IDENTIFICATION and GROUPING of BLOODSTAINS

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In the time that has elapsed since the discovery of the ABO system by Landsteiner, knowledge in forensic serology has expanded tremendously. Excluding hormones and some temporary pathological factors, more than 160 antigens, 150 serum proteins, and 250 cellular enzymes have been found in human blood. Three classes of blood constituents have been chosen by serologists for the analysis of blood samples. They are: (1) The group specific antigens; (2) the cellular enzymes and proteins; and (3) the serum enzymes and proteins.

The Group Specific Antigens

The group specific antigens are macromolecules on or off the blood cell surface which contain, as part of their structure, specific antigenic sites recognizable by their reactions with specific antibodies.

Evidence is available which suggests that at least some of the group specific antigens are present on all three types of the cellular elements of blood—erythrocytes, leukocytes, and platelets; other antigens seem to be cell type specific.

Erythrocytes, or red blood cells, are biconcave discs that are manufactured in the bone marrow. In mammals they lose their nuclei before entering the circulatory system. The average normal red blood cell count is 5.4 million cells/µl in men and 4.8 million cells/µl in women. Each human red cell is about 7.5 µm in diameter and 2 µm thick. The membranes of human red cells contain a variety of antigens called agglutinogens. In the human there are normally 4,000-11,000 white blood cells/µl of blood. Of these, the granulocytes or polymorphonuclear leukocytes are the most numerous. Most granulocytes contain neutrophilic granules (neutrophils); a few contain granules that stain with acid dyes (eosinophils), and some have basophilic granules that stain with basic dyes (basophils). The other white blood cell types found normally in peripheral blood are lymphocytes, cells with large round nuclei and scanty cytoplasm, and monocytes, cells with abundant cytoplasm and kidney-shaped nuclei. Platelets are small, granulated bodies, 2–4 µm in diameter. There are about 300,000 platelets/µl in circulating blood.

According to their incidence of occurrence, antigens can be classified as either a primary blood group system or a secondary blood group system.1
Primary Blood Group Systems

The primary blood groups are those well known and defined blood group systems. This group includes the following systems: ABO, MNSs, P, Rh, Diego, Dombrock, Duffy, I, Kell, Kidd, Lewis, Lutheran, Xg, and Yt.

Secondary Blood Group Systems

The secondary blood group systems are antigens which are either forerunners of new systems or products of rare mutant genes in existing ones. They also include high incident antigens which have not been related to known major systems. This group includes the following antigens: Auberger, August, Batly, Becker, Biles, Bishop, Bg\(^a\)Bg\(^b\), Box, Cavaliere, Chido, Chr\(^a\), Cost, Dp, El, En, Gerbich, Griffith, Good, Heibel, Ho, Ht\(^a\), Jn\(^a\), Kamhuber, Lan, Levay, Ls\(^a\), Mariot, Orris, Ot, Raddon, Dadin, Rm, Stobo, Swann, Torkilden, Traversu, Vel, Ven Webb, Wright and Wolfsherg.

Some of the red cell antigens, such as A and B and I, have been shown to be present on both leukocytes and platelets. Nevertheless, isoantigens representing the majority of blood group systems such as Rh, Duffy, MN, and so forth, seem to be unique antigenic characteristics of the red blood cell. There are also some isoantigens which appear to be unique to leukocytes and platelets. According to the present state of knowledge, there are at least four established leukocyte group antigen systems: HLA-A, HLA-B, HLA-C, and HLA-D.

Red blood cells, like other cells, shrink in solutions with an osmotic pressure greater than that of normal plasma. A 0.85% NaCl solution is isosmotic with plasma. In solutions with a lower osmotic pressure, the cell becomes aspherical rather than disk-shaped. They begin to hemolyse when suspended in 0.48% saline, and in 0.33% saline hemolysis is complete. The hemolyzed red cells release their contents into the solution. More than 250 proteins and enzymes have been found in the red cell, mostly in the soluble portion of the hemolysates of erythrocytes. The predominant erythrocyte protein is hemoglobin (Hb). More than one hundred variants of hemoglobin have been described. Many red blood cell enzymes show genetic polymorphisms, as do a significant number of proteins found in blood serum and other body secretions.

Many of these markers persist in their biological activity even after blood has dried. Therefore, blood can potentially distinguish one person from another. It is estimated that there are well over 500 billion possible known blood group phenotypes, and other undiscovered blood...
group systems undoubtedly exist. In examining blood evidence, the questions which the forensic serologist must answer are: Is it blood? If it is blood, Is it human? If it is human, to what groups does it belong? Is it possible to obtain further information towards individualization? Many techniques have been developed to address the above mentioned questions. The present state of blood evidence examination is summarized in Figure 7-1.

This chapter is concerned only with the identification and grouping of bloodstains. The next chapter will discuss in detail the biochemistry and forensic significance of cellular and serum enzymes and proteins. This discussion does not pretend to be a comprehensive one on the subject. We remain virtually at the infant stage of the forensic serology field.

**FIGURE 7-1** The present approaches to the individualization of bloodstains,
THE IDENTIFICATION OF BLOODSTAINS

The identification of blood is the first and most important step in the examination of a suspected stain. Unless we are certain that a questioned stain is, in fact, a bloodstain, any further analysis will be meaningless. There is considerable literature concerning the identification of bloodstains.7-15 In general, the methods are based upon the presence of blood cells or compounds which are characteristic of blood, such as (a) erythrocytes and leukocytes, (b) blood serum proteins, and (c) hemoglobin and its derivatives.

Microscopic Methods

A number of techniques have been reported for microscopic examination of erythrocytes and leukocytes in bloodstains.16-18 The results obtained with these methods are much affected by the conditions of bloodstains. Aging, environmental factors, or heating can considerably alter blood cells and make it difficult to produce interpretable and reliable results. The microscopic examination of cells found in a fresh stain can not only aid identification of a particular stain as, in fact, blood, but also may reveal other information as well. For example, nucleated red cells may indicate the presence of vertebrate blood; chromatin bodies found in leukocytes can be used to determine the sexual origin of a sample; sickle shaped erythrocytes may indicate that the blood sample originated from a person having sickle cell disease.

Many different techniques have been proposed for the microscopic examination of bloodstains.19-23 In general, the procedure involves two steps: (1) reconstituting the blood cells with a solution in an effort to restore their original shape; and (2) differential staining of the blood with histological stains. One may proceed as follows:

1. Put a fragment of fresh blood crust on the center of a clean slide.
2. Add a drop of the following solution on the crust and mix gently until the crust is dissolved:
   - Albumin 20%
   - Glycerol 20%
   - NaCl 0.85%
   - in GDW (glass distilled water)
3. Place slide in a moist chamber for two hours at room temperature.
4. Prepare a thin film of smear of the mixture.
5. Rapidly air-dry the slide.
6. Without previous fixation, add two drops of Wright’s stain solution, and wait for two minutes. (Wright’s stain is called a polychromatic stain. It is a methyl alcohol solution of methylene blue, eosin, and a basic dye. Wright’s stain is commercially available.)
7. Add two drops of buffer solution on the slide.
   
   Buffer composition (pH 6.4)
   $\text{KH}_2\text{PO}_4$ 0.66gm
   $\text{Na}_2\text{HPO}_4$ 0.25 gm
   GDW to 100 ml

8. Mix the buffer with the staining solution and allow the mixture to remain for four minutes.
9. Float the stain off with a gentle stream of distilled water.
10. Dry the slide by evaporation.
11. Perform a microscopic examination.

Identification of bloodstains by means of chemical methods is based on the detection of hemoglobin and its derivatives. Such tests can be classified under one of two headings: catalytic tests and crystal tests.

Catalytic Tests

All catalytic blood tests depend upon an oxidation reaction in which an oxidant, for example, hydrogen peroxide, oxidizes a colorless material such as phenolphthalein or benzidine to a colored one. Alternately, 3-amino-phthalhydrazide (luminol), a colorless material, can be oxidized to a product which luminesces. Some of the reaction schemes are shown in Figure 7—2.

The heme group of hemoglobin exhibits a peroxidase-like activity which may catalyze the breakdown of hydrogen peroxide. The majority of tests which have been devised for the forensic identification of blood are based upon the peroxide-mediated oxidation of guaiacum, $\text{glu}$, benzidine, $\text{leucomalachite green}$, $\text{phenolphthalein}$, $\text{o-tolidine}$, $\text{luminol}$, $\text{tetramethylbenzidine}$, $\text{fluorescein}$, $\text{eosin hydrate}$, $\text{dimethylaniline}$, $\text{rhodamine}$, $\text{aminopyrine}$, $\text{alizarine}$, $\text{hydrazine}$, $\text{HgI}_2$, and $\text{KI}$. Most of these have only historical interest at the present time.

The tests most commonly employed are benzidine, phenolphthalein, $\text{o-tolidine}$, $\text{leucomalachite green}$, $\text{luminol}$, and $\text{tetramethylbenzidine}$. All of these are highly sensitive to a minute trace of hemoglobin and its derivatives, but all suffer from interference by other materials, such as catalase, peroxidase, cytochromes, strong oxidizing agents, and metallic salts.

The procedure for performing these tests follows.

PREPARATION OF REAGENTS

Benzidine solution (Benzidine is a hazardous and carcinogenic substance)

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<th>Component</th>
<th>Quantity</th>
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<tr>
<td>Benzidine</td>
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<td>Ethanol</td>
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<tr>
<td>Glacial acetic acid</td>
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FIGURE 7—2 The reaction schemes of catalytic color tests.

**Phenolphthalein solution**

*Stock solution*
- phenolphthalein 2 gm
- potassium hydroxide 20 gm
- distilled water 100 ml
The mixture is refluxed with 20 gm of powdered zinc for two hours until the solution becomes colorless. The stock solution should be stored in a dark bottle and refrigerated, with some zinc added to keep it in the reduced form.

*Working solution*
- phenolphthalein stock solution 20 ml
- ethanol 80 ml

**o-tolidine solution**
- o-tolidine 1.6 gm
- Ethanol 40 ml
- Glacial acetic acid 30 ml
- Distilled water 30 ml
Leucomalachite Green

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<tr>
<td>Sodium perborate</td>
<td>3.2 gm</td>
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<tr>
<td>Leucomalachite Green</td>
<td>0.1 gm</td>
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<tr>
<td>Glacial acetic acid</td>
<td>66 ml</td>
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<td>Distilled water</td>
<td>33 ml</td>
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Tetramethylbenzidine

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<td>Tetramethylbenzidine</td>
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<td>Glacial acetic acid</td>
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Luminol

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<tr>
<td>3-aminophthalhydrazide</td>
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<td>Sodium carbonate</td>
<td>0.5 gm</td>
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<td>Sodium perborate</td>
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<td>Distilled water</td>
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TESTING PROCEDURES

The test itself may be performed by one of the following methods:

1. If the stain is on a hard surface, such as a knife, glass, and so on, remove by scraping and dissolving in a few drops of saline. Add two drops of reagent to two drops of the stain extract. Leave the mixture for thirty seconds. Then add two drops of fresh 3% hydrogen peroxide. A color change indicates a positive reaction.

2. If the stain is on cloth or other similar material, pull a thread or cut a small piece of the cloth from the stain and soak in a few drops of saline. Add two drops of reagent to two drops of the stain extract. Leave the mixture for thirty seconds at room temperature. Then add two drops of fresh 3% hydrogen peroxide. A color change indicates a positive reaction.

3. If the stain is visible and absorbed into an object, rub with a clean cotton swab or filter paper and moisten with distilled water. Add two drops of the reagent to the cotton swab or the filter paper. Apply a similar amount of the 3% hydrogen peroxide 30 seconds later. A color change indicates a positive reaction.

4. If the stain is mixed with soil or debris remove first by dissolving the stain in saline and then concentrate by the elution technique recommended by Fiori. Cut a long strip of Whatman No. 1 paper, 1 x 25 cm, to a point at one end. Dip the other end in the material soaked with saline, and concentrate the solution by capillary action on the upper point of the paper. Then air-dry the paper. Carry out color reaction by spraying the paper with the proper reagent.

5. If the stain is invisible it may be brought out by spraying the suspected area with luminol reagent. Remove and concentrate by the filter paper technique devised by Kirk. Place the suspected stained area on a piece of sponge, moistened with water; place a strip of filter paper on top of the stained area, Put a glass plate on the filter paper over the stain with a weight on it to keep it firmly in contact with the cloth and sponge. Suspend the end of the strip a little above the horizontal. The blood will be transferred upward and concentrated at the end of the filter paper by capillary action. Carry out the color reaction by adding or spraying the reagent onto the paper.
6. If the sample is in a very diluted solution it may be concentrated by either lyophilization or by using the filter paper concentration technique suggested by Kirk and then tested for the presence of peroxidase activity.

7. If the stain is aged or denatured dissolve in 2 N potassium hydroxide solution and then neutralize with 2 N HCl before analysis.

**INTERPRETATION OF RESULTS**

Color catalytic tests are very sensitive, but not specific. The positive color test alone should not be interpreted as positive evidence of blood. However, a negative result is proof of the absence of detectable quantities of heme or its derivatives.

When a positive result is obtained, it is necessary to consider carefully whether the test result could have been given by something other than heme from blood. The specificity of various catalytic reagents has been studied extensively. A false positive reaction may be defined as any positive reaction given by any substance other than bloodstains. These substances may be conveniently divided into three groups.

1. **Chemical oxidants and catalysts** Copper and nickel salts are the chemicals which most frequently show false positive reactions. Others are rust, formalin, potassium permanganate, potassium dichromate, some bleaches, hypochlorite, iodine, and lead oxides. Phenolphthalin gives positive results with oxidizing compounds such as copper, potassium ferricyanide, and nickel and cobalt nitrates, and some sulfocyanates. Luminol reacts with cupric ion and some compounds of copper, cobalt and iron. Potassium permanganate and hydrated sodium hypochlorite also give a positive luminol reaction.

2. **Plant sources** Vegetable peroxidases are the most important class of substances which show false positive reaction with chemical color tests. The following plant tissues may react with the benzidine or phenolphthalin reagents and be mistaken for blood: apple, apricot, bean, blackberry, Jerusalem artichoke, horseradish, potato, turnip, cabbage, onion and dandelion root.

   Plant material such as horseradish, beetleaf, garlic, cabbage, tomato, and cucumber reacts positively with tetramethylbenzidine.

3. **Animal origin** The following substances of animal origin may give false positive reactions with benzidine reagent: pus, bone marrow leukocytes, brain tissues, spinal fluid, intestine, lung, saliva, and mucus.

   The false positive reaction caused by material other than blood can be eliminated by the following methods:

   1. **Chemical oxidants and catalysts** The behavior of chemical oxidants is quite different from that of blood. Chemical oxidants will give a discoloration before the addition of the hy-
drogen peroxide or sodium perborate. Therefore, a false positive reaction can be distinguished by use of the two solution test procedure.

A. Add two drops of reagents to two drops of sample extracts.
B. Leave the mixture at room temperature for thirty seconds.
C. If discoloration has occurred, it is possible that chemical oxidants are present in the extract.
D. If no color is developed, add two drops of 3% \( \text{H}_2\text{O}_2 \) to the mixture.
E. A color change indicates the presence of blood or other peroxidases.

2. Plant peroxidases

Heme is stable at high temperatures, while the plant peroxidases are rapidly deactivated. Therefore, heating the sample stain or extract to 100°C for five minutes will differentiate the plant peroxidases from blood sources. Also, it has been found that under electrophoretic conditions using a sodium barbital : barbituric acid : calcium lactate buffer system (0.5 gm 3.5 gm 0.5 gm in 1000 ml) some portions of a bloodstain extract move toward the anode, while the main portion of hemoglobin remains at the origin. On the other hand, most of the plant peroxidases (except tomato) move toward the cathode.42

3. Other substances of animal origin

Microscopic examination of the specimen will distinguish the tissue, pus, and other substances of animal origin from blood.

Crystal Tests

There are several crystal tests that are considered by most authors as a confirmatory test of bloodstains.43-49 All crystal tests are based upon the formation of hemoglobin derivative crystals such as hematin, hemin, and hemochromogen.

HEMATIN (TEICHMANN) TEST50

In 1853, Teichmann reported that by gently heating blood with glacial acetic acid in the presence of salts, crystals were formed. The positive result is due to a combination of a halogen with ferriprotoporphyrin. The crystals are rhombic or prismatic in shape, dark brown in color, and about 10 microns in size (see Figure 7—3a).

**Reagent**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity</th>
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<tbody>
<tr>
<td>potassium chloride</td>
<td>0.1 gm</td>
</tr>
<tr>
<td>potassium bromide</td>
<td>0.1 gm</td>
</tr>
<tr>
<td>potassium iodide</td>
<td>0.1 gm</td>
</tr>
<tr>
<td>glacial acetic acid</td>
<td>100 ml</td>
</tr>
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</table>

**Testing procedure**

1. Place a small amount of the questioned blood material on a slide. Add a cover slip.
2. Let a drop of reagent flow under the cover slip and come in contact with the dried crust. Avoid introducing any air bubbles.
3. With a very small flame, gently heat the slide until bubbles begin to form under the cover slip.
4. Microscopic examination will reveal the presence of hematin crystals.

Many modifications of the Teichmann test have been suggested by various authors. Sutherland reported that overheating, and especially the combination of rust and overheating, interferes with the test. Wood reported any substance or condition which causes hemo-