Variation and Error in Eosinophil Counts of Blood and Bone Marrow

By William R. Best, M.D., and Max Samter, M.D.

Recent interest in changing levels of circulating eosinophils as an index of 11-oxysteroid output from the adrenal cortex demands a critical evaluation of the accuracy of individual counts, of the validity of comparing successive counts, and of physiologic variations in eosinophil level.

The older literature is replete with investigations of eosinophil variation under many conditions. It is questionable, however, how much reliance can be placed on the available data.

Schwarz in his extensive survey of changing levels of eosinophils in blood and tissue examined the validity of various early reports. He cites a study by Tausz who studied the number of eosinophils in a human during a thirty-day fast and found differential counts of 2.7 per cent in the beginning, 3.9 per cent at the halfway mark and 4.7 per cent at the end of the experiment. While Tausz concluded that prolonged fasting results in an increase of eosinophils, Schwarz demonstrates that the absolute numbers were 257, 267, and 197 per cu. mm., respectively; in other words, that the level remained the same throughout the period of observation.

Shortly after the description of a specific stain for the α granules of the eosinophils by Ehrlich, careful observers noted that various stimuli could alter the number of circulating eosinophils. Such stimuli included selective stimulation of the autonomic nervous system, anaphylaxis, acute infectious diseases, and endocrine disorders.

In general early studies concurred that an increase in circulating eosinophils followed anaphylactic reactions, parasitic infestations, certain tumors, splenectomy, postinfectious convalescence, and the administration of parasympathomimetic drugs. Decrease in circulating eosinophils was seen subsequent to acute infections and the administration of sympathomimetic drugs such as epinephrine. From clinical observation, Schwarz proposed an endocrine regulation of circulating eosinophil levels. His postulate was that diminished function of the adrenal or thyroid glands should result in an increase, hyperactivity in a decrease of blood eosinophils.

With the advent of potent adreno-corticotropic hormone (ACTH), extracted from the anterior pituitary, various workers have examined the metabolic and hematologic response which follows its administration. One of the most consistent changes thus induced is a marked fall in circulating eosinophils. The maximal depression occurs at about four hours and subsides within twelve to twenty-

From the Department of Medicine and Allergy Unit, University of Illinois, Chicago, Ill.

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four hours. Cortisone (17-hydroxy-11-dehydrocorticosterone) and Kendall’s compound F (17-hydroxycorticosterone) produce a similar response, but other steroids such as desoxycorticosterone and sex hormones do not. Cortisone and compound F are steroids active in sugar regulation (“S” hormones) and represent the physiologically significant fraction of urinary “Cortin” or “11-oxysteroids.” The mechanism by which eosinopenia is accomplished has not yet been established.

Like ACTH and “S” hormones, epinephrine will produce an eosinopenic reaction in normal subjects. Studies have shown that possibly higher cerebral centers, but at least the hypothalamus, anterior pituitary, and adrenal cortex are successively activated by the drug. The eosinopenia itself, however, depends on the release of “11-oxysteroids” from the adrenal cortex. Higher cerebral centers might well be the site of epinephrine activation (fig. 1).

Thorn and associates have recently proposed a simple test for the integrity of the adrenal cortex utilizing ACTH as the stimulant, the four-hour change of circulating eosinophils as the indicator. Other studies of adrenocortical responses to electro-shock, insulin hypoglycemia, surgery, drugs, labor, and other stresses have utilized changes in absolute eosinophil counts as a parameter.

This study attempts to clarify the sources of error inherent in the technics of the chamber eosinophil count and to establish those physiologic variations which must be taken into account to render its interpretation valid.

METHODS OF COUNTING EOSINOPHILS

Two methods are available for counting eosinophils in blood and bone marrow, the differential count and the chamber count. The differential count has a large margin of
error, and is, therefore, not suitable for enumeration of eosinophils in the peripheral blood. On the other hand, it maintains a place in the study of eosinophils in the bone marrow because the variable degree of sinusoidal dilution in marrow aspirations makes volumetric enumeration valueless. The atypical staining characteristics of certain young forms renders their identification with chamber methods difficult. In studies of induced peripheral eosinophilia we have consistently noted that many young forms present during the early phases could not be readily identified by chamber methods. Furthermore, bone marrow smears contain a sufficient concentration of nucleated elements to make possible the count of a statistically valid number of cells.

A discussion of variation and error in chamber eosinophil counts must be preceded by a discussion of (a) staining methods, (b) blood to stain dilutions, and (c) types of counting chambers.

(a) Staining methods. Several satisfactory stains have been evolved for chamber eosinophil counts:

Dunger described an eosin-acetone stain, which has been modified by Thorn:

\[ 2\% \text{ aqueous eosin} \quad 5 \text{ cc.} \\
\text{acetone} \quad 5 \text{ cc.} \\
\text{distilled water, q.s.a.d.} \quad 100 \text{ cc.} \]

After preparation, the mixture is filtered and may be refrigerated. Blood drawn into the mixture must not be shaken more than thirty seconds, or lysis will occur. Optimal staining occurs in about three minutes. Evaporation at the edges of the counting chamber invalidates counts read after twenty minutes. Counts are reproducible. Occasionally, however, prolonged or too vigorous shaking in the pipet might cause a degree of lysis which invalidates the reliability of the method.

Randolph improved the technic of absolute eosinophil count by substituting phloxine-methylene blue in propylene glycol for Dunger's eosin-acetone mixture. Two stock solutions are used:

A. 0.1\% methylene blue in propylene glycol 50 cc.
\text{distilled water} 50 cc.

B. 0.1\% phloxine in propylene glycol 50 cc.
\text{distilled water} 50 cc.

Randolph has recommended that equal portions of each stock solution be mixed, and filtered for each day's use. Optimal staining requires at least twenty minutes after mixing. Other proportions of these two solutions may be employed according to preference, or Solution B may be used alone or with 0.1 per cent sodium carbonate.

The mixture is isotonic and prevents lysis. It can be kept for at least four hours without precipitation of stain. Filled chambers may be stored under a moist Petri dish cover several hours before cells are counted; this is often helpful if several subjects are under investigation at the same time.

Chambers should be examined with a bright light. The methylene blue stains all leukocytes while the red phloxine identifies the granules of the eosinophils.

Other recent investigators have used acetone-magnal red and May-Grünwald propylene glycol stains. We have had no experience with these technics.

(b) Blood dilutions. Of blood to stain dilutions, two are in general use, the 1:10 and 1:20 dilutions.

For a 1:10 dilution, blood is drawn to the 1.0 mark of the standard white cell pipet, stain to the 11.0 mark.

For the 1:20 dilution, blood is drawn to the 0.5 mark of the standard white pipet, stain to the 11.0 mark.

Wolfson describes a macro-method giving 1:20 dilution which he claims facilitates pipet cleansing.

Oxalated blood, 0.2 ml., is added to 3.8 ml. stain in a Wassermann tube. We prefer the use of 1:10 dilution because twice as many eosinophils are seen for a given determination than with a 1:20 dilution.
(c) Counting chambers. Two counting chambers of different sizes are in general use, an 0.9 cu. mm. and a 3.2 cu. mm. chamber.

The standard Levy counting chamber, which is commonly used for routine red and white counts, has a chamber depth of 0.1 mm. and a ruled area of 3 by 3 mm. Thus the volume of the chamber is 0.1 x 3 x 3 = 0.9 cu. mm.

A special, larger Levy counting chamber has a depth of 0.2 mm. with a ruled area of 4 by 4 mm.; in other words, a volume of 0.2 x 4 x 4, or 3.2 cu. mm.

The larger the chamber, the more eosinophils will be counted. The resulting increase in accuracy recommends the use of the larger chamber for absolute eosinophil counts. Each hemocytometer block contains two chambers. Cells should be counted in the entire ruled area of both chambers. Increase in the number of chambers will increase the validity of the count.

(It is preferable to use the same pipets and chambers for comparative counts on the same individuals. Manufacturing regulations allow a small error in standardization of equipment which can thus be eliminated.)

<table>
<thead>
<tr>
<th>Dilution factor WBC pipet</th>
<th>Chamber volume total ruled area</th>
<th>No. of chambers</th>
<th>Factor x cells per chamber</th>
<th>Factor x all cells counted</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:20</td>
<td>0.9 mm.³</td>
<td>2 (1 hemocytometer block)</td>
<td>22.22</td>
<td>11.11</td>
</tr>
<tr>
<td></td>
<td>3.2</td>
<td>2</td>
<td>6.25</td>
<td>3.12</td>
</tr>
<tr>
<td>1:10</td>
<td>0.9</td>
<td>2</td>
<td>11.11</td>
<td>5.56</td>
</tr>
<tr>
<td></td>
<td>4*</td>
<td>2</td>
<td>11.11</td>
<td>2.78*</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td></td>
<td></td>
<td>1.86</td>
</tr>
<tr>
<td></td>
<td>3.2</td>
<td>2</td>
<td>3.12</td>
<td>1.56</td>
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* Combination used in most of present studies.

In most of our studies, we have counted the number of cells in four 0.9 cu. mm. chambers after a 1:10 dilution. Table 1 lists the factors which are used to convert the actual number of cells observed with different pipet and chamber technic to absolute eosinophil counts.

**Intrinsic Errors of Enumeration**

Berkson and associates have proposed a formula which describes the errors of chamber counts due to chance distribution and to allowable inaccuracies of equipment manufacture.

Coefficient of variation = \[ \sqrt{ \frac{92^2}{\text{number cells counted}} + \frac{4.6^3}{\text{number chambers used}} + \frac{4.7^3}{\text{number pipets used}} } \]

This formula can be applied to eosinophil counts regardless of stain, dilution, chamber size, or number of chambers used. It does not allow for technical er-
rors of measuring, mixing, or chamber filling which vary with the proficiency of the technician.

Using this formula, we have plotted curves defining two standard errors (2σ) of the method for several chamber combinations according to the mean eosinophil level (fig. 2). In statistical determinations of this type a variation of ±2σ should encompass approximately 95 per cent of observations. For example, if the mean eosinophil count is 100 cells/cu. mm., 95 per cent of determinations using four 0.9 cu. mm. chambers and 1:10 dilution (fig. 2) should fall within the limits of 67 and 133/cu. mm. (2σ = 33).

The ±2σ limits for other selected mean counts using this method are: 50 ± 23, 200 ± 46, and 300 ± 62/cu. mm. Figure 2 indicates the normal range of blood eosinophils in nonallergic individuals to be 30 to 250 cells/cu. mm. Counts up to 1500/cu. mm. are not uncommon in allergic states and rare counts of over 4000/cu. mm. are seen.

Comparison of data from each of the four chambers in 143 successive clinical determinations suggests that the theoretic curves are perhaps too stringent (fig. 3). The curve derived from Berkson’s formula encompasses 99 per cent of observations rather than the expected 95 per cent. We believe this is due to the fact that in each determination, a single technician successively filled the four chambers with a single blood-stain mixture using one pipet. Comparison of simultaneous counts from the same blood specimen by different technicians have shown less correlation.

Similar considerations apply to the evaluation of differential counts of bone
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Fig. 3.—Deviation from the mean of four-chamber eosinophil counts for each of 143 determinations. Each circle represents count for one chamber compared with the mean of four such counts. 2σ curve plotted from σ for several representative areas of mean counts. Theoretic curve from formula of Berkson et al.31

Fig. 4.—Change in per cent of bone marrow eosinophils three to five hours after ACTH during peripheral eosinopenia. Ten subjects following ACTH and 4 after no stimulus.
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marrows. As a rule, differential counts from different bone marrow sites are in rough agreement. Assuming a completely random distribution of cells over a smear, the chance variation of percent for a given mean may be calculated with the chi-square ($x^2$) formula. We have counted 2,000 cells for each marrow differential. For this number of cells we constructed from the $x^2$ formula and tables of probability a curve defining the theoretic 5 per cent level of variation from the true mean. In other words, only 5 per cent of repeated determinations should fall outside this curve which we have superimposed as a dotted line on figures 4 and 5. These two figures show preliminary observations concerning the effect of adrenocortical activity on the eosinophils of bone marrow. They are included here primarily for analysis of comparative marrow eosinophil differential counts as a research technic.

Figure 4 compares marrow eosinophils before and three to five hours after ACTH (last dose) in 10 cases, and multiple marrow site examinations in 4 cases receiving no stimulus. Figure 5 illustrates Hartling's data on 15 patients before and three to five hours after electroshock. Significant trends are lacking in these graphs. This suggests that adrenocortically induced peripheral eosinopenia is not accompanied by significant change of marrow eosinophils.

It is evident in figures 4 and 5 that more than the anticipated 5 per cent of observations exceed the limits of the dotted lines. Positive and negative deviations are about equal in frequency. This phenomenon appears to be due to a slight tendency of eosinophils to clump together and to be distributed to the parts of a smear in a non-uniform fashion. Strict interpretation of $x^2$ is thus invalidated. However, if a sufficiently large number of comparative marrow counts are studied for a given problem, any significant trend should become evident in their averages.
PHYSIOLOGIC VARIATION OF EOSINOPHILS

The physiologic fluctuations in the level of circulating eosinophils have been most extensively studied by Rud in Norway. His monograph presents several generalizations pertinent to this discussion: (1) The level of circulating eosinophils fluctuates rapidly and appreciably. The greater the mean count, the greater the degree of fluctuation. (2) Eosinophil levels show a definite diurnal variation which may be masked by short time fluctuations, but will become evident on averaging many daily curves (fig. 6). There is a mid-morning drop of about 20 per cent from the 8 A.M. level with a return to that level shortly after noon and a rise of approximately 30 per cent above it in the middle of the night. Slight dips appear to follow meals. Fasting subjects show a continuous fall extending into the early afternoon. (3) Normal individuals maintain within broad limits the same general level of eosinophils over prolonged periods. This does not appear to be altered by menstruation, minor colds, or season of the year.

Examination of a diurnal curve for each of two normal subjects (fig. 7) demonstrates the marked degree of short term fluctuation as well as the general diurnal trend. It is of interest to examine the upper graph in the light of the significance placed on the four hour period for the evaluation of eosinopenic responses. It is clear that a difference of half an hour in drawing the noon blood sample would make the difference between a 60 per cent drop and a 2 per cent rise.

The scatter of “8 A.M. to noon” blood eosinophil changes in Rud’s normal subjects receiving no stimuli are shown in the first column of figure 8. The noon counts averaged 7.6 per cent below the 8 A.M. level, 15 per cent of the subjects
FIG. 7.—Two representative twenty-four hour blood eosinophil curves with emphasis on possible errors of comparing 8 A.M. and noon eosinophil levels. Data from Rud. Short time fluctuations may be compared with $\sigma$ at different levels.

FIG. 8.—Changes in circulating eosinophils during four hour morning fast following various stimuli. Scatter, mean, standard deviation, and fiducial limits indicated for each group including: Rud's normals, four-hour fall and maximal morning fall; Thorn's normals and Addison's disease after ACTH; and our own data on normals and miscellaneous illnesses following epinephrine, ephedrine, amphetamine sulfate (Benzadrine), and antipyrene.
had an eosinophil drop of 50 per cent or more at noon, and 38 per cent had a
maximal morning fall (second column, fig. 8) of at least 50 per cent. The figure
also indicates standard deviation and fiducial limits of the mean. Columns 3
and 4 compare these random changes due to diurnal variation with Thorn's data,
using ACTH in normals and Addison's disease. Thorn felt that a 50 per cent
fall in eosinophils was a satisfactory dividing point between normal and abnor-
mal response to ACTH. The tendency for counts to show a narrow dispersion
about the no-change level in Addison's disease (fourth column, fig. 8) suggests
that perhaps diurnal eosinophil variations are mediated by diurnal changes of
adrenocortical activity. Thus, an eosinopenic response greater than 50 per cent
would effectively rule out Addison's disease. Figure 8 (columns 5–9) presents

![Graph showing eosinophil counts](image)

**FIG. 9.—Repeat four-hour fasting eosinopenic tests with similar stimuli in subjects under
standard conditions. Data from our own studies.**

our preliminary computations of eosinopenic response to different agents in 113
tests on various subjects.

It is interesting to note that ¼ grain (45 mg.) ephedrine sulphate orally proved
as effective as epinephrine for inducing eosinopenia. On the other hand, subse-
quent tests with placebos, not illustrated here, have failed to show a significant
difference from those obtained with benzedrine or antipyrine.

Each of the scattergrams of figure 8 illustrate the marked variations of re-
sponse to a given stimulus in a group of subjects. We consider this graphic
method of presenting and comparing groups of eosinopenic reactions to be most
helpful to a critical evaluation.

**VARIABLE FOUR-HOUR RESPONSE TO THE SAME OR SIMILAR STIMULI**

The data which we have presented demonstrate a marked variability of eo-
sinopenic response within each group of subjects. Therefore, it seemed necessary
to investigate the repeated response of individuals to comparable stimuli. The similarity of epinephrine and ephedrine was demonstrated in figure 8. Differences from mean per cent change have been plotted against the mean for multiple counts in 27 individuals (fig. 9). Standard deviation from the mean in these tests was 22 per cent. This deviation did not appear to vary with fasting eosinophil level. However, the greater the mean fall, the less the deviation. With less than 40 per cent eosinopenia, standard deviation was 24.6 per cent; with more than 40 per cent it was 19.5 per cent.

This degree of deviation makes it evident that a single eosinopenic response is not an unequivocal test for the integrity of the hypothalamic-pituitary-adrenal chain. The average change in a sufficiently large homogenous group, however, should be significant. Ideally, each individual in such a group should be tested with a placebo as well as with the test stimulus. If fiducial limits of the mean for test stimulus and placebo do not overlap, the stimulus may be considered as active in altering eosinophil levels. It would also be of value to compare this with the response of these subjects to a known eosinopenic stimulant such as ACTH, ephedrine, or epinephrine.

**INTERPRETATION OF INDUCED CHANGES OF CIRCULATING EOSINOPHILS**

The maximal eosinopenia from ACTH or cortisone occurs at about four hours. Eosinopenia of 50 per cent or more four hours after subcutaneous or intravenous administration of epinephrine (0.2 mg. or more) or an equivalent dose per os of ephedrine (45 mg.) would seem to imply adequate function of the hypothalamic-
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pituitary-adrenal chain. Failure to obtain adequate response may be due to any of the following:

1. Inaccuracies of the test due to chance and physiologic variation.
2. Refractoriness to a single dose of ACTH, epinephrine, or ephedrine. Figure 10 shows that in certain diseases a significant number of tests fail to produce a 50 per cent eosinopenic response to epinephrine or ephedrine. Failure of response to single doses of ACTH has also been observed in conditions other than Addison's disease. The reason for this failure is not always apparent. In hypophysectomized animals adrenocortical atrophy prevents an adequate response to single doses of ACTH. Failure of response to epinephrine, ephedrine, or stress, might represent a refractory link anywhere in the chain.
3. The patient might be tested while subjected to stimuli inherent to his disease or his environment which are known to cause a release of adrenocortical hormones. The relatively slight additional stimulation of the test may then be insufficient to induce further eosinopenia, a phenomenon which we have observed while testing patients under stress and in acute illness.

SUMMARY

1. The literature on eosinophil variations under various stresses is reviewed in the light of recent interest in the adrenal cortex as a regulator of blood eosinophil levels. Chamber eosinophil counts are necessary for accuracy in these studies. Phloxine-propylene glycol or eosin-acetone stains with standard white cell pipets and chambers may be employed for simple and satisfactory chamber counts.
2. Statistical formulae are applied to chamber eosinophil counts and marrow differential counts to delineate the theoretic errors. Actual observations differ slightly from the predicted results but substantiate the appreciable intrinsic errors frequently seen.
3. Studies on physiologic variation are reviewed. There are significant minute-to-minute fluctuations as well as a more marked diurnal trend in eosinophil levels with a mid-morning low and a night-time peak. Comparison of single morning eosinophil levels with single noon specimens may be misleading.
4. Oral ephedrine is a potent eosinopenic agent.
5. Repeated tests of the eosinopenic response to ephedrine or epinephrine frequently show significant variation in the same subject.
6. Many patients with various diseases fail to react adequately to ephedrine or epinephrine.
7. Repeated and controlled observations are necessary before conclusions regarding pituitary-adrenal function can be drawn from eosinophil counts alone.

REFERENCES

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