

## Use of Acridine Orange in Lymphocyte Transformation Test (34062)

C. DEAN DUKES, J. L. PARSONS, AND C. A. L. STEPHENS, JR.

*The Tissue Culture Laboratory, Southwestern Clinic and Research Institute, Inc.,  
Department of Microbiology and Medical Technology and Department of  
Agricultural Biochemistry, University of Arizona, Tucson, Arizona 85721*

The lymphocyte transformation test (LTT) is becoming an increasingly valuable tool in investigative immunology. The LTT has been used to study both immediate hypersensitivity (1-3) [pollen, antibiotics, such as penicillin and neomycin, drugs, and proteins]; and delayed or bacterial allergy (4-7) [tuberculin, typhoid, tetanus toxoid, coccidioidin, brucella, and vaccinia]. Depending upon the information desired, the LTT can be used to evaluate either mitogenesis or blastogenesis, or both, by a variety of methods. The most widely used at present is morphologic visualization employing Wright, pyroninophilic and aceto-orcein stains, followed in popularity by scintillometry and autoradiography. The fluorochrome dye acridine orange (AO) possesses the special advantage of cytochemical specificity for RNA and DNA, as well as simplicity, speed, and excellent morphological differentiation of lymphocytes (8-10).

*Materials and Methods. Lymphocyte cultures.* The method employed has been described previously (6). In brief, heparinized venous blood was sedimented gently and the buffy coat and plasma removed by aspiration. Gentle sedimentation of the buffy coat yielded suspensions of cells containing 80-98% lymphocytes. Care was taken to keep the numbers of polymorphonuclear neutrophils to a minimum. Cultures for the LTT generally consisted of  $2 \times 10^6$  cells in 5 ml of Eagle's MEM with glutamine, 20% pooled human cord serum,<sup>1</sup> and penicillin-streptomycin, contained in  $13 \times 100$ -mm Pyrex tubes. The tubes were incubated at 37° for

3-7 days, depending upon the stimulatory material being tested and the expected intensity of reaction of the cells. At completion of incubation, the cultures were sedimented, the supernatant portion discarded, and smears prepared on  $1 \times 3$ -in. slides, or  $22 \times 22$ -mm coverslips. Absolute methanol, acetic acid-methanol (1:3), Carnoy's solution, and Spraycyte<sup>2</sup> were employed at various times for fixation.

*Stains.* A standard Wright stain was used for comparative purposes. The acridine orange<sup>3</sup> used was obtained commercially as a stable 0.1% stock aqueous solution containing 0.2% Tween 80 wetting agent. Dilutions for staining were prepared fresh daily at a 1:10 ratio, using either 2% glacial acetic acid in isotonic saline, or McIlvaine buffer mixture, pH 3.8.

Slides to be stained were immersed in the diluted AO for 5 sec with agitation, excess stain removed by immersion for 12 sec in 2% ethanol in isotonic saline, rinsed for 5 sec in isotonic saline and coverslipped wet. For protracted observation, the coverslips were sealed with synthetic mounting resin.

*Microscopy.* The preparations were examined with a Zeiss microscope equipped with a cardioid darkfield condenser and a Wratten 2B barrier filter. Excitation was provided with an American Optical Fluorolume unit with an Ozram HB 200 mercury lamp and a UG 5 filter. Photomicrographs were taken with a Bausch and Lomb camera unit using Kodachrome II Daylight film with exposures of 20-30 sec at  $400\times$  magnification.

<sup>1</sup> Procured through the generous cooperation of the staffs of St. Joseph's Hospital, St. Mary's Hospital, and Tucson Medical Center.

<sup>2</sup> Spraycyte (R) Fixative, Clay-Adams, Inc., New York, New York.

<sup>3</sup> Acridin Orange and Harleco Synthetic Resin, Hartman-Leddon Co., Philadelphia, Pennsylvania.

*Evaluation.* Blastogenesis was judged by established criteria. Only those cells with markedly increased nuclear-cytoplasmic ratios, reticulated nuclei and prominent nucleoli were recorded as lymphoblastic forms. Cells transitional in morphology were relegated to the "unreacted" category. A minimum of 1000 lymphocytes were counted per slide; disintegrating cells, monocytes, eosinophils, and polymorphonuclear neutrophils were ignored in the tabulations.

*Technique.* Acridine orange is rapidly absorbed by fixed material. When slides are examined before treatment with the alcoholic-saline, or when the removal of excess stain has been inadequate, a uniform orange fluorescence is observed and little internal structure can be detected. When staining is inadequate, the red fluorescence of RNA is minimized or eliminated. In the latter instance, preparations may be restained with impunity. This restainability provides a flexibility virtually unique among histochemical dyes.

*Results.* When properly stained with acridine orange at pH 3.8, leukocytes exhibit characteristic morphological patterns. Nuclei fluoresce a characteristic green, indicative of DNA; and the nucleoli red, specific for RNA. Cytoplasmic staining varies with the type of cell examined. Polymorphonuclear neutrophils show little or no cytoplasmic staining. The cytoplasm of monocytes fluoresces a reddish-brown, while lymphocytes show an intense red cytoplasm, indicative of high RNA content. Unstimulated lymphocytes appear as compact, homogeneously stained green nuclei, with a rim of red cytoplasm. Stimulated lymphocytes in the blastogenic form have a marked increase in cytoplasmic area, staining an intense red, an enlarged reticulated nucleus exhibiting internal structure, marked by variations of the intensity of the green fluorescence, and one or more distinct red nucleoli. Transitional cells—those in the process of undergoing blastogenesis—manifest initial changes by an increase in the amount of visible cytoplasm and in the intensity of the red RNA staining, followed by progressively more distinct nuclear reticulation and appearance of nucleoli. Cells which

pass through blastogenesis to autolysis, in general, display first a loss of cytoplasmic integrity, followed by karyolysis. The nucleoli remain intact until nuclear disintegration is relatively complete.

Background staining with AO with this protocol is dependent upon the degree of contamination of the cultures by platelets and erythrocytes, and upon the fixative employed. With absolute methanol and with the acetic acid-methanol mixture, background fluorescence is minimal. The erythrocytes are unstained and the platelets fluoresce a dull greenish-gray. With Carnoy solution and with Spraycyte, the erythrocytes are outlined by autofluorescence, while the platelets fluoresce a dull brownish color. In our experience, the acetic acid-methanol mixture provides maximum morphologic preservation of the cultured lymphocytes, while minimizing the amount of background fluorescence.

With the distinctive morphology and fluorescence described above, tabulation of lymphoblastic forms is relatively easy and, with experience, quite reproducible. Sampling errors are inherent in any method in which 1000 of  $2 \times 10^6$  cells are being utilized to evaluate the reactivity of the entire culture. Repeat counts on different areas of individual slides have shown a variation of  $1.6 \pm 0.2\%$ . Paired counts of duplicate slides prepared from the same culture tubes gave a variation of  $2.1 \pm 0.4\%$ . Comparable figures for control cultures—in which the number of lymphoblastic cells averaged less than 20/1000—were 0.2 and 0.3%.

Table I illustrates the ranges and comparative values obtained by duplicate counts on cells from cultures subjected to the nonspecific transformation material, phytohemagglutinin (PHA-P), and several immunospecific antigens, as determined by acridine orange and Wright stain, and by autoradiography with tritiated thymidine. The thymidine uptake was included to compare nuclear DNA synthesis with morphologic determination of blastogenesis.

Figure 1 presents a graphic representation of the response and the ranges of variation of a lymphocyte culture to PHA-P, as measured by the three methods utilized in this laboratory.

TABLE I. Determinations of Blastogenesis and Thymidine Incorporation in Nonspecific (PHA) and Immunospecific Systems.<sup>a</sup>

Group	Acridine orange	Wright	Tritiated thymidine	Duplicate no. of counts
Controls	0.0-2.1	0.0-2.0	0.0-2.4	6
PHA <sup>b</sup>	56.9-61.2	45.4-50.1	28.3-32.8	4
Coccidioidin	14.1-14.9	15.3-16.4	20.1-23.9	3
Tetanus	36.5-38.2	29.1-31.7	ND <sup>c</sup>	3
Penicillin	20.0-22.8	18.5-20.1	ND	3

<sup>a</sup> Peripheral blood lymphocytes were obtained from sensitized donors and were incubated in the presence of the specific antigen at 37° for 5-7 days. The numbers refer to the percentage of morphologically recognizable lymphoblasts, as determined with acridine orange or Wright stain. The percentage of autoradiographically labeled cells indicates tritiated thymidine incorporation into DNA.

<sup>b</sup> At 72 hr.

<sup>c</sup> ND = not done.

*Discussion and Summary.* The fluorochrome, acridine orange, has been utilized in this laboratory as a rapid, sensitive, and reliable method for the visualization and tabulation of cells in the lymphocyte transformation test. With careful control of pH of the staining solution, and proper differentiation in ethanol-saline, the staining is highly reproducible and specific for DNA and RNA. Blastogenesis is readily recognizable by both nuclear and cytoplasmic changes. Acridine orange has been used to evaluate the nonspecific effect of phytohemagglutinin (PHA-P), and the specific antigenic stimulation of te-

tanus toxoid, coccidioidin, and typhoid vaccines, penicillin and penicilloyl-polylysine on human lymphocytes from sensitized individuals. The results obtained were comparable to those obtained from Wright-stained preparations and consistent with those from autoradiography of tritiated thymidine incorporation into nuclear DNA. Investigators using blastogenesis as an index for the lymphocyte transformation test may well wish to consider acridine orange for its simplicity and reliability.

The authors thank Miss Janet Barrett and Mrs. Helen Ratajczak for technical assistance.

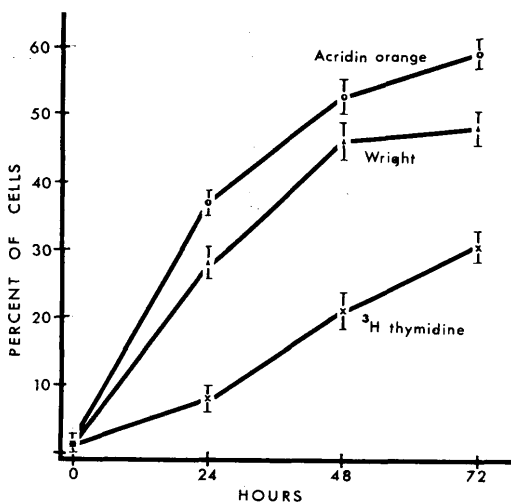


FIG. 1. Blastogenesis and thymidine incorporation into DNA in peripheral blood lymphocyte cultures stimulated with phytohemagglutinin. The vertical bars indicate the ranges of three or more duplicate counts.

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