An Exact Method for the Chamber Count of Eosinophils in Capillary Blood and Its Application to the Study of the Diurnal Cycle

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With the technical assistance of ELEANOR D. HART and DORIS K. DELANOY

The purpose of this paper is to describe a more accurate method for counting the circulating eosinophils. In addition to achieving increased accuracy and reproducibility, the method permits use of capillary blood obtained by finger puncture and allows delay up to several days between collection of blood and actual performance of the count. As an example, the method is applied to a study of diurnal fluctuation of normal subjects and patients.

General Principle

Blood from a skin puncture is taken into a capillary tube containing a dry mixture of double oxalate, saponin and "yellowish" eosin. The saponin hemolyzes the erythrocytes of the oxalated blood, while the eosin stains the eosinophilic granules, for which it has great affinity. The entire amount of blood contained in the capillary tube is transferred to a counting chamber, in several relays, until all the material is exhausted.

Materials and Method

Capillary Tubes

Glass tubes with a length of about 7.6 cm. and an inner diameter of 0.8 mm. are satisfactory. Somewhat shorter tubes may be used. It is not necessary that the bore of the glass tubes be absolutely uniform. A bore smaller than that given would retard the flow of the blood through the tube, and a larger one would permit excessive evaporation of water over a long period.

Saponin

There are several types of saponin varying in hemolytic activity on the market. Merck's Pure Saponin was found to be eminently satisfactory as a hemolyzer, but it is no longer available. Among currently available products Penick's Saponin* most closely approaches the Merck product in hemolytic activity.

Because of this variability in the potency one must test the activity of each lot of saponin. The following method is used to determine the hemolytic activity and consequently the concentration of saponin which is to be employed in the preparation of the capillary tubes:

Prepare aqueous solutions of the saponin in decreasing concentrations: 1.0 per cent, 0.8 per cent, 0.6 per cent, 0.4 per cent, 0.2 per cent.

Add 0.2 ml. of each of these solutions to a small test tube, and place in an oven at a temperature of 40 to 50 C. Remove as soon as dry.

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* Obtainable from S. B. Penick & Company, New York, N. Y.
After the tubes have cooled add to each 0.2 ml. of blood collected in double oxalate. Mix thoroughly with the saponin and then at intervals of about 5 minutes prepare smears from each tube and observe microscopically the degree of hemolysis. The tubes may first be screened macroscopically, so that only those in which hemolysis is grossly apparent are submitted to microscopic examination.

With the Merck Saponin in a concentration of 0.6 per cent the bulk of the erythrocytes are hemolyzed within the first few minutes and hemolysis is complete by the end of 15 minutes. The same result obtains with the use of Penick's Saponin in a concentration of 0.8 per cent to 1 per cent.

Since the volume of blood placed into each test tube is approximately equal to that of the aqueous solution of saponin before drying, the concentration of saponin in the blood is similar to that in the original aqueous solution. Likewise, as it will be pointed out later, the saponin is introduced into each glass capillary tube in such quantity that the concentration in the sample of blood will be approximately that indicated as optimal by the assay for hemolytic activity.

Double Oxalate

A mixture of potassium and ammonium oxalate is used. The usual concentration employed for anticoagulant effect is 200 mg. of the monohydrate crystals per 100 ml. blood. However, while this is enough to prevent coagulation, it is not enough to permit easy flow of the blood within the rather narrow lumen of the tube. The smallest concentration found to be effective is 500 mg. per 100 ml. blood. Hence, the oxalate is introduced into the glass capillary tubes in such concentration that the level in the sample of blood will be close to 500 mg./100 ml.

Eosin-Y

Tests have shown that for good staining of eosinophils in the presence of saponin the concentration of eosin in the blood must be 1:500.

Preparation of the Capillary Tubes

Solution A

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saponin (Penick)</td>
<td>0.8 Gm. (subject to assay)</td>
</tr>
<tr>
<td>Potassium oxalate, monohydrate</td>
<td>0.2 Gm.</td>
</tr>
<tr>
<td>Ammonium oxalate, monohydrate</td>
<td>0.3 Gm.</td>
</tr>
<tr>
<td>Distilled water to</td>
<td>100.0 ml.</td>
</tr>
</tbody>
</table>

Solution B

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eosin-Y</td>
<td>0.2 Gm.</td>
</tr>
<tr>
<td>Distilled water to</td>
<td>100.0 ml.</td>
</tr>
</tbody>
</table>

While the eosin in solution is stable for many weeks, saponin in solution loses almost all of its hemolytic activity after a day or two. The solution should be filtered before use.

Mix well equal quantities of the two solutions, and then completely fill each capillary tube. The solution is frozen and then dried from the frozen state.* A few hundred tubes can be prepared at one time.

The finished product is a glass capillary tube which is filled from end to end with oxalate, saponin, and eosin in a finely divided state. It has the appearance of a pink powder.

The three ingredients remain active for a very long period of time in the dried form. We have used tubes for as long as eighteen months after preparation without any apparent change.

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* When the conventional type of large chamber is used for dehydration, to avoid thawing of the small amount of water while an effective vacuum is being obtained the capillary tubes are placed on the surface of water frozen in a metal pan to a depth of about 0.5 cm.
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Technic

Collection of Blood

Freely flowing capillary blood is required. The blood is taken into the tube until the latter is approximately half filled. By so doing the concentrations of the various components will be similar to those of the original separate solutions, and mixing by tilting is facilitated. Mixing by tilting and rotation is continued until complete clearing of blood by transmitted light has occurred. This operation requires a minute or two. The tube is now placed into a Petri dish, or similar container, with moist gauze or filter paper in order to avoid excessive evaporation. The tube is set aside for at least 30 minutes in order to allow both complete hemolysis and staining.

It is important to take up the blood rapidly and then to mix the blood for a sufficient period of time by tilting and turning. Adherence to this practice will result in more even distribution of the eosinophils.

Loading the Chamber and Counting

An ordinary Neubauer-type counting chamber with a volume of 0.9 cu.mm. is used, but the cover glass must be smaller. The following dimensions appear satisfactory: 20 x 15 x 0.4-0.6 mm. It will be noted that the length has been reduced from the usual 26 to 15 mm. This cover glass can be readily obtained by cutting down one side of the conventional coverslips. The smaller cover-slip permits more complete counting of the eosinophils contained in the capillary tube.

The capillary tube is inverted several times to get as nearly as possible an even distribution of eosinophils. It is then held vertically and both chambers are filled. Because the blood mixture is rather thick it will be found that the drop under the cover-glass can, to a degree, be shifted. After a little practice one becomes accustomed to the high viscosity of the liquid and will be able to charge both chambers rapidly and properly. Occasionally it is necessary to apply a slight degree of pressure at one end of the tube in order to start the flow into the chamber. This may be done with the use of a small rubber bulb.

Microscopically the background is light red and the eosinophilic granules are stained deep red. The eosinophils are distinct and readily spotted. The nuclei are unstained and the cells take various shapes which are usually spheroid or crescentic or annular but at times triangular, and occasionally ovoid or elongated. Due to the viscosity of the blood mixture the eosinophils do not settle and, therefore, are not all on the same level. Proper focusing will permit a clear picture of all of the cells. If the light intensity is very low the non-eosinophilic leukocytes can be seen.

All of the material in a given tube is counted; that is, one continues to fill the chambers and count until the entire amount of blood in the capillary tube is exhausted. Evidence will be presented to indicate the necessity of this procedure. Even the very last small portion of blood mixture, even if it barely covers the ruled area, must be subjected to a count. For each tube three to seven chambers may be filled, the number depending mostly on the size of the tube.

For each chamber all the cells over the entire ruled area of 9 sq. mm. are counted. The results of the counts for a given tube are averaged and this mean value is multiplied by the fraction 10/9 to give the value of eosinophilic leukocytes per cu. mm.

It has been found that the count may be deferred without vitiation of the results for many hours and even days, provided that the capillary tube is maintained in a moist atmosphere, preferably at a low temperature. Dependent upon the number of eosinophils and the volume of blood mixture the count requires 7 to 25 minutes.

If the tube has been set aside for more than an hour or two, a certain amount of debris appears. This is probably the product of break-down of non-eosinophilic leukocytes. While this debris interferes little with the visual definition of the eosinophils, it does make counting a slower and more difficult procedure. If the count of eosinophils cannot be carried out within two hours after collection, the formation of debris can be almost entirely obviated by leaving the capillary tubes filled with blood at room temperature for a short period of
time to facilitate hemolysis, and then placing them in a Petri dish with moist gauze and keeping them in the refrigerator at +4 to 8 C. The tubes should be removed from the refrigerator at least 20 minutes before counting and allowed to stand at room temperature. This is necessary because practically no staining occurs in the cold.

**Evaluation and Discussion**

Relatively few laboratories are equipped to dry from the frozen state, so that the preparation of the tubes must generally be left to laboratories or commercial houses which are properly equipped. The small cover-slips can readily be obtained by cutting down a standard cover-slip (26 x 20 x 0.4 mm.) and then polishing the edge.

Since there is no dilution of the blood the number of eosinophils actually counted is higher than with those methods using dilution. Hence, a longer time is required for each determination. Refrigeration is necessary if the count is not carried out within two hours after collection.

However, the same attributes which cause these disadvantages are responsible also for a high degree of accuracy and reproducibility. This, and the fact that the blood filled capillaries can be stored for as long as two weeks in the refrigerator without loss of accuracy in the count constitute the merits of the method.
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Tables 1, 2 and 3 show results of comparative studies of the accuracy of the capillary tube method, a dilution method and a smear method.

Results in table 1 indicate that among various chambers and from level to level within the column of blood in the capillary tube, the variation of the eosinophil count is great, but that the variation in the final count is much smaller. This is mostly the result of the use of the smaller cover-slip, which barely covers the ruled area of the chamber, and hence permits more complete sampling of the entire column of blood. It should be noted, too, that as long as nearly all the

**Table 3.—Results of Nine Eosinophil Counts Obtained with the Standard Smear Technic. All of the Smears Were Prepared from Freely Flowing Blood from a Single Skin Puncture. Total Leukocytes in the Same Blood Numbered 5400/cu. mm.**

<table>
<thead>
<tr>
<th>Slide</th>
<th>Number of Leukocytes Counted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Eos./cu. mm. 200</td>
</tr>
<tr>
<td>1</td>
<td>378</td>
</tr>
<tr>
<td>2</td>
<td>324</td>
</tr>
<tr>
<td>3</td>
<td>270</td>
</tr>
<tr>
<td>4</td>
<td>351</td>
</tr>
<tr>
<td>5</td>
<td>297</td>
</tr>
<tr>
<td>6</td>
<td>243</td>
</tr>
<tr>
<td>7</td>
<td>270</td>
</tr>
<tr>
<td>8</td>
<td>243</td>
</tr>
<tr>
<td>9</td>
<td>378</td>
</tr>
</tbody>
</table>

Average 306  289  287  284
Stand. dev. ±54.0  ±58.6  ±36.7  ±25.4
Coef. var  ±17.7%  ±20.3%  ±12.9%  ±8.9%

**Table 4.—Comparison of Variability of Distribution of Eosinophils and of Accuracy of Final Count, Using the Standard Smear Method (800 Leukocytes Counted), the Dilution Chamber Count Technic of Dunger, and the Capillary Tube Method, in Terms of the Coefficient of Variation**

<table>
<thead>
<tr>
<th>Method</th>
<th>Eos./chamber</th>
<th>Eos./cu. mm.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smear</td>
<td>—</td>
<td>±8.9%</td>
</tr>
<tr>
<td>Dunger</td>
<td>±10.3%</td>
<td>±4.4%</td>
</tr>
<tr>
<td>Capillary tube</td>
<td>±16.8%</td>
<td>±1.6%</td>
</tr>
</tbody>
</table>

blood in the tube is counted, the number of chambers filled and counted makes little difference in comparative accuracy. This is not the condition prevailing with dilution methods, wherein the accuracy increases with the number of chambers counted. In these dilution technics only a relatively few portions of the entire specimen are sampled. Table 2 shows data obtained with a dilution technic. The C. V. as obtained by us for the dilution technic is somewhat larger than expected from the formula of Berkson, Magath and Hurn.

The two essential features of the capillary tube method are that an indefinite amount of blood is collected and that there is no dilution. Until actual counting is undertaken there is no attempt at exact measurement. Such a procedure eliminates three sources of error common to other methods, viz. those inherent
in the manufacture and calibration of pipets, in the measurement of the sample of blood, and in the dilution. The elimination of these sources of error is reflected in the reduction of the C. V. for the capillary tube method to ±1.6 per cent as compared with ±4.4 per cent obtained by us for the dilution method or the optimal expected value of ±2.4 per cent.7

The example given is for blood in which the concentration of eosinophils is the mode for the population. A comparable accuracy for the capillary tube method has been found with counts as high as 450 eosinophils per cu. mm. and as low as 100 eosinophils per cu. mm. Statistical studies have not been done outside this range. In his extensive monograph Rud5 portrays the fact that as the level of eosinophils increases the coefficient of variation for a method decreases exponentially, so that while the figure is large for values of less than 100 eosinophils per cu. mm., at 150 eosinophils per cu. mm. it is relatively small and then asymptotically becomes smaller as the number of eosinophils per cu. mm. continues to rise.

Table 3 shows results obtained with the smear technic. As previously shown, the decrease in the C. V. with the increase in the number of leukocytes counted on the smear follows an exponential curve.

Recently Bonner4 has stated that eosinophil counts obtained with the smear technic will parallel those with a chamber technic if 800 white cells are counted on the smear. This accord obtains provided the difference between the number

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**Fig. 1.**—Diurnal fluctuations of circulating eosinophils. Composite graph of 5 subjects, each studied for two day period. Note noontime low and nighttime high.
Fig. 2.—Diurnal fluctuation of total circulating leukocytes concurrent with eosinophil fluctuation shown in figure 1. Note independence from pattern of figure 1.

Fig. 3.—Diurnal pattern of eosinophil fluctuation over two day period. The subject was a young, active male (resident physician). In addition to noontime minimum and nighttime maximum, observe generally lower level for second day.
Fig. 4.—Diurnal eosinophil fluctuation, over two day period, for young, active male (technician). Observe noontime minimum and nighttime maximum, slight dip following each evening meal, independence of WBC fluctuation from eosinophil fluctuation, and generally lower level of eosinophils on second day of test.

Fig. 5.—Subject of previous study (see fig. 3), but on this occasion fasting for 24 hours. Note presence of low level until as late as 4 p.m., and rise after eating.
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of eosinophils for each of the two groups of 400 cells for a given determination is small. Rud,⁵ however, points out that with the smear technic, unless thousands of leukocytes are counted, valueless results are obtained when the eosinophil level is low.

Table 4 shows a summary of results of the various technics as carried out in this laboratory.

APPLICATION OF METHOD TO STUDY OF NORMAL SUBJECTS

The capillary tube method was applied to the study of the diurnal fluctuation of circulating eosinophils in normal individuals. Determinations were done every 2 to 3 hours for a period of 48 hours (except 1 A.M. to 7 A.M.) on 5 normal adults leading their daily routines as either laboratory technicians or residents. The composite result is shown in figure 1. Simultaneously with each eosinophil count a total leukocyte count was done; the composite review is seen in figure 2. Typical curves of eosinophil fluctuation for active adult males are shown in figures 3 and 4. Figure 5 shows the effect of fasting upon the eosinophil count.

In addition to these 5 subjects, 9 others were followed for a 24 hour period. The results were similar. In all there are 19 diurnal instances.

The average variation in 24 hours is 64 per cent from the diurnal mean. The most constant variation is the drop occurring between the hour of first activity (8 A.M.) and noontime. This drop averages 35 per cent, from a minimum observed of 11.2 to a maximum of 51.

DISCUSSION AND CONCLUSIONS

As part of an extensive and detailed study by Rud,⁵ eosinophil counts were done at very short intervals of a half-hour or less. These data depict a very rapid oscillation of the eosinophil level, and, in harmony with our observations, the superimposition of this relatively swift oscillation upon a much slower, more general cycle characterized by a midday minimum, an evening upswing and a nighttime peak.

If one is to accept current propositions that eosinophil concentration and fluctuation are functions of adrenal cortical activity,⁸ which in turn is closely associated with the stress incident to the environment,⁹ then one may infer that young, active adults undergo ever increasing stress at the height of the day, and, as the night approaches, they are gradually acclimated.

It seems justified to conclude from these studies that in young, active adults, who work by day and rest at night, there is a diurnal fluctuation characterized by a low at the height of day (noon) and a high late at night. This seems to be generally independent of the fluctuation of total leukocytes.

Since a sharp drop occurs during the same interval which is prescribed for the performance of the eosinophil reduction test,⁹ it must be taken into consideration when interpreting the results of a given test. It has been shown in this study and by Rud⁵ that under normal conditions fasting results in both a bigger drop and a prolongation of the low level much beyond noontime. Further, if the patient is under undue tension, a decrease already in progress before administration of ACTH or cortisone or adrenalin may detract from the magnitude of drop during the test.
Certainly, then, a single eosinophil reduction test is not in every case sufficient to assay adrenal cortical reserve. We endorse the recommendation made by Bonner,4 who, on the basis of 30 afternoon eosinophil reduction tests, concludes that "the Thorn test may be more sensitive as an index of adrenal responsiveness if done on a nonfasting patient from 1 P.M. to 5 P.M."

**Summary**

The paper describes and evaluates a capillary-tube technic for the chamber count of circulating eosinophils. The outstanding merits of the method are ease of collection, accuracy and reproducibility of results and the fact that counting can be deferred for hours and even days without vitiation of the accuracy.

Application of this method to study of normal subjects indicates a diurnal cycle of eosinophil fluctuation, with a low at noon and a high late at night.

**References**

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