Euchrysine, A Supravital Fluorescent Lysosomal Stain: Technic and Application for Hematologic Investigation

By R. S. Blume, P. R. Glade and L. N. Chessin

LYSOSOMES are single membrane bound, subcellular organelles which contain a variety of acid hydrolases and other biologically active materials.1,2 They are usually electron dense, round or ovoid, and 0.2-0.5 microns in diameter (primary lysosomes).3,4 They participate both in normal cellular processes and in disease states,1 following which they are referred to as secondary lysosomes.5 They have been difficult to visualize because of their small size and their limiting membrane. The state of this latter component affects the results of enzymatic staining;6 consequently, the correlation of particulate lysosomal structure with enzyme activity is often erratic.7 The identification of lysosomes and the definition of their cytoarchitectural features are best achieved with the electron microscope and electron microscopic histochemistry,8 but these technics are complex, time consuming, and have a high degree of sampling error.

It has previously been noted in tissue culture cells that euchrysine, an aminoacridine fluorescent supravital dye, is preferentially concentrated in lysosomes.9 In the present study, we report methods for the preparation of this dye into a form suitable for hematologic investigation and its use in the characterization of lysosomes in human peripheral blood, bone marrow and established lymphoid cell lines maintained in vitro.

MATERIALS AND METHODS

Hematologic Specimens

Material for examination was obtained from a variety of sources: 1) peripheral blood from patients with infectious mononucleosis, lymphoblastic leukemia and the Chediak-Higashi syndrome and from normal volunteers; 2) bone marrow aspirates of hematologically normal subjects; 3) long-term continuous suspension cultures of lymphoid cells10 from patients with infectious mononucleosis, unexplained hypergammaglobulinemia, lymphosarcoma, and Burkitt's lymphoma. Heparin (3-5 units/ml.) was added to blood and bone marrow specimens.
Phagocytosis

Lysosomal changes following phagocytosis were assessed with heat-killed *Staphylococcus albus*. Bacteria were added (100 bacteria/phagocytic cell) to leukocyte rich plasma obtained by gravity sedimentation of heparinized blood at 37 C. The mixture was incubated at 37 C. in 40 ml. round bottomed glass test tubes placed in a Dubinoff shaker.

Preparation of the Aminoacridine Euchrysine

The powdered aminoacridine euchrysine (2 GNX, No. 46040, C. T. Gurr, London, England) was diluted in 0.01 M phosphate buffered saline (pH 7.4), isolated chromatographically on a calcium phosphate (hydroxyapatite) column,¹¹ and subsequently purified on a Sephadex G-25 column (2 X 25 cm.). The eluate was monitored by its absorption of ultraviolet light at 280 m(µ)l. Fractions with high absorption were then pooled, dialyzed against 0.01 M phosphate buffered saline (pH 7.4) for 24 hours at 4 C. and subsequently lyophilized. This purified material was diluted in phenol-red free Eagle’s Spinner Minimal Essential Medium to a concentration of 1 µg./ml. The reagent was stored at room temperature in the dark until used, and was stable for at least six weeks.

The purified euchrysine was added to the material to be studied, and incubated in the dark at 37 C. for 30 minutes to one hour. The volume of euchrysine added varied with the material to be examined. An appropriate ratio was essential to achieve adequately uniform and sufficiently intense fluorescence without inducing cell death: 0.2-0.3 ml. euchrysine (1 µg./ml.) to each ml. of peripheral blood; 0.1-0.2 ml. to each ml. of continuous suspension culture lymphoid cultures, 0.4-0.5 ml. to each ml. of bone marrow aspirate. An inadequate quantity of dye resulted in a variable degree of green or yellow lysosomal fluorescence. An excess of euchrysine resulted in diffuse orange nuclear and cytoplasmic fluorescence, without a particulate pattern. Smears or wet mounts were made of the incubated material without counterstain or fixative.

The prepared material was examined on a fluorescent microscope (American Optical Series 10 Microstar with a trinocular body), using a darkfield condenser and a Fluorolume Illuminator (AO Model 645), with excitor filter Schott BG 12 (3 mm. thick) and a colorless Barrier filter E. K. #2A (20 mm. diameter) or orange barrier filter E. K. #15 (20 mm. diameter). Only smears were suitable for photography. Photomicroscopy was performed using a Polaroid land camera microscopic attachment and Polaroid 3000 ASA Black and White Type 107 Land Film Packs. Cell outlines could be better defined for photographic purposes by double exposure adding the orange barrier filter (see Fig. 5).

Results

Euchrysine selectively stained lysosomes of cells in peripheral blood, bone marrow, and continuous suspension cultures. Smears were stable for at least 72 hours in the dark at either room temperature or 4 C. All lysosomes displayed bright orange fluorescence and contrasted clearly from other subcellular organelles. Some differences in the shades of orange were noted and appear to be a function of both cell and lysosomal type (see below). Both nuclei and cytoplasm displayed green fluorescence, but differed in their intensity of staining, nuclear fluorescence being more intense than cytoplasmic fluorescence. Mature erythrocytes did not fluoresce.

Primary Lysosomes

See Table 1.

Peripheral Blood (Fig. 1)

The cytoplasm of normal peripheral neutrophils was completely obscured by the intense pinpoint (0.3 µ) particulate orange fluorescence of the numerous
Table 1.—Characteristics of Euchrysine Stained Primary Lysosomes

<table>
<thead>
<tr>
<th>Cell</th>
<th>Number per Cell</th>
<th>Lysosomal Characteristics*</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophils</td>
<td>Innumerable</td>
<td>0.3</td>
<td>YELLOW TINGED; round; obscuring the cytoplasm</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>100-150</td>
<td>0.5</td>
<td>GREEN TINGED; round; obscuring the cytoplasm</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>1-20</td>
<td>0.3</td>
<td>Pale green cytoplasm; dark green nuclei</td>
</tr>
<tr>
<td>Lymphoblasts</td>
<td>25-75</td>
<td>0.3</td>
<td>Pale green cytoplasm; dark green nuclei</td>
</tr>
<tr>
<td>Lymphoid Cells in</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Continuous Culture</td>
<td>25-75</td>
<td>0.3</td>
<td>Similar to above</td>
</tr>
<tr>
<td>Monocytes</td>
<td>50-100</td>
<td>0.3</td>
<td>Extremely pale green cytoplasm and nuclei</td>
</tr>
<tr>
<td>Platelets</td>
<td>3-10</td>
<td>0.3</td>
<td>YELLOW TINGED; round</td>
</tr>
<tr>
<td>Myelocytes</td>
<td>75-100</td>
<td>0.5</td>
<td>RED TINGED; round</td>
</tr>
</tbody>
</table>

* All euchrysine stained lysosomes fluoresce ORANGE; variations occur in the shades of orange exhibited.

primary lysosomes (specific neutrophilic granules) which outlined the nuclear lobulations. The specific granules in eosinophilic leukocytes exhibited a yellow-green hue to their fluorescence, were distinctly refractile, and were slightly larger than neutrophilic granules. Lymphocytes (small, medium, and large) contained one to twenty pinpoint discrete fluorescent lysosomes, the majority containing less than eight. An occasional lymphocyte contained a single aggregate of fluorescent granules, 2–4 in number. The cytoplasm of the lymphocytes appeared pale green, clearly distinguishable from the black background in darkfield microscopy. Monocytes were identified by their irregular nuclear outlines and by their very pale green nuclear fluorescence. The distribution and number (50–100) of lysosomes in these cells was intermediate to that noted in granulocytes and lymphocytes. Some of the lysosomal granules of monocytes appeared larger than those of neutrophilic leukocytes and lymphocytes. Platelets contained four to ten 0.3 μ discrete orange fluorescing granules in their cytoplasm.

Marrow (Fig. 2)

The fluorescence of lymphoid cells, monocytes, platelets, and mature granulocytes in marrow aspirates was similar to that seen in peripheral blood. The myeloid series in marrow demonstrated a distinct pattern of lysosomal fluorescence. The earliest distinctly myeloid cells seen displayed bright orange granules filling their cytoplasm. These were larger (0.5 μ) than those found in mature circulating neutrophils, less densely packed, and more orange in hue. The granules observed in myeloid cells in marrow in the subsequent stages of maturation were slightly smaller (<0.3 μ), more numerous, and less intensely orange (more yellow) than those in the earlier myeloid precursors.

Maturing (nucleated) erythrocytes closely resembled small lymphocytes, but could be distinguished from them by the relatively greater intensity of
Fig. 1.—Peripheral blood leukocytes and platelets from normal individuals. Nuclei appear black in contrast to the brilliant lysosomal fluorescence. Innumerable pin point fluorescent lysosomal granules are seen in polymorphonuclear neutrophils (A upper; C upper). Larger and more refractile granules are present in eosinophils (A arrow). A small variable number of fluorescent granules are present in lymphocytes (B lower; D arrows). An intermediate number of fluorescent granules are seen in monocytes (B upper left; C lower left). Scattered platelets with a few fluorescent granules are also present (D lower right). Euchrysine stain. Original magnification X1000.
Fig. 2.—Cell from normal bone marrow aspirates. The stages of myeloid maturation between myelocyte and mature granulocyte are accompanied by an increase in the number of the specific fluorescent lysosomal granules until the appearance of confluence occurs (A upper to lower). In addition to the numerous granulocytes with innumerable fluorescent granules, a cluster of erythropoetic cells is seen (B arrow). The degenerating nuclei of the maturing erythroid cells are fluorescent as are a very few granules in their cytoplasm. The size of a histiocytic cell is striking (C). Euchrysine stain. Original magnification X1000.
### Table 2.—Characteristics of Euchrysine Stained Secondary Lysosomes

<table>
<thead>
<tr>
<th>Cell</th>
<th>Number per cell</th>
<th>Lysosomal Size (μ)</th>
<th>Characteristics *</th>
<th>Other Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phagocytic Neutrophil</td>
<td>5-15</td>
<td>0.6-1.0</td>
<td>RED TINGED; round; disappearance of primary lysosomes</td>
<td></td>
</tr>
<tr>
<td>Phagocytic Monocyte</td>
<td>5-10</td>
<td>0.6-1.0</td>
<td>RED TINGED; round; some primary lysosomes remain present</td>
<td></td>
</tr>
<tr>
<td>C-HS Neutrophils</td>
<td>4-12</td>
<td>2.0-4.0</td>
<td>Irregular shape; a few 0.3 μ lysosomes are also present</td>
<td></td>
</tr>
<tr>
<td>C-HS Lymphocytes</td>
<td>1-3</td>
<td>1.0-3.0</td>
<td>Round; a few 0.3 μ lysosomes are also present</td>
<td></td>
</tr>
<tr>
<td>C-HS Monocytes</td>
<td>1-3</td>
<td>1.0-3.0</td>
<td>Round; the normal number of 0.3 μ lysosomes is also present</td>
<td></td>
</tr>
<tr>
<td>C-HS Eosinophils</td>
<td>20-40</td>
<td>1.0-3.0</td>
<td>GREEN TINGED; refractile; irregular; uniformly larger than normal</td>
<td></td>
</tr>
</tbody>
</table>

* All euchrysine stained lysosomes fluoresce ORANGE; variation occurs in the shades of orange exhibited.

† Chediak-Higashi Syndrome

their nuclear fluorescence and the total absence of cytoplasmic fluorescence. Rare punctate orange fluorescing particles were seen in their cytoplasm, particularly in those of relative immaturity. Large ameboid histiocytes 30 micra in size contained a larger number of discrete orange staining normal sized (0.3 μ) fluorescent particles in their cytoplasm. Only a rare megakaryocyte could be discerned. This was the largest cell type seen (<50 μ) and exhibited a pattern of cytoplasmic and particulate fluorescence at its margins similar to that of mature circulating platelets, although the central area of cytoplasm was devoid of lysosomal structures.

**Secondary Lysosomes**

See Table 2.

**Phagosomes (Fig. 3)**

Normal leukocytes were examined during phagocytosis of heat-killed *Staphylococcus albus* to assess the staining of secondary lysosomes by euchrysine. The organisms (incubated with euchrysine in the absence of cells) exhibited a very weak light green fluorescence. Striking changes were observed in the pattern of lysosomal fluorescence in neutrophils and monocytes following phagocytosis. After one, two, and three hours, the normal 0.3 μ orange-yellow fluorescing granules in polymorphonuclear leukocytes were no longer visible. There were, however, 5 to 15 round bright reddish-orange 1.0 micra fluorescent particles scattered throughout the cytoplasm of each neutrophil. Phagocytic monocytes demonstrated similar orange-red structures in all samples observed. As opposed to neutrophils, however, smaller normal primary lysosomes were always present. After three hours of phagocytosis, a
Fig. 3.—Phagocytosis by normal polymorphonuclear neutrophils. The weak fluorescence of the staphylococci is apparent even in this prolonged exposure (A). Phagosomes, in neutrophils, are fluorescent and larger than normal primary lysosomes 1 hour (B), 2 hours (C), and 3 hours (D) after inception of phagocytosis. A few normal size fluorescent lysosomal granules are seen only in the cells examined at 3 hours (D arrow), but are not seen following phagocytosis prior to this time. Euchrysin stain. Original magnification X1000.

Small number of normal sized orange granules were again present in neutrophils. No change was noted in either the lymphocytes or platelets.

Autophagocytic Vacuoles (Fig. 4, E and F)

Abnormal lysosomal structures thought to be autophagic vacuoles are present in circulating leukocytes of patients with the Chediak-Higashi syndrome. Both eosinophilic and neutrophilic granulocytes from affected individuals exhibited distinct, irregular, 2–4 micra diameter granules in their cytoplasm. Those in eosinophils were refractile, greener, and smaller than the intensely orange staining, larger and more irregular structures in the neutrophils. Only a very few normal sized lysosomal structures were present in granulocytes of these patients. Monocytes were normal in appearance except
Fig. 4.—States with abnormalities of lysosomal patterns. Infectious Mononucleosis (A-D). An increased number of fluorescent lysosomal granules (A, B) is present in the large atypical lymphoid cells from peripheral blood of patients with infectious mononucleosis. Distinct aggregates of normal sized lysosomes (C, D) and aggregates of lysosomes which appear almost fused into large fluorescent masses (D arrow) are seen. Chediak-Higashi Syndrome (E, F). The giant fluorescent lysosomal granules characteristic of this syndrome are strikingly apparent in neutrophils (E) as well as being present occasionally in lymphocytes (F arrow) and monocytes (F right). The fluorescence of the granules is so brilliant that the cells in which they are present appear black in this briefly exposed photograph. Euchrysine stain. Original magnification X1000.
Fig. 5.—Acute lymphoblastic leukemia, peripheral blood. The majority of cells present are large lymphoid cells with an increased number of normal sized fluorescent lysosomal granules (A, B). Some aggregates of fluorescent granules (B) are seen. The margins of the erythroid cells are seen when the double exposure technic is used (B). Euchrysine stain. Original magnification X1000.

for an occasional large lysosomal granule 2–4 μ in diameter. All lymphocytes had at least one normal sized fluorescing lysosomal structure. Ten to fifteen percent of the lymphoid cells had one or more abnormally large discrete 1–3 μ orange fluorescing lysosomal particles present in their cytoplasm.

Infectious Mononucleosis (Fig. 4, A-D)

Examination of the peripheral leukocytes from patients with active heterophile-positive infectious mononucleosis revealed that the number of lysosomes in the atypical lymphoid cells was increased, approaching that usually seen in monocytes. The nuclear appearance of these cells, nevertheless, was that of young large lymphocytes. In addition, clumping of lysosomes was frequently seen. Such clusters contained from four to fifteen particles. Some normal monocytes and lymphocytes were also present.

Acute Lymphocytic Leukemia (Fig. 5)

The peripheral blood of a patient with acute lymphocytic leukemia in blastic crisis contained large cells with pale green staining nuclei characteristic of large lymphoid cells. They displayed a greater number of orange fluorescing lysosomes 0.3 μ in size (distributed randomly throughout the cytoplasm) than did normal lymphocytes.

Long Term Suspension Cultures of Cells with Lymphoid Characteristics (Fig. 6)

The findings were similar in all 10 lines studied. The lysosomes were 0.3 μ in size and orange in color, similar to those seen in normal circulating lymphocytes. They were, however, increased in number, closely resembling lymphoblasts in peripheral blood. A variable degree of lysosomal clumping was apparent. The aggregates were present in all lines. In addition to the normal sized lysosomes present in these cultured lymphoid cells, there were one to
Fig. 6.—Four continuous suspension cultures of cells with lymphoid characteristics derived from patients with Burkitt's lymphoma (A), lymphosarcoma (B), infectious mononucleosis (C) and hypergammaglobulinemia of undetermined etiology (D). A large number of fluorescent lysosomal granules are present in the cells from all the lines and aggregation of these particles can be quite striking (C arrow). Euchrysine stain. Original magnification X1000.

five larger (0.5-0.8 μ) orange fluorescing lysosomal structures present in some cells (5 per cent).

DISCUSSION

Morphologic studies have demonstrated the presence of a single membrane limiting all lysosomal structures as a unifying characteristic independent of
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type, cell origin, and content.\textsuperscript{13} This membrane has been used to explain the "latency" of lysosomal acid hydrolases,\textsuperscript{2} and appears to have a significant lipoprotein content.\textsuperscript{14} Lysosomal staining by aminoacridines including euchrysine appears to be a function of the affinity of these cationic dyes for lysosomal membranes,\textsuperscript{15} not a function of lysosomal content. This affinity exists at low temperatures, after freeze-thawing, and can be shown with isolated lysosomes, indicating a passive process dependent on the chemical composition of the membrane, perhaps due to the presence of an insoluble lipophilic polyanion.\textsuperscript{16} It is possible to achieve intense selective and uniformly reproducible lysosomal fluorescence with euchrysine. The superiority of euchrysine to the other aminoacridines is due to its greater relative affinity for lysosomes than that exhibited by the other supravital fluorescent stains.\textsuperscript{9}

The sephadex gel and calcium hydroxyapatite column purifications yielded a preparation with which uptake by and fluorescence of nonlysosomal structures was minimal. The dilution to 1 \(\mu\)g./ml. of purified substance was sufficient to achieve reliable staining of blood cells without causing cell death. The use of buffered diluent to maintain a neutral pH increases the affinity of dye for lysosomes,\textsuperscript{17} reduces the cellular toxicity of the final preparation, and in conjunction with dark-storage, reduced the decomposition and quenching of dye.

Euchrysine was originally used to stain and examine viable tissue culture cells.\textsuperscript{9} Blood cells are considerably more difficult to study. They are smaller than fibroblasts, amnion or kidney cells in culture, free-floating, and except for granulocytes, have only a small number of fluorescent particles. However, once the supravital stained blood cells were spread on glass slides, they could easily be examined, identified, and photographed. The integrity of the lysosomal fluorescing particles was not destroyed by the preparation of slides, subsequent air drying, and storage for up to 72 hours. Agar embedding as previously described\textsuperscript{17} was not found to be necessary.

The volume of dye necessary for adequate staining varied greatly with the material to be stained and is probably a function of the numbers of available cells and particles per cell capable of accumulating the dye. There was also a minor variability in the appropriate amount of dye needed for any one material. This often could be assessed only by trial and error. It was also important to limit the duration of cellular exposure to euchrysine prior to making slides since at least one other aminoacridine can induce lysosomal swelling following prolonged exposure.\textsuperscript{18}

The application of this technic to blood cells has yielded several pieces of information. The lysosomes of all cell varieties observed (lymphoid, monocytoid, granulocytic, erythroid, platelets, and lymphoid cells in continuous culture) exhibited remarkably similar staining characteristics. Furthermore, the lysosomes of hematopoietic cells stained similarly to those of fibroblastic, renal, and amnionic origin as previously described.\textsuperscript{9,19,20} There is also a striking similarity between the fluorescence of primary lysosomes and secondary lysosomes, and also between different types of secondary lysosomes. Although exhibiting minor differences in fluorescent coloration (e. g., specific vs. nonspecific myeloid granules), all lysosomes appear to selectively accumu-
late euchrysine. This uniform behavior appears to be related to the presence of similar lipophilic polyanions in the limiting membranes of all lysosomes.

Lysosomes in cultured lymphoid cells have been noted previously, but their abundance has not been appreciated. Euchrysine stained cells of these culture lines and circulating lymphoblasts contained a larger number of lysosomes than the atypical lymphoid cells of patients with infectious mononucleosis. The latter cells, however, contained more lysosomes than normal. The occasional larger than normal fluorescent particles seen in these lines probably represent the secondary lysosomes of the myelin figure variety previously noted in these lines. The most striking abnormality in both the cultured cells and infectious mononucleosis cells, however, was the widespread appearance of lysosomal clumping. This pattern is similar to that seen in a variety of virus-infected culture systems. However, the specificity of this pattern for virus-infected cells has not been determined.

The present study demonstrates that euchrysine is relatively easy to use and affords excellent definition at the light microscopic level of both primary and secondary lysosomes, as well as distinguishing between several varieties of lysosomes. Any single histochemical or non-enzymatic stain (e.g., phosphatase, peroxidase, PAS, oil red 0, and Sudan black) can not be applied to the identification of all lysosomes, nor are any of these specific for lysosomes. However, euchrysine appears to be a reliable lysosomal marker since all lysosomes (independent of species of origin, cell or organ type, lysosomal type and state of lysosomal membrane integrity) are selectively stained with this dye.

Euchrysine fluorescent staining would therefore appear to be a significant technical advance for the investigation of hematologic cells. It makes possible the easy assessment of the presence, type, and distribution of a subcellular particle, heretofore relatively inaccessible except to the electron microscope. In addition, the technic can be applied to conditions either in vivo or in vitro during which there are changes in the nature and state of activity of these particles, e.g., phagocytosis, autophagy, viral infection, and possibly malignant change. There is reason to believe that it may be a dependable marker for following isolated lysosomes or lysosomal membranes during cellular fractionation. Also, euchrysine staining of cells may be of value in performing routine laboratory procedures involving peripheral leukocytes, platelets, or bone marrow aspirates by increasing the ease and dependability of cell counting, or by altering the material to a form better suited to the development of automated counting systems.

**Summary**

Euchrysine, a fluorescent aminoacridine dye which is selectively accumulated in lysosomes, has been used in an investigation of cells of hematopoietic origin. The technics for its preparation and use are described as well as the characteristics and patterns of lysosomal fluorescence for 1) primary lysosomes in peripheral blood and bone marrow cells; 2) secondary lysosomes; and 3) lysosomes and lysosomal aggregates in states of altered lymphoid proliferation. Euchrysine fluorescent staining appears to be a convenient and reliable technic for the identification of lysosomes in hematologic investigation.
Euchrysine, un fluorescente colorante aminoacridimc que es accumulate selectivemente in le lysosomas, esseva usate in un investigation de cellulas de origine hematopoietic. Le technicas de su preparation e de su application es describite como etiam le characteristicas e configurationes de fluorescentia lysosomal pro (1) lysosomas primari in cellulas de sanguine peripheric e de medulla ossee, (2) lysosomas secundari, e (3) lysosomas e aggregatos lysosomal in statos de alterate proliferation lymphoide. Coloration fluorescente a euchrysina pare esser un convenibile e fidedigne technica pro le identification de lysosomas in investigationes hematologic.

REFERENCES

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