Brief Report

An Evaluation of Human Lymphocyte Nuclear RNA with Acridine Orange

By David M. Johnson, Peyton T. Pratt and Perry G. Rigby

THE IMPORTANCE OF lymphocytic RNA in immune function has received widespread attention in recent years.[1] A case in point is the work of Mannick and Egdahl showing that adoptive immunity can be transferred from one animal to another by means of lymphocytic RNA.[2] The RNA of the lymphocyte can be evaluated and compared with the DNA by selective staining with acridine orange for fluorescent microscopy.[3-5] Therefore, a morphologic investigation of human lymphocytic RNA has been undertaken in normal and diseased individuals.

Materials and Methods

Human blood was obtained from patients and normals by venipuncture with a heparinized syringe and by fingerstick. Smears were made on a No. 1½ coverglass (Gold Seal) and immediately fixed in absolute methanol for 5 minutes at room temperature. These smears were stained with acridine orange according to Schiffer.[5] After the stained smears were dry, they were mounted on glass slides with Permount. Fluorescent microscopy was accomplished with an American Optical Fluorolume unit. The oil immersion studies utilized Cargille's Type A, Very Low Fluorescence oil. Photomicrographs were taken with exposure times varying from 10 to 30 seconds. Wright's-stained smears were evaluated on each patient, and confirmatory observations were accomplished on selected smears with pyroninmethyl green.

The lymphocytes were evaluated on the basis of the staining qualities of RNA (orange) and DNA (green). On each peripheral blood smear, the lymphocyte population as a whole was rated on the basis of the amount of nuclear RNA (Table 1). The acridine orange nuclear (AON) rating adopted used a 0 to 4+ scale (5 AON units), with a normal smear (normal small lymphocytes) being rated 1+. Rating the nuclei of the lymphocyte population as a whole was found to be in agreement with a quantitation of the nucleolar RNA and extranucleolar RNA of each cell separately (50 lymphocytes of each of 12 patients).

In order to minimize the subjective aspects of such an evaluation, each slide was coded and rated by three observers. The ratings were consistent among the observers, varying no more than one scale unit per slide. The AON ratings were correlated with the patient's diagnosis established by standard clinical methods.

The lymphocyte cultures were performed using a method similar to that described by

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From the Eppley Research Institute, University of Nebraska College of Medicine, Omaha, Nebraska.

Presented in part at the International Congress of Hematology, August 26, 1966, Sydney, Australia.

Supported by Grants #868 and #906 from the Damon Runyun Memorial Fund.

First submitted Nov. 1, 1966; accepted for publication Jan. 30, 1967.

David M. Johnson, B.S.: *Research Assistant, Department of Anatomy, University of Nebraska College of Medicine.* Peyton T. Pratt, M.D.: *Associate Professor of Medicine, University of Nebraska College of Medicine.* Perry G. Rigby, M.D.: *Associate Professor of Medicine and Anatomy, University of Nebraska College of Medicine.*

Table 1.—*AON Rating System for Nuclear RNA of the Lymphocyte Population on Peripheral Blood Smears*

0 —Nearly all cells lack stainable nuclear RNA.
1+—Many cells have a faint amount of nuclear RNA. Occasional cells have a nucleolus.
2+—Most cells have a moderate amount of nuclear RNA, some with nucleoli.
3+—Most cells have increased nuclear RNA and bright nucleoli.
4+—Nearly all cells have increased nuclear RNA and bright nucleoli.

Table 2.—*Evaluation of Lymphocytic Nuclear RNA in Various Conditions*

Number Patients	Diagnosis or Condition	Lymphocyte AON Rating	Comment on Nuclear RNA
19	Normal	1-2	
1	Pertussis	3	Nucleoli prominent
8	Chronic lymphocytic leukemia	3	Nucleoli prominent
2	Acute lymphoblastic leukemia (ALL)	4	Highest
4	ALL/treated	0-1	Low
4	Uremia	0-1	Below normal
1	Acquired hypogammaglobulinemia	0	Lowest
5	Cancer	1-2	
5	Lymphoma Myeloma Paraproteinemia	1-2	
12	Miscellaneous	1-2	

Bach and Hirschhorn.[6-8] Heparinized human blood was allowed to sediment at room temperature (25 to 27 C.) in a screw-capped glass tube for 3 hours. The supernatant plasma was removed and a WBC count performed; 8.5×10^5 cells were pipetted into flat glass prescription bottles containing 3 cc. of media (MEM Spinner medium supplemented with 20 per cent fetal calf serum, penicillin, streptomycin, and glutamine).

This basic suspension was varied by the addition of (a) phytohemagglutinin (PHA), 0.02 ml./ml. culture medium (General Biochemicals, lot number 661, 601); (b) yeast RNA, 1–4 mg./ml. (Mann Research Laboratories, Inc., lot number L1941); (c) actinomycin D, 0.1 μg./ml. (Merck, Sharp and Dohme, lot number 1271E).

The cultures were incubated at 37 C. under 5 per cent CO_2 and harvested at various intervals. The cells were obtained by centrifugation and evaluated under light and fluorescent microscopy by the methods described above. WBC counts were performed initially and at harvest, after the cells had been deagglutinated on a Vortex (Scientific Products Co.), using a hemocytometer.

Results

Acridine orange-stained lymphocytes showed a wide range of stainable RNA under fluorescent microscopy. The nuclei were especially variable, seeming to form a continuum from a virtual absence of RNA to an abundance. The cells from the patient with acquired hypogammaglobulinemia, for example, ex-

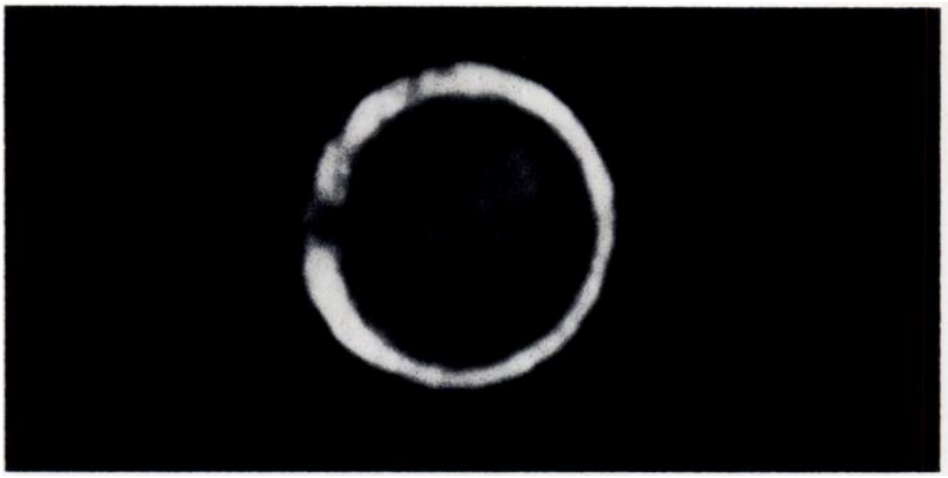

Fig. 1.—Normal lymphocyte (acridine orange stain, 930×).

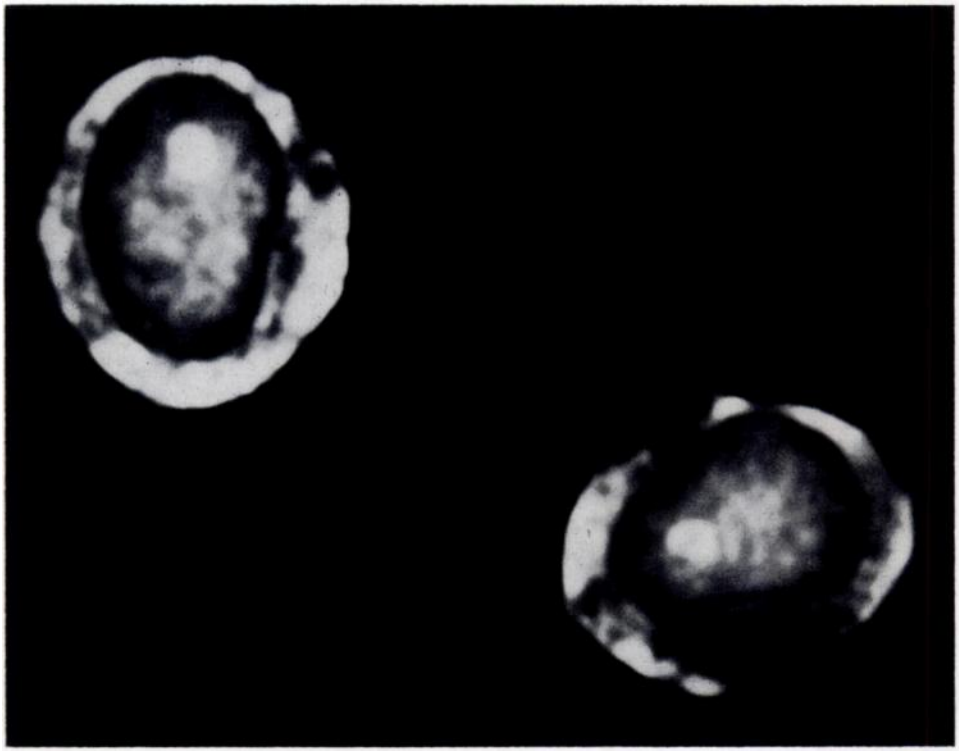

Fig. 2.—Lymphocytic cell from a patient with chronic lymphocytic leukemia.

hibited essentially no stainable nuclear RNA (Table 2). Lymphocytes from normals showed only faint nuclear RNA with an occasional faint nucleolus (Fig. 1). At the other end of this continuum are the lymphocytes of patients with chronic and acute lymphocytic leukemia (Fig. 2) or pertussis (Fig. 3).

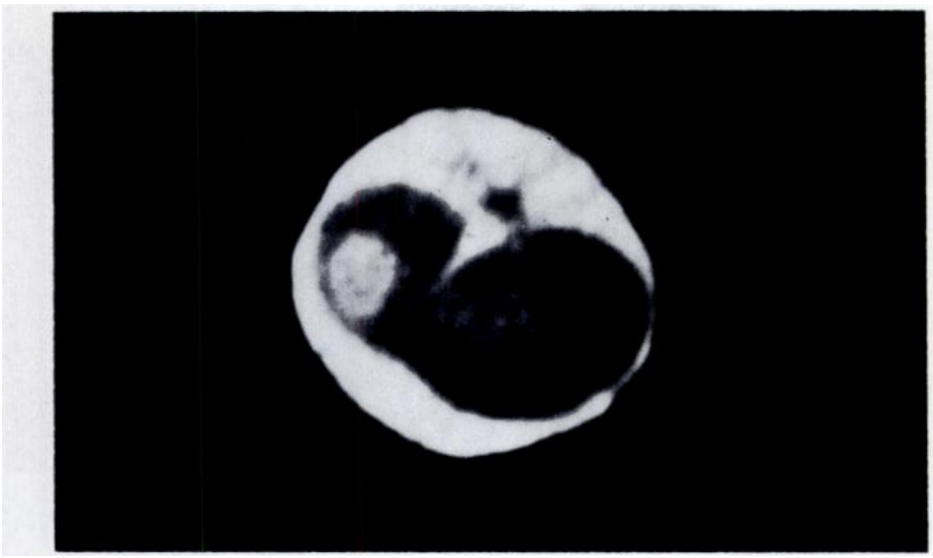

Fig. 3.—Lymphocytic cell from a patient with pertussis.

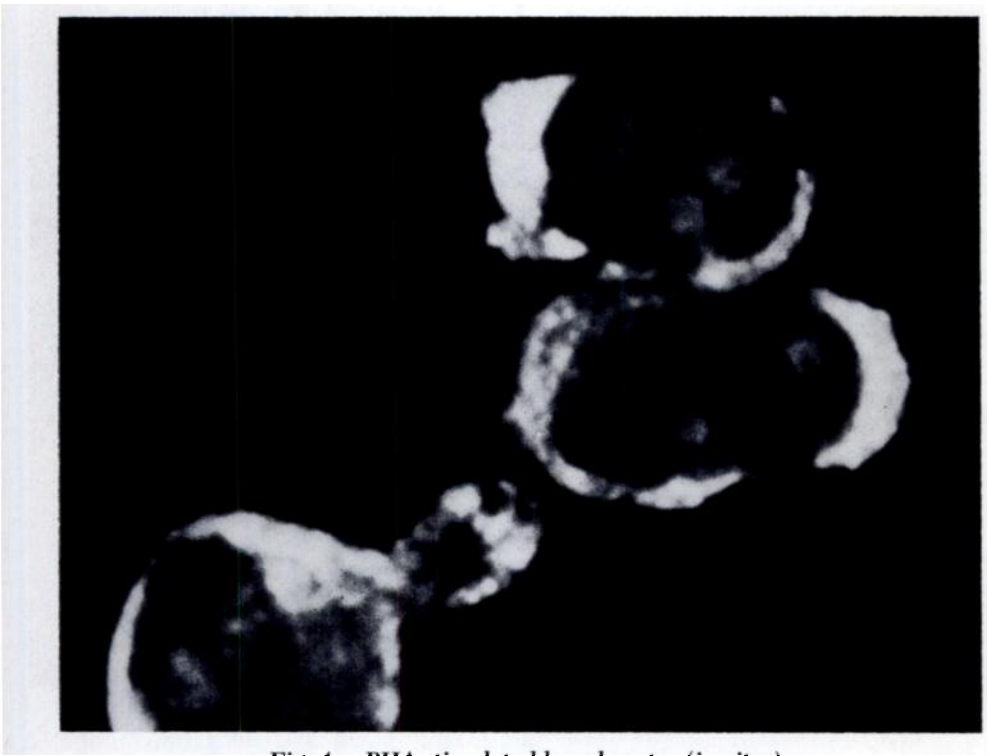

Fig. 4.—PHA-stimulated lymphocytes (in vitro).

Generally, these cells were larger and showed increased nuclear RNA and large, bright, distinct nucleoli. The PHA-stimulated lymphocytes also had an increased amount of RNA and were larger and blast-like (Fig. 4).

Table 3.—*Lymphocyte Nuclear RNA in Tissue Culture.* AON Rating 0 to 4+ Scale*

No. of Cultures†		Immediate	48 Hrs.	96 Hrs.	144 Hrs.	Comment
5	Normal	1+	1+	1+	1+	—
7	PHA‡	1+	4+	4+	4+	Dedifferentiated
9	PHA + RNA§	1+	1+	2+	3+	Inhibition
4	RNA	1+	1+	±	±	Slight inhibition
2	PHA + Act. D‖	1+	0	0	0	Inhibition
2	Act. D	1+	0	0	0	Inhibition

*Method of Bach and Hirschhorn.
†Two normal individuals on separate occasions.
‡Phytohemagglutinin 0.02 ml./ml. (Lot #661, 601 General Biochemicals).
§Yeast RNA 1 mg./ml.
‖Actinomycin D 0.1 μg./ml.

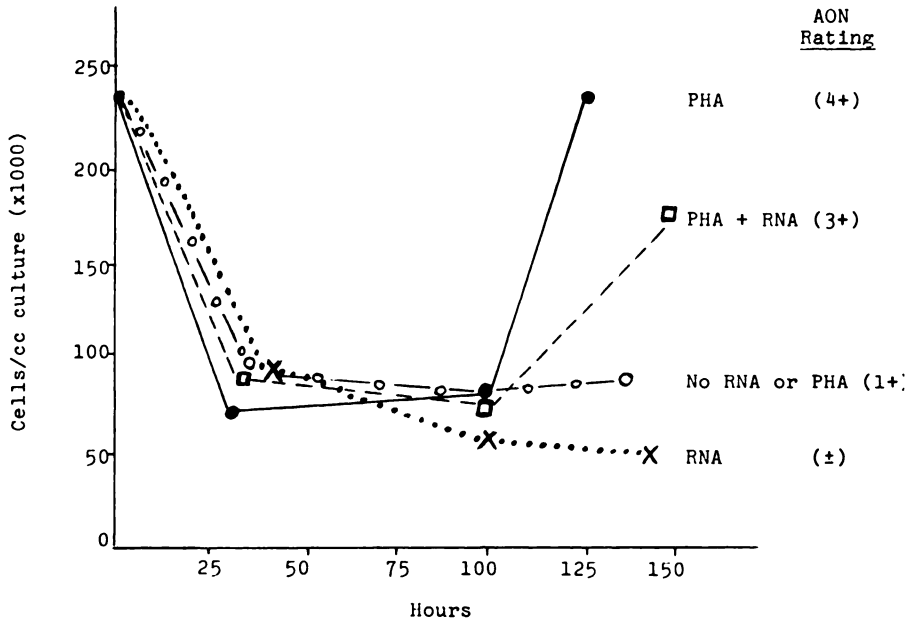

Fig. 5.—Average number of cells per culture upon incubation with PHA and/or RNA.

Normal human lymphocytes in tissue culture without mitogenic stimulation exhibited little change in AON rating during 6 days incubation, as can be observed in Table 3. In the cultures which contained PHA, dedifferentiation occurred rapidly and was accompanied by a corresponding increase in AON rating (Fig. 5). Parallel cultures containing yeast RNA and PHA initially resembled the nonstimulated cells, thus exhibiting marked inhibition in the expected increase in nuclear RNA when compared to PHA-stimulated cell cultures. A lessening of the inhibition with time occurred, but fewer cells and a lower AON rating were still evident in the RNA and PHA cultures at the sixth day. The effect of RNA on the cultured PHA-stimulated normal

lymphocytes was dependent on the concentration of RNA, showing a more prolonged inhibition with increasing RNA (amounts of 1, 2, and 4 mg./ml. of culture). The cultures containing yeast RNA, but without PHA, also showed some inhibition, but this appeared less pronounced. When actinomycin D, a known inhibitor of DNA-directed synthesis, was added to the cultures with and without PHA, inhibition was observed as expected of both cell numbers and AON rating.

Discussion

The technic here presented has proved useful as a relatively simple method for the direct quantitative evaluation of nuclear RNA in the lymphocyte. In cases where the cytoplasmic RNA stains well, it is more difficult to quantitate this RNA because of its even distribution and the variation in the size and configuration of the cell. The nuclear RNA shows differences in the intensity of fluorescence and can be definitively related to the nucleolar structure and to the relative distribution of DNA. The AO-stained smears were stable for several months and showed noticeable fading only upon prolonged or repeated excitation with ultraviolet light. Heparinized blood, smeared within 5 minutes, appeared to have a slightly higher AON rating compared to fingerstick smears prepared immediately.

The ability to respond to an antigen appears to depend on the presence of the normal small lymphocyte.[9-11] The observations made by examination of acridine orange-stained peripheral blood smears would suggest a possible connection between the amount and/or type of nuclear RNA and the participation of the lymphocyte in immune reactions. On this basis, three categories of lymphocytes may be seen:

1. *Normal Nuclear RNA.* The normal small lymphocytes, which presumably will respond to an appropriate antigenic stimulus, had some nuclear RNA. These were given an AON rating of 1+.

2. *Reduced Nuclear RNA.* The "depressed" lymphocytes of patients with uremia, hypogammaglobulinemia, or those undergoing antimetabolite therapy (6-mercaptopurine, methotrexate, or prednisone) showed less nuclear RNA (0 rating) than the normal small lymphocyte. It has been shown that in hypogammaglobulinemia lymphocytes show depressed protein synthesis,[12] which might correlate with reduced nuclear RNA. Also, in cases of uremia or in antimetabolite therapy, lymphocytes show depressed protein synthesis under phytohemagglutinin stimulation in vitro.[13]

3. *Increased Nuclear RNA.* The lymphocytes which showed increased nuclear RNA (3–4+ rating) were observed in two different situations.

   A. "Stimulated" lymphocytes, having encountered an antigenic (pertussis) or mitogenic stimulus (PHA) had increased nuclear RNA.

   B. Neoplastic lymphocytes of leukemic patients also had increased nuclear RNA.

Ordinarily, increased RNA would be expected to result in increased protein production. However, normal or reduced serum protein levels are observed

frequently in chronic lymphocytic leukemia. This may suggest a block in the pathways of RNA-protein metabolism in leukemic cells and is perhaps related to the "immunologic incompetence" described in this condition. Also, the protein production of these leukemic cells has been shown to be lower than normal and does not rise appreciably under the influence of phytohemagglutinin in vitro.[13]

The findings in the tissue culture experiments showed increased RNA in phytohemagglutinin-stimulated dedifferentiated lymphocytes. The protein production of phytohemagglutinin-stimulated lymphocytes in vitro has been shown to be increased, but the type of protein produced apparently lacks specificity.[13] The synthesis of nonribosomal RNA by PHA-stimulated lymphocytes has been demonstrated by Cooper and Rubin, a situation differing from that of cells responding to known antigens.[14]

The addition of RNA to lymphocytes in tissue culture in these studies, either with or without phytohemagglutinin, appears to inhibit "dedifferentiation." Whether this specifically interferes with RNA metabolism in the nucleus or elsewhere, or whether it inhibits the cells in some general way, is as yet undetermined. One might speculate that in neoplastic and, possibly, other conditions, RNA from malignant or other cells could interfere with host immune responses by inhibiting lymphocyte nucleic acid function, resulting in immunologic incompetence. Mannick has suggested a similar hypothesis with regard to RNA inhibition of transplantation immunity.[15] In these situations, the function of cellular and tissue ribonucleases is of obvious importance and could be relatively increased or decreased, depending on the load presented.[16]

Summary

Lymphocytes from human peripheral blood have been evaluated by fluorescent microscopy using acridine orange-stained preparations. The nuclear RNA has been quantitatively rated and correlated with the diagnoses. These observations were performed on normals and patients with chronic lymphocytic leukemia, pertussis, hypogammaglobulinemia, acute leukemia, uremia, other malignancies, and a miscellaneous group. RNA added to in vitro normal lymphocyte cultures showed an apparent inhibition of cell proliferation in PHA-stimulated cells.

SUMMARIO IN INTERLINGUA

Lymphocytos ab human sanguine peripheric esseva evalutate per microscopia fluorescentic con le utilisation de preparatos tincturate a orange acridinic. Le acido ribonucleic del nucleos esseva estimate quantitativemente e correlationate con le diagnoses. Iste observationes esseva effectuate in subjectos normal e in patientes con chronic leucemia lymphocytic, con pertusse, con hypogammaglobulinemia, con leucemia acute, con uremia, con altere malignitates, e un gruppo miscellanee. Acido ribonucleic addite a culturas in vitro de lymphocytos normal monstrava un apparente inhibition del proliferation cellular in un population stimulate per phytohemagglutinina.

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