Fluorescence Microscopy with Acridine Orange: A Study of Hemopoietic Cells in Fixed Preparations

By Lewis M. Schiffer

The use of acridine orange (AO) as a fluorochrome dye has recently gained some popularity in the disciplines of exfoliative cytology, tissue culture, and viral research. Its importance as a cytochemical reagent for nucleic acids has been emphasized by several investigators, and its specificity for ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) established by the use of their respective nucleases. The application of AO to the study of the blood-forming organs, particularly with regard to supravital staining, has also been recorded. This report describes a simple, reproducible fluorochrome technic for the study of fixed preparations of the blood and bone marrow. Variation of intracytoplasmic RNA content during the maturation sequence is described. In addition, the paper will give an account of the variation of color between the acridine orange-ribonucleic acid (AO-RNA) complexes of the erythroid, myeloid, and plasmacytic cells.

Materials and Methods

Thin smears of peripheral blood and bone marrow aspirate were made on one mm. thick non-fluorescent slides. Wherever possible, anticoagulants were avoided (heparin increases the background fluorescence without altering cell fluorescence). The slides were air dried and fixed in absolute methanol for three minutes. Fixation with Carnoy's fluid for three minutes gave comparable results. After fixation the slides could be stored for months. The staining procedure was a modification of the one devised by Dart and Turner with the major difference being a change in hydrogen ion concentration of the buffer and AO solution from a pH of 3.8 to 6.4. Trials were conducted varying the pH between 0.87 and 7.68. A pH of 6.4 for the fluorochrome solution was selected because of optimal color development and definition of cellular structure. The staining procedure was as follows:

1. Ethyl alcohol 80 per cent—5 dips
2. Ethyl alcohol 70 per cent—5 dips
3. Ethyl alcohol 50 per cent—5 dips
4. Distilled water—5 dips
5. Acetic acid 1 per cent—4 dips
6. Distilled water—2 minutes
7. McIlvane's buffer, pH 6.4—5 minutes

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*Erie Scientific Corp., Buffalo, N. Y.

1. Disodium hydrogen phosphate 19.66 Gm. and citric acid 6.46 Gm. diluted to one liter in distilled water.
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8. Acridine orange* (3,6-tetramethylindolinoacridine) 0.01 per cent in buffer pH 6.4—2 minutes
9. McIlvane's buffer, pH 6.4—4 minutes
10. McIlvane's buffer, pH 6.4—4 minutes

Fresh buffer was then placed on the slide and a no. 1 non-fluorescent coverslip applied. Most of the fluid was then withdrawn with absorbent paper. The latter procedure materially reduced fading during the microscopic examination. A Leitz Laborlux microscope was used with an Osram HBO 200 mercury vapor lamp as the illuminator. The primary or exciting filter was a Jena 4 mm. BG-12, and the secondary or barrier filter a Jena 2.5 mm. OG-1. This combination of filters effectively transmits blue and long ultraviolet light illumination to the slide but eliminates this part of the spectrum from the eyes of the observer. A bright field condenser was used for screening purposes; this was replaced with a dark field condenser for detailed examination and photography. Photomicrographs were made with high speed daylight Ektachrome (EH-135) using 30-second exposures with magnifications of 950X, and 25-second exposures with magnifications of 540X. All film and prints were processed at the Eastman Kodak Laboratories, Rochester, N. Y. After examination, the coverslips were removed under a gentle stream of water and the slides destained with methanol for five minutes. Wright's stain was applied after the slides had dried thoroughly. Using the vernier coordinates of a graduated mechanical stage, the same cells were observed and photographed under tungsten illumination. Accurate identification of cells was made possible by this procedure.

Pretreatment with ribonuclease was performed on a number of normal and abnormal smears. The slides were incubated for one hour at 37 °C. in a solution of protease-free ribonuclease, 1.0 mg./ml., in glass distilled water. Pretreatment of slides with deoxyribonuclease, 1 mg./ml., or streptodornase, 1000 units/ml., was similarly carried out. Control slides were treated identically except for the respective nucleases. The slides were then carried through the staining procedure.

RESULTS

Pretreatment with ribonuclease prevents orange and red cytoplasmic fluorescence (fig. 1). Pretreatment by deoxyribonuclease or streptodornase likewise removes all green or yellow fluorescence from the nuclei of cells (fig. 2). It can be concluded, therefore, that orange and red fluorescence in the cytoplasm and nucleolus is indicative of an AO-RNA complex, and the green or yellow fluorescence demonstrates an acridine orange-deoxyribonucleic acid (AO-DNA) complex. Using the AO fluorescent technic, 134 normal and abnormal marrow aspirates and 54 peripheral blood smears were examined.

The cytoplasm of the rubriblast of the normal erythroid series fluoresces intensely. With increasing maturity the fluorescence decreases (fig. 3). The reticulocyte has a fine, barely visible red fluorescence which is sometimes reticular in form, but more often appears stippled (fig. 4). The mature erythrocyte does not fluoresce. The greatest diminution in cytoplasmic fluorescence appears to occur between the prorubricyte and early rubricyte stages.

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1 Stock stain of AO 0.1 per cent in distilled water is diluted 1:10 with buffer. Tween 80, 2 ml., is added to each liter of stock stain.
2 Corning Glass Co., Corning, N. Y.
3 Eastman Kodak Co., Rochester, N. Y.
4 Mann Research Laboratories, New York, N. Y., and Sigma Chemical Co., St. Louis, Mo.
5 Mann Research Laboratories, New York, N. Y.
6 Varidase, Lederle Laboratories, Pearl River, New York.
Figs. 1–12.—See legends, facing page.
In the megaloblastic sequence of vitamin B₁₂ deficiency, the preceding pattern is altered to a certain extent. These erythroid elements appear to have more cytoplasmic fluorescence than normal cells of comparable maturation (fig. 5). The greatest decrement in fluorescence occurs later in the maturation sequence, at the late rubricyte level. In non-megaloblastic erythroid hyperplasia, the cytoplasmic fluorescence throughout maturation appears to be very slightly increased over normal.

In the normal myelocytic series, the cytoplasm of the myeloblast is intensely fluorescent. In this respect it is identical to the rubriblast. With maturation the fluorescence decreases until it is no longer apparent in the granulocyte (fig. 6). The greatest decrease in fluorescence appears to be between the promyelocyte and myelocyte stages. In acute myeloblastic leukemia, the predominant cell has intensely fluorescent cytoplasm and very distinct nucleolar fluorescence. There appears to be a very slight increase from normal in cytoplasmic fluorescence in cells of similar maturation of chronic myelocytic leukemia, polycythemia vera, and in leukemoid reactions.

Mature plasmacytes are easily recognized in the marrow smears by their vivid orange cytoplasmic fluorescence (fig. 7). This is the only mature cell in the marrow with such fluorescence. Immature forms, such as found in multiple myeloma, may appear to be even brighter and more vivid (fig. 8). It is apparent that maturity in this series is not accompanied by very significant decreases in cytoplasmic RNA.

Mature megakaryocytes are found to have a granular orange-red cytoplasmic fluorescence, while more immature forms display less granularity (fig. 9). Platelets are distinguished by their very faint pink fluorescence.

Lymphocytes in the bone marrow and peripheral blood usually have cytoplasmic fluorescence of intermediate intensity. The cytoplasm of the immature cells of acute lymphoblastic leukemia, however, are intensely fluorescent and can be equated to other "blast" forms (fig. 4). There appears to be little or no increase in the fluorescence of cells from patients with chronic lymphocytic leukemia. The atypical lymphocytes of infectious mononucleosis may exhibit
intense cytoplasmic fluorescence in addition to occasional well defined nucleolar fluorescence.

Monocytes display approximately the same fluorescent color and intensity as do myelocytes, and in one instance of monocytic leukemia the cytoplasmic fluorescence of the predominant cell was similar to that of a myelocyte in chronic myelocytic leukemia.

Reticulum cells are distinguished by their very distinct nucleoli. Cytoplasmic fluorescence is not remarkable and is often dull (fig. 10).

It has been shown that the intensity of fluorescence is proportional to the RNA content of cells. An attempt to quantitate the amount of RNA as a function of the intensity of fluorescence is illustrated in table 1. Cytoplasmic fluorescence was graded from zero to four plus, with the latter representing the fluorescence of the “blast” and zero representing no fluorescence. The table is a subjective evaluation of all cells observed during the study. Also included in the table are Thorell’s findings of the percentage of RNA found in the cytoplasm by means of ultraviolet spectrophotometry.

No consistent pattern of variation in nuclear fluorescence, from one cell to another, could be found during this investigation. Mitotic figures (fig. 11) furnished the sole exception to the above statement, since it was often apparent that there had been an increase in nuclear material. Nuclei which appeared more reticular and “open” often fluoresced green, while the more dense (such as plasma cell nuclei) fluoresced yellow. A not unexpected finding was that LE inclusions fluoresced green (fig. 12) indicating the presence of DNA.

The variation in cytoplasmic hue between the AO-RNA complexes of the erythroid, myeloid, and plasmacytic cells deserves attention. This variation will be referred to as cytoplasmic metachromasia. This is in contrast to the usual interpretation of the term “metachromasia,” as referring to a different color in the nucleus and cytoplasm produced by the same dye. It was observed that cytoplasmic metachromasia appears fairly constant for individual cell lines throughout maturation. Thus, the AO-RNA complex formed in the erythroid series is distinctly red (figs. 3 and 11), and this is just as apparent in the megakaryoblastic series (fig. 5). The most immature cells of the line, however, do have a tendency to fluoresce with a more orange hue. The AO-RNA complex in the granulocytic series fluoresces dull orange to orange-brown (fig. 6). This is evident throughout maturation once the myeloblast stage has been passed. Plasmacytes are easily distinguished by their vivid orange color (fig. 7). This is also apparent in the plasmacytes of patients with multiple myeloma (fig. 8).

DISCUSSION

It is evident that chemical procedures for the determination of the nucleic acids of the blood-forming elements suffer from several drawbacks. Notably, one cannot differentiate between the various cell lines, and population mixtures are invariably encountered. It is equally apparent that measurement of the nucleic acids of individual cell types at various phases of maturation must be performed cytochemically. In 1947, Thorell published his classic
monograph of the cytoplasmic nucleic acids of the bone marrow as measured by ultraviolet microspectrophotometry. From this and subsequent reports it became evident that RNA was at its maximum in the “blast” forms and decreased during maturation. It was also found that the mature plasma cell contained large amounts of cytoplasmic RNA. Although this paper does not propose quantitation of the RNA of blood elements as Thorell has so elegantly done, it does provide essentially the same type of data (table 1). This technic provides a basis for investigation with microspectrophotometric methods for both RNA and DNA.

Recent studies of the physical chemistry of the acridine orange-nucleic acid complexes have shown that the dye molecules are bound to nucleic acids as a function of the number of binding sites on the acid and the number of dye molecules available. It is believed that the aggregation or stacking of the dye molecules on the nucleic acid results in metachromasia. RNA apparently has more available binding sites and promotes stacking, whereas DNA has fewer

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Table 1.—Cytoplasmic Fluorescent Color and Intensity of Individual Cells

<table>
<thead>
<tr>
<th>Cell</th>
<th>Cytoplasmic Color</th>
<th>Fluorescent Intensity</th>
<th>RNA in Cytoplasm (after Thorell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rubriblast</td>
<td>orange-red</td>
<td>++++</td>
<td>5%</td>
</tr>
<tr>
<td>Prorubricyte</td>
<td>red-orange</td>
<td>+++ - +++++</td>
<td>2%</td>
</tr>
<tr>
<td>Rubricyte</td>
<td>red</td>
<td>+ - +</td>
<td>0.5%</td>
</tr>
<tr>
<td>Metarubricyte</td>
<td>red</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>Reticulocyte</td>
<td>red</td>
<td>trace</td>
<td></td>
</tr>
<tr>
<td>Erythrocyte</td>
<td>none</td>
<td>none</td>
<td></td>
</tr>
<tr>
<td>Megaloblast (pernicious anemia)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>large</td>
<td>orange-red</td>
<td>++++</td>
<td>5%</td>
</tr>
<tr>
<td>small</td>
<td>red</td>
<td>+++</td>
<td>3%</td>
</tr>
<tr>
<td>Myeloblast</td>
<td>orange</td>
<td>++++</td>
<td>5%</td>
</tr>
<tr>
<td>Proerythroblastic</td>
<td>orange-brown</td>
<td>+++ - +++++</td>
<td>3%</td>
</tr>
<tr>
<td>Myelocyte</td>
<td>orange-brown</td>
<td>+</td>
<td>0.5%</td>
</tr>
<tr>
<td>Metamyelocyte</td>
<td>orange-brown</td>
<td>trace - +</td>
<td></td>
</tr>
<tr>
<td>Band</td>
<td>orange-brown</td>
<td>0 - trace</td>
<td></td>
</tr>
<tr>
<td>Segmented cell</td>
<td>none</td>
<td>none</td>
<td>0 ± 0.5%</td>
</tr>
<tr>
<td>Myeloblast of acute myeloblastic leukemia</td>
<td>orange</td>
<td>++++</td>
<td>5-7%</td>
</tr>
<tr>
<td>Plasmacyte</td>
<td>orange</td>
<td>+++ - +++++</td>
<td>3%</td>
</tr>
<tr>
<td>Plasmacyte of multiple myeloma</td>
<td>orange</td>
<td>++++</td>
<td>3-5%</td>
</tr>
<tr>
<td>Lymphocyte</td>
<td>orange-red</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>Lymphoblast of acute lymphoblastic leukemia</td>
<td>orange</td>
<td>++++</td>
<td>5-7%</td>
</tr>
<tr>
<td>Monocyte</td>
<td>orange-brown</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Megakaryocyte</td>
<td>orange-brown</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Reticulum cell</td>
<td>red-orange</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>
such sites and does not promote stacking. The hydrogen ion concentration of the medium is also an important variable. Interestingly, not only the AO-RNA but also the AO-DNA complex fluoresces metachromatically at pH 6.4. This accounts, in part, for the green and yellow colors which are observed in the nucleus.

An intriguing aspect of this study was the repeated observation that the AO-RNA complexes of the erythroid, myeloid, and plasmacytic series fluoresce with different colors. This difference is most apparent in cells beyond the "blast" stage of maturation. The color variation is reproducible and allows for differentiation of cells by means of cytoplasmic fluorescence rather than by more classical methods. There are a number of possibilities to consider as the cause of the cytoplasmic metachromasia. It may represent a variation in the chemical species of RNA, and therefore a difference in their stacking capabilities. For example, one can speculate that a large portion of the RNA of the maturing erythroid cell is primarily concerned with the formation of hemoglobin, and that of the plasma cell with immune globulin. Considering the sensitivity of this system to changes in pH, cytoplasmic metachromasia may represent a difference in intracellular hydrogen ion concentration. The surrounding or coating materials may also influence cytoplasmic metachromasia. Hemoglobin, for example, quenches fluorescence to a certain extent and this may account for the variation produced in the cytoplasm of the erythroid cell.

**Summary and Conclusions**

1. A technic for the study of fixed preparations of blood and bone marrow by means of fluorescence microscopy with acridine orange is presented.
2. The distribution of RNA and DNA within the blood cells can be demonstrated cytochemically.
3. The RNA content of the erythroid and myeloid cells decreases with maturation. Mature plasma cells contain large quantities of RNA.
4. It is possible to demonstrate cytoplasmic metachromasia between the AO-RNA complexes of the erythroid, myeloid, and plasmacytic cells.

**Summary in Interlingua**

1. Es presentate un methodo pro le studio de fixate preparatos de sanguine e de medulla ossee per medio de microscopia fluorescentic con orange acridinic.
2. Le distribution de acido ribonucleic e de acido disoxyribonucleic pote esser demonstrate cytochimicamente.
3. Le contenu de acido ribonucleic del cellulas erythroide e myeloide declina con le maturation de ille cellulas. Matur plasmocytos contine grande quantitate de acido ribonucleic.
4. Il es possibile demonstrar metachromasia cytoplasmic inter le complexos de orange acridinic e acido ribonucleic in le cellulas erythroide, myeloide, e plasmatic.

**Addendum**

An article, by Prof. Alberto Marmont, on the supravital staining of the formed blood elements with AO should be included among the references. It can be found in the Pro-
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11. Ibid.: p. 916.


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