SEPARATION OF HUMAN LYMPHOID CELLS INTO G_1, S, AND G_2 CELL CYCLE POPULATIONS BY USE OF A VELOCITY SEDIMENTATION TECHNIQUE

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Human lymphoid cell suspension cultures are stable homogeneous cell lines that are readily available for use in examining many lymphocyte functions and characteristics. Such cell lines have been increasingly used for studies of immunoglobulin synthesis (1, 2), HL-A antigens (3–5), surface immunoglobulin (6), viral antigen expression (7), and insulin receptors (8), to name a few. In addition, these lymphoid cell lines have been shown to produce a substance with migration inhibitory factor (MIF)[1]-like activity (9).

The life cycles of these cell cultures have also been analyzed. The duration of the different phases of the cell cycle in human lymphoid cell lines can be determined by established techniques in which labeled DNA precursors with autoradiography are used (10–15). However, relating the varied lymphoid cell functions to specific cell cycle phases has proven more difficult, largely because of difficulties involved in obtaining synchronized cell populations.

Partially synchronized cultures have been obtained using chemical methods based on reversible inhibition of a process that is specific for one phase of the cell cycle, such as DNA synthesis (16–19). Such methods have been used to demonstrate that immunoglobulin synthesis takes place during the late G_1 and S phases in human lymphoid cells (1). Another technique for obtaining partially synchronized lymphoid cells is based on the tendency for cells in high density and with nutrient shortages to dwell in G_1 (1). Other methods of chemical synchrony have recently been reviewed (20). Both chemical and growth limitation methods have the advantage of preparing large numbers of partially synchronized dividing cells. However, the degree of synchrony often is not very great; and the cellular metabolism is modified, producing an unbalanced growth of cells no longer corresponding to that of normal uninhibited cell cultures.

Techniques based on single-cell analysis have been successfully used to correlate immunoglobulin production, cell surface immunoglobulin, and HL-A antigen expression with cell cycle parameters. However, these methods are extremely laborious and permit examination of only relatively small numbers of cells.

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[1] *Abbreviations used in this paper:* BSS, balanced salt solution; FCS, fetal calf serum; MEM, minimal essential medium; MIF, migration inhibitory factor; [^{3}H]TdR, tritiated thymidine.

Another group of methods used for the preparation of synchronous cultures is based on the separation of cells in one specific phase of the life cycle from the remainder of the cell population by physical means. These methods offer the potential advantage of producing a highly synchronized and metabolically unaltered cell population.

Recently the use of gradient techniques for the production of synchronous cell cultures has received more attention (21–28). In this paper we describe a method for obtaining relatively pure populations of G_1, S, and G_2 cells from exponentially growing human lymphoid cell cultures by using a Ficoll gradient velocity sedimentation technique.

Materials and Methods

Cell Line and Culture Techniques.—Continuous suspension cultures in vitro of human diploid lymphoid cells were used. Cell line 8866 was obtained, at Roswell Park Memorial Institute, from the peripheral blood of a patient with acute myelocytic leukemia (29). Cell line IM-1 was obtained from a gingival biopsy of a patient with lymphoblastic lymphoma (29). Cell line IM-9 was obtained from the bone marrow of a patient with multiple myeloma having an IgG3-type kappa paraprotein (1). The general culture techniques, in which Eagle's no. 2 minimal essential medium (MEM) supplemented with penicillin, streptomycin, L-glutamine, and 10–20% heat-inactivated (56°C for 30 min) fetal calf serum (FCS) (Grand Island Biological Co., Grand Island, N. Y.) is used, have been previously described (29).

Cell Cycle Characteristics.—Cell cycle characteristics for the 8866 cell line were determined by means of autoradiographic techniques. The S phase was derived from serial autoradiographic data on exponentially growing cell lines, a double [^{14}C]- and [^{3}H]thymidine ([^{3}H]TdR) labeling technique being employed (12). Direct estimation of the length of G_1 and G_2 was made by a modification of the method of Maekawa and Tsuchiya (14).

Reagents and Buffers.—Ficoll (Sigma Chemical Co., St. Louis, Mo.) was prepared to a starting concentration of 40% wt/wt with glass-distilled water. It was subsequently adjusted to a final concentration of 20% Ficoll wt/wt with an equal volume of Hanks' double-strength (570–580 mosmol) balanced salt solution (BSS) or double-strength Eagle's no. 2 MEM with penicillin, streptomycin, and L-glutamine, which had previously been adjusted to pH 7.2–7.4. This 20% wt/wt Ficoll was diluted 1:4 with isosmolar (285–290 mosmol) Hanks' BSS or Eagle's no. 2 MEM to obtain a 5% wt/wt Ficoll solution. Refractive index and equivalent sucrose concentration were determined with a Bausch and Lomb refractometer (Bausch & Lomb, Inc., Rochester, N. Y.).

Sterile aqueous [^{3}H]thymidine (Methyl-3H) (sp act 1.9–6.0 Ci/μmol) was obtained from Schwarz/Mann, Division of Becton-Dickinson & Co., Orangeburg, N. Y.

Cell Counting, Cell Volume Analysis, and Cell Viability.—An electronic cell counter and pulse-height analyzer (Coulter Model B Counter and Coulter "Channelyzer," Coulter Electronics Inc., Hialeah, Fla.) were used for both cell counting and cell volume determinations. Exclusion of 0.05% trypan blue in isotonic saline was utilized as the criterion for cell viability.

Autoradiography.—TdR-labeled cells were washed, resuspended in Hanks' BSS with 10% fetal calf serum to a final concentration of 2 × 10^5 cells/cm^3, and placed on microscope slides by use of a cytocentrifuge (Model CF-12, Sakura Finetechnical Co., Japan). The slides were dried and then fixed for 10 min in 95% methanol. They were then dipped in Kodak nuclear track emulsion NTB-2 (Eastman Kodak Co., Rochester, N. Y.), dried in a stream of warm air, and stored at 4°C in a refrigerator. After exposure for 5 days, the slides were developed with Kodak DK-19 developer. After this they were stained with Giemsa stain at half strength for 8 min, washed in distilled water, and dried. Cells were then examined for the appearance of developed silver grains over their respective nuclei.

Mitotic Index.—Fixed slides were stained with Giemsa stain 4 min, washed with distilled water, and dried. The cells were then examined under a microscope; and the percentage of cells in prophase, metaphase and early anaphase was determined as a measure of "mitotic index."

Incorporation of TdR.—(*a*) Exponentially growing cells were collected by centrifugation (800–1,000 rpm, 10 min) and resuspended to a final concentration of 1×10^7 cells/cm^3 in 10 cm^3 of Hanks' BSS + 10% FCS solution containing 10 μCi of [^{3}H]TdR. This cell suspension was then incubated with agitation at 37°C for 30 min. These cells, termed "prelabeled cells," were washed twice in BSS and applied to the Ficoll gradient at a concentration of 1×10^7 cells/cm^3 in BSS.

(*b*) To measure thymidine incorporation during synchronous growth or in pooled samples from the gradient, samples of 5×10^4 cells were similarly exposed to 10 μCi of [^{3}H]TdR in a total volume of 10.0 cm^3 of BSS + FCS and incubated at 37°C for 30 min. The extent of the [^{3}H]TdR incorporation into DNA was determined by filtering the sample through 0.45 or 0.65-μm Millipore filters (Millipore Corp., Bedford, Mass.), rinsing with 4 cm^3 of ice-cold, phosphate-buffered, normal saline, washing with 4 cm^3 of 5% ice-cold TCA, and, finally, washing twice with 2-cm^3 vol of 95% ice-cold ethanol. The Millipore filter with precipitate was then transferred to a glass counting vial, and 10 cm^3 of Aquasol (New England Nuclear, Boston, Mass.) were added. Subsequent disintegrations per minute were registered on a Beckman Model LS-255 liquid scintillation system (Beckman Instruments, Inc. Fullerton, Calif.).

Microspectrophotometry of Fuelgen-stained Cells.—Slides were prepared and placed on microscope slides with a cytocentrifuge as described in the section entitled Autogradiography and were stained by the Fuelgen reaction (30). The slides were equilibrated to water and hydrolyzed in 1.0 N HCl at 60°C for 12 min, chilled in cold 1.0 N HCl for 1 min, and stained with Fuelgen reagent for 1 h. These slides were then rinsed for 4 min in running tap and distilled water. 400–500 cells were examined individually for DNA content, using the integrating microdensitometer type GN-2, built by Barr and Stroud Ltd. (London, England).

The 75-W tungsten lamp was used at 9 V. A wavelength of 546 nm was selected, the circular graded spectrum filter having a range of 400–700 nm. A 100 $\times$ oil immersion objective was employed throughout in examination of each cell.

Preparation of Gradient, Conditions of Centrifugation, and Collections of Fractions.—A 5–20% wt/wt Ficoll linear continuous gradient was generated in a Buchler Instruments (Fort Lee, N. J.) density gradient generator, resulting in a total volume of 80 cm^3 contained in a 3 cm (diameter) $\times$ 10.5 cm (length) cylindrical polycarbonate tube (International Equipment Co., Needham Heights, Mass.; no. 2086). On top of this gradient 10 cm^3 of 5% wt/wt Ficoll was layered to modify the initial slope of the gradient. This technique was based on a somewhat similar system of "buffered step" gradient preparation used in velocity sedimentation at unit gravity for separation of sheep blood and mouse spleen cells (31) and in rate zonal centrifugation (32). This manipulation increased the streaming limit so that more cells could be effectively applied to the gradient.

The suspension of cells to be separated was then carefully layered on top of this 5% Ficoll buffer zone. The cell load varied between 3 and 5×10^7 cells, and was suspended in Hanks' BSS to a final concentration of 1×10^7 cells/cm^3. A typically prepared and loaded "modified" gradient is illustrated in Fig. 1 *b* and is contrasted to an unmodified gradient in Fig. 1 *a*.

The centrifugation was subsequently carried out at 4°C in a PR-2 centrifuge (International Equipment Co.), using a swing-out rotor at 80 g, average, for 20–25 min. Fractions were then collected by carefully placing a 2 mm (diameter) $\times$ 20 cm (length) stainless steel tube through the center of the gradient to the bottom and sampling via polyethylene tubing in a Buchler Instruments polystactic pump. Alternatively, a sampling apparatus of the type used by Boone et al. (33) was employed in collecting fractions.

The various fractions and/or pools of fractions were then analyzed for cell number, mean

cell volume, [^{3}H] TdR incorporation, and, by autoradiography and Fuelgen staining, for DNA content of cells.

Collection and Characterization of Synchronously Growing Cells.—All procedures were carried out aseptically at room temperature to permit subsequent culturing of cells. Pooled fractions from the upper, middle, and lower portions of the gradient were washed twice in supplemented Eagle's no. 2 MEM. These pools of cells were then suspended to a final concentration of 1–2 × 10^5 cells/cm^3 in growth media and transferred to three separate spinner flasks. The cell suspensions were incubated at 37°C. Samples were removed at frequent intervals and analyzed for cell number, [^{3}H]TdR incorporation, and mitotic index, and were subjected to autoradiography.

RESULTS

Cell Cycle Characteristics.—The cell cycle characteristics for the cell line 8866 are presented in Table I. The doubling time for 8866 cells is approximately 17.5–20.5 h. The lengths of G$_1$ and G$_2$ were found to be approximately 8–9 and 3–4 h, respectively. The duration of the S phase was 6–7 h.

Effect of Type of Gradient on Streaming Limit.—One of the most important parameters in determining the success of the cell separation is the concentration of cells in the starting band. A phenomenon called "streaming," which results in poor resolution of cell separation, occurs above a critical cell concentration called the "streaming limit" (31, 32, 34). The exact value of this limit depends on both the type of cells in the cell band and the shape of the gradient.
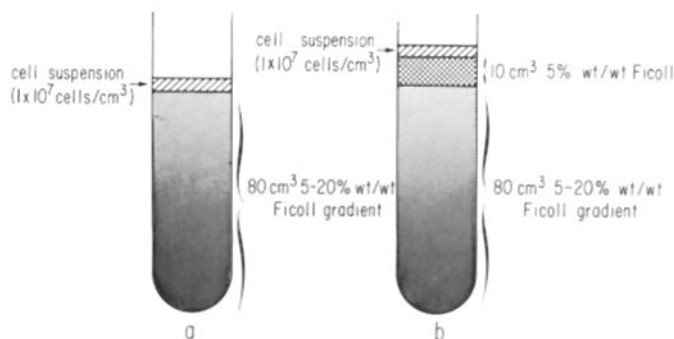

FIG. 1. Unmodified linear (a) and modified linear (b) Ficoll gradients.

TABLE I

Cell Cycle Characteristics of the Human Lymphoid Cell Line 8866

Cell cycle phase	Mean duration
	h
G$_1$	8–9
S	6–7
G$_2$	3–4
M	0.5
Cell doubling time	17.5–20.5

This effect is also apparent in our system, and by "modifying" the initial slope of the gradient we can apply 7 times more cells on the gradient without appreciable admixture of the different cell subpopulations. Fig. 2 shows an approximation of the streaming limit for two different gradient slopes. The
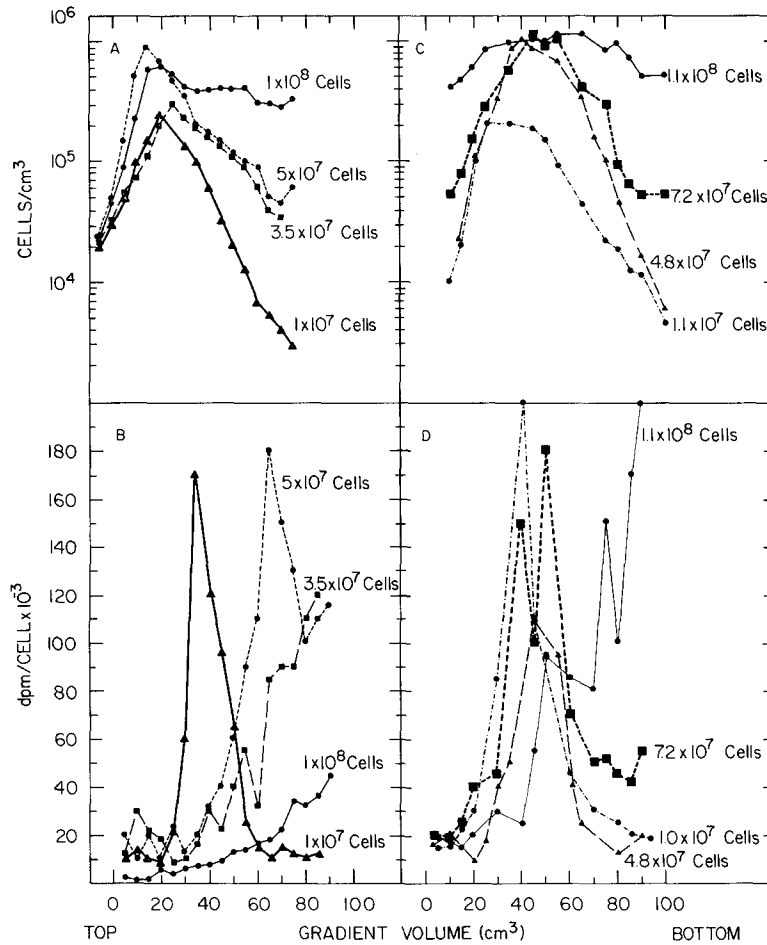

Fig. 2. Cell separation characteristics of 8866 cells as a function of cell load using modified and unmodified linear Ficoll gradients. (A) Cell concentration on an unmodified gradient. (B) Distribution of [^{3}H]TdR-labeled cells on an unmodified gradient. (C) Cell concentration on a modified gradient. (D) Distribution of [^{3}H]TdR-labeled cells on a modified gradient.

continuous "unmodified" linear 5–20% wt/wt Ficoll gradient is depicted in Fig. 1 a. Its separation characteristics of cell concentration and [^{3}H]TdR incorporation are presented in Fig. 2 A, B. The "modified" type of gradient is depicted in Fig. 1 b, and its separation characteristics are shown in Fig. 2 C, D.

The approximate streaming limits for the modified and unmodified gradients are, respectively, 7 × 10^7 cells vs. 1 × 10^7 cells. Thus, the modified linear

gradient, compared with the unmodified linear gradient, appears to give a 7-fold increase in streaming limit.

Distribution of Differences in Cell Volumes, [³H]TdR Incorporation, and Cell Concentration in the Modified Gradient.—Prelabeled exponentially growing cells were separated by velocity sedimentation, as described; and the fractions were analyzed for cell volume, cell concentration, percent Ficoll, and TCA-precipitable [³H]TdR incorporation per cell. Representative gradient separations using IM-1, IM-9, and 8866 cells are shown graphically in Figs. 3 and 4 A, B.

There is a steady increase in mean cell volume (approximately 170–560 μm^3) with respect to increasing percent Ficoll within the gradient (Fig. 3). Most of the cells have a relatively low volume (range of approximately 170–350 μm^3), and are found in the upper regions of the gradient.

In addition, cells synthesizing DNA, as judged by the incorporation of [³H]TdR, are found in the middle of the gradient. Maximum radioactivity in the middle of the gradient was 124 dpm $\times$ 10^{-3}/cell, compared with minimum values of 10 dpm $\times$ 10^{-3}/cell for the upper and lower portions of gradient.

These experiments demonstrated a good separation of cells with differing volumes. Strong evidence was obtained that cells in the S phase of the cell cycle were clearly in the middle of the gradient, and presumably G_1-phase cells were on top and G_2-phase cells were on the bottom of the gradient.

This separation of cells within the gradient has been seen in more than 10 experiments, and gives a characteristic distribution of cell volume, [³H]TdR incorporation, and cell concentration, as shown in Figs. 3 and 4.

Evidence That Gradient Separation of Cells Correlates with Differences in Cell Cycle Parameters.

(a) *Autoradiography of cells from pooled fractions:* Fig. 5 presents data from three experiments on TCA-precipitable [³H]TdR incorporation and on autoradiographs of three separate pools of cell fractions taken from the gradient. These pooled cell fractions are taken from the upper, middle, and lower portions of the gradient, as shown in Fig. 3, and presumably correspond to the G_1, S, and G_2 phases of the cell cycle. The results support the evidence for separation of cells into their respective cell cycle phases.

Fig. 5 shows that the percent contamination of G_1 and G_2 phases with S-phase cells was relatively small. Autoradiographs reveal that at the time of pooling, only 6–11% of the G_1 pool of cells were synthesizing DNA, thus representing 6–11% contamination of the G_1 pool with S-phase cells. Also, approximately 5–10% of G_2 pool cells were contaminated with S-phase cells; and in the S pools from three experiments, the labeling index ranged from 66 to 80%. Comparative data showing TCA-precipitable [³H]TdR incorporation into these three pools are illustrated in Fig. 5 and support the autoradiographic evidence for relatively little S-cell contamination of the G_1 and G_2 pools.
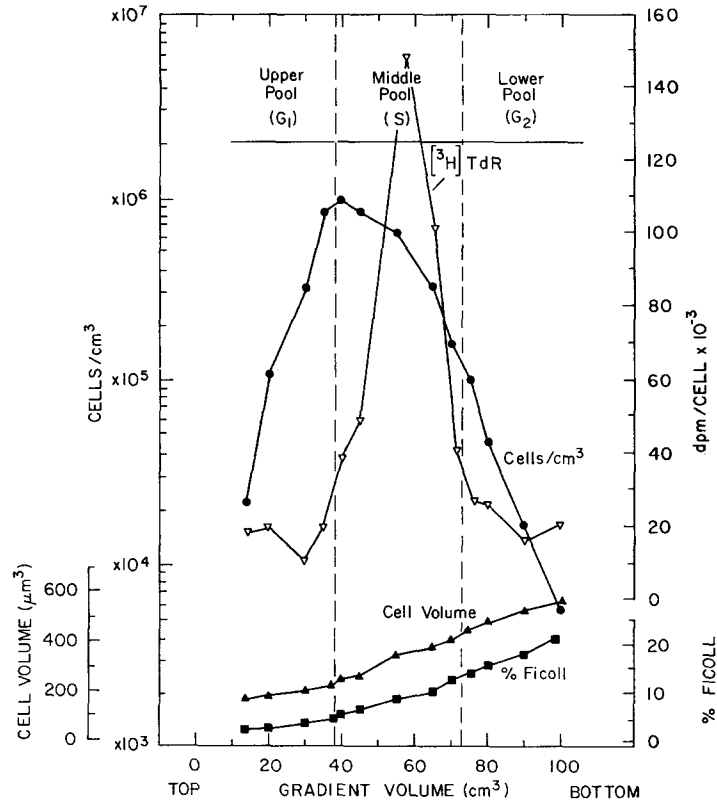
Fig. 3. Modified linear Ficoll gradient separation of 5×10^7 8866 cells. ●, cells/cm^3; ▽, [^{3}H]TdR dpm/cell $\times$ 10^{-3}; ▲, cell volume, μm^3; ■, percent Ficoll.

(b) *DNA content of cells in pooled fractions:* The three histograms in Fig. 6 represent the relative amounts of Fuelgen-stained DNA per nucleus, as determined by a microspectrophotometric method. The first peak represents cells with a DNA content corresponding to 2 N chromosomes, and is found almost exclusively in the upper pooled fractions of the gradient (G$_1$ pool of cells). The 4 N chromosomal population of cells with twice the amount of DNA is found almost exclusively in the lower pool of cells (G$_2$ pool). The transition values for amounts of DNA occur between the 2 N and 4 N peaks and mostly are present m the S-phase pool of cells.

It may also be seen from Fig. 6 that there are probably a few polyploid cells in the G$_2$ pool. Also, random clumps of cells resulting from streaming effects may be found in this lower region of the gradient. This is evidenced by the slightly increased [^{3}H]TdR uptake in cells found in the bottom-most fractions (Figs. 3, 4).
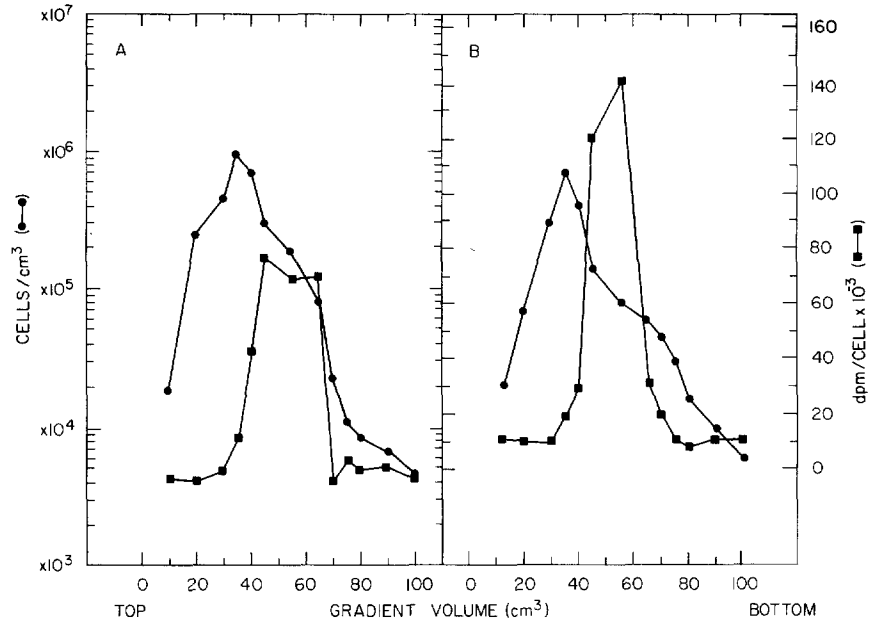
Fig. 4. (A) Modified linear Ficoll gradient separation of 5×10^7 IM-9 cells. (B) 5×10^7 IM-1 cells. ●, cells/cm^3; ■, [^{3}H]TdR dpm/cell $\times 10^{-3}$.

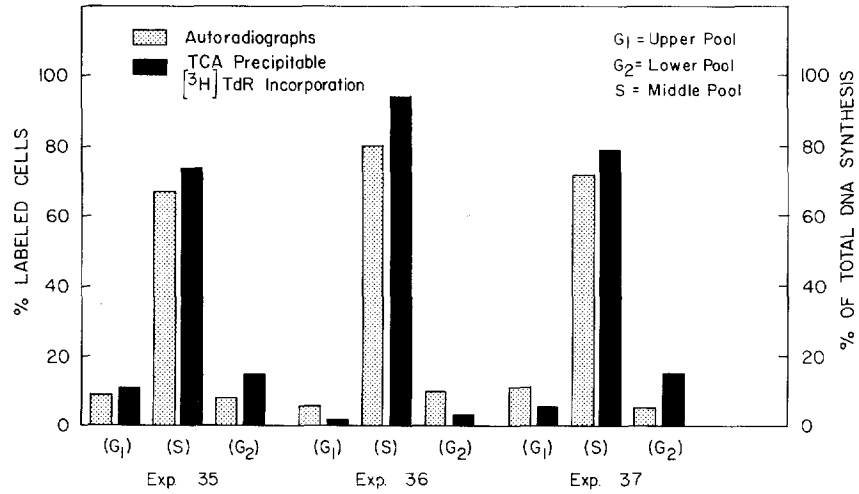

Fig. 5. Distribution of S-phase cells in pooled fractions from three experiments as measured by autoradiography (shaded area) and TCA precipitable [^{3}H]TdR incorporation (black area).
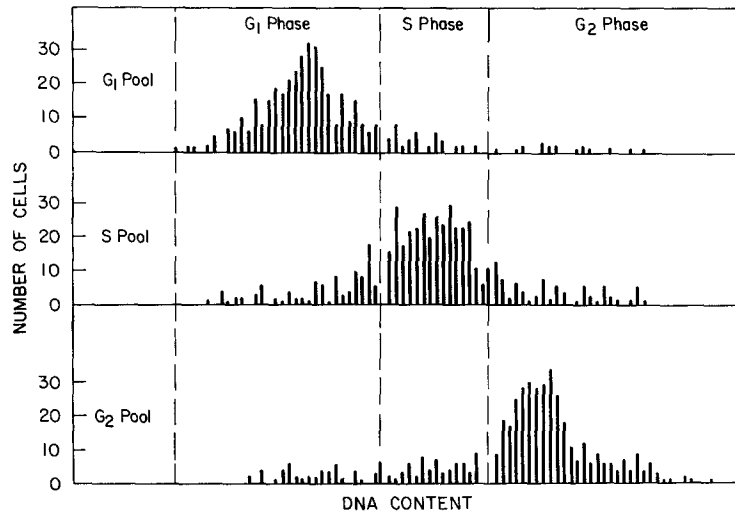
FIG. 6. DNA content of individual 8866 cells in pooled fractions from a modified linear gradient as measured by microspectrophotometry of Fuelgen-stained cells.

(c) *Growth characteristics of cells from the gradient:* 8866 cells separated by this method, when explanted as pooled fractions into growth media, grow synchronously (Fig. 7). Cells taken from the upper region of the gradient (G_1 pool) have the growth characteristics of G_1-phase cells, with an approximate lag period of 11–12 h before cell division, whereas cells from the lower region (G_2 pool) behave in a manner expected of G_2-phase cells, with approximately a 1-h lag period before cell division. Cells taken from the middle part of the gradient (S pool) are intermediate in growth characteristics between G_1 and G_2 and behave as S cells.

In the G_1 pool, the proportion of cells synthesizing DNA increases to reach a maximum at 7 h and then decreases to a minimum at a time when mitotic activity is at a maximum. The expected patterns are also seen for the S and G_2 pools.

As shown in Fig. 7, measurement of the mitotic index also confirms the state of synchrony: it is maximum during the period of cell growth when thymidine incorporation is at a minimum. These rhythmic changes in the proportion of DNA-synthesizing cells, which are the inverse of those of the mitotic index, correspond well to the cell cycle characteristics of the 8866 cell line, as illustrated in Table I.

Taken together, these three methods, autoradiography and determination of DNA content of individual cells and growth patterns of the explanted cell pools, provide conclusive evidence that exponentially growing cells, separated accord-
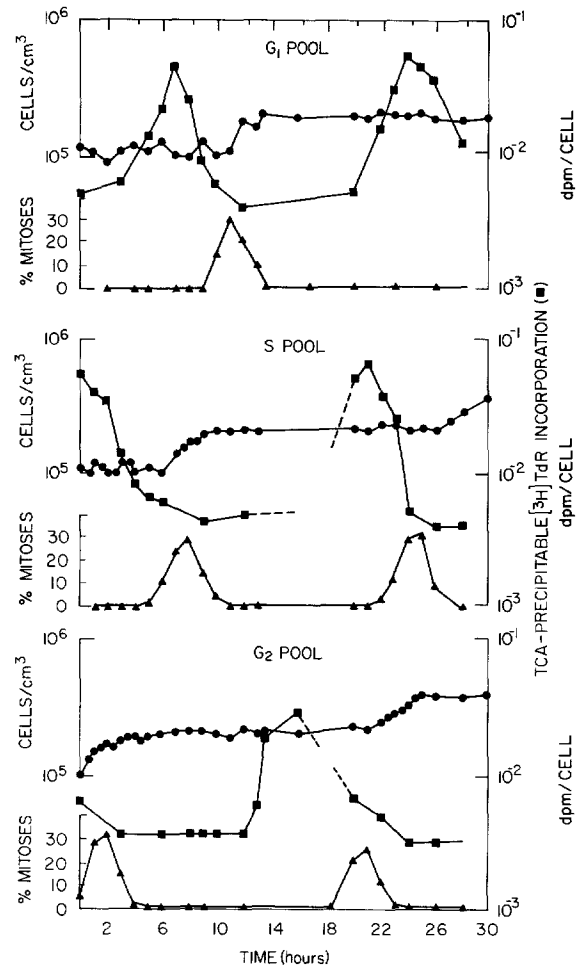
Fig. 7. Growth characteristics of explanted gradient separated pools of 8866 cells. ●
cells/cm³; ■, [³H]TdR dpm/cell; ▲, mitotic index.

ing to volume by velocity sedimentation, are indeed separated into discrete cell
cycle phases.

*Estimation of Degree of Cell Cycle Admixture in the Different Pooled Frac-
tions.*—Within its limitations, the Fuelgen microspectrophotometric technique
for estimation of DNA content per cell nucleus is the only method that has the
advantage of yielding percent contamination of one cell cycle phase by the other
two phases. It is difficult, however, to separate the extent of error due to the
histochemical technique itself from the extent of admixture of the various cell
cycle populations.

The boundaries for these various pools are drawn in Fig. 6 and include

two-thirds of all the cells from each pool. This manipulation is admittedly somewhat arbitrary, but is probably indicative of the maximum degree of contamination with these respective cell cycles.

These data do show that the G_1, S, and G_2 pools of cells contain relatively pure populations representing the cell cycle phases. Table II presents a comparison of data from experiments using the different methods described to

TABLE II

Comparison of Data from Three Methods Showing Degree of Cell Cycle Admixture in Pooled Fractions

Pool	Exp. no.	[³H]TdR	% total DNA synthesis	Pool	Exp. no.	Cells labeled	Total cells counted	Cells labeled
		dpm/cell						%
A. TCA-precipitable [³H]TdR Incorporation				B. Autoradiographs				
G_1	35	0.013	11.5	G_1	35	22	252	9.6
G_1	36	0.001	0.8	G_1	36	31	508	6.1
G_1	37	0.007	5.3	G_1	37	33	300	11.0
S	35	0.082	72.6	S	35	267	400	66.7
S	36	0.122	96.8	S	36	220	275	80.0
S	37	0.060	78.2	S	37	252	350	72.0
G_2	35	0.018	15.9	G_2	35	25	300	8.3
G_2	36	0.003	2.4	G_2	36	30	300	10.0
G_2	37	0.015	16.5	G_2	37	12	250	5.0

Pool	Exp. no.	G_1 cells	G_1	S cells	S	G_2 cells	G_2	Total cells
			%		%		%	
C. Microscpectrophotometry of Fuelgen-stained Cells								
G_1	35	354	84.4	65	14.9	16	3.7	435
	36	365	84.1	51	11.8	18	4.2	434
S	35	69	20.7	184	55.4	79	23.8	332
	36	98	17.1	328	67.2	75	15.6	489
G_2	35	26	5.1	75	14.8	306	80.1	507
	36	28	5.8	72	15.0	380	79.2	480

estimate the percent contamination of pooled fractions with their respective "excluded" pools.

The amounts of admixture in these pools as delineated from these various methods is quite small. For example, in experiment no. 36, the G_2 pool of cells contains only 5.8% G_1-phase cells and 2.4–15% S-phase cells; the G_1 pool of cells contains only 0.8–11.8% S-phase cells and 4.2% G_2-phase cells; and the S pool of cells contains 17.1% G_1-phase cells and 15.6% G_2-phase cells.

Cell Yields from the Gradient.—The amounts of recoverable cells in these various pools, their percent viability as measured by trypan blue exclusion, and the percent yield of cells in these pools relative to the total amount of cells

originally applied to the gradient are summarized from four separate experiments and shown in Table III. The total percent yield of cells in these experiments varies from 60 to 81.8%. This 20–40% loss of cells is probably due to the adherence of cells to the sides of the centrifuge tubes and subsequent multiple washings during the preparation of pooled fractions. However, the viability of these cells ranged from 92 to 98% and was essentially identical with the viability of cells before gradient application.

There is a paucity of cells available for recovery in the lower portion of the gradient. These cells have characteristics of the G_2 phase. The low numbers of separated cells probably reflect the small G_2 population of cells present in the original exponentially growing cell load. However, although the cell number is small, this group of cells does represent a fairly pure population of the G_2 cell cycle phase.

TABLE III

Cell Yield and Percent Cell Recovery in G_1, S, G_2 Pools from Four Different Experiments

Exp. no.	Total cells applied	Viability	Total cells recovered in G_1 pool	Viability	Total cells recovered in S pool	Viability	Total cells recovered in G_2 pool	Viability	Total cells recovered	Total yield
		%		%		%		%		%
41	5.1×10^7	98	4.1×10^6	98	2.6×10^7	95	3.9×10^6	97	3.4×10^7	65.2
42	5.0×10^7	94	5.7×10^6	94	3.1×10^7	93	4.3×10^6	92	4.1×10^7	81.8
45	5.1×10^7	98	5.1×10^6	98	2.9×10^7	96	3.9×10^6	97	3.8×10^7	72.3
46	5.4×10^7	99	2.6×10^6	98	2.7×10^7	97	2.4×10^6	98	3.2×10^7	60.0

DISCUSSION

The results clearly show that velocity sedimentation centrifugation is a useful tool for separating and analyzing human lymphoid cell lines by cell cycle phases. The method is based on the separation of cells according to differences in volume by a 5–20% Ficoll gradient. It is known that the volume of a cell appears almost to double as it moves through the G_1 to M phases of the cycle. If one assumes that the density of the cell remains constant, the sedimentation velocity would increase $2^{2}/_{3} = 1.49$ (31) according to the formula:

$$S = \frac{2}{9} \frac{(p - p^1)gr^2}{\eta} ,$$

where S is the sedimentation velocity of spherical cells falling through a fluid under the influence of gravity; η is the coefficient of viscosity; p and p^1 are densities of the cell and fluid medium, respectively; g is acceleration due to gravity; and r is the radius of the cell. This effect is modified by the increasing density and viscosity of the gradient so that in a velocity sedimentation technique, cells are clearly separated on the basis of size.

The smallest cells represent, for the most part, G_1-phase cells, and are found toward the top of the gradient. Presumably, those at the very top are in the

early G_1 phase, the later stages of G_1 cells being found farther down the gradient. S-phase cells are found in the middle of the gradient, and G_2-phase cells are found toward the bottom of the gradient. These findings correspond to those in experiments of other investigators using murine peritoneal mast and lymphoma cells (22, 25). Pooled fractions from upper, middle, and lower portions of the gradient are, respectively, 84–99% rich in G_1 cells, 55–96% rich in S cells, and 79–97% rich in G_2 cells, as compositely measured by three different techniques.

In these experiments, it should be noted, the cell pools were obtained by combining fractions that were sampled adjacently without excluding the intervening fractions between pools. This necessarily maximizes the chances for contamination with other cell cycle phases. Purer populations of cells can be obtained by narrowing the number of fractions collected in each pool. This, of course, can be done only with subsequent sacrifices in the cell yield in each pool, but would definitely increase the relative purity of the G_1- and S-phase populations, perhaps approaching $>90\%$. It is doubtful whether increased homogeneity of the G_2 pool could be obtained, since the presence of random cell clumps and polyploid cells (larger volumes) certainly contributes to the decreased purity of this cell population.

This procedure should be particularly valuable for separating human lymphoid cells for biochemical study. Cellular viability is not adversely affected by this procedure; and, presumably, no modification of cellular metabolic events occurs as no chemical manipulation is used. This is a distinct advantage over chemical methods of synchronization, which result in unbalanced cell populations.

Also, synchronization is achieved in all phases of the cell cycle at the same time. This avoids the obvious disadvantage of desynchronization of recultured, chemically synchronized cells.

A drawback of using gradient centrifugation for separation of cells is the resultant low yield of synchronously dividing cells. This is true even though the cell load can be increased 7-fold, with modification of the initial linear slope of the gradient. Although the pools of cells reported in these experiments are relatively free of contamination from other cell cycle phases, the final recovery of cells is only 60–81.8% of the starting cell load applied to the gradient. However, because cell suspensions may be cultured on a large scale, synchronous pools of cells may be obtained by running a number of gradient separations simultaneously. In addition, the use of large zonal centrifugation rotors may make possible an increase in the cell load and the production of even larger numbers of synchronous cell populations, and with possibly better resolution between S- and G_2-phase cells (24).

Finally, the method described in this report is both simple and versatile. It appears to have much promise for further investigation of metabolic changes and cell surface changes relative to the life cycle of lymphoid cells. Such studies are presently under way in our laboratory.

SUMMARY

Human lymphoid tissue culture cells can be separated according to cell size and corresponding cell cycle phase with a velocity sedimentation centrifugation method employing a continuous 5–20% wt/wt Ficoll gradient. A 7-fold increase in streaming limit was achieved by placing a buffer zone of isosmolar 5% Ficoll on top of the gradient before application of the cell load.

The various pooled populations of cells from upper, middle, and lower areas of the gradient were characterized using autoradiographic, TCA-precipitable ^{3}H]thymidine incorporation, and Fuelgen microspectrophotometric methods. The upper range of the gradient contains cells in the G_1 cell cycle phase; the lower range, cells in the G_2 phase; cells found in the middle of the gradient belong largely to the S phase of the cell cycle.

These gradient-separated cell pools contained relatively little contamination with cells from other phases of the cell cycle and, when explanted from the gradient into fresh growth media, showed growth patterns characteristic of synchronized cell populations. This system of cell separation provides a useful tool for investigating the relationship of the cell cycle to surface membrane and metabolic characteristics in human lymphoid cell culture systems.

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