Cell Cycle-related Changes in Nuclear Chromatin of Stimulated Lymphocytes as Measured by Flow Cytometry

Zbigniew Darzynkiewicz, Frank Traganos, Thomas K. Sharpless, and Myron R. Melamed
Cytology Service, Memorial Sloan-Kettering Cancer Center, New York, New York 10021

SUMMARY

Flow cytometric techniques have been developed to assay lymphocyte stimulation as reflected by the increase in the cell transcriptional activity and cell progression through the cell cycle. The metachromatic fluorescent dye, acridine orange, is used to (a) stain DNA and RNA differentially in individual cells, and (b) stain nuclear chromatin after removal of cellular RNA by RNase and cell pretreatment at acidic pH.

Stimulated cells with diploid DNA content (G1) have an increased content of stainable RNA that makes it possible to distinguish them from nonstimulated (G0) cells. G0 cells can also be distinguished from G1 cells based on differences in stainability of their nuclear chromatin after treatment with acid. Mitotic indices can be scored automatically, inasmuch as the metaphase chromatin stains differently than does chromatin in the interphase cells. Altogether, the numbers of cells in the G0, G1, S, G2, and M phases may be obtained rapidly and with great accuracy. The cell transcriptional activity can be correlated with changes in nuclear chromatin (e.g., during the transition from G0 to G1). The two independent techniques may also prove to be useful in recognizing and quantitating noncycling cells in other cell systems.

The possible mechanisms responsible for differential stainability of nuclear chromatin in cells at different phases of the cell cycle are discussed.

INTRODUCTION

We have shown previously that lymphocyte stimulation can be rapidly assayed by flow cytometry after differential staining of cellular DNA and RNA with the fluorescent, metachromatic dye AO3 (8, 23–25). In particular, subpopulations of G0, G1, S, G2 + M cells can be discerned and quantitated. The accumulation of stainable RNA per cell, for each of these subpopulations, can also be measured (8).

We now propose a new method of analyzing lymphocyte stimulation based on differential stainability of nuclear chromatin with AO (10, 11). This method, like the previous method (8, 23, 25), allows one to distinguish stimulated (G1) from nonstimulated (G0) cells and to quantitate cells in other phases of the cycle (S and G2). In addition, the new method makes it possible to recognize and quantitate cells in mitosis and thus to determine mitotic indices automatically. The distinction between G0 and G1 cells is based on an entirely different principle than before (8, 23). The methods, therefore, complement each other and, when used together, provide information regarding the positions of cells in the cell cycle, their transcriptional activities (i.e., accumulation of RNA), and changes in nuclear chromatin.

MATERIALS AND METHODS

Cells

Cell collection, lymphocyte separation, and culture conditions were described in detail previously (5, 8, 23, 25). Briefly, the blood was collected by venipuncture of healthy volunteers. Leukocytes were separated from heparinized blood by gradient centrifugation on Ficoll:Isopaque (LymphoPrep; Nyegaardo, Oslo, Norway). The mononuclear cells, rinsed with phosphate-buffered saline [0.15 M NaCl, 0.15 M NaH2PO4-Na2HPO4 (pH 7.4)], were suspended in Eagle’s basal medium containing 15% fetal calf serum and subcultured on plastic dishes to remove most of the monocytes (5). The nonadhering cells were then suspended in Eagle’s basal medium containing 15% fetal calf serum and incubated at 37°. Lymphocytes were stimulated either by addition of PHA-P (2 µg/ml; Grand Island Biological Co., Grand Island, N. Y.) or by addition of irradiated allogeneic lymphocytes from nonrelated donors (“mixed-lymphocyte cultures”), as prepared as described previously (25). Cultures with an increased number of mitotic cells were obtained by treatment with Colcemid (0.05 µg/ml) for periods of time as specified in the chart legends. For the synchronization of cells in G1 phase, the cultures incubated with PHA for 24 hr were treated for the next 24 hr with 2 mM hydroxyurea or 2 mM thymidine, as described Ref. 8.

Cell Staining

Simultaneous Staining of RNA and DNA. For simultaneous staining of cellular RNA and DNA, the following procedure was used. Cell suspensions (0.2 ml) (0.2 to 0.4 x 10^8 cells), withdrawn directly from tissue cultures, were mixed with 0.4 ml of 0.05 N HCl:0.15 N NaCl:0.1% Triton X-100 (v/v

Received June 9, 1977; accepted September 20, 1977.
Fluorescence Measurements

Fluorescence of individual cells was measured in the FC200 Cytofluorograf (Ortho Diagnostic Instruments; Bio/Physics Systems, Westwood, Mass.) interfaced to a Nova 1220 minicomputer (Data General Corp., Southboro, Mass.). The cells, suspended in AO solution, were transported through the instrument at rates of about 200/sec. Fluorescence and light scatter signals were generated by each cell as it passed through the focus of a 488 nm argon ion laser beam. The red fluorescence emission (F<sub>red</sub>) and green fluorescence emission (F<sub>green</sub>) for each cell were separated by optical filters and recorded by separate photomultipliers, and the integrated values of the cell were stored in the computer. Background fluorescence of the AO solution in which cells were suspended was automatically subtracted. The pulse width, i.e., the time taken by the cell to pass through the illuminating beam, was also recorded and was used to distinguish single cells from cell doublets, as well as to estimate cell size, as described previously (19). More detailed description of the instrument and the computer data-handling system are given elsewhere (7, 9, 19, 23-25). The data presented were based on a total of 5 x 10<sup>3</sup> cells/sample. Cell doublets and higher aggregates were excluded. Each experiment was repeated at least 5 times.

RESULTS

Differential stainability of DNA versus RNA in individual cells makes it possible to discriminate several subpopulations of lymphocytes in stimulated cultures (8, 23, 25). Since a detailed description of the technique and its application in studies of lymphocyte stimulation are given elsewhere (8, 23), we present here only an example of the data obtained by this method (Chart 1). Thus, using DNA stainability, one can discriminate among cells with diploid DNA content (G<sub>e</sub> and G<sub>i</sub>), those with tetraploid DNA content (G<sub>4</sub> and M), and cells with intermediate values of DNA, i.e., cells in S phase. The specificity of DNA staining (F<sub>DNA</sub>) is rather good as reflected by a low variation coefficient for the mean value of F<sub>DNA</sub> of the peak of G<sub>e</sub> + G<sub>i</sub>. An additional parameter, namely, RNA stainability (F<sub>RNA</sub>) enables one to discriminate between nonstimulated G<sub>i</sub> cells and cells with increased RNA but with the same DNA content (G<sub>e</sub> cells). Altogether, this simple measurement provides information regarding: (a) total number of cells in culture; (b) number of nonstimulated G<sub>i</sub> cells; (c) total number of stimulated cells, i.e., cells that initiated RNA synthesis as determined by F<sub>RNA</sub> values that exceed those in control cultures; (d) number of G<sub>i</sub>-stimulated cells; (e) number of cells in S phase; (f) number of cells in the G<sub>2</sub> + M phases; (g) number of macrophages in cultures in which monocytes are not removed (25); (h) the extent of RNA accumulation per cell in particular classes of stimulated cells; (i) the ratio of stainable RNA to stainable DNA per cell; (j) number of dead cells [very low stainability (8, 25)]. This method was applied in studies of lymphocyte stimulation by mitogens (8, 23) and in allogeneic mixed-lymphocyte cultures (25).

While Chart 1 illustrates staining of DNA versus RNA,
Charts 2 to 4 represent differences between lymphocyte subpopulations based on staining of nuclear DNA after specific extraction of cellular RNA and partial denaturation of DNA by acid (see "Discussion"). Nonstimulated lymphocytes (G₀) are represented on scattergrams or frequency histograms as a single cluster of cells with minimal F₅₃₀ and with minor variation in F₅₃₀ and F₆₀₀ values. Cell transition from G₀ to G₁, which occurs during the first day of stimulation in cultures (for reviews, see Refs. 15 and 16), is reflected by an increase in F₅₃₀ and a decrease in F₆₀₀ (Chart 2B). This change occurs in the absence of any DNA synthesis; we have presently observed that suppression of DNA synthesis by 2 mM hydroxyurea, as described in Ref. 8, or by 2 mM thymidine (not shown) does not prevent this change. The transition from G₀ to G₁ is already evident after 6 hr in culture with mitogen and continues during the first 2 days after stimulation (Chart 2B). At that time, there is no clear separation of cells into 2 distinct clusters as on the third day and later (Chart 2, compare B with C and D). Apparently, the recruitment of cells from the G₀ to G₁ phase, like recruitment into S phase (15,16), is asynchronous. After 2 days in culture with PHA, the number of cells in transition from G₀ to G₁ drops, and G₀ and G₁ appear as distinct clusters.

When cells initiate DNA synthesis, there is a simultaneous increase in their F₅₃₀ and F₆₀₀ values (Chart 2, C and D). After 3 days of incubation with PHA, clusters of cells in G₀, G₁, S, G₂, and M phases may be distinguished. At that time the peaks of G₁ and G₀ cells are separated (Chart 3). Cells in mitosis, in comparison with G₂ cells, have markedly increased F₆₀₀ and lowered F₅₃₀ values. The number of cells in mitosis increases in proportion to the time of incubation with Colcemid, and this coincides with the lowering of cell number in the G₁ cluster (Chart 2D). The change in DNA stainability during the transition from G₁ to mitosis has been described recently for other cell types (10,11); in this respect, the difference between G₂ and M cells, as presented here for stimulated lymphocytes, is of the same magnitude.

Chart 3. Computer-drawn frequency histogram showing various subpopulations of lymphocytes in the PHA-stimulated cultures. Cells from the 3-day-old PHA culture were mixed in a 1:1 proportion with cells from a culture maintained with PHA for 3 days and then blocked with Colcemid for 12 hr; compare cell clusters as identified in Chart 2 with the peaks of the histogram.

In such a cell mixture, not only is the peak representing cells in mitosis apparent (i.e., as it is in the Colcemid-treated culture alone) (Chart 2D), but also the peak of G₁ cells is clearly evident due to the enrichment by G₁ cells, which are in large proportion in the culture not treated with Colcemid (Chart 2C). There appears to be an increase of cells in the mid-S phase in such a mixture.
Z. Darzynkiewicz et al.

Chart 4. Fluorescence values and size of the cell nucleus of lymphocytes in stimulated cultures. Computer-drawn scattergrams illustrating $F_{530}$ and $F_{610}$ values (left) and also $F_{530}$ and $F_{610}$ pulse width ($W$) values (right, arbitrary units) of lymphocytes from the 3-day-old PHA cultures. The conditions of culturing and the scattergram of $F_{530}$ and $F_{610}$ values are described in Chart 2C. The $G_0$ cells (i.e., cells with the lowest $F_{530}$ and $F_{610}$ pulse width (nuclear diameter) value (19).

character and magnitude as in the case of the human leukemic (SK-L7) or murine erythroleukemia cell lines described before (10, 11).

Cell staining under conditions as described in Chart 2 allows one to discriminate between cells in $G_2$ phase and cells in mitosis as well as between $G_0$ and $G_1$ cells. This pattern of cell stainability, as illustrated in Charts 2 and 3, is highly reproducible; it was repeated in over 20 different sets of lymphocyte cultures, including cultures incubated with various mitogens (PHA, pokeweed mitogen, allogeneic mixed lymphocytes), studied at various times after stimulation (0 to 6 days), and with the use of various methods of cell synchronization (i.e., cell blocking in the $G_1$ phase by 2 mM hydroxyurea or 2 mM thymidine, by the Colcemid block in mitosis, or by cell release from these blocks).

Chart 3 illustrates the respective positions of the detectable subpopulations of lymphocytes from stimulated cultures. In this case, cells from 2 different cultures were mixed together before staining. One of these cultures was exposed to Colcemid while the other was asynchronous. As a result of this mixing, the peaks representing particular subpopulations are emphasized. Since accumulation of cells in mitosis (in the absence of $G_0$ to $G_1$ transition) lowered the $G_1$ peak (compare $G_1$ clusters in Chart 2C and D) in the population of mixed cells (Chart 3), the $G_1$ peak is restored by the $G_1$ cells from the asynchronous subpopulations in which the $G_1$ peak prevails. As a result of such mixing, there is also apparent enrichment of cells in mid- and late $S$ phase.

In addition to measurements of the intensity of cell fluorescence, the pulse width of individual cells was also recorded. The pulse width, or "time of flight" of the fluorescent object through the narrow focus of the laser beam, is directly proportional to the diameter of that object (19). Chart 4 illustrates the distribution of $F_{530}$'s and pulse width values of cells in stimulated cultures. Nonstimulated cells that exhibit minimal $F_{530}$ also have the smallest nuclei; as a matter of fact, they are the only cells with nuclear sizes below a value of 30 units. Additionally, these data confirm that small, nonstimulated lymphocytes are the cells located in the peak (cluster) with the lowest $F_{530}$ values.

Experiments were also performed in which both methods of cell staining were applied to the same PHA-stimulated cultures to correlate changes in RNA stainability with chromatin changes and to compare both methods. The results of such a comparison are included in Table 1.

Observation of cells stained with AO (after RNase and acid treatment) under UV fluorescence microscopy reveals that only cell nuclei (or chromosomes) were stained, that the staining is rather granular, and that some areas in the nuclei fluoresce green while others emit red fluorescence. When the same cells are viewed in phase contrast and then in UV light, it becomes apparent that areas of condensed chromatin (including metaphase chromosomes) fluoresce red while the green fluorescence is confined to the euchromatic regions. Since the extent of euchromatin in small nonstimulated lymphocytes is much lower than in blast cells, the former exhibit much less green fluorescence. Both the green and red fluorescence disappear after treatment with DNase.

DISCUSSION

Lymphocytes are dormant cells that can be stimulated in vitro by a variety of agents to differentiate into blastoid cells and undergo mitosis (18). In the course of stimulation, RNA synthesis is initiated at an early stage and is followed by protein and DNA synthesis (see Refs. 15 and 16 for review). Assays of lymphocyte stimulation are widely used in clinical and experimental immunology as well as in basic biological research.

We propose 2 entirely different cytofluorometric methods to analyze lymphocyte stimulation. Method 1, based on differential staining of RNA versus DNA, has already been described in detail (8, 23-25) and was used here with minimal modifications. In this method, pretreatment of cells with Triton X-100 at low pH makes them permeable to the dye while at that same low pH nucleic acids remain insoluble in these unfixed cells. Subsequent staining with AO in the presence of chelating agents (EDTA, citrate) results in the denaturation of all cellular RNA that stains metachromatically (7) due to electrostatic binding of AO and dye stacking (4), while native DNA intercalates the dye and stains orthochromatically (12). All the details of this procedure are given elsewhere (8, 23-25).

An example of the discrimination obtained by this method is presented here (Chart 1) to be compared with the discrimination provided by Method 2.

Method 2 is based upon differential stainability of nuclear DNA after cell treatment at low pH. Several lymphocyte subpopulations may be distinguished because they differ
Comparison of the main features of 2 different methods of flow cytometric analysis of lymphocyte stimulation

<table>
<thead>
<tr>
<th>Features</th>
<th>Method 1 (DNA/RNA staining)</th>
<th>Method 2 (chromatin staining)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of cell subpopulations that may be discriminated*</td>
<td>4: G0, G1, S, G2 + M</td>
<td>5: G0, G1, S, G2, M</td>
</tr>
<tr>
<td>Measurement of G0 to G1 transition</td>
<td>Based on RNA content</td>
<td>Based on chromatin changes</td>
</tr>
<tr>
<td>Earliest time the G0 to G1 transition observed**</td>
<td>8 hr after addition of PHA</td>
<td>6 hr after addition of PHA</td>
</tr>
<tr>
<td>Measurement of G1 to M transition*</td>
<td>Not possible with this method</td>
<td>Possible, based on chromatin changes</td>
</tr>
<tr>
<td>Measurement of transcriptional activity*</td>
<td>Possible, based on the increased accumulation of DNA; may be calculated for the particular cell subpopulation</td>
<td>Not possible by this method</td>
</tr>
<tr>
<td>Recognition of dead cells*</td>
<td>Based on lower stainability in F550</td>
<td>Based on lower stainability in F550 and F430</td>
</tr>
</tbody>
</table>

* There was less than 3% difference in the number of cells in G0, G1, S, and G2 + M subpopulations when the same 3-day-old culture of PHA-stimulated lymphocytes was examined by both methods simultaneously, and the results of the first method were compared with those of the second.

** By both methods, 20 to 25% of the lymphocytes showed changes at that time (i.e., 6 and 8 hr). During the first 48 hr a continuous transition to G1 was observed; on frequency histograms the G0 and G1 peaks were not fully separated, since many cells had intermediate fluorescence values. Nearly full separation of G0 and G1 subpopulations was seen in cultures incubated with mitogens for 3 days and longer.

While differential stainability of DNA and RNA, which allows one to distinguish the lymphocyte subpopulations (as evidenced in Chart 1), is quite well understood (7, 8, 23, 25), the changes in nuclear chromatin that are responsible for the differences in the stainability of nuclear DNA (Charts 2 to 4) are less clear. It is likely that F550 reflects the extent of denatured DNA that reacts with AO electrostatically (4, 9) while F430 represents AO intercalating into double-stranded DNA (9, 12). The experiments presented previously strongly suggest that the DNA in metaphase chromatin in situ is more sensitive to heat (9) or acid (10, 11) denaturation than it is in interphase chromatin. It is likely that acid pretreatment at pH 1.5 that removes factors that restrict intercalation of AO into DNA also initiates DNA denaturation (11). Subsequent cell staining at pH 2.6 either potentiates this denaturation or maintains the degree of denaturation occurring at pH 1.5. Present data suggest, therefore, that denaturation of DNA at pH 1.5 is more extensive in the dormant, condensed chromatin of metaphase or G0 cells than in the active, dispersed chromatin of the cycling, interphase cells. There are other numerous differences in situ between condensed chromatin of dormant cells and the dispersed chromatin of cycling cells, as reflected by the template activity or sensitivity to various intercalating probes [see review by Baserga (1)].

The molecular mechanisms and factors associated with chromatin packing that influence the sensitivity of DNA to acid or heat denaturation are unknown (13, 21). These factors, however, appear to be lost during chromatin isolation, since DNA denaturation in chromatin isolated from metaphase and interphase cells is identical (20). However, other features of the chromatin structure, i.e., as reflected by the increased ellipticity in circular dichroism spectra or binding of intercalating dyes, remain different in chromatin preparations isolated from active versus dormant cells (1).

The difference between dormant noncycling (G0) and cycling cells (G1) in other cell systems (i.e., between noncycling and cycling fibroblasts) may not be of the same character as the difference between nonstimulated (G0) and stimulated lymphocytes presently described. For in-
stance, the chromatin changes during the G_0 to G_1 transition that we present here or the changes during transition from interphase to metaphase (9-11) are of an entirely different character than the chromatin changes in fibroblasts described recently by Nicolini et al. (17). Namely, we (22) and others (2, 3, 14) have been unable to find any difference between nonstimulated and stimulated lymphocytes in the extent of DNA accessible for intercalation of AO or ethidium bromide measured over a wide range of dye concentration and dye:DNA ratio, i.e., under conditions described by Nicolini et al. In addition, the difference that we present is observed only after cell treatment with acids at pH's below 1.9 (10, 11). In contrast, chromatin changes described by Nicolini et al. are reflected by differences in the accessibility of DNA to intercalating dyes in untreated cells (17).

Regardless of the mechanisms that might be responsible for the different stainingability of G_1 versus G_0 or G_2 versus M cells, the method provides an opportunity to investigate in a very detailed way the kinetics of the cell cycle in lymphocyte cultures. Subpopulations of lymphocytes in the G_0, G_1, S, G_2, and M phases of the cell cycle may be assayed with great accuracy. Accumulation of cells in mitosis may be scored easily, based on measurements of several thousand cells per sample. In conjunction with Method 1, which in addition to the cell cycle also measures the content of stainable RNA per cell, it is possible to analyze cell transcriptional activity, which can then be correlated with changes in nuclear chromatin. Such parameters as the ratio of stainable RNA to stainable DNA per cell, or sensitivity of chromatin to acid treatment, may also prove to be useful parameters for discriminating cell subpopulations in other cell systems. These parameters are presently being evaluated in this laboratory for subclassification of human leukemias and as a possible indication of response to treatment or guide in the choice and timing of drug therapy.

ACKNOWLEDGMENTS

We are greatly indebted to Dr. John Hansen for providing us with lymphocyte cultures during the initial phase of our work. We also thank Robin Nager for her assistance in the preparation of the manuscript.

REFERENCES

Cell Cycle-related Changes in Nuclear Chromatin of Stimulated Lymphocytes as Measured by Flow Cytometry

Zbigniew Darzynkiewicz, Frank Traganos, Thomas K. Sharpless, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/37/12/4635

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.