Nuclear Volume Distribution of Phytohemagglutinin-Stimulated Human Lymphocytes[1] (35046)

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The quantitation of lymphocyte blastoid transformation is being used with increasing frequency to study normal and pathological lymphocytes. Parameters which have been measured include morphological changes and functional characteristics, such as DNA, RNA, and protein synthesis. Electronic measurement of the variation in mean cell volume during transformation has been used with both animal (1) and human lymphocytes (2). Experience with the quantitation of changes in nuclear volume during lymphocyte transformation is described.

Methods and Materials. Isolation of lymphocytes. Fifty to 100 ml of heparinized peripheral blood were obtained under sterile conditions from normal subjects. The blood was allowed to sediment at 37° for 2 hr. The supernatant plasma was transferred to a Nylon reticulum column and incubated for 20 min, as described previously (3). The effluent plasma was centrifuged at 150*g* for 10 min. The supernatant fluid was discarded. The sedimented cells were washed once with 10 ml of TC 199 culture medium, resuspended in 5 ml TC 199 with 15% fetal calf serum (FCS), and counted.

Preparation of cultures. Aliquots of cell suspensions were added to culture media containing TC 199 with 15% FCS, in final concentrations of 6×10^6 cells/10 ml culture, in 25-ml screw-capped prescription bottles. Stimulated lymphocyte cultures were prepared by adding phytohemagglutinin-P (PHA-P)[4] (0.02 ml of reconstituted solution/10 ml of culture); unstimulated cultures were identical except for the absence of PHA-P.

Morphological studies. Lymphocyte transformation was identified in Wright-stained, fixed smears of lymphocyte suspensions. Parameters of transformation used were those described by Bach and Hirschhorn (4).

Nuclear-volume determination. Freshly separated lymphocytes and cultured PHA-P-stimulated and unstimulated lymphocytes, harvested at 2- to 3-day intervals, were examined. Aliquots of the lymphocyte suspension in TC 199 with 15% FCS were centrifuged at 150*g* for 10 min. The sedimented cells were resuspended in 0.5 ml of supernate to which 10 ml of Cetrimide[5] counting solution (1) were added. The cell suspension was counted in a Model F-Coulter Counter[6] using an aperture current setting of 64, amplification control setting of 1 and a 50μ orifice. The nuclear volume distribution was obtained by pulse-height analysis, using a 400-channel analyzer.[7] The counter-analyzer device was calibrated before each experiment, with a fixed red cell suspension.[8] The calibration factor was set at 1μ^3/channel, and the results were expressed as modal volumes (in $\mu^3 \pm 1$ SD). The volume-distribution data from the multichannel analyzer were relayed to an

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[4] Bacto-Phytohemagglutinin-P, Difco Laboratories, Detroit, Mich.

[5] Cetavlon, Cetrimide BP. Gift of ICI America, Inc., Stanford, Conn.

[6] Coulter Electronic Inc., Hialeah, Fla.

[7] Packard Instrument Company, Chicago, Ill.

[8] Celltrol, Pfizer Diagnostics, New York, New York.

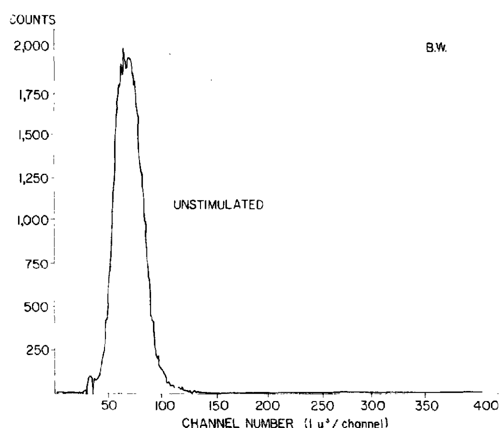
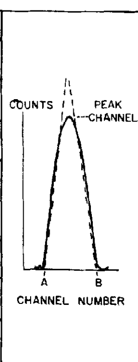


FIG. 1. Nuclear-volume distribution of freshly isolated, unstimulated lymphocytes.

XY-recorder which plotted the distribution curves.

Results. Nuclear volumes of unstimulated ("off column") lymphocytes. A representative nuclear volume-distribution curve of freshly prepared control lymphocytes prior to culture or PHA stimulation is shown in Fig. 1. On the left side of the curve, two small components of variable heights are demonstrable. These are made up of particles smaller than $50\mu^3$. The central component, comprising most of the lymphocytic nuclei, ranges between $43.7 \pm 3.4\mu^3$ and $95 \pm 6.9\mu^3$ (Table I), with a modal distribution at $66.6 \pm 3.3\mu^3$. This central segment is followed by a small component on the right, suggesting a population of larger nuclear particles.

TABLE I. Values of Volume Distribution Curves of Freshly Isolated, Unstimulated Lymphocytes in Cetrimide Counting Fluid, from Seven Subjects ($1\ \mu^3$/channel).

Subject	A	B	Peak Channel	
B.G.	44	100	64	
J.D.	39	83	64	
E.H.	51	102	72	
F.M.	41	100	71	
B.W.	44	100	65	
S.T.	44	93	67	
L.C.	43	87	63	
	43.7±3.4	95±6.9	66.6±3.3	

Two of the nine healthy control subjects exhibited a moderate degree of lymphocytosis (3612 and 4100 lymphocytes/mm^3). The "off column" lymphocyte nuclear-volume distribution of these samples was similar to that of the other seven subjects except for the presence of slightly larger nuclei ("right shift"), with a modal distribution of $80\mu^3$ in one of the two subjects.

Sequential changes of nuclear volumes of unstimulated normal lymphocytes. Figure 2 depicts a representative pattern of the distribution of nuclear volumes of lymphocytes from one healthy subject. After culture for 3 to 5 days, the curves were essentially identical to those from freshly isolated unstimulated cells. After 72 hr, there was, in some cultures, an increase in the number of small particles ($<50\mu^3$) and a slight increase in the larger particle component. Morphological evidence of cell death and the presence of cellular debris were associated with a marked increase in the number of small particles.

Nuclear volumes of stimulated normal lymphocytes. The nuclear volume distribution of stimulated lymphocytes was characterized by a marked increase in the number of large particles evident at 72 hr of incubation (Fig. 3). The maximum volume increment was variable in intensity and occurred between 5 and 8 days of culture. These changes were associated with definite morphological evidence of transformation. A variable increase in the number of small particles occurred coincidentally with morphological evidence of cell death or nontransformation, as well as with pH changes of the medium to the alkaline range.

Discussion. Quantitation of lymphocyte transformation after antigenic stimulation is essential for the clinical evaluation of certain immune disorders, as well as in immunological research. Morphological parameters, using fixed and stained cells, are subjective and time consuming. A rapid electronic method employing unstained and viable lymphocytes would provide a rapid and objective method of analysis. The value of such a system would be additionally enhanced by relaying the electronic signals to a programmed computer

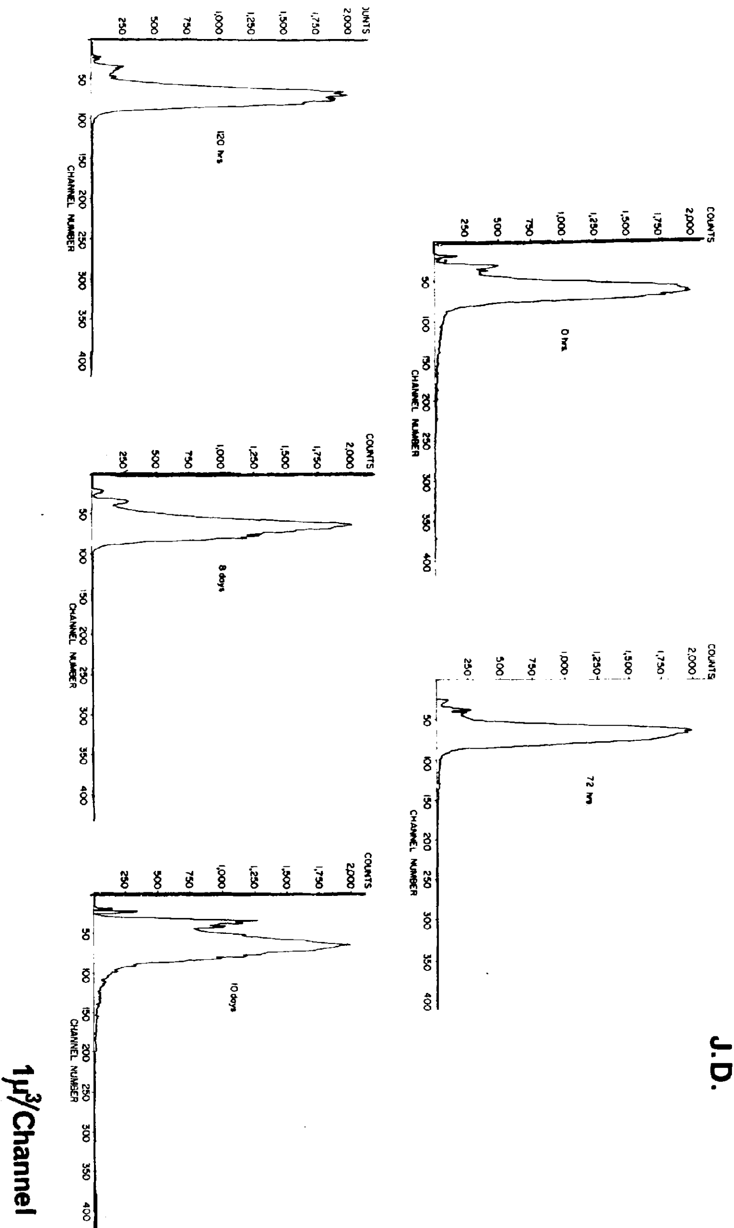


FIG. 2. Serial determinations of nuclear-volume distribution of unstimulated lymphocytes in culture.

for immediate data analysis. The application of such a method involves a number of mechanical problems. When in contact with PHA, there is a tendency for lymphocytes to form clumps; there is paucity of knowledge about the influence of flow kinetics and little is known about the morphologic and impedance changes occurring in cells subjected to rapid motion through a narrow orifice across an electric field (5).

This method is a modification of one described previously (2) which has been used in the study of lymphocytes (3) and erythrocytes (6) from normal persons and from uremic and burn patients. Stewart and Ingram (1) described their experience with Cetrimide in the measurement of transformation of canine lymphocytes. They observed that

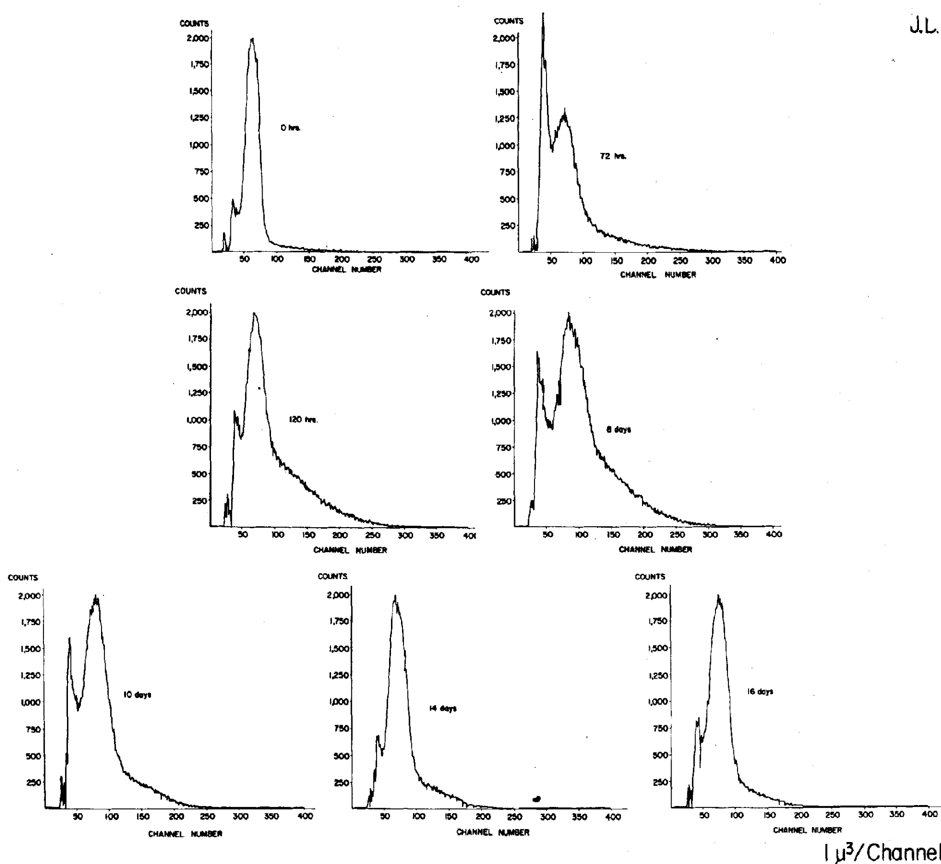


FIG. 3. Serial determinations of nuclear-volume distribution of PHA-P-stimulated lymphocytes in culture.

clumps of stimulated cells were dispersed and the cells were stripped of their cytoplasm after exposure to Cetrimide.

Cetrimide is a mixture of tetradecyl alcohols widely used in industry. Its main application is as an antiseptic and detergent, but it has been used for leukocyte counting (7) with the Coulter electronic counter. When used in concentrations of 5 mg/ml of fluid, the cytoplasm of human lymphocytes is stripped off the cells leaving the nuclei morphologically intact (Fig. 4). The proteolytic enzyme, pronase, was used in our earlier experiments for the purpose of digesting cellular debris. There was no significant difference between the distribution curves of lymphocytes treated with enzyme and those of untreated ones and its use was discontinued.

Peripheral blood lymphocytes, cultured in the presence of phytohemagglutinin, undergo a series of changes. RNA, DNA, protein synthesis, and lysosomes increase, and morphological alterations occur (8, 9). These changes are characterized by the appearance of intermediate "transitional" blastoid cells at 24 hr, followed by the appearance of larger blastoid cells after 48 hr of culture. At 72 hr, a spectrum of small, transitional and blast cells is present. The larger blast cells constitute nearly one third of all cells (8). The diameter of these cells may be more than five times that of small unstimulated lymphocytes. The nuclear–cytoplasmic ratios vary, but, in general, the nuclear diameters increase in proportion to the cytoplasm.

Small lymphocytes from normal subjects rarely undergo significant transformation in unstimulated cultures. Upon stimulation, *in*

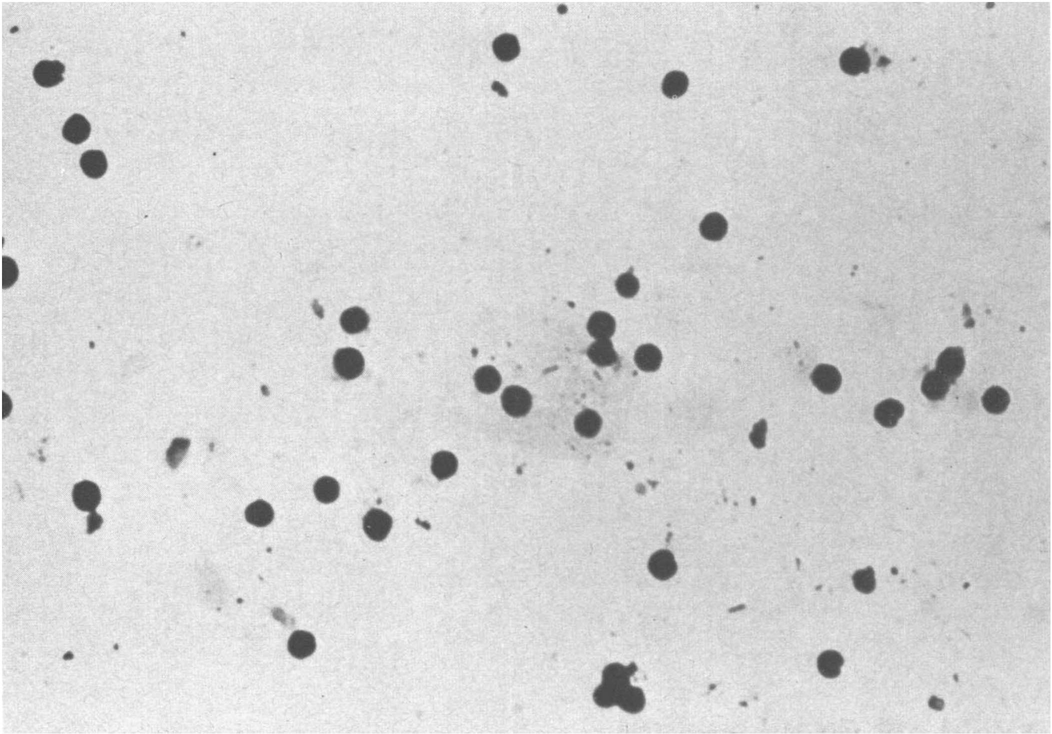


FIG. 4. Nuclei of unstimulated lymphocytes treated with Cetrimide (160×).

vitro with PHA, these cells rapidly transform during the first 72 hr of incubation. From the fifth to the ninth day of incubation, the percentage of large blastoid cells either remains constant (10) or increases (11, 12). Other relevant changes are an increase in transitional and degenerating cells after 7 days of incubation (12). The number of degenerating cells continue increasing until by the seventeenth day most cells show changes associated with cellular death (13).

The distribution patterns of nuclear volume correlate well with previously described morphological findings. The "off column" lymphocyte suspensions produce a curve with slight skewing to the right, suggesting a relatively homogeneous particle population with the presence of a small number of large cells and, possibly some degree of coincidence counting. At 24 hr, nuclei of stimulated lymphocytes are not markedly different from the unstimulated "off column" cell nuclei, again correlating well with the morphological data. At 48 hr, a small increase in number and volume of lymphocyte nuclei is evident, and at 72 hr, a conspicuous increase in the number of large particles is demonstrable. This trend continues up to the eighth day of culture. These data are in agreement with those described in previous reports (8). In stimulated cultures, a regression of the right component of the curve was frequently observed after 8 days, correlating with the presence of smaller particles as suggested by the results of Yoffey *et al.* (8). Evidence of cell death was paralleled by an increase in the number of small particles.

Cellular volume is determined by the Coulter counter in an indirect way by measuring the variations of an electrical field produced by the cellular displacement. It is possible that, besides measuring changes in cellular volume, variations in the electric conductivity or pliability of the cells, with or without transformation, are being recorded. Unpublished preliminary results of detailed studies

of lymphocytes by Wilkins *et al.* indicate that such changes are less marked than those observed with erythrocytes (5). Correlation of known morphological alterations with changes of volume distribution of lymphocyte nuclei indicates the feasibility of measurement of nuclear volume distribution for the detection and quantitation of transformation of human lymphocytes. Recent data of nuclear-volume changes of PHA-stimulated lymphocytes obtained by split-image measurements of Townsend *et al.* correlate well with the presented electronically obtained data, and support the feasibility of this system. The authors, in conjunction with the center for cybernetic studies of the University of Texas, are developing an automated computer procedure for the quantitation of the volume distribution data (14).

Among the numerous applications of this rapid and objective semiquantitative technique are screening of mitogenic agents, characterization of disorders of cellular immunity, assay of antilymphocyte globulin (16), etc.

Summary. Unstimulated and phytohemagglutinin-stimulated lymphocytes from nine human subjects were suspended in Cetrimide solution before and after tissue culture. Their nuclear volumes were measured serially with a particle counter—400 channel analyzer device. Variations of the volume distribution curves correlated well with the morphological changes associated with blastoid transformation. This method permits rapid and objective assay of lymphocyte transformation.

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1. Stewart, C. C., and Ingram, M., Blood **29**, 628 (1967).

2. Townsend, C. M., Jr., Ritzmann, S. E., and Levin, W. C., Texas Rep. Biol. Med. **26**, 445 (1968).

3. Daniels, J. C., Cobb, E. K., Sakai, H., Remmers, A. R., Jr., Sarles, H. E., Fish, J. C., Levin, W. C., and Ritzmann, S. E., Amer. J. Med. Sci. **259**, 214 (1970).

4. Bach, F. H., and Hirschhorn, K., Sem. Hematol. **2**, 68 (1965).

5. Wilkins, B., Frondolig, J. E., and Fischer, C. L., J. Ass. Adv. Med. Instr. **4**, 99 (1970).

6. Blocker, T. G., Jr., Lewis, S. R., Lynch, J. B., Larson, D. L., Ritzmann, S. E., and Lanier, B. Q., "The Use of Blood in Burns," Trans. N.Y. Acad. Sci., in press (1970).

7. Hatch, A., and Balazs, T., Amer. J. Clin. Pathol. **36**, 220 (1961).

8. Yoffey, J. M., Winter, G. C. B., Osmond, D. G., and Meek, E. S., Brit. J. Haematol. **11**, 488 (1965).

9. Elves, M. W., "The Lymphocytes," 303 pp. Lloyd-Luke, England (1966).

10. Bouroncle, B. A., Clausen, K. P., and Aschenbrand, J. F., Blood **34**, 166 (1969).

11. Haveman, K., and Rubin, A. D., Proc. Soc. Exp. Biol. Med. **127**, 668 (1968).

12. Yam, L. T., Castoldi, G. L., and Mitus, W. J., J. Lab. Clin. Med. **70**, 699 (1967).

13. Bender, M. A., and Prescott, D. M., Exp. Cell Res. **27**, 221 (1962).

14. Charnes, A., Klingman, D., Stutz, J., Houston, E. W., and Muñiz, F. J., Communication at Ann. Symp. Biomath. Computer Sci. Life Sci. 8th (1970).

15. Townsend, C. M., Jr., Vyvial, T. M., Wood, A. J., Remmers, A. R., Jr., Sarles, H. E., Canales, C. O., Beathard, G. A., Fish, J. C., Muñiz, F. J., Levin, W. C., and Ritzmann, S. E., Texas Rep. Biol. Med. **27**, 497 (1969).

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