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Differential staining of normal and leukemic cells with acridine orange and its possible application in acute lymphocytic leukemia

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THE DIFFERENTIAL STAINING OF NORMAL AND LEUKEMIC CELLS WITH ACRIDINE ORANGE AND ITS POSSIBLE APPLICATION IN ACUTE LYMPHOCYTIC LEUKEMIA

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INTRODUCTION

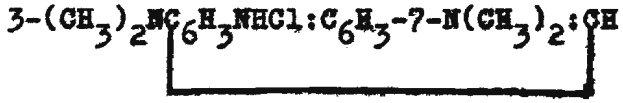
It is known that differentiation of the various forms of leukemia on a clinical basis is impossible and often, if not always, difficult even on blood smear examination (1). For Wright stained smear examination, uniform, well stained, thin smears are essential; otherwise, often the predominant cell types seen may be considered to be simply lymphocytes. Experienced, learned personnel are a prerequisite to slide examination, since even with perfect slides nuclear differences are not easily recognizable, and cytoplasm is usually minimal in quantity. It would indeed be valuable if a method for cell staining were available which gave simple enough results for unskilled persons to be quickly trained in examination of such slides. This paper is an evaluation and presentation of one possible method.

HISTORY OF AO

One possible solution to this problem has arisen in the past 10 years; namely the metachromatic, nucleic acid, fluorescing stain, acridine orange (AO).

Acridine orange and the other acridine dyes are closely related to quinoline dyes and are isolated from coal tar (2). The acridines were originally used for bacteriostasis in the urinary tract since they are excreted through the kidneys. While studying excretion, nuclear in vivo labeling of frog liver and kidney was noted (3, 4, 5). Farr (6) was the first to apply this discovery by supravitally staining leucocytes with acriflavine HCl and transfusing them in animals to determine their fate by later sacrificing and sectioning the animals. DeBruyn, Robertson, and Farr (7) experimented again with the acridines in an effort to determine which of them produced the most stable complex with the nucleus. Farr (8) utilized the acridines to further investigate the fate of the lymphocyte. Kosenow (9) used acridine orange specifically to supravitally demonstrate the basophilic nucleoproteinoid substance of young erythrocytes. DeBruyn, Farr, Banks and Morthland (10) continued work on the in vivo and in vitro affinity of diamino-acridines for nuclei, finding that specimens stained with the acridines and observed under the ultraviolet microscope have a similar staining pattern to those same cells stained with methyl green-pyronin,

a stain specific for nucleoproteins. From this observation they concluded that the staining ability of acridines on nuclei is based on the cellular content of nucleoproteins. They achieved essentially the same results with the Feulgen method of staining nucleoproteins as another control. They concluded that possibly the directing effect for the staining reaction of the diamino-acridines may be found in their steric configuration and the placement of amine groups on this ring structure, since only those acridines with amines in either the 3 or 6 positions (acridine orange has an amine in the 3 position), or both, have good in vivo nuclear staining reactions.



Acridine Orange

Kosenow (9) and Strugger, Krebs and Gierlach (11) used AO in attempts to determine whether or not it was capable of differentiating between living and dead cells. Strugger noticed when examining blood stained with this material that fresh white blood cells fluoresced green, and as the preparation aged and the cells died, more and more red fluorescent forms appeared while the green fluorescent forms disappeared. He, thus, theorized that living cells concentrated the proper amount of dye to yield green fluorescence when optically

activated, and dead cells concentrated a different amount, yielding red fluorescence when optically activated. Gierlach again upheld this idea (12).

The suspected "Strugger Concentration effect" was refuted by Bertalanffy and Bickis (13) who concluded that this effect is not applicable to animal tissues. They reported having seen green fluorescent nuclei in dead animal tissues, a color attributed to living tissue according to Strugger. They also reported that staining with AO will yield an indication of cytoplasmic ribonucleic acid (RNA) by the prescence of cytoplasmic secondary fluorescence after staining. They reported on the appearance of these colors in various tissues. Leucocytes, in particular, showed green nuclei, with occasional red cytoplasmic granules that can be removed by treating the slides with ribonuclease. Occasionally they observed a faint greyish rim of cytoplasm about lymphocytes and monocytes. Red blood cells remained unstained always. Bertalanffy, Masin, Masin (14) with Kaplin (15) applied this stain to cervical smears and found that malignant cells fron squamous cell carcinoma of the cervix showed brilliant orange-red fluorescence of the cytoplasm in contrast to the more subdued rust fluorescence of the normal cells on the smears. Nuclei still had green fluorescence but this was overshadowed by the brilliant cytoplasmic coloration. Slides were restained by the Papanicolaou technique and cells

that were called malignant when stained with AO were individually checked and again found to be malignant on the routine smears in all cases. Dart and Turner (16) studied AO stained cervical smears also and arrived at the same conclusion, finding, in addition, that the lesser time necessary for processing AO smears, plus the ease with which they could be read by relatively unskilled personnel, necessitated the institution of routine cytologic examinations with AO stain in place of Papanicolaou stain at Walter Reed Army General Hospital. Dart and Turner (16) and Bertalanffy (17) showed that the AO stain could be used for other cytologic examinations as well as cervical smears, with malignant cells always appearing with the typical bright orange-red cytoplasmic fluorescence.

LEUKEMIA, NUCLEIC ACIDS AND AO

Much work has been done with the various leukemic cells to determine their metabolic pathways in order to find better means of interrupting these paths as a form of therapy. As a portion of these cellular metabolic studies, the desoxyribonucleic acid (DNA) and ribonucleic acid (RNA) of normal and leukemic leucocytes has been studied since they are basic to cellular life. These experiments assume importance to this paper because AO is a proven nucleic acid stain.

Kelsall and Crabb (18) presented a working hypothesis showing that the lymphocytes function is to synthesize, store and transport nucleoproteins for use by other cells. Their hypothesis is upheld by Thorell (19), Hamilton (20) and others.

Petermann and Schneider (21) studied leucocytes in their entirety in peripheral blood of normal and leukemic persons with respect to nucleic acid content. Leukemic cells were found to show no change in the amount of DNA, while RNA proved to be 1.6 times that found in normal cells. Davidson, Leslie and White (22) performed similar studies, including the marrow as well, and confirmed these findings, showing that DNA remained similar in normal and leukemic white blood cells. Both DNA and RNA in the normal and leukemic patients showed decreased concentration in peripheral blood cells when compared with marrow cells, causing them to associate

increased DNA and RNA with cellular immaturity among the white blood cells. They found a fall in the marrow and peripheral blood cells RNA when the patients were in apparent remissions. These studies were again upheld by Hale and Wilson (23).

It was on the basis of these considerations, namely:

1. The need for a staining technique for acute leukemias which would make peripheral blood smears easier to read and perhaps even differentiate between them to the relatively unskilled worker.
2. The possibility that since AO has been proven to be a differential stain for nucleic acids, as well as a differential stain for normal and malignant cells, that it might differentially stain the abnormal cells appearing in the peripheral blood in acute lymphocytic leukemia, since lymphocytes are normally more associated with the nucleic acids than are the other white cells.

that this study was undertaken, in the hope that an easier method might be found for diagnosing and following acute lymphocytic leukemia. I believe the following results bear this hope out to some extent.

MATERIALS AND METHODS

Universe

Blood specimens were obtained at varying times in the course of their disease from 10 patients undergoing therapy for diagnosed acute lymphocytic leukemia. These patients were obtained from the University of Nebraska, College of Medicine, Hematology Clinics and from the private practice of Dr. Peyton Pratt. Both males and females were in this group.

Control studies were performed on the peripheral blood of 10 persons, all of whom were considered to be normal in health with no evidence of recent or present serious illness. Further controls were obtained by studying smears from a patient with chronic granulocytic leukemia, one with acute granulocytic leukemia suspected to be in a monocytic phase, and 5 patients with chronic lymphocytic leukemia. These patients were all chosen, both leukemias and controls, because of their availability to me and for no other reason.

Stain and Staining Technique

Peripheral blood was obtained by finger puncture and droplets of blood were placed on clean glass slides and drawn across the surface of the slides, as if for a Wright-stain film examination. Films were subsequently allowed to dry completely and were then fixed in 100% Methanol for 5 minutes prior to staining.

Acridine orange has been used in various ways by previous authors to best suit their experiments. In earlier experimentation (not reported) I have found that a blood film can be well stained with higher concentrations of AO and the only solvent needed for the stain is a physiologic solution, such as Ringer's or Tyrode's solution. Physiologic, isotonic saline proved to be the easiest, least expensive solution to prepare to meet this requirement and staining solutions of AO in concentrations of 1:20,000 were prepared. More physiologic saline was used to wash excess stain from the slides after the staining period.

Apparently there are only so many available binding sites on the nucleic acids for AO and prolonged times in the staining solution do not seem to overstain the cells. I experimented with staining times from 30 seconds to 5 minutes, and found that all times in this range with the concentration of AO at 1:20,000 gave similar results. Therefore a staining time of 45 seconds was arbitrarily selected, being within the range of good results so understaining would not occur. All slides were fixed exactly alike, stained for the same period of time, washed thoroughly in physiologic saline following staining, and allowed to dry completely before examination of them was undertaken. Slides prepared thusly and examined numerous times have been found to be uniform in reaction and to have kept strong fluorescence for at least 3 months at the time of writing this paper.

Technical Apparatus for Microscopy

In earlier experiments I had attempted to use a General Electric "black light" bulb as a light source for fluorescence microscopy. However, this soon proved to be inadequate for good visualization of AO-tagged cells. The Scopicon was then turned to and has been used again for these experiments. The Scopicon is basically a high intensity vapor arc lamp in a water cooled jacket and yields a large quantity of ultra-violet light rays in the wavelengths ranging from 360 mU to 505 mU in addition to much visible light which must be filtered out before viewing the slides.

Various ultra-violet light filters have been used, but the best visualization of AO-tagged cells has been achieved with a Wratten "M" # 49 filter and a liquid copper sulfate-ammonia coolant filter (24) between the light source and the specimen. The Wratten "M" # 49 filter transmits light in the range of 360 mU to 505 mU with a peak transmission at 450 mU. To avoid burning the retina with ultra-violet light a Wratten "G" # 15 filter has been placed in the ocular of the microscope. This filter has a range of 510 mU into the infra red spectrum with a peak transmission of 95 percent from 540 mU into the infra red spectrum. This combination of filters yields a black background against which any fluor-escent nuclei are brilliantly seen. Quartz optics are not necessary in a set up of this sort. A standard light

microscope with conventional optics can be used for cell visualization.

Previous workers with AO have not been primarily interested in the peripheral blood and therefore have not with great accuracy recorded the colors of the fluorescence of the various blood elements. Therefore after I had stained and examined smears from the normal controls with AO, I restained the slides with Wright's stain. Cell maps were made under the fluorescent microscope, and these cells were then later identified under ordinary light microscopy with the Wright's stain. Apparently there is no competition for staining sites between Wright's stain and AO since AO slides were not destained before using Wright's and later comparison between these slides and ordinary Wright's stained smears did not reveal any visible difference in staining reaction. Under the fluorescent microscope cell counts were made of the normal and abnormal forms appearing. Peripheral blood cells were counted by experienced hematology technicians under Wright's stain. One hundred cells per smear were counted and percentages of each type cell are based on these counts.

Photography

It was desirable to record cell appearances for comparison of individual abnormal cells. Any camera with microscope attachment may be used for this purpose. I used an Edixa Reflex with microscope attachment and Kodak Ektachrome

daylight film with an ASA rating of 32. Exposure times of 10 minutes for high power dry field and 15 minutes for oil immersion pictures were used. Film was processed through commercial channels. Identical printing times were requested so that color differences might be more apparent.

RESULTS

In all the following studies, the white, green white, or greenish fluorescence indicates DNA and any orange fluorescence indicates RNA. Therefore, an increase in orange fluorescence, as in the abnormal cells, indicates an increase in cellular RNA. This study tends to corroborate previous findings (21,22). No increase in DNA was found. This, too, seems to support previous studies (21,22,23).

Appearance of Individual Cells - Normal Controls

Low Power

Fluorescent colors tended to appear differently under low power when compared with higher magnifications. Therefore, I feel it is worthwhile to record the cells as seen with 150x magnification. As Bertalanffy and Bickis (13) reported, the majority of the leucocytes on the normal smears showed green nuclei. In addition, close scrutiny of the numerous cells visible with this power revealed some cells appearing as faint red-brown dots of light. Later Wright's stains showed the green appearing leucocytes to be of the neutrophilic series and the red-brown cells to be of the lymphocytic-monocytic series. Tiny pin points of orange-white light proved to be platlets. Occasionally on the normal smears brilliant orange fluorescing cells were seen. These cells, when examined with Wright's stain, had large nuclei with very dense chromatin, no visible nucleoli, a

slight nuclear halo and a thin rim of very basophilic cytoplasm. They were approximately twice the size of the normal small lymphocyte and were considered to be atypical lymphocytes.

High Power and Oil Immersion

Neutrophilic leucocytes when observed under powers of 500x and higher did not appear green distinctly, but now the nucleus fluoresced a greenish white. No cytoplasmic granulations were seen in these neutrophiles. Lymphocytes and monocytes fluoresced a dull rust color diffusely. It was not possible to distinguish between the nucleus and cytoplasm. The occasional atypical lymphocytes still persisted in fluorescing a brilliant orange. This cytoplasmic fluorescence was so brilliant that it was not possible to ascertain if the nucleus was fluorescing, although the nuclear shadows were well visualized. Eosinophiles fluoresced a diffuse grey white with no distinction between the nucleus and the cytoplasm. In all the slides examined none of the various fluorescent forms which were distinctive proved to be basophiles, so it must be presumed that either they fluoresce similar to the segmented neutrophiles or to the eosinophiles, or simply that none were found.

Appearance of Individual Cells - Acute Lymphocytic Leukemia

Cells of the neutrophilic series appeared to fluoresce in the same manner as those of normal smears with one excep-

tion. Instead of the green white fluorescence of the normals,the cells had a diffuse orange cast to them. Specific granulations were again not visualized. Greater numbers by far of the brilliant orange fluorescing cells were visible, and counterstaining with Wright's showed that the exceptionally brilliant cells were prolymphocytes, blast cells and myelocytes. Lymphocytes that seemed normal in size seemed to have a greater degree of cytoplasmic orange fluorescence. These cells were considered abnormal whenever the degree of greater fluorescence was obvious after comparison with the controls and were included in the cell counts. Platlets appeared as on routine smears. No monocytes were found.

Acute Granulocytic Leukemia

Here again many brilliantly fluorescent orange cells were visualized. It was impossible to differentiate these cells from those few cells seen in the normal control, or those cells seen in acute lymphocytic leukemia. Cells which fluoresced thusly were myelocytes or younger. Mature segmented neutrophiles again showed an unusual diffuse orange coloration. Lymphocytes and monocytes fluoresced similar to the normal controls.

Chronic Lymphocytic Leukemia

The cases studied were all small cell chronic lymphocytic leukemia. The count of abnormal cells was very high, and under the fluorescent microscope the whole slide appeared

to be filled with them. Segmented neutrophiles fluoresced with an orange white color. No monocytes were seen.

Chronic Granulocytic Leukemia

As in the other leukemias the brilliant orange cells were seen and were all seen to be again myelocytes or younger. More mature segmented neutrophiles showed orange white fluorescence. Lymphocytes and monocytes, though hard to find amid the numerous other cells, appeared to be normal.

STATISTICAL DATA

Since abnormally fluorescing cells were found in the peripheral blood of the normal control, I feel that a statistical analysis of the counts from both groups is necessary at this point. In the following table average numbers of abnormal cells per 100 cells counted in the peripheral blood of the acute lymphocytic patients are analyzed with the average numbers of abnormal cells per 100 cells counted in the peripheral blood of the normal controls.

Acute lymphocytic leukemia		Controls	
C.B.	55.0	B.J.	0.0
W.	57.5	Wa.	1.0
S.K.	72.5	W.W.	5.0
E.B.	59.0	F.	0.0
A.	39.0	L.W.	3.0
D.H.	24.0	T.H.	4.0
B.	21.0	Fu.	2.0
J.H.	25.5	F.F.	3.0
D.	30.8	V.D.	2.0
R.A.	43.8	M.	0.0
	428.1 mean = 42.81		20.0 mean = 2.00
	Variance = 290.9432		Variance = 3.1111
	Standard Deviation = 17.057 cells		Standard Deviation = 1.761 cells
	Probable Error = 11.5050 cells		Probable Error = 1.1878 cells

Standard Error of the Mean = 5.394 cells	Standard Error of the Mean = 0.557 cells

Standard Error of the difference between these two Means = 5.423 cells.

If one standard error is 5.423 the observed difference between these two means amounts to 7.33 standard errors which is definitely of statistical significance. The chance of a common universe ever throwing two samples such as these is infinitesimally small.

Slightly less than 2½ % of the time will the abnormal cell count of acute lymphocytic leukemia fall below 9 cells per 100 cells counted, and 99.73 % of the time the average patient without obvious known disease will not show an abnormal cell count above 7 cells per 100 cells counted. Of course, I realize that the values obtained from the patients with acute lymphocytic leukemia represent random sampling throughout the course of their disease, and do not represent the figures that might have been obtained on their first visit to their physician. Initial visit values might throw samples which reflect less of a difference.

COMPARATIVE COUNTS

The following counts are comparisons of AO stain and Wright's stain results on smears from acute lymphocytic leukemia patients. Cells counted as abnormal were those in which a definite increase in cytoplasmic RNA could be seen.

Patient 1.

11-3 5700 WBC's, 17 segs, 14 bands, 26 lymphs, 1 mono, 34 blasts, 5 myelocytes, 3 youngs, 1 nuc. RBC.
Fluorescence microscopy - 37% normal cells, 63% abnormal cells (brilliant orange).

11-7 5500 WBC's, 10 segs, 9 bands, 36 lymphs, 1 mono, 24 blasts, 13 prolymphs, 4 myelocytes, 3 youngs, 2 nuc. RBC.
Fluorescence microscopy - 37% normal cells, 63% abnormal cells.

11-10 1900 WBC's, 8 segs, 12 bands, 34 lymphs, 42 blasts, 1 plasma cell, 1 myelocyte, 3 youngs, 2 nuc. RBC.
F.M. - 44% normal, 56% abnormal.

11-21 1100 WBC's, 10 segs, 2 bands, 70 lymphs, 2 blasts, 16 prolymphs, 4 nuc. RBC.
F.M. - 38% normal, 62% abnormal.

11-28 1800 WBC's, 24 segs, 18 bands, 40 lymphs, 2 monos, 2 blasts, 12 prolymphs, 1 myelocyte, 1 plasma cell, 5 nuc. RBC.
F.M. - 42% normal, 58% abnormal.

12-5 5700 WBC's, 38 segs, 22 bands, 26 lymphs, 4 monos, 3 prolymphs, 6 myelocytes, 1 young, 3 nuc. RBC.
F.M. - 71% normal, 29% abnormal.

12-12 7200 WBC's, 28 segs, 20 bands, 24 lymphs, 6 blasts, 13 prolymphs, 4 myelocytes, 5 youngs.
F.M. - 48% normal, 52% abnormal.

12-15 6400 WBC's, 38 segs, 21 bands, 6 lymphs, 2 monos, 10 blasts, 18 prolymphs, 4 myelocytes, 1 young, 2 nuc. RBC.
F.M. - 66% normal, 34% abnormal.

Patient 2.

12-7 48700 WBC's, 4 bands, 53 lymphs, 5 blasts, 38 prolymphs.
F.M. - 69% normal, 31% abnormal.

12-9 5900 WBC's, 4 segs, 7 bands, 82 lymphs, 1 eosin., 6 prolymphs.
F.M. - 71% normal, 29% abnormal.

12-13 1200 WBC's, 8 segs, 8 bands, 84 lymphs.
F.M. - 65% normal, 35% abnormal.

12-19 950 WBC's, 7 segs, 20 bands, 67 lymphs, 6 eosin.
F.M. - 62% normal, 38% abnormal.

12-23 2550 WBC's, 6 segs, 28 bands, 62 lymphs, 4 monos.
F.M. - 79% normal, 21% abnormal.

Patient 3

11-8 4550 WBC's, 28 segs, 13 bands, 49 lymphs, 6 monos, 3 eosin., 1 baso.

F.M. - 51% normal, 49% abnormal.

11-18 5600 WBC's, 28 segs, 5 bands, 55 lymphs, 4 monos, 2 eosin., 6 prolymphs.

F.M. - 59% normal, 41% abnormal.

12-5 7600 WBC's, 44 segs, 14 bands, 31 lymphs, 10 monos, 1 eosin.

F.M. - 50% normal, 50% abnormal.

12-16 3550 WBC's, 24 segs, 20 bands, 38 lymphs, 17 monos, 1 eosin.

F.M. - 54% normal, 46% abnormal.

12-23 6000 WBC's, 25 segs, 10 bands, 51 lymphs, 13 monos, 1 eosin.

F.M. - 67% normal, 33% abnormal.

Patient 4

11-21 2500 WBC's, 2 segs, 4 bands, 65 lymphs, 1 baso., 12 blasts, 16 prolymphs.

F.M. - 32% normal, 68% abnormal.

12-13 1200 WBC's, 6 segs, 14 bands, 78 lymphs, 1 mono, 1 myelocyte.

F.M. - 23% normal, 77% abnormal.

Patient 5

11-18 5000 WBC's, 30 segs, 14 bands, 46 lymphs, 10 mono.

F.M. - 41% normal, 59% abnormal.

Patient 6

12-7 2250 WBC's, 21 segs, 13 bands, 60 lymphs, 3 monos, 3 eosin.

F.M. - 53% normal, 47% abnormal.

12-21 3150 WBC's, 17 segs, 10 bands, 69 lymphs, 3 monos, 1 eosin.

F.M. - 69% normal, 31% abnormal.

Patient 7

11-18 14800 WBC's, 44 segs, 42 bands, 6 lymphs, 5 monos, 3 eosin.

F.M. - 85% normal, 15% abnormal.

Patient 8

12-9 3400 WBC's, 30 segs, 44 bands, 22 lymphs, 4 mono.

F.M. - 83% normal, 17% abnormal.

12-22 3450 WBC's, 10 segs, 25 bands, 52 lymphs, 12 monos, 1 young.

F.M. - 75% normal, 25% abnormal.

Patient 9

12-9 4100 WBC's, 46 segs, 13 bands, 34 lymphs, 7 mono.

F.M. - 78% normal, 22% abnormal.

12-14 5550 WBC's, 15 segs, 16 bands, 63 lymphs, 5 monos, 1 myelocyte.

F.M. - 71% normal, 29% abnormal.

Obviously, I didn't have the time to check every one of

the abnormally fluorescing cells by Wright's stain on my own,but the ones I did check were immature lymphocytes, some myelocytes, blasts with nucleoli, and as previously stated some which appeared as normal small lymphocytes except for an increase in cytoplasmic orange fluorescence.

If the individual counts are taken separately and the cells which should fluoresce abnormally are totaled and the cells which should fluoresce normally are totaled, sometimes they match the fluorescent counts of normal and abnormal cells and sometimes they don't.

Examples:

Patient 1

11-3 17 segs + 14 bands + 3 youngs + 1 mono = 35%
26 lymphs + 34 blasts + 5 myelocytes = 65%
F.M. - 37% normal, 63% abnormal.

11-10 8 segs + 12 bands + 3 youngs = 23%
34 lymphs + 42 blasts + 1 myelocytes = 77%
F.M. - 44% normal, 56% abnormal.

Of course, it must be remembered that on occasion the small lymphocytes were very difficult to place in either the normal or abnormal fluorescent group and this factor makes quite a difference in the count under the fluorescent microscope. In checking counts by both methods however, I do feel there seems to be a correlation. Actually there should be whether I found one or not if it is assumed that AO accurately

reflects the cellular content of DNA and RNA. Variations in the total normal and abnormal lymphocytes can account for the differences seen. For example, in Patient 1, 11-10; if 21 of the 34 lymphocytes counted are normal and the rest abnormal, this explains the difference in results. This indicates that AO is accurately recording the natural cycles of the disease.

DISCUSSION

As has been stated previously AO selectively stains the nucleic acids (10). When stimulated with ultraviolet light DNA will fluoresce in the white to green range and RNA will fluoresce in the orange to red range. It has been shown that in leukemic cells circulating in the blood there is a measurable increase in RNA while there is no consistent measurable quantitative change in DNA (21,22). This is evident in my experiments also. This increase in RNA apparently reflects a rapid rate of growth. It has been utilized with the AO staining technique in other cytologic studies involving rapidly growing tissues where an increase in cytoplasmic RNA has been shown.

The AO technique is presented now as a possible diagnostic procedure for acute lymphocytic leukemia. The fact that less than 2½% of the time will the abnormal cell count fall below 9 cells per 100 cells counted, and 99.73% of the time the average patient without obvious known disease will not show an abnormal cell count above 7 cells per 100 cells counted, is highly significant and worthy of consideration. To fully evaluate this method, however, as a diagnostic test, it will be necessary to screen large segments of the general population and check the method with Wright's stained smears.

It is equally difficult at this point to state the value of this technique in regard to prognostic value. Theoretically

it should be ideal as presented with comparative counts. Whenever an increased number of immature cells with high cellular content of RNA appear in the peripheral blood, AO staining should accurately reflect this increase, and it does. One of the problems at present is that 6 patients in an apparent clinical and Wright's stained smear remission for 2 to 18 months continue to show a high number of abnormal cells when stained with AO. The only possible significance of this is that even though clinically and apparently hematologically the patients are in remission, they are continuing to show cells with high cytoplasmic RNA content, and thus rapid growth and immaturity of the circulating blood cells. Often these patients showed abnormal counts while in remission clinically that were as high as when they were actively showing symptomatology. It is possible that this phase of the leukemic process never does return to normal. At present this is a limiting factor to the usefulness of the stain as a prognostic test.

An attempt was made to see if cortisone therapy induced a decrease in the number of abnormally fluorescing cells, but none of the patients in this series on cortisone were followed long enough to elucidate any information. This problem is available for future study. Cortisone's anti-inflammatory properties should cause a decrease in proliferation of leukemic cells and therefore a decrease in cellular RNA and in abnormal

cells when stained with AO.

A distinct increase in cytoplasmic RNA was noted in neutrophiles in acute lymphocytic leukemia. The nuclei of these cells in normal smears fluoresced a green-white, which they also did in ALL, but in addition the cytoplasm of these cells in ALL showed a diffuse orange fluorescence which was not seen on the normal smears. This suggests that though ALL manifests itself primarily in the lymphocytic cell series it is a pancellular disorder. This may or may not be applicable to the other leukemias.

Lymphocytes which appear in acute lymphocytic leukemia as normal small lymphocytes under Wright's stain are occasionally showing excessive amounts of cytoplasmic RNA when stained with AO. This factor coupled with the increased cytoplasmic RNA of the neutrophiles proves that an organic disorder is still present even though clinically the patient may be in remission. This finding refutes previous reported information stating that a fall in RNA in peripheral blood cells in the leukemias can be demonstrated when the patient is in remission clinically (22,23). However previous studies were quantitative and this is qualitative. The reported fall in RNA was not completely to normal levels.

Abnormal cell counts were performed on patients with acute lymphocytic leukemia, acute granulocytic leukemia, chronic granulocytic leukemia, and chronic lymphocytic leukemia.

All had similar counts except for chronic lymphocytic leukemia which averaged 80 abnormal cells per 100 cells counted, while the other leukemias studied averaged about 40 abnormal cells per 100 cells counted. This indicates little except that the large numbers of apparently normal lymphocytes seen with Wright's stain in chronic lymphocytic leukemia are definitely not normal, but have a marked increase in cytoplasmic RNA indicating rapidly growing and immature cells.

The significance of these abnormally fluorescing cells in normal smears is little. It is generally accepted that practically everyone has a few circulating atypical lymphocytes, the origin of which is uncertain. These cells under Wright's stain do appear abnormal and may indicate a chronic low grade infection or past infection in the body. Several conditions which should be studied which relate to this are infectious mononucleosis, infectious lymphocytosis, pertussis, etc. These are disorders which are not malignant, but do show high lymphocyte counts, often atypical. They have not been studied with AO stains, but should be since it is possible for them to show high abnormal cell counts and may detract from AO as a diagnostic stain for leukemias.

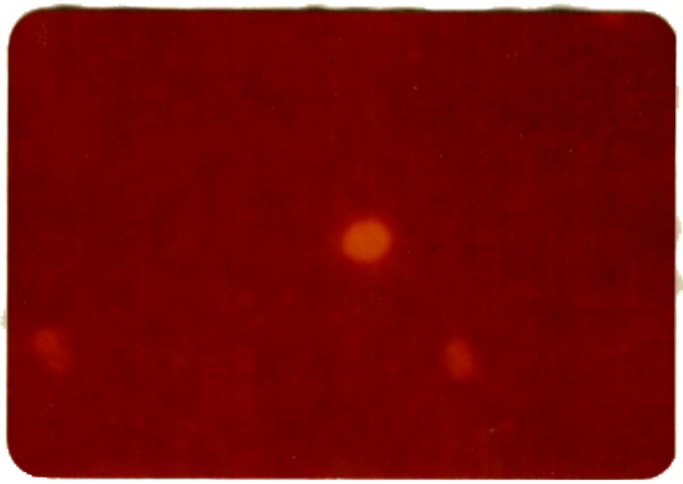


Figure 1. Normal smear. High, dry study. An abnormally fluorescing atypical lymphocyte is seen centrally. Two more faintly fluorescing neutrophiles are seen inferiorly to the left and right. Small nearly invisible points of light are platlets. RNA appears brilliant orange. DNA appears faintly orange, as in the neutrophiles. Ektachrome prints in the orange range more than white or green.

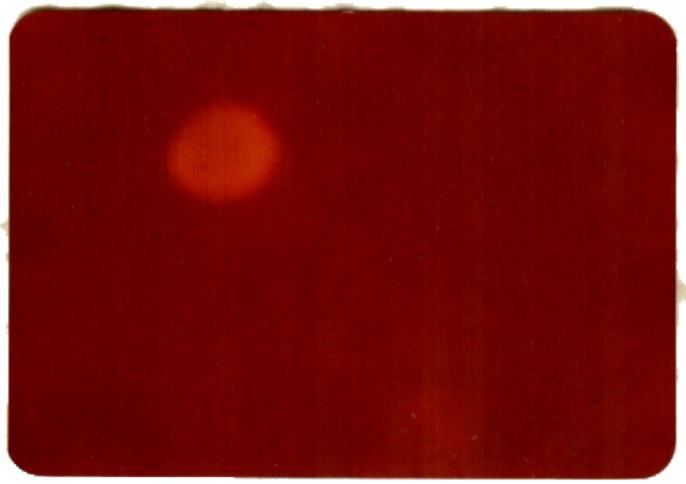


Figure 2. Normal smear. Oil immersion study. Large abnormally fluorescing atypical lymphocyte upper left. Nucleus is seen as dark shadow. Neutrophile is seen in lower right as large faint spot of light.

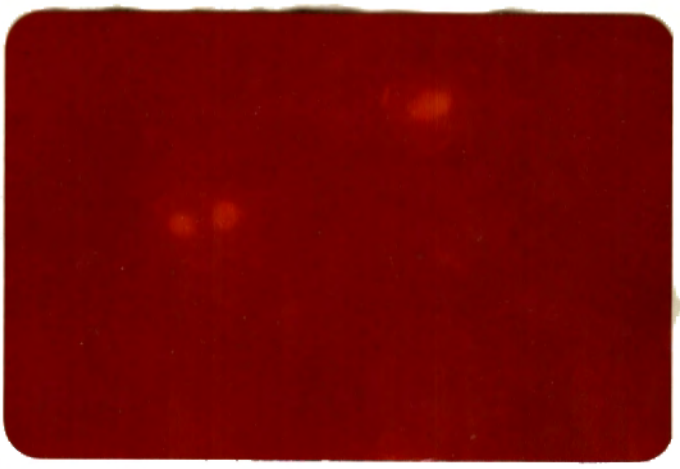


Figure 3. Acute lymphocytic leukemia. High, dry study. An abnormally fluorescing immature lymphocyte is seen upper right of field with brilliant rim of cytoplasm at right pole of cell. Two small lymphocytes are seen inferiorly to the left, one with a brilliant rim of cytoplasm.

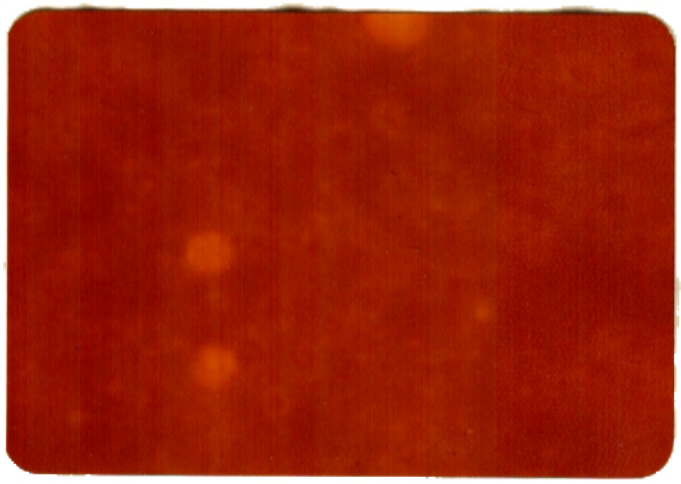


Figure 4. Acute lymphocytic leukemia. High, dry study. An abnormally fluorescing lymphoblast or prolymphocyte is partially seen at top of picture. Two neutrophiles showing abnormal cytoplasmic orange fluorescence are seen lower left. Round orange white spot of light lower right is a platlet. Shadows in background are red cells which do not stain with AO.

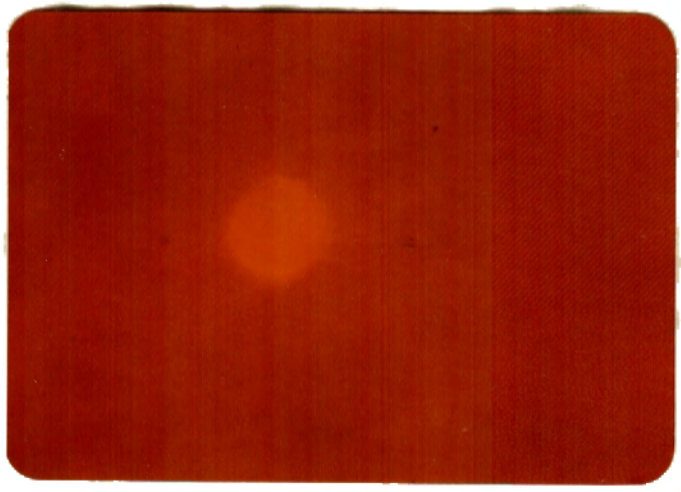


Figure 5. Acute lymphocytic leukemia. Oil immersion study. Only cell shown is abnormally fluorescing lymphoblast or prolymphocyte. Note faint shadow centrally indicating nucleus of cell. Cytoplasmic fluorescence is so brilliant fainter fluorescence of DNA does not appear in most of these figures.

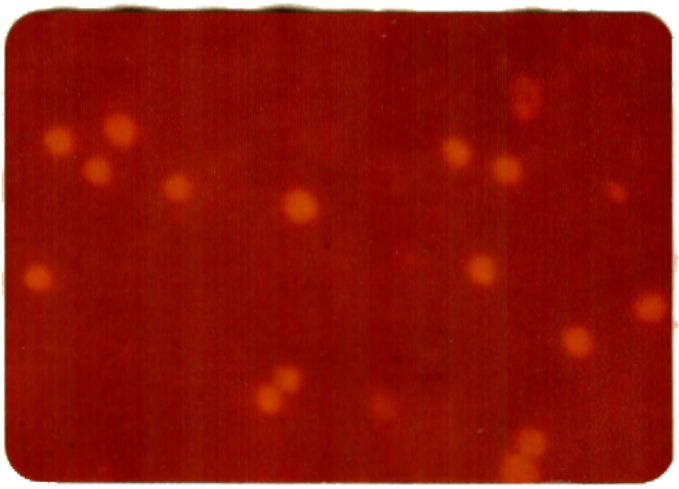


Figure 6. Chronic lymphocytic leukemia. High, dry study. Small cell leukemia. All cells are apparently normal lymphocytes under Wright's stain. Neutrophiles appear more faintly upper right corner and lower centrally. Note uniformity of cells in fluorescence and size.

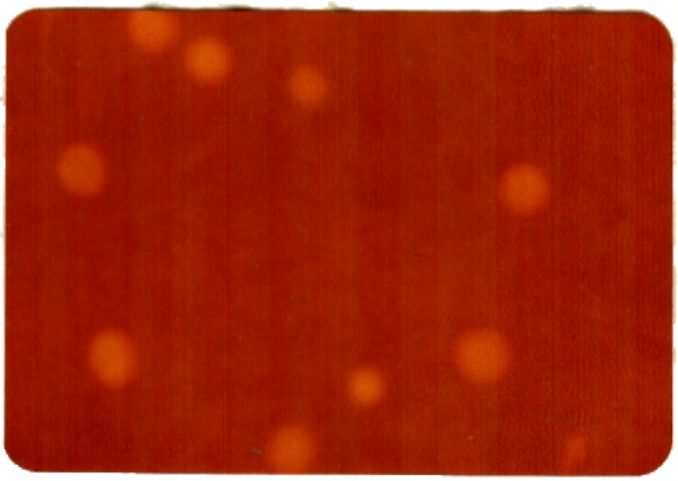


Figure 7. Acute granulocytic leukemia. High, dry study. All cells are immature granulocytes. It will be noted after comparison with other figures that these cells cannot be differentiated from immature lymphocytes.

SUMMARY

The various leukemias are very difficult to differentiate and follow for the unaccustomed worker. An easier method than Wright's stain smears seems desirable. Acridine orange has been used in malignant cytologic studies from other portions of the body because of its affinity for nucleic acids. It is a fluorescent stain under ultra violet stimulation and accurately records increases in cellular RNA which occur in malignancies along with rapid growth and immaturity of the cells. It suited itself to the purpose of searching for easier diagnosis and followup in acute lymphocytic leukemia, for lymphocytes are directly related with nucleic acid metabolism normally, and should show greater abnormalities between normal and leukemic cells when stained with AO.

Ten apparently healthy individuals and ten patients with diagnosed acute lymphocytic leukemia were selected, their peripheral blood smears stained with AO, and the results compared. Healthy individuals were found to have 0 to 7 per cent abnormally fluorescing blood cells per 100 cells counted, while the patients with acute lymphocytic leukemia had 9 to 100 per cent abnormally fluorescing cells per 100 cells counted, suggesting this stain as a diagnostic screen.

Another important finding was that neutrophiles showed cytoplasmic RNA in acute lymphocytic leukemia while neutrophiles on normal smears showed no or non-readable RNA.

This suggests a pancellular disorder. In addition lymphocytes which appeared normal on Wright's stain in acute lymphocytic leukemia showed increased cytoplasmic RNA and fluoresced abnormally when stained with AO.

Counts with the two staining techniques were compared and were found to have a degree of correlation except for the change in supposedly normal lymphocytes. It was also discovered that patients in clinical remissions showed abnormal cell counts as high as when they were not in remission. Previously in quantitative studies blood cytoplasmic RNA has been shown to fall when patients are in remission, but not to normal levels. My opinion is based on qualitative results but I feel a fall in RNA has not been demonstrated in remissions with the AO staining technique.

Staining variations in lymphocytes, monocytes, and neutrophiles between normal and leukemic smears are presented. Findings in acute granulocytic leukemia, chronic granulocytic leukemia, and chronic lymphocytic leukemia when stained with AO are presented also.

CONCLUSIONS

1. Blood cell nuclei of all series fluoresce green to white when stained with AO and stimulated by ultra violet light. Lymphocytes have a bright orange cytoplasmic fluorescence in normal smears. In many lymphocytes in acute lymphocytic leukemia this orange fluorescence is increased indicating an increase in cytoplasmic RNA. Neutrophiles from normal smears contain no or non-readable cytoplasmic orange fluorescence whereas neutrophiles from acute lymphocytic leukemia patients show diffuse orange cytoplasmic fluorescence in supposedly normal cells. This indicates cytoplasmic RNA where it was not readable before and suggests that acute lymphocytic leukemia affects more than the lymphocytic series of cells. Monocytes from normal smears showed dull rust cytoplasmic fluorescence and greenish white nuclei. No monocytes were found in the acute lymphocytic leukemia patients.
2. The presence of high numbers of abnormally fluorescing cells in 6 patients in clinical remissions of 2 to 18 months is significant, indicating that the disease process is still active even though no symptomatology is apparent. Therefore it is also reasonable to suspect a diagnosis of acute lymphocytic leukemia in a patient in

which more than 9% abnormal fluorescent forms appear in the peripheral blood since less than 0.5% of the time will the average healthy person without obvious known disease have an abnormal cell count of more than 7%. This may also apply to the other leukemias since in the few patients studied, high counts of abnormal cells were obtained; but these were not analyzed statistically.

3. After further study revealing the cause of large numbers of abnormal fluorescent forms in patients in remission, this stain may be of value as a prognostic test in acute lymphocytic leukemia. It must first be shown that the number of these cells seen is an accurate reflection of the severity of the disease process.
4. The question has been presented regarding the effect of cortisone on these abnormally fluorescing forms when compared with other antileukemic agents. This must be studied further.
5. Supportive evidence has been presented that there is an increase in RNA in the leukemic cell, and little or no detectable change in DNA.
6. Evidence has been presented that RNA does not decrease in amount markedly in the peripheral blood cells when leukemic patients are in remission. Previous studies indicated a fall, but not to normal levels.

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