400)	(1/24,000)		
Approx.	3	0.3	0.03	3	3 BDH units

Figures in parenthesis give the dilutions of the original semen proteins

Units of Neuraminidase (BDH) per test well included in plates 1 and 2 as follows;

(g) i. Test solutions were immobilized for 2.5hr at 37°C

ii. The MCA/conjugate was incubated for 1.5hrs at 20°C

iii. The substrate (pNPP) was incubated at 20°C for 30mins and the plates were then stored at 5°C overnight before being stopped with 3M NaOH.

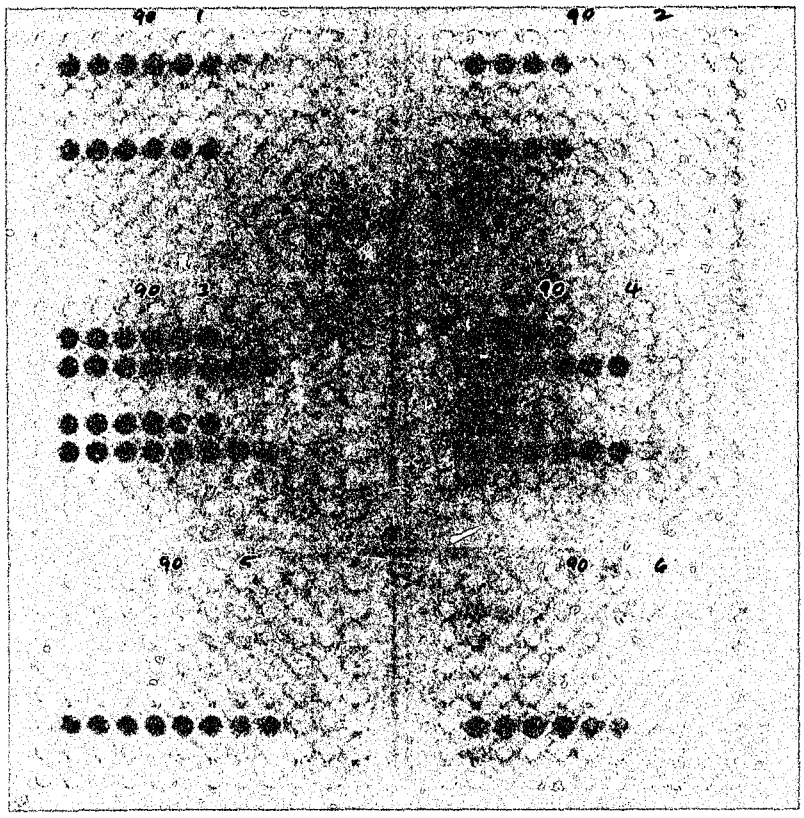

Figure 15. Le^a and Le^b activities detected by ELISA in extracts of 3m old stains from an 0 Le a+b– and an 0 Le a–b+ pair of individuals after neuraminidase treatment at either the immobilization or antibody/conjugate stage of a simultaneous ELISA compared with no neuraminidase treatment. The arrangement of the tests is similar to Plate 1 except as follows;

(a) Approximately 0.5 units (BDH) of neuraminidase were added to all wells (except outside wells) of microtiter plates 1–4 either at the immobilization stage (rows B–D) or at the antibody/conjugate stage (rows E–G)

(b) No neuraminidase was added to plates 5 and 6

(c) Rows B–D contained monoclonal antibody to Le^a CB, Le^b CB and a no MCA (diluent) control respectively. This was repeated for rows E–G.

CB=Chembiomed, MCA=monoclonal antibody,

(d) Each 20 microliter stain was extracted in 600 microliters of PBS pH 7.4, giving a 1/30 dilution of original semen proteins.

(e) Columns 2–11 contained four dilutions of semen stain extract and either a dilution of a cloth control extract or a no antigen (diluent) control as follows;

	Semen stain extract				Cl. Con. or No antigen
Cols:	2 & 3	4 & 5	6 & 7	8 & 9	10 & 11
	1/10	1/100	1/1,000	1/10,000	1/10
	(1/300)	(1/3,000)	(1/30,000)	(1/300,000)	

Figures in parenthesis give the dilutions of the original semen proteins. Fifty microliters of semen stain extract dilutions were immobilized per test.

(f) Columns 2–9 of Plates 1 and 2 and rows B–D of Plates 5 and 6 contained an 0 Le a+b– stain extract. Columns 2–9 of Plates 3 and 4 and rows E–G of Plates 5 and 6 contained and 0 Le a–b+ stain extract.

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DISCUSSION

Question: Does the discrepancy, which we have noted, between the A:H ratios and B:H ratios in your autoanalyzer study compared with the results by the manual method, indicate a more fundamental problem? We do not have any absolute reference standards for blood group substances. There are chemically produced ones, but these are not used in anything that we do. When we are performing much of the quantitative work, we are working on relative scales, but we do not have an absolute standard to compare the scales with each other or to compare with another investigator's scales.

Werrett: I think that was the point I was trying to make, and it is regrettable that it was billed as a conflict. I hope that in the future, with the availability of carbohydrate sugars (the sort of sugars that Dr. Oriol has been using), we could use them as standards. Then we would be much better off and know exactly with what we are dealing.

Oriol: I think it is a very important point. If you look at different monoclonals or at different polyclonals, the reactivity will vary in the different systems. It is very important to have standards, but you need to have standards specific for each of the different things you want to test. For example, the standards for the red cell stain would be different than the standards for semen and the Le^a structure. Sialic acid is not a problem for cells, but it presents a problem for semen. You can take it as an advantage to have a difference between semen and other secretions that will not have large amounts of sialic acid. Whenever you get a monoclonal that only reacts with a given body fluid, it will help you detect the difference between the body fluids.

All the comparisons of titers and different systems are dangerous because it all depends on the affinity of each reagent for the given structure.

Werrett: Yes, in this particular test system, that should be emphasized. With these semen stains, we

hope that we are showing the worst possible situation for Le^a because we did make sure the samples were collected and frozen immediately to avoid any degradation during liquefaction.

Schanfield: In your last example where the monoclonal Le^b reacted with the a-negative b-negative secretor, the reagents were characterized by red cell agglutination and had not been characterized for testing saliva or body fluids.

Werrett: Yes.

Schanfield: Which means that when you tested the red cells of this person, they were Le^a negative Le^b negative and the saliva reacted with the Le^b.

Werrett: Yes. Dr. Oriol has some data on this.

Question: How often will people have Le antigens in their saliva but not on their red cells?

Oriol: I cannot give you an answer, but I do not think in terms of Le^b. The antibody was supposed to be an anti-Le^b because it was raised with Le^b and when it was tested in red cells, it looked like an anti-Le^b. This particular antibody reacts with H type 1, Y, X and others. The question depends on the molecules and on the relative amounts of the different sugars present in each sample you use. For red cells, you only see Le^b, but in saliva you may see other things that are not present in red cells.

Werrett: I think we are beginning to realize that more and more. With our tests, we are demonstrating qualitative differences that may be determined by the sensitivity levels of the assay.

Lincoln: Concerning the table showing saliva samples taken from individuals at various times, you took a number of samples from each particular individual and looked at the A, B and H levels. Can you give us an idea of whether there was any pattern of the levels going up or down at any particular time? Was there a correlation between the level of the substance and the time the samples were taken?

Werrett: This table is taken from Marion Dorrill's work, and I do not believe a pattern was mentioned.

APPLICATIONS AND IMPLICATIONS OF ELISAS IN FORENSIC IMMUNOLOGY

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LIMITATIONS OF CURRENT IMMUNOLOGIC TECHNIQUES

A wide variety of immunologic methods are used in forensic biology. A few basic hemagglutination techniques have been extensively modified and refined for use with many different sample types, and a similar range of precipitation, diffusion–in–gel and immunoelectrophoretic methods are in use for relatively few antigens of interest. This proliferation of methodology is a healthy sign that workers in our field are striving continuously to improve the performance of methods in terms of sensitivity and in terms of the range of sample types and conditions to which they can be applied. It also testifies to the dissatisfaction which all must feel when the methods fail to provide interpretable data.

From the outside, the number of different methods used to detect relevant antigens might appear somewhat excessive. The reason for so many is partly historical, and in the pursuit of the most sensitive technique available for a particular antigen, the significance of adding another technique and then another to the biologist's tool kit has been largely ignored. In scientific terms and from the researcher's viewpoint, the actual cost of establishing a fact is often irrelevant. In the legal arena, where the consequences of a fact can be far–reaching, it is easy to justify costly practices because of the results of sporadic cases. The practical consequences of this single–minded pursuit of information is, however, a logistics problem, both in the training of scientists and in the amount of time and effort consumed in stain analysis.

A second consequence of the pursuit of extreme sensitivity is that methods are sometimes pushed beyond safe limits and applied in situations where interpretation of results becomes difficult and susceptible to bias on the part of the scientist. This problem is widely recognized, of course, and measures to eliminate personal bias are used, normally by independent assessment by two or more workers, which also adds to the logistic burden already mentioned. This measure does not eliminate bias entirely, since individuals are influenced by local factors and practices and not least by the personal responsibility of the criminalist for the evidence and interpretation submitted to court. The element of subjectivity in reading the results of hemagglutination methods, in particular, remains an important barrier to the achievement of good quality control of these methods.

One of the constant concerns of the serologist is the securing of supplies of good antisera for each of the methods in use. It is rare that one can order another sample of the same batch of antiserum, which means that each new sample must be checked. For some antisera, this is a simple matter of running it in a series of dilutions against a single local standard. For others, it means first establishing a coating level of standard antigen followed by determination of antiserum titer using several stains of homologous antigen. This burden of testing is further complicated because, in general, the antisera available have been characterized by different methods from those used by the forensic scientist. Variations occur in the average avidity of sera and in the range of avidities and specificities of their individual antibody components which affect their performance in different methods to different degrees. This is particularly true in the area of ABO/Lewis (Le) grouping where it is not possible to predict from the results of adsorption–inhibition how well an antiserum will perform in adsorption–elution. For species–specific antisera, the serologist more often than not relies on the assumption that antisera behave identically (regarding specificity) regardless of methodology. Although this is probably true most of the time, some methods may be so finely tuned that minimal crossreactivities could be expressed. The consequence of this is that assay sensitivity, and perhaps occasionally assay specificity, changes every time a new batch of serum is introduced.

Finally, remarkably, no positive control standard is available from commercial sources for any of the antigens routinely searched for. Some purified materials are available, such as A and B substance, Le substances and prostatic acid phosphatase, and some workers have produced purified materials for immunization and other purposes. The use of these as positive controls has largely been rejected by serologists,

sometimes on the grounds of cost, but more often on the grounds that locally produced controls (for example, volunteer blood and semen and saliva stains) are much more appropriate than is purified material. Simulated case stains do have an important role in checking the response of methods from time to time, but they do not substitute for the positive control type of standard of high stability which should be available to all laboratories. This type of control allows realistic interlaboratory method comparability and tight quality control of sensitivity levels. The fact that the standard does not reflect the type of sample routinely analyzed is not in dispute, but the suggestion that it has no role to play in assay control is profoundly mistaken.

The foregoing should not be taken as a condemnation of current methods as unsound but to highlight the most important limitations of the methods, in general terms, in order to clarify which assay features we should concentrate on improving.

NEW DEVELOPMENTS IN IMMUNOLOGIC METHODOLOGY

Over the last decade, two important developments have emerged which have produced a new impetus in research on immunologic methodology, the successful development of enzyme labeled immunoassays for a wide variety of macromolecules and the advent of monoclonal antibodies (MCAs) (Fletcher and Davie 1980). The use of enzymes as labels for antibodies and, to a lesser extent, other proteins has produced an array of analytic methods that have vastly extended the scope of immunoanalysis. The first successful conjugation of enzyme and antibody was reported by Avrameas (1970), followed a year later by the first reported enzyme linked immunosorbent assay (ELISA) for human immunoglobulin (Engvall and Perlmann 1971). The use of antigen–coated polystyrene tubes in an ELISA was reported by the same authors a year later (Engvall and Perlmann 1972). At about the same time, Van Weeman and Schuurs (1971, 1972) were working along the same lines. From this beginning, ELISA methods were quickly developed for the diagnosis of a variety of infectious diseases of human and veterinary interest. Assays for substances other than specific antibody were, however, much more difficult to achieve.

A number of enzyme immunoassays that employ enzyme labeled antigen in heterogenous systems analogous to radioimmunoassay (RIA) have been reported, but this method has the disadvantage of needing a mechanical separation stage, and it has not proven popular. Over the last decade, developments in assay methodology have focused on improvements in the reproducibility and capacity of protein–adsorbent surfaces and on the production of better antibody enzyme conjugates. This research has resolved many of the early limitations, and the ELISA technique is now approaching maturity, with a large literature, a range of assay configurations for different analytic problems and the availability of reagents and automated instrumentation (including turnkey systems) from many commercial sources.

The potential advantage of ELISA methodology in forensic immunology was recognized at the Central Research Establishment (CRE) in the mid–1970's. Some success from our first attempts (Werrett and Dolton 1978) was achieved in grouping liquid saliva, but the materials and antisera available at that time proved unsuitable for the development of a sensitive method. In 1980, it became clear that substantial improvements had been made in solid–phase technology and in the reduction of nonspecific binding. At the same time, the significance of the MCA developments had become apparent and made us resume research on the method.

The achievement of Kohler and Milstein (1975) in producing stable hybrids of antibody secreting lymphocytes with tumor cells was barely appreciated by most immunologists at the time. Now the consequences of their work have transformed the face of immunology and have produced the means for the development of a new generation of immunoanalytic methods.

Monoclonol antibody is the product of a cell line derived originally from a single spleen cell from a mouse immunized with antigen. It differs from conventional experimental animal antisera (polyclonal antibody) in containing a single type of antibody molecule, effectively a pure single protein apart from the tissue culture medium in which the cell line is grown. In contrast, polyclonal serum contains a number of different antibodies, each with the ability to bind to the specified antigen in a different way. Thus, the specificity and avidity of a polyclonal serum is an "average" property, a fact that has important consequences for its suitability for use in different types of assay.

The advantages and limitations of MCAs as reagents for immunologic analysis are outlined in Table 1. The advantages derive from their ease of production (and reproduction) and the more narrowly defined specificities achievable. This availability of unlimited supplies of a relatively pure antibody has spurred research into solid phase coating and enzyme conjugation methods, and rapid advances in antibody technology can be confidently expected during the

Table 1. ADVANTAGES AND LIMITATIONS OF MCA

ADVANTAGES OF MCA
Unlimited quantity
Single antibody species
Constant affinity and specificity
Relatively clean preparations
Easy to purify
Fine specificity easier to achieve
Purified immunogen is unnecessary
LIMITATIONS OF MCA
Poor precipitating characteristics
Single isotype
Isotype modifiable with difficulty
Specificity may be too fine
pH can affect specificity markedly
Affinity restricts application

next decade. It is already possible to switch the antibody class of most MCAs to any class desired. This last development offers the opportunity of using two different antibodies with an identical specificity, which opens up an exciting new set of analytic possibilities.

Not least of the advantages of MCAs is their immortality, which now makes it possible to standardize the most important component of any immunoassay. In general, it is not possible to make meaningful comparisons of numeric data from assays performed in different laboratories on the same sample, simply because they use different antisera and establish their sensitivity levels with different control material. Quality assurance, in such a situation, is concerned mainly with specificity (that is, qualitative). However, in this area the evidential value of negative results is often an issue, and effective quality assurance must seek to establish a minimum level of sensitivity. One of the limiting factors, the availability of antisera, can now be eliminated by the use of MCAs, leaving the problem of common antigen standards as the main barrier to the design of quantitative quality control schemes.

The main limitation to the use of MCA results is that they have a much more sharply defined character than do polyclonals. The affinity constant is a true value, not an average; there is often a marked pH optimum and in some cases a dramatic change in specificity with pH. The IgG MCAs are generally very poor precipitating and agglutinating reagents. The IgM MCAs agglutinate red cells well but are also poor precipitin reagents. The tissue culture medium in which the hybridomas are grown has a very different composition from polyclonal sera, and the relatively low protein concentration prevents the direct substitution of MCAs in some of the finely tuned methods used in forensic immunology. All these factors have contributed to a somewhat disappointing early experience with MCAs in current methods, although it is only a matter of time before suitable MCAs are identified and experimental conditions modified to permit their use.

Fortunately, the limitations outlined above hardly apply at all to the use of MCAs in ELISA methods. The monovalency of MCA is no disadvantage, and the affinity requirements for a particular method can be established by experiment and MCA of suitable type selected. Direct conjugation of IgG MCAs with enzymes and other markers can be achieved by a number of reliable methods, although IgM MCAs are extremely difficult to conjugate. It is not in fact necessary to conjugate a MCA directly, since it is possible to detect the specific binding of a MCA by a commercial antimouse Ig antibody enzyme conjugate. In this way, a large number of MCAs can be used in the same assay format.

THE IDEAL METHOD

No attempt will be made here to define the characteristics of an ideal method or explain how to achieve it. However, a number of objectives are uncontroversial, although their relative importance is not. First, it would be desirable to reduce the number of different types of immunoassay in use in forensic immunology, if possible to one, in order to reduce the amount of time spent acquiring technical competence in analytic methods. Second, the reagents and controls for each method should be highly defined, of constant composition and stable. Quality control should be such that each batch of analyses would be accepted or rejected on the basis of control results by reference to rigidly defined rules and levels. The highest degree of automation should be used to reduce transposition error, subjective bias, transcription error and inconsistent interpretation. Finally, if at all possible, methods should be rapid, robust and simple to learn and perform.

A COMPROMISE APPROACH

The ELISA method development work at CRE has focused on meeting as many of the objectives just outlined as possible. Instead of seeking to optimize assays for each antigen system for each type of sample, we have sacrificed some sensitivity in order to keep the same assay format for all analyses. In most instances,

more sensitivity is achievable for individual analyses, but the high sensitivity of ELISA methods in general has allowed us to make this compromise and still retain a considerable sensitivity advantage over conventional methods. Not only does this meet the objective of a single methodology, it also has the effect of producing a more robust assay.

The type of ELISA variant that we have concentrated on is variously called "indirect ELISA for antigen" or "direct ELISA." It is not a widely used variant, but for samples in which the antigen of interest is present in significant amounts, it is the simplest assay type as regards development. Stains are extracted by appropriate means and the extract diluted. This solution is pipetted into microtiter plate wells and left for 1–2 hours at 37° C. Proteins and polysaccharides are bound by the polystyrene, and the excess is washed away with phosphate buffer containing a small amount of detergent (Tween 20™), with a short blocking stage with 0.5% casein in wash buffer. Appropriately diluted MCA and antimouse Ig enzyme conjugate are then added and incubated for 1–2 hours at 37° C. After washing, substrate solution (*p*–nitrophenylphosphate) is added, and after 30 minutes, the adsorbance of individual wells is read at 405 nm.

All ELISA work carried out at CRE in the last 6 years has been with MCAs or affinity–purified *Ulex europaeus* lectin (Harris–Smith and Fletcher, 1983a, 1983b). A series of 10 commercial antimouse Ig enzyme conjugates has been tested against 24 MCAs in order to identify a suitable conjugate with which to pursue development (Fowler, personal communication). The same conjugate (Tago antimurine μ–chain alkaline phosphatase conjugate) has now been used for 18 months and has varied little from batch to batch. The conjugate works well with most mouse IgM MCAs, and with a view to retaining a simple assay protocol, we have rejected MCAs that do not react with it.

To simplify assay protocol further, we tested the effect of adding MCAs and conjugate to microtiter plates at the same time and also with a 30–minute preincubation period before addition to the plate. Most MCAs work well when added at the same time as conjugate, and as this reduces incubation time and overall complexity, we now work only with MCAs that perform well in this protocol.

The compromise approach described has allowed us to screen blood and secretion stains with as many as 15 different MCAs in the same assay, using automated washers, dispensers and readers. The use of these instruments has improved the reproducibility of results and has enabled a much greater range of conditions and concentrations to be tested than would have been possible with manual methods. In particular, the interfacing of the microtiter plate reader to a computer and the use of commercial software (spreadsheet) has made data analysis much easier and less vulnerable to transcription error. The reader interface supplied by the manufacturer was rudimentary, and the only commercial software available was designed for routine quantitative applications of ELISA; we therefore found it necessary to write suitable software for our own purposes. The experience gained in the development of this hardware/software interface has led us to develop an assay concept that includes bar coding of samples and reagents, automation of sample and reagent placement, computer–guided assay protocol with bar code progress chasing and computer interpretation of assay controls and sample results. This type of system is now at an advanced stage of development at CRE and should be complete within 12–18 months.

ABO AND LEWIS ELISA

The main problem encountered in the development of an ELISA method for ABO grouping has been the solubilization of blood group substances from bloodstains. The carriers of blood group activity on the red cell surface are tightly bound and are not eluted by saline. Aqueous ammonia does remove some activity, but it proved to be too variable in practice. Recent work at CRE, however, suggests that ammonia is a useful extractant for solubilization of bloodstains older than 4 or 5 years. Good extraction was finally achieved by the use of a detergent, octyl glucose, in alkaline buffer (Harris–Smith and Fletcher, personal communication). The choice of detergent was important, as it was subsequently found that only detergents with a high critical micelle concentration are effective in our system. The amount of detergent used to extract stains must be roughly matched to the amount of protein in stains to avoid a large excess of free detergent which interferes with subsequent stages of the assay. In contrast, the extraction of soluble ABH and Le substances from saliva and semen stains and from vaginal swabs has presented no problem.

A considerable number of MCAs specific for A, B, H, X, Le^a, sialylated Le^a and Le^b antigens have been investigated as potential grouping reagents for blood, saliva and semen stains and for vaginal swabs. Several good anti–A and anti–B MCAs have been identified, together with two anti–H MCAs, one anti–X MCA, one anti–Le^a MCA and two anti–Le^b MCAs. A recent trial with the best of these was carried out with

experimental stains prepared from blood and secretions from volunteers. The results of this study, given in Tables 2–5, are expressed in terms of the highest and lowest values recorded, together with the average value. The bloodstain results were obtained with an effective 1:500 dilution of original blood volume; the secretion results were obtained from 1:1,000 dilutions of original sample. Where a value is not quoted, it was below 0.01 adsorbance units. All values are means of duplicate determinations corrected by subtraction of a no–MCA control value for each sample.

It is not possible at this stage of development to fix the level of substrate conversion which represents a true positive, but we have adopted a conservative working criteria of 0.1 adsorbance units as our threshold. The average no–MCA control result was 0.074, so our threshold is more than double the background.

The ABO grouping results for bloodstains were in complete agreement with the known group apart from the two A_2B individuals, where no A reaction was seen. The B results for the three A_1Bs were rather weak, but the other groups gave very satisfactory values. An interesting and potentially useful finding was that stains from the A_2 individuals all gave good anti–H reactions, whereas stains from A_1 individuals gave no such reaction. On this evidence, it appears that it may be possible, under some circumstances, to distinguish A_1 and A_2 bloodstains.

Results with secretor saliva stains were not as good as those with the bloodstains. No mistypings were made with O, A or B stains, but seven group O, two group A^2 and one group B stain failed to give any positive reaction at all. These fall into the category "no result" and represent about 10% of the stains tested. One of the two A_1B stains typed as a group A, but the other A_1B and an A_2B both gave the correct result. Thus, one A_1B was mistyped as an A. The semen stain results for group O, A, B and AB secretors were in complete agreement with known blood type, with large amounts of H substance detected also in stains from A, B and AB individuals. None of the nonsecretors gave a positive reaction.

The Le grouping results for 140 saliva stains are given in Table 5. Correct grouping results were obtained with 32 out of 34 Le positive nonsecretors and with 102 out of 108 Le positive secretors. The eight remaining stains gave negative results. Of the Le negative stains, all gave no reaction except one from a secretor; this gave a positive reaction with one anti–Le^b MCA (Chembiomed™) but not with a

Table 2. SUMMARY OF RESULTS FOR ABH TRAIL WITH BLOOD

ABO Group	No. of Stains	Adsorbance at 405 nm	Reaction With MCA		
			a–A	a–B	a–H
0	64	Max.	–	–	1.5
		Min.	–	–	0.324
		Mean	–	–	1.024
A_1	53	Max.	1.5	–	–
		Min.	0.210	–	–
		Mean	1.42	–	–
A_2	14	Max.	1.198	–	1.5
		Min.	0.258	–	0.343
		Mean	0.582	–	0.77
B	13	Max.	–	1.5	0.410
		M			
		Mean	–	1.101	0.100
		Mean	–	1.101	0.100
A_1B	3	Max.	1.5	0.22	–
		Min.	1.158	0.149	–
		Mean	1.336	0.193	–
A_2B	2	Max.	0.017	1.042	–
		Min.	0.01	0.608	–
		Mean	0.014	0.825	–

Abs = corrected mean adsorbance at 450 nm

Table 3. SUMMARY OF RESULTS FOR ABH TRIAL WITH SALIVA

ABO Group	No. of Stains	Adsorbance at 405 nm	Reaction With MCA		
			a–A	a–B	a–H
0	46	Max.	–	–	1.5
		Min.	–	–	0.01
		Mean	–	–	0.238
A_1	42	Max.	1.5	–	0.40
		Min.	0.173	–	–
		Mean	1.021	–	0.07
A_2	8	Max.	1.021	–	0.298
		Min	0.012	–	0.041
		Mean	0.894	–	0.144
B	9	Max.	–	0.707	–
		Min.	–	0.040	–
		Mean	–	0.325	–
A_1B	2	Max.	1.5	0.235	–
		Min.	0.711	0.063	–
		Mean	1.105	0.149	–
A_2B	1	Max.			
		Min.	1.251	0.817	–
		Mean			

Abs = corrected mean adsorbance at 405 nm
All nonsecretors gave negative results

Table 4. SUMMARY OF RESULTS FOR ABH TRIAL ON SEMEN STAINS

ABO Group	No. of Stains	Adsorbance at 405 nm	Reaction With MCA		
			a–A	a–B	a–H
0	25	Max.	–	–	1.5
		Min.	–	–	0.2
		Mean	–	–	0.97
A	12	Max.	1.5	0.07	1.42
		Min.	0.37	–	0.38
		Mean	1.24	–	0.84
B	1	Max.	–	0.38	1.5
		Min.	–	0.38	1.5
		Mean	–	0.38	1.5
AB	2	Max.	1.5	0.74	1.37
		Min.	1.5	0.18	1.03
		Mean	1.5	0.46	1.2

Ten nonsecretors all less than 0.01

second (Messiter™). The samples were also tested with an MCA directed against X–antigen (sometimes mistakenly designated Le^x), and it was found that most of the stains from nonsecretors and some from secretors gave positive results. The Le negative secretor stain that gave a positive anti–Le^b result also gave a positive result with anti–X, suggesting that the possibility of secretor status determination by anti–X and anti–Y ELISA may not be clear cut. It is quite probable that the X and Y antigens in secretions are sialylated, and this probability must be taken into account in the development of such a system.

Table 5. SUMMARY OF RESULTS FOR LEWIS TRIAL WITH SALIVA

Lewis Group	No. of Stains	Adsorbance at 405 nm	Reaction With MCA			
			Le^a	Le_1^b	Le_2^b	Le^x
a+b–	34	Max.	1.5	0.076	0.024	0.799
		Min.	0.08	–	–	–
		Mean	0.43	0.017	–	0.23
a–b+	108	Max.	0.756	1.5	0.651	0.347
		Min.	–	0.07	–	–
		Mean	0.18	0.656	0.208	0.047
a–b–	6	Max.	0.042	0.772	0.023	0.539
		Min.	–	–	–	–
		Mean	0.018	0.167	0.016	0.098

Further work has been done in recent months on ABO and Le grouping of casework stains and vaginal swabs, with highly encouraging results. As a result, the U. K. Forensic Science Service is now conducting parallel trials of ABO and Le grouping and also human species identification ELISA on casework material in operational laboratories. After the trials are completed and if the methods continue to perform as they have to date, they will be integrated into routine casework.

One of a number of interesting phenomena observed during the development of these assays has been the finding of MCAs directed against group A and H substance which show specificity for the secretion of origin. One anti–A and two anti–H MCAs reacted only with bloodstain substance, and one anti–H reacted only with secretory substance. The markedly uneven distribution of type 1 and type 2 chain substances between blood and secretions may explain the behavior of some of these MCAs. However, the secretion–specific ELISA characteristics of the anti–H (Chembiomed™), which is a good hemagglutinating antibody, require some other explanation than chain–type specificity. When more of these narrow–specificity MCAs become available, it should be possible to group blood–saliva, blood–semen and blood–vaginal secretion mixtures reliably. This area of our work is currently being given high priority. Given that there must be qualitative differences in the carrier molecules of blood group activity in semen and vaginal secretion, it may one day also be possible to dissect the ABO and Le groups in these mixtures.

SPECIES IDENTIFICATION ELISA

The application of ELISA to the identification of human stains was an early offshoot of our work (Fletcher *et al.* 1984). The MCAs directed against human immunoglobulin proved to have adequate human specificity, and we have recently begun to investigate commercial anti–human globulin MCA–enzyme conjugates for sensitivity and species specificity. One commercial product appears to have the right characteristics and has been included in the trial mentioned above. The methodology is exactly the same as that for soluble blood group substance.

Gm/Km ELISA

An ELISA method for Gm(3) grouping of bloodstains which used an MCA was developed (Fletcher *et al.* 1983). The method was sensitive but took about 36 hours to complete. The MCA and assay details were supplied to other investigators (Newall and Hoste) who in turn did further work on the assay. Newall essentially followed our methodology and obtained similar results (1984), but Bernadette Hoste (personal communication) developed a sandwich type of assay in which polyclonal antihuman Ig was coated on the surface of microtiter plate wells and used to capture IgG from stain extracts. This assay was more sensitive than our original development and also took less time to complete.

The number of MCAs with allotype specificity is still very small. Some work has been done with anti–Gm 1, 17 and 21. The anti–Gm 1 does not show strict specificity except under a very narrow range of conditions. The other two work only in the inhibition mode, which has very poor sensitivity. Production of good allotype specific MCA is evidently a much more difficult task than was first thought, and it will be some time before these become available.

THE FUTURE

A large number of soluble antigens in stains and other evidential traces can be identified and measured

by ELISA methods. Some work is also in progress on M and N grouping by ELISA, and no doubt there is some interest in Rhesus grouping. An alpha-fetoprotein ELISA has also been used recently for forensic purposes.

Over the next 5 years, we should see the rapid adoption of ELISA methods for the antigens listed above, and probably others, and a continuing process of improvements in sensitivity and specificity of the methods. It is likely that the need for at least some immunoassays will remain when DNA typing finally becomes a routine technique in the forensic laboratory, so the future of some of these assays is secure.

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DISCUSSION

Benjamin: Have you checked to see whether there is endogenous alkaline phosphatase that is responsible for that reactivity in the sample concerning Eel crossreactivity?

Fletcher: Yes, there is none. We do a control by immobilizing the Eel serum and omitting the monoclonal. That control is negative.

Sensabaugh: Would your laboratories be using the conventional procedures in parallel on the same samples, if they were going to use the enzyme linked immunosorbent assay (ELISA) methods for several months?

Fletcher: Yes, the laboratories analyze all the stains, by ELISA, that they can get, but they would not attempt conventional grouping on all of those stains. One laboratory that has completed its evaluation has obtained a high success rate and convincing results from ELISA on samples they would not have attempted by conventional methods.

Oriol: The problem is of standards and of putting just the synthetic sugars linked to a protein as the first layer of ELISA. This works. You get wonderful results and it is a perfect standard because it will be constant all the way across. However, in the kind of work you are doing I would prefer to have something that comes from the biologic fluid you want to test, as a standard for the antigen, because the structures present in each of these biologic fluids are different. If we use the synthetic sugar, we will get a crossreaction with synthetic sugar that is standardized, but our result may be lower in certain secretions as compared with others with red cells.

With an antibody, I still think you have to run all the controls and run a lot of them in parallel. For example, the best anti-A I have for secretion is an anti-A that does not agglutinate red cells at all because it reacts with a molecule that contains two fucose residues, which are very fully represented in red cells. In addition, for the B system, it seem that the acquired B is a frequent problem in some laboratories. Certain monoclonals will not recognize the acquired B. So these are wonderful reagents.

Fletcher: To my knowledge, there is only one that does recognize it and that is an anti-AB produced by Steven Moore.

Oriol: I have a second one, but more often the monoclonals will not recognize the acquired B. This is particular to blood, so I think that each secretion has some unique antigenic determinants, perhaps because the molecule is longer or there are other sugars; even the carrier and other factors may differ.

Fletcher: I am sure you are right. In a routine test, you would already have done all that groundwork and you would have established the specificity of the monoclonals. What you really want on the plate is a fairly simple substance that is stable and which will give you a positive result. You are not actually going to be asking questions about that plate concerning the specificity of the antibody; you want to know if it is behaving the same as it did yesterday. You could have a mixture of all those blood group substances, ABH, Le^a, Le^b, all together as one standard. Running that against each monoclonal will give a positive result that should be the same from day to day.

Schanfield: If you dilute saliva, seminal stains and bloodstains to a lower level of dilution, 1:100, instead of 1:500 or 1:1,000, do you see a change in the reaction pattern of your readings? Will you decrease the failure rate by increasing your concentration of antigen?

Fletcher: We have not tested extracts at relatively strong concentrations. What we are looking for is a system where you make an extract, preparing enough dilutions to do either 16 or 32 individual tests. Working from 10 μl of blood, at a 1:500 dilution, gives enough for 50 tests. The greatest dilution that we have tried is 1:500. If we used a lower dilution, I have no doubt that we would see more positive readings. It is a compromise. I am currently looking at a large number of monoclonals and comparing them. From a certain amount of bloodstain I can only get so much antigen, so I have to make a 1:500 dilution to get enough volume.

Also, I feel that if you cannot achieve that kind of sensitivity, you are going to have to look for another monoclonal that will give it to you or go to another extract and another test. What we would like to do is save time and make one extract and do a lot of tests at once. That is why we have not tested them at lower dilutions. Bloodstains still give reasonable results at 1:2,000. Saliva stains and semen stains give good results for ABH at 1:4 to 1:5,000 dilutions and for Le can be diluted to 1:30,000. For specification of bloodstains, we can dilute to 1:10,000.

Werrett: I think you made the point that the presence of Le^x in secretors would be expected. We know that Le^x does not get silylated, and we have not explored the possibility of using neuraminidase with Le^x.

Fletcher: There is published evidence of silylated Le^x, so it would have been present.

QUALITY CONTROL OF IMMUNOLOGY REAGENTS

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Forensic laboratory professionals work in an environment that requires careful design and execution of tests. The use of appropriate controls and confirmatory testing are required to provide a degree of certainty, or at least an idea of the probability, that conclusions reached are correct. These tools are needed to meet the strict rules of evidence within which one must work or to permit the investigation to proceed in the right direction and to the next logical step. Professionals working under these constraints have more than a casual interest in knowing that there is a high probability, to use the words of the Code of Federal Regulations, that the reagents used can be expected beyond a reasonable doubt to yield their specific results and retain their safety, purity and potency, in other words, that they will be suitable for their intended use.

This paper will discuss the tools of quality control and will emphasize those systems or practices that make up the comprehensive quality control or assurance program used by manufacturers when dealing with reagent quality control. The program starts with the design and development of the product and continues through manufacturing and distribution until it is used and even after it has reached its expiration date. This program is the real source of confidence in a product, not the inspections and tests run on a batch or lot sample before it is released for sale. Such programs have evolved in parallel with the regulation and monitoring by government agencies, now mostly at the Federal level and primarily by the Food and Drug Administration (FDA).

The day-to-day tools of quality control are well developed. Sampling and inspection plans are an example. General Diagnostics has an Inspection Department that samples and inspects all incoming raw materials, packaging components and labeling and other finishing supplies. Line inspections during manufacture are documented and detailed to insure that samples delivered to the laboratory are representative. We have statistically validated sampling plans including Military Standard 105D (Department of Defense 1963), the Military H53 Handbook for Sampling Inspection and the American National Standards Institute (1980) Sampling Procedure and Tables for Inspection prepared by the American Society for Quality Control. These provide plans for tightened and reduced inspection levels, when testing for single, double or multiple dependent or independent variables or attributes. Properly used, these tools will result in a high probability that good material will not be rejected and a low probability that material of poor quality will be accepted. Yet sampling plans are still misused. Suppose a sample comes to the laboratory (either representative or random) to be tested. If it passes the test, everything is all right, but what happens if it fails? A resampling is done, the worst thing that can happen from a control point of view. The original sample is retested to better define the problem. If the test on the resample passes, questions still remain. For example, is the sample bad? Is the lot bad? Is the test bad? Is there a contamination or lot mixup problem? Over the years, resampling has created more problems in sterility testing of reagents and pyrogen testing of biologicals than it has ever solved.

Norman Augustine, the president of Martin Marietta Corporation, has pinpointed this problem in his book *Augustine's Laws* (1986). According to his "law" on the amplification of agony, the unexpected should be expected and therefore can be prevented. In his example, the company developing a new aircraft engine suffered a number of destructive failures caused by foreign objects, such as small tools and parts left inside test engines. To correct the problem, inspection was enhanced and required an inspector to physically enter the engine inlet before a test run and, using a flashlight, inspect for extraneous objects and then certify in writing that no objects were found. As could be expected, it was not long before another destructive failure occurred because a flashlight was left in the engine.

Our industry is based on the clinical diagnosis and treatment of disease or injury. The technology of our business is that of drugs and pharmaceuticals, serums, antitoxins and other biologicals. The regulation of these products is based on a clear public health and safety need, but the forensic scientist is not in the health care business. Even when reagents were still regulated as drugs, Federal regulations always had exclusionary statements exempting a product from

many requirements if it was used for valid teaching, law enforcement or research purposes. Similar statements still exist in current regulations covering *in vitro* diagnostic products. It is obvious that use of reagents does not come under the prevention, diagnosis, treatment or cure of disease or injury. In theory, these products could be treated as general laboratory chemicals and reagents or labeled for research and investigation purposes only, but in practice, it is not economically or logistically practical to differentiate as to whether the use of a product is clinical or investigational or to have a two tiered set of standards based on this distinction. This fact should be readily evident when one considers that many of the reagents in use are those that are used clinically. A good example is Blood Grouping Serum.

Therefore, the quality control program that drives manufacturers is based on the regulations covering "*in vitro* diagnostic products" intended for use in the diagnosis of disease. They are used in the collection, preparation and examination of specimens taken from the human body. They are regulated by the Federal Food, Drug and Cosmetic Act, including the 1976 Medical Device Amendments, and they may also be biologic products subject to licensure under section 351 of the Public Health Service Act.

Even for a product without a clear or evident clinical use, the responsible manufacturer will clearly designate intended uses of the product and any appropriate limitation. As an example, in the instructional information from the current catalog of a maker of immunochemical materials and supplies including antisera and animal proteins, whole serum antisera raised by immunization against pooled normal serum are qualified by the following: "Species cross reactivity is a quality of all polyclonal antisera to animal proteins. These antisera are not recommended for forensic use without prior screening or treatment to render them species specific." There are then four literature references to guide the user.

A comprehensive quality control program has been described as three tiered or the pyramid of quality control.

1. At the top is the monitoring by regulatory agencies, including the Center for Drugs and Biologics, the Center for Devices and Radiological Health, the Center for Disease Control, state health agencies and quasi- or self-regulatory bodies such as the National Committee for Clinical Laboratory Standards, the College of American Pathologists (CAP) and the American Association of Blood Banks. These agencies deal with Good Manufacturing Practice (GMP) guidelines coupled with on-site inspection, product licensure, reference standards, proficiency testing and accreditations. Sometimes reluctantly, industry must admit regulation has been a strong force to improve quality and quality control.

2. At the second level is corporate involvement or commitment. This is a little harder to define but represents the Quality Assurance Group in a corporation which is close to top management and sets overall quality control policy in a large company with many product groups such as cosmetics, drugs, medical devices or diagnostic reagents. This group is the tangible evidence that management has recognized that for good quality to exist, it must be management supported. This group provides internal audit surveillance of individual quality control operations in the corporation, much like the inspection and policing function of the FDA. They insure a standardized, up-to-date system of documentation, revision and publication of all written procedures, directions, policies, test methods and specifications used in quality control operations. They provide expert consultation when problems arise and a supportive link to management if competitive or economic considerations place unreasonable pressure on individual quality control operations. They are a source of training and publicity materials.

3. At the bottom or foundation is the local quality control operational unit. Their work is primarily to implement appropriate sampling plans, qualify suppliers, test and approve raw materials, test product, document, analyze and report results, release product, carry out a product stability testing program and investigate and review customer complaints. They also have GMP monitoring and random auditing responsibility, certainly within the Quality Control Department, but often also in manufacturing and packaging operations.

In our industry, quality control generally is organized into three functional units: Laboratory Services, Release or Administrative Services and Inspection. The laboratories are responsible for the actual testing of finished product lots, in-process bulk, or incoming raw materials and other components. They are charged with conducting tests that are relevant, doing them correctly, using proper controls, keeping their equipment in control and in calibration and reporting out reliable and statistically valid test results. The tests may be for potency, performance, specificity or stability; they may be qualitative or quantitative. Part of this unit will be a group responsible for assay research and development, although organizationally, it may be part of another department. They initially establish and document the accuracy sensitivity, specificity and reproducibility of test methods. They also determine the accuracy and

precision needed in instruments and other measuring devices. Release Services is an administrative and audit function. Their responsibility is to review the laboratory data, review manufacturing records, reconcile quantities and yields and actually carry out the paper work or documentation of individual lot release or rejection. They prepare certificates of analysis and lot submissions to outside agencies for approval, where required. They will audit operations and records and generally perform a policing function and deal with government inspectors in the plant. Included here is Quality Control Engineering, which insures that there are adequate provisions for monitoring the reliability, accuracy, precision and performance of laboratory test procedures and instruments.

The Inspection function has already been touched on. They sample incoming materials, in-process bulk and finished products. They proofread labeling and monitor filling and packaging lines. The employees here generally come from skilled production or packaging operating jobs rather than from the laboratory.

The Quality Control Department usually reports to a Plant Manager or Director of Operations, thus avoiding conflict with those directly responsible for manufacturing. Often there is also a dotted line responsibility to the corporate Quality Assurance or Regulatory Compliance Group. In some cases, reporting is to a Technical Operations or Research Group. A very consistent industry ratio of one quality control person for every three to four people in the production or operating departments has been found. This high ratio is high because we are in a high technology industry. If thought of only as overhead, it is expensive, but it telegraphs a message that adequate quality control is important to our industry.

It is important at this point to clarify what is meant by quality. Quality to the manufacturer means the product has been made to certain specifications. There is no other definition. The specifications may have been created for good or poor reasons, but as long as the product conforms and is within specifications, it has, or is of, good quality. Quality control is applying the criteria that demonstrate that the product meets the specifications.

Now that these specifications are set, reliability should be defined. Reliability of a reagent or reagent system results from a combination of specificity, accuracy and precision. The hardest lesson for those in the laboratory to learn, whether in the manufacturer's Quality Control Laboratory or in the customer's laboratory, is that accuracy is far more important than precision. Biologic materials have inherent variability, which is seldom well defined. Large random errors can be caused by inherent instability or deterioration of the biologic sample being tested, the same instability of the test reagent once it has initiated the test reaction, complex or difficult equipment manipulations, and too many steps in the procedure and intricate calculations. A less precise analytical technique free of large test variables or cumulative errors is always to be preferred and will give the accuracy and reliability needed in all but the most specialized of studies. Finally, there almost always has to be a trade off between specificity and sensitivity. A simple antiserum reagent, such as would be used on an Ouchterony plate to identify the characteristics of a dissolved stain, usually has multiple specificities. The time and temperature of the test become very critical as does the potency or titer of the reagent. The sensitivity may also have been increased by adding polyethylene glycol to the agarose. If additional precipitin lines form with time, then false positive results can occur. Specificity can be increased by diluting the reagent. The reduced sensitivity but increased specificity should make a more reliable reagent. This observation is based on the rule that a false negative result is better than a false positive. Some may dispute this in forensic science, but it generally holds in clinical science. A reagent system that gave a false positive test for pregnancy, acquired immune deficiency syndrome or antigens related to certain cancers will result in problems that will not occur with false negative tests. In blood banking, there have been arguments for years over a too sensitive antihuman globulin serum which detects clinically insignificant antibody and creates a problem finding compatible blood donors.

At a meeting of the GMP advisory committee of the FDA, Dr. Edward McDonnell, Director of Compliance Programs at the Center for Devices and Radiological Health, noted the number of product quality problems manufacturers have, as measured by product recalls. These numbered 367 in fiscal year 1983–84, 599 in 1984–85, and 426 for the first half of the fiscal year 1985–86 (for an annual rate of 852). Almost half of these (45.6%) could be directly traced to preproduction manufacturing deficiencies. What this means is there was a design or development flaw either in the manufacturing, testing, packaging or labeling of the product which was not uncovered before the product was introduced. This demonstrates the importance of product and process validation, which will be discussed later, in a comprehensive quality control strategy. The other half of the recall problem (49%) was caused by GMP deficiencies. Good Manufacturing Practice is another key element of quality control strategy: GMP represents, for the most part, common sense guidelines on operating an enterprise so that there is a high probability that the

products manufactured will be effective and otherwise in compliance with the Federal Food Drug and Cosmetic Act.

These accepted practices and requirements for the facilities and controls used for the manufacture, packaging, storage and distribution of diagnostic reagents have developed into codified regulations over the years. Drug GMP at one time was applied to reagents, but since the device amendments of 1976, specific GMP regulations for *in vitro* diagnostic devices have been published in the Code of Federal Regulations (FDA, 21 CFR 820). Those diagnostic devices subject to licensure under the Public Health Service Act have additional specific GMP requirements in Parts 600 through 610, as well as detailed laboratory standards for release testing in part 660 (FDA 1985). These regulations are, in reality, a handbook of how we should operate and control our business. Items included are:

Organization. How you will be organized to manufacture and test your product. How your Quality Assurance Program remains independent and not responsible to an individual directly responsible for manufacturing operations.

Personnel training and personnel health and cleanliness.

Suitable design of building and laboratories for proper cleaning and to prevent mixups.

Environmental controls. In the laboratory, both humidity and temperature can be critical. If the reagent you manufacture and test requires incubation at room temperature not over 24° C, the area must be controlled to maintain that temperature; you cannot assume ambient will be a suitable room temperature.

Calibration standards, schedules and procedures. These are required for all measuring equipment with any secondary standards traceable to a National Standard of the National Bureau of Standards.

Adequate written procedures for manufacturing, packaging and testing.

Recordkeeping adequate to provide a batch or lot history and a compilation of the complete production history of a reagent. These will be based on a device or Product Master Record compiling records containing the design, formulation, specification, manufacturing procedure, quality assurance requirements and the labeling and stability of a product. Each revision or change will be appropriately documented.

Adequate distribution and warehousing. Stock will be properly rotated, storage will be at the proper temperature and records of customers will be maintained for batch recall purposes. For example, for perishable reagents, a maximum shipping stress has been established by survey and delivery records. This is 5 days in transit with maximum temperature exposure of 44° C (111.2° F). In the final release testing, there is a test exposing the product to these conditions and then reconstituting and testing with the unexposed lot as a control. A typical specification would be not more than a 2 second increase in activation time.

About half the serious problems encountered in the field could be eliminated by proper design and development. This is accomplished through Product and Process Validation, which over the last 5 years or so has become very important to both quality control and regulatory compliance. For many years, the need to validate sterilizing equipment and procedures has been recognized. No sampling plan can assure sterility, but sterilizing equipment can sterilize a given product or material when loaded in a particular configuration and when carrying a particular bioburden or level of contamination. Once proven, it is not really necessary to do sterility testing, and many products sterilized by ethylene oxide, radiation or steam under pressure can be released directly to distribution. This concept has been extended to other manufacturing equipment and processes including performance measurement and testing. Performance qualification of a reagent product means establishing confidence through appropriate simulated use testing that the product produced by a specified process meets all release requirements, that is, it will do what you claim it will do. Obviously, the starting materials also have to be qualified and the critical specifications identified. The performance testing should simulate actual user conditions in an environment similar to that in which the reagent is expected to be used.

Sometimes performance testing is not enough to insure suitability. If components are critical to stability, for example, then appropriate tests must be added. Osmolarity might be tested to confirm that critical materials were included during manufacture. If an activity is removed by adsorption or affinity chromatography, then it should be tested for. If an animal plasma component is added to a product and its presence is not readily evident, then its presence should be confirmed by a precipitin test or test for protein. Some of these tests may be specified as "omit routinely" or as "for information only." This is especially true if ranges or limits are not yet well documented.

This validation should, wherever possible, be on a prospective basis. This means establishing the documented evidence based on a preplanned protocol. Retrospective validation means establishing evidence of performance based on an analysis of product test data history. Prospective validation provides greater

confidence that a process is under control. The FDA feels strongly that industry should regard validation as a preproduction quality activity. When product and process have been validated this way, in-process and finished product testing properly serves as a confirmation of consistent product quality. Finished product testing by quality control should not serve as an elaborate inspection and evaluation process to validate individual batches of a product, that is, prove that they work.

For the traditional reagents of interest to the forensic investigator, closer attention to assay methods and their validation became necessary 8–10 years ago when the Additional Standards for Blood Grouping Serum were published. More recent published proposals (50 FR 8743, 1985) to remove specific test procedures from the regulations should not be interpreted as easing up on requirements. By keeping the specifics of tests and product requirements as guidelines or recommended methods, it is much easier to make changes and keep them up to date with advances in the science. The advent of monoclonal antibody (MCA) is an example. Once regulations are published, even minor changes require a lengthy publication as proposals, followed by a comment period and final review. Cost and environmental impacts also have to be evaluated.

These Blood Grouping Serum standards, especially those for the ABO system, are well defined and predictable products that have been under good regulatory control since publication of the first Minimum Requirements in 1948. In a comprehensive study of blood grouping sera (Hoppe 1976), the Division of Blood and Blood Products, Office of Biologics, concluded that the number of product problems was amazingly small considering the biologic variability of human and animal sera. Individual donors can be immunized in a variety of ways, and each may respond differently to an antigenic challenge. Stimulation may have been natural or more likely in a program of immunization with blood group substances or with red cell antigens in a Source Plasma Center. Even this step in the supply of antisera, which used to be an extension of the manufacturer's responsibility, is now under direct FDA control through licensure. Both to insure good quality starting material and to protect the donor, strict guidelines have been imposed, and immunization protocols must be approved by FDA before a Source Plasma License is granted.

Since the effective date of the additional standards in 1976, manufacturers have had to prove reagent specificity. This required demonstrating that hemolyzing or agglutinating activity against an extensive list of red cell antigens had been excluded or that activity against them is absent by all test methods recommended by the manufacturer's directions for use. Sometimes, such as in human leukocyte antigen testing, probable exclusions must be relied upon.

In the attempt to exclude more and more specificities from the common Blood Grouping Sera, some ingenious laboratory adsorption steps and enzymatic treatments have been devised. An example of some of these techniques is applicable to anti-M and anti-N serum raised in rabbits. These reagents over the years have had a number of specificity problems from contaminating antibodies. The antigens St^a and Dantu are difficult to test for and exclude because their antisera are very rare. With *Vicia graminea* lectin (anti-Nvg), red cells pretreated with 0.1% ficin would react strongly if they were positive for the St (a+) and Dantu+ antigens. If cells testing positive are then pretreated with papain-cysteine and retested, according to Vengelen-Tyler and Mogck (1986), only Dantu+ cells react. Through such identification of appropriate cells, this activity could be confirmed as absent in a serum being tested for release.

Obviously, the identification of antibody or the confirmation of red cell antigen based on agglutination of red cells modified by enzymes or chemicals must be done carefully and with the use of adequate controls. It is for the experienced specialist only. More often, rare antisera or well characterized frozen rare red cells are exchanged between manufacturers or obtained from frozen cell banks to exclude contaminating antibody and fully characterize each source plasma donor or each antiserum pool made from these donors. This information is generally made available by the Quality Control Group through the Professional or Educational Services Department of a company.

Quality control of the traditional reagents used in forensic investigations has evolved as an efficient, validated system. But what of the recent reagent products using the newer immunoassays? Included here is the more traditional radioimmunoassay (RIA) to which has been added the enzyme linked immunosorbent assay (ELISA) and in some cases, the fluorescence immunoassay (FIA). These newer tests are usually characterized as having high sensitivity and can detect, for example, very small quantities of the target antigen, protein or drug in body fluids often more than a week after it would normally disappear. This high sensitivity must usually be balanced with a lack of total specificity because of crossreactivity with substances of similar molecular structure. In addition, these newer tests are often performed in a series of critical steps that are subject to technician error. They, therefore, require high skill and care to perform.

Most of these products are not licensed biologicals and are regulated by the Center for Devices and Radiological Health. If the product represents new or unique technology, it is subject to premarket approval and must be accepted by an FDA advisory committee of experts in the clinical field involved. In the submission for approval, there will be a laboratory data section with test protocols, test results, analysis of data and conclusions. There will be a clinical data section with similar information from outside studies. There will be a section covering manufacturing and testing which gives details of the control system for components, the quality control procedures, packaging, sterilization and labeling controls and the finished product inspection procedures.

An example of these new products is the ELISA kit manufactured by General Diagnostics. It is an enzyme immunoassay for the direct quantitation of platelet associated antibodies and the indirect quantitation of plasma antibodies that bind to platelets. It is of peripheral forensic interest in that the immune mediated destruction of platelets which it detects may be drug induced. It is a complex product with nine components including antihuman IgG coated microtiter trays, an IgG alkaline phosphatase conjugate, a substrate tablet, lyophilized platelets and IgG coated positive platelet control, buffers and buffer concentrates. Probably the most important element of quality control for this type of product is in the initial design and validation by research and the assay development group. Here, through clinical trials, the capabilities of the reagent system or test are determined.

In this case, each of three geographically diverse hospital centers tested 20 normal donor plasma samples and 40 abnormal samples. The objective was to determine the within-plate precision and plate-to-plate or day-to-day precision by confirming acceptable coefficients of variation at various antibody levels. It was determined that the positive platelet control curve would be unreliable below the 8 ng level. It identified the critical procedural steps to be emphasized in the instructions for use, such as the importance of 22°–24° C incubation temperature, timing of IgG dilutions for the standard and use of a plate washing manifold instead of a plastic wash bottle, which is difficult to control. The study also established that the nanogram value of the positive control can vary 30%–40% in a daily quality control program and that the assay is technique dependent and should be used by skilled and experienced technicians only.

As a result of this preproduction validation work, the critical items for quality control testing are identified, and reasonable specifications and ranges can be established. Most important, the test limitations and sources of error can be identified and quality control procedures for the daily user recommended and included in the directions for use.

One tangible benefit of the 1976 Device Amendments is the regulation (FDA, 21 CFR 809.10) covering labeling for *in vitro* diagnostic products, in particular, those covering the package insert or directions for use. The new mandated format and detail of information required on performance characteristics and limitations, quality control, calibration, expected values and interpretation of results, along with test principles and meaningful references, were in stark contrast to the brief and relatively uninformative inserts of years ago, even those for licensed biologic reagents.

Quality control continues to monitor products after they have been released and distributed in two ways. Both are essential elements of GMP, and the plant Quality Control Department is normally responsible for both. Stability testing of selected lots of each product is carried out through the expiration dating period assigned to the product. Generally, the first three production lots of new or modified products will be placed on the stability testing program. Lots that are released on waiver or by an exception notice will also be monitored through their dating period. Modifications to product which require stability studies are formula changes, a process modification or changes in the immediate container or closure. Periodically, routine lots are selected for stability surveillance. The number of lots selected each year will be based on frequency of production. The basic test protocol will be a use test in support of label claims.

The studies normally continue for 1 year beyond the lot expiration date on samples held at the recommended storage temperature. All new products are routinely held under the recommended storage condition and at least one temperature increment above and below. When refrigeration at 2°–8° C is recommended, samples will also be held frozen (–20° C) and at controlled room temperature (20°–25° C). Tests on lots held above recommended temperature are valuable in establishing deterioration rates for establishing and supporting expiration date extensions. Copies of all test results and their analyses are filled both in Quality Control and the stability portion of the Master Device Record.

The complaint file is prominently mentioned in FDA regulations and is probably one of the best assurances the user of the product has that the lot received is in fact satisfactory. It is required that all customer complaints be logged and investigated, a record made of the study and the customer notified of the results. If warranted, a replacement or field

correction is made. If serious deterioration of a lot or lots is clearly evident, then a product recall is required. Sometimes the complaints are resolved by telephone with little or no laboratory involvement. More often, performance testing is done in parallel with normal retention samples of the lot in question. With this system, Quality Control is able to watch for the pattern of complaints which is the certain indicator that something is wrong with a reagent lot. Without this pattern, the testing is directed to correcting a user methodology problem, a reagent storage or delivery problem or some other isolated error of mixup.

The qualification of suppliers and vendors has become an important function in the reagent quality control organization. It is no longer economically sound or practical to maintain a fully integrated manufacturing operation for many of the high technology test kits and reagents available today. Unless this is prohibited by a biologic license requirement, production of large quantities of highly purified, avid and specific MCA tends to migrate to organizations with the expertise and resources to provide these materials under contract. A good example is Celltech in England which supplies antibody to some of the leading reagent manufacturers. They have a unique serum free medium that greatly reduces crossreactivity. Even raising antisera to various species in animals is often contracted to the expert animal handling firm.

Validation of these special or custom raw materials, like contract sterilization, must follow the checklist used within the company. Recognizing that there are proprietary interests and trade secrets, the work is best accomplished in a collaborative way by both parties. The agreement on the validation protocol should be confirmed by both parties in writing. When a new supplier is looked at, validation is best evidenced by written procedures, both for production and testing of the material. Records of production and laboratory testing should be complete and available for inspection and analysis. Where legitimate confidentiality concerns limit availability of records, there should be written agreement that they will be available for FDA examination. Also very important is written agreement that changes in processing, testing or controls will not be made without notification and, where possible, prior agreement. Once validation has been accomplished, routine followup will be through periodic GMP vendor inspections by quality control audit personnel.

As regards accuracy and precision, the quality control laboratory, just like the clinical laboratory, must monitor daily laboratory results by using control reagents or a standard lot run in parallel with the material being evaluated. From the results, quality control charts are constructed to indicate that assay values, means, ranges and linearity are not drifting or out of control. Traditionally, these were maintained as wall charts, but now the microprocessor and personal computer are widely used in laboratory instrumentation and the charts can be called up as needed on a monitor or printout.

In the same way, proficiency testing specimens are not just for the user's laboratory but are routinely evaluated by quality control to monitor the manufacturer's performance. Commercial proficiency and control programs are available from several manufacturers of reagents in addition to the well known CAP program or that of the American Association of Clinical Chemistry for therapeutic drug monitoring.

With this type of overall program in place and supported by management, reagent problems will seldom result from intrinsic defects. Error in use of a reagent are more often the source of a problem. If proper controls are used and manufacturer's directions followed, even these false results will be rare.

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SAFETY IN THE FORENSIC IMMUNOLOGY LABORATORY

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This paper addresses the safety concerns of personnel such as those working in a forensic science laboratory where it is common to encounter on a daily basis potentially hazardous biologic material in either moist or dried states, for example, blood, semen, saliva or items stained by these fluids. Of the bloodborne diseases, the two having the highest levels of concern today are hepatitis B virus (HBV) and acquired immune deficiency syndrome (AIDS). Since HBV, like AIDS, is a bloodborne virus but is present in much higher titer and is also more stable to physical or chemical stresses than is AIDS, it serves as a "worst case" scenario with respect to recommendations for preventing transmission of either infectious agent. Current safety recommendations for AIDS are virtually identical to those for HBV (Centers for Disease Control 1982, 1985) and are also discussed by Bond (1982).

Hepatitis B is an occupational risk among personnel working in health care fields, especially those who are employed in clinical and forensic laboratories and is the most commonly reported laboratory–acquired infection. Laboratory workers who frequently handle blood or blood–contaminated bodily products are at higher risk than is the general population for acquiring infection (Maynard 1978a; Pike 1979; Levy *et al.* 1977). Although non–A/non–B hepatitis has not been reported as a major laboratory–acquired infection, its epidemiology appears to be very similar to HBV in that it is a bloodborne disease (Francis and Maynard 1979); specific information on high risk groups is not available because of the lack of a serologic test. It is reasonable to assume, however, that like HBV, non–A/non–B hepatitis constitutes a significant risk among workers in forensic and clinical laboratories.

The basic reservoir of HBV is the human chronic or transient carrier of hepatitis B surface antigen (HBsAg). In addition to its presence in the blood of these individuals, HBsAg has also been detected in other body fluids, excretions and secretions, including urine, feces, saliva, nasopharyngeal washings, breast milk, bile, semen, synovial fluid, sweat, tears, peritoneal fluid and cerebrospinal fluid. Since these substances contain detectable HBsAg, it has been hypothesized that they may be infectious. There is general agreement, however, that a fecal–oral route of HBV transmission does not exist (Favero *et al.* 1979; Maynard 1978b). Transmission by these other fluids may be possible; however, blood is acknowledged to be the most commonly effective vehicle of HBV transmission.

The efficiency of the various mechanisms of HBV transmission within a laboratory environment is related significantly, if not solely, to the extraordinary amounts of circulating HBV in the blood of infected individuals. Blood diluted to such an extent that HBV is present in relatively small inocula or on laboratory environmental surfaces in the absence of visible or even chemically detectable blood can still be infectious. It has been shown, for example, that human serum containing both HBsAg and HBV e antigen (HBeAg—a serologic marker closely associated with infectivity) can be diluted to 10^{-8} and still produce HBV infection when injected into susceptible chimpanzees (Shikata *et al.* 1977). It has also been shown that HBV in plasma will survive and cause infection after being dried and then stored at 25° C and 42% relative humidity for 1 week (Bond *et al.* 1981).

ROUTES AND MECHANISMS OF HEPATITIS TRANSMISSION IN LABORATORIES

The following modes of HBV transmission can occur in a variety of epidemiologic settings including laboratories and are listed in the probable order of efficiency of disease transmission.

Direct Contact

1. Direct percutaneous inoculation of contaminated serum or plasma such as occurs by accidental sticks or cuts by needles or other objects.
2. Percutaneous transfer of infective serum or plasma in the absence of overt puncture, such as contamination of minute scratches, abrasions, burns or any other lesions.
3. Contamination of mucosal surfaces by infective serum or plasma or infective secretions or excretions such as occurs with accidents associated with mouth pipetting, label licking, splashes or splatterings or other means of skin–to–mouth or skin–to–eye contact.

In a recent study (Bond *et al.* 1982), a chimpanzee was infected 63 days after inoculation of its corneal surfaces with 50 μl of plasma known to contain HBV.

Indirect Contact

Hepatitis B transmission can occur by indirect means via common environmental surfaces in a laboratory such as test tubes, laboratory benches, laboratory accessories and other surfaces contaminated with infectious blood, serum, secretions or excretions that can be transferred to the skin or mucous membranes. The probability of disease transmission with a single event in this category may be remote, but frequency of such events makes this mechanism of transmission potentially an efficient one over a long period of time. Activities in laboratories, such as nail biting, smoking, eating and a variety of hand–to–nose, hand–to–mouth and hand–to–eye actions, may contribute to indirect transmission.

Airborne Transmission

Although HBV transmission by means of the airborne route has been hypothesized, it has never been documented. The results of two studies showed that true airborne transmission of HBV from infectious blood or saliva is not likely. In one study (Petersen *et al.* 1976), a filter–rinse technique capable of detecting low levels of aerosolized airborne HBsAg was devised, evaluated and used in a hemodialysis center serving a patient population with a high prevalence of HBsAg seropositivity and one in which conditions favored the production of aerosols; HBsAg was not detected in any of 60 air samples collected. In the second study (Petersen *et al.* 1979), air samples were collected in a dental surgery at an institution for the mentally retarded where residents had a high incidence of HBsAg seropositivity. Although gingival swab samples from nearly all of patients showed the presence of HBsAg, air samples collected during procedures of scaling, extraction, high speed drilling and other procedures favoring aerosol production were likewise uniformly negative for HBsAg. However, in a separate study concentrating on HBsAg contamination of environmental surfaces in and around hemodialysis centers, 15% of frequently touched surfaces were shown to be contaminated when a swab/rinse sampling technique (Bond *et al.* 1977) was used. Consequently, true aerosols, that is, particles less than 100 μm in diameter, appear to present a less significant hazard than contaminated surfaces. It should be pointed out, however, that events such as splashing, centrifuge accidents or removal of rubber stoppers from tubes can account for disease transmission by means of large droplet transfer into the mouth, eyes, or minor cuts or scratches or onto abraded skin. This is not true airborne (aerosol) transmission but rather transmission by direct droplet contact.

SPECIFIC PRECAUTIONS FOR PREVENTING LABORATORY–ACQUIRED VIRAL HEPATITIS

The primary strategy that should be used in preventing laboratory–acquired HBV is the consistent practice of blood precautions. This is true not only in laboratories where blood, serum and other specimens are processed from patients who are known to be infected with hepatitis but in all clinical laboratories involved in the biochemical, hematologic and microbiologic assay of blood and blood products. The prevalence of HBsAg in sera of patients whose blood is being assayed varies with the population being served by the laboratory. However, one can assume that this prevalence would be approximately 1% in the total population, whereas in specific high risk groups such as hemodialysis patients, parenteral drug abusers or male homosexuals the HBSAg carrier rate may be significantly higher. Consequently, laboratories that process hundreds to thousands of blood samples per day will, without doubt, handle a blood specimen that contains HBV but is not labeled as such. It is common practice to prominently identify specimens and specimen containers with a "hepatitis" label when dealing with known hepatitis patients. Unfortunately, two sets of blood precautions are sometimes practiced in the laboratory: very careful ones with those tubes labeled "hepatitis" and fairly lax ones with unlabeled specimens from other patients. It is emphasized that blood precautions should be employed at all times in the laboratory environment.

The following guidelines are designed and presented specifically for the prevention of laboratory–acquired HBV but could be considered applicable in routine laboratory practices to limit the acquisition of other infectious diseases as well.

Safety Officer

The responsibility for laboratory safety resides ultimately with the director of the laboratory; however, from an operational standpoint, a safety officer who is familiar with laboratory practices and biohazards should be appointed from among the laboratory staff. The safety officer should be responsible for giving advice and consultation to the laboratory staff

in matters of biohazards, instructing new members in safety procedures, procuring protective equipment and supplies, developing and maintaining a laboratory accident reporting system, periodically reviewing and updating safety procedures and monitoring serologic surveillance data for the laboratory staff if such data are collected.

Reporting of Accidents

Accidents, such as cuts, needle sticks and skin abrasions with instruments possibly contaminated with blood, soiling of broken skin or contamination of the eyes or mouth, must be reported promptly to the safety officer who should maintain records and make sure that proper medical consultation and treatment, if necessary, are available. Spills of high risk specimens such as documented or suspected HBsAg–positive blood, even if not associated with personnel contamination, should also be reported to the safety officer.

Handwashing

Frequent handwashing is an important safety precaution that should be practiced after contact with specimens and performance of laboratory procedures, especially those associated with blood or blood products. Hands should always be washed before eating, drinking or smoking and after completing analytical work. Frequent handwashing should be performed even if gloves are used for particular procedures. Handwashing facilities should be conveniently located for frequent use, and a handwashing product that is widely acceptable to personnel is desirable. Liquid or granule soaps are preferable to bar soaps.

Gloves

All laboratory personnel who have direct or indirect contact with blood or articles contaminated with blood should wear gloves. It should be realized, however, that gloves can become contaminated in use and should not contact surfaces such as telephones, door knobs, marking pens and laboratory equipment frequently touched by other ungloved laboratory workers. Disposable single–use, nonsterile gloves are preferred because they can be changed frequently.

Protective Clothing

A gown with a closed front, a coat with an overlapping front or a disposable plastic apron should be worn in any laboratory area. Disposable gloves should be used when opening or processing specimens. Gowns, apron and gloves must be removed, and hands should be washed before a staff member leaves the laboratory for any purpose. Disposable gloves and aprons should be worn only once and placed in an impervious bag for safe disposal. Gowns and coats should be placed in a laundry bag at the end of each appropriate period of work. If the gown or coat is accidentally contaminated, it should be discarded in the laundry bag at once and a fresh one obtained. A face shield or protective eyeglasses and mask should be worn when it is anticipated that there is a potential for blood and other types of specimens being spattered into the worker's face (Bond *et al.* 1982).

Personal Hygiene

Smoking, eating and drinking in the laboratory should be prohibited. Care should be taken not to put fingers, pencils or other objects into the mouth, specimen tube labels must not be licked and hands should be washed after every procedure in which they may have become contaminated.

Pipetting

Mouth pipetting should be prohibited; automatic pipets with disposable plastic tips are recommended. If disposable pipet tips are used, they should be employed as such and discarded after each specimen is pipetted; they should not be rinsed several times in water between each specimen. Other pipets should be used with rubber bulbs or an automatic suction device, and fluids should never be drawn up to the top of pipet. Contaminated pipets must not be placed on the laboratory bench; they should be placed gently in a flat discard pan and later completely submerged in disinfectant. Any rubber bulb that may have become contaminated internally during use should be placed into disinfectant and subsequently discarded.

Receipt of Specimens

Incoming blood specimens should be received in a designated area of the laboratory and examined closely to be sure they have been properly packed. Soiled or leaking containers should be brought to the attention of the safety officer to decide whether they should be autoclaved and discarded without being unpacked. Disposable gloves should be worn during the unpacking procedure.

Labeling, Processing and Storage of Blood Tubes

Blood, serum and biologic specimens from patients who are known to be infected with hepatitis or

for whom HBV or serum enzyme tests are being performed should be identified with special labels marked "hepatitis." It should be reemphasized, however, that precautions employed by laboratory workers when handling these types of high risk specimens should be no different when handling specimens that are not labeled as such. Because blood tubes may be contaminated on the outside (Centers for Disease Control 1980) as well as contain infectious blood, they must be handled and stored with care. If blood tubes must be refrigerated, they should be capped and placed in a designated refrigerator or in a designated portion of a refrigerator. Blood tubes should never be kept in a refrigerator that contains food or beverages.

Needles and Syringes

Special precautions should be taken with needles and syringes that are blood contaminated. Disposable needles and syringes should be used and should then be discarded after a single use. Used needles should not be recapped; they should be placed in permanently labeled, leak and puncture–resistant containers designed for this purpose. Needle nippers should not be used and needles should not be purposely bent or broken by hand, since accidental needle punctures are likely to occur during this procedure. Used syringes should be placed in a leak–resistant bag, and all containers or needles or syringes should be either incinerated or autoclaved before being discarded.

Disposal of Waste Specimens and Contaminated Material

All blood and most biologic specimens from humans or from primates used in hepatitis research must be viewed as potentially infectious. Accordingly, when these materials become waste, they must be disposed of in a safe manner. Each laboratory should have special receptacles for these wastes. Preferably, the wastes should be autoclaved in the laboratory or transported in double impervious bags for terminal processing in an autoclave or approved incinerator. Where it is not feasible to autoclave blood or other potentially infectious fluids, they may be poured down a single sink drain designated for this purpose. Gloves should be worn during the procedure, and care should be taken to prevent splashing onto the walls of the sink. After a sink drain is used for fluid disposal, it should be thoroughly flushed with water (minimizing splashing), and the sink and drain should be treated with an appropriate liquid disinfectant.

Centrifuging

Specimens containing blood should be centrifuged with tubes tightly capped. If a tube breaks in the centrifuge, the bucket containing the spilled blood and broken glass should be placed gently in a pan of disinfectant; the surfaces of the centrifuge head, bowl, trunnions and remaining buckets should be swabbed with an appropriate disinfectant; alternately, the trunnions and buckets can be autoclaved. Micro–hematocrit centrifuges and blood bank serofuges should be cleaned daily.

Automated Equipment

Automated equipment capable of performing a number of biochemical assays simultaneously are becoming commonplace in clinical laboratories. However, it is not evident that any instrument or class of instruments pose significant risks of hepatitis transmission due to external or internal design. Rather, the potential risks of hepatitis transmission are associated with procedures involving specimen handling, specimen preparation and delivery of the specimen to the automated equipment. Gloves should be worn by operators of this equipment at all times. If blood or serum ultimately is collected in a reservoir that is not piped into the sewer system, the contents of the reservoir should be autoclaved before disposal. If this is not feasible, the contents should be poured into a designated sink drain as described previously for fluid wastes.

Care of Laboratory Bench Tops

Each working area should be supplied with a wash bottle containing an appropriate disinfectant. The disinfectant solution should be mixed and renewed according to the direction on the manufacturer's label, and the bench surface must be cleaned and wiped with disinfectant at the beginning and end of each day or more frequently as spills or contamination occur. Since accidents and errors are most likely to happen when the laboratory work area is crowded with equipment and materials, care should be taken to keep the laboratory work area tidy. Tubes and other containers should be placed only in the appropriate rack or tray, never directly on the bench. Disposable, absorbent, plastic–backed pads can be used to protect laboratory bench tops where spattering or spills are common or anticipated.

IMMUNE PROPHYLAXIS

The Advisory Committee on Immunization Practices (ACIP) of the U. S. Public Health Service periodically publishes recommendations and strategies for use of the HBV vaccine as well as immune globulins for protection against several types of viral hepatitis (Recommendation of the Public Health, ACIP 1981). These recommendations are updated periodically in the Centers for Disease Control's Morbidity and Mortality Weekly Report.

The HBV vaccine licensed for use in 1982 is currently available for both preexposure and postexposure prevention of HBV. The vaccine, given in a three dose series, is over 90% effective in preventing HBV infection, has minimal side effects and is recommended for the preexposure prevention of HBV. Laboratory personnel, especially those who frequently handle human or primate blood, are at high to moderate risk of HBV infection and should consider receiving HBV vaccine. No vaccines are available for other hepatitis viruses.

Immune globulins are sterile solutions of antibodies prepared from large pools of human plasma, and Ig (formerly referred to as "immune serum globulin," ISg or "gamma globulin") produced in the United States since 1977 contains relatively stable amounts of antibodies against HBV and hepatitis A virus (HAV). Hepatitis B immune globulin (HBIg) is prepared from plasma preselected for high titer of HBV antibodies, and the cost of HBIg on a per dose basis in approximately 20 times that of Ig.

Personnel who frequently handle blood should be considered as candidates for the HBV vaccine. Postexposure prophylaxis may be warranted in the event of percutaneous or permucosal exposure to blood or body fluids known or thought to contain HBV. The final decision to provide prophylaxis must take into account whether the source of the blood or fluids is known or unknown, whether the HBsAg status of the blood or fluids is known or unknown and the anti-HBs or HBV vaccination status of the exposed person. If postexposure prophylaxis is to be given, it should be done as soon as possible after exposure (no later than 7 days). The reader is referred to the complete ACIP guidelines for details (Recommendation of the Public Health, ACIP 1981). It is important to recognize, however, that after the use of a vaccine, a natural immunity to HBV, or any other disease for that matter, should never be considered as a substitute or replacement for proper techniques (asepsis, cleaning, disinfection or sterilization) in the laboratory.

DISINFECTION, STERILIZATION AND DECONTAMINATION

Since HBV cannot be propagated in tissue culture, comparative virucidal testing has not been performed as it has been for other types of viruses that can be conveniently cultured and tested in the laboratory. Consequently, little is known about the precise inactivation kinetics of HBV by physical and chemical agents, and this situation has led many experts to recommend nothing less than a sterilizing treatment when dealing with contamination by this virus. Unfortunately, this type of recommendation has fostered the concept that HBV is some sort of a "super virus" in terms of its resistance to chemical and physical agents. Bond *et al.* (1977) pointed out that although HBV may be comparatively more resistant to a variety of physical and chemical agents than most viruses, it is unreasonable to assume that the resistance level is equivalent to that of bacterial endospores. Until additional data become available, they proposed that the resistance of the human hepatitis viruses should be considered to be greater than that of the tubercle bacillus but less than that of bacterial spores and probably closer to the former. They further pointed out that conventional sterilization treatments will inactivate HBV. With disinfection processes, however, one must rely on empirical observations. The HBV is not more resistant nor does it approach the resistance levels of bacterial spores; this is known because boiling for 2 minutes inactivates HBV in serum. Therefore, a physical or chemical treatment known to exhibit sporicidal activity should also be virucidal for HBV. Furthermore, because of the exhibited high stability of the HBsAg, a treatment that fully or partially inactivates the immunologic reactivity of this antigen should also inactivate HBV. This rationale and strategy are reviewed at length by Bond *et al.* (1977) and are beyond the scope of this article. However, they did recommend that environmental HBV contamination be dealt with using chemicals, concentrations and contact times capable of producing at least an intermediate level of disinfectant action. Table 1 lists a number of chemical germicides that, if used correctly, can be considered effective for the inactivation of HBV. The liquid chemicals are used primarily for purposes of decontamination for spills of known HBsAg-positive blood and are not generally recommended for routine housekeeping purposes. Additionally, it has been shown that the resistance level of HBV to liquid disinfectant chemicals is not as high as once believed (Bond *et al.* 1983; Kobayashi *et al.*

1984). In fact, although even the intermediate–level disinfectants listed in Table 1 (iodophors, hypochlorite) in addition to 70% isopropyl or ethyl alcohol have been shown effective in killing large numbers of HBV in plasma or serum, alcohols are still not recommended for general disinfecting purposes, since effective contact times are difficult to achieve due to rapid evaporation.

DECONTAMINATION

In high risk areas such as laboratories, one is confronted with the problem of decontaminating large and small blood spills on large, smooth surfaces such as floors or bench tops. Also, frequently touched surfaces, for example, instrument control knobs, racks or precision pipets, may play a role in environmentally mediated hepatitis if they are manipulated in an improper manner. The strategies for applying the principles of HBV inactivation vary according to the item or surface being considered, its potential role in the risk of hepatitis B transmission and, to a certain extent, the thermal and chemical lability of the surface or instrument. For example, if a blood spill occurred on the floor or a counter top in a laboratory, the objective of the procedure to inactivate the HBV would be one of decontamination or disinfection and not sterilization. Consequently, in such an instance, it is recommended that gloves be worn and the spill be absorbed with disposable towels. The spill site should first be cleaned of all visible contamination, and then the area should be wiped down with clean towels wetted with an appropriate high level or intermediate level disinfectant (Favero 1980), for example, a dilution of commercially available household bleach (sodium hypochlorite). All soiled towels should be placed in a container that can either be placed with the infectious waste of that particular department or, since it is sometime easier, autoclaved. The concentration of disinfectant used will depend primarily on the type of surface that is involved. For example, in the case of a direct spill on a porous surface that cannot be physically cleaned before disinfection, 0.5% sodium hypochlorite (5,000 μg available chlorine per liter) should be used. Conversely, if the surface is hard and smooth and has been cleaned appropriately, then 0.05% sodium hypochlorite (500 μg available chlorine per liter) is sufficient.

For purposes of illustration, if an item in question is a medical instrument that is classified as semi–critical (Favero 1980), such as a flexible fiberoptic endoscope (Bond and Moncada 1978), it is extremely important that meticulous physical cleaning precede the sterilization or high level disinfection

Table 1. GERMICIDAL CHEMICALS RECOMMENDED FOR HEPATITIS B VIRUS DECONTAMINATION[a]

Chemical	Concentration	Activity Level
Ethylene oxide gas[b]	450-800 mg/L	High
Glutaraldehyde, aqueous (acid to alkaline)	2 %	High
Stabilized hydrogen peroxide	6-10 %	High
Formaldehyde[c]	3-8 %	Intermediate to high
Hypochlorite[d]	500-5000 mg/L available chlorine	Intermediate
Iodophors[e]	70-150 mg/L available iodine	Intermediate

[a]Except for ethylene oxide (a sterilizing treatment), contact times should be 10–30 minutes. Quaternary ammonium compounds are not recommended for specific site decontamination since these compounds are not broad spectrum in germicidal activity and their effects on HBV or "AIDS virus" are unknown. Although alcohols (ethyl and isopropyl) are known to inactivate HBV and "AIDS virus," they are not generally recommended since rapid evaporation prohibits effective exposure times. Chemicals listed as "high activity level" are used primarily for total immersion of instruments or objects. In addition to the chemicals listed above, heat in the form of boiling water (98°–100° C) for 10–30 minutes is an effective high–level disinfecting treatment for heat–stable materials.
[b]In autoclave–type equipment at 55° to 60° C according to manufacturer's instructions.
[c]Because of the ongoing controversy of the role of formaldehyd as a potential carcinogen, its use is recommended only in limited circumstances under carefully controlled conditions, that is, fixation of tissue specimens or disinfection of "closed systems" such as fluid pathways of hemodialysis systems.
[d]Use 500 to 5000 mg/L depending upon the cleanliness of the surface being treated. These concentrations are easily prepared by diluting sodium hypochlorite (household bleach) 1:100 or 1:10, respectively, in tap water. Ideally, the solution should be made fresh daily. Hypochlorite solutions are corrosive to some metals, especially aluminum.
[e]Use only those iodophors registered with the EPA as disinfectants. Follow manufacturer's directions closely regarding dilution. Antiseptic iodophors (commonly povidone–iodine) should NOT be used as disinfectants. See Favero, in press, for details of the current iodophor controversy.

procedure. This would be true whether or not ethylene oxide gas sterilization is employed or a high level chemical disinfection procedure with a contact time of 10–30 minutes is used.

Other types of frequently touched environmental surfaces could be classified as intermediate between noncritical and semi–critical (Favero 1980) such as control knobs on hemodialysis systems. Ideally, in these types of environments, gloves should be worn to avoid "finger painting" of blood contamination and to avoid percutaneous exposure. Further, these surfaces should be routinely cleaned and disinfected using cloths or, if necessary, swabs. In any case, the objective is to reduce the level of HBV contamination to such an extent that disease transmission is remote. In a practical sense, this could mean that, after cleaning, a cloth soaked in either 0.05% sodium hypochlorite or a disinfectant–detergent can be used. In this context, the element of physical cleaning is as important as, if not more important than, the choice of the disinfectant. It obviously would not be cost effective or in many cases even feasible to attempt to achieve sterilization or high level disinfection in all instances.

GENERAL HOUSEKEEPING PROCEDURES

As a common rule, routine daily cleaning procedures that are used for general microbiologic laboratories can be used for laboratories where blood specimens are processed. Obviously, special attention should be given to visibly contaminated areas or items. Further, cleaning personnel must be alerted to the potential hazards associated with such contamination. Floors and other environmental surfaces contaminated in this manner should be thoroughly cleaned with a germicide–disinfectant registered with the U. S. Environmental Protection Agency as a "hospital disinfectant" that is mycobactericidal. Germicides that are mycobactericidal are preferred because mycobacteria represent one of the most resistant groups of microorganisms; therefore, germicides that are effective against mycobacteria will be effective against most other bacterial, fungal and viral pathogens as well. Gloves should be worn by cleaning personnel doing these duties. However, in the case of large blood spills as mentioned above, this type of procedure may have to be augmented by specific site decontamination with a more effective chemical agent.

SUMMARY

Type B hepatitis is one of the most frequently reported laboratory–associated infections, and clinical laboratory workers involved with handling blood or serum are at increased risk of acquiring this disease. The primary modes of transmission are direct contact with blood and serum specimens or by indirect contact with contaminated environmental surfaces. The presence of even small amounts of blood or serum on hands, whether from direct or indirect sources, can result in the HBV gaining access to the vascular system by needle sticks, cuts and abrasions or via nasal, oral or ocular exposure. Infection control strategies should stress proper techniques for handling blood and containers, appropriate use of gloves, protective clothing and eye protection, frequent handwashing, good personal hygienic practices and the effective use of cleaning, disinfecting and sterilization techniques.

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DISCUSSION

Adama: There has been some concern about the vaccination for hepatitis B virus (HBV). Do you have any comments on that?

Bond: There is absolutely no epidemiologic evidence that it is dangerous, only efficacious.

Question: Those to whom I have talked have said there is a possibility of getting hepatitis from it.

Bond: The vaccine is chromatographically purified material that goes through at least three disinfecting sterilizing steps. There is absolutely no epidemiologic evidence that HBV vaccine is anything but efficacious. It is not dangerous.

Question: How about acquired immune deficiency syndrome (AIDS) contamination of the HVB vaccination?

Bond: None.

Keener: The California Department of Justice checked into whether our health maintenance organization would pay for the HBV vaccination for those who belong to that organization. The health maintenance organization established that we were in a high risk category so we are getting the vaccines now.

Bond: Very good.

Gordomer: What is the estimated incubation period for AIDS at the moment?

Bond: For the infection, we can detect the AIDS antibody within 3-6 weeks. But for clinical AIDS to appear, which occurs after destruction of the immune system and subsequent invasion by secondary invaders, it takes approximately 7 or 8 years.

Barrett: In some of your slides, you showed hepatitis infection coming from centrifuges. Could you elaborate on that?

Bond: I got splattered from centrifuges. If you fill a centrifuge cup too full and put the lid on, the centrifugal force will drive the fluid over the edge, or the tube can break. You can have a drop of blood so small you can not see it with the naked eye and have hundreds and thousands of viruses in it. I can detect with my swab rinse radioimmunoassay technique about 10^{-4} or 10^{-5} ml.

Jones: What is your recommendation for disposal of liquid blood samples that have been analyzed by the laboratory?

Bond: If it is convenient, autoclave the liquid samples and then pour them down the drain. If it is not convenient to autoclave them, pour the liquid samples very carefully down the drain and then clean up the work area.

The sanitary sewer system has a way of cleaning itself up. We have done epidemiologic studies on sewage workers and plumbers, and they do not seem to have a higher rate for antibody to hepatitis A or HBV than does, for instance, somebody who is not around blood all the time.

Question: How are carriers of HBV identified and how dangerous are they to their coworkers?

Bond: Carriers of HBV surface antigen are identified by a serologic screening. You should not be sharing eating utensils or food with a person who has any infectious disease. In a normal work situation, carriers of HBV are not dangerous to their fellow employees. The disease is transmitted by blood or serum.

Question: Although there is no vaccine for AIDS, would you recommend that all forensic workers have periodic testing for AIDS?

Bond: No, I would not. What would be done with the data if you are screened? It might be a good idea to have a baseline serum bank if there is a question of AIDS exposure and it goes on to a workman's compensation related question.

I would like to get an idea of how many of you routinely wear gloves in the laboratory. How many people have had the HBV vaccine? Are there still people afraid to take the vaccine because they think they might get AIDS? I will reemphasize that the only reactions the HBV vaccine has been known to elicit are immunologic side reactions.

Question: At the beginning of the presentation you gave the name of a publication. Are all the guidelines and information that you presented today available in that book?

Bond: The Center for Disease Control biologic guidelines are in there. The paper I wrote which was published in Crime Laboratory Digest is in that book in an expanded form and there are about 30 other articles that have to do with other types of infectious agents.

PERSPECTIVES ON THE FUTURE OF FORENSIC IMMUNOLOGY

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The application of immunology to forensic problems dates almost from the beginnings of immunology as a distinct field of scientific inquiry. The development of the precipitin test for species identification in 1901, for example, followed shortly on the heels of the discovery that antibodies could be made against animal cells and proteins (Gaensslen 1983). Similarly, Landsteiner's first major paper on the ABO blood group system (1901) describes experiments on dried bloodstains and notes the forensic potential. These early applications made clear the inherent specificity and sensitivity of immunologic reactions, and over the years the forensic armamentarium has come to include a wide range of immunologically based procedures.

We stand now at what may be the threshold of a new era for forensic immunology. Developments in immunology over the past decade, particularly the development of monoclonal antibody (MCA) technology and the development of enzyme linked immunosorbent assay (ELISA) systems, have altered the face of immunology, and the carryover to forensic immunology is already apparent. This new technology must be put into perspective.

PROSPECTIVE DIRECTIONS FOR ADVANCEMENT

The current state of forensic immunology needs to be assessed, for example, how well does it serve our needs and what kinds of advances are needed? Table 1 outlines some of the diverse applications of immunology to forensic problems; virtually all involve the use of specific antibodies to detect target molecules or molecular structures. Current procedures vary in their specificity, sensitivity and ease of use. It almost goes without saying that improvements would be welcomed in each of these areas.

A second front for advancement is to expand the arsenal of immunologic methods for the detection of genetic markers and body fluid markers. Only a few examples need be noted. There is a range of red cell blood group antigens, Duffy and Kell to name but two, which are not currently used for bloodstain typing but which might be. The immunoglobulin markers gamma (Gm) and kappa (Km) and the human leukocyte antigen (HLA) markers are known to be stable in biologic evidence and would find much greater application if simpler test systems could be developed. The detection of body fluids at dilution or in mixtures with other body fluids poses problems that might be overcome with new immunologic approaches. It is obvious that the field is wide open.

A third direction for advancement is the development of more objectively quantitative immunologic approaches. Such development would benefit evidence analysis in three respects. First, quantitative data can be evaluated statistically; analytic variation within and between samples can be measured and confidence limits can be assigned to analyses in individual cases. Moreover, statistical analysis provides the only solid basis for distinguishing between background and meaningful data. Even if our interest is with qualitative tests, qualitative tests are but quantitative tests with built in significance thresholds, and the quantitative data base tells us where the thresholds should be set. A second benefit of quantitative analysis is that

Table 1. SOME APPLICATIONS OF IMMUNOLOGY IN FORENSIC SCIENCE

Detection of Antigens for Body Fluid Identification:
Hemoglobin (blood)
Acid phosphatase (semen)
Prostatic antigen p30 (semen)
Seminal vesicle antigen MHS–5 (semen)
Detection of Antigens for Species Identification:
Blood plasma proteins
Detection of Genetic Markers after Electrophoresis:
Group specific component (Gc)
Transferrin
Other genetically polymorphic plasma proteins
Detection of Serologically Defined Genetic Markers:
ABO on cells and in secretions
Lewis antigens for secretor status
MN and other blood group markers
Immunoglobulin markers – Gm and Km
Histocompatibility antigens – HLA
Detection of Drugs of Abuse:
Opiates
Barbiturates
Amphetamines

quantitative assessment of marker levels in evidence samples aids in the interpretation of analytic findings. To give an example, quantitative assessment of semen levels (by acid phosphatase and/or P30 assay) provides a background for the interpretation of genetic marker findings (Blake *et al.* 1981; Sensabaugh 1984a); genetic marker quantitation adds yet more information to the interpretative nexus (Baechtel 1985). The third benefit of quantitation is that it provides a data base describing the world with which we work. Again with semen evidence as an example, measurement of semen marker levels in vaginal fluids as a function of postcoital interval is beginning to provide a picture of the dynamics of semen loss from the vagina (Davies and Wilson 1974; Sensabaugh 1984a, 1984b). This picture in turn provides a sense of what is probable, what is possible and what is beyond a reasonable limit of expectation, thus benefitting the capacity to interpret analytic findings in individual cases.

If quantitative approaches are to be pursued, meaningful quantitative standards must be developed. The critical issue is that without quantitative standards, it is impossible to compare quantitative data generated by different methods or by different laboratories. A statement that a particular sample has an agglutination–inhibition titer of 64 has real meaning only in the context of the particular methods and reagents used for the determination. The situation would be significantly improved if antigen levels could be defined in terms of standard units such as gram mass or molar equivalents.

The final direction for advance to be noted here has the objective of reducing ignorance of the inner workings of immunologic test systems. At present, many immunologic test systems have a black box aspect; we know how they work in principle, but the internal variables are not defined with any kind of chemical precision. The adsorption–elution procedure for blood group typing in stains illustrates this point; although the operational parameters have been well studied (Lincoln and Dodd 1973), there remain significant ignorance gaps. For example, intact red cells are known to possess about 10^6 ABH determinant sites per cell (Economidou *et al.* 1967), but it is not known, even to an order of magnitude estimate, how many of these are accessible to antibody in a bloodstain. Of those antibodies that are bound, it is known what proportion are eluted off or whether those that are eluted off represent a specific subset of the total antibody population. With regard to specificity, the picture of ABH antigen expression on red cells is not complete (Oriol, these proceedings); the extent of variability in the precursor structures or to what extent antibody binding may be affected by this variability are not known. In short, although we know that the adsorption–elution procedure is based on sound principle and that it works, we lack detailed knowledge of the guts of the system. Similar ignorance gaps exist for other immunologic test systems. If these gaps can be reduced or, perhaps better, alternative test systems with fewer ignorance gaps can be developed, in the end we will have more control over our work product.

MONOCLONAL ANTIBODIES

Arguably, the most significant single advance in immunology over the past decade has been the development of MCAs as tools for immunologic analysis. From a practical standpoint, MCAs have two major virtues. First, their specificity and affinity are constants and can be defined with chemical precision, since each MCA is a single molecular species. Second, an MCA can be produced indefinitely and in virtually unlimited amounts; a monoclonal from a particular cell culture line will be the same in the future as it is today. The conjunction of these two virtues provides the promise of standard "reagent grade" antibodies, which would allow control of the antibody variable in immunologic test systems. The benefit for quality control is obvious.

Efforts to substitute MCAs for conventional polyclonal antisera in forensic test systems have met with mixed success. In retrospect, this should have been anticipated, since most immunologic test systems are predicated on the collective behavior of the population of antibody molecules in an antiserum. To use the adsorption–elution procedure again as an example, with a conventional antiserum, it is important only that some proportion of the specifically bound antibodies elute; it is not of concern that perhaps high affinity antibodies do not elute or that some antibodies may be inactivated by the heat step. These factors become relevant, however, when attempting to use a monoclonal, for if the monoclonal is of too high affinity or is heat labile, it will not work in the test system. The lesson is clear: each monoclonal must be investigated on its own terms. Some may be found to behave as the polyclonal sera, although it is likely that most will not. These monoclonals should not be rejected, however; rather, the specific properties of the monoclonals should be looked at and used in whatever way possible.

This premise leads to perhaps the most exciting prospect of MCA use, the prospect of exploiting the exquisite specificity of MCAs to detect fine structure

differences in antigens (Benjamin *et al.* 1984; Benjamin, these proceedings). This prospect is already beginning to be realized with the ABH and Lewis (Le) antigens. As detailed by Oriol (these proceedings) and by Werrett and Lang (these proceedings), recent studies with monoclonals have revealed interesting complexities in the core polysaccharides on which the ABH and Le antigenic determinants reside. It is possible, as suggested by Oriol, that each tissue and body fluid will be found to have characteristic ABH polysaccharide chain structures. For example, evidence of differences in the core chain structures in saliva and semen is developing (McGinnis and Sensabaugh, unpublished). It is also possible that characteristic differences between individuals will be found. Suggestive evidence of this latter possibility appears in the data of Sakamoto *et al.* (1984) shown in Table 2; saliva from 33 individuals can be subdivided into 8 groups based on the pattern of reactivity with six monoclonals that distinguish mono– and di–fucosyl type 1 and type 2 chain structures. Even in the unlikely event that none of these monoclonal defined differences turns out to be of practical value, there will still be a benefit from having a more detailed picture of the ABH–Le antigen structures.

The fine structure specificity of monoclonals also applies with protein antigens; the potential exists to distinguish proteins differing in sequence by but a single amino acid (Benjamin *et al.* 1984a). This opens the way for reliable immunodifferentiation of closely related proteins and of genetically variable proteins. An example of closely related proteins differentiated by monoclonals are the salivary and pancreatic amylases (Herr, personal communication); these two proteins differ by only 6% in sequence (Nakamura *et al.* 1984) and cannot be distinguished by conventionally prepared antisera (Merritt and Karn 1977). With regard to monoclonals for genetic typing, work is progressing on several fronts. A few monoclonally defined genetic polymorphisms have been discovered, and certainly more will be recognized as more proteins are studied with monoclonals (Harris 1983; Smith 1985). Immunogeneticists are actively searching for monoclonals against the highly variable Gm and HLA antigens, but progress has not been as rapid as had been initially hoped. The mouse immune system seems not to be homing in on the variant antigenic sites. The Gm and HLA typing antibodies are of human origin, and the generation of human monoclonals may be a productive path to follow. Although human monoclonals are more difficult to produce than are mouse monoclonals, the technology does exist and is being actively worked on as an approach to cancer immunotherapy (Engleman *et al.* 1985). The success of

Table 2. PATTERNS OF SALIVARY BLOOD GROUP SUBSTANCE REACTIVITY WITH SIX MONOCLONAL ANTIBODIES
(Sakamoto et. al. 1984)

Monoclonal Specificity	Red Cell Lewis Type							
	a^-b^+		a^+b^-		a^-b^-			
H1	0	+	0	0	0	0	0	0
Le^a	0	0	+	+	0	0	0	0
Le^b	+	+	0	0	0	0	+	0
H2	0	0	0	0	0	0	0	0
X	0	0	+	0	+	0	0	0
Y	+	+	0	0	0	+	0	0

monoclonal production against other variant proteins rests on the same contingency: the ability of the immune system to respond to the variant sites. It is clear that genetic typing monoclonals is coming, although it may take time.

It is important to appreciate that the significance of MCA use in species identification, body fluid identification and genetic marker typing extends beyond the promise of improved operational specificity. The use of monoclonals opens the way to a detailed definition of the most important test system variable, the target antigen. The use of monoclonals in species identification illustrates this point. Heretofore, species test antisera have been prepared against a complement of blood serum proteins; operational specificity is achieved by adsorbing crossreacting antibodies on sera of other species and/or by dilution. The result is considerable batch–to–batch variation in the number and identity of the protein antigens recognized by a "specific" species test antiserum (Figure 1). A monoclonal, in contrast, recognizes a small patch on the surface of a single protein; this patch is capable of definition in terms of local peptide chain sequence and conformation. In the context of species identification, this antigenic site, that is, its sequence and conformation, is defined by the evolutionary history of the protein. Its specificity can be assessed by comparison with the corresponding surface patch on the homologous proteins from closely and distantly related species. Thus, although it has always been clear that molecular evolution provides the scientific foundation for species identification, the use of monoclonals brings the two into direct contact, as it can be with the other applications of monoclonals. The test systems can be brought closer to their scientific foundation.

Potential applications of MCAs have been described, and it is clear that we are only at the beginning of the monoclonal era. In looking forward, we must keep in mind the several practical hurdles that must be passed before MCAs can become commonplace in forensic practice. The first hurdle any

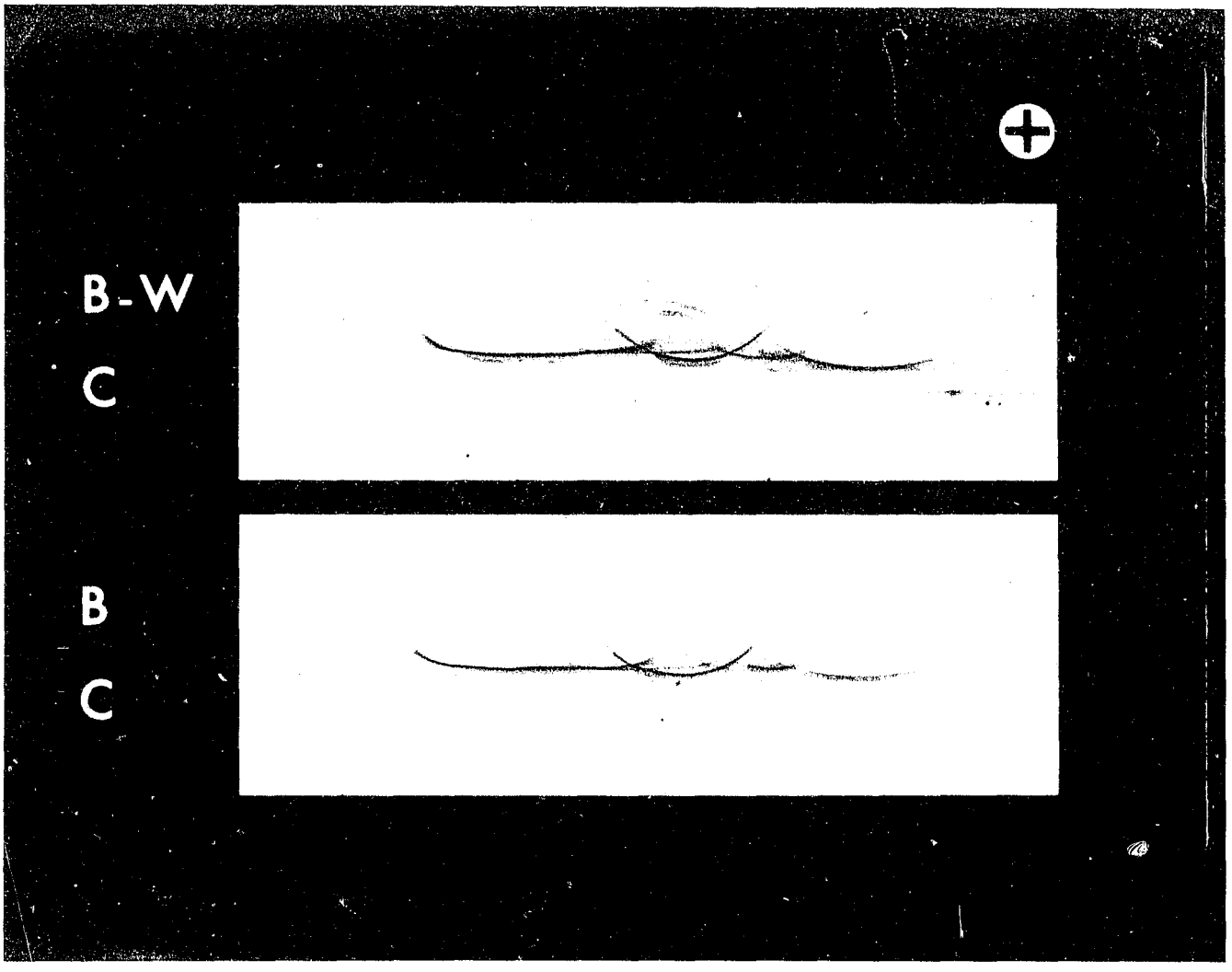

Figure 1. Differences in the antigens recognized by three antihuman test sera prepared for forensic species identification. The three antisera have been compared by immunoelectrophoresis against whole human blood plasma. The B and B–W antisera recognize fewer plasma proteins than the C antiserum and differ also from each other. Photo from Sensabaugh (1975).

monoclonal must pass is the full definition of its operational specificity. As noted earlier, the specificity of monoclonals is narrower than the specificity of conventional polyclonal antisera, and although this narrowness may be useful, it also may be idiosyncratic. For monoclonals that define new specificities, as many are likely to do, the validation testing will have to be well thought out. The second hurdle is the development of test systems that are compatible with the properties of the monoclonal. Test conditions that work well with one monoclonal may not with another. Finally, and perhaps most important, if a monoclonal is to be useful, it must be accessible. Some monoclonals, particularly those that may be useful in blood banking, will be commercially viable and hence will be readily available. However, most monoclonals are prepared for research purposes and are available only as long as the researcher maintains them. Should some of these have forensic utility, the question of availability could become a real problem, since the forensic market may not be large enough to justify transfer to commercial production. It is perhaps appropriate to begin thinking about the formation of a forensic monoclonal cell line culture collection. Such a collection would protect against the loss of useful monoclonal cell lines and could be used to provide "starter" cultures when new production is needed.

ENZYME LINKED IMMUNOSORBENT ASSAY

The ELISA is characterized by the use of solid phase supports to capture antigen antibody complexes and the use of enzymes linked to the antibodies or antigens to measure the antigen antibody reaction. The practical concept of ELISA was developed by

Voller and colleagues (1974, 1979). They sought a robust immunoassay system with a sensitivity comparable to radioimmunoassay (RIA) which could be used in the field in undeveloped countries. From this core, ELISA has become increasingly prominent all areas of clinical immunodiagnostics, and, as might be expected, an extensive technology has come into existence. Faced with automated ELISA washers and computerized ELISA readers, it is easy to forget that ELISA was developed originally for use under primitive conditions.

Some advantages of ELISA based test systems are outlined in Table 3. Several of these advantages follow from the use of solid phase supports. Notably, solid phase systems are a virtual requirement for assays using MCAs, for MCAs are rarely precipitating and are not always good agglutinators. Since in ELISA the solid phase traps the antigen antibody reaction complex, the antibodies used need be neither precipitating nor agglutinating. The ELISA can be used with conventional antisera as well as with monoclonals.

Another benefit of ELISA is the simplicity of the reaction format and the ease of procedural manipulation. Because the reaction products are held in the solid phase, the reagent addition and washing steps are straightforward. The result is that the test system variables are relatively easily defined and controlled, a clear improvement over the situation with some of the classical immunologic procedures. These features recommend ELISA for procedural standardization and automation as described by Fletcher (these proceedings). They also make possible high sample throughput. Even with manual ELISA methods, it is almost as easy to run 100 assays as 10, and this number can be fit comfortably into a working day. With automation, hundreds of samples can be assayed in a day, the rate limiting step being the preparation of samples for assay.

The use of enzyme labels for detection provides sensitivity approaching that of RIA with none of the liabilities associated with the use of radioactive isotopes. The sensitivity gain with ELISA ranges from 10 $\times$ to 100,000 $\times$ over traditional immunologic methods. This sensitivity gain does not have to be exploited, however; a benefit of ELISA is that operational sensitivity can be regulated by changing system parameters.

The sensitivity potential of ELISA has prompted an undercurrent of concern that it may be too sensitive; ELISA may pick up background noise from the samples that will confuse interpretation of results. This concern comes, perhaps, from our general ignorance of the sample world with which we work. Because current methods are relatively insensitive, the question of the significance of low level signals has not had to be faced. A benefit of this insensitivity is reliability; only the strong, meaningful signals give positive results. We fear then an apparent loss of reliability as sensitivity increases. The solution to this concern is to learn about the sample universe by measuring background levels and true positive levels and learning how to distinguish them. This means looking at the sample universe quantitatively, and fortunately one of the virtues of ELISA test systems is that they provide objective quantitative output. The general benefits of quantitation and of thinking quantitatively have been noted previously and need not be belabored further.

Table 3. ADVANTAGES OF ELISA

- Can be used with both monoclonal and polyclonal antibodies
- Simplicity of reaction format
- Potential for standardization and automation
- High sample throughput potential
- High intrinsic sensitivity
- Objective output
- Quantitative
- Cost effective

Concern has been expressed at this meeting that getting started with ELISA may require extensive training and capital investment. This concern is misplaced. Since basic ELISA methods are at least as easy to learn as are electrophoretic methods, there should be no concern about training. The cost of getting started with ELISA is quite reasonable; one can get by with a few micropipets, some plastic ELISA plates and commercially available antibodies and reagents. At the upscale end of the spectrum, one can invest in automated ELISA washers, automated ELISA plate readers and computers to analyze and store the data. The cost of such a setup is, nevertheless, likely to be less than the cost of equipment for gas chromatography or mass spectrometry. Whether manual or automated ELISA systems are used, the analytic cost per sample is low. Supplies and reagents contribute minimally to the cost. Reagents have a good shelf life and are used in small volumes, usually at great dilution. The cost of equipment, if any, can be amortized. The major cost factor, as for any other laboratory analysis, is labor. The ELISA is not as labor intensive as are most immunologic methods in current forensic use, and so the per sample costs are probably lower. The significance of automation is that it diminishes labor costs proportionately to the increase in sample throughput. For high volume operations, automation is cost effective almost regardless of the capital outlay that goes into it. For low volume operations, the cost per analysis will inevitably be higher, regardless of the balance of labor and instrumentation.

These cost considerations give rise to another concern: the general cost effectiveness of ELISA systems relative to other immunologic methods compounded by the specific cost effectiveness of automated ELISA systems argues for the formation of highly centralized immunologic testing laboratories. Small laboratories doing only a few samples per week or per month may find it difficult to justify the cost of doing their own analyses and will be faced with the choice of not doing the analyses at all or of sending the samples to a central laboratory. Most forensic analysts wish to do their own testing to maintain control over the samples.

CONCLUSION

As stated, forensic immunology appears at the threshold of a new era. It is likely that within 5 years many if not most forensic immunologic procedures will exploit MCA technology in one way or another. Concomitantly, ELISA or other solid phase test systems will come into broad use. These developments should lead to both improved analytic and interpretative capabilities but may also force changes in the structure of forensic laboratory operations.

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SECTION II

EXTENDED ABSTRACTS

DETECTION OF SEMEN BLOOD GROUP SUBSTANCES BY ADSORPTION–INHIBITION, ADSORPTION–ELUTION AND ENZYME LINKED IMMUNOSORBENT ASSAY

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Presently, a donor's ABO phenotype can be determined from body fluid stains only by checking for the presence or absence of soluble ABH antigens. Because the ABO phenotype from body fluid stains cannot be confirmed by testing for a second independent marker, as is possible with bloodstains, some laboratories require that both adsorption–inhibition and adsorption–elution assays be used to group these stains. To conclusively group body fluid stains, both assays must produce consistent results. This report relates the results of a comparative study of the sensitivities of the microplate adsorption–inhibition (micro–AI) assay, the microplate adsorption–elution (micro–AE) assay and an enzyme linked immunosorbent assay (ELISA) for detecting soluble blood group substances in human semen.

MATERIALS AND METHODS

The micro–AI assay was performed according to established methods (Baechtel 1985). The micro–AE was performed in V–bottom microplates (Dynatech Laboratories, Alexandria, VA). The adsorption and elution steps of the micro–AE were conducted in flexible plates in which seminal blood group substances had been previously immobilized. After heat or acid elution, the eluates were transferred to a rigid plate and the hemagglutination portion of the assay was completed.

The ELISA method was performed with flat bottom Immulon IITM plates (Dynatech Laboratories, Alexandria, VA) in which the seminal blood group substances had been previously immobilized. Blood group monoclonal reagents from Dakopatts (Santa Barbara, CA) were used to detect the immobilized blood group antigens after the addition of an appropriate enzyme conjugated antibody.

RESULTS AND DISCUSSION

In this study, 111 semen specimens were assayed by the different assay techniques. The micro–AE and ELISA techniques provided the greatest sensitivity for the detection of A and B antigens (twentyfold to eightyfold increase in sensitivity) as compared with the inhibition assay. Although an increase in assay sensitivity was not observed with the micro–AE when testing for the H antigen using an affinity purified Ulex I lectin (Sigma Chemicals, St. Louis, MO), the ELISA assay exhibited a 134–fold to 392–fold increase in sensitivity for this antigen. Although more sensitive than the micro–AI assay, the micro–AE and ELISA were more prone to false negative results because of the failure of some monoclonal reagents to recognize the B antigen (ELISA) or the occurrence of a prozone phenomenon due to antigen excess (elution). Additionally, attempts to use the monoclonal reagents in the micro–AE were unsuccessful because these reagents failed to elute by either the heat or acid procedure.

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PHENOTYPING OF ABO SUBGROUPS FROM CLOTH THREADS

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The accurate ABO phenotyping of bloodstain evidence is very important in forensic serology. Currently, little information is available on the phenotyping of subgroups of A and B from dried bloodstains. The ABO subgroups represent a diverse group of genetic variants within the ABO blood group system. They are characteristically weaker in their phenotypic expression when liquid anticoagulated blood samples are used. Some of these rare genetic variants may appear to be of Group O origin when tested with potent ABO antisera. This study was based on the supposition that these subgroups may be mistyped when less sensitive forensic techniques are used on dried bloodstains (Gaensslen 1983).

We present a test model of this hypothesis using dried ABO subgroups phenotyped by a conventional adsorption–elution technique. This approach also included the use of monoclonal ABO antibodies that are known to give stronger reactions with ABO subgroups than conventional antisera.

MATERIALS AND METHODS

Preparation of Test and Control Blood

Fresh and frozen red cells samples representing known controls and select ABO subgroups were dried onto cloth that had been carefully laundered to remove residual sizing and detergent. The stains were allowed to dry for at least 24 hours at ambient room temperature before phenotyping. The frozen red cells were deglycerolized in graded salt solutions and concentrated to approximately a 40% hematocrit in AB plasma. All blood samples used in this study were evaluated by the direct antiglobulin test. At least 10 different ABO determinations were performed with a standard adsorption–elution technique. Control threads (nonbloodstained) were run in parallel with the test material.

Adsorption–Elution Procedure

Individual cloth threads from the test and control stains were carefully teased and cut to lengths of approximately 7–10 mm. The threads were attached to standard ring slides with nail polish. Standard and special ABO monoclonal antisera (Ortho Diagnostics, Raritan, NJ) were adsorbed onto the respective stains and heat eluted at 64° C. The eluates were tested against indicator red cells (0.2%). The ABO phenotyping was assessed by grading hemagglutination reactions with the indicator cells (Gaensslen 1983).

RESULTS

Phenotyping of the various ABO subgroups is summarized in Table 1. The A1, A intermediate, A2 and A3 subgroups were typed as being of Group A origin. When the hemagglutination reactions of these respective eluates were graded against Group I indicator cells, the following results were obtained: A1>A intermediate >A2 >A3. The group A2B and A3B variants were phenotyped as group AB. The group A2B eluate (adsorbed with anti–A and anti–A,B) had a stronger reaction with the A1 indicator cells than that derived from A3B threads. The B3 variant was identified as group B by the adsorption–elution technique. This rare variant appeared to be quantitatively weaker in its eluate reactions than those derived from known Group B threads.

The A^x, A^{el} and B^x subgroups consistently phenotyped as Group O. Attempts to enhance the amount of ABO antibody in these eluates by increasing the number of threads in the test system were not successful. The use of anti–H did not allow the discrimination of the subgroups from Group O bloods (Table 2). We could not obtain consistent reactions with control blood samples using the monoclonal ABO antibodies produced by Ortho Diagnostics (Raritan, NJ).

Table 1. ADSORPTION-ELUTION STUDIES[a]

ABO PHENOTYPE	ADSORPTION WITH ABO ANTISERA	HEMAGGLUTINATION REACTION OF ELUATE WITH INDICATOR RED CELLS[b]	
		A_1	B
A_1	Anti-A	3^+-4^+	0
	Anti-B	0	0
	Anti-A,B	3^+-4^+	0
A_2	Anti-A	2^+-3^{+w}	0
	Anti-B	0	0
	Anti-A,B	2^+-3^+	0
A_2B	Anti-A	2^+-3^+	0
	Anti-B	0	3^+-4^+
	Anti-A,B	2^+-3^+	3^+-4^+
A INTERMEDIATE	Anti-A	2^+-3^{+w}	0
	Anti-B	0	0
	Anti-A,B	2^+-3^{+s}	0
A_3	Anti-A	1^+-2^{+w}	0
	Anti-B	0	0
	Anti-A,B	1^+-2^{+s}	0
A_3B	Anti-A	1^+-2^{+w}	0
	Anti-B	0	1^+-2^+
	Anti-A,B	1^+-2^{+s}	1^+-2^{+s}
A_x	Anti-A	0	0
	Anti-B	0	0
	Anti-A,B	0	0
A_{el}	Anti-A	0	0
	Anti-B	0	0
	Anti-A,B	0	0
B	Anti-A	0	0
	Anti-B	0	3^+-4^{+w}
	Anti-A,B	0	3^+-4^{+w}
B_3	Anti-A	0	0
	Anti-B	0	1^+-2^{+w}
	Anti-A,B	0	1^+-2^{+s}
B_x	Anti-A	0	0
	Anti-B	0	0
	Anti-A,B	0	0

[a]All red cells were found to be negative for direct antiglobulin test

[b]0=Negative 1^+-4^+=Hemagglutination Reaction w=Weak s=Strong

Table 2. ELUTION OF ANTI-H WITH SELECT ABO SUBGROUPS[a]

ABO TYPE - ADSORPTION WITH ANTI-H	HEMAGGLUTINATION REACTION OF ELUATE WITH INDICATOR RED CELL
	0
A_1	$0-1^{+w}$
A_2	1^+-2^{+s}
A INTERMEDIATE	1^+-2^+
A_3	1^+-2^{+s}
A_2B	2^+-3^{+w}
A_3B	2^+-3^{+s}
A_x	3^+-4^+
A_{el}	3^+-4^+
B	1^+-1^{+s}
B_3	2^+-2^{+s}
B_x	3^+-4^+
0	3^+-4^+

[a]Standardized Anti-H from *Ulex europaeus*

CONCLUSIONS

Some of the weaker subgroups of A and B (such as A^x, A^{el} and B^x) were misidentified as Group O. The anti–A,B gave quantitatively stronger reactions than did anti–A and anti–B with select subgroups. The anti–H was not useful in delineating the weaker ABO subgroups from group O controls. Select monoclonal ABO antibodies (Ortho Diagnostics, Raritan, NJ) were not found useful in routine adsorption–elution of bloodstains.

The ABO subgroups are characterized by their lower antigen density when compared with A^1 and B cells (Issitt 1986). This difference explains the weaker or negative hemagglutination reactions observed with these genetic variations. We believe that the failure to

phenotype A^x, A^{el} and B^x accurately is based on a simple model of antigen excess in which there is insufficient eluted antibody to induce hemagglutination.

The possible misidentification of an ABO subgroup could be a serious problem, but forensic serologists should remember that many of these variants occur at a very low incidence (for example, A^x 1 in 40,000). Strategies should be developed to maximize the detection of subgroups in blood specimens drawn from criminal suspects (for example, extended forward and reverse groupings). If a subgroup is identified in this typing, it may be useful to prepare threads from this sample to compare with the submitted dried bloodstain evidence.

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TWO-DIMENSIONAL ADSORPTION-INHIBITION

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Three different adsorption–inhibition methods have been used to determine the soluble ABH antigenic substances in secretor semen, saliva and other physiologic fluids (Lee 1982; Gaensslen 1983). The simplest procedure, often called "one–tube" or "all–or–none," is seriously limited by its use of a single body fluid stain extract dilution that cannot take the known variations in ABH content of body fluids into account. Use of this procedure may lead to erroneous conclusions. Inhibition–titration (Holzer 1937) (I–T3) and titration–inhibition (Hirszfeld and Amzel 1932; Kind 1955) (T–I) procedures give more complete results because the tests are arranged to detect the antigens over a range of ABH concentrations in body fluids. A two–dimensional (2–D) inhibition procedure has been devised which takes advantage of the best features of both the I–T and T–I procedures. It requires little additional effort and no additional (often limited) stain extract in comparison with I–T.

An illustrative way of comparing sensitivities of the different procedures is to compare agglutination reaction patterns resulting from the presence of soluble A, B or H antigen sufficient to remove defined fractions (1/2, 1/4, 1/8, 1/16 and so on) of the corresponding antibody present. Such an analysis shows that 2–D is more sensitive than T–I, which in turn is more sensitive than I–T in detecting smaller quantities of soluble ABH antigens.

MATERIALS AND METHODS

Known control body fluids from healthy donors and casework stains were examined with all four procedures. Antisera and reagent red cells obtained from standard commercial sources were employed. Some anti–H was prepared from *Ulex europaeus* seeds (Wiener *et al.* 1958; Kind 1962). All four procedures were carried out with several different initial antiserum titers. The 2–D procedure was performed by first setting up a T–1 series. Each tube in the row was then titrated out by doubling dilutions in the fashion of an 1–T procedure.

RESULTS AND DISCUSSION

Table 1 shows the number of correct results obtained with the four different procedures in a series of 31 saliva and 12 semen samples, some of which

Table 1. NUMBER OF CORRECT RESULTS OBTAINED WITH THE FOUR DIFFERENT INHIBITION TECHNIQUES ON 31 SALIVA AND 12 SEMEN SAMPLES

Type of Sample	ABO Group and Secretor Status	N	Number of Correct Results Obtained With One-Tube	I–T	T–I	2–D
Saliva						
	A secretor	7	0	6	7	7
	B secretor	6	1	5	6	6
	O secretor	8	5	8	8	8
	Nonsecretor	10	10	10	10	10
Semen						
	A secretor	1	0	0	1	1
	B secretor	2	2	2	2	2
	O secretor	3	3	3	3	3
	AB secretor	2	1	1	2	2
	Nonsecretor	4	4	4	4	4

were known controls and some of which were stains. A "correct" result was one that clearly indicated the secretor status and the correct ABO blood group in the case of secretors. In this series, both T–1 and 2–D procedures gave correct results in every instance but the one–tube and I–T techniques did not. Table 2 shows the results obtained with five problematic casework samples. Here, correct results were obtained in every case only with the 2–D procedure.

The data collected on both known control and casework samples and experience with the four procedures show that one–tube and I–T methods do not always yield correct results in secretor samples containing smaller quantities of soluble ABH substances. The T–I method gives correct results most of the time, but there were occasional samples for which only the 2–D technique provided unequivocal results. These results are in accord with the predictions from the theory underlying the methods. Since T–I yields correct results most of the time and the 2–D set–up is in effect a T–I test, the 2–D test can be carried out more efficiently. After inhibition, an aliquot of material from each tube is set aside for possible titration and the first row is read.

The first row is itself a T–I test, and if unequivocal results are obtained upon reading it, the second dimension titrations need not be performed. This procedure offers caseworkers a routine inhibition protocol that has been shown to yield maximal correct information from every sample examined.

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Table 2. RESULTS OBTAINED USING THE FOUR INHIBITION TECHNIQUES WITH CASE SAMPLES EXHIBITING LOW LEVELS OF SOLUBLE ABH BLOOD GROUP SUBSTANCES

Type of Sample	ABO group and Secretor Status	Antigen(s) Detected Using[a] One-Tube	I–T	T–I	2–D
Saliva					
	A secretor	H	(A),(H)	A,H	A,H
	AB secretor	B	(B),(H)	(B),(H)	A,B,H
	AB secretor	A	(A),(B)	A,B,H	A,B,H
Semen					
	A secretor	H	(A),(H)	(A),(H)	A,H
	AB secretor	A,H	A,(B),(H)	A,B,H	A,B,H

[a]Antigens shown in parentheses represent weak reactions which would be regarded as inconclusive for reporting purposes

ABH TYPING OF URINE BY A TWO-DIMENSIONAL ADSORPTION-INHIBITION PROCEDURE

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Urine samples are now routinely screened for the presence of drugs or drug metabolites by the Armed Forces, police departments and others, but the accused may challenge the identity of positive samples or the authorities may question negative results. The need for a straightforward reliable test for checking sample identity is met by the ABO grouping procedure.

Many researchers have found ABH antigenic substances in urine (Yosida 1928; King *et al.* 1961; Lundblad 1967). The concentration of these antigens is significantly lower in urine than in many other body fluids, and adsorption–inhibition techniques often fail to detect these antigen carrying molecules. Lee and Gaensslen (1980) obtained reliable ABO grouping by concentrating urine. However, even concentrated samples may not yield convincing results, and bacterial or other contaminants in the urine, introduced during sample manipulation or concentration, may cause problems.

Lee *et al.* (1986) used a two–dimensional inhibition procedure that combines the best features of the Holzer (1931) and Hirszfeld and Amzel (1932) techniques. This method was examined for its ability to detect ABH antigenic substances in urine.

Seventy–one urine samples were collected from normal, healthy donors of known secretor status. Each sample was centrifuged at 3,000 rpm for 5 minutes, and the resulting supernatant and precipitate were tested with a chemical presumptive test for blood. The supernatant was then tested for the presence of ABH antigenic substances using the two–dimensional procedure. Each urine sample was also tested by three more classical adsorption–inhibition procedures: one–step (one–tube) (Gaensslen 1983), inhibition–titration (Holzer 1931), and titration–inhibition (Hirszfeld and Amzel 1932; Kind 1955). These results were compared with those obtained with the two–dimensional technique.

As shown in Table 1, results obtained by inhibition of secretor urine samples were dependent on the procedure used. Nine of the 60 samples from secretors failed to reveal ABH antigens by the one–tube method, but antigens corresponding to the known ABO blood group of donors were detected in 40 specimens. Thirty–four samples showed antigens corresponding to the ABO blood group of urine donors when inhibition–titration was used. Although blood group antigens were detected in all but one urine sample with this method, 19 specimens yielded inconclusive results. A result was considered inconclusive if less than a three–tube reduction in inhibition was noted. Titration–inhibition of the 60 secretor urine samples resulted in four inconclusive results. Conclusive results were obtained with all samples when the two–dimensional method was used. Urine from nonsecretors failed to reveal the presence of antigenic substances with all four inhibition procedures used in this study.

Table 2 summarizes the results obtained with the secretor urine samples tested. The detection of antigens not corresponding to the known ABO blood group of donors and the failure to detect antigens were designated as incorrect results. Incorrect results were noted with both the one–tube and the inhibition–titration methods. Correct, conclusive results were obtained for all secretor urine specimens when the two–dimensional method was used. This two–dimensional technique not only eliminates the need for sample concentration but also yields more conclusive results.

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Table 1. ANTIGENS IN SECRETOR URINES

	Blood Group	ANTIGEN DETECTED A	B	H	A,H	B,H	ABH	NAD[a]	INC[b]
One-tube	A	8	0	5	5	0	0	1	0
	B	0	3	2	0	9	0	1	0
	O	0	0	8	0	0	0	7	0
	AB	0	0	0	2	2	7	0	0
Inhibition-titration	A	1	0	5	6	0	0	0	7
	B	0	0	0	0	10	0	0	5
	O	0	0	9	0	0	0	1	5
	AB	0	0	0	0	1	8	0	2
Titration-inhibition	A	0	0	0	18	0	0	0	1
	B	0	0	0	0	14	0	0	1
	O	0	0	14	0	0	0	0	1
	AB	0	0	0	0	0	10	0	1
Two-dimensional	A	0	0	0	19	0	0	0	0
	B	0	0	0	0	15	0	0	0
	O	0	0	15	0	0	0	0	0
	AB	0	0	0	0	0	11	0	0

[a]NAD = No antigens detected
[b]INC = Inconclusive

Table 2. NUMBER OF SECRETOR SAMPLES INCORRECT OR INCONCLUSIVE

	ABO BLOOD GROUP A	B	O	AB
Number of Samples	19	15	15	11
One-tube				
Incorrect	6	2	7	4
Inconclusive	NA	NA	NA	NA
Inhibition-titration				
Incorrect	5	0	1	1
Inconclusive	7	5	5	2
Titration-inhibition				
Incorrect	0	0	0	0
Inconclusive	1	1	1	1
Two-dimensional				
Incorrect	0	0	0	0
Inconclusive	0	0	0	0

Total samples tested = 60

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ABO TYPING OF DRIED BLOODSTAINS MIXED WITH BODY FLUIDS BY METHANOL EXTRACTION

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Forensic serologists have had difficulty typing dried bloodstains that have been mixed with other body fluids, such as semen, vaginal secretions, perspiration and saliva. The ABO bloodgroup system is the one most widely used in the crime laboratory. The ABH blood group substances are alcohol soluble in erythrocytes and organs and are water soluble in body secretions (Gaensslen 1983). The protein portion of soluble substances may affect serologic reactivity but not blood group specificity (Cote 1970). A and B blood group activity can be demonstrated in the glycolipids. The lack of H activity would imply a biosynthetic process for H antigen which is different from that of A and B antigens found on erythrocytes and from that in the secreted mucoids. The A and B active glycolipids are located on the cell surface, and H activity is due to a glycoprotein or some similar substance (Prokop and Uhlenbruck 1969).

Using these principles, Howard and Martin (1969) extracted in methanol dried bloodstains mixed with various body fluids and left the water soluble fraction behind. The methanol extracted fraction was then typed for ABO by adsorption–elution.

MATERIALS AND METHODS

All antisera used in this study were polyclonal anti-A and B (Ortho Diagnostic Inc., Raritan, NJ). Anti H was prepared in–house. The saliva specimens were collected on clean filter paper and allowed to air dry. The perspiration specimens were collected on cotton sheeting, and vaginal secretions were collected on tampons that were worn for 10 hours. The semen samples were collected directly into the tubes. The dried bloodstains were mixed with extracts of various body fluids (saliva, semen, vaginal secretions or perspiration). One hundred and ten mixed stains were prepared in which the ABO type was known for the bloodstains and body fluids. In each case, the mixed stain was typed for ABO by adsorption–elution. A sample from each mixed stain was extracted in methanol and typed for ABO by adsorption–elution. The same piece of stain was extracted in water for the water soluble fraction and typed by adsorption–inhibition for ABO. Fourteen of the samples that showed false negatives were tested by adsorption–elution for prozone effect using serial dilutions.

RESULTS AND DISCUSSION

Of the 110 samples extracted, 53 were typed accurately for bloodstain (that is, the influence of the body fluid antigen was eliminated). Thirty–one bloodstains were mixed with saliva, and 13 were typed accurately for ABO following methanol extraction. Seventeen type O bloodstains gave no H activity after methanol extraction, and this finding supports the statement that H activity cannot be demonstrated in alcohol soluble glycolipids (Prokop and Uhlenbruck 1969). One bloodstain of type A showed no ABH activity. Twenty–nine bloodstains were mixed with perspiration, 24 bloodstains were mixed with vaginal secretions and 26 bloodstains were mixed with semen. All of the A, B or AB samples were typed accurately in ABO for the bloodstain following methanol extraction. None of the type O bloodstains gave any activity. Nine of the mixed stains demonstrated false B activity. These blood samples were morgue blood specimens, and we believe that bacterial growth may be responsible for the spurious "B" activity. However, no spurious "B" activity was detected after methanol extraction.

No bloodstains mixed with perspiration, vaginal secretions or saliva demonstrated the ABH activity by adsorption-inhibition after water extraction. Twenty–two of the 26 blood/semen stains were typed accurately in ABO for the semen by adsorption-inhibition after water extraction. Three of the water extracts gave negative results and one showed H activity for a type A semen. Of the 110 samples extracted, 53 typed

accurately for bloodstains, eliminating the influence of the body fluid antigens. This suggests that suspected bloodstains mixed with other body fluids can be extracted in methanol before typing.

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SEPARATION OF BLOOD GROUP SUBSTANCES ABH AND LEWIS IN MIXTURES OF BODY FLUIDS

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Mixtures of body fluids from more than one person are difficult to type for ABO and Lewis (Le) blood groups. A secretion from an individual whose group may be known can mask the characteristics of the group of the donor of the other fluid (Davies 1982). This problem occurs in two–thirds of all mixtures of body fluids from two different donors (Table 1). Masking could be reduced by separating different blood group substances. When separation is used, only one–third of cases in which the two secretions are of the same group are masked.

Watkins and Morgan (1956) coprecipitated A and B activity from saliva of an AB secretor, using anti–A or anti–B serum. An artificial mixture of A plus B showed no coprecipitation. Every blood group active molecule secreted by an individual is supposed to carry all the determinants encoded in the genome of that person (Watkins and Morgan 1957, Brown *et al.* 1959). Each molecule has about 300 short, branched carbohydrate chains whose terminal sugar residues determine the blood group specificity. The blood group activities are produced by sequential addition of sugar residues and are governed by enzymes produced by blood group genes. The enzymes compete for precursor chains: some are converted to Le^a and some to H active determinants. The Le^a terminates a chain, but H substance may be further converted to A, B or Le^b active structures. The conversions are not complete, and so each molecule is expected to carry all possible determinants (Watkins 1980).

MATERIALS AND METHODS

Immunoadsorbent columns were made with cyanogen bromide activated Sepharose 4BTM (Pharmacia Fine Chemicals, Sweden) coupled to ascitic anti–A, anti–B or anti–H antibodies (Dakopatts, Denmark) according to *Affinity Chromatography* (1979). For each column, analysis of which blood activities come straight through and which are bound ought to reveal which activities are carried together and therefore originate from the same source. Blood group substances were bound from samples of saliva which had been boiled, clarified and stored frozen. When the columns had been thoroughly washed and 1 ml fractions retained, bound substances were eluted with

Table 1. MASKING

KNOWN "VICTIM'S" GROUP	%	MASKING: Probability of two secretions of same group	MASKING: Other Cases	MASKING: %	MASKING: Total
A Secretor	34	12%	All other groups	22	34%
B Secretor	6	0.36%		5.64	6%
AB Secretor	2	0.04%		1.96	2%
O Secretor	33	11%	Non-secretor	8	19%
Non-secretor	25	6%	None		6%
Total		30%			

buffers of pH 7.5 and pH 4.5 in two cycles. All the fractions were diluted in coating buffer and assayed by indirect enzyme linked immunosorbent assay (ELISA) (Bolton and Thorpe 1986). The anti–Le^a, anti–Le^b and anti–H were incorporated into this assay. Originally, only anti–A and anti–B were used. All the antibodies were culture supernatants from mouse hybridomas (Biotest Folex Ltd, Germany). The optical densities in ELISA were compared with the activities present in a dilution series of the original saliva sample.

RESULTS

We measured specific binding and elution of blood group substances. By comparing the void and eluate fractions with the standard dilutions, we determined that not all the blood group active molecules of an individual are identical (Figure 1). For example, with an anti–A column and saliva from an AB secretor, the eluate showed more A activity relative to Le^b or B than could be expected (Figure 2). The first fraction from the column, though still containing A activity, showed more B, Le^b, H and Le^a activity relative to A than did the original sample.

A spectrum of molecules seems to exist which carry different relative amounts of each blood group activity. If such a spectrum exists, then identifying the components of a mixture via immunoseparation may be difficult in operational casework.

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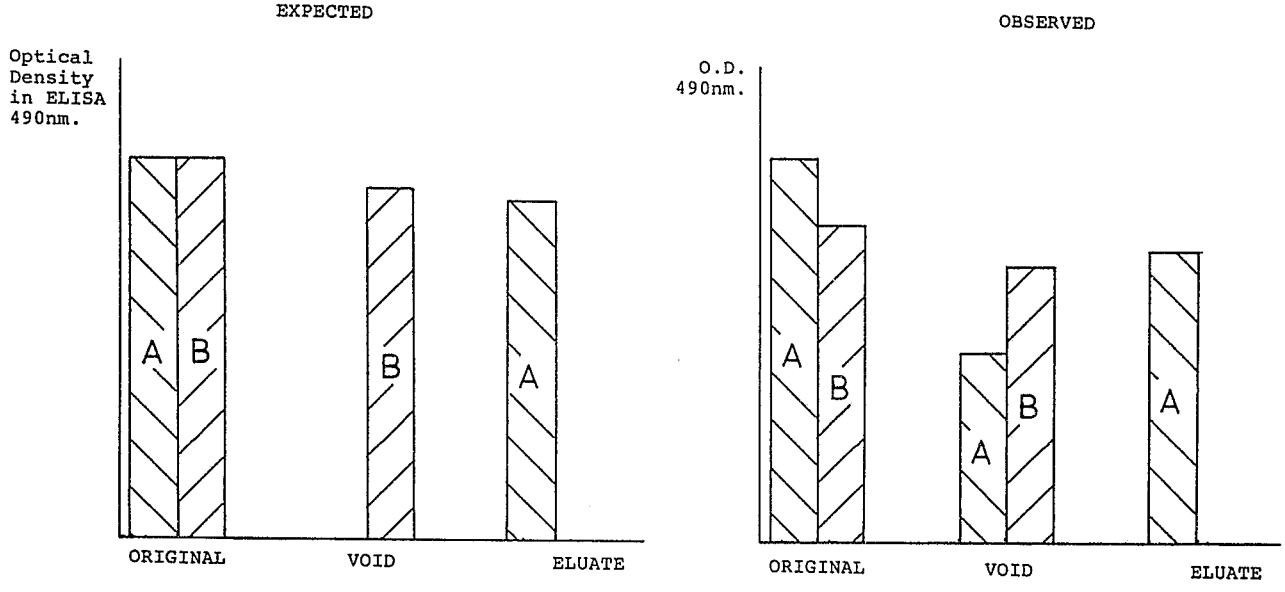

Figure 1. Mixture of A and B Saliva Through an Anti-A Column

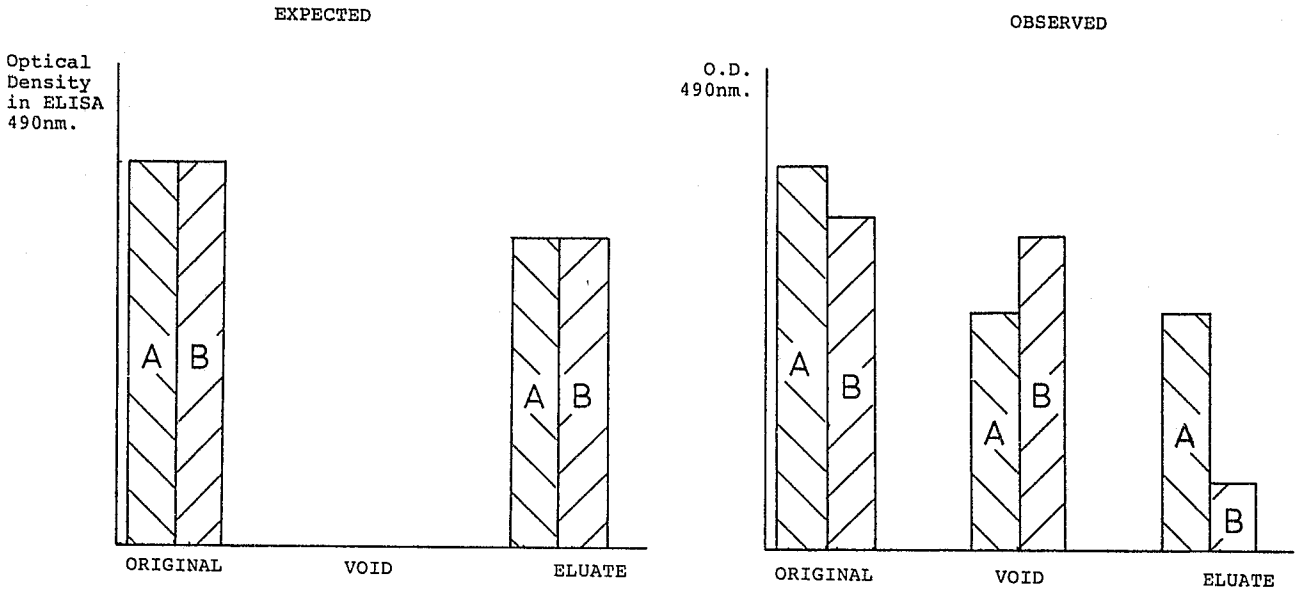

Figure 2. AB Saliva Through an Anti-A Column

ABH BLOOD GROUP SUBSTANCE LEVELS IN MATCHED BODY FLUID STAINS: A PRELIMINARY INVESTIGATION

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This study examined matched body fluid stains (semen or vaginal samples, saliva, urine and perspiration) for the ABO blood group substances from 18 donors using both the adsorption–inhibition and adsorption–elution techniques. The 18 donors included male donors who were ABO type A, B and O secretors and nonsecretors and female donors who were ABO type A, B and O secretors and type A and O nonsecretors. Although analysis of semen, saliva and vaginal samples for the ABO blood group substances is routinely performed in crime laboratories, little information is available about levels of ABO blood group substances in urine and perspiration stains. Adsorption–inhibition and adsorption–elution were run in parallel to evaluate the suitability of the two techniques to type accurately body fluid stains for the ABO type.

Stain extracts and dilutions of these extracts (ranging from 1:5 to 1:1000) were prepared from dried stains. The extracts and dilutions were then analyzed by both the inhibition and elution techniques. The inhibition procedure was carried out with equal volumes of cell–free extract and anti–sera or lectin (Dade anti–A diluted 1:32, Dade anti–B diluted 1:64 and laboratory–prepared anti–H diluted 1:8 and 1:16) in disposable microtiter plates. Adsorption was carried out at 4° C overnight, 0.3% A_2, B and O indicator cells were added to the appropriate wells and the results macroscopically scored.

The extract containing the cellular debris and dilutions prepared from the "neat" cell–free extract then was analyzed by the elution technique. The extract was spotted onto three circled areas of a clear plastic plate. Undiluted anti–H was dropped onto one of the dried stain extract spots and allowed to adsorb for 4 hours. Dade anti–A and anti–B (both diluted 1:2) were then dropped onto the two remaining dried stain extract spots and allowed to adsorb for 1 hour. The plate was washed overnight in normal saline at 4° C. Elution was carried out at 60° C for 25 minutes in normal saline. After elution, A_1, B and O indicator cells (a 0.3% concentration of cells made up in saline containing 1% bovine serum albumin) were added to the saline eluates, and the plates were rotated and microscopically scored. The short adsorption time and long wash period resulted in a less sensitive but more specific test.

Semen stains from the secretor donors displayed the highest levels of ABO blood group substances when compared with the other body fluids. Vaginal swabs from secretor donors showed the greatest variation in blood group substances levels. The A and B blood group substances from type A and B secretors were detected in both urine and perspiration stains by both the inhibition and elution techniques. The H blood group substances were never detected in urine or perspiration stains from any of the donors tested. In general, saliva and vaginal stain extracts displayed lower levels of blood group substances than did semen stains but higher levels than did urine or perspiration stains. No clear, consistent trends were seen in the relative levels of blood group substances from a series of body fluid stains from any one individual. Thus, for example, relatively high levels of blood group substances in saliva would not guarantee that blood group substances would also be detected in the corresponding urine or perspiration stain. Body fluid stains from nonsecretor individuals displayed blood group substances levels that were never detected by the inhibition technique and only rarely by the elution technique.

The criteria selected for reporting the ABO results from the elution test produced a number of "no call" results, but the inhibition and elution tests conflicted only once. A vaginal swab from an A secretor donor revealed no blood group substances by inhibition and B blood group substances by elution. No spurious results were encountered with the inhibition test. The elution test infrequently revealed a reportable result that was not detected by the inhibition test. However, the analysis of the data generated from this somewhat limited study suggested that the elution test might be used primarily to resolve a questionable inhibition result.

ABH AND LEWIS TYPING: APPLICATION TO SELECTED PROBLEMS IN MIXTURES OF SEMEN AND VAGINAL SECRETIONS

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Two difficult interpretive problems frequently arise during the analysis of mixtures of seminal and vaginal fluids: interpreting the significance of ABH blood group substances that are qualitatively nonforeign to the female and confirming the nonsecretor semen donor. The 99% threshold levels of significance for endogenous ABH blood group substances in vaginal secretions serve as a guide as to when ABH levels in semen/vaginal mixtures are higher than expected for vaginal fluid alone. By knowing the range of ABH levels in semen and estimating the minimum amount of semen in the specimen under consideration, one can predict whether the sample has the potential to exceed the 99% vaginal ABH threshold. The relationships among antigen titer, estimated semen dilution and 99% vaginal threshold can be illustrated graphically for each of the ABH blood group substances (Figures 1–3). In each figure, zone 1 defines those titers of qualitatively nonforeign antigen which exceed the 99% vaginal threshold, indicating that semen is contributing to the antigen pool. Semen samples that are more dilute than 1:100 are unlikely to possess enough antigen to exceed the vaginal threshold.

Zone 2 defines those titers of antigen which are lower than expected for concentrated semen (that is, no more dilute than 1:100). Antigen titers in this zone are more likely to be contributed by vaginal secretions. These results suggest that the semen donor is either an unusually low level secretor or a nonsecretor. Zone 3 defines those titers of antigen for which no attribution of source can be made. Antigen titers falling within zone 3 may be from vaginal fluid, semen or both.

Periera and Martin (1977) and others have suggested that Lewis (Le) typing may be used to identify the nonsecretor semen donor. With the typing technique employed in this study, nonsecretor women would be expected to have Le^a in their vaginal secretions. Some of the Le^a activity could be attributed to a nonsecretor semen source if the 99% vaginal threshold for Le^a was exceeded. However, few nonsecretor semen samples appear to have levels of Le^a that exceed the vaginal threshold (Kearney *et al.* 1983).

When the woman is a secretor, a different approach is used and the likelihood of identification is improved. Our study indicates that secretors and nonsecretors can be distinguished on the basis of their Le^a:Le^b ratios, with nonsecretors showing ratios exceeding 1 and secretors showing ratios less than or equal to 1. A secretor woman will be expected to have

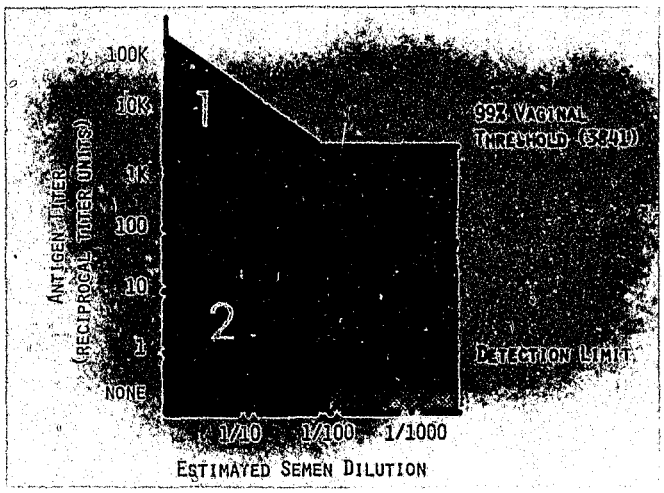

Figure 1. Zones of significance: A and B blood group substances.

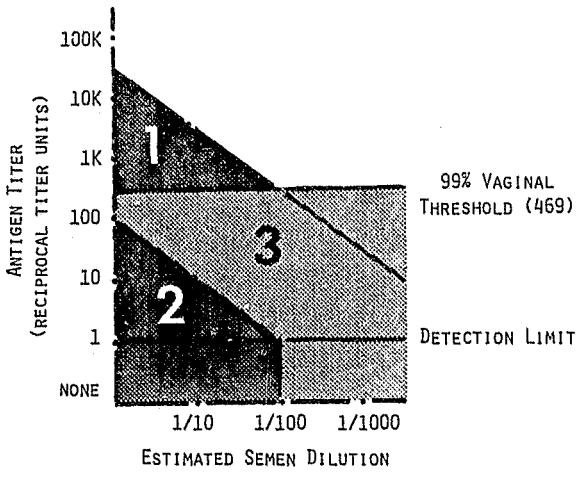

Figure 2. Zones of significance: H in O-secretors.

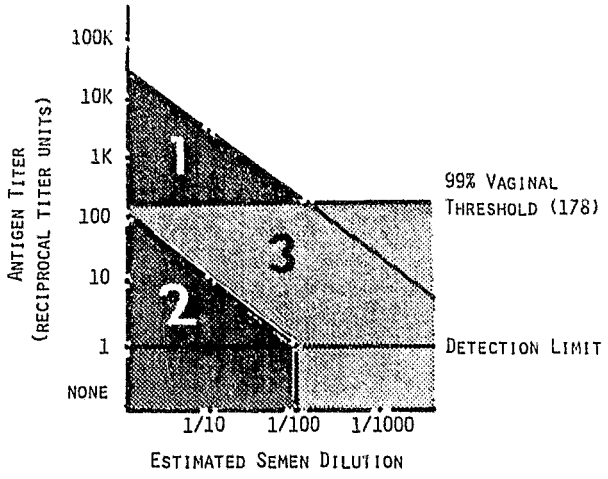

Figure 3. Zones of significance: H in non-O-secretors.

an Le^a:Le^b ratio in her vaginal secretions which is less than or equal to 1 in the absence of any semen. If the normal Le^a:Le^b ratio became greater than 1, it would suggest that the semen present was contributing preferentially to the Le^a pool, but not to the Le^b pool, and would indicate a nonsecretor semen donor.

Threshold levels can be used to analyze vaginal swabs and samples from a single drainage event, such as stains on bedding. They would not be appropriate to analyze samples that represent multiple drainage events such as stains in the crotch of undergarments where blood group substances activity may be elevated because of repeated drainage and drying. Approaches based on ratios are applicable to both types of stains, since ratios should not be influenced by concentration effects.

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LEVELS AND DISTRIBUTION OF ABH AND LEWIS BLOOD GROUP SUBSTANCES IN VAGINAL SECRETIONS

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Semen–free vaginal swab extracts were studied to quantitate the levels of secreted ABH and Lewis (Le) blood group substances and to determine how the substances are distributed. If the blood group substances are distributed according to a standard statistical distribution, such as the normal distribution, the proportion of blood group substance values falling above or below any given value can be accurately stated. Statistically characterizing the endogenous vaginal blood group substances would allow the distinction of elevated values in semen/vaginal mixtures at defined levels of significance or thresholds and would thus provide a means by which the significance of qualitatively nonforeign blood group substances might be assessed.

Extracts prepared from semen–free vaginal swabs collected from complainants in sexual assault cases were prepared by the method described by Blake *et al.* (1981). The ABH and Le substances in serial dilutions of the extracts were measured by microscopically monitored adsorption–inhibition techniques described by Gibbons (1983).

A chi–square statistical analysis of the three largest groups, Le^a and Le^b in secretors of all ABO types and H in O secretors, indicates that these substances appear to be lognormally distributed (P=0.63, 0.13 and 0.63, respectively). We expect that the lognormal distribution will apply to the other antigens for which our sample sizes are smaller. Accordingly, we have calculated the means, standard deviations and 99% threshold levels for the ABH and Le blood group substances in vaginal swab extracts (Table 1).

Significant differences in the 99% threshold levels for A and B blood group substances are expected on biochemical grounds and are probably the result of small sample sizes. Thus, we expect variation in B to be as great as variation in the A substance. Ninety–nine percent of females will be expected to have ABH and Le blood group substance activity in their vaginal

Table 1. DISTRIBUTION PARAMETERS OF ABH AND LEWIS BLOOD GROUP SUBSTANCES (reciprocal titer units)

	n	Mean	Range	99% Threshold
A	18	50.3	0–1024	3841
B	14	23.7	0–256	1368
H (non–O–secretors)	21	5.0	0–50	178
H (O–secretors)	29	9.5	0–512	469
SECRETORS				
Le^a	29	10.1	0–1024	682
Le^b	37	42.3	0–1024	6522
NONSECRETORS				
Le^a	9	17.2	1–256	1009

secretions at levels less than the 99% threshold. Unusually high levels of qualitatively nonforeign ABH activity in vaginal swab extracts (those exceeding the 99% threshold) would indicate that semen is contributing a portion of the blood group substances detected and would further reduce the population of potential semen donors.

In vaginal swab extracts from A and B secretors, the levels of A or B substance (the nominal antigen) usually exceeded greatly the level of H, producing A:H and B:H ratios that were frequently significantly greater than 1. Vaginal secretions differed in this regard from semen, which generally does not exhibit large quantitative differences in the levels of the nominal and H antigens (Sensabaugh *et al.* 1980). Thus, ratios might be useful for distinguishing between vaginal secretions and semen as to the source of nonforeign antigen in mixtures.

The patterns of Le activity detected by the adsorption–inhibition method used in this study differ from classical patterns of expression for several of the Le types and have been described previously (Gibbons 1983). Secretors can be distinguished from nonsecretors on the basis of their Le^a:Le^b ratio. Secretors, regardless of their Le blood type, produced Le^a:Le^b ratios in their secretions which were generally less than or equal to 1. Nonsecretors, regardless of Le blood type, were characterized by Le^a:Le^b ratios greater than 1 (Table 2). This finding can be applied to the analysis of semen/vaginal mixtures and provides a potential mechanism by which the nonsecretor semen donor can be independently confirmed.

The threshold levels described are operationally defined by the specific adsorption–inhibition technique used in this study. They would not be applicable to other inhibition methods that differ in sensitivity. The general ratio relationships between blood group substances levels would be expected to apply regardless of the procedure used in testing.

Table 2. Le^a/Le^b RATIOS IN VAGINAL SWAB EXTRACTS (reciprocal titer units)

Secretors (n=43)			Nonsecretors (n=9)		
Mean	Standard Deviation	Range[a]	Mean	Standard Deviation	Range
0.45	1.22	0–8	16.1	11.5	1–32

[a]97% of samples from secretors showed ratios that were less than 1.

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INTERPRETATION OF SEROLOGIC RESULTS

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Bloodstains may be typed for ABO by detecting the isoagglutin in serum or by detecting the isoagglutinogen on red blood cells. At present, serologists commonly use four different methods to detect ABH antigens of serologic evidence: Lattes Crust, adsorption–elution, adsorption–inhibition and mixed agglutination. These procedures are relatively standard and straightforward and, with proper controls, a reproducible result can be obtained. However, the interpretation of these serologic grouping results is still not totally standardized.

The interpretation of the results of Lattes Crust, adsorption–elution, adsorption–inhibition and mixed agglutination tests is incomplete and oversimplified in much of the literature. Typically, it is suggested that an ABO group may be inferred based on the pattern of agglutination with test cells (Table 1).

In reality, interpretation is more complex, and the data depicted in Table 1 are incomplete and oversimplified in representing the possibilities for interpretation. Interpretation of results using oversimplified schemes could lead to incorrect conclusions. Tables 2–5 represent a more complete picture of the interpretive possibilities in Lattes Crust, adsorption–inhibition, adsorption–elution and mixed agglutination procedures.

Table 1. INTERPRETATION OF LATTES CRUST, ADSORPTION-ELUTION, ADSORPTION-AGGLUTINATION AND MIXED AGGLUTINATION TESTS

LATTES CRUST PROCEDURE

Stain Extract Agglutination of:	Implies Bloodgroup
A Cells	B
B Cells	A
A Cells and B Cells	O
None	AB

ADSORPTION-ELUTION PROCEDURE

Eluate Agglutination of:		Implies Bloodgroup
A Cells	B Cells	
+	–	A
–	+	B
+	+	AB
–	–	O

ADSORPTION-INHIBITION

Inhibition with:			Implies Blood Type
Anti-A	Anti-B	Anti-H	
+	–	+	A Secretor
–	+	+	B Secretor
+	+	+	AB Secretor
–	–	+	O Secretor
–	–	–	Non-Secretor

MIXED AGGLUTINATION

Eluate Agglutination with:			Implied Blood Type
αA/A	αB/B	αH/0	
+	–	+/–	A
–	+	+/–	B
+	+	+/–	AB
–	–	+	O

Table 2. LATTES CRUST METHOD

Reaction with RBC	Implies Blood Type
A	B
B	A
A and B	O, A + B
None	AB, INC

Table 3. INHIBITION TESTS WITH BODY FLUID STAINS

Inhibition With			Observed Result	
Anti-A	Anti-B	Anti-H	(BGS Detected)	Interpretation[a]
yes	no	no	A	A secretor with undetected H; foregoing + nonsecretor
yes	no	yes	A + H	A secretor; A secretor + O secretor; foregoing + nonsecretor
no	yes	no	B	B secretor with undetected H; foregoing + nonsecretor
no	yes	yes	B + H	B secretor; B secretor + O secretor; foregoing + nonsecretor
no	no	yes	H	O secretor; O secretor + nonsecretor
yes	yes	no	A + B	AB secretor with undetected H; A secretor + B secretor with undetected H; foregoing + nonsecretor; other mixtures
yes	yes	yes	A + B + H	AB secretor; many mixture combinations
no	no	no	negative	nonsecretor; any secretor with undetected BGS; other mixtures of foregoing

[a]Adventitious or "acquired" BGS not considered in Interpretation column of table

Table 4. ELUTION TESTS ON BLOODSTAINS

Agglutination With			Observed Result	
A Cells	B Cells	O Cells	(Antigen Detected)	Interpretation[a]
+	–	–	A	A blood
+	–	+	A + H	A blood; A + O blood; A bl + O bfl; O bl + A bfl; A bl + A bfl
–	+	–	B	B blood
–	+	+	B + H	B blood; B + O bl; B bl + O bfl; O bl + B bfl; B bl + B bfl
–	–	+	H	O blood; O bl + O bfl
+	+	–	A + B	AB blood; A bl + B bl; AB bl + A and/or B bl; A and/or B from bfl without detection of H
+	+	+	A + B + H	AB blood; AB bl + any other bl or bfl mixture
–	–	–	negative	inconclusive
+	–	–	A	A
+	–	+	A + H	A or A + O
–	+	–	B	B
–	+	+	B + H	B or B + O
–	–	+	H	O
+	+	–	A + B	AB
+	+	+	A + B + H	AB or mixtures
–	–	–	negative	inconclusive

[a]bl = blood; bfl = body fluid; adventitious or "acquired" antigens not considered in Interpretation column of table

Table 5. AGGLUTINATION TESTS WITH BODY FLUIDS

Inhibition Of			Observed Result	
Anti-A	Anti-B	Anti-H	(BGS Detected)	Interpretation
yes	no	no	A	A secretor fluid
yes	no	yes	A + H	A secretor fluid
no	yes	no	B	B secretor fluid
no	yes	yes	B + H	B secretor fluid
no	no	yes	H	O secretor fluid
yes	yes	no	A + B	AB secretor fluid
yes	yes	yes	A + B + H	AB secretor fluid
no	no	no	negative	nonsecretor body fluid

APPLICATION OF THE IMMUNOHISTOCHEMICAL TECHNIQUE TO THE ABO BLOOD GROUPING OF HUMAN HEAD HAIR

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The examination of ABO blood groups of hair offers a useful criterion for establishing its personal origin. Japanese police laboratories have used the adsorption–elution technique to examine the ABO blood grouping in single hair strands, which can be used for blood grouping of a hair sample more than 3 cm long. The present study was undertaken to develop a new method for examining the ABO blood grouping in a hair sample less than 3 cm long. The unlabeled antibody immunoperoxidase (PAP) technique was applied, and the efficiency of this technique in demonstrating blood group substances in hair was investigated.

Hair samples were collected from healthy adult donors of blood group A, B, AB and O, respectively, and subjected to longitudinal or cross-sectioning for the immunostaining. Furthermore, scalp skin sections were used to investigate the immunohistochemical localization of blood group substances in hair roots.

The immunostaining procedure used in the present study, summarized in Table 1, is based on the PAP method by Sternberger *et al.* (1970) which uses three kinds of antibodies. The primary antibodies used were anti–A (diluted 1:2000) and anti–B (diluted 1:1500) sera produced in rabbits immunized with human red blood cells. To demonstrate blood group substances at the electron microscope level, two kinds of electron immunocytochemical approaches (that is pre– and postembedding PAP techniques) were used with hair shafts and roots. To evaluate the specificity of the immunostaining, the following three control experiments were performed:

1. Adsorption of the primary antibody (anti–A or anti–B) with the corresponding antigen (erythrocytes of group A or B).
2. Omission of the primary antibodies.
3. Incubation of the sections with diaminobutyric acid (DAB) solution alone. No positive staining was revealed in any control experiments.

With the present technique, the blood groups of hair samples were correctly determined. The group–specific stainings, which are revealed as dark brown precipitates under a light microscope, were clearly observed within the medullae of hair shaft regardless of secretor status of hair donors (Figure 1). For black and brown hair samples, positive stainings on medullae could be clearly identified by mildly treating samples in the bleaching reagent (10% hydrogen peroxide solution, pH 9.5). Furthermore, the electron immunocytochemical approach enabled us to

Table 1. IMMUNOSTAINING PROCEDURE FOR THE LIGHT MICROSCOPICAL DEMONSTRATION OF BLOOD GROUP SUBSTANCES IN HUMAN HAIR

1	Rinse in PBS (0.02 M phosphate buffer containing 0.9% NaCl, pH 7.4)	5 min
2	Treatment with 2.0% BSA in PBS	60 min
3	Rinse in PBS	5 min
4	IMMUNOSTAINING	
	i) Primary antisera, 4° C Rabbit anti-A (1:2000) Rabbit anti-B (1:1500)	12 h[a]
	ii) Rinse in cold PBS	20 min
	iii) Goat anti-rabbit IgG (1:150), room temp.	30 min[a]
	iv) Rinse in cold PBS	10 min
	v) PAP complex (1:100), room temp.	30 min[a]
	vi) Rinse in cold PBS	10 min
5	Incubation with 0.003% DAB and 0.002% H_2O_2 in 0.05 M Tris-HCl buffer (pH 7.6)	20 min
6	Rinse in distilled water	
7	Post fix with OsO_4 vapor	
8	Dehydrate, clear and mount slides	

[a]Immunoreaction was carried out in moist chamber.

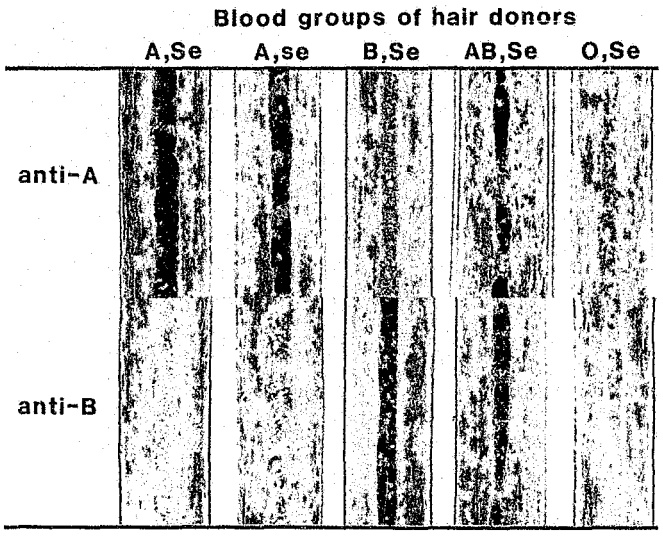

Figure. 1. Longitudinal sections of bleached hair samples immunostained with anti-A and anti-B sera. Respective blood groups of hair samples were successfully determined depending on the presence or absence of positive stainings on hair medullae regardless of secretor status of hair donors; Se:secretor, se:nonsecretor.

demonstrate blood group substances in the cortex of hair shaft which could not be detected by the light microscopic immunostaining. These immunoreaction products, revealed as electron-opaque DAB deposits, were mainly localized in the intermacrofibrillar matrix in the cortex. At the hair root, the ABH blood group substances could be successfully detected within the cytoplasm of medullary cells and cortical cells by both light and electron immunohistochemical techniques. Especially in the keratogenous zone, the positive staining of cortical cell cytoplasm was very conspicuous. These findings suggest that these antigenic substances were intrinsically produced in both medullary cells and cortical cells in hair roots.

The present technique has enabled us to determine ABO blood groups from minute hair samples that cannot be examined with the conventional adsorption-elution technique. This immunohistochemical technique can be useful for the ABO blood grouping of a fragment of medullated hair sample.

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USE OF AN ENZYME LINKED IMMUNOSORBENT ASSAY FOR THE DETECTION OF P30 IN QUESTIONED SEMEN STAINS

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Many laboratories rely on tests for P30 to identify semen stains when microscopic searches for spermatozoa have failed. Several test procedures for P30 have been described (Baechtel 1985; Graves *et al.* 1985) which use polyclonal antisera to detect this protein. The commercial availability of a monoclonal antibody (MCA) has facilitated the development of an enzyme linked immunosorbent assay (ELISA) method for the detection of P30 in stain extracts which avoids the inherent shortcomings of polyclonal antisera reagents. This study describes the results of using ELISA to test more than 2,500 case–evidence stains.

MATERIALS AND METHODS

The P30 ELISA was configured as a capture assay in which immobilized MCA to P30 (Boehringer Mannheim Biochemicals, Indianapolis, IN) was used to bind soluble P30 from questioned stain extracts. Bound P30 was detected after the sequential addition of polyclonal antiserum to P30 and enzyme conjugated antimouse IgG. The assay was standardized against purified P30 and could measure P30 in the nanogram per milliliter range.

The P30 concentration in 2,665 questioned stains was determined using ELISA. From data on the distribution of P30 (Brown *et al.* 1983) and the soluble blood group antigens (Baechtel 1985) in semen, an algorithm was developed that could be used to estimate the likelihood of detecting blood group antigens in stain extracts according to their P30 concentrations. The P30 ELISA result for each extract was compared with the examination results for semen presence and blood group antigens present as carried out by the Federal Bureau of Investigation Laboratory Serology Unit (SU).

RESULTS AND DISCUSSION

There were 459 stains shown to contain semen by the current methods employed in the SU. The P30 ELISA detected P30 in 450 of these stains. By contrast, of the 2,206 stains in which semen was not identified by the SU, the P30 ELISA detected P30 in 289 stains. Of these 289 stains, 78% contained P30 at concentrations beneath the detection limit of the radial immunodiffusion method used currently in the SU for P30 assay. These results suggest that the P30 ELISA is a reliable and sensitive method for the detection of P30 in questioned stains.

The likelihood of detecting blood group antigens in a stain extract that was foreign to the victim appeared to be independent of the P30 concentration of the extract. However, the number of specimens that possessed low concentrations of P30 were predicted to be ungroupable. Similarly, the number of specimens for which ABH grouping results were available was too small to permit decisive conclusions to be drawn concerning the accuracy of the developed algorithm. Thus, additional study will be required to establish this point with certainty.

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PURIFICATION OF SEMINAL VESICLE SPECIFIC ANTIGEN BY IMMUNOAFFINITY CHROMATOGRAPHY ON BOUND MONOCLONAL ANTIBODY MHS–5

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Seminal vesicle specific antigen (SVSA) is a protein secreted by the seminal vesicle epithelium into the lumen of the seminal vesicle (Evans and Herr 1986). Immunocytochemistry had shown that the predominant cell type within the seminal vesicle epithelium, the principal cell, is the source of this antigen. At the time of ejaculation, seminal vesicle fluid mixes with prostatic secretion. Together, the secretions of these glands are composed of the bulk of the fluid components of semen and interact to form a soft coagulum, which normally liquefies within 20 minutes of ejaculation (Tauber *et al.* 1980). From 50% to 80% of the volume of an average human ejaculate comprises fluids originating in the seminal vesicles (Table 1, after Mann and Lutwak–Mann 1981).

A monoclonal antibody (MCA), mouse anti-human semen–5 (MHS–5), has been produced to the SVSA protein. This MCA recognizes a conserved epitope present in all humans thus far tested (more than 500). This conservation of structure among individuals has led to the suggestion that assay systems employing the MHS–5 monoclonal probe would be appropriate for semen identification (Herr *et al.* 1986).

Monoclonal antibody MHS–5 has shown no cross-reactivity with any other human body fluid or with semen from common domestic animals and monkeys. However, close human relatives, chimpanzees, orangutans and gorillas (Pongids), also secrete seminal proteins bearing the SVSA epitope (Herr *et al.* 1986). This crossreactivity with semen from Pongids should not detract from the usefulness of the MCA in forensic science or clinical chemistry applications for identifying semen.

The SVSA in semen exhibits polymorphism on reduced sodium dodecyl sulfate (SDS) gels when analyzed by Western blots (Figure 1). The antigen also undergoes changes in mass during semen liquefaction (McGee and Herr 1986). When ejaculates are collected directly into a buffer containing SDS to inhibit enzymes involved in liquefaction, two major antigenic seminal proteins of 69–71 and 58 kilodaltons (kd) are recognized by the MHS–5 MCA Y. In addition, several antigenic peptides of lower molecular weight are recognized. As early as 5 minutes after ejaculation, when liquefaction is allowed to proceed at room temperature, the two major antigenic proteins of 69–71 and 58 kd are no longer detected and new

Table 1. CONTRIBUTIONS OF ACCESSORY SEX GLANDS TO EJACULATE VOLUME[a]

Epididymis	5%–15%
Prostate	15%–30%
Seminal vesicles	50%–80%

[a]After Mann and Lutwak-Mann 1981.

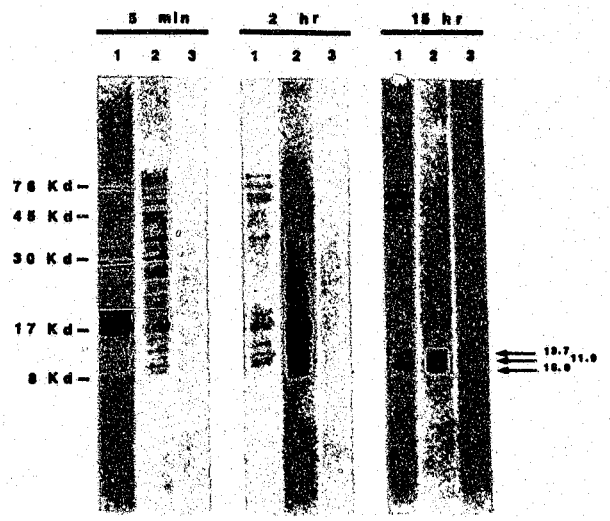

Figure 1. Immunoblot identification of polypeptides reacting with monoclonal antibody (MCA) MHS–5 during the course of semen liquefaction. The nitrocellulose strips contained proteins electrophoresed after 5 minutes, 2 hours or 15 hours of liquefaction. Lanes marked 2 were incubated in MCA MHS–5. Seminal proteins stained with amido black for total protein are present in lanes marked 1. Control lanes (3) were incubated with another IgG1 MCA at the same concentration as MHS–5. The seminal vesicle specific antigen identified with MCA MHS–5 exhibits considerable polymorphism in the fresh ejaculate (5 minutes postejaculation) and undergoes transformation in molecular weight with liquefaction, resulting in a triplet of immunoreactive peptides with molecular masses ranging from 10 to 13.9 kd after 15 hours of liquefaction.

immunoreactive peptides below 15 kd appear. This finding suggests that major immunoreactive proteins at 69–71 kd and 58 kd are converted by proteolysis to lower molecular weight peptides within minutes of ejaculation (McGee and Herr 1986).

After longer periods of liquefaction, there is a general loss of immunoreactivity in higher molecular weight peptides and a persistence of immunoreactivity in low molecular weight peptides. For example, after 16 hours of semen liquefaction at room temperature, three peptides of 10, 11.9 and 13.7 kd contain the preponderance of immunoreactivity (Figure 1).

Molecular weight transformations in the mass of the SVSA that occur during liquefaction have made purification of seminal vesicle specific antigen difficult. Our current efforts at isolating antigenic material from liquefied semen use samples liquefied for 15–24 hours, and they are designed to recover lower molecular weight antigenic peptides that arise during the liquefaction process. The method employed is immunoaffinity chromatography on bound MCA MHS–5.

METHODS AND RESULTS

Monoclonal antibody MHS–5 was purified on a Protein–A sepharose column, and SDS–polyacrylamide gel (PAGE) demonstrated single heavy and light chain immunoglobulin bands, indicating a high degree of antibody purity (data not shown). Twenty–five milligrams of the purified MCA was conjugated to a column of Affi–Gel 10 (4 ml final volume). Twenty–five milligrams of seminal fluid protein was cycled over the column four times at a flow rate of 1 ml/min. Elution of the MHS–5 antigen was initiated in 0.1 M glycine buffered saline, pH 3.0. Fractions from four column runs were pooled, dialyzed against ammonium carbonate for 72 hours and lyophilized. Antigen from the first purification was then reconstituted in 0.1 M Hepes buffered saline and recycled on the column for a second purification.

Fractions were collected from the second column and evaluated by enzyme linked immunosorbent assay (ELISA) for enrichment of immunoreactive antigen and analyzed for purity by SDS–PAGE. Coomassie blue–stained gels revealed eluted material predominantly in the 10–13 kd range, with a band of 76 kd also present (lane G, Figure 2). The 76 kd band comigrated with purified lactoferrin but did not immunoreact with MHS–5 on western blots, although the 10–13 kd material recovered from the column was strongly immunoreactive on western blots (lane H,

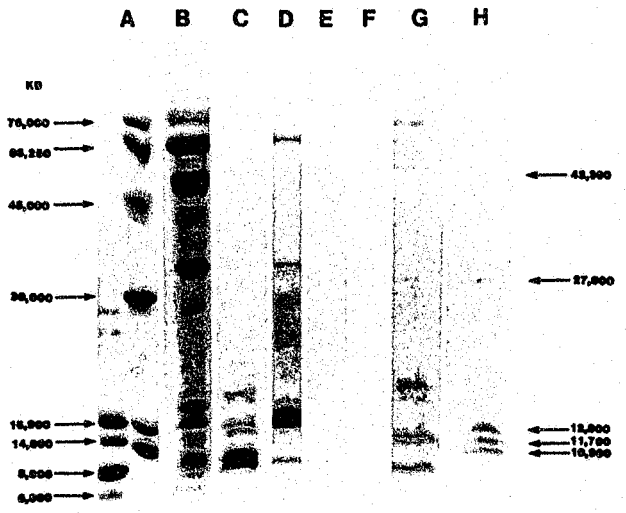

Figure 2. Results from a purification protocol for seminal vesicle specific antigen using an immunoaffinity column constructed with bound MCA MHS–5. The two lanes under A are amido black stained low and high molecular weight standards. Lane B shows the proteins (stained by amido black) present in seminal fluid (liquefied for 10 hours) used as the starting sample for the purification procedure. Lane C in an immunoblot of lane B after staining with MCA MHS–5. This lane shows the low molecular weight immunoreactive species, typical of seminal vesicle specific antigen, present after 10 hours of liquefaction. Lane D contains an amido black stain of the material that passed through the affinity column after two cycles. Next to this lane, in lane E is an immunoblot of lane D, showing that no immunoreactive material is leaving the affinity column. In lane F the secondary antibody peroxidase conjugate is tested in the absence of MCA for nonspecific reactivity with seminal proteins. This lane is negative for any immunoreactivity. Lane G contains the amido black stain of the proteins eluted from the immunoaffinity column. When lane G is compared with the starting material in lane B, considerable reduction in the complexity of the proteins present in seminal fluid is observed. Lane H is an immunoblot of the recovered antigen. The predominant triplet of low molecular weight antigens is present as well as an immunoreactive band at 27 kd (possibly a dimerized form of the 12,800 band).

Figure 2) (Towbin *et al.* 1979). A immunoreactive band at 27 kd was also recovered from the column. These findings indicate that affinity chromatography on bound MCA MHS–5 is significantly enriching for seminal vesicle specific antigen (compare lanes G and B, Figure 2). The presence of the 76 kd band, which is not immunoreactive, suggests that further steps of molecular exclusion are necessary to provide a preparation pure enough for amino acid analysis.

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ELISA ASSAY FOR HUMAN SEMEN IDENTIFICATION USING A MONOCLONAL ANTIBODY TO A SEMINAL VESICLE SPECIFIC ANTIGEN

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In an enzyme linked immunosorbent assay (ELISA), experimental samples were coated on microtiter plates and probed with the biotinylated form of the mouse antihuman semen–5 (MHS–5) monoclonal antibody (MCA) and a streptavidin–horseradish peroxidase (HRP) complex. A complete description of this assay method can be found in Herr and Woodward (1987).

MATERIALS AND METHODS

Monoclonal antibody MHS–5 was produced as an ascites tumor in BALB/c mice and purified from the ascites fluid with 50% saturated ammonium sulfate. Following two cycles of precipitation, the precipitate was resuspended on 0.01 M sodium phosphate (pH 6.8) and the suspension applied to a Bio–gel P6–DG column (Bio–Rad, Rockville Centre, NY). Fractions containing protein from this column were pooled and applied to a hydroxyapatite column according to the methods of Stanker and coworkers (Stanker *et al.* 1985). The eluted proteins were analyzed for purity on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) stained with Coomassie blue.

Approximately 2 mg of MHS–5 were biotinylated with *N*–biotinyl–ω–aminocaproic acid–*N*–hydroxysuccinimide ester (ENZOTIN, Enzobiochem, NY) at a protein to ENZOTIN ratio of 1:22 (Hofmann *et al.* 1982). The stoichiometry of biotin to antibody in the conjugate was not determined.

The newly biotinylated antibody probe was employed on immunoblots of liquefied semen to determine if the seminal vesicle specific antigen (SVSA) was recognized. Immunoblot analysis was done with 15% polyacrylamide gels. Low molecular weight standards (Sigma and LKB) were used for molecular weight estimation. After electrophoresis, proteins were transferred to nitrocellulose (100 mA for 103 hours at room temperature) using 25 mM TRIS–192 mM glycine containing 10% methanol (Towbin *et al.* 1979). Unoccupied sites on the nitrocellulose were blocked by incubating the blots in 1% bovine serum albumin (BSA)–50 mM TRIS–150 mM sodium chloride for a minimum of 1 hour. Subsequently, the blots were washed twice in 0.05% TWEEN 20–50 mM TRIS–NaI (TTN) and then incubated with a soluble biotinylated MHS–5–streptavidin–HRP complex for 1 hour at room temperature. After five rapid washes with TTN, bound MHS–5 was localized with 1 mM diaminobenzidine–100 mM TRIS (pH 7.0)–0.01% H_20_0.

The basic steps in the ELISA are presented in Figure 1. Immulon II plates were coated with seminal fluid, forensic samples or tissue homogenates for 1 hour at room temperature. Then 100 μl sample volumes were applied per well. Protein concentration was determined by the method of Bradford (1976), and samples were placed in the wells at various protein concentrations ranging from 5000 to 0.005 μg/ml. Plates were then emptied, filled with TTN plus 0.1% BSA and incubated at room temperature for 10 minutes. Next freshly prepared biotinyl MHS–5–streptavidin–HRP was added and the plates incubated for 30 minutes. After five washes, the presence of bound MCA–biotin–avidin–peroxidase complex was detected with 1 mM ABTS–citrate–phosphate buffer–0.03% H_20_2. Plates were read in an automated plate reader (Multiskan, Flow Laboratories, McLean, VA) after 30 minutes and then photographed.

RESULTS

The MHS–5 antibody was purified with ammonium sulfate precipitation and hydroxyapatite chromatography. The protocol yielded approximately 2 g of antibody from 180 ml of ascites fluid. The SDS–PAGE of the purified immunoglobulin showed single heavy and light chain bands indicative of a high degree of purity (Figure 2).

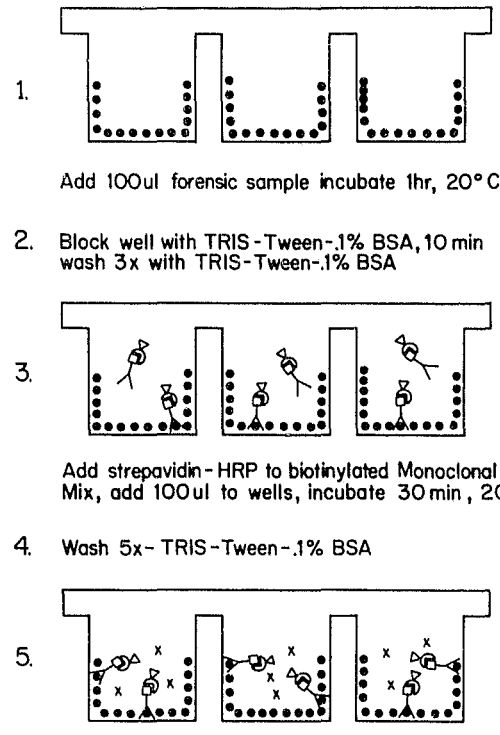

Figure 1. Summary diagram of the ELISA protocol based on monoclonal antibody MHS–5 and streptavidin–HRP added as a single complex.

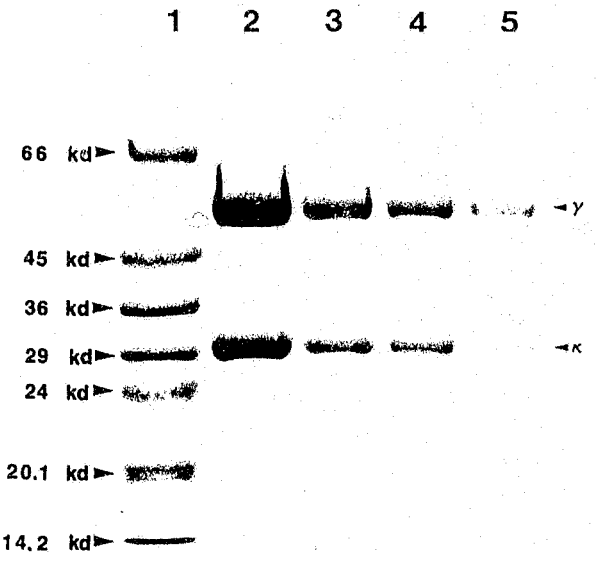

Figure 2. SDS–PAGE of various concentrations (10 μg/lane –1.2 ug/lane) of purified MHS–5 antibody. Heavy and light chains are seen free of contaminants. Lane 1 contains m.w. standards.

Previous evidence (Herr *et al.* 1986) has indicated that the epitope recognized by MHS–5 occurs on many proteins in nonliquefied seminal fluid but that liquefaction considerably reduces this complexity to peptides with molecular weights from 11 to 13 kilodaltons (kd). Immunoblots of liquified semen, when reacted with the biotinylated probe, exhibited a complex pattern of bands between 10,500 and 20,000 daltons with strongly reactive bands of apparent molecular weights of 10,500, 11,500 and 13,500 daltons (Figure 3).

In ELISA, both the primary biotinylated MCA and the secondary streptavidin–peroxidase were mixed together as a single step reagent being added to the sample. This procedure reduced assay time by eliminating a step that would be necessary if reagents were added as single analytes.

The biotinyl MHS–5–streptavidin HRP complex detected as little as 10 ng of seminal fluid protein per well (Figure 4). This is a maximal estimate of antigen, since we have not determined what percentage of the 10 ng of seminal fluid protein added to the assay well is actually bound to the plastic plate. The optimal concentration of MHS–5 in this assay appears to be between 0.25 and 0.5 μg/ml (Figure 5) when 0.25 μg of streptavidin–HRP is used. This optimum value is in good agreement with the optimum observed by Herr *et al.* The decrease in signal above 0.5 μg/ml (a prozone–like effect) is likely due to subsaturating levels of streptavidin–HRP in the system. Negative control wells coated with 7 μg/ml of human serum consistently give optical densities less than 0.05. It has been our experience that an optical density greater than 0.100 exceeds the mean negative control optical density plus three standard deviations.

If this assay is to be useable in forensic analysis, it should have minimal nonspecific interactions with human tissues or fluids other than semen. Table 1 lists the various human tissues and fluids tested with this assay system. A range of antigen concentration was employed in these crossreactivity tests to ensure that there was no artifactual blocking of plate binding. No nonspecific reactivity was observed with any tissue tested, even after several hours of color development.

DISCUSSION

The ELISA described in this report is rapid and inexpensive, and it shows specificity for human seminal fluid and lack of crossreactivity with other biologic

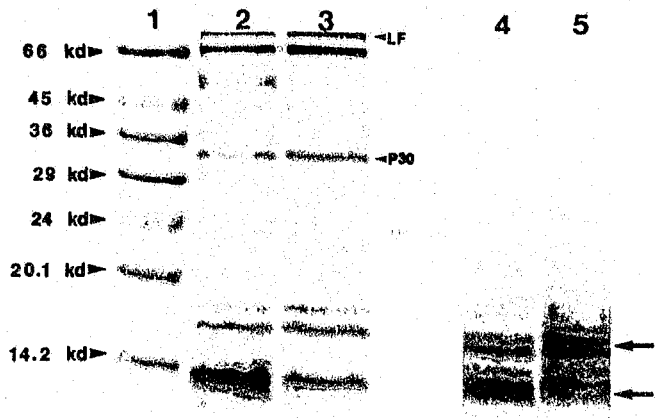

Figure 3. Immunoblot of liquefied seminal fluid using biotinyl MHS-5-streptavidin-HRP complex. Seminal fluid samples were allowed to liquefy for either 2 or 24 hours at room temperature after which they were reduced in 1% SDS-5% b-mercaptoethanol by heating at 95° C for 2 minutes. Approximately 90 μg of seminal fluid proteins per well were separated on a 15% polyacrylamide gel and either stained with Coomassie blue (a) or transferred to nitrocellulose and visualized with biotinyl-MHS-5-streptavidin-HRP (b). Lane 1 shows low molecular weight standards (66 kd, 45 kd, 36 kd, 29 kd, 24 kd, 20.1 kd and 14.2 kd). Lanes 2 and 3 are Coomassie stained seminal fluid proteins following the 2 and 24 hours of liquefaction respectively. Seminal fluid following 2 or 24 hours of liquefaction (lanes 4 and 5 respectively) was immunoblotted. The blot was probed with (0.4 ug/ml biotinyl MHS-5-0.25 μg/ml streptavidin-HRP- and bound MHS-5-streptavidin-HRP was detected with 1 mM diaminobenzidine. The major immunoreactive peptides are indicated at the arrowheads. LF = lactoferrin; P30 = prostate-specific antigen.

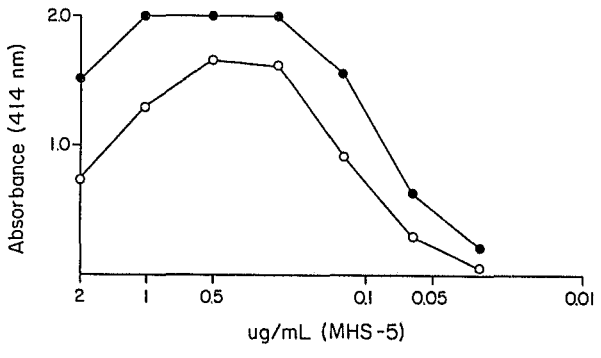

Figure 5. Determination of optimal MHS-5 concentration in the assay system: Twofold dilutions of biotinyl MHS-5 (starting concentration 2 μg/ml) were assayed on an Immulon II plate coated with 100 microliters of seminal fluid over a wide concentration range (2-0.01 μg/ml). Values for 0.88 and 0.44 μg/ml of seminal fluid are plotted.

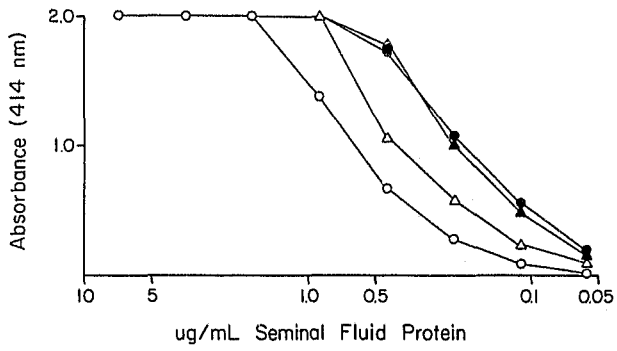

Figure 4. Sensitivity of the biotinylated MHS-5-streptavidin-HRP based ELISA: The binding of the biotinylated MHS-5 streptavidin-HRP complex to adsorbed seminal fluid proteins was titrated. An Immulon II plate was coated with twofold dilutions of seminal fluid proteins (starting concentration 7 μg/ml) and then probed with 4 concentrations of the biotinylated antibody. In this experiment, the concentration of biotinylated antibody was varied between 2 and 0.03 μg/ml and the streptavidin-HRP concentrations held constant 0.25 μg/ml. Four concentrations of the biotinylated antibody are plotted: 2, 1, 0.5 and 0.25 μg/ml.

Wells lacking antigen served as reagent blanks. These values were subtracted from the experimental points. These control wells had optical densities less than 0.05.

Table 1. FLUIDS AND TISSUES TESTED FOR MHS-5 BINDING

	Range Tested μg/well	Result
Swine serum	400–0.13	–
Orangutan serum	450–0.14	–
Gorilla serum	450–0.14	–
Chimpanzee serum	450–0.14	–
Human serum	350–0.11	–
Human liver	50–0.016	–
Human pancreas	250–0.08	–
Human kidney	500–0.16	–
Human milk	200–0.065	–
Human vaginal secretion[a]		
(2D)	1600–0.5	–
(3D)	2000–0.65	–
(5D)	2500–0.8	–
Seminal fluid	0.7–0.01	***

[a]2D, 3D and 5D refer to days elapsed since last coital contact.

fluids. Because the MHS-5 MCA has been shown to react with peptide antigens secreted by the seminal vesicle epithelium (Herr *et al.* 1986), it is a useful probe for detecting vasectomized or azoospermic seminal fluids.

Use of the biotinylated MCA streptavidin-HRP complex on western blot analysis of seminal fluid peptides after 24 hours of semen liquefaction demonstrated major immunoreactive bands in the 10–13 kd

range. These findings are comparable to the results previously reported using a secondary antibody–HRP conjugate (Herr *et al.* 1986), and they verify that the specificity of the antibody is retained after the biotinylation procedure. Herr and coworkers reported the dominant immunoreactive peptides to be 10, 11.9 and 13.7 kd in semen liquefluid for 15 hours. The calculations made in this study, which give 10.5, 11.5 and 13.5 kd, are within the expected range of variability in molecular weight determinations from SDS–PAGE.

In its current format, the assay can be performed in approximately 2 hours. We have observed that the plate coating step using semen samples or forensic evidence can be reduced to 30 minutes without substantial loss of sensitivity. Our preliminary experiments with extracted forensic specimens and laboratory generated semen stains indicate that positive signals generally develop within 5–10 minutes, with optical densities of 2 (the maximum reading on the spectrophotometer) being reached in 15 minutes. The assay we have developed can detect the equivalent of 1 ml of seminal fluid protein diluted in 200 liters.

The method we employ involves adding streptavidin–HRP to the MCA in a mixing step adding it to the unknown sample. The streptavidin–HRP is added to the antibody at concentrations below a 1:1 ratio. The dissociation constant for the avidin–biotin complex is of the order of 5 M. Because of this high dissociation constant and the use of subsaturating amounts of streptavidin–HRP/MCA–biotin, we assume that little free streptavidin–HRP is available in the system to give false positive results on endogenous biotin in biologic samples. Together these findings indicate that this ELISA, based on a biotinylated MCA to a seminal vesicle–specific antigen, may be useful for semen identification.

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ENZYME IMMUNOASSAY FOR M ANTIGEN IN BLOODSTAINS

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The MN typing of bloodstains has great potential in forensic science. The adsorption-elution method can now be used to detect the presence of M antigen, but no weight can be attached to the failure to detect M. The enzyme immunoassay (EIA) method offers another way to detect the MN blood group antigens. We here investigated a monoclonal based EIA method that distinguishes M positive (M and MN) bloodstains from M negative (N) bloodstains. The EIA method is also used to develop a quantitative test that allows MN typing to be more objective. The EIA also allows quantitative studies to be made of marker loss under different storage conditions.

MATERIALS AND METHODS

Bloodstains are washed free of water soluble material and then extracted with *n*-butanol selectively to remove glycophorins, the carrier molecules for the MN determinants. The lower phases of the butanol extracts are diluted in pH 9.6 carbonate buffer and coated in the wells of a microtiter plate. Replicate wells are probed with anti-M monoclonal, anti-glycophorin A (GpA) monoclonal and nonimmune mouse immunoglobulin. The monoclonal antibodies were detected with an antimouse enzyme conjugated second antibody.

RESULTS

Strong reactions with both anti-M and anti-GpA indicate an M positive reaction. A stain was called M positive if the anti-M/Ig ratio was greater than 3.0. A strong reaction with anti-GpA but not with anti-M indicates a true M negative reaction. A stain was called M negative if the anti-GpA/Ig ratio was greater than 3.0 and the anti-M/anti-GpA ratio was less than 0.25. Weak or negative reactions with both anti-M and anti-GpA are considered to be indeterminant. We did not observe a strong reaction with anti-M coupled with a weak or negative reaction with anti-GpA. The binding of nonimmune mouse IgG provides a measure of background nonspecific binding. Stains of as little as 5 μl of blood have been successfully extracted, although the frequency of typeable results increases with stain volume. Of the over 100 stains that were typed blind (Tables 1 and 2), no incorrect calls were made, and only 13 stains were not able to be called. The parameters of M antigen decay have also been characterized. Many of the stains stored at room temperature are not typeable beyond 1 week's time, whereas most of the stains stored frozen could be typed after 4 weeks.

DISCUSSION

This EIA using butanol extracts has been successful only with monoclonal anti-M. Polyclonal anti-M and anti-N and monoclonal anti-N exhibit either unacceptable levels of background binding or do not bind significantly. However, all antibodies tested bind to detergent extracted glycophorin, and an EIA employing detergent extracted stain material is under development. We have been able to distinguish between M positive stains and true M negative stains by using this EIA. We have also been able to tentatively distinguish between the three MN types based on the value obtained for the ratio anti-M/anti-GpA. This idea needs to be explored further, as our study was limited to only 104 stains.

Table 1. BLIND TRIAL RESULTS[a]

		M+	M–	No call
Set 1	M (19)	16	0	0
	MN (22)	19	0	3
	N (6)	0	4	2
Set 2	M (9)	9	0	0
	MN (25)	21	0	4
	N (23)	0	22	1
Total	M (28)	25	0	3
	MN (47)	40	0	7
	N (29)	0	26	3

[a]M positive or M negative calls based on results of enzyme immunoassay blind trials on two sets of bloodstains.

Table 2. MN BLIND TRIAL – RATIOS[a]

Stain type	Anti-M/Ig	Anti-Gp/Ig	Anti-M/Anti-Gp
M	7.5–145	1.7–74	1.3–3.6
MN	3.0–59	2.5–46	0.7–2.6
N	0.3–1.5	3.1–19.4	0.03–0.23
No Call	0–2.7	0–2.9	0–1.75

[a]Enzyme immunoassay ratios for MN stain types.

DETERMINATION OF ABO BLOOD GROUPS FROM HUMAN BLOODSTAINS AND BODY FLUID STAINS WITH MOUSE MONOCLONAL ANTIBODIES

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This study elucidates the serologic characteristics of mouse monoclonal antibodies (MCAs) against chemically synthetic A, B and H antigens and evaluates the suitability of these MCAs for use as ABO blood grouping reagents for human bloodstains and body fluid stains by the adsorption-elution technique. In addition, this study also discusses the usefulness of these MCAs as ABO blood grouping reagents for human body fluid stains by the indirect enzyme linked immunosorbent assay (ELISA) method.

MATERIALS AND METHODS

Mouse monoclonal anti-A, anti-B and anti-H antibodies produced by use of the synthetic blood group A, B and H oligosaccharide haptens were supplied by Chembiomed-Unicom Co., Ltd. Human anti-A and anti-B antibodies (Ortho Diagnostics Systems Inc.), chicken anti-H antibody and *Ulex europaeus* anti-H lectin were used as polyclonal antibodies.

Adsorption-inhibition, adsorption-elution and agglutination-inhibition techniques for ABO blood grouping of bloodstains and body fluid stains were carried out following the procedures of Outteridge (1965). The ABO blood grouping of hair samples followed the procedure of Mukoyama and Seta (1986). The determination of ABH antigens in body fluid stains by using MCAs was carried out according to the indirect ELISA method using an avidin-biotin-peroxidase complex system (Vectastain ABC kit, PK-4010).

In the serologic studies of antibody specificity, agglutinin titer, avidity and stability of the monoclonal anti-A and anti-B antibodies, these MCAs were found to have serologic characteristics comparable to those of human polyclonal antibodies. Results of the adsorption-elution and hemagglutination-inhibition techniques using the MCAs for human bloodstains and body fluid stains from secretor and nonsecretor individuals were consistent with those for conventional polyclonal antibodies (Tables 1 and 2). All of these MCAs were of the IgM class. The monoclonal anti-H antibody prepared against Type 2 H antigen was shown to be superior to *U. europaeus* anti-H lectin in ABO blood grouping of human bloodstains and saliva stains as well as red cells.

The ABO blood grouping of human hair samples was performed by an adsorption-elution technique using these MCAs. Eluates obtained with MCAs from the hair samples were unreactive or very weak in saline and slightly improved in 1% albumin medium. The MCAs also seemed to be suitable for use as ABO blood grouping reagents of the body fluid stains by the indirect ELISA method.

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Table 1. RESULTS OF ABO BLOOD GROUPING OF BLOODSTAINS BY THE ADSORPTION-ELUTION TECHNIQUE USING MONOCLONAL ANTI-A, ANTI-B AND ANTI-H ANTIBODIES

Human Bloodstains[a]		Adsorption-Elution Reaction					
Known Blood Groups	Age of Stains (mo)	Monoclonal Anti-A	Monoclonal Anti-B	Monoclonal Anti-H	Human Anti-A	Human Anti-B	Human Anti-H
A	1	+	–	+	+	–	+
	6	+	–	+	+	–	+
	12	+	–	+	+	–	±
B	1	–	+	+	–	+	+
	6	–	+	+	–	+	+
	12	–	+	+	–	+	+
O	1	–	–	+	–	–	+
	6	–	–	+	–	–	+
	12	–	–	+	–	–	+
AB	1	+	+	+	+	+	+
	6	+	+	+	+	+	±
	12	+	+	+	+	+	±

[a]Blotted on gauze

Table 2. COMPARATIVE QUANTITATION OF ABH SUBSTANCES IN HUMAN WHOLE SALIVA BY HEMAGGLUTINATION-INHIBITION TECHNIQUE USING MONOCLONAL AND POLYCLONAL ANTI-A, ANTI-B AND ANTI-H ANTIBODIES

	Hemagglutination-Inhibition Reaction					
Saliva Samples (Known Blood Groups)	Monoclonal Anti-A[a]	Monoclonal Anti-B[a]	Monoclonal Anti-H[a]	Human Anti-A[a]	Human Anti-B[a]	Human Anti-H
A, secretor	11[b]	–[c]	8	10	–	9
A, nonsecretor	3	–	–	2	–	2
B, secretor	–	10	6	–	10	7
B, nonsecretor	–	1	–	–	1	1
O, secretor	–	–	9	–	–	10
O, nonsecretor	–	–	–	–	–	1
AB, secretor	9	9	3	9	9	6
AB, nonsecretor	1	1	–	1	1	1

[a]Agglutinin titer 1:8
[b]Inhibition titer 2^n
[c]No inhibition

THE FORENSIC APPLICATION OF ANTI-A AND ANTI-B (MURINE MONOCLONAL BLEND) BIOCLONE™ ANTISERA TO THE EXAMINATION OF BLOOD AND BODY FLUIDS IN THE CRIME LABORATORY

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Ortho Diagnostic Systems Inc. (Raritan, NJ), a manufacturer and supplier of reagents used for the analysis of blood, is presently marketing anti-A and anti-B (murine monoclonal blend) Bioclone™ antisera. These reagents are marketed as highly specific antisera for the identification of the rare subgroups of the A, AB and B blood groups, as well as the more common variants of these groups. Before these highly specific antisera were developed, it was difficult, if not impossible, to identify certain rare forms of the ABO blood groups. An examination of the use of these monoclonal antisera versus the polyclonal antisera in adsorption-elution and adsorption-inhibition analysis of blood and body fluids has provided useful information to the forensic serologist.

The antisera and the blood group indicator cells used in this study were purchased from Ortho Diagnostic Systems Inc. (Raritan, NJ). The H lectin was purchased from Seri (Emeryville, CA) and the "normal" saline was purchased from American Scientific Products (McGraw, PA).

The procedure for adsorption-elution included attaching bloodstained threads to a glass plate that contained 12 ceramic rings and adding neat anti-A and anti-B sera and H lectin (Figure 1). The plates were placed in a moisture chamber and incubated overnight at 4° C, and the excess antisera were removed with cold tap water. The plates were washed by suspending them in a 1,000 ml beaker that contained cold tap water and a magnetic stir bar. The beaker was placed on a magnetic stir plate and allowed to stir for 1 hour, and the plates were removed and blotted dry. One drop of "normal" saline was added to each sample, and the plate was placed in a 64° C moisture chamber for 15 minutes. After elution of the antibodies, a 0.2% dilution of A_1, B and O indicator cells were added to the respective wells. The plates were gently agitated for 1 hour before being examined microscopically for agglutination. Every test plate was run simultaneously versus a control plate with the polyclone A and B antisera.

When this method was used, the anti-A monoclone failed to agglutinate on all samples, including the blood group A standards that were run on each plate. The anti-B monoclone agglutinated in the samples that contained B blood group information. The degree of agglutination was highly variable and usually mixed field in nature.

The procedure for adsorption-inhibition was the standard tube method, with A and B antisera diluted 1:32 with "normal" saline and H lectin diluted 1:16 with "normal" saline. The samples incubated overnight at 4° C, after which the samples were removed from the tubes, leaving behind the antisera. One drop of 3% A_1, B and O indicator cells was added to each of the respective tubes. The samples were allowed to stand at room temperature for 5 minutes before being centrifuged at 3000 rpm for 5 seconds. The tubes were then gently agitated and examined for agglutination. Every test sample was run simultaneously versus control samples using 1:32 dilutions of the polyclone A and B antisera. To date, 40 body fluid samples have been examined, and the test results indicate no difference between the monoclonal and the polyspecific antisera.

The failure of the monoclonal A antisera and the variable reactivity of the B antisera in adsorption-elution analysis of bloodstains indicates a potential problem area that may affect their usefulness in forensic serology. The causes of these problems have not been determined and are the subjects of current studies.

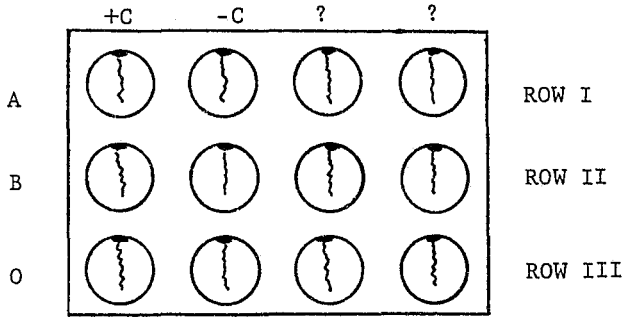

Figure 1. Diagram of an adsorption-elution plate.

USE OF MONOCLONAL ANTIBODIES AGAINST ABO(H) ANTIGENS: DETECTION OF SOLUBLE BLOOD GROUP SUBSTANCES IN SEMEN

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In the last 10 years, the field of immunohematology has rapidly expanded in the area of monoclonal antibody (MCA) techniques. The development of MCAs to the ABH blood group antigens has been very important for forensic pathologists. Although these reagents have been thoroughly evaluated for clinical use in red cell typing, their suitability for routine use in forensic blood grouping procedures has not been established. This paper reports on a study undertaken to assess the forensic applicability of three commercial sources of monoclonal reagents.

MATERIALS AND METHODS

A direct binding enzyme linked immunosorbent assay (ELISA) was used to evaluate monoclonal reagents from Ortho Diagnostics (Raritan, NJ); Allotype Genetic Testing (AGT) (Atlanta, GA) and Dakopatts (Santa Barbara, CA). The blood group antigens were immobilized, and the antigens were detected after the sequential addition of the specific mouse monoclonal and enzyme conjugated secondary antibody.

RESULTS AND DISCUSSION

The Dakopatts and AGT monoclonals (anti-A, anti-B and anti-H) were single clone reagents composed of a homogenous population of antibodies. The Ortho Bioclone reagents (anti-A and anti-B) represent a blend of two or three separate monoclonal reagents.

Titers of soluble blood group substances were determined for 47 semen specimens using the Dakopatts, Bioclone and AGT monoclonal blood grouping reagents. Of the six specimens identified as having originated from group B secretors, the Dakopatts anti-B failed to detect the B antigen in three specimens, whereas the AGT anti-B failed to detect the B antigen in one specimen. The Bioclone anti-B successfully detected the B antigen in all six group B specimens.

When the various monoclonal anti-B reagents were tested with four semen specimens classified as blood group AB, all three reagents produced clear positive results for three of the four specimens. With the fourth specimen, only the AGT anti-B produced a positive signal. However, the signal was weak, being just above the O. D. threshold considered as a positive result by ELISA. Neither the Dakopatts and AGT anti-A and anti-H or Bioclone anti-A failed to recognize the corresponding blood group antigen in semen.

These results indicate that monoclonal blood grouping reagents (particularly anti-B) must be carefully selected and used and that all monoclonal blood grouping reagents should be thoroughly evaluated before being used in forensic grouping procedures.

AN IMPROVED EXTRACTION TECHNIQUE FOR GROUP SPECIFIC COMPONENT (Gc) DETERMINATION USING 6M UREA

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Group specific component (Gc) is a polymorphic serum protein. The population distribution of Gc makes it a good discriminator, but the success rate of phenotyping bloodstains was only 29% in our laboratory. Whole serum samples usually gave excellent results, but dried bloodstains extracted in gel buffer gave poor, if not negative, results. Haddad and Walgate (1976) and Haddad (1982) discovered that Gc protein binds to other blood constituents such as vitamin D, vitamin D metabolites and actin, and that these Gc complexes have different electrophoretic mobilities. To combat these problems, Kimura *et al.* (1983) proposed an extraction procedure with 4M guanidine hydrochloric acid requiring dialysis. Budowle and Gambel (1984) proposed the reading of Gc complex electrophoresis patterns. Westwood (1984) reported on the use of 6M urea on stains before isoelectric focusing. This process dissociates the bound complexes and allows for the typing of the free Gc. Our paper applies this treatment to a conventional electrophoretic method (Wraxall *et al.* 1978).

MATERIALS AND METHODS

Bloodstains were extracted in 13 μl of buffer, the extract was pipetted into the gel and voltage was applied until the hemoglobin migrated 5 cm. The protein was then immunofixed with anti-Gc serum (SERI, Emeryville, CA) and eventually stained using Coomassie blue followed by silver staining/blue toning when necessary.

A blind study was conducted with 14 laboratory prepared bloodstains of various ages. All stains were on white cotton and had been frozen. Each stain was analyzed by two different extraction procedures: extraction in gel buffer only and extraction with 6M urea in gel buffer followed by a chloroform wash.

Four stains gave typeable Gc results when extracted with buffer alone. These gels showed areas of precipitated protein anodic to the normal Gc banding. All 14 samples were successfully typed for Gc when extracted with 6M urea with a chloroform wash. In fact, Gc bands on these gels were easily distinguished when stained with Coomassie blue, and silver staining was not necessary. No precipitated proteins were present anodic to the normal Gc banding area.

DISCUSSION

Group specific component is a good, discriminating marker for forensic serology; however, previous extraction techniques were tedious or yielded poor results, and so Gc typing was rarely worth the effort. A 6M urea extraction with a chloroform wash breaks Gc protein complexes and allows Gc typing in stains that previously gave poor or negative results. Using this extraction technique, we can determine a Gc phenotype on over 90% of the stains tested.

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COMPARISON OF IMMUNOPRINT TECHNIQUES FOLLOWING ISOELECTRIC FOCUSING OF THE Gc PROTEIN: IMMUNOPRINTING ON CELLULOSE ACETATE AND ON LARGE PORE ULTRATHIN-LAYER REHYDRATABLE POLYACRYLAMIDE GELS

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Isoelectric focusing (IEF) on polyacrylamide gels is normally carried out on gels prepared in-house. However, these gels have several undesirable characteristics:

1. Gel polymerization by-products, such as unpolymerized acrylamide monomers, linear polymers, breakdown products of acrylamide, ammonium persulfate breakdown products and TEMED remain in the gel.

2. Polymerization kinetics in the presence of synthetic carrier ampholytes depend on the ampholyte and the pH range chosen. The need for more TEMED in acid range gels and less in basic range gels further complicates the characterization process.

3. Incorporation of additives, such as urea, during the polymerization process may also affect polymerization kinetics, eventual pore size and structure.

4. Reproducible results are difficult, although not impossible, to achieve if different gels or gel types are used, even when the process includes purified reagents and freshly recrystallized monomer.

5. Polymerization by-products may inhibit subsequent reagents from impregnating gels with substances, such as antibodies, into the gels.

Ultrathin-layer gels are desirable, since rehydration equilibration time depends on the gel thickness, and gels greater than 500 microns require a longer time to rehydrate. In addition, ultrathin-layer gels have a higher resolution potential than those with a thickness of over 500 microns because higher voltage gradients may be used and less Joule heat produced at any given voltage gradient. This advantage is found when focusing is carried out on a precisely controlled Peltier controlled cooling device (E.C. I.C.E. apparatus) that uses isothermically controlled electrophoresis. Thermoelectric cooling systems are more effective than are liquid cooling systems in removing Joule heat generated in ultrathin-layer gels, and they allow higher ampholyte concentrations to be used to further improve resolution.

Dried gels produced by Allen GelBond™ Pag and glass plates may be stored for over a year at room temperature without apparent loss of structural functionality. This stability was measured by assessing pattern quality using test proteins and enzymes as assay systems and by determining the ability of gels to adsorb other materials such as substrates, dyes and antibodies without loss of activity on storage. Rehydrated polyacrylamide gels were backed on glass or polyester film as both a support medium for electrophoresis and as a visualization medium for immunoprinting following impregnation with antibody for group specific component (Gc).

The specific antibody for Gc protein (Dako) was diluted serially in twofold dilutions in physiologic saline and impregnated into cellulose acetate membranes and dried gels covalently bound to GelBond™ Pag by rehydration. These gels were used directly for overlaying on Gc proteins already separated by IEF on a pH 4.5–5.4 Pharmalyte™ gradient. The overlay acetate membrane and the gel containing antibody remained in contact with the focused Gc gel for 5 and 3 minutes, respectively, at 23° C. The overlay acetate or gel was then washed through three or more 10 minute changes of physiologic saline, and the resultant precipitin bands were fixed in 20% trichloroacetic acid for 10 minutes and stained with Coomassie brilliant blue R250 or with silver diamine. Measurements were performed on a Biomed SL-2D computer operated Densitometer with the 2D-Stepover program with a slow scan rate and a soft laser source for the Coomassie brilliant blue R250 and silver stained Gc precipitin bands. All data were then quantified and migration distances determined with a Video-phoresis II (Biomed Institute) program with superimposition capability, and data were analyzed with MicroSoft file

Table 1. POSITION LOCATION OF GC PROTEIN BANDS DETERMINED BY DENSITOMETRY WITH DISTANCE EXPANDED FROM A TOTAL SEPARATION DISTANCE OF 1.2 CM TO 6 INCHES (12.7X) BY COMPUTERIZED MICRODENSITOMETRY

Scan/Type	1F Anodal	1F Cathodal	1S Anodal	1S Cathodal	2
A 2					5.344
N 2					5.591
E 2-1S			1.219	3.516	5.426
H 1S			1.251	3.457	
B 1S		1.301	3.316		
L 1S			1.301	3.492	
V 1F-1S	0.926	3.001	1.172	3.223	
T 1F-1S	0.973	3.047	1.242	3.375	
Z 1F-1S	0.984	3.105	1.219	3.328	
X 1F-1S	1.031	3.035	1.184	3.293	
U 1F	1.031	3.001			
W 1F	1.031	3.023			
D 2-1F	1.031	3.094			5.367
F 1F	1.031	3.094			
G 1F	1.078	3.176			
Mean	1.013	3.064	1.236	3.375	5.432
SD Inches	±0.044	±0.058	±0.048	±0.104	±0.111

for determine the average and standard deviation from the mean.

Comparison of the two support media for the immunoprint indicated that both post and prozonal reactions were apparent on the rehydratable gel but not on the cellulose acetate membrane. Further, wash time requirements were longer on the cellulose acetate before clear backgrounds were obtained.

Immunoprints produced in the rehydratable gel overlays containing Gc antibody were washed free of unreacted antibody in only 30 minutes. Conventional polyacrylamide gels, on the other hand, retained a background of unremovable antibody that will stain with Coomassie brilliant blue R250 or with silver. Thus, the sensitivity of the system was so greatly reduced that it was impractical to use.

Densitometric scanning of the separations allows migration values to be assigned from an index point for each of the allele products of the Gc proteins. These values also may be assigned confidence limits so that hard data can be presented to identify the allele products. This approach appears possible not only for Gc proteins but also for any system in which the allele products can be separated by the electrophoretic method and visualized by protein, enzyme or specific immunochemical methods. If more sophisticated computer programs are used, it should be possible to identify phenotypes directly from the scan and the subsequent computer output of the scan data.

IMPROVED RESOLUTION AND SENSITIVITY IN THE SUBTYPING OF Gc

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Several methods have been used in an attempt to attain good resolution and sensitivity for forensic subtyping of group specific component (Gc). Among the methods tried, the best resolution was achieved by Immoboline™ gel followed by Edwards' ultrathin polyacrylamide gel isoelectric focusing (IEF) with MOPS and HEPES used as separators (Westwood, personal communication). It was also reported that ultrathin IEF provided better sensitivity.

Initially, we developed a modified Edwards' method that provides a resolution better than that obtained by the Immoboline™ gel method. The modified method removed sucrose from the gel to induce cathodic drift and used a 15 cm electrode gap (Westwood, personal communication). However, the long run time used (4 hours) did not always provide reproducible resolution. Also, the direct silver staining of the polyacrylamide originally described worked only part of the time.

The method presented here is a further modification of the original polyacrylamide IEF method. This method provides a reproducible resolution in the order of the Immoboline™ gel method (Go 1A1 resolved) while using a shorter run time (Figure 1). This combination is achieved by shortening the electrode gap and decreasing the ampholine concentration to shorten the time for the onset of cathodic drift. Serum Gc diluted 1:160 can be detected by the immunoprinting method used. This sensitivity is achieved by titering the antiserum to obtain an optimum and economical antiserum concentration. An agarose IEF method also provides a similar resolution (Figure 2).

The polyacrylamide gels (180 X 130 X 0.25 mm) are cast by the flap method using a solution containing 7.7 ml acrylamide stock (5.7%T 3%T), 0.21 g MOPS, 0.07 g HEPES, 1.7 mg APS and 0.0 ml pH 4.5–5.4 Parmalyte™. The gel is prefocused on a cooling plate at 10° C, at 10 ma limit to reach 1900 V. Samples are placed 1 cm from the cathode and focused for 90 minutes. The immunofixation method used is a modified method of Westwood (1985) visualized by Coomassie blue.

The agarose gels (160 X 110 X 0.5 mm) are cast by the flap method using 14 ml of 1% IEF grade agarose, 128 mg MEPES, 385 mg MOPS and 0.7 ml pH 4.5–5.4 Pharmalyte™. The gel is prefocused at 300 V initial voltage, unlimited wattage, with a 2000 V limit for 30 minutes. Samples are placed 2 cm from the cathode. Voltage is slowly ramped up to 2000 V with 3 ma limit for 2 hours. The immunofixation method used is similar to the method used in conventional Gc typing. Subtyping of Gc by the methods described in this paper has been demonstrated to be sensitive, reliable and reproducible.

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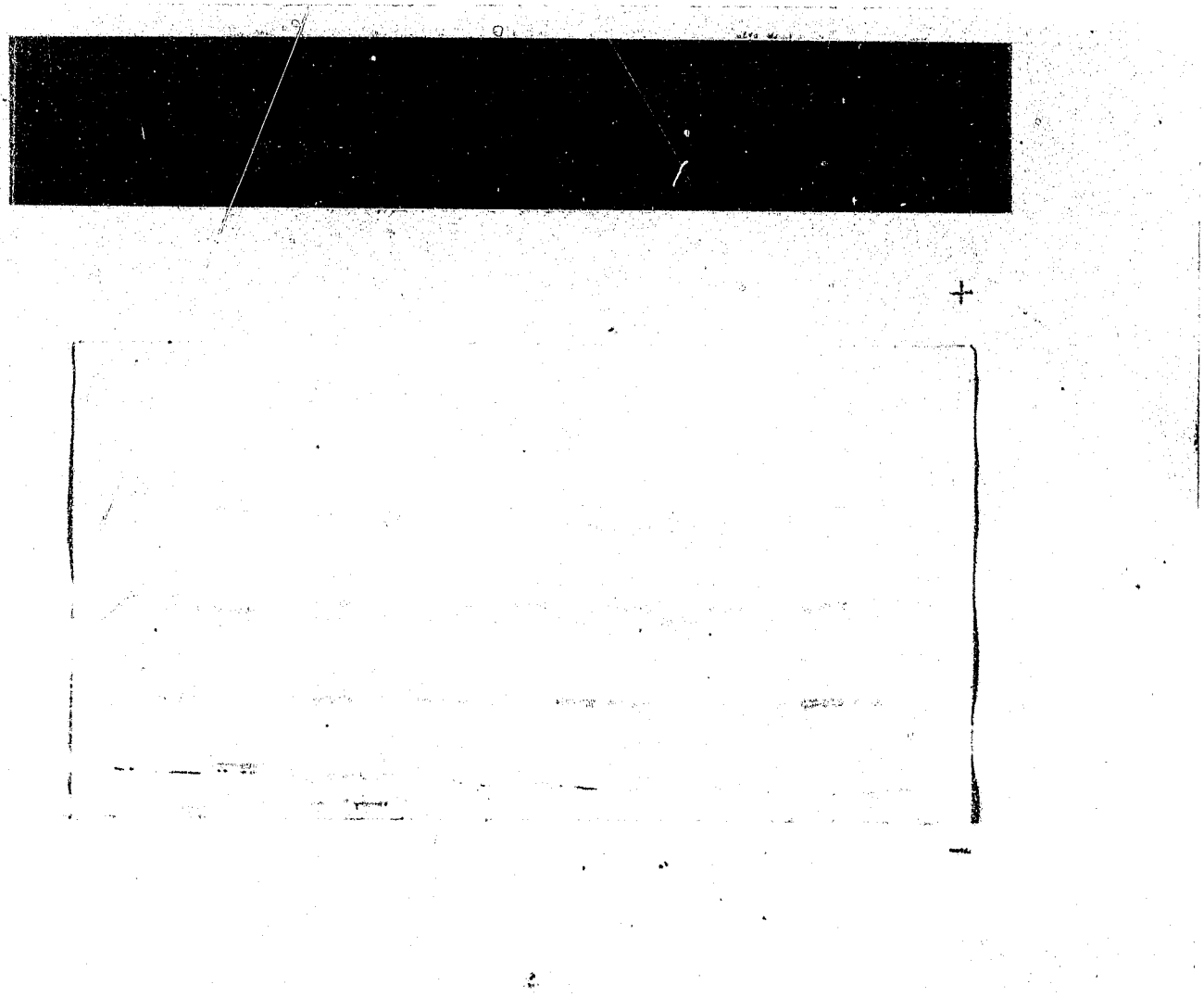

Figure 1. CAM Immunoprint from acrylamide gel showing serum samples diluted 1:30. Samples from left to right are: 1F-iC10 variant, standard mix, 1S-2C2 variant, 2, standard mix, 1S-1A1, 2-1C variant, standard mix, 1F, 2-1S, standard mix, 1S, 1F-1S, standard mix, 2-1F, 2 and standard mix. The anode is at the top.

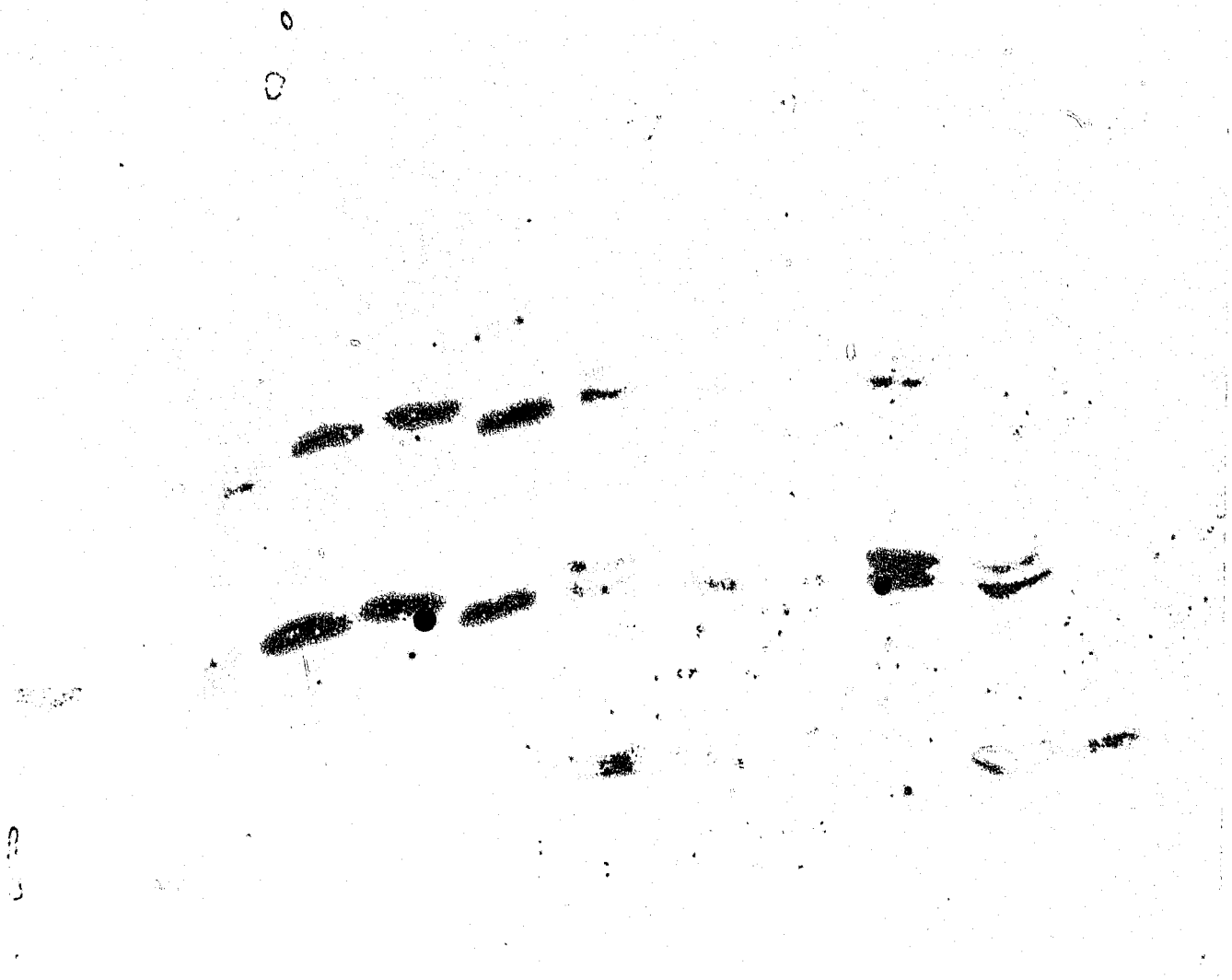

Figure 2. Agarose gel showing serum diluted 1:30, after immunofixation, Coomassie blue, silver staining and blue toning. Samples from left to right are: standard mix, 1F-1C10, 1F, 1S, standard mix, 2-1S, 2-1S, 1F-1S, standard mix and 2. The anode is at the top.

COMPARISON OF IMMUNOBLOT AND IMMUNOFIXATION FOR THE DETECTION OF GROUP SPECIFIC COMPONENT (Gc) IN BLOODSTAINS

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The identification of group specific component (Gc) by using an immunofixation technique (Johnson *et al.* 1982) after isoelectric focusing (IEF) has been very successful in the forensic laboratory. However, results may be difficult to obtain from samples that showed decreased activity. The immunoblot technique (Gershoni and Poladi 1983) proved to be more sensitive than immunofixation, and it can detect activity that would otherwise could not be detected.

MATERIALS AND METHODS

Polyacrylamide IEF gels were made on Gel Bond film (FMC, Rockland, ME) using the flap technique (Table 1). An LKB Ultrophor system (LKB, Rockville, MD) was used for focusing. Electrode solutions were 0.2 N sodium hydroxide (cathode) and saturated L-aspartic acid (anode). The gels were prefocused for 30 minutes at 250 V. The samples, soaked in one drop of 6 M urea, were applied to the gels using LKB application tabs and placed about 2 cm from the cathode wick (Table 2). At the end of the run, one half of the gel was overlaid with a nitrocellulose acetate strip (Bio Rad, Richmond, CA) soaked in a tris buffered saline (TBS) solution. The anti-Gc (goat) was diluted 1:200 in a 1% gelatin (Norland Products, New Brunswick, NJ) TBS (Bio Rad) and allowed to react with the membrane for 1–2 hours. The membrane was then washed in TBS and subsequently allowed to react with rabbit anti-goat alkaline phosphatase (Bio Rad) diluted 1:3,000. After another washing, the membrane was developed with an alkaline phosphatase color development reagent (Bio Rad). The other portion of the gel was overlayed with a cellulose sheet (Shandon, Sewickley, PA) soaked on a 1:3 dilution of anti-Gc. The sheet was then washed for 1 hour in 0.15 M sodium chloride. A Coomassie blue stain was then used to visualize the bands.

The immunoblotting technique using bloodstains stored at 4° C for more than 1 year gave excellent results that were not difficult to interpret. The immunofixation technique was less sensitive when old stains were tested. Sample extraction with 36 M urea may have to be processed overnight, depending on the age of the stain and the material.

In this study, Gc identification using an immunoblot method greatly improved success in bloodstain analysis. The procedure used less anti-Gc per plate and can be completed in 1 working day. This sensitive technique could be important when small samples must be analyzed.

IMMUNOFIXATION

After the run, one half of the gel was stained using the immunofixation technique. Using 0.5 ml anti-human Gc-globulin (goat) as a 1:4 dilution in saline, one side of the cellulose acetate membrane was soaked. The reverse side was then applied to the gel. The membrane was covered with several layers of paper towels and overlayed with a glass plate, and a 5 kg weight was applied. After 20–30 minutes, the membrane was washed for 1 hour in a 0.15 M sodium chloride solution. The membrane was then washed off in distilled water, stained for 5–7 minutes and then destained for 5 minutes.

The stain was composed of 1.5 g Coomassie brilliant blue R, 225 ml methanol, 50 ml glacial acetic

Table 1. GEL PREPARATION

Acrylamide gel (.5 mm):	
6.25 ml	[a]acrylamide/bis stock
16.7 ml	(distilled) H_2O
1.9 ml	4–6.5 Ampholyte
	Degas 5 mins, then add
150 µl	[b]Ammonium persulfate stock
25 µl	TEMED

[a] 2.91 gm acrylamide, 0.09 Bis make up to 10 ml dist. H_2O
[b] 0.1 gm ammonium persulfate 1.0 ml dist. H_2O

Table 2.

	TIME (mins)	Volts	mA	Watts
Prefocus	30	250	unlimited	unlimited
Samples on	30	2,000	unlimited	5
Sample tabs off	30	"	"	7
	30	"	"	10
	120	"	"	12

acid and 225 ml deionized water. The destain contained 450 ml methanol, 100 ml glacial acetic acid and 450 ml deionized water.

IMMUNOBLOT

Buffer Preparation

The TBS contains 4.84 g tris and 58.48 g sodium chloride made up to 2 liters with distilled water and adjusted to pH 7.5 with hydrochloric acid. To prepare the TBS, 0.5 ml of Tween-20 was added to 1 liter of TBS (above).

Soak the nitrocellulose membrane in TBS. Place the opposite side on the second portion of gel. Place several layers of paper towels and glass plate over membrane and cover with a 5 kg weight. Allow the solution to react for 30 minutes. Remove the membrane and wash in 100 ml of a 3% gelatin-TBS blocking solution for 30 minutes. Gently agitate. Next wash twice for 5 minutes each in TBS, with agitation. Prepare the antibody buffer by adding 1 ml gelatin in 100 ml TBS. Add anti-Gc to make a 1:200 dilution. Put the membrane in antibody buffer and slowly agitate for 1 hour. After 1 hour, remove the antibody solution and wash the membrane twice, 5 minutes each, in TBS on a rocker platform. Remove the wash solution and add 33 μl of alkaline phosphatase (rabbit anti-goat IgG) to 100 ml antibody buffer. Allow the solution to react for 1 hour with gentle agitation. The membrane is then washed twice for 5 minutes each in TBS. Rinse once for 5 minutes in TBS.

Color Development Solution (For Alkaline Phosphatase) from Bio Rad

Stock solutions:

1. Carbonate buffer (0.1 M sodium bicarbonate and 1.0 mM magnesium chloride, pH 9.8). For 1 liter, add 8.40 g sodium bicarbonate to 0.20 g magnesium chloride and bring to 1 liter with distilled water. Adjust to pH 9.8 with sodium hydroxide.
2. *p*-Nitro blue tetrazolium chloride (NBT) color development stock solution. Make 1 ml of 70% N, N-dimethylformamide (DMF) solution by mixing 0.7 ml of DMF with 0.3 ml of distilled water. Dissolve 30 mg of NBT in this 70% solution.
3. 5-Bromo-4-chloro-3-indolyl phosphate disodium salt (BCIP) color development stock solution. Dissolve 15 mg of BCIP in 1 ml DMF.

Color development solution:

Just before using, mix 1 ml of the 30 mg/ml NBT stock and 1 ml of the 15 mg/ml BCIP stock into 100 ml of the carbonate buffer.

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SECOND ANTIBODY FIXATION, SILVER STAINING AND BLUE TONING: A SUMMARY OF TECHNIQUES FOR THE ENHANCEMENT OF IMMUNOCHEMICAL REACTIONS IN AGAROSE GELS

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Many forensic laboratories currently make use of immunodetection of proteins in agarose gels. However, some of these methods, such as immunofixation of group specific component (Gc) globulin after electrophoresis and the detection of the seminal protein P30 by immunoelectrophoresis, produce weak staining patterns by conventional Coomassie brilliant blue staining methods that are frequently difficult to see and more difficult to preserve photographically.

The study evaluates methods used to enhance protein bands and applies these methods to the immunodetection of Gc and P30 proteins to provide increased sensitivity for these proteins and to produce gels with higher contrast. Such improvements could lead to better photographic recording of electrophoresis results.

MATERIALS AND METHODS

Double Antibody Fixation

Enhancement of antigen-antibody reactions with secondary antibodies was suggested by Rumke and Breekveldt-Kielich (1969) for radial immunodiffusion experiments. White (1983) and Podleki and Stolorow (1985) suggested using the method to detect Gc via immunofixation.

After immunofixation with anti-Gc antibody (goat; Atlantic Antibodies, Scarborough, ME), the gel is washed overnight in 1 M saline and then immunofixed with the second antibody, rabbit anti-goat (Atlantic Antibodies, 0S8-01) for 1.5 hours. The gel is pressed, washed in saline for 3 hours, pressed, dried and stained for protein.

Silver Staining

Silver stain methods were initially designed for acrylamide. A silver stain for agarose was devised by Willoughby and Lambert (1983) and has been modified by Budowle (1984) and Dykes (1985):

1. Stain with brilliant blue as usual; destain.
2. Put in 5% glutaraldehyde 5 minutes.
3. Wash 20 minutes in water with one change.
4. Prepare silver stain by mixing 1 part solution A to 2 parts solution B and add to gel (Table 1).
5. Mix and observe 5–7 minutes.
6. When developed, pour off and fix with 1% acetic acid.

Formulary Reducer I

If gels remain in contact with the silver staining reagent too long, increased background color develops. This background has been thought to be irreversible (IsoLabs Silver Stain Kit Protocol), but it can be reversed by using a darkroom technique, Formulary Reducer I (Lester 1979). This technique has been mentioned by Heukeshoven and Dernick (1985) under its proprietary name Farmer's Reducer.

To prepare Formulary Reducer I:

1. Mix 1 part solution C:4 parts solution D:20 parts water (see Table 1).
2. Pour on gel and mix until background color is reduced.
3. Wash with water.

Blue Toning:

A method of achieving additional sensitivity after silver staining has been suggested by Berson (1983):

1. Following silver staining, rinse with water.
2. Mix 10 ml each solutions I, II and III, and take to 100 ml with water (see Table 1).
3. Pour on gel. Reaction takes place in 0.5–3 minutes.
4. Rinse in water.
5. Rinse 10 minutes in 20% methanol and 5% acetic acid (v/v).

CONCLUSION

The sequential application of these techniques can provide a stepwise increase in the sensitivity of immunologic detection. Samples with high protein levels may be recorded and photographed early in the brilliant blue/silver/blue toning series, and weaker samples may be detected by these enhancement steps.

Table 1. CHEMICAL SOLUTIONS FOR ENHANCEMENT TECHNIQUES IN AGAROSE GELS

Silver staining:

Solution A	Solution B
5% anhydrous Na_2CO_3	1 g ammonium nitrate 1 g silver nitrate 5 g tungstosilicic acid 7 ml 37% formaldehyde to 500 ml H_2O

Formulary reducer I:

Solution C	Solution D
7.5% potassium ferricyanide	24% sodium thiosulfate

Blue toning:

Solution I	Solution II	Solution III
5% $FeCl_3$	3% Oxalic Acid	3.5% $K_3Fe(CN)_6$

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DETERMINATION OF SECRETOR STATUS IN BILE

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Interpretation of typing results from body fluid swabs or associated stains requires evidence of the secretor status of the patient under study. Conventional techniques are often uninformative on autopsy specimens due to deterioration, such as for Lewis (Le) in blood, or contamination (such as a spurious "ABH" substance on most "oral swabs" from autopsy). To study more nonsecretor specimens of bile than were reported in the limited literature (Hartman 1941; Gaensslen 1983), paired blood and bile samples were typed for ABO and Le types. By including Le typing and type "O" secretors identified by *Ulex europeaus* in the study, we sought to assess the predictive value of bile for secretor status.

MATERIALS AND METHODS

Specimens of paired blood and bile, preferably in red top vacutainers, were obtained from autopsy kits and stored at 4° C until analyzed. Ortho (Raritan, NJ) test cells and polyclonal antibodies, along with anti-H lectin made in-house from *U. europeaus* seeds, were used throughout this study. These were diluted and titered as appropriate to the technique. Conventional direct and back-typing techniques were used for red cell ABO typing. Adsorption-inhibition using a modification of Culliford's (1971) technique was used with dilutions of bile to 1:1000 or more in saline. Where lysis occurred at low dilutions of bile (1:8 to 1:32), dilute albumin was added (to approximately 0.01%) to the test cells. Lewis typing of ficinated red cells and inhibition of bile dilutions was conducted by the capillary tube method of Mudd (1983), as used previously by Konzak and Long (1983). Saliva standards of appropriate ABO types and Le(a+b–), Le(a–b+) and Le(a–b–) saliva, along with similarly typed red cells, were used as controls and test materials. Since we did not have a true Le(a–b–) bile control, anti-D was used to eliminate totally nonspecific inhibition.

RESULTS

Results were obtained on 14 bile and blood pairs thus far, with 4 nonsecretors identified (via Le[a+b–] blood of caucasian origin). All nonsecretors were type A (Tables 1 and 2). In the bile of secretors, titers of over 1,000 were present for A, B and apparent Le^b antigens for the appropriate types. Titers of H substance were significantly lower in bile of type O secretors (averaging 32), but those tested show high Le^b versus Le^a inhibition (Gibbons 1983). The titers of H in the type A nonsecretors were similar. This similarity may cause confusion if the ABO type is not otherwise known because of the low levels of A antigen in the nonsecretor bile. The Le^a inhibition, or antigen activity, is higher than that in Le^b in these nonsecretors, confirming the nonsecretor character, but unexpectedly high Le^b inhibition is present. The H titers and possible Le^b crossreactivity may be better assessed when type O nonsecretors and true Le(a–b–) bile are examined or other assays or reagents compared.

CONCLUSION

The typing of bile and other fluids not generally tested for secretor status in the laboratory must not be attempted without first running sets of control specimens of that particular fluid in all common types. The results of this initial study suggest that bile ABH levels should not be used alone to identify secretor status. When the ABO type is confirmed in blood and other tissue and both Le and ABH inhibitions in the bile correlate, bile may still be a useful material for secretor status determination.

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Table 1. "SECRETOR" SUBSTANCE TYPING IN BILE

#	Blood Types ABO	Blood Types Le	Apparent Secretion Status	ABH Titer A	ABH Titer B	ABH Titer H	Lewis Titers a	Lewis Titers b
683	O	a–b+	Secretor	–	–	100		
1021	O	a+b+ (child)	Secretor	–	–	10	100	>1000
1023	O	a–b+	Secretor	–	–	32	100	>1000
1024	O	a–b+	Secretor	–	–	32		
1026	O	a–b+	Secretor	–	–	32		
1027	O	a–b+	Secretor	–	–	64		
1018	A_1	a–b+	Secretor	>1000	–	–	1000	>1000
1030	A_2	a–b– (inc.)	(Secretor)	>1000	–	–		
1025	B	a–b+	Secretor	–	>1000	–		
1031	B	a–b+	Secretor	–	>1000	–		
1032	B	a–b– (inc.)	(Secretor)	–	>1000	–		

Table 2. "NONSECRETOR" SUBSTANCE TYPING IN BILE

#	Blood Types ABO	Blood Types Le	Apparent Secretion Status	ABH Titer A	ABH Titer B	ABH Titer H	Lewis Titers a	Lewis Titers b
682	A_1	a+b–	non-secretor	16	–	8	>1000	100
1020	A_1	a+b–	non-secretor	16	–	32	>1000	10
1022	A_1	a+b–	non-secretor	32	–	8	>1000	100
1028	A_2	a+b–	non-secretor	none	–	–		

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ENZYME LINKED IMMUNOSORBENT ASSAY FOR Gm TYPING

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Gamma marker (Gm) blood groups are inherited antigenic determinants present on immunoglobin heavy chains, and they are specific for one IgG subclass. The Gm system is valuable for its stability, discrimination potential and usefulness in determining the racial origin of a stain donor (Hoste and Andre 1978; Blanc *et al.* 1971). Adsorption-inhibition procedures are available for Gm(1), (2), (5), (10) and (11) for blood and semen stains (Khalap *et al.* 1976; Khalap and Divall 1979a, 1979b; Tahir 1984). We have been typing bloodstains for these markers in our laboratory for several years, and their importance has been significant. Adsorption-inhibition is used, but it is believed to be less sensitive and thus requires a large size stain. Since forensic serology rarely has large stains for analysis, we have to develop a more sensitive technique. Enzyme linked immunosorbent assay (ELISA) is more sensitive and needs a smaller sample size than does adsorption-inhibition. The procedure is described for Gm(1) in serum and bloodstains.

MATERIALS AND METHODS

Titertek flat-bottomed polystyrene plates and a Multiskan microplate reader were used (Flow Laboratories, McLean, VA). All the solutions were made with analytical grade reagents, and anti-Gm(1) was used (Molter Heidelberg, West Germany and Allo-Type Genetic Testing, Inc., Atlanta, GA) as well as anti-IgM horseradish peroxidase (HRP) and *o*-phenylenediamine (Sigma).

RESULTS AND DISCUSSION

Serum or bloodstain extract was diluted in carbonate/bicarbonate buffer pH 9.6 and coated onto the flat-bottomed microtiter plate (100 µl/well) at room temperature for 2 hours or overnight at 4° C. The plate was washed three times, for 5 minutes each time, with phosphate buffered saline (PBS) and Tween PH 7.4.

The anti-Gm(1) diluted 1:50 in PBS, Tween and BSA is coated to the wells (50 µl/well) at 4° C for 2 hours. The plate is washed with PBS and Tween three times as previously described, then 50 µl of anti-human IgM HRP diluted 1:500 in PBS, Tween and bovine serum albumin (BSA) is coated to each well at 4° C for 2 hours. The plate is again washed three times. Next, 50 µl of substrate (40 mg *o*-phenylenediamine plus 50 ml of phosphate citrate buffer pH 4.5 and 20 µl of water) was added to each well and incubated at 37° C for 30–60 minutes. At the end of the incubation period, 25 µl of 2 N hydrochloric acid was added and the plate was read at 490 nm with the Flow Multiskan microplate reader. The results indicate that the difference between Gm+1 and –1 is fourfold, and the procedure is workable up to the dilution of 1:30,000. The procedure also gave satisfactory results with dry bloodstains. The effect of the age of the bloodstains has not been determined at this time but is under investigation.

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DEGRADATION OF THE LEWIS ANTIGEN DURING STORAGE AND METHODS TO ENHANCE DETECTION

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The Lewis (Le) antigens on human erythrocytes are soluble glycospingolipid components acquired through adsorption from plasma. These antigens are determined by two alleles. One, the *Le* gene, produces a fucosyltransferase that attaches fucose to the subterminal *N*-acetyl-galactosamine of Type I precursor chains (Rolih 1983). The resulting structure has Le^a activity. Le^b factor is an immunologically reactive structure, the result of interactions between the secretor gene, the ABH *H* gene and the *Le* gene. It has been demonstrated that upon storage, red blood cells experience considerable loss of Le^b activity (Myhre *et al.* 1984).

Even though an antigen's concentration has decreased, hemagglutination can be enhanced through a variety of methods, such as proteolytic enzymes and the use of capillary tubes (Crawford 1978). Monoclonal antibodies have been recently developed that are superior to polyclonal sera (Messeter *et al.* 1984). In this study, samples were examined for the degradation of the Le antigen while techniques and antisera, which may improve detection, were evaluated.

Ten milliliters of clotted whole blood was drawn from each of 43 volunteer donors from laboratory personnel into preservative-free Venoject tubes (Terumo Medical, Elkton, MD). Two milliliter aliquots of whole blood were removed from each tube. The serum was separated and the cells washed three times in Celline isotonic saline (Fisher Scientific Co., Fairlawn, NJ) before resuspending to a working concentration of approximately 3%. The tubes of whole blood were resealed and stored at 2°–8° C for the remainder of the study.

Forward ABO typing was completed with A, B, AB antisera (Ortho Diagnostics, Raritan, N.J.) and lectins H (*Ulex europaeus*; Clyde Robin Seed Co., Inc., Castro Valley, CA) (Tsutsubuchi *et al.* 1983) and A1 (Dolichos Biflorus, Ortho). Reverse grouping studies using commercially prepared A1, A2, B, and O cells (Ortho) were conducted. Lewis antigens were phenotyped using Ortho goat antisera. All testing for ABO and Le was accomplished according to the manufacturer's instructions using appropriate antisera from a single lot.

Working dilutions for capillary testing were determined by titering with appropriate commercially prepared reagent cells (Ortho Resolve Panel A) that had been treated with 4% ficin (Sigma Chemical Co., St. Louis, MO). Dilutions of antisera were made with phosphate buffered saline (PBS). All capillary tubes were 30 µl, (1.0 mm (O.D.) x 7.5 cm) (Applied Science, State College, PA) and were sealed with Critoseal (Lanier Division of Sherwood Medical, St. Louis, MO). The titer values for the antisera are in Table 1.

Subsequent testing with both monoclonal (Immucor, Norcross, GA) and Ortho goat antisera was conducted after 45 days using both tube and microcapillary methods (Mudd, 1983). All tube testing was completed according to the manufacturers' instructions. Thirty-seven of the original 43 samples were available for analysis. The Le^a antigen had greater reactivity than the Le^b and was easily detected with either the tube or micro technique using the different sera. Six group A1 specimens previously identified as Le(a–b+) were typed as Le(a–b–) using the monoclonal tube method. Seven other A1 samples, also Le(a–b+), exhibit diminished Le^b activity when tested with monoclonal antisera. Further investigation (Technical Services, Immucor, personal communication) revealed that the mouse monoclonal Le^b sera was Le^bH rather than Le^bL. The Le^bH recognizes both the H and the Le^b structures; therefore, individuals with limited H substance (that is, an A1 person) could give a false negative reaction. On the other hand, it would be especially effective on O and A2 individuals. The Le^bL reacts to the same form of Le^b on all erythrocytes, regardless of ABO blood type. No other discrepancies in tube typing were noted when the goat or monoclonal sera were used. Several samples, however, gave weakened expressions of their Le^b antigens. Although no discrepancies were noted using the

Table 1. LEWIS TITERS

Antiserum	Capillary Titer
Ortho anti-goat Le^a:	1:6
Ortho anti-goat Le^b:	1:12
Immucor monoclonal Le^a:	1:16
Immucor monoclonal Le^b:	1:32

capillary methods with either antisera, one A1 Le(a–b+) gave inconclusive results with the goat. Even though the monoclonal anti-Le^b is regarded as superior to polyclonal reagents by Longworth *et al.* (1985), it appears that the preparation of the monoclonal antisera, the age of the samples, analytical technique and the ABO type of the individual may have an effect on Le phenotyping in the crime laboratory.

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INHERENT UNRELIABILITY OF THE LATTES TECHNIQUE DEMONSTRATED BY ENZYME IMMUNOASSAY

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A confirmatory test is available for typing liquid blood samples for ABO groupings. Iso-antibodies in the serum follow a pattern: –O serum contains anti-A and anti-B, B serum contains anti-A, A serum contains anti-B and AB serum contains neither type of iso-antibody (Race and Sanger 1975). In 1913, Leone Lattes established the grouping of several persons' blood by the pattern of agglutination and nonagglutination of the various red blood cells by the various sera (Gaensslen 1983). He further reported using extracts of bloodstains made on blotting paper to agglutinate cells from known B, but not A, red blood cells. He judged the stains to be of type A, containing anti-B iso-antibodies. Sensitive enzyme linked immunosorbent assay (ELISA) methods for ABO antigen detection in saliva have been recently developed (Bolton and Thorpe 1986; Fletcher, personal communication), and it may be possible to develop a modified Lattes technique using immobilized soluble ABO antigens as a target instead of red blood cells, and detection by enzyme labeled antibody instead of agglutination. This development would permit the confirmation of grouping.

Saliva from a number of donors was coated onto plastic microtiter plates (Bolton and Thorpe 1986), and extracts from different stains were incubated in the wells. Immobilized iso-antibodies were detected with an anti-human IgM enzyme conjugate (Sigma, Poole, Dorset).

No correlation between ELISA test results and different extract saliva combinations was observed. The range of values obtained from 10 O type extracts combined with O type saliva of an individual overlapped with the range from the same 10 extracts combined with A saliva and the range from B saliva.

The technique can be improved by making stains more uniform, but the forensic analyst rarely has control over the nature of the stain. Confirming stain typing by iso-antibody identification via ELISA remains unreliable. This view agrees with earlier findings by Bolton and coworkers of nonspecificity of polyclonal antisera and of disparity between ABO antigens in blood and in body secretions in some individuals (Bronnikova *et al.* 1984).

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SECTION III

PANEL DISCUSSIONS

PANEL DISCUSSION
SEROLOGY REPORTING PROCEDURES AND COURTROOM PRESENTATION

MODERATOR: *Joseph J. Errera*, FBI Laboratory

PANEL MEMBERS: *Graham B. Divall*, Metropolitan Police Forensic Science Laboratory
Mary M. Gibbons, Oakland Police Department
Terry L. Laber, Minnesota Department of Public Safety
Hermann Schmitter, Bundeskriminalamt
Richard L. Tanton, Palm Beach County Sheriff's Office

This panel discussed the history, purpose and types of serology reporting methods, procedures used, pretrial preparation and the development of effective courtroom communication skills.

Panel members briefly described the reporting format and methods used in their laboratory and the reasons for selecting those particular methods. There was a general consensus among the panel members as to the purpose of the laboratory report. The report was viewed as a vehicle that should clearly and concisely convey the results of scientific tests to the report recipient. The laboratory report should also communicate to the defense attorney, prosecutor and investigator the conclusions or opinions of the examiner based on an interpretation of the test results.

Use of either a tabular or narrative format for the presentation of test results, conclusions and statistical information was discussed. Generally, it was felt that a report that conveyed the essence of the examiner's future testimony would provide valuable information to the report recipients and allow them to make an intelligent assessment of the potential impact of the analyses on future judicial proceedings and an informed decision as to additional investigative efforts.

Many critical decisions are based on laboratory reports without consultation with the examiner for an interpretation of the test results. Conclusionary statements effectively prevent misrepresentation of the serology analyses during judicial proceedings. It is often impossible to include within the confines of the report all of the possible implications, considerations and alternative explanations that may be derived from the test results. The opinions contained in the report need not restrict the scope of future testimony if the examiner maintains an honest, straightforward approach without becoming defensive. The witness can explain that any additional interpretations are based on recent information, such as a new suspect or a rape victim's sexual history, that has been developed since the original report was issued.

Laboratory reports should include the following:

1. What, if any, body fluids and/or mixtures of body fluids can be identified on the items examined?
2. Where on the item is the body fluid located?
3. Can the quantity of the body fluid be determined?
4. If conclusive genetic marker analyses are performed, are the results consistent or inconsistent by comparison with the victim(s) and/or suspect(s).
5. If the suspect(s) is unknown, what information can be obtained from the genetic marker analyses as to the blood type of the source of the body fluid?
6. What percent of the population possesses the same combination of genetic markers found both in a stain and in the victim and/or suspect?

A major controversy in forensic serology report writing is the change in function of the laboratory report from that of strictly reporting test results to that of including tables, lists of evidence, statistics and conclusions. In some jurisdictions, the known standards from the defendant are not submitted to the laboratory until a trial is imminent, whereas the results of the analyses of the other physical evidence have been previously reported. Some attorneys regularly depose forensic experts before trial and, therefore, have the opportunity to obtain the analyst's opinions, conclusions and explanations. Many serologists maintain regular contact with investigators and attorneys, particularly in serial murder, rape and assault cases, and advise them of the test results and their conclusions on a continuing basis.

Even though a report contains conclusions, it may still be misinterpreted by the recipients in support of their own theories. A function of our judicial system is to elicit information from the forensic scientist concerning his conclusions, assumptions, biases and interpretations during the adversarial presentation of physical evidence by the prosecution and defense in court. Conclusionary statements may admit to more than one interpretation and show a bias unless

the report also includes a very thorough, detailed description of the examiner's assumptions, prejudices and less probable interpretations of the test results.

The serologist should report and testify to the results of genetic marker analyses in terms of phenotypes and not genotypes. The forensic serologist does not normally conduct paternity tests and family studies in the laboratory and, therefore, has no scientific basis for conclusive genotyping of body fluids.

Contact with investigators should be initiated, if necessary, to ascertain additional background information pertaining to a case, to determine the source of any evidence submitted, to request known standards from the parties involved or to discuss the nature of the scientific tests requested and/or the capabilities of the laboratory.

Prior sexual history of the rape victim provides important information to the serologist that can influence the interpretation of test results and possibly initiate requests for known standards for elimination purposes. However, the point was made that in some states rape shield laws can limit the amount of information that can be obtained from a rape victim regarding sexual history. In fact, some sexual assault reporting forms may be very intrusive into the victim's rights.

Cases involving alleged sexual assault may involve the issue of consent and not identification of the offender. Also, the serologist should not assume that every offender ejaculates. In many cases, all of the elements of a criminal sexual assault are present, including vaginal penile penetration, but due to sexual dysfunction no semen is deposited.

A thorough, comprehensive pretrial conference with the prosecutor and defense attorney, if requested, is important. The attorney should have a complete understanding of the expert's qualifications, the testing methods employed, the test results and the basis for any interpretations or conclusions. The use of demonstrative exhibits such as charts, tables or diagrams during a trial are generally found to facilitate effective courtroom presentation of serology results to the jury.

Manipulation of an expert witness by an attorney who elicits specific information from the witness that gives a false impression of the expert's actual conclusions and opinions is a major concern for forensic serologists. An expert witness must be able to take control of his testimony if necessary. Experts must explain their opinions to the jury in a manner that is not misleading. Some successful strategies that can prevent manipulation of the expert witness include:

1. Rephrasing a misleading question in the reply.
2. Advising counsel that the question as posed is misleading.
3. Advising the judge that yes or no answers may not be accurate or truthful and that an explanation may be necessary.
4. Asking for previous testimony to be reread.
5. Asking for lengthy or complex questions to be repeated.
6. Pausing before replying to a question to allow time for appropriate objections to be made.
7. Refusing to testify regarding subject matter beyond the scope of expertise.

PANEL DISCUSSION
IMMUNOLOGY REAGENT USE

MODERATOR: *Moses S. Schanfield*, ALLOType Genetic Testing, Inc.

PANEL MEMBERS: *F. Samuel Baechtel*, FBI Laboratory
Steven M. Fletcher, Central Research Institute
Pamela J. Newall, Centre For Forensic Science
George F. Sensabaugh, University of California, Berkeley

The purpose of this panel was to discuss changes in immunologic technology in forensic serology including use of the enzyme linked immunosorbent assay (ELISA) and monoclonal antibodies (MCAs). Reasons for changing procedures or methods and problems of implementing or evaluating the new procedures were discussed. Generally, new methods or procedures are tried when the old methods do not work for particular case samples. Often descriptions of new methods are inadequately presented in the literature or at meetings, which prevents rapid laboratory setup for implementing new procedures. Therefore, authors and lecturers are urged to provide as much detail as possible when describing methods. If a specific grade of reagent is specified, it would be useful to explain why.

Most forensic laboratories do not have time to research new methods and prefer to wait until the new procedures have been published. There was some agreement that it would be useful to have a national library of approximately 200 body fluid stains so that when a system is being set up in different laboratories, there would be a standard for comparison.

The concept of procedural drift was addressed. Although forensic scientists may perform adsorption-inhibition or adsorption-elution, these procedures are not necessarily performed the same way. When reports come out describing the use of the same reagents but with different results, the first concern is the possible differences in the procedures used. Enzyme linked immunosorbent assays for blood group substances do not work with human polyclonal antisera but do work with MCAs. Some forensic scientists have encountered difficulties using MCAs for traditional adsorption-elution tests. These difficulties lead to a concern about the appropriate use of serologic reagents.

The blood bank reagents used for forensic serology are licensed for typing wet blood but may not be appropriate for forensic applications. Commercial wet typing reagents are used in inhibition studies and adsorption-elution studies for blood banking. The adsorption-elution studies are performed on intact red blood cells or stroma. These reagents have been used in a wide array of tests that are analogous to forensic applications.

Polyclonal reagents do differ substantially from MCAs however. Monoclonal antibodies are a single homogeneous population of antibodies against a single epitope and generally have a higher binding affinity. Thus, though MCAs may work, they may not elute as well as a mixed population of lower affinity antibodies. Monoclonal antibody reagents generally being used were selected for their capacity to agglutinate red blood cells. Thus, they may have more in common with commercial polyclonal reagents than is realized. Some of the new reagents may be better for some tests but not as good for others.

Monoclonal antibody reagents have lot-to-lot consistency that polyclonal reagents lack. Polyclonal reagents consist of a blend of donors, which will change from lot to lot, whereas MCAs are constant in specificity and titer. Since the specificity is more restricted, some properties have already appeared, including the fact that MCA anti-B does not recognize the acquired B structure produced by certain bacteria. Also, the availability of identical reagents creates the possibility of standardization of procedures among laboratories. Finally, it is possible the MCAs will enable testing for specific body fluids using reagents selected because MCAs will only react with structures found in specific tissues.

The new methods using MCAs will mainly be ELISA tests. These tests are significantly more sensitive than existing tests, some of which are already noted for problems, adsorption-elution for example. At very high levels of sensitivity there is the possibility of detecting sex performed several days previously by means of an ELISA P30 assay, or other problems with substrate ABH activity, but the quantitative nature of the tests will allow for the development of trash thresholds, or more accurately, thresholds of substrate and other background activity that do not represent

areas of valid interpretation, much as the background levels for the high-performance liquid chromatography. Thus, the problem of a bed sheet with multiple stains, some fresh and some quite old, may be interpretable when quantitative data can be applied.

One concern with the introduction of new technology is whether the adsorption-elution technique will become obsolete. In many cases, use of adsorption-elution is perceived as more of a problem than a salvation because it is more sensitive than adsorption-inhibition, but interpretation of results not in agreement can be problematic. Adsorption-elution faces many of the problems that ELISA will face if ELISA is to gain acceptance. The increasing awareness of new technology appears to be a valid basis for the re-evaluation of existing methods and procedures.

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