THE EFFECTS OF SIZE OF SAMPLING AREA AND DILUTION ON LEUCOCYTE COUNTS IN A HEMOCYTOMETER[1]

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ABSTRACT

With nearly 5000 areas examined, the comparison of a Poisson distribution to the distribution of leucocytes in a hemocytometer chamber at both 1:100 and 1:25 dilutions indicated a poor fit ($P = <0.0001$), a non-random distribution. In a series of leucocyte determinations from a young, male university student comparing 28 counts from 18 mm^2 (both chambers) with 28 from 9 mm^2, and 28 from 4 mm^2 (the usual standard), the mean from the 18 mm^2 counts was not significantly different from that of the 9 mm^2 counts. However, both the means of the 18 mm^2 counts and the 9 mm^2 counts were significantly different from that of the 4 mm^2 counts ($P = 0.02$ and 0.035 respectively). Forty-two counts at 1:100 dilution had a mean not significantly different from that of 42 counts at 1:20 dilution ($P = 0.06$).

It appears that the counts from the 18 mm^2 area at 1:100 dilution are the most reliable and are therefore recommended for critical research determinations. Fourteen such counts had the most restricted range and the lowest standard deviation of any of the six combinations.

INTRODUCTION

In studies involving the effect of hypoxic stresses on the number of circulating white blood corpuscles in man, some skepticism has arisen, on the writer's part, in regard to the accuracy of standard hemocytometer techniques in making counts and accompanying calculations for leucocytes per mm^3. The basis for such skepticism has been the occurrence of wider fluctuations than expected in a number of instances.

As stated by "Student" (1906–1907) many years ago, there are two main sources of error in counting blood corpuscles with a hemocytometer. One of these is that the drop taken may not be representative of the bulk of the liquid. This source of error can be dealt with by determining the probable error of random sampling and by averaging the counts from a series of drops. Such procedure is desirable in hemocytometer techniques, as indicated by some of the data which follow. The other source of error is that the distribution of corpuscles over the hemocytometer slide is never completely uniform. Thus in standard examinations, where only a fraction of the available slide area is used in the counting, a non-random distribution may appreciably distort accuracy. Of course, a marked leucocytosis or leucopenia may readily be detected regardless of some degrees of distortion in accuracy. However, in critical research measurements where electronic counting is not employed, it may be difficult to evaluate whether a difference of 2000–5000 leucocytes is due primarily to chance fluctuation or to some other factor.

In recent years, modifications of hemocytometer techniques have appeared. Some of these involve greater dilutions than the standard 1:20, and also involve utilizing greater sampling areas than the four-corner 1 mm squares which are standard for leucocyte counts. One such innovation is the Unopette disposable blood-diluting pipette made by Becton, Dickinson, and Company, of Rutherford, New Jersey. For leucocytes, a dilution of 1:100 is recommended, and a measured 1.3 ml of diluent (1% acetic acid) in disposable vials is filled with 13 microliters of blood from a disposable, capillary pipette holding that exact amount. Nine of the 1 mm squares (all of one side of the double-chambered slide with Neubauer ruling) serves as the area for the count.

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It would appear that the greater the area sampled, the less chance of error in leucocyte counts. Likewise, greater dilution should diminish chances of error, as well as promote ease of counting. Accordingly, this study represented an effort (1) to establish the effect of dilution on the randomness of distribution of leucocytes in the chamber and (2) to compare results, and the significance of their differences, obtained from a series of counts using sampling areas of 18 mm^2, 9 mm^2, and the standard 4 mm^2. In addition, each of these series was counted at 1:100 dilution and at the standard 1:20 dilution.

PROCEDURE

A healthy male university student twenty years of age, from which preliminary, daily, white-cell counts at 1:100 dilution from a chamber area of 18 mm^2 indicated a minimum of fluctuation (maximum = about 2000 corpuscles), was selected as donor for all blood samples utilized in the study. Blood was obtained from the finger tip, and, for convenience, Unopette disposable vials and pipettes were used. Double-chambered AO Spencer Bright-Line hemocytometer slides with improved Neubauer ruling were employed (0.1 mm. deep). Counts were made through binocular research LG Officine Galileo microscopes at 100× magnification, with the aid of an automatic hand counter.

In an effort to evaluate the corpuscle distribution at two different dilutions, the number of white cells in each of 2,415 one mm squares (16 subsquares) at 1:100 dilution was compared to that of a Poisson distribution. For the lesser dilution, a ratio of 1:25 was employed, so that an exact one fourth (four subsquares of the sixteen) comprised the area of examination. The four subsquares were not in a line, but formed a square of their own. At this dilution, 2,500 such units were examined. At both dilutions, different 1 mm squares or 0.25 mm squares were used, so that all parts of the chamber had equal representation.

At a later date, seven leucocyte counts were made from each of six different combinations of sampling area size and dilution. These were 18 mm^2 (both chambers), 9 mm^2, and 4 mm^2 (corner squares) at both 1:100 dilution, and 1:20 dilution. One fifth of the 1.3 ml of the disposable vial's diluent was used for the 1:20 dilution. The other four-fifths were withdrawn with needle and syringe. A day later, at about the same time of day, seven more examinations of each of the same six combinations were counted from the same donor, making 84 counts in all. Leucocyte numbers were calculated by the formula,

$$\frac{\text{corpuscles x dilution x 10}}{\text{no. of 1 mm squares counted}} = \text{leucocytes per mm.}^3$$

At no time during the study was differentiation among types of leucocytes attempted.

RESULTS

The results of the determination of uniformity of distribution at each dilution are listed in Table 1. The fit indicates that the distribution was not random at either dilution, but that 1:100 dilution has a slightly higher probability value. From these results, it is decidedly apparent that counting in a greater area may be more reliable.

A comparison of the series of counts at the three sizes of sampling area and at the two dilutions is given in Table 2. Though there are no great differences among the six means listed, the ranges and standard deviations are quite revealing. The 18 mm^2 sampling area at 1:100 dilution has the most restricted range and the smallest standard deviation. The 4 mm^2 sampling area at both dilutions has the widest ranges and greatest standard deviations. In addition, the 4 mm^2 values show the lowest means.

In considering the matter of sampling area alone, the mean from all 28 counts from 18 mm^2 (6,811) is not significantly different from that of the 28 from 9 mm^2

(6,722). However, the difference in means between all 18 mm² counts and all 4 mm² counts (6,811–5,969) is significant at the 2% level (P=0.02). Furthermore, the difference in means between the 9 mm² counts and the 4 mm² counts (6,722–5,969) is also significant at less than the 5% level (P=0.035). When considering the dilution factor alone, the mean of all 42 counts at 1:100 (6,780) is not significantly different (P=0.06) at the 5% level from the mean of the 42 counts at 1:20 dilution (6,256).

TABLE 1

Actual distribution of leucocytes at 1:100 and 1:25 dilutions compared to Poisson distributions

A. 1:100 dilution							
No. of cells in 1 mm²	0	1	2	3	4	>4	Total
No. of 1 mm² examined	1791.0	492.0	111.0	19.0	2.0	0.00	2415.00
Poisson distribution	1746.6	565.9	91.7	9.9	0.8	0.05	2414.95
Chi-square=25.317							
P=<0.0001							
B. 1:25 dilution							
No. of cells in 0.25 mm²	0	1	2	3	4	>4	Total
No. of 0.25 mm² examined	1907.0	470.0	107.0	16.0	0.0	0.00	2500.00
Poisson distribution	1864.3	547.2	80.4	7.8	0.6	0.04	2500.34
Chi-square=30.603							
P=<0.0001							

TABLE 2

Comparison of leucocyte counts from different dilutions and sizes of sampling area

	Sampling Area	No. of Counts	Cell Count		
			Range	Mean	Standard Deviation
1:100 Dilution					
	18 mm²	14	5,888–7,944	6,978	± 691.22
	9 mm²	14	5,544–8,666	7,237	± 977.16
	4 mm²	14	3,600–8,500	6,124	±1,728.31
1:20 Dilution					
	18 mm²	14	4,955–8,277	6,645	±1,310.04
	9 mm²	14	4,968–8,866	6,307	±1,340.62
	4 mm²	14	3,300–8,055	5,815	±1,715.89

DISCUSSION AND SUMMARY

It would appear that, for critical research determinations of leucocyte counts with the hemocytometer chamber of the Neubauer ruling, it is certainly more accurate to employ the 18 mm² area (both chambers), preferably at 1:100 dilution, or at least the 9 mm² area at 1:100 dilution. The old standard of using 4 mm² areas is not advisable, in view of the fact that distribution of white corpuscles in the chamber deviates considerably from randomness, as part of this study clearly implies. In addition, it is advisable to take an average from a series of counts representing a number of blood drops in order to approach more closely the true numerical value representing a larger volume of blood. The 1:100 dilution provides more ease in counting and greater accuracy.

In evaluating erythrocyte counts, Berkson, Magath, and Hurn (1935) found that the red corpuscles were not completely random in their distribution in a

hemocytometer, but were close enough to random that applications of fit against a Poisson distribution were rather good, as revealed by a subsequent chi-square analysis. In fact, they proposed that the error of estimate was not significantly diminished by increasing the sampling area beyond 80 squares (smallest squares), though greater accuracy might be expected by increasing the number of specimens examined. Even here, their calculations of expected maximum differences, when compared with those in standard treatises on hematology, indicate that values that are beyond standard expectancy of maximum differences and are therefore disallowable can be anticipated normally from 50 percent to more than 90 percent of the time.

The situation with leucocytes is apt to be even less reliable, because of their greater size and shape plasticity. As mentioned by Athens (1963), the specific gravity of lymphocytes and monocytes is comparatively great, and he also points out the surface adhesiveness of granulocytes. In addition, there is the extra weight of ingested particulates. As a matter of fact, Hynes (1947) has shown that leucocyte distribution on a hemocytometer slide is not random, which is in agreement with the results in this study. As he emphasizes, the work implying random cell distribution was done with the old, well-type hemocytometer. With the advent of the Bürker-type chamber with the Neubauer ruling, new and less predictable variations in random sampling are bound to occur. In using the four-corner 1 mm squares, as is routinely done for leucocytes, Hynes (1947) found that the two squares nearest the filling point gave totals of 3.5 percent below the mean for all four squares. The difference, statistically, was highly significant. Hynes (1947) concluded that this variation in leucocyte density on the counting chamber was not due to bending of the cover-slips under capillary tension, nor did he attribute it to uneven cell distribution in the capillary stem of the pipette. Rather, he considered the cause to be the drift of the leucocytes along the chamber. Having a greater density than the diluting fluid, they continue to flow after the fluid has stopped.

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