STUDIES ON HUMAN PERIPHERAL BLOOD LYMPHOCYTES IN VITRO

II. Morphological and Biochemical Studies on the Transformation of Lymphocytes by Pokeweed Mitogen

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The transformation of human peripheral blood lymphocytes in vitro to large "blastlike" cells following incubation with phytohemagglutinin (PHA) has been studied extensively (1–6). The PHA-transformed cells display a primitive undifferentiated morphology and bear resemblance to the hemocytoblast (7), the hematopoietic reticular cell (8), and the "transitional cell" of Fagraeus (9).

Recently, extracts of the plant *Phytolacca americana* (pokeweed) have been reported to be capable of inducing the transformation of human lymphocytes in vitro (10). In individuals who had received accidental injections of the pokeweed mitogen (PWM) typical plasma cells as well as large immature pyroninophilic lymphocytes were observed in their peripheral blood (11). In the preceding paper (12) we have shown that the mitogenic principle derived from pokeweed possesses biologic activities distinct from those of phytohemagglutinin (PHA). This report described the morphologic changes occurring in lymphocyte cultures treated with purified PWM; the kinetics of RNA and DNA synthesis; and sedimentation analysis of the classes of rapidly synthesized RNA seen in response to PWM stimulation.

Methods

Pokeweed Mitogen Fraction (PWM).—The preparation and characterization of pokeweed mitogen fractions have been described in the preceding paper (12). In this study, the 0.05 M front fraction (F) derived from calcium phosphate chromatography was used.

Human Peripheral Blood Lymphocyte Cultures.—

Method for the collection and separation of peripheral blood lymphocytes: Blood donors used

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for these studies were normal adult volunteers, who were screened to rule out a history of recent immunization, illness, and consumption of specific medications. One unit (500 ml) of venous blood was withdrawn aseptically into a Fenwal pack containing 2250 units heparin sodium, USP. (Fenwal Laboratories, Inc., Morton Grove, Illinois). All procedures following venesection were carried out at 37°C. The blood was transferred to 250 ml graduated cylinders which had been pretreated with 1 ml heparin (Liquaemin sodium "50" heparin, Organon, Inc., West Orange, N. J.), and allowed to sediment at a 45° angle for approximately 2 hr.

The leukocyte-rich plasma supernate was removed, diluted with one volume of Eagle's MEM, Spinner modification (13), supplemented with fresh *l*-glutamine (0.02 M), and filtered at 30 drops/min through a 100 ml syringe, prepacked with 3 g of sterile Pyrex glass wool filtering fiber (No. 3950) Corning Glass Works, Corning, New York. The column was then washed with an additional 100 ml of medium and the eluate pooled with the original harvest. The pooled eluates were centrifuged for 10 min at 250 *g* and the cell pellet was washed once with Dulbecco's phosphate-buffered saline (PD) deficient in calcium and magnesium ions (14) containing 0.2 cc heparin/100 ml.

Lymphocytes were cultured at 37°C in 85% Eagle's Spinner MEM containing 0.02 M *l*-glutamine, penicillin 100 units/ml, streptomycin 100 μg/ml and either 15% heat-inactivated calf serum or 15% autologous serum. A standard inoculum of 3×10^6 lymphocytes in 5 ml of growth medium was cultured in 16 × 125 mm screw cap disposable glass culture tubes (Demuth Glass Works, Inc., Parkersburg, West Virginia).

Measurement of the rate of RNA synthesis: Tritiated uridine was used as a precursor to measure the rate of RNA synthesis by radioautography. Lymphocyte cultures were harvested at intervals from 0 to 72 hr and 2 μc/ml of uridine-H^3 (specific activity, 2.7 c/mmole, Schwartz Bio-Research, Inc., Orangeburg, New York) were added for the terminal 2 hr of incubation of each culture. The cells were then washed three times with 0.01 M phosphate-buffered saline at pH 7.4, and resuspended in medium containing serum. Smears were made on gelatin-coated slides, fixed in absolute methanol for 10 min, and then in methanol-acetic acid (3:1) for 1 hr at 4°C. The slides were coated with NTB-3 Kodak emulsion at 43°C (two parts emulsion and one part distilled water) and stored at 4°C for 7 days in light tight boxes containing desiccant. Slides were developed and stained with Giemsa according to the method of Epstein and Stohlman (15). Grain counts were performed on 1000 cells in each preparation, and tabulated into three classes: unlabeled (<3 grains); lightly labeled (4 to 10 grains); and heavily labeled (>10 grains).

RNA sedimentation studies: After incubation with PWM, lymphocyte cultures containing 5×10^7 cells were pulsed for 20 min with uridine-5-H^3 (10 μc/ml, specific activity, 4.4 c/mmole, New England Nuclear Corp., Boston). RNA was then extracted with hot phenol-sodium dodecyl sulfate as described by Cooper and Rubin (16) and sedimented at 20,000 RPM (Spinco Model L ultracentrifuge, Rotor SW 25) across a 5 to 20% sucrose gradient for 17 hr. To locate the sedimentation markers (provided by unlabeled rabbit liver RNA) the contents of the centrifuge tube were first allowed to pass through a recording spectrometer (Gilford Instruments Company, Oberlin, Ohio) and the absorption at 260 mμ determined. Thirty serial fractions were collected and aliquots of each sample were assayed for radioactivity in a Packard 4000 TriCarb Liquid Scintillation Spectrometer.

Measurement of the rate of DNA synthesis: The rate of DNA synthesis was determined by radioautography and assay of radioactivity incorporated into DNA of lymphocytes grown continuously in the presence of 1 μc/ml of thymidine-H^3 (1.9 c/mmole, Schwartz BioResearch, Inc.). DNA was extracted as described in the preceding paper (12). Assays were performed on at least 4 replicate cultures.

Morphological studies: The per cent transformed cells was determined from both Giemsa and acetic orcein stained preparations. PAS staining was performed on replicate slides accord-

ing to the method of Lillie (17). To determine diastase sensitivity, methyl alcohol-fixed control slides from each culture were digested for 1 hr at 37°C with a 0.1% diastase solution in normal saline (Isomaltase, Armour Pharmaceutical Co., Chicago) Alcian blue staining was performed according to the method of Spicer and Meyer (18).

RESULTS

Morphological and Biochemical Characteristics or Human Peripheral Blood Lymphoctyes Prepared from Glass Wool Columns. The passage of leukocyte-rich plasma through the columns effectively removed phagocytic cells (polymorphonuclear leukocytes and monocytes) which adhere to glass wool. The final eluate contained only 1 to 3% of phagocytic cells as determined by incubation with polystyrene particles.[1]

The lymphocyte population obtained after processing on the glass wool column consisted of 95 to 97% small lymphocytes (cell diameter 7 to 10 μ); 3 to 4% medium lymphocytes (10 to 12 μ); less than 1% were large lymphocytes (12 to 15 μ). The cells showed the characteristic features described by Downey (19) when stained with Giemsa a high nucleus:cytoplasm ratio (N:C), a thin rim of pale blue cytoplasm with occasional aurophilic granules, and a densely stained nucleus with a coarse chromatin network. Nucleoli were seen in 20 to 35% of cells when stained with acetic orcein. Diastase resistant PAS-positive granules were found in 7 to 10% of lymphocytes.

To determine the RNA synthetic activity in the unincubated ("resting") lymphocyte population 1×10^7 freshly harvested cells were pulse labeled for 30 min with 10 μc/ml uridine-H^3. Radioautograph preparations were made, exposed for 7 days to nuclear track emulsion and analyzed by grain counts. The largest class (68% of cells) showed insignificant labeling (<3 grains/cell), 22% of cells showed light but significant labeling (4 to 10 grains/cell), and approximately 10% of cells showed heavy labeling (>10 grains/cell).

Radioautographs made from freshly harvested lymphocytes (1×10^7 cells) pulse labeled with 10 μc/ml thymidine-H^3 and exposed for periods up to 3 wk showed virtually no incorporation of radioactivity (< 1/1000 cells displayed >3 grains/cell). Thus DNA synthesis was virtually absent in unincubated ("resting") lymphocytes prepared from glass wool columns. Control cultures incubated for as long as 120 hr in autologous serum-MEM continued to be composed of 98 to 99% small lymphocytes with less than 2% of cells displaying significant labeling (4 to 10 grains/cell) with thymidine-H^3.

Morphological and Histochemical Changes in PWM-Stimulated Lymphocyte Cultures. In the preceding study (12), the optimal concentration for the 0.05 M

[1] 0.1 ml of 1:100 diluted polystyrene latex particles (1.3 μ diameter, Dow Chemical Co., Midlands, Michigan) were added to 5 ml of culture medium containing 3×10^6 cells harvested from the glass wool column incubated for 2 hr at 37°C, and the per cent phagocytic cells determined under a hemocytometer.

front fraction (F) of the purified PWM was determined to be 0.05 μg/6 $\times$ 10^5 lymphocytes/ml. Incubation of lymphocytes with PWM at this concentration resulted in progressive morphologic changes in cellular morphology ("transformation"), first detectable after 16 to 18 hr. At the peak of transformation,

TABLE I

Morphologic Features of PWM-Transformed Lymphocytes

Criterion	Cell type	
	PWM blast cell type I*	PWM intermediate cell type II
Cell size (largest diameter), μ	20–35	25–30
N:C ratio	Decreased	Decreased
Nucleus		
Positions	Central or slightly eccentric	Eccentric
Chromatin	Mainly euchromatin with small amount of heterochromatin	Euchromatin but with increased heterochromatin
Nucleoli	1 to 2 in number, macronucleoli seen in less than 5% of cells	1 to 3 in number, 10 to 15% of cells have macronucleoli
Cytoplasm		
Perinuclear clear zone	Infrequent, small area when present	Frequent, very prominent and at times circumscribing the nucleus
Vacuoles	Prominent	Absent to few
Basophilia (pyroninophilia)	Prominent	Intense
PAS granules	Coarse, granular (PAS reaction maximum between 24 to 48 hr, negative at 72 hr) diastase sensitive	Fine, diffuse granular (PAS reaction maximum at 72 hr) diastase resistant
Alcian blue	Positive	Negative

* Features indistinguishable from PHA blast cell.

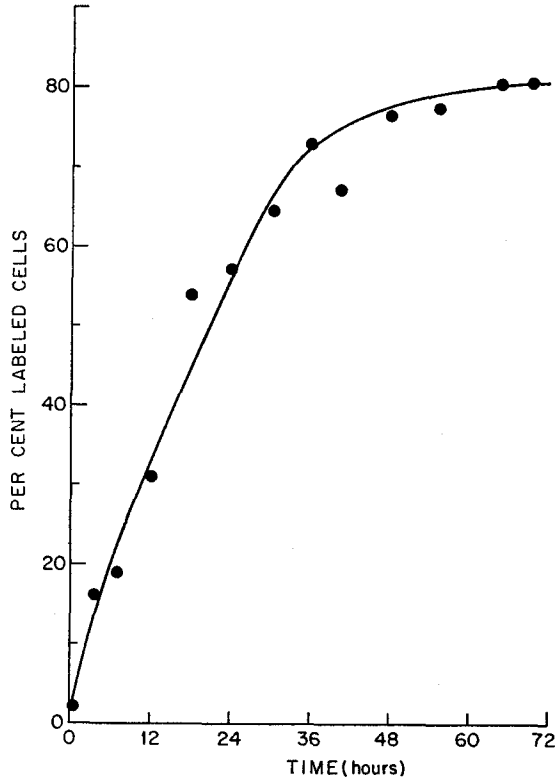
66 to 78 hr after incubation with PWM, 3 distinct types of cells could be distinguished by morphological and histochemical criteria (Figs. 1–15):

1. Typical small peripheral blood lymphocytes comprising 40 to 50% of the population.

2. A PWM type I ("blast cell"), comprising 30 to 35% of the cell population, which is morphologically indistinguishable from transformed cells found following exposure to phytohemagglutinin (1–6). The characteristics of these cells during their development are sumarized in Table I.

3. A distinctive cell type, not found in PHA cultures, a PWM type II ("in-

termediate cell"). These cells comprised 25 to 30% of the cell population at 72 hr, and are first distinguishable after 36 to 60 hr of incubation. These cells are either round or oval; the cell size is increased to 25 to 30 μ in the larger diameter and 8 to 15 μ in the smaller diameter, the N:C ratio is diminished; the cyto-

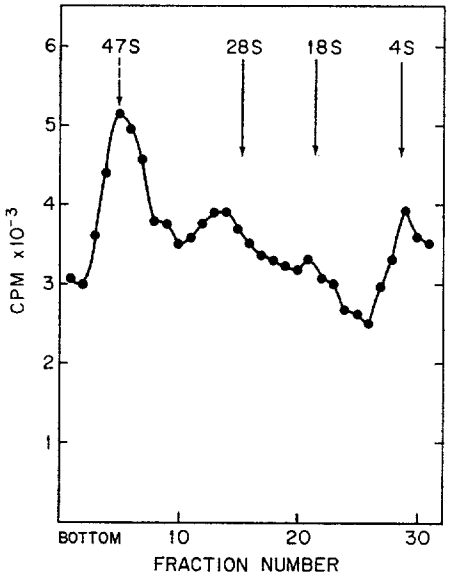


TEXT-FIG. 1. Rate of RNA synthesis in PWM-stimulated lymphocyte cultures. After varying periods of incubation the per cent lymphocytes becoming labeled during a 30 min pulse with uridine-H^3 (2 μc/ml) were scored (>10 grains/cells). Control cultures harvested at the same time showed a range of 3 to 8% of cells in the >10 grain class. Each point represents the mean of 4 replicate cultures.

plasm is extremely basophilic, with little or no vacuolization; a perinuclear clear zone is prominent and in some cells a clear space is seen circumscribing the nucleus; the nucleus is eccentric in position with a lacy nucleoplasm containing 1 to 3 nucleoli. These cells were Alcian blue negative and showed finely granular PAS-positive, distase-resistant material at 72 hr. These latter histochemical reactions were the primary means of differentiating type I and type II cells (see Table I).

Kinetics of RNA Synthesis.—The RNA synthetic activity of PWM-stimulated

peripheral blood lymphocytes was studied by means of radioautography and grain counting after exposure to uridine-H^3 (Text-fig. 1). The per cent of PWM-stimulated cells displaying significant labeling after 2 hr of exposure to radioactive precursor increased during the first 12 to 30 hr of incubation and reached a plateau after 48 to 64 hr.

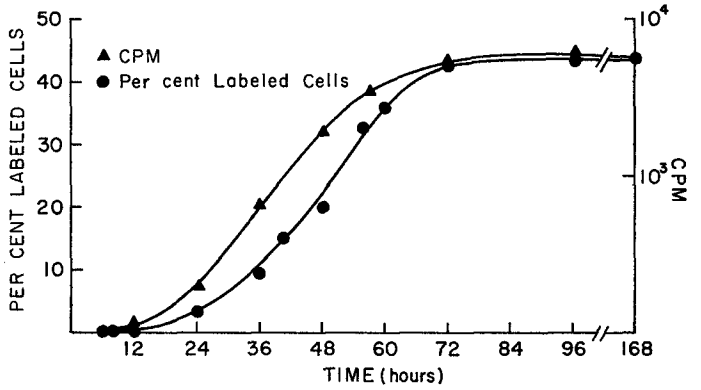


TEXT-FIG. 2. Sedimentation distribution of RNA newly synthesized by lymphocytes stimulated with PWM. 5×10^7 cells were incubated with 100 μg of PWM for 40 hr, then exposed to uridine-5-H^3 for 20 min. RNA was extracted and centrifuged for 17 hr at 20,000 RPM (Spinco Rotor SW-24) across a 5 to 20 per cent sucrose gradient. 30 serial 1 ml fractions were collected after passage through a recording spectrophotometer. 0.5 ml of each fraction was assayed for radioactivity. Ordinate indicates CPM per 0.5 ml aliquot. Solid arrows indicate position of the unlabeled sedimentation markers determined by absorption at 260 mμ; broken arrow indicates calculated relative S value.

RNA Sedimentation Profile.—Newly synthesized RNA from PWM-stimulated lymphocytes was characterized by means of sucrose density gradiant sedimentation of the pulse-labeled RNA which had been extracted with hot phenol and sodium dodecyl sulfate (16) (Text-fig. 2). A large peak of pulse-labeled RNA sedimented rapidly, at 45 to 50 S. The remainder of the newly synthesized RNA was polydisperse, sedimenting in a broad band across the entire gradient. Results were essentially similar after 24 and 40 hr of incubation with PWM.

Kinetics of DNA Synthesis.—The rate of DNA synthesis in PWM-stimulated lymphocyte cultures was measured by assay of radioactivity incorporated into DNA from thymidine-H^3 and by radioautography of labeled cells. Continuous exposure to thymidine-H^3 (Text-fig. 3) revealed that the proportion of labeled cells and the amount of thymidine -H^3 incorporated into the DNA reached

a maximum between 66 to 78 hr. The per cent transformed cells and the mitotic index at different times correlated with the isotope studies (Table II).



TEXT-FIG. 3. Rate of DNA synthesis in PWM-stimulated lymphocyte cultures. Replicate cultures containing 3×10^6 lymphocytes were incubated continuously in the presence of thymidine-H^3 (5 μc/ml). Cultures were harvested and assayed for radioactivity by autoradiography (expressed as per cent labeled cells) and liquid scintillation counting (expressed as CPM). Assays were performed on 3 to 5 replicate cultures for each time interval.

TABLE II

Transformation Rate and Mitotic Index in PWM-Stimulated Cultures

Parameter	Time (hours)				
	18	36	48	56	72
Transformed cells, %.....	11	25	33	41	58
Mitotic index‡.....	§	0.03	0.18	0.32	2.4

* Values are expressed as per cent transformed cells upon examination of 1000 cells from Giemsa and acetic orcein–stained preparations.

‡ Velban (Vincoleukoblastine, Eli Lilly and Co., Indianapolis) 1.0 μg/culture was added at times noted. Specimens were examined after 4 hr of incubation in the presence of Velban. Average values are expressed as per cent mitoses/hour and are based on examination of 1000 cells from replicate slides.

§ None detected.

DISCUSSION

Study of the kinetics of RNA and DNA synthesis of PWM-stimulated lymphocytes revealed that augmentation of RNA synthesis preceded the onset of DNA synthesis by approximately 24 hr and that DNA synthesis was maximal between 66 to 78 hr. This sequence of events has also been described for the transformation of lymphocytes induced by PHA (15, 20) antiallotype sera (21), and staphylococcal filtrate (22).

Radioautographic studies performed on PWM-stimulated cells revealed that both the blastlike cells (type I) and the type II cells were labeled with thymidine-H^3 and showed that maximum incorporation of label coincided with the peak of transformation as previously described for PHA (23). These findings were in good agreement with the liquid scintillation data, indicating that the rate of DNA synthesis may be used as a quantitative measure of the degree of transformation.

Resting lymphocytes (unincubated) were heterogeneous with regard to their RNA synthetic activity, with approximately 10% of cells actively engaged in RNA synthesis. From inspection of tables for the cumulative Poisson distribution (24) it may be seen that for a homogeneous population with 10% of cells in the >10 grain class, less than 7% of cells may be expected to occur by chance in the 0 to 3 grain class. This is clearly different from the observed value of 68% found for the 0 to 3 grain class, and allows us to reject the hypothesis of homogeneity of the resting lymphocyte population with respect to RNA synthesis. Cooper and Rubin (25) reached a similar conclusion with regard to the RNA synthetic ability of lymphocytes prepared from cellulose fiber columns.

The sucrose density gradient sedimentation pattern for newly synthesized RNA in these cells was similar to that reported for HeLa cells during logarithmic growth (26), and at various stages during phased growth (27). The large peak of 45 to 50 S material in these systems has been shown to be a precursor which is subsequently converted to ribosomal RNA (28). This same pattern has been demonstrated in cultured African (Burkitt) lymphoma cells (29), in human lymphocytes stimulated by specific antigens in vitro (16, 30) and in rat lymphoid cells stimulated by antigen in vivo (31).

Stimulation of lymphocytes by PWM thus produces an acceleration of RNA synthesis in which the size-distribution of newly produced RNA molecules resembles that found in other rapidly growing cells (26, 27) and in lymphocytes responding to a specific antigen (16, 30, 31). The large peak of material at 45 to 50 S suggests that a major portion of the RNA synthetic activity is involved in the production of ribosomal RNA. In this, PWM-stimulated lymphocytes differ from lymphocytes treated with PHA, since in the latter case newly synthesized RNA has been found to be predominantly nonribosomal (16, 32).

In this study we have demonstrated that two morphologically distinct transformed cell types are formed after stimulation of lymphocytes by pokeweed mitogen. The PWM type I cell is a large pyroninophilic lymphoblastoid ("blastlike") cell with structural features indistinguishable from those described for PHA stimulation (1–6). Cells with similar morphology have been demonstrated in vivo at sites of antibody synthesis (33–41) and have been reported to produce immunoglobulin in vitro (42–44).

The other cell type we have observed, the PWM type II cell, bears a closer resemblance to the plasma cell series than does the type I cell. In another paper (45) this resemblance is affirmed at the fine structural level. A large body of

experimental work has implicated the plasmacytic series in antibody synthesis (46–54), but the precise roles of the lymphocyte versus the plasma cell in antibody synthesis has been disputed (41). Harris et al. (55) have provided direct evidence that cells of both types can produce antibodies.

Stimulation of lymphocytes with PWM results in the appearance, in vitro of classes of cells resembling both of the cell types now generally associated with antibody production. Whether PWM possesses the capability of transforming cells in both directions with the fate of any particular cell depending on a random event, or whether the type of transformation is predetermined by the prior immunologic history of individual cells cannot be resolved from our current knowledge. The present demonstration that the resting lymphocyte population is heterogeneous with respect to RNA synthetic activity supports the latter possibility. The fact that after PHA stimulation 80 to 90% of resting lymphocytes have the type I appearance, suggests that nearly all lymphocytes possess the capacity for this type of transformation. It may be that the type I transformation is within the overall capability of lymphocytes generally, while the type II transformation is restricted to a segment of the population with appropriate prior history, and may occur only in response to a specialized stimulus.

Alternatively, it is possible that a segment of the lymphocyte population with the potentiality for type II (plasmacytic) transformation is eliminated in PHA cultures and that only the type I cells remain to be scored after 72 hr. In that event, PWM may be acting as a more generalized stimulus for lymphocytic growth.

SUMMARY

A study of the kinetics of RNA and DNA synthesis in PWM-stimulated lymphocytes revealed that RNA synthesis preceded the onset of DNA synthesis by approximately 24 hr and that DNA synthesis and transformation was maximal between 66 to 78 hr. Histochemical and radioautographic studies on PWM stimulated cultures indicated that at 72 hr 50 to 60% of the cell population had been transformed by PWM, and that a distinct cell type bearing cytologic resemblance to the early plasma cell had emerged. The RNA sedimentation profile for newly synthesized RNA in PWM-stimulated cells showed that a large peak of 45 to 50 S material was formed after 24 and 40 hr. PWM thus produces a distinctive transformation of human peripheral blood lymphocytes.

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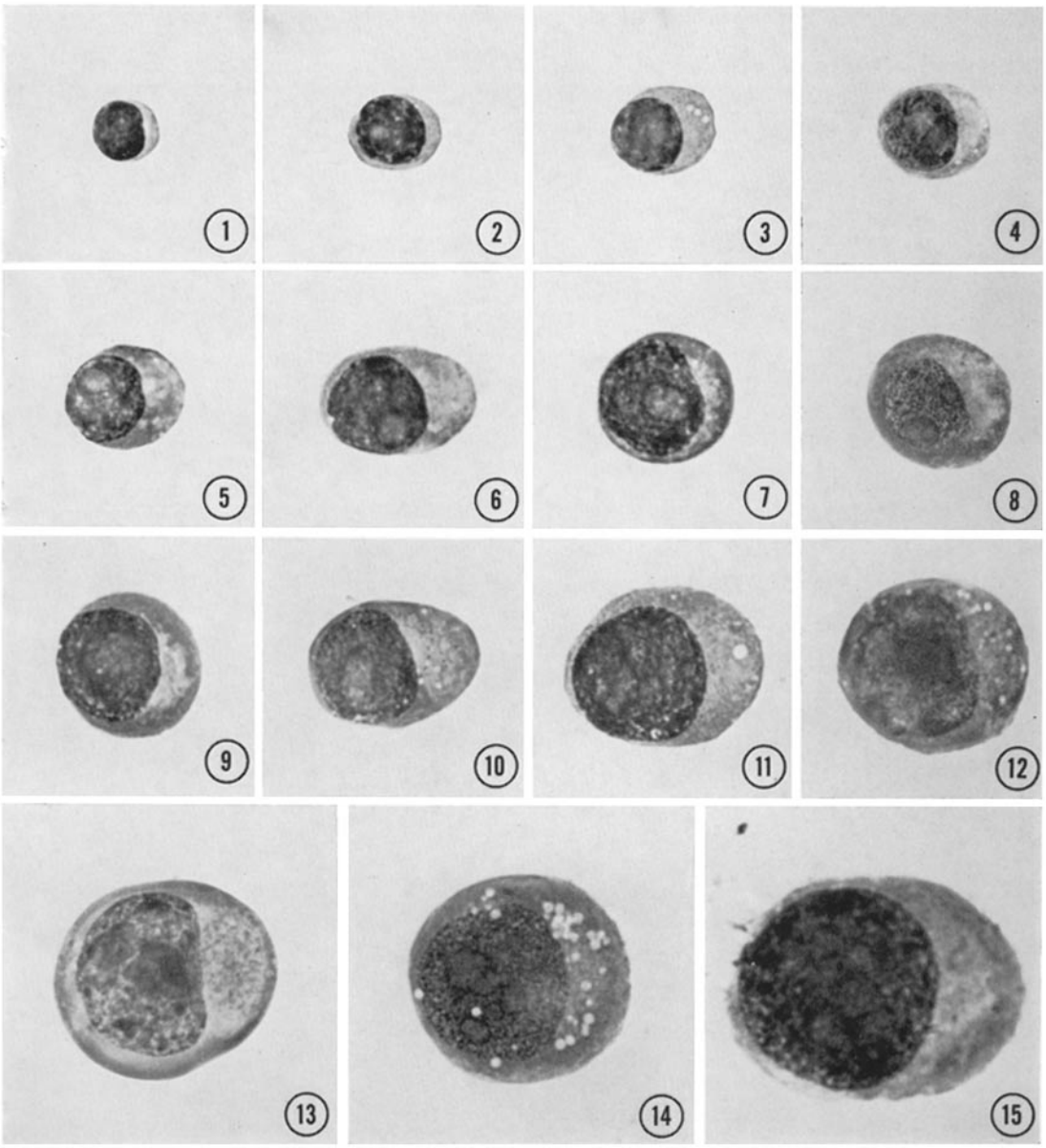
EXPLANATION OF PLATE 87

FIGS. 1 to 15. Composite photomicrograph of PWM-transformed human peripheral blood lymphocytes; time course study (stained with Giemsa). × 1600.

Fig. 1, unincubated small lymphocyte; Figs. 2 to 4, transformed lymphocytes seen after 24 hr, and Figs. 5 to 12, transformed lymphocytes seen between 24 to 72 hr. This group of cells illustrates the morphological spectrum of PWM type II cells, which can be distinguished from the PWM type I cells by their diastase resistant finely granular, PAS-positive reactivity. Giemsa staining alone is inadequate for making this distinction.

Figs. 13 to 15, large blastlike cells seen at 72 hr. Figs. 13 to 15 may be either PWM type I (PAS-negative) or PWM type II (PAS-positive) cells.



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