LYMPHOCYTE CELL-CYCLE ANALYSIS BY FLOW CYTOMETRY

Evidence for a Specific Postmitotic Phase Before Return to G_0

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ABSTRACT

We studied the cell cycle of lectin-stimulated human lymphocytes, making use of a flow cytometer. The RNA and DNA content of large numbers of individual cells was determined by supravital staining with acridine orange. The present study confirmed previous observations by others of a progression from G_0 through G_1 and S phase to G_2/mitosis during the first 3 d in culture. It was also found that on subsequent days stimulated cells, before their return to G_0, remained stationary in a state in which they contained the G_0 complement of DNA and approximately twice the G_0 complement of RNA.

Cell-cycle manipulation with vinblastine and 5-bromo-2-deoxyuridine (BUdR) revealed that previous passage through both S phase and mitosis was required for entry into this newly observed late phase. In addition, there was high correlation ($r = 0.973$, $P < 0.001$) between the number of cells in the late phase and measured [3H]thymidine uptake. It therefore appears that, in this system, stimulated cells remain in a distinct cell-cycle phase for a number of hours before their return to the resting state.

Only a small proportion of lymphocytes obtained from the circulation or from lymphoid organs spontaneously divide in vitro, but the vast majority retain the ability to enter the cell cycle when exposed to lectins in culture. Specific antigens, either soluble or cell-surface associated, will also stimulate lymphocytes in vitro. Nondividing lymphocytes therefore satisfy the definition of G_0 cells (16). Advantage has been taken of these properties with the employment of lymphocytes to probe the mechanisms and control of cell proliferation in general.

Considerable effort has been expended on the study of two crucial aspects of the regulation of cell proliferation: entry into and exit from the cell cycle. In lymphocytes, it is convenient to view the existence of two states: resting or G_0 cells (2, 16, 21) and cycling cells, which may be in the G_1, S, G_2, or mitosis phases of the cell cycle (14). The entry into the cell cycle from G_0 appears to occur primarily into G_1 and, conversely, exit from the cycle is from G_1 (12, 13).

It has been suggested that so-called G_0 cells are merely traversing G_1 phase at a very slow rate and, hence, that the G_0 phase does not exist (9, 24). Though this holds true for some systems, no data to support this contention are available for lymphocytes. A possible reconciliation of this view with the classical concept of G_0 is provided by the probabilistic model in which all cells have a finite probability of entry from G_0 into the cell cycle, some cells having much smaller probabilities than others (3, 26). It has been proposed that the event determining the transition from G_0 to G_1 always

occurs at a particular, rate-limiting point in G_1, called the "restriction point" (20).

Less information concerning the mechanism of exit of cells from the cycle is available. Because most, but not all (12), resting cells are postmitotic and contain a diploid content of DNA, they are assumed to have entered G_0 from the G_1 phase. Studies of the restriction point in G_1 suggest that, at least in some systems (4, 18, 20), many growth-inhibiting factors act at the same point in mid-G_1. If blocked at that site, cells exit from the cycle to enter G_0. Little is known of the mechanism or time-course of this transition (1, 11; reviewed in 2).

Most studies of cell-cycle regulation have made use of morphologic changes or measures of the uptake of radio-labeled DNA and RNA precursors. These methods are used either on large cell populations as a whole or on small numbers of individual cells. The recent use of flow cytometry has allowed more efficient study of cycling cells by examining individual members of a population, but at rapid rates. The pioneering work of Darzynkiewicz, Sharpless, Melamed, and collaborators (7, 8) on development of staining and on-line computing methods to be used in conjunction with flow cytometry has provided a particularly useful technique for cell-cycle analysis. Data on each cell in a large population can be obtained so that statistically significant observations may be made. The technique can also be employed on extremely small samples, such as the cells obtained from 5-ml specimens of spinal fluid (19).

The present study has used the flow cytometric determination of the DNA and RNA content of cultured lymphocytes as the means of cell-cycle analysis. A modified acridine orange staining procedure (27) was carried out, in which single-stranded nucleic acids fluoresce red and double-stranded nucleic acids fluoresce green. Double-stranded RNA was selectively denatured by EDTA so that the DNA and RNA content of each cell could be quantified by simultaneous measurement of the red and green fluorescence emitted. Application of this technique showed that stimulated lymphocytes exit from the cell cycle in a distinctive and reproducible manner. At approximately 4-5 d after stimulation by lectins, cells remain stationary for a number of hours in a postmitotic state with a diploid content of DNA but with increased RNA, after which they return to the G_0 state.

MATERIALS AND METHODS

Pharmacologic Agents

Concanavalin A (Con A), 5-bromo-2-deoxyuridine (BUdR), uridine, 5-fluorodeoxyuridine, α-methyl-D-mannoside, vinblastine sulfate, and Triton X-100 were all obtained from Sigma Chemical Co. (St. Louis, Mo.); phytohemagglutinin-M (PHA) from Grand Island Biological Co. (Grand Island, N. Y.), acridine orange from Polysciences, Inc. (Warrington, Pa.) and RNase A from Worthington Biochemical Corp. (Freehold, N. J.).

Cell Isolations

Mononuclear cell (MNC) fractions were isolated from the peripheral blood of normal adult donors by centrifugation on discontinuous Ficoll/Hypaque gradients (Pharmacia Fine Chemicals, Div. Pharmacia, Inc., Piscataway, N. J.) (specific gravity, 1.078) by methods previously described (22). This fraction contained 80-90% lymphocytes, 10-20% monocytes, and <5% polymorphonuclear leukocytes (PMN). PMN fractions were isolated by Dr. S. Uberoi by a method described previously (25) or by gravity sedimentation of peripheral blood specimens from patients with leukocytic reaction to sepsis.

Cell Cultures

All cells for culture were suspended at a final concentration of 1×10^6 cells/ml in tissue-culture medium consisting of Eagle's minimum essential medium, 4 mM fresh 1-glutamine, 100 mg/ml of gentamicin, and 20% heat-inactivated fetal calf serum. Cells were cultured in 25-cm^2 tissue-culture flasks at 37°C in a 5% CO_2/95% air humidified atmosphere.

In each experiment, cells were cultured both in the presence and in the absence of either 30 μg/ml Con A or 10 μg/ml PHA (concentrations found previously to be optimal for the lots of lectin used in these experiments). In some experiments, vinblastine (final concentration: 0.2 μg/ml) was added to the cultures on day 3 (i.e., after 72 h of culture) and in others BUdR with uridine and 5-fluorodeoxyuridine (final concentrations, respectively: 0.09 mM, 6 μM, 4 μM) were added on day 1. In other experiments, the cells were washed either with medium or with 0.3 M α-methyl-D-mannoside on day 2 and resuspended in final culture medium alone or culture medium with fresh Con A.

Flow Cytometric Studies

Because it was found that the concentration of protein in the cell suspension medium affected relative DNA/RNA staining, all specimens were suspended in culture medium before staining. The staining procedure was a modification of that previously described (27). A 0.2-ml aliquot of cell suspension was mixed with detergent solution at low pH (0.1% Triton X-100, 0.05 N HCl, and 0.15 N NaCl) at 4°C for 15 s and then mixed with the chelating agent and acridine orange at room temperature (final concentrations: acridine orange, 12 μg/ml; EDTA-Na, 10^{-3} M; NaCl, 0.15 N; and phosphate-citrate buffer, pH 6.0). In some experiments, the cells were then incubated with RNase, 2,000 units/ml for 30 min at 37°C before cytometric measurements.

Stained cells were analyzed in a model FC200/4800A Cytofluorograf (Ortho Instruments, Westwood, Mass.) modified with a 50-mW argon-ion (488-nm) laser. Two bands of emitted fluorescence as well as small-angle light scattering were measured simultaneously for each cell as it passed through the focused laser beam. Green fluoresence emission (F_{530}) was measured in

a band from 515 to 575 nm and red fluorescence emission ($F_{>600}$) in a band from 600 to 650 nm. Each of the three measured signals was integrated over the time of cell passage through the beam, and the values were stored on-line in a NOVA 1200J minicomputer (Data General Corp., Westboro, Mass.). For each specimen, 5×10^3 cells were analyzed and the values for each of the three measurements were displayed on an arbitrary scale of 0–120, which was approximately linear. For each experiment, photomultiplier tube gains remained constant so that the data from different days in culture were comparable. Calibrations were done daily using standard fluorescent spheres.

[^3H]Thymidine Uptake

For some cultures, quadruplicate 0.2-ml (2×10^5 cells) aliquots obtained at various times were pulsed for 5 h with [^3H]thymidine (New England Nuclear, Boston, Mass.; specific activity, 6.7 Ci/mmol) at 5 μCi/ml and harvested onto fiberglass filters in an automated cell harvester (22). Filters were counted in a liquid scintillation counter.

RESULTS

MNC Fraction

The MNC fraction, stained immediately after isolation, was examined in preparations from >30 donors. A two-parameter histogram displaying the $F_{>600}$ (RNA) and F_{530} (DNA) values of the individual cells from a representative specimen is shown in Fig. 1. Two distinct populations are noted. The identity of each was determined by the following experiment. The PMN cell fractions obtained from whole blood were added in various
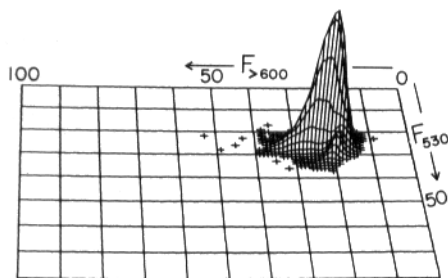

FIGURE 1 Two-parameter computer-generated histogram displaying the simultaneously measured emission red ($F_{>600}$) and green (F_{530}) fluorescence (measures of RNA and DNA content, respectively) of 5×10^3 cells from an MNC fraction freshly isolated from peripheral blood. The height of the peak above the $F_{>600}$–F_{530} plane represents the number of cells with $F_{>600}$–F_{530} values in that region (with this system, the distance of each peak from the origin can be varied by adjustment of the gain settings of the $F_{>600}$ and the F_{530} photomultiplier tubes). Note the presence of a major peak and a second smaller peak with larger F_{530} and smaller $F_{>600}$ values.

proportions to the MNC fractions from the same donors. As a larger percentage of PMN was added, an increase in the peak containing slightly larger amounts of F_{530} and smaller amounts of $F_{>600}$ was seen, identifying the smaller peak in Fig. 1 as PMN.

Mitogen Stimulation

The results of mitogenic stimulation by Con A (Fig. 2) was studied through daily analysis of MNC cultures from 19 donors. Identical results (data not shown) were found for PHA stimulation. By day 2, there was an increase in the percentage of cells containing a marked increase in $F_{>600}$ (with unchanged F_{530}). The percentage of cells in this group peaked by day 3. Because one of the early and crucial changes that occurs in the G_0 to G_1 transition is an increase in RNA content (2), it seems reasonable to conclude that the original population, with small amounts of DNA and RNA, consists of G_0 cells and that the second population, with increased RNA content, comprises G_1 cells. On days 2–4, the percentage of cells containing increased $F_{>600}$ and F_{530} values between one and two times the G_0 value also increased, usually peaking on day 3. On days 3 and 4, cells with increased $F_{>600}$ and F_{530} exactly twice the G_0 value were also noted. (Many cells, however, remained in G_0 throughout the entire culture period.) These observations confirm those made previously by Darzynkiewicz et al. (8) and strongly suggest that the latter two populations represent, respectively, S phase and then a mixture of G_2 phase cells and cells undergoing mitosis.

On days 4–6, as the percentage of cells in S and G_2/mitosis was decreasing, there was a sharp increase in the number of cells containing moderately increased RNA, with the G_0 amount of DNA. This population differed from the G_1 cells seen on days 1–3 in that it formed a rather sharp peak in the two-parameter histogram. The early G_1 cells, in contrast, formed a broad ridge with $F_{>600}$ values ranging from slightly greater than the G_0 value to many times that value. The sharp peak of the day-5 cells occurred at $F_{>600}$ values approximately twice those of G_0 cells. The cells in the early G_1 region made up ~15% of the total population in culture on that day, whereas the day-5 cells in this region averaged 40% of the day-5 total. This, along with the continued presence of this peak over 2–3 d, suggests that mitogen-stimulated
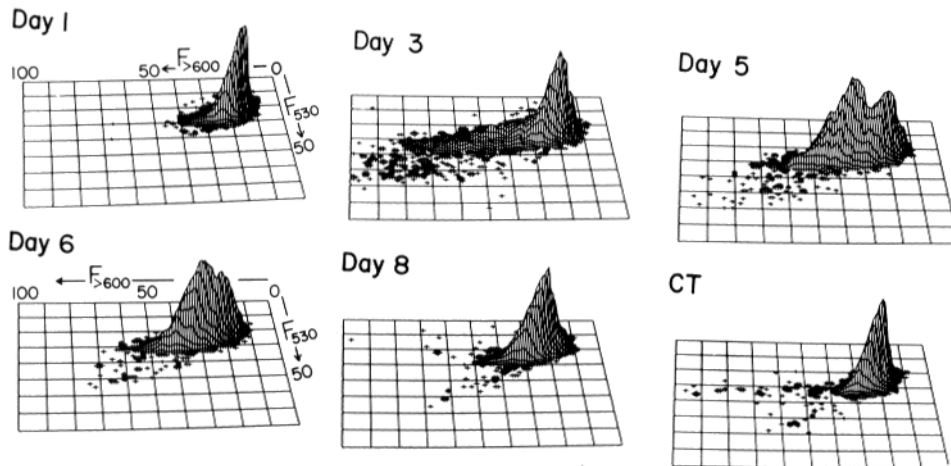
FIGURE 2 Representative two-parameter histograms of Con A stimulated cells over 8 d (e.g., day 1: cells cultured for 24 h) in culture (gain settings approximately those of Fig. 1). Control (CT) is a day-5 specimen of cells from the same donor cultured simultaneously but in the absence of Con A.

cells remain in this later region for a much longer period of time than do the early G_1 cells.

On subsequent days in culture, the cells in this late peak decreased in number while the number of cells in G_0 markedly increased. The number of cells in G_1, S, and G_2/mitosis remained small, although occasionally there was a second round of progression through the cycle, again with the latter peak in the increased RNA region as a prominent feature. [^{3}H]Thymidine uptake correlated only roughly with numbers of cells in the standard G_1, S, or G_2/mitosis regions (see below).

The Late Peak

The cells included in the late peak of increased $F_{>600}$ values occurring on day 5 (range: day 3–day 6) after Con A stimulation were studied in greater detail. The increased $F_{>600}$ was eliminated by preincubation of the cells with RNase (Fig. 3). Addition of vinblastine on day 3 to stimulated cultures from six donors resulted in arrest of cells in G_2/mitosis on days 3 and 4 and elimination of the late peak on day 4 and subsequent days in culture (Fig. 4). That the late peak required progression through the cell cycle was further confirmed by a study of the effect of BUdR that was added to Con A-stimulated cultures on day 1. BUdR, which is incorporated into newly synthesized DNA, results in quenched F_{530} staining of those strands into which it is incorporated (15, 17). Therefore, cells synthesizing DNA during the period of exposure to BUdR will have decreased F_{530}
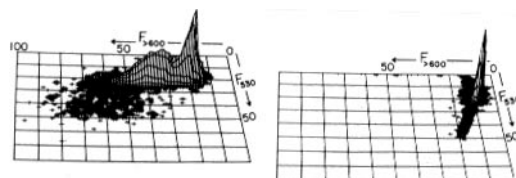

FIGURE 3 Two-parameter histogram (gain settings as in Fig. 1) of cells taken from day-4 Con A–stimulated cultures. Left, untreated; right, RNase treated.
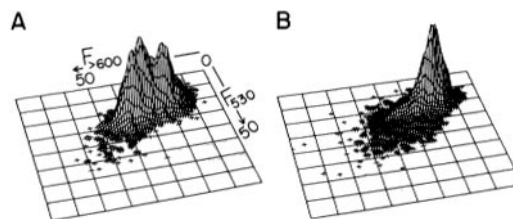

FIGURE 4 Two-parameter histogram of Con A–stimulated cultures from the same donor examined on day 6 (gain settings as in Fig. 1). A, untreated cells; B, vinblastine added to cultures on day 3.

values. Addition of BUdR resulted in a shift of the entire late peak to lower F_{530} values (Fig. 5).

The possibility that the late peak was the result of a cell-cycle blocking effect by Con A present in the later days of culture was addressed by the following experiments. Con A cultures were washed, either with medium or with 0.3 M α-methyl-D-mannoside, and then recultured either with or without Con A. These treatments produced

no change in the pattern observed on subsequent days. Specifically, the late peak still occurred.

Correlation of Percentage of Cells in the Late Peak with [³H]Thymidine Uptake

The percentage of cells in the late peak was compared with the 5-h [³H]thymidine uptake. The data for the percentage of cells in the late peak on days 3–6 and for [³H]thymidine uptake for days 2–5 are shown in Table I for the seven donors for which both are available. Because of the wide variations from donor to donor in the day of maximal late-peak activity and in the day of maximal [³H]thymidine uptake, the highest correlation ($r = 0.973$, $P < 0.001$) was seen when the values for each were summed over the respective 4-d periods (Table I). The wide variation from donor to donor in Con A response, as measured by summed late-peak content or by summed [³H]thymidine uptake, should also be noted.
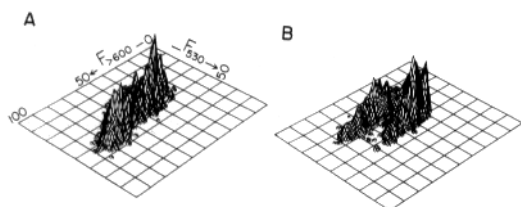

FIGURE 5 Two-parameter histogram of Con A–stimulated cultures from the same donor examined on day 5. (The gain settings are somewhat higher than in Fig. 1, and the orientation has been rotated to better display F_{530} differences). A, untreated cells; B, BUdR added on day 1.

DISCUSSION

The present study makes use of the supravital staining method of Darzynkiewicz et al. (27), in which double-stranded RNA is selectively denatured to single strands by chelating agents. The staining occurs in detergent-treated but intact cells and produces roughly quantitative measures of DNA (F_{530}) and RNA ($F_{>600}$).

The MNC fraction freshly isolated by centrifugation on Ficoll/Hypaque is homogeneous with respect to DNA and RNA content except for a small group of cells with slightly more DNA and slightly less RNA. This latter population was found to be the small number of contaminating PMN present after the isolation procedure. The explanation for the difference in red and green fluorescence between these cells and lymphocytes is being investigated at present. One explanation is an increased resistance of PMN double-stranded RNA to denaturation. Alternatively, it is possible that a small amount of lymphocyte DNA is denatured by the staining procedure and that the DNA of the highly differentiated and noncycling PMN is more resistant than the lymphocyte DNA (6).

Culture of the MNC in the presence of mitogen results in the appearance of additional cell populations: first, cells with increased RNA and, subsequently, cells with increased DNA. These results confirm the observations of Darzynkiewicz et al. (8) of a progression of stimulated cells through G_1, into S phase, and ultimately into G_2 phase and mitosis.

The present study has focused on an observation not previously noted in this experimental system. On days 4–6 of culture, with either Con A or PHA,

TABLE I

Comparison of 5-h [³H]Thymidine Uptake with Percentage of Cells in Late Peak

Donor	[³H]Thymidine uptake					Late peak				
	Day 2	Day 3	Day 4	Day 5	Σ*	Day 3	Day 4	Day 5	Day 6	Σ‡
	cpm × 10⁻³					%				
1	1.6	2.2	3.4	1.8	2.3	0	0	4.8	1.6	1.6
2	3.9	13.2	22.0	7.0	11.5	0	6	24	30	15.0
3	13.8	34.1	37.6	11.1	24.1	5	24	42	23	23.5
4	26.4	47.4	34.0	8.2	29.0	10	36	45	57	37.0
5	8.9	83.0	33.7	10.0	33.9	15	35	56	40	36.5
6	31.2	55.5	38.5	15.0	35.1	14	40	54	46	38.5
7	26.5	31.5	102.7	21.4	45.5	10	51	68	43	43.0

* cpm Index = Sum of cpm of 5-h uptake on days 2–5 divided by 4.
‡ Percentage index = Sum of percentage of cells in late peak on days 3–6 divided by 4.

there is a distinct population of cells with the G_0 complement of DNA and with increased RNA. This population differs from the classical G_1 phase seen earlier in the culture, in that the RNA values fall in a narrowly restricted range (about twice the G_0 amount) and the cells represent a much larger percentage of the cells present in culture on that day: ~40%. As the cells in this region diminish in number on days 6–8, the number of cells in G_0 increase. Throughout this period the number of cells in other portions of the cycle remains extremely small. It therefore appears that this late peak occurs just before the return of cells to G_0.

One possibility is that these cells are stimulated cells that have been blocked in the midportion of classic G_1, perhaps at the restriction point, and that the phenomenon observed is merely one of cell-cycle blockade. However, if the phenomenon represents only blockade, then cells stimulated to divide for the first time on day 4 would also be blocked. This is not the case, because vinblastine completely eliminates the late population, suggesting that mitosis, and not merely stimulation, is a prerequisite for entry into this stage. In addition, all cells in this stage incorporated BUdR added to the cultures, as manifested by decreased F_{530} values, implying a prerequisite for S phase as well. There is a strong correlation between the total number of cells that appear in this late stage and the total [^{3}H]thymidine uptake of the culture, which suggests that all the cells that enter S phase also enter this later stage. A second implication that may be drawn from these high correlations is that individual cells remain in this stage for <24 h, because cells that remain longer would be counted twice in the summed percentages.

From the above data, it appears that the late cell-cycle stage is in fact different from the G_1 phase and is not the result of cell-cycle blockade. An alternative explanation is that cycling cells destined to return to G_0 enter a specific state after their final mitosis, during which RNA content is relatively uniform among cells and remains constant for at least several hours. If the latter explanation holds, then this stage represents a separate cell-cycle phase that one might designate "G_3."

On subsequent days, this population of cells is replaced by increases in the G_0 population, cells with less RNA. A major biochemical change in the "G_1 to G_0 transition" that has been noted previously is a decrease in RNA synthesis (10, 23).

Whether the phenomenon observed here is the result of simple blockade of the cell cycle within G_1 or a more complex cell-cycle phase, it was important to exclude the possibility that its appearance is a result of the continuing presence of lectin in the cultures. The lack of a change in this stage in Con A–stimulated cultures produced by extensive washing of the cells with medium and with α-methyl-D-mannoside to remove Con A suggests that this is not the case, but it is still possible that some lectin remained even after the above procedures. Alternatively, lectin may stimulate some cells in culture before washing to continue to produce a factor after washing that results in the observed effect. Therefore, it remains uncertain whether the observation made here holds only for stimulation by lectins or whether it occurs with stimulation by other means. The data from a flow cytometric study of the mixed lymphocyte reaction (5) show a histogram on day 6 similar to those illustrated in Fig. 2, raising the possibility that this late peak occurs in that system as well. In addition, it is not known whether this phenomenon occurs in cycling cells other than human lymphocytes.

One application of the observations in the present study relates to the strong correlation between the number of cells in the late peak and [^{3}H]-thymidine uptake. Similar correlations were not found for the cytometric determinations of the number of cells in the other cell-cycle phases. Determination of the percentage of cells in the late peak on days 3–6 appears to give a reasonable measure of the number of cells that have cycled. It thus could replace standard [^{3}H]thymidine uptake methods with the advantage of increased sensitivity of 5- to 50-fold.

The present study confirms the usefulness of flow cytometric techniques for investigation of cell-cycle regulation. Further adaption of the staining and cytometric techniques to other cell types will help determine whether the observations on this lympocyte model hold in a more general setting.

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