

Basophil Counting With a New Staining Method Using Alcian Blue

By Harriet S. Gilbert and Leonard Ornstein

Difficulties in obtaining reproducible and accurate enumeration of circulating basophils with existing techniques have hampered investigation of this infrequent cell population. A new basophil staining method is described that employs alcian blue dye for staining of heparin within basophils at low pH and in the presence of lanthanum ions. Basophil recognition is facilitated by reducing nonspecific nuclear staining. This objective is achieved because of the differences in stability of alcian blue-heparin, alcian blue-nucleic acid, lanthanum-heparin, and lanthanum-nucleic acid complexes. Reduction of pH after staining also favors solubilization of leukocyte cytoplasmic proteins, providing greater contrast between stained and unstained cells by reducing light scattering of the unstained leukocytes. The alcian blue staining method is suitable both for chamber basophil counting and automated basophil counting using continuous-flow sam-

pling and electro-optical detection. The new staining method was evaluated by comparing it with the chamber counting method using toluidine blue in a triple-blind study in which the results of basophil counting by the alcian blue chamber method, alcian blue automated instrument method, and the toluidine blue chamber method were analyzed for reproducibility and compared with an indirect basophil count obtained from a 1000-cell leukocyte differential and a total leukocyte count. Both alcian blue staining methods gave greater reproducibility than toluidine blue and were more accurate, as evidenced by a significantly higher correlation with the indirect basophil count. The improved reproducibility, accuracy, and convenience of this method over existing methods should facilitate the collection of more meaningful information about circulating basophil levels in health and disease.

ALTERATIONS IN THE number of circulating basophils are associated with hypersensitivity and allergic states, endocrinopathies, and hematologic disorders.¹⁻⁴ Although correlations between the absolute basophil count and the status of the patient have been observed, the basophil count rarely is utilized in clinical diagnosis due to the difficulties in obtaining reliable values with existing techniques of enumeration. The indirect method of quantitating this infrequent cell population by examination of stained preparations of peripheral blood and the total white cell count is prohibitive, since satisfactory precision requires the classification of at least 1000 cells in the leukocyte differential. Methods for direct counting of basophils in suspension using a hemocytometer and specific basophil stains such as toluidine blue and neutral red have been described⁵⁻⁷ and have the advantage of increased statistical accuracy, as several thousand cells are examined. However, these methods are tedious,

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since high-magnification microscopy ($400\times$) is required to distinguish the stained granules of the basophil from partially stained nuclei of other leukocytes with toluidine blue staining and from eosinophils with the neutral red stain.

In order to investigate the value of basophil counting in diagnosis and monitoring of pathologic states associated with basophilia or basopenia, an improved method for basophil staining was sought which would maximize accuracy and minimize tedium. Such a method was developed employing an alcian blue dye for staining basophils at low pH.⁸ The improved contrast between stained basophils and unstained leukocytes obtained with this method has been exploited in the design of an automated system employing continuous-flow sampling and electro-optical recognition for basophil counting.⁹ This report describes the alcian blue method for basophil staining and presents the results of an evaluation of basophil enumeration by toluidine blue chamber count (TBC), alcian blue chamber count (ABC), and an automated basophil counting method that employs the alcian blue staining procedure (alcian blue instrument count, ABI).

MATERIALS AND METHODS

Patient material was drawn from the population of the Mount Sinai Medical Center and included blood drawn from normal subjects, patients selected at random from the hospital population, and patients being treated for thyroid and myeloproliferative diseases. Blood was collected by venipuncture into 7-ml tubes containing 0.06 ml of 15% EDTA (K_3) solution (B-D Vacutainer No. 3204QS). The time from blood collection to basophil counting of the sample varied from 1 to 18 hr. However, in order to eliminate basophil age as a variable in this comparative study, the basophil count of each sample was determined using the three different methods within a 2-hr time period.

Toluidine blue chamber counts were performed using the method of Cooper and Cruickshank,⁷ modified by substituting an equimolar amount of lanthanum chloride for aluminum sulfate. In order to reduce errors introduced by pipetting during the various dilution and staining steps, the method was further modified to employ a larger volume of blood (0.1 ml), and the amounts of diluent and staining mixture were increased in proportion.

The alcian blue chamber count was performed using the following solutions: (1) solution A, 0.1% EDTA in saline; (2) solution B, deionized water containing 0.076% cetyl pyridinium chloride, 0.7% lanthanum chloride $\cdot 6 H_2O$, 0.9% NaCl, 0.21% Tween 20, 0.143% alcian blue 8 GN (color index No. 74240), filtered through a 1- μ filter; and (3) solution C, 1 N HCl.

Samples for chamber counting were prepared by diluting 0.1 ml blood with 0.4 ml solution A. To the diluted blood was added 0.45 ml solution B. After 1 min of gentle agitation, 0.05 ml of solution C was added, and the mixture was again agitated gently. Counts were performed in a Fuchs-Rosenthal hemocytometer, and the number of basophils counted in each chamber was multiplied by 3.13 to give the total number of basophils per cubic millimeter of blood.

Automated basophil counting was performed using an instrument designed by the Technicon Corporation, Tarrytown, N.Y.⁹ In the automated basophil counting method, the solutions used for alcian blue staining (described above) were proportioned in a continuous flow manifold. Maleic acid (0.188 M) was substituted for hydrochloric acid to buffer the solution at pH 2.3. (This pH produced the optimal signals in the electro-optical detection system and also prevented protein film build-up on the manifold glassware.) The resulting mixture was metered through a flow cell for electro-optical enumeration of basophils. The detection scheme⁹ was based on the spectrally distinct optical scattering properties of the alcian blue-stained basophils.¹⁰ The flow cell metering volume was calibrated daily with a normal sample of blood previously counted on a Coulter Model S. Basophils were detected and enumerated per unit volume and per 10,000 leukocytes by electronic counting circuitry.

A triple-blind study was designed in which each of 106 blood samples was divided into four parts by the technician performing the instrument basophil counts. Each aliquot was assigned a random number and was given to another technician who performed the toluidine blue and alcian blue chamber counts. A single basophil count, determined from the number of basophils present in one chamber (3.2 cu mm) of the hemocytometer was obtained on each sample. Two samples of each blood were counted by each technique, but duplicates were not identified, and the technician recorded each result according to sample number. Duplicate instrument basophil counts were performed serially from a single tube containing the remainder of the blood from which the chamber count samples had been obtained. Following the completion of all counts, results were decoded and collated.

Five blood smears were prepared from the anticoagulated blood of 43 study subjects using a Perkin-Elmer Coleman Model 90 Uni-Smear Spinner and stained with an Ames Hema-Tek slide stainer using the Hema-Tek Stain-Pak. The percentage of basophils was determined from differential counting of 1000 leukocytes by counting 200 cells on each of five slides. Indirect basophil counts (IBC) were calculated from the per cent basophils in the 1000-cell differential and the white cell count, as determined by a Coulter Model S.

RESULTS

The reproducibility of the TBC, ABC, and ABI methods was determined in 106 blood samples containing a range of basophils from 0 to 360 cu mm, and the results are summarized in Table 1. Paired results obtained with all three methods were highly correlated. The mean basophil counts/cu mm for TBC, ABC, and ABI were 44.3, 56.7, and 59.1, respectively. Paired t tests (using the means of the duplicate determinations) showed that basophil counts obtained by the toluidine blue chamber method were significantly lower than those obtained by both methods which employed alcian blue. The difference in means between each alcian blue method and the toluidine blue method was significant at the 99.95% confidence level, while the difference in alcian blue methods was not significant.

The relation of basophil counts in whole-blood suspensions to those identified morphologically as basophils on stained smears was studied in a subset of 43 blood samples selected at random from the larger population. TBC, ABC, and ABI results were compared with the indirect basophil count (IBC) calculated from a 1000-cell leukocyte differential and the total leukocyte count/cu mm. The results of this comparison (Table 2) showed a statistically significant (at 99.95%, using a test devised by Hotelling¹¹) higher correlation of IBC with basophil counts obtained by alcian blue staining using either the

Table 1. Reproducibility of Basophil Counting Methods (106 Samples)

Method of Counting	Pooled Standard Deviation*	Correlation Coefficient (r) of Duplicate Counts
Toluidine blue chamber	16.9	0.84
Alcian blue chamber	14.1	0.95
Alcian blue instrument	10.5	0.97

*Pooled standard deviation calculated as

$$SD = \sqrt{\frac{1}{N} \sum_{i=1}^N \frac{(x_1 - x_2)^2}{2}}$$

where, x_1, x_2 are duplicate counts for the i th sample. $N = 106$.

Table 2. Correlation Coefficient Between Basophil Counting Methods (43 Samples)

Counting Method*	1	2	3	4
1	1.0			
2	0.45	1.0		
3	0.81	0.84	1.0	
4	0.77	0.84	0.98	1.0

*1, indirect from stained smears (1000 cell counts); 2, toluidine blue chamber count (mean of duplicate counts); 3, alcian blue chamber count (mean of duplicate counts); 4, alcian blue instrument count (mean of duplicate counts).

chamber method or automated counting method than with counts obtained by the toluidine blue chamber method.

The increased reproducibility and accuracy of basophil counts using alcian blue staining is attributable to the improved contrast between stained basophils and unstained leukocytes. This fact is best demonstrated by examination of the leukocyte population using electro-optical detection. The automated basophil counting instrument was equipped with an oscilloscope which displays as a dot the signal generated by each leukocyte passing through the flow cell. Figure 1 shows the distribution of the leukocyte population after alcian blue staining.

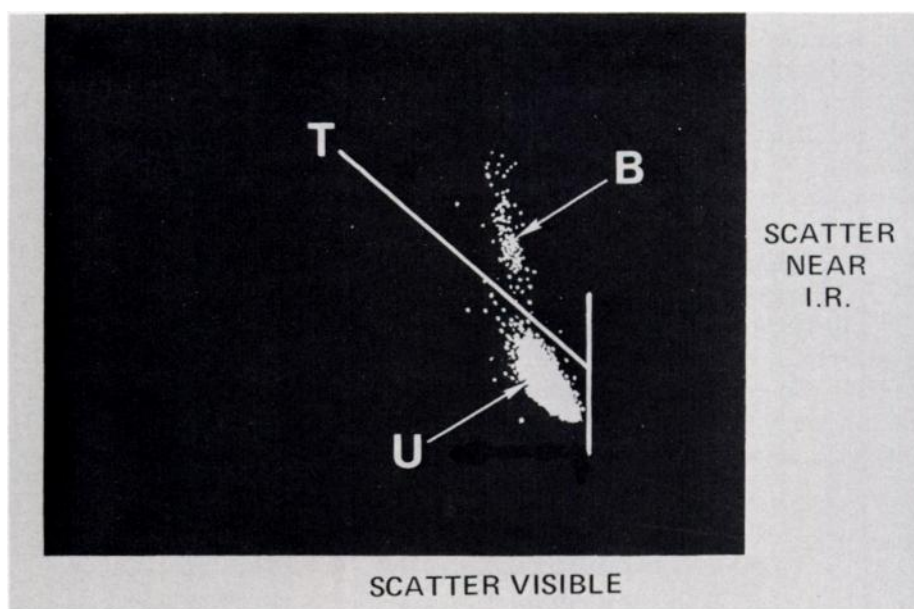


Fig. 1. Photograph of an oscilloscopic display of 10,000 leukocytes after alcian blue staining. Each dot represents the two signals generated by the passage of a single leukocyte through the electro-optical detection station. The vertical axis is proportional to the scatter intensity in the near infrared spectrum (730–1200 $m\mu$). The horizontal axis is proportional to the scattering intensity in the visible spectrum (510–730 $m\mu$). The angular threshold line separating basophils (B) from unstained cells (U) is indicated by the line T. A horizontal threshold separates signals generated by stained and unstained leukocytes from those produced by stroma, platelets, and electronic noise. The detection logic classifies all cells producing signals exceeding the horizontal threshold as leukocytes and all signals exceeding both the angular and horizontal thresholds as basophils.

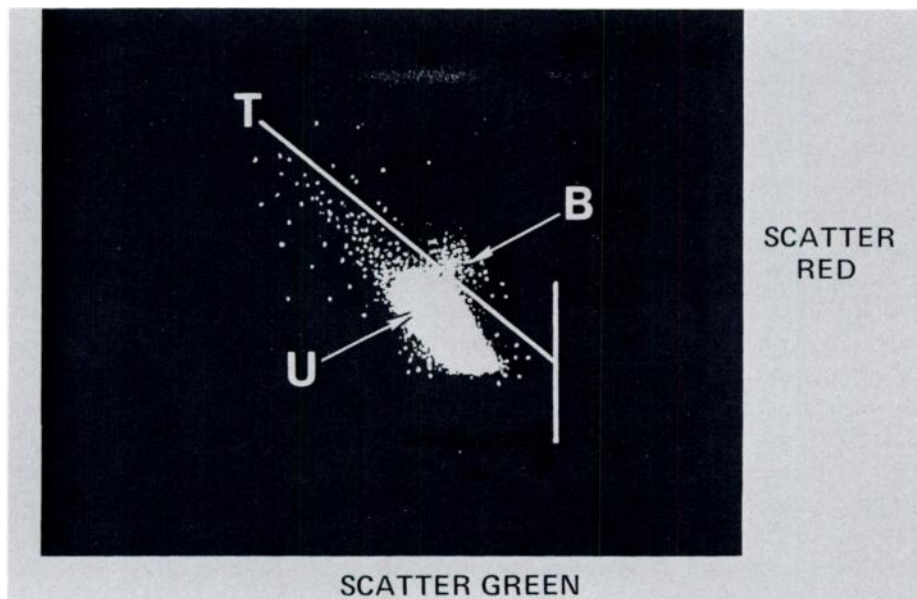


Fig. 2. Photograph of an oscilloscopic display of 10,000 leukocytes from the same blood as depicted in Fig. 1, after staining with toluidine blue. Each point represents the two signals generated by the passage of a single leukocyte through the electro-optical detection station. The vertical axis is proportional to the scatter intensity in the red spectrum (630–800 $m\mu$). The horizontal axis is proportional to the scattering intensity in the green spectrum (460–630 $m\mu$). Stained basophils are indicated by B and unstained cells by U. The threshold line separating basophils from unstained cells is indicated by the line T. Photograph kindly provided by W. Groner, Technicon Corporation, Tarrytown, N.Y.

Attempts to enumerate basophils stained with toluidine blue, in which the wave lengths employed were selected to optimize the signal of the toluidine blue-stained basophils, produced a distinct population in a two-dimensional display (Fig. 2), but the overlap between basophils and other leukocytes made the selection of an appropriate threshold difficult. In contrast, the electro-optical separation of unstained and stained cells obtained with the alcian blue staining method was sufficiently great to facilitate accurate threshold setting.

DISCUSSION

Methods for specific staining of basophils in whole-blood suspensions have been based upon the ability of cationic dyes to bind to the sulfate groups of heparin. Because the basophil granules are extremely soluble in water, staining in suspension requires that the granules be rendered insoluble rapidly. In the metachromatic staining method devised by Moore and James⁵ this was attempted with toluidine blue in 20% alcohol. Saponin was used to hemolyze the erythrocytes. Cooper and Cruickshank⁷ improved the basophil staining method by using EDTA as an anticoagulant to eliminate platelet clumping and by substituting cetyl pyridinium chloride, both to insolubilize the mucopolysaccharides and to act as a hemolytic agent. Aluminum sulfate was added, since it was reported to accentuate the metachromatic staining of the basophils by acting as a mordant.¹²

The alcian blue staining method introduces several modifications in basophil staining which confer greater specificity and facilitate recognition of basophils by chamber counting or with optical detection devices. Lanthanum chloride is used in place of aluminum sulfate. Lanthanum and aluminum phosphates are less soluble than their sulfates, and the difference is considerably greater for lanthanum than aluminum compounds. Since alcian blue competes with other cations (i.e., La^{3+} , cetyl pyridinium, H_3O^+ , Na^+) for binding sites on both heparin and nucleic acids,^{13,14} the lesser stability of lanthanum-heparin complex compared to lanthanum-nucleic acid complex¹⁵ increases the contrast of basophil granule staining relative to nuclear staining. In addition, alcian blue (in comparison with toluidine blue) has a greater affinity for sulfated than phosphorylated compounds,¹⁶ further favoring the staining of heparin over nucleic acids. Once staining has occurred, the pH of the staining mixture is reduced. This step permits further increase in staining contrast because at low pH alcian blue-nucleic acid complexes are less stable than alcian blue-heparin complexes. Low pH also favors the solubilization of leukocyte cytoplasmic proteins, reducing the light scattering of the unstained leukocytes and facilitating the recognition of basophils by providing still greater contrast between basophils and unstained cells.

In this study the use of the alcian blue staining method for basophil counting afforded greater reproducibility and accuracy than the toluidine blue staining method. The lower values obtained by toluidine blue staining are believed to be due to undercounting of basophils, since basophil enumeration by alcian blue staining showed better correlation with the number of basophils identified morphologically on stained slides than did the toluidine blue staining method. Undercounting may be due to the presence of lightly stained basophils which are difficult to distinguish from nonbasophil leukocytes with nuclear staining. These indeterminate cells are usually omitted from the basophil count because of this uncertainty. Our observation of a comparison of the Cooper and Cruickshank toluidine blue method with our toluidine blue modification indicated that the lightly-stained-basophil problem was even more severe in the original method which employs aluminum sulfate instead of lanthanum chloride. However, the alcian blue method essentially eliminated the problem of nuclear staining.

Another benefit conferred by the improved contrast of the alcian blue stain was the ability to perform chamber counts using a lower magnification. For this study a magnification of $250\times$ was employed for ABC, whereas $400\times$ was required for TBC. As a result, the time required for ABC counting was less than that for the TBC method, and the technician found the former method less of a visual strain. This was attributed to the greater contrast between stained and unstained cells, the lighter color of the suspending dye solution, and the lower magnification at which the count was performed.

The enumeration of basophils by alcian blue staining and electro-optical detection gave somewhat greater reproducibility than the alcian blue chamber count. This finding is due, in part, to the difference in sample size in the two techniques. In the instrument basophil count, all the leukocytes contained in 1 cu mm of blood are counted. This is approximately three times the number of leukocytes examined in a chamber count. The automated basophil counter has

the capacity to perform basophil counts at the rate of 60 samples per hr. With the chamber counting method, approximately 24 samples can be studied hourly. However, visual fatigue limits the number of consecutive hours that counting can be performed by a single technician. The choice of the chamber or automated method will depend on the extent of the work load, as the precision and accuracy of both methods permit the detection of small deviations from normal in the blood basophil count.

The ability to generate reliable information about the basophil should expedite the study of this cell population in various pathologic states, just as the quantitation of eosinophils by chamber counting has provided insight into certain endocrine dysfunctions. Preliminary studies of the value of basophil counting using the alcian blue staining method in the diagnosis and monitoring of thyroid dysfunction have been conducted¹⁷ to explore the previous observations of a reduction in basophil count in Graves' disease and an elevation in hypothyroidism.² The distribution of basophil counts in 83 normal subjects was such that 95% of the counts fell between 10 and 80 basophils per cu mm. In a population of 60 untreated patients with Graves' disease, 53% of the counts were below 10 basophils per cu mm, and in 24 patients with untreated idiopathic hypothyroidism, 50% had basophil counts above 80 per cu mm. Both populations of patients with thyroid disease differed from normal at a probability level of 0.001. Serial studies revealed restoration of normal basophil levels with effective treatment of the thyroid dysfunction. These results, confirming the relationship of circulating basophil levels to the state of thyroid function, suggest that basophil counting may be a valuable adjunct to other more time-consuming and costly methods for assessing and monitoring thyroid disease. Another clinical application of hormone-induced changes in circulating basophils recently has been described¹⁸ in which serial basophil counts have been used to predict the time of ovulation in patients being treated for infertility.

The capability of recognizing small changes in the basophil population should facilitate the study of basophil regulation and stimulate further investigation of the utility of basophil counting in clinical medicine.

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REFERENCES

1. Shelley WB: The circulating basophil as indicator of hypersensitivity in man. *Arch Dermatol* 88:759, 1963
2. Boseila AWA: Hormonal influence on blood and tissue basophilic granulocytes. *Ann NY Acad Sci* 103:394, 1963
3. Fredricks RE, Moloney WD: The basophilic granulocyte. *Blood* 14:571, 1959
4. Gilbert HS, Warner RRP, Wasserman LR: A study of histamine in myeloproliferative disease. *Blood* 28:795, 1966
5. Moore JE III, James GW III: A simple direct method for absolute basophil leukocyte count. *Proc Soc Exp Biol Med* 82:601, 1965
6. Shelley WB, Parnes HM: The absolute basophil count. *JAMA* 192:108, 1965
7. Cooper JP, Cruickshank CND: Improved method for direct counting of basophil leukocytes. *J Clin Pathol* 19:402, 1966
8. Gilbert HS, Ornstein L: A new method for basophil counting with alcian blue. *Proceedings of the American Society of Hematology Sixteenth Annual Meeting, Chicago, December 1973, p 99*

9. Mansberg HP, Saunders AM, Groner W: Hemalog D white cell differential system. *J Histochem Cytochem* 22:711, 1974
10. O'Brien R, Coyne L: Spectral characteristics of human leukocytes and their relevance to automated cell identification. I. Basophils (in preparation)
11. Williams EJ: Significance of difference between two non-independent correlation coefficients. *Biometrics* 15:135, 1959
12. Heath ID: Staining of sulfated mucopolysaccharides. *Nature* 191:1370, 1961
13. Scott JE: Aliphatic ammonium salts in the assay of acidic polysaccharides from tissues, in Glick D (ed): *Methods of Biochemical Analysis*, vol 8. New York, Interscience, 1960
14. Mowry RW: Alcian blue technics for the histochemical study of acidic carbohydrates. *J Histochem Cytochem* 4:407, 1956
15. Hammarsten E, Teorell T: Studies on microchemical reactions. II. Precipitation of protein nucleic acid with lanthanum and sulfosalicylic acid ions. *Acta Med Scand* 68:226, 1928
16. Spicer SS: A correlative study of the histochemical properties of rodent acid mucopolysaccharides. *J Histochem Cytochem* 8:18, 1960
17. Gilbert HS, Karpel J, Gilbert PL: The basophil count as a test of thyroid function. *Proceedings of the Fifteenth Congress of the International Society of Hematology*. Jerusalem, September 1974
18. Mettler L, Shirwani D: Direct basophil count for timing ovulation. *Fertil Steril* 25:718, 1974