Staining of Leukocytes by the Tetrazolium Method:
Correlation of Results with the Leukocyte Count
and with the Clinical Classification

By PETER M. MARCUSE AND JOAN COCHRAN

M ANY CYTOCHEMICAL methods have been used in hematologic studies. As applied to leukocytes, these include the demonstration of phosphatase activity,\(^1\) the visualization of phosphorylase,\(^2\) and the staining of intracytoplasmic glycogen.\(^3\) Intracellular visualization of dehydrogenase activity in leukocytes can be achieved by the use of the tetrazolium salts, which are reduced to colored, water-insoluble formazans. Wachstein\(^4\) used the tetrazolium method to visualize the reducing activity in blood cells and in the bone marrow under normal conditions as well as in leukemia. Additional observations on the reduction of tetrazolium by leukocytes were recorded by Vercauteren.\(^5\) Bajuz and Szirmai\(^6\) used triphenyltetrazolium chloride solutions for the vital staining of leukocytes in peritoneal exudates of experimental animals. The following account deals with the technical details of the tetrazolium method as applied to laboratory studies and also with the significance of the results obtained by this procedure. In addition to a basic method, a number of modifications were employed.

Materials

Blood Samples

Specimens of whole blood, each measuring 7 ml., were obtained by vein puncture from 41 patients with leukemia and from 324 persons in categories other than leukemia.

Tetrazolium Solutions

Solution A.—1 ml. of neotetrazolium chloride (General Biochemicals), 1 per cent in distilled water; 4 ml. of phosphate buffer (pH 7.4).

Solution B.—1 ml. of neotetrazolium chloride, 1 per cent in distilled water; 3 ml. of 0.2 M sodium succinate in phosphate buffer (pH 7.4); 1 ml. of phosphate buffer (pH 7.4).

Solution C.—1 ml. of neotetrazolium chloride, 1 per cent in distilled water; 3 ml. of 0.2 M sodium succinate in phosphate buffer (pH 7.4) containing sodium malonate in 1.26 per cent solution; 1 ml. of phosphate buffer (pH 7.4).

Glucose Solution.—1 gm. of glucose, dissolved in 100 ml. of phosphate buffer (pH 7.4).

Glycerol Solution.—1 ml. of commercial glycerol diluted with 5 ml. of phosphate buffer.

Standard Procedure

Seven ml. of blood were immediately mixed with 7 mg. of sodium ethylenediamine tetraacetate (Cambridge Chemical Products) in a sealed tube (10 x 1.3 cm.). After a delay of not more than 5 hours the tube was inverted twice and the erythrocytes were then allowed to settle for about 30 minutes. The remaining suspension was removed by pipetting and was centrifuged at 1000 r.p.m. for 6 minutes. The sediment, representing a concentrate of

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leukocytes with an admixture of platelets and of a few red blood cells, was diluted with 8 drops of the plasma after this had been centrifuged at 3500 r.p.m. for 10 minutes. Two drops of the resulting suspension were mixed in a test tube (7.5 × 1 cm.) with 2 drops of solution A and with 2 drops of phosphate buffer. The tube was briefly flushed with nitrogen admitted through a narrow cannula. The preparation was then sealed with a rubber stopper and was incubated at 37° C. for 45 minutes. Upon completion of the incubation, a drop of the suspension was removed after shaking and was placed on a slide beneath a cover slip (22 × 22 mm.). The cells were examined under high magnification and the results were indicated by the percentage of leukocytes that contained within their cytoplasm either any number of large formazan particles or at least 5 punctate deposits (figs. 1 and 2). The number of leukocytes per cu.mm. of whole blood was determined as each sample was obtained.

MODIFICATIONS

The leukocyte suspension was divided into four equal portions. One of these was processed according to the standard method without substrate. The second was similarly processed, but glucose solution was substituted for the 2 drops of phosphate buffer. For the third portion, solution B (succinate) was employed instead of solution A, while the fourth fraction was diluted 1:4 with plasma and was then subjected to the standard procedure.

Fig. 1.—Fresh preparation obtained upon completion of the standard procedure. Three leukocytes contain formazan deposits. × 600.
Other modifications included the use of malonate as an inhibitor (solution C), freezing on dry ice for 10 minutes, and substitution of heparin for EDTA (1 mg. per 1 ml. of blood).

Permanent preparations were initiated upon completion of the incubation by mixing a drop of the suspension with a trace of a 1 per cent EDTA solution. A film was made from this mixture on a slide and was allowed to dry for 6 to 12 hours. The preparation was then fixed in methanol for 30 seconds and, after drying, was stained in working Giemsa's solution for 3 minutes. It was subsequently washed in distilled water and allowed to dry.

Storage prior to processing. Specimens, prior to processing, were set aside for various periods of time at room temperature and at 4° C.

**Results**

The formazan was visualized in the form of black and purple crystals that were either granular or needle-shaped and were intracytoplasmic in location (fig. 2). The reaction seemed strongest in the polymorphonuclear leukocytes but positive results were also observed in some of the lymphocytes (fig. 4). The strength of the reaction was indicated by the percentage of leukocytes stained and also by the quantity of formazan deposited within the individual cells. Storage of the blood sample or of the leukocyte suspension for 5 hours had...
no appreciable effect on the outcome of the reaction. The omission of nitrogen in the standard procedure resulted in a considerable decrease of the reaction. Among the anticoagulants heparin proved to be the most satisfactory, but its suitability was approached closely by EDTA. Defibrination was less acceptable and oxalate was detrimental to the reaction. Washing of the leukocyte suspensions prior to staining had no inhibitory effect on the deposition of formazan within the cells but seemed to decrease their tendency to clump. Variations in the incubation time were reflected by weak staining when the tubes were incubated for less than 30 minutes. The addition of malonate had, in most instances, no appreciable inhibitory effect. Freezing, on the other hand, caused a marked decrease in staining and was often followed by the complete absence of formazan deposits within the leukocytes. The use of permanent smears was made difficult by the solubility of the formazan in alcohol. Some degree of protection was afforded by the chelating effect of the EDTA that was employed as the anticoagulant and that was added again to the leukocyte suspension prior to preparing the films (figs. 3 and 4).
The role of the anticoagulant

The choice of ethylenediamine tetra-acetate (EDTA) was based on the literature dealing with its adaptability to various laboratory procedures. The effect of EDTA on dehydrogenase activity is not yet fully known. Tyler states that EDTA protects some enzyme systems against inactivation. The observations reported herewith indicate an overall increase in the percentage of stained cells when heparin is substituted for EDTA. A selective depression of any specific reaction by EDTA seems unlikely; the use of this anticoagulant is acceptable for comparison of different procedures as well as for a correlation of the results with hematologic or clinical data.

Significance of results

Succinate was employed in one tube of each set in order to detect any noticeable effect of succinic dehydrogenase. The use of glucose in the third tube seemed to be indicated, since Barka and Dallner have shown that the formazan within a lymphocyte.
azan production of ascites tumor cells was greater with glucose than with succinate. However the percentage of stained cells resulting from the individual procedures in our series reflect only a slight enhancing effect of glucose. The presence of succinate failed to raise the results above those obtained with tetrazolium alone. The reaction is nonspecific in type and indicates merely the existence of endogenous reductase within the leukocytes. The complex nature of the dehydrogenase system, as explained by Farber et al.\textsuperscript{13} makes it impossible to identify the degree of the reduction with any particular enzyme even if one could demonstrate a dependence on a specific substrate. Table 1 indicates that there is, in the nonleukemic category, a relation of the reaction to leukocytosis since results below 20 per cent are infrequent in this group when the leukocyte count exceeds 13,000 per 1 cu.mm. The explanation for the greater percentage of stained cells in the presence of leukocytosis may be found in the metabolic state of the leukocytes, as generally the function of the dehydrogenase system is increased under conditions of heightened metabolic activity.\textsuperscript{14} The immature white blood cells in leukemia were found by Wachstein\textsuperscript{4} to stain poorly with the tetrazolium. This observation is in accordance with our results, which reveal a striking discrepancy between the high leukocyte count of most leukemic specimens and their low percentages of cells stained by the formazan deposits (tables 1 and 3). The enhancing action of glucose was also absent in the leukemic group (table 2). No significant differences in staining were noted between lymphocytic leukemia and the granulocytic type, or between the acute forms and the chronic variants. Most disease categories other than the leukemias failed to show any noteworthy variations in the degree of staining, except for the increase ordinarily observed with a rise in the leukocyte count (table 3). The increase in the ratio of stained cells was particularly noticeable in the presence of marked leukocytosis and of leukemoid reactions. In these cases the differential count regularly revealed stab forms and often also juveniles and occasionally myelocytes, while blast forms were not observed. In the lymphoma

<table>
<thead>
<tr>
<th>Leukocytes (thousand/cu.mm.)</th>
<th>Number of cases</th>
<th>Mean result (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nonleukemic</td>
<td>Leukemic</td>
</tr>
<tr>
<td>4.0-7</td>
<td>107</td>
<td>34</td>
</tr>
<tr>
<td>7.1-10</td>
<td>88</td>
<td>29</td>
</tr>
<tr>
<td>10.1-13</td>
<td>60</td>
<td>31</td>
</tr>
<tr>
<td>13.1-16</td>
<td>26</td>
<td>38</td>
</tr>
<tr>
<td>16.1-20</td>
<td>21</td>
<td>35</td>
</tr>
<tr>
<td>20.1-25</td>
<td>9</td>
<td>37</td>
</tr>
<tr>
<td>over 25</td>
<td>13</td>
<td>60</td>
</tr>
</tbody>
</table>

\*Three hundred and eighty samples of whole blood were processed by the standard method without substrate. Specimens were divided into groups according to their initial leukocyte count.

\†Represents leukocyte count of whole blood prior to processing.

\‡Represents results expressed as the percentage of leukocytes that contained formazan at the end of the procedure. Mean values were computed for each group by calculating the arithmetic sum of the results and then dividing by the number of specimens examined in that group.

\§In 15 instances two samples were obtained from the same patient on different dates.
Table 2.—Comparison of Results Obtained by Modified Procedures*

<table>
<thead>
<tr>
<th>Modification†</th>
<th>Nonleukemic</th>
<th>Leukemic</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>34</td>
<td>20</td>
</tr>
<tr>
<td>Dilution of leukocyte suspension (1:4)</td>
<td>29</td>
<td>18</td>
</tr>
<tr>
<td>Succinate added</td>
<td>34</td>
<td>20</td>
</tr>
<tr>
<td>Glucose added</td>
<td>37</td>
<td>19</td>
</tr>
</tbody>
</table>

*Suspensions of leukocytes were prepared from 258 specimens, including 41 instances of leukemia. Each suspension was divided into 4 equal portions which were simultaneously subjected to different procedures.
†Refers to modification of standard procedure.

Table 3.—Comparison of Results Observed in Different Disease Categories*

<table>
<thead>
<tr>
<th>Clinical classification</th>
<th>Number of cases</th>
<th>Mean WBC/cu.mm.†</th>
<th>Mean result†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Miscellaneous</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(normal &amp; unclassified)</td>
<td>63</td>
<td>9.100</td>
<td>32</td>
</tr>
<tr>
<td>Elective surgery</td>
<td>18</td>
<td>9.770</td>
<td>29</td>
</tr>
<tr>
<td>Cardiovascular disease</td>
<td>42</td>
<td>10.420</td>
<td>28</td>
</tr>
<tr>
<td>Inflammatory disease</td>
<td>100</td>
<td>12.770</td>
<td>34</td>
</tr>
<tr>
<td>Neoplasms (exclusive of lymphomas)</td>
<td>16</td>
<td>15.050</td>
<td>48</td>
</tr>
<tr>
<td>Malignant lymphomas</td>
<td>45</td>
<td>5.710</td>
<td>39</td>
</tr>
<tr>
<td>Leukemia, lymphocytic</td>
<td>21</td>
<td>117.000</td>
<td>18</td>
</tr>
<tr>
<td>Leukemia, granulocytic</td>
<td>20</td>
<td>83.000</td>
<td>14</td>
</tr>
</tbody>
</table>

*Three hundred and twenty-five specimens from persons in different clinical classifications were subjected to the standard procedure.
†Represents leukocyte count of whole blood prior to processing. Mean values were computed for each category by calculating the arithmetic sum of the leukocyte counts and then dividing by the number of specimens belonging to that category.
‡See legend to table 1.

group the white blood cell count exceeded 10,000 per cu.mm. in only a few instances. A relatively high percentage of stained cells was observed in some cases of lymphoma having a leukocyte count of 4,000 per cu.mm. or less. In the remaining lymphoma cases the results were either in proportion to the total white count or somewhat higher than the total count would suggest. The presence of atypical or immature cells in the peripheral blood did not usually depress the staining ratio. The lymphoma category thus stood in contrast to the leukemias that had a low staining ratio even if blast forms were not prominent in the peripheral blood. In none of the disease categories could the tendency toward a strong formazan reaction be traced to any specific cell type; neither was weak staining regularly associated with any particular forms. The low staining ratio of the leukemias was as noticeable in the relatively differentiated variants as in the cases with many atypical cells. No special type of medication could be shown to have any constant effect on the tetrazolium staining of the leukocytes.

The advantage of the tetrazolium method lies primarily in the direct visual-
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ization of dehydrogenase activity. This permits a rapid appraisal of the intensity of the reaction and also allows localization of the sites at which the reduction takes place. The deficiency in quantitative evaluation is minimized when the observations are interpreted only on a relative basis by comparison between results obtained from different specimens under identical procedures. If this limitation is kept in mind the tetrazolium method may be regarded as suitable for use as a laboratory procedure. Its diagnostic value lies chiefly in the differentiation of the leukemias from nonleukemic conditions associated with an elevated leukocyte count. The discrepancy between the total count and the percentage of cells stained by formazan deposits is similar in significance to the low alkaline phosphatase activity observed in leukemia.¹

SUMMARY AND CONCLUSIONS

1. A method is described for the supravital staining of leukocytes with neotetrazolium chloride, using EDTA as the anticoagulant.
2. The reaction, as demonstrated by this method, is not dependent on any particular substrate and cannot be regarded as a function of specific dehydrogenase. It permits, however, the direct visualization of endogenous reductase activity.
3. The results, expressed as the percentage of leukocytes stained, show a relation to leukocytosis. Readings below 20 per cent are infrequently obtained with nonleukemic specimens when the leukocyte count exceeds 13,000/cu.mm. In leukemia low ratings are the rule, even in the presence of a high white blood count. In other diseases, including malignant lymphomas, the number of stained cells roughly parallels the elevation of the white blood count.

SUMMARIO IN INTERLINGUA

1. Es describite un methodo pro le tincturation supravital de leucocytos con chloruro de neotetrazolium, in le presentia de tetra-acetato ethylendiaminic como anticoagulante.
2. Le reaction que es demonstrate per iste methodo non depende de un substrato particular e non pote esser reguardate como function de dishydrogenase specific. Tamen, illo permitte le visualisation directe de activitate de reductase endogene.
3. Le resultatos, exprimite como procentage de leucocytos tincturate, revela un correlation con le leucocytose. Lecturas de minus que 20 pro cento es obtenite rarmente in specimens non-leucemic quando le numeration leucocytic exceede 13,000 per mm³. In leucemia, basse lecturas es le regulas, mesmo in le presentia de un alte numeration leucocytic. In altere morbos (include maligne lymphomas), le numero del tincturate cellulas es grossiermente parallel al elevation del numeration leucocytic.

ACKNOWLEDGMENT

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