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The Rehydration and Isolation of Leukocytes from Dried Bloodstains

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ABSTRACT: The extraction of leukocytes from dried bloodstains on a variety of surfaces was explored in terms of percentage of recovery. A glycerol-containing solution produced excellent results for many of the surfaces. The outstanding exception was cotton and related cloths, for which a moderate to good result was obtained with a 2-h incubation at 4°C using human serum. A major factor affecting the yield was the blood's ability to form a fibrin network (clotting). In treated or cadaveric blood, 80 to 100% recoveries could be easily obtained from dried crusts or pellets; in blood obtained from a finger prick, a dried crust or pellet yielded only 10 to 25%. A fibrous network containing a large number of entrapped leukocytes was observed under a microscope. This network was identified as fibrin, which acted to collect the cells. The "fibrin-concentrated" leukocytes may be used directly for testing or they can be released by the action of the enzyme plasmin under carefully controlled conditions. Leukocytes may be concentrated from the extraction solution by centrifugation. This step must be done at acidic pH. Leukocyte yields have been high enough to make sex determinations, polymorphic enzyme typing, and human lymphocyte and surface antigen typing feasible in the future.

KEYWORDS: pathology and biology, blood, leukocytes

Although blood has traditionally been important as evidence in forensic science cases, red blood cells (erythrocytes) rather than the white blood cells (leukocytes) have been of interest. This is not surprising since they outnumber their white cell counterparts 500 to 1 and because their heme component serves as the basis for the presumptive and confirmatory tests for blood identification.

Recently, leukocytes have been used with some success to determine the sexual origin of dried bloodstains in cases where the genetic marker phenotypes were the same for both victim and suspect. In these cases the alleged perpetrator, a male, claimed bloodstains found on his clothing and elsewhere were his own and not the victim's, a female. This claim could not be discredited on the basis of available phenotyping, but sexing the disputed blood resolved the issue [*1,2*]. Sexual determination, however, requires the use of a large number of leukocytes so that a statistically valid assessment of chromosome staining can be made.

The use in the forensic sciences of leukocytes derived from dried bloodstains has been limited to sexing and species determination. Their immunological properties (human lymphocyte antigen [HLA] and other surface antigens) as well as enzymatic phenotyping have been largely ignored despite the availability of well-established techniques that could provide

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the forensic serologist with a highly individualized profile, a "serological fingerprint," of any bloodstain of interest. The major hurdle to the use of a number of these powerful tools has been the inability to obtain large numbers of morphologically intact leukocytes from dried stains.

This study was undertaken specifically to address the problem of yield. All of the methods and procedures used were evaluated quantitatively so that optimum conditions were determined. The goal was to provide the forensic serologist with an approach that will consistently give high-quality leukocytes in good yield from all types of dried bloodstains.

Background

A solution designed for the rehydration of dried blood was described as early as 1865 by Roussin [*3*]. It consisted of a mixture of sulfuric acid and glycerin and had a specific density of 1.028 at 15°C. Contemporary scientists modified this solution; Pacini (1872) retained the glycerin, eliminated the acid, and added the chloride salts of sodium and mercury [*4*]. Hayem (1889) eliminated the glycerin and used only the sodium and mercury salts [*5*]. In 1907, Sutherland [*6*] recommended the use of an aqueous solution containing equal parts of gum arabic and sodium salts. In the 1920s a transfer method using a celloidin film came into use. The celloidin was pressed against the dried stain and the leukocytes became entrapped in the matrix [*7*].[2]

The glycerin-containing solutions were rediscovered in 1954 by Kerr [*8*]. Glycerin's ability to protect cellular preparations against low-temperature damage had found use in the laboratory during this time period.

In 1958, Erik Undritz, a Swiss hematologist, who since his discovery of the stability of leukocytes had done extensive studies on dried blood, entered the notorious Jaccound case. Teamed with Pierre Hegg of the Swiss police, he was able to identify very small spots found on Jaccound's clothing and the cord of his ceremonial sword as human blood. He extracted leukocytes from the dried stains with human serum and was able to confirm the origin on the basis of shape and nuclear morphology [*9,10*].

Recently pooled human serum has been used for the rehydration of old bloodstains by an Italian group interested primarily in species identification based on leukocyte morphology. No attempt was made to determine yield or to collect concentrated cells [*11*].

Most recently (1976), the scanning electron microscope has been used as an aid to the identification of dried blood. Under the conditions described, the authors were unable to make a definite identification solely on the basis of morphology, and an additional chemical analysis was needed [*12*].

Materials and Methods

The blood used in all of the major experiments was from one source and was obtained in one of two ways: (1) from a venipuncture using ethylenediaminetetraacetic acid (EDTA) as an anticoagulant or (2) from a finger prick containing no anticoagulant. The leukocyte count was determined before the blood was dried by diluting it 1:20 in a standard leukocyte pipet with Turk's solution (3% acetic acid) and counting on a clinical hemacytometer [*13*].

A typical procedure is as follows: 25 μL of blood were pipetted with a very accurate pipet (3% reproducibility) onto the test surface. The samples were covered (petri dish) and allowed to dry at room temperature (25°C) for at least two days. A drop of rehydration solution (50 μL) was added to the dried blood, and the covered sample was stored at 4°C for between 2 and 12 h, depending on the age of the sample. At the end of this period, the sample

[2]A more complete review of the early work in the reconstitution of dried blood can be found in Dr. Robert E. Gaensslen's soon-to-be-published *Source Book in Forensic Serology, Immunology, and Biochemistry*. We used the author's rough draft as a guide to the early literature.

was diluted with Turk's solution to a final 1 to 20 dilution by using a conical centrifugation tube graduated in tenths of a millilitre. The resulting solution was counted in the same way as the control (fresh blood), and the percentage of recovery calculated. The sample could then be concentrated by centrifugation for 2.5 min at 250*g*.

All chemicals were reagent grade. The plasmin (Lot 118E-0284, Sigma Chemical Co., St. Louis, Mo.) had an activity of 0.04 units/mg. The enzyme concentration was determined spectrophotometrically using an $E_{280}^{1\%} = 17$, in 1*N* sodium hydroxide. Concentrated solutions (10 mg/mL) were stored frozen in a tris(hydroxymethyl)aminomethane (Tris)-lysine buffer, pH = 9.0, because diluted solutions at neutral pH are highly unstable [*14*].

When morphological studies were made, the cells were collected via centrifugation at 250*g* for 2.5 min. The centrifugation medium was either 3% acetic acid, or 0.05*M* citrate or acetate buffer, pH 5.5. After centrifugation the supernatant was carefully removed and the packed cells were resuspended in human (AB) serum or 3.3% bovine serum albumin (BSA), air-dried, and stained with either Wright's stain or orcein. All photomicrographs were taken with a Zeiss photomicroscope, Model 52037. The film used was Kodachrome 135, ASA 25 for color slides, and Kodak Tri-X Pan, ASA 400 for black and white prints.

Results

The results obtained when fibrin-containing blood (a 25-μL sample size was used and the average leukocyte count was 10 000 cells/mm^3) (finger prick) was rehydrated with a solution of 50:50 v/v glycerol/normal saline are presented in Table 1. Recoveries ranged from less than 1% for cotton and gaberdine to 73% for water-repellent cloth. Good recoveries were obtained from wood (45 to 68%) and leather (25 to 30%). When serum was substituted for the glycerol as the extracting solution, good recoveries were also obtained from cotton (20 to 60%).

Unexpectedly low recoveries (5 to 26%) were obtained from plastic and glass in this series of experiments. Previously, very high yields, ranging from 60 to 100%, had been obtained from cadaveric and EDTA-chelated blood on these surfaces. It was apparent that factors other than surface type were acting to influence the leukocyte recoveries. When the solution obtained from finger-prick blood was carefully examined, small, fine clumps were discerned. Microscopic examination of the clumps revealed a large number of leukocytes.[3]

A comparison study was made between EDTA-treated dried blood (defibrinated) and finger-prick blood (containing fibrin) (Fig. 1). Concentration ranges of glycerol/saline from 0 to 100% were explored in terms of cell recovery. Although the maximum recoveries were obtained at the same composition, 50:50 glycerol/saline, representing a 20% improvement for the fibrinated blood and a 27% increase for its defibrinated counterpart compared to normal saline alone, a highly significant difference in total recovery existed between fibrinated and defibrinated bloods at all concentrations. At the 50:50 level, fourfold more leukocytes were recovered from the chelated blood (100% versus 25%).

The reduced yield obtained with the fibrinated finger-prick blood can be attributed to a fibrin network that acts to entrap leukocytes on drying [*15*]. The amount of fibrin formed depends on the drying time, which is related to the thickness of the stain, air movement, and surface area of the stain. Thus a better recovery (26%) was obtained in the case of the thin smear on glass than from the thicker drop or pellet (9%).

Various methods for freeing trapped leukocytes from the fibrin matrix were explored. The effects of pH on leukocyte recovery were studied over a pH range of 3.5 to 9.5 (Fig. 2). The maximum recovery was obtained at pH 5.5 and represented a total of 75 000 cells or 33%. Although a threefold improvement in yield is achieved at this pH over the acidic and basic pH, no real increase has been obtained since the pH of the unbuffered 50:50 glycerol/saline is close to 5.5 (5.8).

[3]K. Hillman, unpublished data.

TABLE 1—*The extraction of leukocytes from dried bloodstains from various sources using (A) 50:50 glycerol/saline and (B) human serum.*

Source of Stain	Total Cells Recovered	% Recovery
A. With 50:50 glycerol/saline		
(1) Wood (scraped)	112 500	44.6
(2) Wood (soaked)	171 000	67.8
(3) Leather (smooth)	85 000	33.6
(4) Leather (rough)	63 125	25.0
(5) Cloth (shiny surface)	183 600	72.7
(6) Wool	31 000	12.4
(7) Gaberdine	...	< 1.0
(8) Cotton	...	< 1.0
(9) Glass (drop)	26 328	9.7[a]
(10) Metal (knife blade)	67 856	25.0
(11) Glass (smear)	70 570	26.0
B. With human serum		
(1) Cotton		
(a)	121 000	41.7
(b)	56 250	19.4
(c)[b]	187 500	64.6

[a]This value varied from 4 to 25%.
[b]The incubation time was 2 h at 4°C.

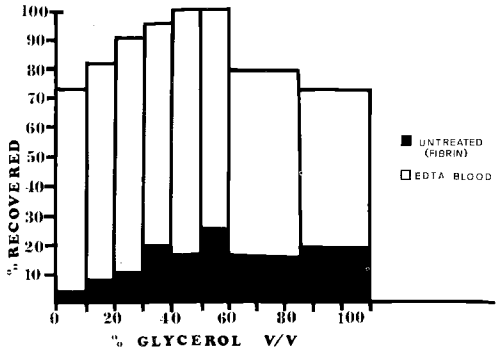

FIG. 1—*The effects of coagulation on leukocyte recovery as a function of glycerol concentration. The fibrin-containing blood was obtained from a finger prick, the EDTA-chelated blood from a venipuncture. Normal saline was used to dilute the glycerol.*

Treatment with high ionic strength salt solutions ("chaotropic" agents), such as 3*M* potassium chloride or urea, was ruled out because of the potential damage to cells. An enzymatic treatment with plasmin (fibrinolysin) was chosen, and results are presented in Figs. 3a and 3b. It is apparent that both curves are a summation of two events: (1) the release of leukocytes from fibrin and (2) the enzymatic lysis of the released cells. It therefore follows that conditions must be carefully controlled so that the maximum recovery can be obtained and that these conditions must be empirically determined for each plasmin preparation. The best results in the experiment cited were for an enzyme concentration of 1.2 mg/mL at pH = 7.6, at 37°C, for 15 min. Shorter times showed unresolved clumps, while times longer than 45 min led to large amounts of cellular debris. Concentrations lower than 0.6 mg/mL gave poor activity because of enzyme instability [*14*]. The total number of leukocytes

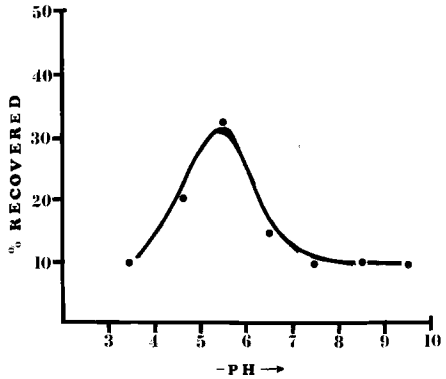

FIG. 2—*The recovery of leukocytes from fibrin-containing dried blood as a function of pH. The 0.05*M *buffers in normal saline were as follows: phthalate, pH 2.5-5.5; phosphate, pH 6.5-7.5; and Tris for pH 8.5-9.5. The buffers were mixed with glycerol to 50% v/v.*

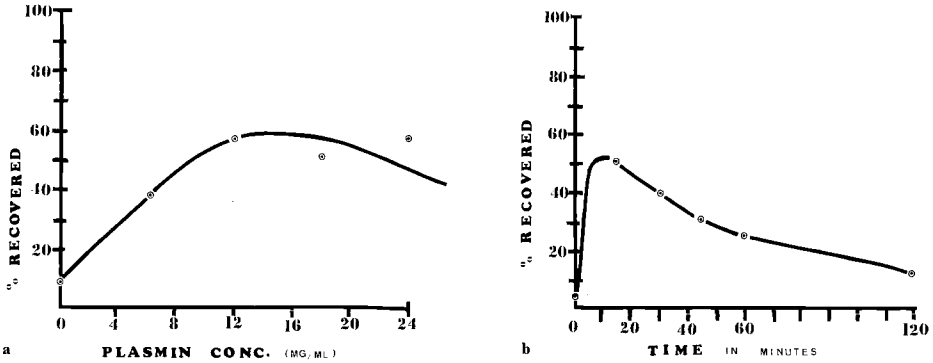

FIG. 3—*The enzymatic release of leukocytes from fibrinated blood. The reaction was run at pH = 7.6 ± 0.1 in 0.068*M *phosphate after the glycerol was removed from the rehydration step, at 37°C, and terminated by diluting with 3% HAC.* (a) *Plasmin concentration versus recovery: the reaction time was 20 min.* (b) *Reaction time versus recovery: the plasmin concentration was 12 mg/mL.*

recovered from 25 μL of dried blood was 167 000 at the maximum for Fig. 3a and 146 000 for Fig. 3b.

The morphological integrity of the recovered cells is as important as high yields and should serve as a criterion in selecting a procedure. Although the preservation of structure generally parallels the recovery determined in the counting stage, moderate changes as well as biochemical changes cannot be detected. Likewise, shifts in the distribution of leukocyte types can go unnoticed. In this respect, the polymorphonuclear cells are much more fragile than the very resistant lymphocyte, and preparations containing large numbers of polymorphonuclear cells and especially eosinophils suggest good procedural handling [*11*].

Most forensic science uses of leukocytes will require a concentration step. This may be accomplished by centrifugation or filtration; in either case, the cells are exposed to mechanical stress. Microscopic examination involves drying and staining, further sources of distortion. A study of the media used during this stage demonstrates the importance of selecting the proper composition. When normal saline was used for rehydration, collection, and drying, cells were distorted beyond recognition. If the collection step was carried out in an acid

media (3% acetic acid), a marked improvement was noted. Some cells were still swollen and broken, but many morphologically intact neutrophils and lymphocytes were then discernible. The use of a 50:50 glycerol/saline solution during rehydration yielded a slightly better preparation. However, the best results were obtained when an acid solution, pH 5.5, 0.05*M* acetate or citrate, containing 3.3% BSA is used for all three stages (rehydration, collection, and drying).

These morphological and quantitative observations led to the construction of a flow chart (Fig. 4). When the treatment described in that chart is applied to the dried bloodstains from

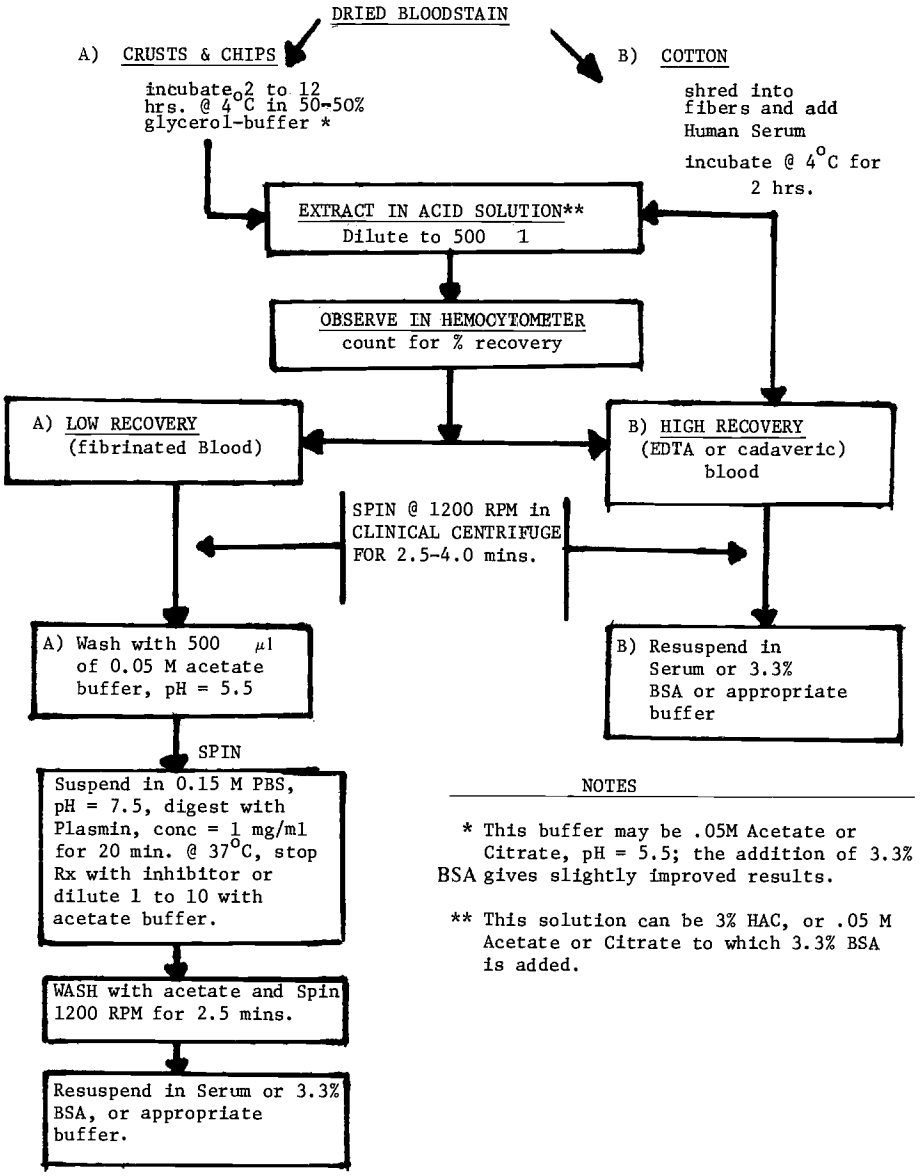

FIG. 4—*Suggested procedure for extracting leukocytes from dried bloodstains.*

most surfaces, high quality leukocytes in large yields can be obtained. An example of the number and condition of leukocytes recovered in this way from dried stains is given in Fig. 5.

The ability to extract leukocytes from dried bloodstains depends to some extent on the age of the bloodstain. Stains up to three months old are easily extracted, and the incubation period at 4°C can be reduced to 2 h. Stains from three to eight months old require a longer incubation period; 12 h at 4°C has been found satisfactory. Bloodstains older than nine months are extracted with difficulty, but the longer 12-h incubation is appropriate.

The extraction of leukocytes from cotton cloth is difficult but not impossible. Recently, we have gotten moderate to good results (see Table 1) by cutting the stained area into threads and incubating them for 2 h with human (AB) serum. The morphology of the cells obtained after an acid centrifugation was excellent.

The choice of glycerol-containing solutions was based on glycerol's known properties of preserving cells during freezing [*16*], its competition with other polyols to interact with surface glycoproteins of cells [*17*], and the need to attempt to slow the entry of water into the dried cells and minimize rehydration damage.

Our results show that glycerol solutions provide some protection for rehydrating cells, improve morphological integrity, and give increased extractability for select surfaces.

The step in which leukocytes are concentrated is critical, and without an acid suspension medium great amounts of cellular damage are incurred. The exact action of the acid solution is unknown. It may act to strengthen the cells (tanning), thus minimizing the effects of mechanical stress. At present, we are investigating the possibility of substituting a 6% v/v formalin solution for the acid in this step. Surface antigens are known to be preserved by this treatment (bacterial attenuation), and the hydrolytic enzymes associated with neutrophils are inactivated, thus removing a potential source of cellular damage while increasing the

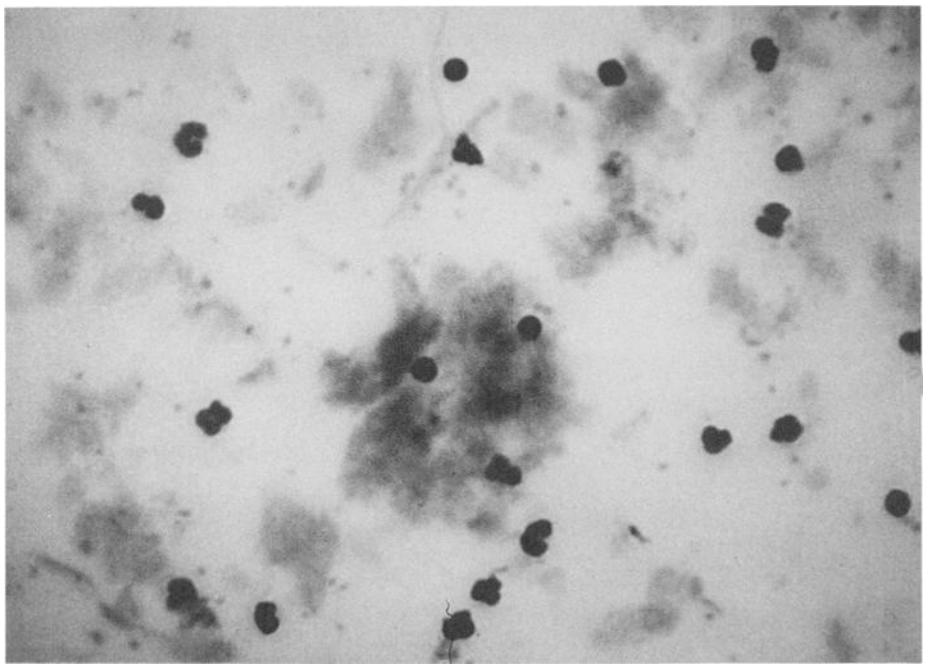

FIG. 5—*The morphology of leukocytes recovered from 14-day-old dried blood. The rehydration was carried out in a 50:50 glycerol/saline buffer. The buffer used in the collection step was pH = 5.5, 0.05*M *citrate, containing 3.3% BSA. The cells were suspended in human serum when slides were made.*

mechanical strength of the cell. However, this type of preparation may not be suitable for enzyme phenotyping.

The procedure recommended in Fig. 5 provides a step in which a quantitative evaluation of leukocyte recovery can be made. In general our approach has involved a quantitative basis for the evaluation of every test procedure, a criterion often missing in past studies. However, we have not evaluated many of the solutions and procedures put forth in the literature, but we recommend the procedure described in the Materials and Methods section as a tool for further investigation.

The fact that fibrin entraps large numbers of leukocytes is not surprising in light of the physiology of blood clotting. Since fibrin acts to collect leukocytes, and has been so used in the past [*15*], it may not be necessary to release the cells from the entrapping matrix for tests such as enzyme phenotyping or for the determination of the surface HLA antigens. If this strategy is not appropriate, carefully controlled enzymatic treatment can be employed. Some degree of cellular damage is inherent, but it can be minimized by controlling time and enzyme concentration. The use of highly purified plasmin from a human source was not investigated, but it may eliminate some of the nonspecific proteolytic activity of the commercial nonhuman preparations.

Cotton and other types of cloth present a special challenge. The use of glycerol solutions is inadequate, and only low yields have been obtained (Table 1). However, moderate to good results have been obtained when human serum, as suggested by the work of Undritz and Hegg in the Jaccound case [*10*], is substituted for the glycerol mixtures, and no more than 2 h of incubation is used. We are still in the process of evaluating and modifying this approach, and only preliminary results are given in this report.

In general, we have shown that it is now possible to extract and collect leukocytes from dried bloodstains in yields high enough to make several important forensic serological and cytogenetic tests feasible, including (1) sex determination; (2) typing of the polymorphic enzymes found associated with the leukocytes such as phosphoglucomutase (third locus), cytidine deaminase, and α-L-fucosidase; and (3) histocompatibility antigen (HLA) profiles.

Of these, histocompatibility antigens are by far the most promising and important. They can theoretically provide the forensic serologist with a better identification of a blood source. The next step, therefore, is to apply this wealth of knowledge to the area of forensic serology, where it is much needed and can serve an important role. We think that with the establishment of procedures to extract leukocytes from dried stains an important first step towards this goal has been taken.

Acknowledgment

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