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The authors evaluated the Coulter® S-Plus three-part differential and compared it with the total of component cells obtained by manual slide differential. The patient population (N = 989) was strikingly abnormal, in that 109 samples contained nucleated red blood cells, 118 samples contained one or more abnormal cells (blasts, promyelocytes, or lymphoma cells), and 95 samples had >10% monocytes. The GRAN and LYMPH fractions were accurately measured in the specimens that did not display region (R) flags. The MONO fraction correlated poorly with the manual differential and underestimated monocytes when they were increased. Not all samples with small numbers of abnormal cells or nucleated red blood cells were flagged by the three-part differential. Specimens with eosinophilia and reactive lymphocytosis likewise did not always show R-flags. The following findings (prevalence = 23.9%) were arbitrarily defined to constitute an "abnormal" manual differential: nrbc > 0%, blasts > 0%, promyel > 0%, lymphoma cells > 0%, baso > 5%, myel > 5%, meta > 5%, eos > 10%, reactive lymphs > 10%. Sensitivity of R-flagging for these abnormalities was 81.7% and specificity 73.3%. The predictive value of an R-flagged ("positive") result was 48.3% and of an unflagged ("negative") result, 93.0%. Efficiency (accuracy) was calculated to be 75.5%. (Key words: Leukocyte sizing differential; Automated differential; Three-part differential) Am J Clin Pathol 1985; 84: 620-626

AUTOMATION of leukocyte differentials has been achieved by flow cytometry and pattern-recognition methods. Both techniques require expensive instruments and often cannot identify abnormal cells. Thus, these instruments provide screening differentials, flagging a certain proportion of suspect samples for further review.

Recently, a three-part screening differential based on leukocyte volume analysis has been added to Coulter® S-Plus series instruments. Hemolysis of red blood cells (RBCs) is accomplished with a weaker lysing reagent; this reagent leaves the white blood cells (WBCs) intact but shrinks the WBC cytoplasm. Lymphocytes are reduced to one-half their original volume, while granulocytes, because of their cytoplasmic granules, shrink to two-thirds original size.2,3 Monocytes and other mononuclear cells are reduced to a volume in between that of lymphocytes and granulocytes.

The resulting WBC sizing histogram is seen in Figure 1. The instrument sizes approximately 20,000 WBCs and determines the percentage of cells contained in the lymphocyte (LYMPH), mononuclear (MONO), and granulocyte (GRAN) fractions.2,3 The LYMPH fraction appears in the region between approximately 35–90 fl and is claimed to contain mature lymphocytes, atypical lymphocytes, and lymphoma cells.2,3 The GRAN fraction, in the area between 160–450 fl, contains polymorphonuclears, bands, metamyelocytes, eosinophils, and basophils.2,3 The MONO fraction is said to contain monocytes, blasts, promyelocytes, and myelocytes.2,3

The divisions between the fractions are individualized for each sample. However, if these valleys are not located within the expected region, an R-flag is displayed on the data terminal next to the result for the affected fraction. These R-flags indicate an abnormal WBC volume distribution, and, because the three-part differential may not be valid, these flagged results are not printed on the report form. When the WBC distribution is severely distorted, or when the WBC count is less than 1.0 or greater than 100.0 × 10^3/μL, the fraction percentages are not computed at all.

Backlighting, which shows as a darkened color behind the parameter on the data terminal, also serves to identify potential abnormal specimens and erroneous results. Backlighting of the WBC count indicates potential interference at 35 fl, which can be caused by nucleated RBCs, clumped platelets, giant platelets, megakaryocytic fragments, cryoglobulin, fibrin strands, or unlysed RBCs. The MONO fraction also shows backlighting if its absolute value is greater than 1.5 × 10^3/μL as a warning that a significant number of mononuclears may be present.

Ideal performance of the three-part differential would include accurate categorization and estimation of normal cells while R-flagging the samples with abnormal cells. To assess how closely these goals are met, we evaluated the performance of the Coulter S-Plus IV with three-part differential as compared with the conventional manual

Received November 16, 1984; received revised manuscript and accepted for publication April 29, 1985.
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EVALUATION OF LEUKOCYTE SIZING DIFFERENTIAL

Vol. 84 • No. 5

Fig. 1. Illustration of three-part differential histogram and component cells of the fractions. Volumes along x-axis are approximate points or "regions" where valleys are expected. When valleys are not found, an R-flag is placed next to the affected fraction(s).

slide differential in a population with a high prevalence of abnormalities.

Materials and Methods

The Coulter® S-Plus IV with three-part differential (IV-D) was installed and operated according to the manufacturer's instructions. The instrument was calibrated to reference manual methods for WBC and hemoglobin (Hgb), to historic moving averages for other RBC parameters, and to the Clay-Adams Ultraflo® for platelets. Calibration of the leukocyte sizing differential was performed by the manufacturer.

Since no assayed commercial control existed for this instrument at the time of evaluation, the IV-D was compared daily with a Coulter S-Plus IV in operation in the laboratory. This S-Plus IV was calibrated in a similar manner and controlled by using moving averages, 4C+, Technicon TQC® platelet-rich plasma (self-assayed), and by cross-referencing to manual WBC and Hgb methods.

Samples were collected in K3EDTA Vacutainers® (Becton Dickinson) or K2EDTA Microvettes® (Sarstedt) and processed between one to five hours of collection. Results from the IV-D three-part differential were compared with 100- or 200-cell manual slide differentials, with manual fractions consisting of the cells stated by the manufacturer (Fig. 1). When the manual and instrument differentials were discrepant, using the 2 SD limits Rumke,7 both were repeated. Repeat manual differentials were performed on 200 WBCs, and these new results were used for data analysis. When the WBC count was less than 1.5 X 10^3/µL, a buffy coat slide was prepared for manual differential.

Sorting and tabulating data, as well as regression analysis and scatter plots, were performed using Minitab®, a computerized statistical spreadsheet.9 Regression was performed using the IV-D results as the independent variable (because of increased precision), then solving the resulting equation for the IV-D as the dependent variable. Statistical significance was assessed using a two-sided paired t-test or by testing for differences between two proportions.10 Graphic illustrations were constructed on a Macintosh® computer.

Results

Precision

Measurement precision estimated on 14 samples by the method of duplicates showed coefficients of variation (CVs) of 1.1% for the GRAN fraction (mean 84.2%), 3.6% for the LYMPH fraction (mean 11.0%), and 12.3% for the MONO fraction (mean 4.8%). Since the IV-D uses a different lyse reagent than previous S-Plus instruments, assessment of WBC and Hgb precision is also important. Using three levels of 4C+ control over 20–30 days, the

Table 1. Comparison of White Counts on Samples with Backlighting

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>IV-D</th>
<th>IV</th>
<th>ZBI</th>
</tr>
</thead>
<tbody>
<tr>
<td>All samples</td>
<td>107</td>
<td>14.1*</td>
<td>13.3</td>
<td></td>
</tr>
<tr>
<td>nRBC &gt; 5</td>
<td>20</td>
<td>18.9*</td>
<td>17.4†</td>
<td>18.1</td>
</tr>
<tr>
<td>nRBC ≤ 5</td>
<td>87</td>
<td>13.0*</td>
<td>12.4</td>
<td></td>
</tr>
</tbody>
</table>

* P < 0.05 for IV-D minus IV equals 0.
† P > 0.05 for IV-D minus ZBI equals 0.
‡ P < 0.05 for IV minus ZBI equals 0.

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CV for WBC ranged from 1.0 to 2.5% and Hgb from 0.5 to 1.0%.

Comparison of Hemogram Parameters

Comparison of WBC and Hgb, as well as RBC parameters and platelets, showed excellent agreement between the S-Plus IV-D and the S-Plus IV. Regression analysis on 535 paired samples gave slopes ranging from 0.98 to 1.01, intercepts less than 2% of the mean of the data set, and correlation coefficients all greater than 0.99. However, samples with backlighting of the WBC count due to interference at the lower counting threshold gave higher WBC values on the IV-D (Table 1). This positive bias
existed whether or not significant numbers of nRBC were present. The samples without nRBC that showed a greater uplifting of the S-Plus IV-D histogram at the lower WBC threshold were most often from patients with giant platelets (myeloproliferative syndrome) or from patients with RBCs more resistant to lysis (sickle cell disease, severe liver disease with acanthocytes, and neonates). Thus the WBC count on the S-Plus IV-D may be more susceptible to interference by giant platelets and unlysed RBCs because of the weaker lysing reagent and a decreased lower counting threshold.

In 20 cases with NRBC ≥ 5/100 WBC, the WBC count was compared with a ZBI with a lower threshold setting of 20 fL. As expected, the IV (with lower threshold of 45 fL) had a lower total WBC count for these samples, as all of the smaller nucleated RBCs were not included. However, the IV-D (with lower threshold of 35 fL) actually had a higher total WBC count than the ZBI. Review of individual patient cases revealed that many of the samples with nRBC also contained giant platelets or RBCs more resistant to lysis. Thus, it was impossible to assess whether or not the IV-D included all of the nRBC in the WBC count.

Flagging Rate

A total of 989 samples analyzed on the IV-D were compared with the manual differential. These consisted of 13% from hematology clinic, 6% from oncology clinic, 8% from radiation therapy clinic, 8% from intensive care units, 11% in Microvettes® (mostly neonates), and 54% random patients.

The all-over flagging rate (samples with any R-flag) in the study was 39%, resulting in 387 flagged and 602 unflagged specimens. Region flagging by patient category is shown in Figure 2. The Microvettes samples (mostly neonates) have a very high flagging rate, which, on inspection of the histograms and review of cell morphology on the

Table 2. Comparison of Percentages in the IV-D Differential versus the Sum of the Constituent Cells in the Manual Differential

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Y*</th>
<th>X</th>
<th>BIAS</th>
<th>bY, X</th>
<th>aY, X</th>
<th>r</th>
<th>Sxy</th>
<th>d &gt; 10%</th>
</tr>
</thead>
<tbody>
<tr>
<td>LYMPH</td>
<td>602</td>
<td>21.3†</td>
<td>21.0</td>
<td>0.3</td>
<td>0.96</td>
<td>1.1</td>
<td>0.95</td>
<td>4.5</td>
<td>7 (1.2%)</td>
</tr>
<tr>
<td>MONO</td>
<td>602</td>
<td>5.4</td>
<td>7.4</td>
<td>-2.0</td>
<td>1.16</td>
<td>-3.1</td>
<td>0.53</td>
<td>4.0</td>
<td>14 (2.3%)</td>
</tr>
<tr>
<td>GRAN</td>
<td>602</td>
<td>73.2</td>
<td>71.6</td>
<td>1.6</td>
<td>0.97</td>
<td>3.9</td>
<td>0.94</td>
<td>5.6</td>
<td>30 (5.0%)</td>
</tr>
<tr>
<td>GRAN (R-FLAG)</td>
<td>219</td>
<td>55.8</td>
<td>47.9</td>
<td>7.9</td>
<td>0.98</td>
<td>8.9</td>
<td>0.82</td>
<td>16.0</td>
<td>108 (49.3%)</td>
</tr>
<tr>
<td>GRAN‡ (OTHER R-FLAG)</td>
<td>42</td>
<td>58.4</td>
<td>55.2</td>
<td>3.2</td>
<td>0.95</td>
<td>5.8</td>
<td>0.96</td>
<td>8.4</td>
<td>6 (14.3%)</td>
</tr>
</tbody>
</table>

* Y = mean of IV-D fraction; X = mean of constituent cells in manual differential; BIAS = Y – X; Regression: Y = bY, X + aY, X; r = correlation coefficient; Sxy = Standard error of regression; d = individual Y – X differences.
‡ GRAN fraction not flagged, but LYMPH and/or MONO fraction flagged.
† All values expressed as percent of total white blood cells.

Fig. 3. (Continued)
smear, resulted from larger lymphocytes entering the R₂ region. Other sample categories had a similar flagging rate to the random patients. To ensure that our flagging rate was not biased through purposeful selection of abnormal samples, all samples received in the laboratory on three afternoons were run on the IV-D. The 683 random samples processed on these three days showed a lowered rate of R-flagging (23%).

MONO fraction backlighting (MONO > 1.5 × 10³/μL) can also serve to flag the presence of abnormal cells. However, only ten samples were found in our study to have MONO fraction backlighting without the presence of an R-flag. Thus, this indicator was not considered further in this study.

Quantitative Accuracy

Regression analysis of the IV-D fraction percentages versus the manual differential results is shown in Table 2 and scatter plots in Figure 3. For the 602 unflagged samples (unflagged in any R-region), the LYMPH and GRAN percentages agreed well with the corresponding cell totals in the manual differentials. Regression coefficients and scatter plots (Figs. 3A and 3B) were as expected for a line of identity; bias was small, and only a few sample pairs showed absolute differences of more than 10%.

Regression analysis also was performed on 219 samples with R-flags on the GRAN fraction. These flagged samples had a lower mean total of manual granulocytes (47.9%) than the unflagged group (71.6%). A higher positive bias was reflected by the higher least-squares intercept. Of note was the increase in scatter of the results (Fig. 3C), reflected by the increased standard error of regression and lower correlation coefficient. Nearly one-half of the samples showed absolute differences of more than 10% from the manual differential. Thus, the % GRAN is not accurate when the result is R-flagged.

Forty-two cases occurred where the LYMPH and/or MONO fraction was flagged, but the GRAN fraction unflagged. Results for this group (Table 2 and Fig. 3D) were comparable to that for the completely unflagged samples (Fig. 3B).

Results for the MONO fraction were less satisfactory. Correlation coefficient was 0.53. Considering that the mean value for the manually calculated MONO fraction was 7.4%, the bias of ~2.0% was high, as was the standard error of regression of 4.0%. The scatter plot (Fig. 3E) also showed increased variability and suggested that IV-D often may give lower values than the manual differential.

Ninety-five unflagged samples with high monocytes (>10%) were included in our study. These samples had a mean of 12.4% monocytes, but the mean of the IV-D fraction was only 7.4% (P < 0.001). In Figure 5F, the percent in the IV-D MONO fraction is plotted versus the percent monocytes. The correlation coefficient is 0.15, and no linear relationship is evident. Thus, the MONO fraction does not reflect the percent of monocytes present in the sample and should be thought of as a remainder fraction rather than a measure of mononuclear cells.

Of the unflagged 602 samples, only 9 samples for the LYMPH fraction, 11 samples for the MONO fraction, and 26 samples for the GRAN fraction had differences of more than 10% from the expected results. Causes for these discrepancies are shown in Figure 4. Unflagged lymphoid blasts that the IV-D sized in the LYMPH fraction but were tallied in the manual differential as part of the MONO fraction, and myelocytes that sized in the GRAN fraction, but were tallied as belonging to the MONO fraction, accounted for eight discrepant cases. Twelve more discrepancies resulted from high percentages of monocytes, a portion of which were included by the IV-D in the GRAN fraction. But the largest number (28) of discrepancies occurred when the WBC count was low. In these cases, the IV-D counted a higher number in the GRAN fraction and less in the MONO and LYMPH fraction than the manual differential. We speculate that the manual differential, rather than the IV-D, may be erroneous when the WBC count is low because of non-random distribution on the wedge smear. A study comparing the IV-D differentials with flow cytometry, which also classifies large numbers of cells, could resolve this issue.

Causes of R-Flagging

Comparison of the proportion of flagged and unflagged samples with various abnormalities is seen in Figure 5. Significantly higher (P < 0.001) proportions of the following abnormalities were seen in the flagged group: nRBCs > 0%, blasts > 0%, promyelocytes > 0%, basophils > 5%, myelocytes > 5%, metamyelocytes > 5%, monocytes > 10%. However, not all instances of these abnormalities were flagged. The proportion of the following ab-
normalities was not higher ($P > 0.05$) in the R-flagged samples: lymphoma cells $> 0\%$, bands $> 10\%$, eosinophils $> 10\%$, reactive lymphocytes $> 10\%$.

**Flagging of Increased Eosinophils and Reactive Lymphs**

The rate of R-flagging of eosinophils and reactive lymphocytes is dependent on the percent present, as shown in Figure 6. Although our total number of cases with $> 10\%$ eosinophils (30) or reactive lymphocytes (14) is small, it is evident that many cases of eosinophilia and reactive lymphocytosis will not be detected.

**Flagging of Abnormal Cells**

R-flagging of various percentages of abnormal cells (blasts, promyelocytes, or lymphoma cells) is shown in Figure 7. A high proportion (13/41 or 32%) of samples with 1–5% abnormal cells were not flagged by the IV-D. In addition, 6/77 or 7% of samples with larger numbers of abnormal cells (small-sized lymphoblasts or lymphoma cells) were not flagged.

**Flagging of nRBC**

Samples with nRBC frequently cause WBC backlighting because of interference at the lower counting threshold for WBC. Also, it is possible that the presence of nRBC would be linked to other WBC abnormalities that would result in R-flags. As seen in Figure 8, combination of R-flagging with WBC backlighting added to the ability of the IV-D to detect nRBCs. However, for the 53 cases of one to two nRBCs, 19 (36%) samples still were not detected.

**R-Flag Sensitivity and Specificity**

The following findings were considered to constitute an “abnormal” differential, that is, one that should be flagged by the three-part differential screen for manual review: NRBC $> 0\%$; blasts $> 0\%$; promyelocytes $> 0\%$;
Table 3. Predictive Value of R-Flagging in Three-Part Differential

<table>
<thead>
<tr>
<th></th>
<th>Flagged (positive)</th>
<th>Unflagged (negative)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>&quot;Abnormal&quot; diff*</td>
<td>Sensitivity (True pos)</td>
<td>(False neg)</td>
<td>Prevalence</td>
</tr>
<tr>
<td>187 (81.7%)</td>
<td>42 (18.3%)</td>
<td>229 (23.9%)</td>
<td></td>
</tr>
<tr>
<td>&quot;Normal&quot; diff</td>
<td>Specificity (true neg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>200 (26.3%)</td>
<td>560 (73.7%)</td>
<td>760</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>387</td>
<td>602</td>
<td>989</td>
</tr>
</tbody>
</table>

Predictive value of positive: True pos/total flagged = 48.3%; Predictive value of negative: True neg/total unflagged = 93.0%; Efficiency (Accuracy): (True pos + True neg)/Total = 75.5%.

* "Abnormal" differentials are defined in text.

Discussion

The differential leukocyte count is a labor-intensive and expensive laboratory test. Although limited in effectiveness for screening or casefinding, it is of use in detecting or following acute infectious processes, hematologic disorders, and allergies, and in monitoring therapies with hematologic side effects. A variety of decisions can be made from a screening differential that accurately enumerates total neutrophils and lymphocytes. Our evaluation shows that the Coulter S-Plus three-part differential is capable of accurate enumeration of these cells, when the sample is not R-flagged. However, in a predominantly inpatient population, such as represented in our study, a high proportion of samples may be R-flagged.

In addition, a screening differential should flag samples with abnormal cells or increased numbers of normal cells. Our study shows that, based on R-flag criteria alone, this is not always true for small numbers of blasts, lymphoma cells, or nucleated RBCs, or increased numbers of reactive lymphocytes or eosinophils. The presence of abnormal cells or increase in reactive lymphocytes may be linked to other criteria in the complete blood count (CBC) (e.g., leukocytosis, neutropenia, lymphocytosis, or low platelet count) that would identify the samples requiring manual review. However, such linkage is not likely for increased eosinophils, and a manual differential or total eosinophil count must be performed when eosinophilia is suspected.

The Coulter leukocyte sizing differential will not separate bands from segmented neutrophils or flag an increase in bands. However, counting of bands in a 100-cell manual differential is limited by poor reproducibility. In a recent study, cytochemical determination of total neutrophils was found to be a more sensitive test for the diagnosis of acute appendicitis than the manual determination of bands. Similarly, the determination of total granulocytes on the IV-D, which examines large numbers of cells, may prove to be a more precise indicator of acute infectious or inflammatory conditions than the manual enumeration of bands.

It may indeed be true that all "abnormals" as arbitrarily defined in our study could have been identified if we had imposed high and low value limit flags for all of the CBC parameters in addition to the R-flags. However, in an inpatient environment, as opposed to an outpatient screening situation, requiring the complete CBC to be within normal range would lead to an unacceptably high rate of specimens needing blood smear examination. In a population such as ours, with a high prevalence of abnormalities, the best use of the Coulter S-Plus three-part differential will be for patient follow-up rather than for screening. Once it has been established that the patient is free of abnormal cells, the three-part differential can replace the manual differential in monitoring the percentage or absolute number of granulocytes.

Acknowledgments. The authors thank Coulter Electronics, Inc., for their support during this evaluation. They also thank all the technologists in the Stanford Hospital Hematology Laboratory for their help.

References