The Principles of Eosinophil Diluents

By R. S. Speirs, Ph.D.

The determination of the number of eosinophil cells and the change in the number of these cells in the peripheral blood following injections of ACTH or epinephrine is becoming a routine clinical procedure in many hospitals and laboratories. Rud1 reported in 1947 a thorough investigation of the techniques of performing total eosinophil counts and noted the variations in these cells throughout the day, week, month and year. However, the significance of the short term variation of eosinophils was not understood until Thorn's group reported the eosinopenic response to ACTH and epinephrine. They were able to incorporate this response into a clinical diagnostic test for adrenal cortical hormone secretion.2-4 In experimental animals these cells are also used to indicate quantitatively the amount of stress, ACTH and adrenal cortical hormones applied.5-8

This increased interest in the eosinophils has stimulated several investigators to evaluate the various procedures for enumerating these cells.1, 5, 8-11 The blood film procedure involves a great variation in cell counts and is impractical especially when the eosinophil counts are low.1, 5 It seems to be generally agreed that provided proper precautions are taken, the direct counting procedure is preferable for rapid determinations and is more accurate. On the other hand, the direct procedures also have inherent difficulties that should be known to everyone using them. This paper is a report on the materials that make up the various eosinophil diluents and the action of these substances on the blood cells.

Eosinophil Diluting Fluids

A. Acetone Diluents

1. Danger's original solution12
   Aqueous Eosin 0.1 Gm.
   Acetone 10 cc.
   Distilled Water 100 cc.

2. Modification A (Thorn9)
   Aqueous Eosin 0.1 Gm.
   Acetone 5 cc.
   Distilled Water 95 cc.

3. Modification B (Rud9)
   Magdala Red (possibly phloxine) 0.02 Gm.
   Acetone 12 cc.
   Sodium Carbonate 10% 1 to 2 cc.
   Distilled Water 90 cc.

4. Modification C (Speirs)
   Phloxine B 0.02 Gm.
   Acetone 15 cc.
   Distilled Water 85 cc.
   Detergent (Alconox) 0.02 Gm.
B. Propylene Glycol Diluent

Modified Randolph Diluent
g

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
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<tbody>
<tr>
<td>Phloxine</td>
<td>0.05 Gm.</td>
</tr>
<tr>
<td>Propylene Glycol</td>
<td>50 cc.</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>50 cc.</td>
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C. Urea Diluent (Manners)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
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<tbody>
<tr>
<td>Urea</td>
<td>50 Gm.</td>
</tr>
<tr>
<td>Trisodium Citrate</td>
<td>0.06 Gm.</td>
</tr>
<tr>
<td>Phloxine</td>
<td>0.1 Gm.</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>100 ml.</td>
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With the above diluents, 1 part of blood is mixed with 20 parts of diluent, usually in a white blood cell pipet. If proper precautions are taken other proportions such as 1 to 10 or 1 to 100 may be used. The acetone solutions must be kept stoppered to prevent loss of acetone by evaporation. It is usually convenient to make up stock solutions to which acetone may be added just before using.

At the present time there are a number of different types of slides available which may be used for counting eosinophils. In general, the error of a determination is proportional to the square root of the number of eosinophils counted, therefore large chambers having a depth of 0.2 ml. are preferable. The Fuchs-Rosenthal slide has been commonly used although recently an improved slide specially designed for counting eosinophils has been described.

The compositions of the eosinophil diluents is based upon the specific staining properties of the eosinophil granules and the relatively increased resistance to lysis of eosinophil cells over other white and red blood cells.

A. Acetone Diluents

The acetone diluents consist of the following materials:

1. An acid dye, such as eosin or phloxine. This is used to stain the eosinophil granules.

2. Water. This is mixed with the blood to produce a swelling and rupturing of the blood cell membranes. The eosinophils are more resistant to lysis than other blood cells.

3. Acetone. This has an inhibiting effect on the lytic action of water and the effect is proportional to the concentration used:

   0 to 10 per cent solutions: All the RBCs lysed and most of the WBCs ruptured. Membranes of eosinophils usually ruptured on standing.

   10 to 20 per cent solutions: All the RBCs lysed, most of the WBCs weakened or ruptured. Eosinophils do not rupture on standing.

   20 to 50 per cent solutions: Some RBCs not lysed. All the WBCs intact.

   50 per cent solutions: RBCs and WBCs both intact.

4. Detergent. This is added to reduce the surface tension of water. It permits more rapid mixing of blood and diluent and increases the rate of staining of the eosinophils.

5. Small amounts of an alkaline solution will increase the lytic action of water especially on the RBCs and neutrophils. It also increases the staining rate of eosinophil granules, possibly by affecting the permeability of cell membranes.

6. In some cases a small amount of diethylene glycol or propylene glycol may
PRINCIPLES OF EOSINOPHIL DILUENTS

be added to the diluent to increase its viscosity. This has an advantage in situations where the acetone may evaporate rapidly.

It may be seen that the dye, water and acetone make up the chief ingredients of this type of diluent. The lytic action of water varies depending upon the proportions of water to blood. Complete lysis of the RBCs does not always occur when dilutions of 1:10 are used. However at 1:20 and higher dilutions, complete lysis occurs. The amount of acetone added to the water depends in part upon the ratio of diluent to blood. Approximately a 15 per cent solution is optimal for normal human blood in a 1:20 dilution, but the amount may be varied depending upon the fragility of the cells. The criterion of a good eosinophil diluent is a solution which will produce the following:

(1) The eosinophil cells will stand out as distinct units with unruptured membranes and granules stained for easy identification.
(2) The WBCs (except the eosinophil) should be seen only as ghost cells or pale cells with wrinkled or broken cell membranes.
(3) There should be no RBCs or precipitated dye present.

The following chart may be helpful in overcoming difficulties in the staining and breakdown of blood cells when the acetone type of diluents are used:

<table>
<thead>
<tr>
<th>Difficulty</th>
<th>Cause</th>
<th>Cure</th>
</tr>
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<tbody>
<tr>
<td>Cell membrane of eosinophils broken. Few or no pale WBCs in background.</td>
<td>A. Acetone concentration too low or B. Diluent may be too alkaline.</td>
<td>A. Increase Acetone or B. Decrease amount of alkali.</td>
</tr>
<tr>
<td>All WBCs distinct. A few RBCs usually seen. Eosinophil granules poorly stained or require a long time to stain properly.</td>
<td>Acetone concentrations too high.</td>
<td>A. Decrease Acetone or B. Add a few drops of a 10% sodium carbonate solution.</td>
</tr>
<tr>
<td>Incomplete lysis of RBC with a great deal of precipitated material in the background.</td>
<td>Solution not alkaline enough. This often occurs when 1:10 mixtures of blood and diluent are used.</td>
<td>A. Add a few drops of 10% sodium carbonate solution or B. Dilute blood 1:20.</td>
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<tr>
<td>Clumping of cells.</td>
<td>A. Clotting started before blood was diluted or B. Citrated blood mixtures will clump on standing.</td>
<td>Avoid using blood which has been exposed to air for more than a few seconds.</td>
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In practice when using acetone diluents, fresh blood is taken as it oozes from the wound. The blood should flow freely without squeezing and it should not be exposed to air for more than a few seconds. The pipet is wiped free of excess blood and lightly touched on the tip to draw the blood down to the exact marking on the pipet stem. Care should be taken to prevent blood from leaking out of the pipet especially when it is first put in the diluent. The pipet is rotated between the fingers as the diluting fluid is drawn up into the bulb. The blood mixture is
shaken for approximately 10 seconds, and then loaded immediately into the
counting chamber after the usual procedure of discarding the first 3 drops. The
loaded chamber should stand for at least 3 minutes to permit lysis, staining and
settling of the cells. The slides may be counted several hours after loading providing
care is taken to reduce evaporation by keeping them in a moist atmosphere.
This may be done by moistening the inside of a slide box, or similar container and inverting it over the slide.

Cells are most recognizable and easiest to count at a magnification of 150
times and a field of vision which includes a one square millimeter area. This is
accomplished by using a 15X wide field or hyperplane ocular and a 16 ml. objective. It is sometimes useful to have a yellow filter over the light source (Watten G # 15).

B. Propylene Glycol Diluents

This diluent is a modification of a solution developed by T. G. Randolph. It is a particularly useful diluent when speed is not important or when citrated blood is sampled over prolonged periods. The propylene glycol solution is isotonic and does not rupture cell membranes. When equal parts of propylene glycol and water are mixed with blood, usually in a 1:20 dilution, the RBCs fade away, while the white blood cells remain distinct. Eosin or phloxine may be added to stain the eosinophil granules and to differentiate the cells. In Randolph's original solution, methylene blue was added to stain the nuclei of all the cells. Recently it has been suggested that a 0.1 per cent sodium carbonate solution may also be added to aid in differentiating the eosinophils by weakening the membranes of the other WBCs.

This diluent is a viscous solution requiring 15 minutes or longer for adequate settling and staining of the cells. Counting is slower, chiefly because of the presence of other cells in the counting field.

The substitution of urea for propylene glycol or acetone has recently been suggested by Manners. He was able to obtain satisfactory eosinophil counts after allowing the pipet to stand for 30 minutes before shaking and counting.

SUMMARY

The various eosinophil diluents have been outlined and the action of each component discussed. A chart of difficulties often experienced with acetone diluents has been presented along with possible causes and cures.

REFERENCES

3 — and —: Metabolic changes in man following adrenal and pituitary hormone administra-
4 HILLS, A. G., FORSHAM, P. H. AND FINCH, C. A.: Changes in circulating leukocytes in-
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