MOUSE SPLEEN LYMPHOBLASTS GENERATED IN VITRO

Recovery in High Yield and Purity After Floatation in Dense Bovine Plasma Albumin Solutions*

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Ever since the work of Gowans (1), it has been evident that the transformation of a small lymphocyte into a proliferating lymphoblast is a critical feature of many immune responses *in situ*. Subsequently, it has been found that blast transformation occurs in vitro in response to antigens (2, 3), and to "nonspecific" stimulants or mitogens (4; reviewed in 5). However, there is still very little direct information on the physical, surface, and functional properties of lymphoblasts.

There would appear to be two problems in the direct study of lymphoblasts. One is that they have not been isolated in high yield and purity. In the past, velocity sedimentation techniques have been used to obtain enriched populations, particularly those arising in transplantation reactions (6–8). The present technology for velocity sedimentation (9) is relatively difficult and time consuming, and the gradients have a low capacity in terms of the numbers of cells that can be separated. Velocity sedimentation protocols assume that all blast-transformed cells are large. However, the yield and purity of lymphoblasts separated by these methods have not been fully documented, nor has the functional capacity of the individual cells vs. cell fractions been fully analyzed.

A second problem is that transformation of small to large lymphocytes is not a synchronous event, at least in vitro, where it is clear that cells can blast transform over a several day period (10–12). To our knowledge, there is no method available for isolating cells in cell cycle from resting cells, at any given time.

In this paper, we use an isopycnic or equilibrium density technique to isolate proliferating mouse spleen lymphocytes that have been stimulated to divide with several agents in vitro. These include lipopolysaccharide (LPS;[1] 13) and fetal calf serum (FCS; 14, 15; reviewed in 16) which are primarily B-cell stimulants, and concanavalin A (con A; 13, 17) and mixed leukocyte culture (MLC; 18) which are primarily T-cell stimulants. In all cases, cells proliferating in these cultures at early time-points (1–2 days) can be recovered in high yield and purity simply by floatation on bovine plasma albumin (BPA) columns of a suitable density. Under appropriate conditions, cells entering their first prolif-

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[1] *Abbreviations used in this paper:* BPA, bovine plasma albumin; FCS, fetal calf serum; FMF, flow microfluorometry; LPS, lipopolysaccharide; MLC, mixed lymphocyte culture; sCon A, succinylated concanavalin A.

erative cell cycle can be obtained in bulk. The cytologic features, size distribution, and distribution within the cell cycle of these lymphoblasts are reported. Finally, we show that the floatation procedure does not alter the subsequent behavior of the separated spleen cell populations when they are returned to tissue culture.

Materials and Methods

Mice. DBA/2J (The Jackson Laboratory, Bar Harbor, Me.) mice, 3–10 mo old, were used in all experiments recorded here. However, inbred F_1 mic (DBA/2 × C57B1) and outbred NCS mice of The Rockefeller University colony behaved similarly.

Bovine Plasma Albumin (BPA) Solutions. Dense BPA solutions, with a density of 1.082 (determined from a standard curve relating refractive index to density), and approximate concentration of 27–28% wt/vol, were prepared as previously described (19). Generally, we sprinkled 100 g of BPA powder (fraction V, Armour Pharmaceutical Company, Chicago, Ill.) onto 280 ml of solvent comprised of 186 ml phosphate-buffered saline without calcium and magnesium salts, 29 ml of 1.0 N NaOH, and 65 ml of 2 × glass distilled water. The powder dissolved overnight, the pH was checked (7.2–7.4), and the refractive index adjusted when necessary. The solution was filtered through successive prefilters (AP 2504700; Millipore Corp., Bedford, Mass.) and 0.45-μm millipores in a Nalgene Filter Unit (Nalge Co., Nalgene Labware Div., Rochester, N. Y.) and stored at 4°C. We have only used albumin from this one commercial source, although we have used dozens of different lots over a several year period. We have not found it necessary to dialyze out any salts present in the BPA, and we have not manipulated the pH, osmolarity, or divalent cation composition of the dense BPA.

Spleen Cells. Mouse spleens were teased with a fine forceps in cold RPMI-1640 medium (Grand Island Biological Co., Grand Island, N. Y.) and transferred to a stainless steel sieve for further disruption with the bottom of a sterile tube. Cell clumps were allowed to settle, and the single cell suspension then washed twice by centrifugation (200 *g*, 10 min) to remove cell debris. 12–15 × 10^7 cells were then cultured in 5% CO_2-air in 100-mm Petri dishes (Falcon Plastics, Oxnard, Calif.) in 12–15 ml of RPMI-1640 medium (Grand Island Biological) containing penicillin, 5 × 10^{-5} M 2-mercaptoethanol (Matheson, Coleman & Bell, East Rutherford, N. J.) and various stimulants for 24–48 h.

Stimulation of Blastogenesis. Four stimulants were used: lipopolysaccharide 100 μg/ml (LPS-W *Escherichia coli* 055:B5, Difco Laboratories, Detroit, Mich.); fetal calf serum (FCS) 5% vol/vol (Flow Laboratories, Inc., Rockville, Md.; Grand Island Biological; and Microbiological Associates, Bethesda, Md.); concanavalin A (Con A) 3 μg/ml (crystallized three times, Miles Yeda, Rehovot, Israel), or its succinylated derivative (sCon A) at the same concentration, prepared according to Gunther et al. (20); and mixed leukocyte culture (MLC) generated by co-cultivating 15 × 10^7 DBA/2 × C57B1 F_1 cells and 15 × 10^7 DBA/2 parent cells. Because FCS is itself mitogenic (14–16), we omitted serum supplementation for LPS cultures and used 1–2.5% heat-inactivated (56°C; 1/2 h) isologous mouse serum, obtained by cardiac puncture, for con A and MLC cultures.

Isolation of Low Density Cells from Stimulated Spleen Cultures. After 20–48 h in vitro, the cultures were harvested with a Pasteur pipette leaving behind only a few adherent macrophages. The cells were spun at 200 *g* for 10 min, and cultured for 1 h in a fresh Petri dish. During this hour, additional typical macrophages were adhered to the dish, and where necessary the cells could be radiolabeled (see below). The cells were reharvested with a Pasteur pipette, spun at 200 *g* for 10 min, decanted, and resuspended by pipetting in 5 ml of dense BPA, final density with cells of 1.080. The cells in BPA were transferred to small centrifuge tubes, usually 10-ml cellulose nitrate tubes (no. 302235; Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) overlain with 1.5 ml of dilute BPA, ρ 1.060, and spun to equilibrium in a swinging bucket head at 10,000 *g* av for 20 min (both a Lourdes LRA centrifuge with an SCR rotor and a Sorvall RC 2-B centrifuge with an HB-4 rotor were used). 5–50 × 10^7 cells can be separated in this volume of BPA with identical results. The floating or low density cells were harvested with a curved Pasteur pipette, the BSA column decanted, and 5 ml of RPMI-1640 added to resuspend the cell pellet or high density cells. Cell recovery was excellent (80–100%) in dozens of experiments. More than 90% of the floating cells rebanded if the cells were respun on BPA, ρ = 1.080, whereas only

30–50% floated on BPA, $\rho = 1.076$. When further culture was necessary, cells harvested from the columns were washed twice by centrifugation to remove the BPA and were then cultured at 1–3 $\times 10^6$ viable cells/ml medium in 16-mm diameter wells (tissue culture dish FB-24 TC, Linbro Chemical Co., New Haven, Conn.).

This density cut procedure was initially devised for the enrichment of dendritic cells, a new, nonproliferating cell type, harvested directly from spleen (21). Subsequently, it was noted that lymphoblasts generated in vitro also float. Dendritic cells were present in the low density populations from stimulated spleen, but they were a minor component (about 5% or less of the total).

Cell Smears and Light Microscope Autoradiography. Cell smears were prepared by centrifuging 2–5 $\times 10^5$ cells at 60 *g* for 10 min onto 12- (no. M-7210; SGA Scientific, Inc., Bloomfield, N. J.) or 13-mm (Gold Seal 3550, Clay Adams, Div. Becton, Dickinson & Co., Parsippany, N. J.) circular coverslips placed on the bottom of small 13 × 45-mm glass cylinders (Rochester Scientific Co., Rochester, N. Y.). This centrifugation technique, taught to us by Doctors M. Fedorko and J. Hirsch, The Rockefeller University, produced a uniform distribution of cells in which 80% of the expected number of cells were recovered per unit area. The coverslips were removed with a fine forceps, drained, quickly (30 s) air dried with a hair dryer, and fixed in absolute methanol for at least 5 min. For autoradiography, the smears were dipped in Ilford L4 emulsion (Ilford Ltd., Ilford, Essex, Eng.) at 30°C, or NTB-3 emulsion (Eastman Kodak, Co., Rochester, N. Y.) at 43°C, and exposed 1–7 days before development. Giemsa staining was performed at a 1:50 dilution of a stock solution (Fisher Scientific Co., Pittsburgh, Pa.) in 0.02 M McIlwaine's phosphate citrate buffer pH 5.75 for 2 min.

Electron Microscopy. Cells were suspended in balanced salts or medium, and fixed with an equal volume of 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, for 30 min at room temperature. At this stage, we could visualize endogenous peroxidase activity according to Graham and Karnovsky (22) using a 10-min exposure to freshly prepared 50 mg/100 ml (wt/vol) diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, Mo.) in 0.1 M tris buffer, pH 7.6 with 0.01% (vol/vol) hydrogen peroxide. This technique visualizes the reactivity of myeloperoxidase-containing granules in myeloid cells, and cytoplasmic hemoglobin in erythroid cells, thus distinguishing these cell types from lymphoblasts. Postfixation was in 1% (wt/vol) osmium tetroxide in 0.1 M cacodylate buffer, pH 7.4, for 1 h on ice, followed by en bloc staining with 0.5% uranyl acetate in saline, pH 5.0. The cells were pelleted in agar, dehydrated in alcohols, and embedded in Epon.

Cell Sizing. Cell populations were sized by low angle, laser light scattering in a cytograph machine (model 6300 A, Bio/Physics Systems, Inc., Mahopac, N. Y.). The size distributions of low and high density cells were reported in terms of the channel number on the distribution analyzer (no. 2102, Bio/Physics), instead of absolute cell sizes. The cytograph was set at high sensitivity and the analyzer at a conversion of 512.

Flow Microfluorometry (FMF). Quantitative measurements of relative DNA content of individual cells were made after staining of cell nuclei with Krishan's propidium iodide method (23) according to Fried et al. (24). Laser flow microfluorometry was performed in a cytofluorograf (model 4802, Bio/Physics) using an argon ion laser at 488 nm. Data were stored on a Northern model NS-602 512-channel, pulse height analyzer (Tracor Northern, Middleton, Wis.), recorded on paper tape on a printer, model ASR 33, and analyzed on an IBM 1800 digital computer using the method of Fried et al. (25–27).

[^{3}H]Thymidine Radiolabeling. Cells were radiolabeled with [^{3}H]thymidine (Schwarz/Mann Div. Becton, Dickinson & Co., Orangeburg, N. Y.; spec act 6.0 Ci/mM) in three different situations. To follow the distribution of S phase cells during floatation in BPA, the cells were radiolabeled for 1 h at 0.3–1.0 μCi/ml either before or after floatation. To determine what proportion of cells were capable of synthesizing DNA during an 18- to 20-h interval in vitro, we continuously labeled cultures at a final concentration of 0.05 μCi/ml. To follow proliferative activity of cells maintained in tissue culture for several days, we administered 0.05 ml of an 8.0 μCi/ml [^{3}H]thymidine solution to 0.15 ml of washed cells (15% of the culture volume) for 2 h after varying periods of culture. In all instances, [^{3}H]thymidine incorporation was assessed either by autoradiography or by liquid scintillation counting. For the latter, cells were aspirated with a multisample harvester onto glass fiber filters (no. 5601, Whatman, Inc., Clifton, N. J.), washed in water, and counted in Aquasol-2 scintillation fluid (New England Nuclear, Boston, Mass.) at

40% efficiency in a Nuclear-Chicago Mark II liquid scintillation counter (Nuclear-Chicago, Corp., Des Plaines, Ill.). This technique gave identical results to the traditional approach of precipitation and washing in 5% cold trichloroacetic acid.

Results

Floatation of Proliferating Spleen Cells: Enrichment and Yield Assessed by [^{3}H]Thymidine Uptake. Spleen cells were cultured for 1–2 days in vitro in the presence of four different kinds of blastogenic agents: LPS, FCS, con A (both tetravalent and the divalent succinylated derivative, sCon A), and MLC. At any time-point after stimulation, the cells were harvested, radiolabeled, suspended in dense BPA, and spun to equilibrium. Generally, 40–50% of the initial viable cells were recovered after 1–2 days of culture.

Some 5–35% of the viable cells floated after stimulation in vitro, the percentage varying with the type and duration of stimulation (Table I). LPS- and tetravalent Con A-treated cultures usually gave the highest yield of low density cells, followed by FCS and MLC. The yield was similar in the presence of either BPA or mouse serum supplementation, except for LPS where mouse serum reduced yields 30–50%. Few low density cells were present in spleens cultured without stimulant, i.e., with low doses of BPA or mouse serum (Table I), and we have evidence (manuscript in preparation) that these cells are predominantly lymphocytes proliferating in the animal at the time the spleens were taken for culture. In all cases, red blood cells and dead (trypan blue positive) cells pelleted through the BPA, so that the low density population was virtually 100% viable and nucleated.

Quantitation of [^{3}H]thymidine present in the low and high density fraction showed that proliferating cells floated after all types of stimulation, except for tetravalent con A (experiment B, Table I, and see below). We attributed this to the presence of many large, lectin-mediated, cell aggregates which could not be dissociated by vigorous pipetting. Pretreatment of cultures before floatation with 0.1 M alpha-methyl mannoside, a specific competing sugar for Con A binding (28), reduced cell aggregation and allowed for the floatation of most [^{3}H]thymidine-labeled cells (data not shown).

More direct and precise data on the distribution of proliferating cells were obtained by autoradiographs of cell smears (Fig. 1, Table I). The labeling index (labeled cells/total cells, or specific activity) was often increased 100-fold in the low density population, and more than 90% of the labeled cells (total activity) were recovered in this fraction. In most instances, some 20–35% of the low density cells were in S phase.

We conclude that mouse spleen cells stimulated to synthesize DNA in vitro can be floated on dense BPA columns. The columns accommodate large numbers of cells and provide excellent cell recoveries.

Floatation of Proliferating Spleen Cells: Purity of the Low Density Fraction. [^{3}H]Thymidine radiolabeling is an excellent probe to follow the floatation of cells synthesizing DNA, but it does not provide information on the purity of the floating cell population. Two other approaches were taken to show that most low density cells were lymphoblasts.

CYTOLOGY. On smears, some of the low density cells were large and most of the large cells were radiolabeled (Fig. 1 A and B). Few large lymphocytes were

TABLE I
Floatation of Proliferating Cells Induced by Different Stimuli In Vitro

Culture medium	Total cells/fraction ($\times 10^{-6}$)		[^{3}H]Thymidine uptake*		[^{3}H]Thymidine labeling index‡	
	Low	High	Low	High	Low	High
Experiment A – 26 h in culture						
No stimulant	2.0	46	ND§	175	26	0.22
100 μg/ml LPS	12	53	6,545	135	32	0.23
3 μg/ml sConA	11	50	7,146	203	31	0.58
5% FCS	6.1	60	7,560	107	29	0.23
Experiment B – 48 h in culture						
No stimulant – DBA/2 cells	1.2	20	ND	202	25	0.90
No stimulant – DBA/2 × C57Bl F_1 cells	0.6	14	ND	243	22	0.63
No stimulant – DBA/2 and F_1 cells (MLC)	5.6	55	4,114	178	23	1.0
100 μg/ml LPS	20	50	11,693	254	39	1.3
3 μg/ml ConA	14	64	5,461	1,754	24	7.3
5% FCS	17	48	8,382	271	32	0.25

12 (experiment A) or 15 (experiment B) × 10 spleen cells were cultured for 26 or 48 h in the presence of various stimuli of blastogenesis and 30 μg/ml BPA (A) or 2.5% mouse serum (B). FCS cultures had no BPA or mouse serum supplement. Before floatation, the cells were radiolabeled. The low and high density fractions were analyzed for cell bound [^{3}H]thymidine (liquid scintillation counts) and number of labeled cells (autoradiographs).

* Cpm/3.10^5 cells.

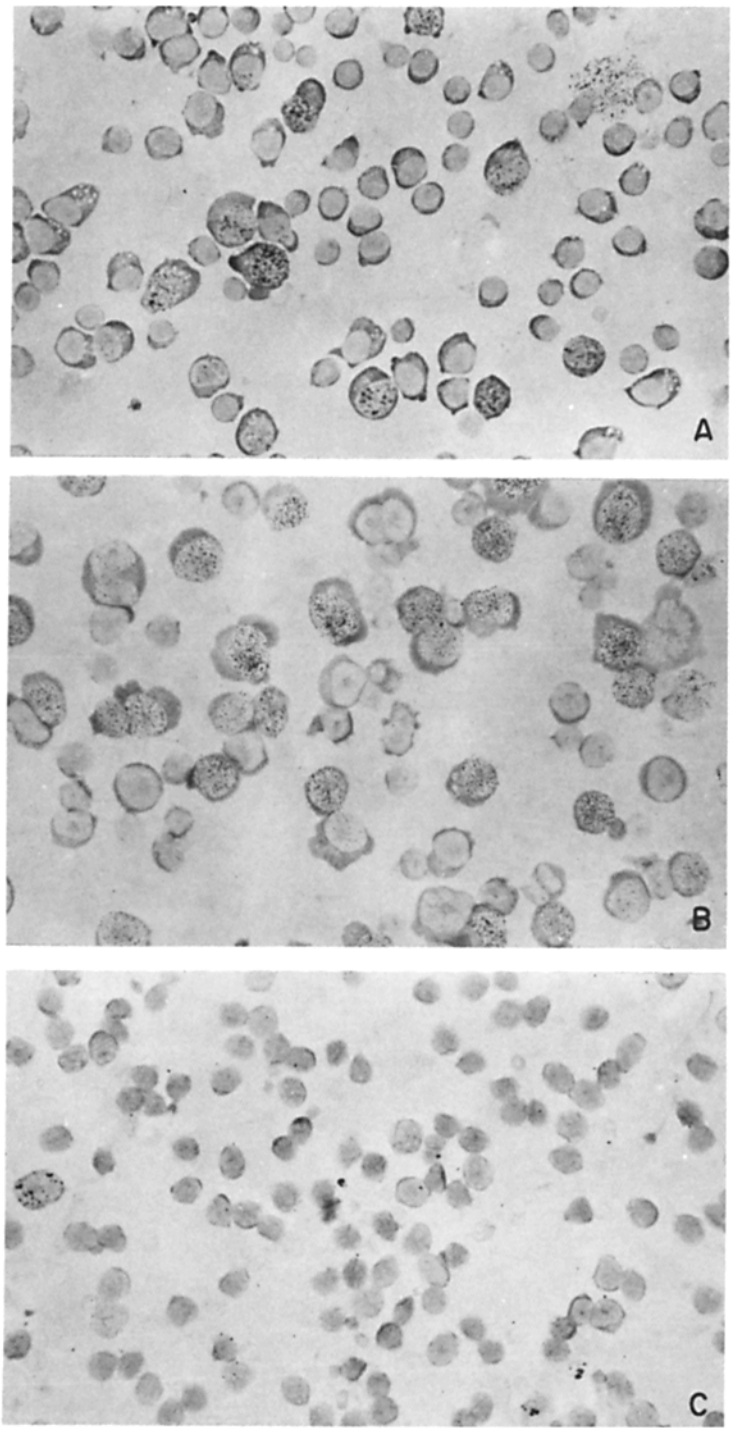
‡ Labeled cells/total cells × 100.

§ Not determined.

noted in the high density fraction (Fig. 1 C). Many low density cells did not appear that much larger than their high density counterparts, especially in preparations obtained within 1 day of stimulation. However, all these cells had obviously basophilic cytoplasm, and in some preparations, distinct nucleoli. In contrast, the small, high density lymphocytes showed little cytoplasmic basophilia under our staining conditions (Fig. 1 C).

By electron microscopy, 80–90% of the low density cells, obtained after LPS, FCS, or con A stimulation, had the typical cytologic features of lymphoblasts (Fig. 2 A–C; references 29, 30). The nuclei contained relatively little condensed or heterochromatin. The nucleoli were enlarged, and the cytoplasm had many scattered polyribosomes which were free rather than membrane-bound. The Golgi zone exhibited few associated vesicles and lysosomes. High density cells (Fig. 2 D) consisted almost entirely of small lymphocytes with abundant nuclear heterochromatin and single, nonmembrane-bound, cytoplasmic particles, presumably ribosomes.

Other cell types were identified in much lower frequencies. Macrophages, large cells with abundant vesicles and lysosomes, were almost entirely removed by adherence during the 1 h used to radiolabel the cells. Dendritic cells, a newly described cell type identified by large, irregularly shaped nuclei, many cytoplasmic processes, and paucity of cytoplasmic organelles other than mitochondria (21), comprised less than 5% of the low density fractions. Granulocytes, with endogenous peroxidase-positive granules, and erythroid cells, with diffuse


A
B
C

endogenous peroxidatic activity, were largely found in the high density fraction. Red blood cells, dead cells, and mature plasma cells were in the latter fraction as well.

We conclude that the low density population is almost entirely lymphoblasts by cytologic criteria. However, the size of these blasts varies considerably.

CONTINUOUS [^{3}H]THYMIDINE RADIOLABELING. 24-h, LPS- and sCon A-stimulated, low and high density cells were cultured continuously in 0.05 μCi/ml [^{3}H]thymidine, spec act 6.0 Ci/mM, in the presence of the appropriate stimulant (LPS or sCon A). The percentage of labeled low density cells increased with time, reaching a level of 80% or more by 18–20 h (Table II and Fig. 3 A). After 20 h, viable cell recoveries were generally 20–25% higher than present initially and another 10–20% of the cells were trypan blue positive. After 20 h of culture, a fraction of the high density cells had blast transformed and some were radiolabeled (5–15% of the total recovered cells; Table II and Fig. 3 B). These blasts could again be floated in high yield and purity on dense BPA (data not shown).

Conceivably many high density cells were prevented from blast transforming because they lacked some accessory cell depleted by floatation. Mixtures of LPS- and sCon A-induced low and high density cells were therefore cultured for 20 h in 0.05 μCi/ml [^{3}H]thymidine in the same proportion as they existed in unseparated spleen cultures. By both autoradiography and liquid scintillation counting, the mixtures behaved according to the sum of their low and high density components. Also, proliferative activity in the low and high density mixtures was identical to that seen in unseparated spleen cells (data not shown).

We conclude that most low density cells obtained after just 1 day of stimulation are capable of entering S phase of the cell cycle. Some high density cells can be blast transformed after further culture.

Other Properties of Low and High Density Cells. Because we had highly enriched populations of lymphoblasts in good yield, we could use laser flow techniques to further characterize the size and cell cycle distribution of these cells.

CELL SIZE. The distribution of individual cell sizes in suspension was displayed quantitatively by low angle, laser light scattering in a cytograf

FIG. 1. Giemsa-stained autoradiographs of [^{3}H]thymidine, pulse radiolabeled, LPS-stimulated cells. These smears demonstrate three features: cell size; cytoplasmic basophilia, i.e., dark staining with the blue dye of the Giemsa mixture; and number of radiolabeled cells or cells in S phase of the cell cycle. × 360. (A) Low density cells after 20 h of stimulation. Most cells are blasts as indicated by their increased content of basophilic cytoplasm. At this time, most blasts are relatively small in size, and only a small percentage (7% in this experiment) have entered S phase. (B) Low density cells after 44 h of stimulation. Many cells are large, and most of these are radiolabeled (38% of the low density cells were in S phase in this experiment). The smaller blasts are probably in G_1 phase. (C) High density cells after 24 h of stimulation (smears of the high density fraction would appear similar after 20–48 h of stimulation). Most of the cells are small lymphocytes with little basophilic cytoplasm. Only a few cells are in S phase (arrow), 0.3% in this experiment.

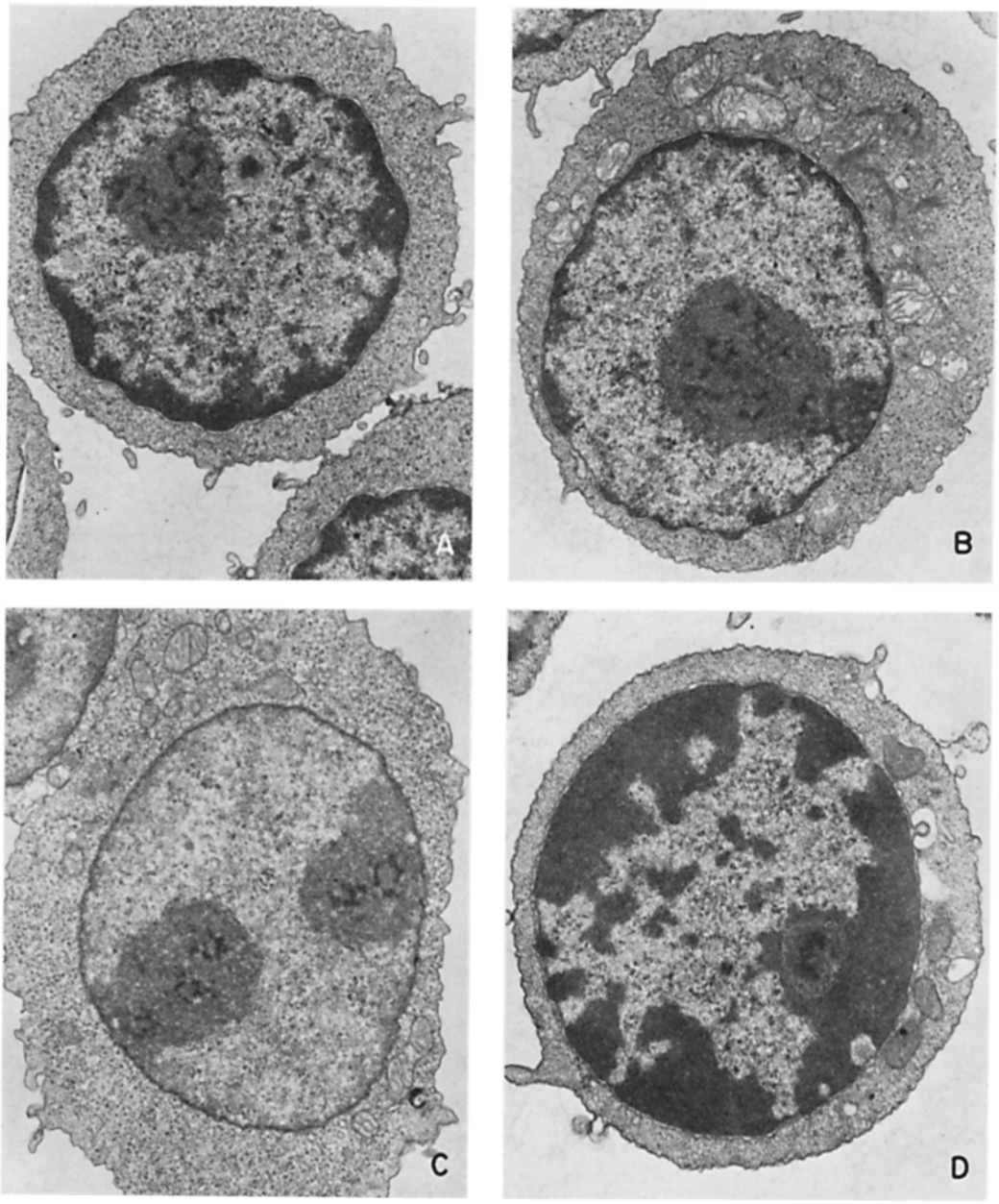


FIG. 2. Typical cells in low (A–C) and high (D) density fractions from stimulated mouse spleen cultures. (A) 20-h LPS-induced lymphoblast with decreased nuclear heterochromatin and increased nucleolar size relative to small lymphocytes. The cytoplasm contains many polyribosomes. × 9,500. (B) As in A. The section passes through the Golgi region, but few vesicles or lysosomes are evident. × 8,600. (C) 25-h Con A-induced lymphoblast with similar cytologic features to LPS blasts. × 7,200. (D) Small lymphocyte with abundant nuclear heterochromatin, small nucleolus and scanty cytoplasm lacking polyribosomes. × 13,300.

TABLE II
Continuous Culture of Low and High Density Cells in [^{3}H]Thymidine

Cells	Time in culture	Labeling index (labeled/total)	
		LPS stimulated	sConA stimulated
	h		
Low density	1	29.9	23.2
"	4.5	43.5	33.9
"	9	59.4	51.6
"	19	79.6	73.3
High density	19	5.6	10.2

Low and high density fractions from 25-h spleen cultures were maintained for varying times in the same stimulant used to induce them (LPS or sConA), plus 0.05 μCi/ml [^{3}H]thymidine. At varying times, cell smears were prepared for autoradiography. Labeling indices were measured after 7 days of autoradiographic exposure.

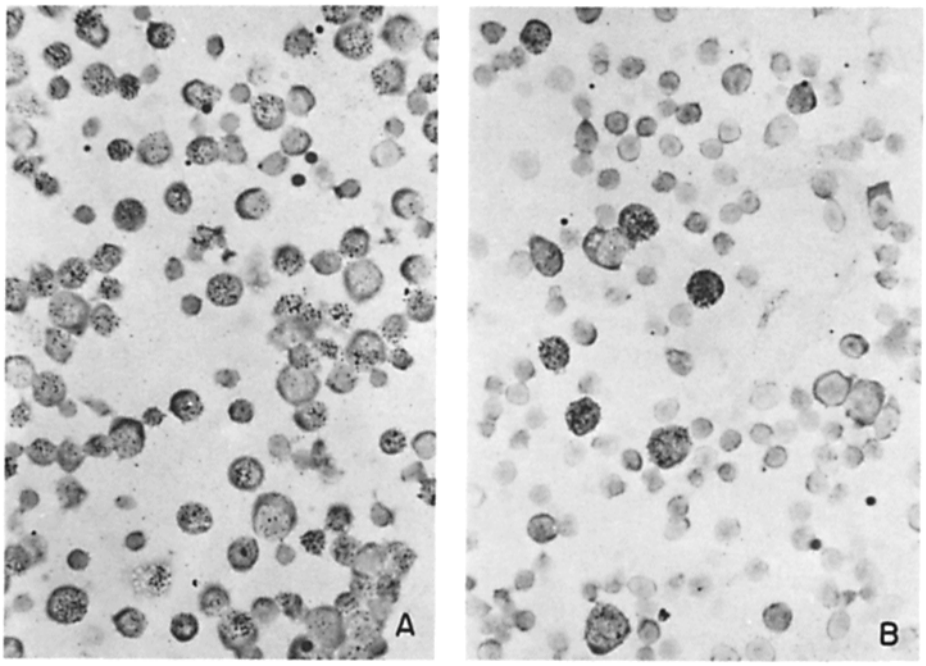


FIG. 3. Low (A) and high (B) density cells obtained after 20 h of stimulation with LPS (same preparation as Fig. 1 A), and then recultured an additional 24 h in LPS with 0.05 μCi/ml [^{3}H]thymidine. At least 75% of the low density cells have entered S phase because they are detectably radiolabeled. Unfortunately, the plane of focus of this micrograph does not reveal grains on some of the labeled cells. About 10% of the high density cells became radiolabeled during the 24-h interval. × 345.

device (Fig. 4). The distributions were remarkably similar in cells obtained from all four kinds of stimulated cells, and were largely symmetrical. The peak frequency for low density cells was at channel 310–324 with most cells falling in a range of channels 220–450. For high density cells, the peak frequency was

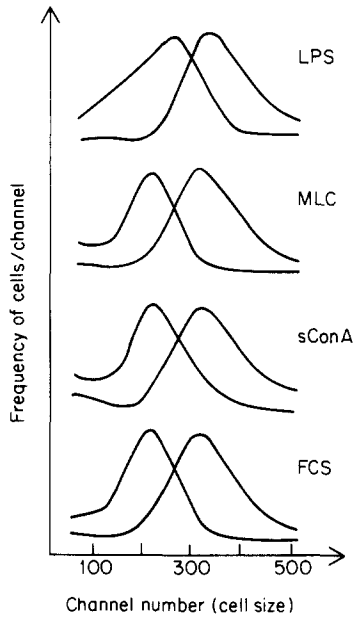


FIG. 4. Size distribution (low angle, laser light scattering) of low (right-hand curve) and high (left-hand curve) density cells obtained after 25 h of stimulation with LPS, FCS, sCon A, and MLC. The patterns are similar for each stimulant. The low density population is clearly larger, but overlaps the high density distribution.

220–245 with most cells falling between channels 145 and 365. We did not try to measure the absolute cell sizes that correspond to these channel numbers, but it is clear that roughly 50% of each fraction falls within the size range of the other—after 24 h of stimulation (Fig. 4). After 48 h, the low density size distribution was displaced to slightly higher values, but the overlap was still about 30% (data not shown). The smaller low density blasts were likely in the G_1 phase of the cell cycle (see below).

FLOW MICROFLUOROMETRY (FMF). This technique was used to quantitate the amount of fluorescence in individual cells after staining with the fluorescent, DNA-intercalating dye, propidium iodide (23, 24). Proliferating populations contain cells with distributions of fluorescence corresponding to DNA levels and cell cycle stages of 2n (G_1), 2n–4n (S), and 4n (G_2+ M). Therefore, cells in all stages of the cell cycle can be enumerated simultaneously.

Simple visual examination of fluorograms performed on various low and high density populations revealed that the low density cells were enriched in cells exhibiting more than 2n fluorescence, i.e., S and G_2+ M (Fig. 5). FMF distributions of low density cells, performed from cultures stimulated with any of the four stimulants for a single time interval, were indistinguishable (data not shown). The percentages of cells in the various phases of the cell cycle were determined by the technique of Fried et al. (25–27), and in the case of S phase, were compared with the autoradiographic labeling indices. Examples of these data are shown in Table III. In all cases, FMF and autoradiography gave similar results for the frequency of low density cells in S phase, but FMF gave higher values for this frequency in high density cells. The FMF data for the

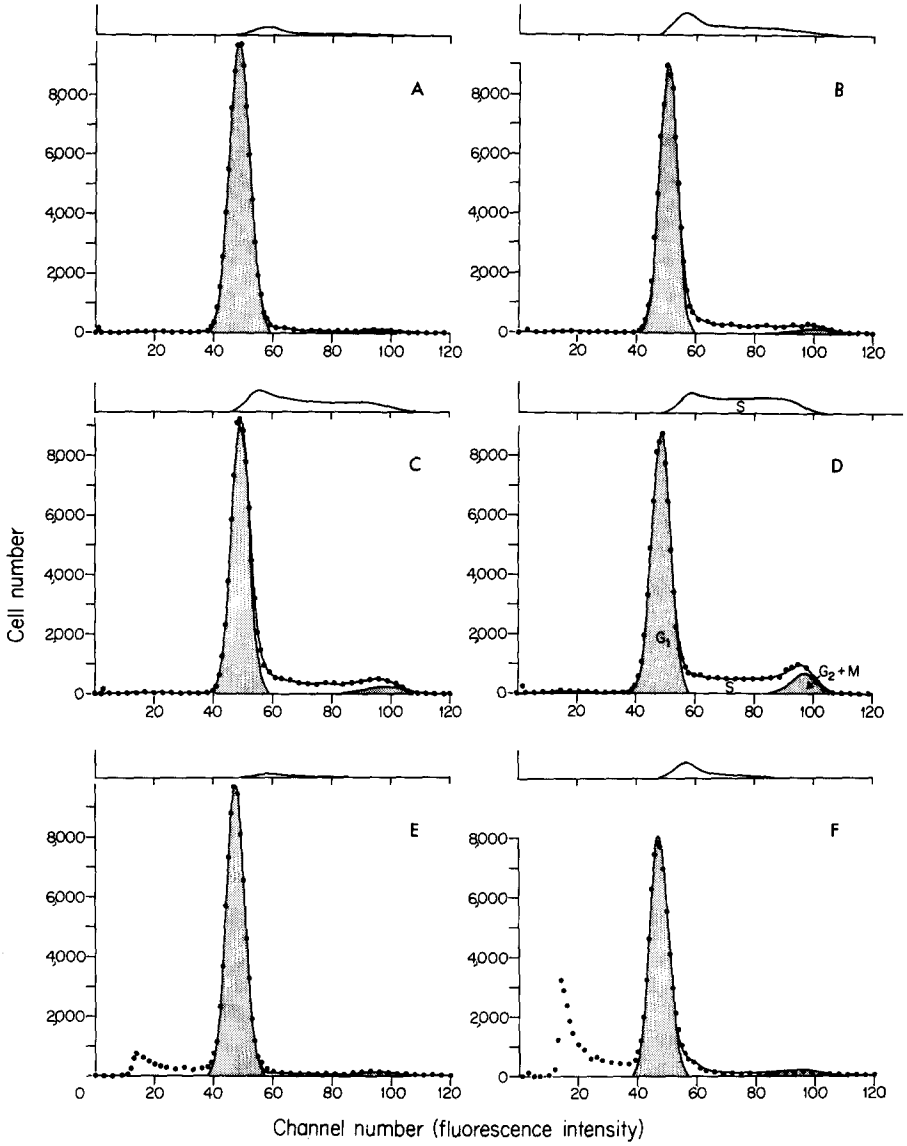


FIG. 5. Laser flow microfluorograms of low (A-D) and high (E, F) density cells obtained after varying times of LPS stimulation (A, E—20 h; B—25 h; C—30 h; D, F—44 h). The dots are actual data points, and the continuous line represents a mathematical fit to the data. Above each fluorogram is the mathematically derived distribution of S phase (2n–4n) cells, whereas the calculated G_1 and G_2+ M distributions are shaded within the fluorogram. With an increasing time in LPS (A-D), more of the low density cells are in S and G_2+ M (data for these figures are given in Table V). Most high density cells (E, F) are in G_1 at all time-points. The dead cells and cell fragments in the high density fraction account for the component exhibiting less fluorescence than the G_1 cells.

high density cells may be overestimates. First, there is a region of fluorescent cell debris to the left of G_1, which may extend into the G_1 and S regions. Second, clumped cells or cell fragments adhering to intact cells may artifactually produce cells in the S or G_2+ M regions.

TABLE III
Cell Cycle Analysis with FMF

Stimulant	Time in culture	Fraction	FMF – cells			Autoradiography cells in S
			G_1	S	G_2+ M	
	h			%		%
LPS	24	Low density	88	10	2	12
LPS	24	High density	96	3	1	0.2
LPS	44	Low density	61	33	6	35
LPS	44	High density	93	4	3	0.8
sCon A	48	Low density	51	39	10	41
sCon A	48	High density	91	6	3	1.4

Mouse spleen cells were stimulated with mitogens for varying periods of time. Before floatation, the cells were radiolabeled for 1 h with [^{3}H]thymidine. After floatation, the cell cycle distribution of low and high density fractions was analyzed by FMF and by autoradiography.

At 20–25 h in culture, most of the blasts were in G_1 by FMF (Fig. 5 A and B), but they were still relatively small in size on Giemsa-stained cell smears (Fig. 1 A). We conclude that the G_1 lymphoblast would not be classified as a large lymphocyte. We do not know whether this conclusion applies only to G_1 cells in their first proliferative cell cycle, or for all lymphoblasts in G_1.

LYMPHOBLASTS CAN BE ISOLATED IN THEIR FIRST CYCLE OF CELL DIVISION. Our FMF studies had indicated that cells obtained 1 day after mitogenic stimulation contained few cells in G_2+ M (Fig. 5 A and B, Table III), so that they probably were in their first proliferative cell cycle. We therefore studied the same batch of spleen cells cultured for varying periods of time in LPS – 20, 25, 30, and 44 h. With increasing stimulation intervals, there was an increase in the number of cells in the culture that floated (Table IV). But in all cases, the floating cells were lymphoblasts by cytologic criteria (Giemsa staining and electron microscopy), and most cells radiolabeled if exposed continuously to [^{3}H]thymidine for an additional 20 h after floatation. In contrast, the high density cells were primarily small lymphocytes (as in Figs. 1 C and 2 D), but on continued culture a small proportion would blast transform.

We then determined the number of low density cells in various stages of the cell cycle at each time-point after LPS stimulation. FMF distributions were first analyzed mathematically to give the percentage of cells in G_1, S, and G_2+ M. This percentage times the total number of low density cells gave the number of cells in each phase of the cell cycle. We found that the number of low density S and G_2+ M cells per culture increased progressively (Fig. 5 A–D, Table IV), whereas high density cells contained G_1 cells at all time-points (Fig. 5 E and F). Because few cells were in S or G_1+ M at 20 h, we knew that the low density cells were entering their first proliferative cell cycle during the first day of mitogen stimulation. By 44 h, many had proceeded through their first cycle, and new cells in their first cell cycle must have been recruited as well. In the accompanying paper, we analyze this problem directly by following the fate of lymphoblasts isolated after 1 day of stimulation and maintained further in vitro.

TABLE IV
Cell Cycle Analysis of Low Density Cells Obtained after Varying Intervals in LPS

Time In LPS	Total cells/fraction $\times 10^{-6}$		Low density cells in various stages of the cell cycle (FMF)			Total low density cells in various stages of the cell cycle		
	Low density	High density	G_1	S	G_2+ M	G_1	S	G_2+ M
h				%				
20	15	45	93	6 (7)*	1	14	0.9	0.1
25	17	39	78	20 (19)	2	13	3.4	0.3
30	25	35	74	22 (22)	4	19	5.5	1.0
44	28	29	70	22 (26)	8	20	6.2	2.2

12×10^7 spleen cells were stimulated for varying times in LPS without serum. Low and high density cells were obtained, and cell cycle analysis performed using FMF. Data for the low density cells are given. At all time-points, more than 95% of the high density cells were in G_1.
* Data in parentheses are percentages of cells that radiolabeled with [^{3}H]thymidine during the hour before floatation.

Viability of Cells Obtained from BPA Columns. Low and high density cells were obtained after 25 h of stimulation with FCS, LPS, and sCon A. Their viability was assessed by following their ability to proliferate upon continued culture in vitro. Low density cells were highly enriched in proliferative activity, illustrating that they represent a major proportion of the mitogen-induced response, even when obtained after 25 h in mitogen. The proliferative activity of cells that were of high density at 25 h clearly expanded with time (Fig. 6), showing that new cells were recruited into mitogenesis after the first day. For all three stimulants, appropriate mixtures of low and high density cells proliferated identically to unseparated spleen cells, indicating that the floatation procedure per se did not alter subsequent proliferative capacity.

Discussion

The transformation of small lymphocytes into lymphoblasts is an essential component of immune responses both in vivo (1) and in vitro (2, 3). These lymphoblasts, however, have not been subject to extensive direct study. As yet there are no protocols for obtaining these cells in high yield and purity. The problem is further compounded by the fact that blast transformation is not a synchronous event. Rather, it can occur continually over a several day period, with new cells constantly being recruited into proliferative activity and mingling with cells stimulated at earlier times (10–12; this paper).

In this paper, we have approached these problems using isopycnic, centrifugation techniques. We found that mouse spleen lymphocytes acquire a low cell density when stimulated to divide by a variety of agents in vitro. These included LPS and FCS, presumed B-cell mitogens (13–16), and con A and MLC, primarily T-cell mitogens (13, 17, 18). More than 90% of the lymphoblasts present in stimulated cultures at any single time could be recovered by

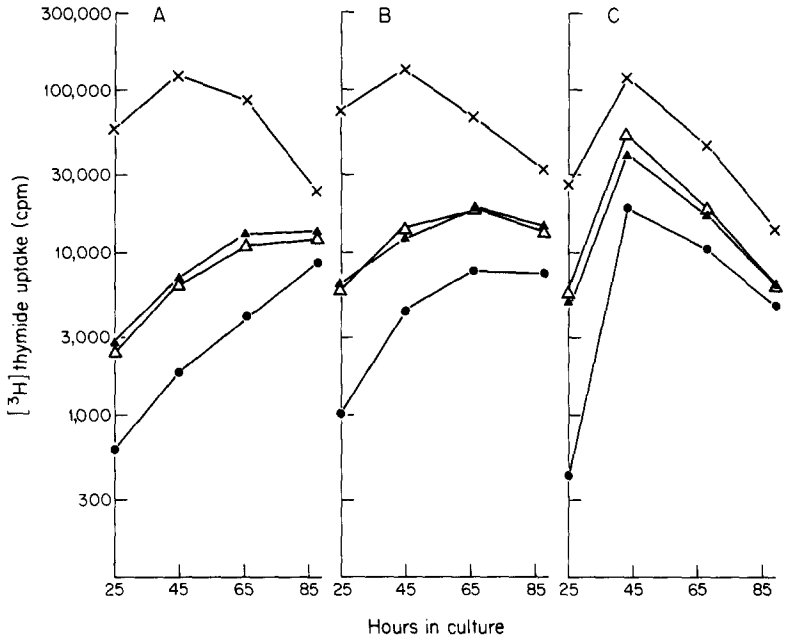


FIG. 6. Proliferative activity (uptake of [^{3}H]thymidine during a 2-h pulse; cpm/10^6 initially plated cells) of cells that have been stimulated for 25 h with FCS (A), LPS (B), or sCon A (C), and then returned to culture with the appropriate stimulant for another 60 h. In each case, the low density cells (×- - -×) are clearly enriched in proliferative activity relative to the high density cells (●- - -●). Suitable mixtures of low and high density cells (▲- - -▲) behave identically to unseparated cells (△- - -△), indicating that the separation procedure in BPA columns does not alter this parameter of cell function in vitro.

floatation in dense BPA; and we estimate at least 80% of the floating cells were lymphoblasts. The floatation procedure could then be performed on successive days in culture to obtain blasts generated during that day. We have not yet looked in detail at other lymphoid organs, other species, or other antigenic stimulants, but we have preliminary data that lymphoblasts obtained in these other situations also have a low density.

The low density of large lymphocytes has been documented previously (31, 32), but most workers have used velocity sedimentation to separate lymphoblasts (e.g. 6–8). This approach separates cells on the basis of size differences. It has provided enriched preparations, but often the purity and yield have not been fully documented. One difficulty is that [^{3}H]thymidine radiolabeling has been the main criterion for blast transformation, so that cycling lymphocytes in the G_1 phase have not really been identified or characterized. It may well be that the increase in cell size which allows for separation by velocity sedimentation is more related to the presence of cells in the S and G_2+ M phases of the cell cycle, rather than to blast transformation per se. In fact, most cell types enlarge considerably as they prepare for mitosis (e.g. 33–35).

Much of our data pertain to the identification and characterization of lymphoblasts in the G_1 phase of the cell cycle, particularly the first cell cycle

after administration of the stimulant. What we found is that stimulated spleen cultures had large numbers of low density cells within 20–24 h of stimulation, most of which had not entered S or G_2+ M. These cells were typical blasts by cytologic criteria, i.e., they had basophilic cytoplasm and a readily identifiable nucleolus on Giemsa staining, and typical nuclei and abundant cytoplasmic polyribosomes by electron microscopy (29, 30). Also they would eventually synthesize DNA if placed in culture for 18–20 h. However, they were not markedly enlarged, and by light-scattering techniques, we could show that many overlapped in size with the noncycling component of the culture. It is therefore unlikely that velocity sedimentation could separate these blast-transformed cells in high yield and purity. Further, any properties of lymphoblasts that would be purified on the basis of increased size might reflect properties of S or G_2+ M cells rather than properties peculiar to lymphoblasts per se.

What we do not know is why or when, during its initial cell cycle, the transformed lymphocyte acquires a low density. Possibly changes in the condensation of chromatin or in the water content of the cell (? nucleus) are involved. It is likely that the density shifts long before DNA synthesis begins. We found that lymphoblast populations, most of which are in the G_1 phase of their first cell cycle, enter S phase continuously at a rate of just 4–5%/h. That is, many of the G_1 cells take many hours before they begin DNA synthesis. Gunther et al. (36) obtained information that stimulated cells in G_1 are irreversibly committed to entering S phase many hours before they actually synthesize DNA. They noted that removal of much of the administered con A load after 20 h of stimulation did not prevent the cells from entering S after a day of additional culture. Removal of con A at earlier times (before 20 h) reduced the number of cells that would subsequently label with [^{3}H]thymidine.

Another general finding of interest was that lymphoblasts stimulated by a variety of agents were very similar in all parameters measured: physical (cell size and density), cytologic, and cell cycle distribution (percentage of cells in G_1, S, and G_2+ M) after any one interval of stimulation. Inasmuch as LPS and FCS, and con A and MLC, probably stimulate B or T cells, respectively, or even subpopulations of B and T cells, we would assume that various classes of lymphoblasts are indistinguishable by the criteria used in this paper. We did not establish directly that the blasts were specifically B or T. We are now looking to see whether markers that distinguish small B from small T lymphocytes also apply to their blast-transformed counterparts. So far, this does not appear to be the case, e.g., presumptive B blasts show considerably reduced expression of the complement (C3) receptor, and susceptibility to killing with anti-immunoglobulin agents and complement. We are also interested in the fate of the blasts in vitro, and in the accompanying paper, we demonstrate that most FCS- and LPS-induced blasts mature into typical plasma cells in vitro.

What appeared to vary with the various stimulants we employed was the efficiency of blast transformation, as reflected in the number of cells that acquire a low density. With LPS stimulation, we routinely recovered 2×10^7 blasts after 24 h of stimulation of 15×10^7 spleen cells in serum-free medium, whereas with MLC, some 0.3×10^7 blasts were obtained. We cannot estimate

the absolute efficiency of blast transformation for several reasons: transformation is not synchronous in that new cells appear to be recruited continually for several days in vitro; many cells are dying in these cultures, so we do not know the number of cells available to be transformed; and new responsive cells may be generated in vitro particularly as mouse spleen is a marrow (central) as well as a peripheral lymphoid organ. But, in any case, a change in cell density may be a universal property of lymphoblasts. Isopycnic procedures may therefore be used to quantitate the extent of transformation in response to a particular stimulus or set of stimulation conditions. We are also using this approach to obtain lymphoblasts stimulated by specific antigens *in situ*.

In conclusion, the floatation method described here will help characterize further the properties and behavior of proliferating lymphocytes. In the accompanying paper, we analyze the proliferative potential and differentiation of LPS- and FCS-induced B blasts in vitro.

Summary

Mouse spleen lymphoblasts, stimulated to divide in vitro, acquired a low cell density and could be separated by isopycnic techniques. Cultured cells were suspended in BPA columns, ρ = 1.080, and spun to equilibrium. The method was simple, fast, accommodated large numbers of cells, and was reproducible. It provided lymphoblasts in high yield and purity (at least 80% of the low density cells were blasts). It allowed for the recovery of proliferating cells in their first cell cycle, and did not alter the subsequent ability of cells to proliferate when recultured in vitro.

Certain properties of mouse spleen lymphoblasts were analyzed in detail. Lymphoblasts induced by LPS, FCS, con A (tetravalent and succinylated), and MLC were very similar except in the absolute numbers that were induced. The blasts exhibited the classic cytologic features of enlarged nucleoli and abundant cytoplasmic polyribosomes (basophilia). As a population, they were enlarged in size relative to nondividing cells, but this seemed to apply primarily to cells in the S and G_2+ M phase of the cell cycle rather than G_1. The cell cycle distribution of lymphoblasts was analyzed by flow microfluorometry. By analyzing low density cells obtained at varying intervals after mitogen stimulation, FMF indicated that lymphoblasts enter the S phase of their first cell cycle beginning at 20–24 h after stimulation.

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