Lymphocyte stimulation: A rapid multiparameter analysis
(cytocfluorometry/RNA and DNA measurements/cell cycle)

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ABSTRACT Several parameters of stimulation of individual lymphocytes are measured simultaneously by flow-cytocfluorometry after differential staining of cellular DNA and RNA with the metachromatic fluorescent dye acridine orange. The method provides a means of analyzing the progression of stimulated cells through the cell cycle (G1, S, and G2 + M), in addition to the measurement of RNA per cell; the RNA parameter is useful in distinguishing G1 from G0 cells. The multiparameter analysis may be of unique value in discriminating cases where the transcriptional and proliferative responses of lymphocytes to stimulants are not correlated.

Lymphocytes can be stimulated in vitro by a variety of agents to transform into blastoid cells and undergo mitosis (1). During stimulation, RNA synthesis is initiated at an early stage and is followed by protein and DNA synthesis (see reviews, refs. 2 and 3). Assays of lymphocyte stimulation are widely used in clinical and experimental immunology as well as in basic research, where the stimulation provides a model of cell differentiation involving rapid activation of the genome. The most common methods of assaying stimulation are based either on measurements of the synthetic activities of cell DNA by radioactive tracers or on changes in cell morphology as visualized by light microscopy (3).

We propose a simple cytocfluorometric assay of lymphocyte stimulation. It was developed from earlier efforts in this laboratory to differentially stain DNA and RNA in fixed cells by use of a metachromatic dye, acridine orange (4), and applied to distinguish stimulated from nonstimulated lymphocytes (5). Under appropriate conditions, acridine orange preferentially stains nucleic acids, among other cellular polyamions. The dye intercalates into the double helix as a monomer that fluoresces green (530 nm) (6), while electrostatic dye binding to the phosphates of single-stranded nucleic acids involves dye aggregation ("stacking") and dye–dye interactions that result in red fluorescence (640 nm) (7). To obtain differential stainability of DNA compared to RNA in situ, it is necessary to selectively denature any double-stranded dye–DNA prior to staining (4, 5), because otherwise the binding of acridine orange to double-stranded RNA is indistinguishable from binding to native DNA (4). This is achieved by treatment of cells with the chemating agent EDTA, which is known to unfold ribosomes (8).

RESULTS AND DISCUSSION

The present method involves initial treatment of unfixed cells with chemulating agents (citrate, EDTA) in the presence of the nonionic detergent Triton X-100, at pH 3.0, followed by staining with acridine orange at pH 3.8. Control experiments on cells first treated with DNase or RNase (Table 1) reveal that the detergent treatment makes cells permeable not only to the dye but also to nucleases, and that at least 88% of the green fluorescence (F550) is the result of acridine orange interaction with cellular DNA, while at least 87% of the red fluorescence (F600) of stimulated cells is due to RNA. This relatively good specificity in differential staining of DNA and RNA by acridine orange may be due to the following: (i) Exposure of permeable cells to chemulating agents and the dye, as in the case of fixed cells (4, 5) or isolated ribosomes (8), denatures double-stranded RNA in situ. (ii) Cell staining is done at pH 3.8, i.e., near optimal for the discrimination of DNA from RNA (11). In fact, morphological recognition of stimulated lymphocytes, based on RNA stainability with acridine orange, was shown to be optimal at pH 3.8 (12). (iii) The cells are stained in the presence of salt, at moderate ionic strength. This is expected to additionally increase the specificity of binding of acridine orange to DNA by intercalation, since in the presence of counterions electrostatic interaction of basic dyes with polyamions is markedly impeded while intercalation is affected only minimally (13). Nonstimulated lymphocytes stain very uniformly with acridine orange; they are represented by a single locus on the
Increasing these number of cells day first increases, but the cultured after intensities the accumulation to identify after hr Go-G1-S-G2 + (f) days. The presence of all and GRN values, dead cells excluded in all and G0 cells excluded in b, c, and e) is given below each scattergram. (a) Lymphocytes cultured in the absence of phytohemagglutinin for 3 days. The lymphocytes stained prior to culturing had distribution similar to the above except that the number of dead cells (with low F530, see f) was minimal (below 1%). (b) Lymphocytes cultured in the presence of phytohemagglutinin for 3 days. (c) Lymphocytes cultured in the presence of phytohemagglutinin for 3 days; 2 mM hydroxyurea was added 24 hr after phytohemagglutinin to inhibit DNA synthesis. Note the accumulation of cells in G1 phase. (d) Lymphocytes cultured with phytohemagglutinin for 2 days; 0.05 µg/ml of actinomycin D was added 24 hr after phytohemagglutinin. Minimal increase of cellular F>600 increased number of dead cells. The cells do not enter S phase. (e) Lymphocytes cultured with phytohemagglutinin for 3 days. Vinblastine (0.2 µg/ml) was added 16 hr before cultures were terminated. There is a marked increase of cells at G2 + M phase. (f) Lymphocytes cultured with phytohemagglutinin for 3 days. The thresholds for F530 and F>600 are masked to discriminate cell subpopulations. The control experiment, designed to block, or to synchronize cells in particular phases of cell cycle, were used to identify the positions of these subpopulations and the thresholds, as presented. In the absence of the blocks, there is a continuity in the G0→G1→S→G2 + M transition; the subpopulations thus are not fully separated from one another.

scattergrams in which individual cells are plotted according to their respective values of F530 and F>600 (Fig. 1a). During the first day of stimulation, the F>600 of some of the lymphocytes increases, but the F530 of all the cells remains unchanged. On the second day of stimulation, there is a further increase in the number of cells with higher F>600; the mean value of F>600 of these cells also increases (Fig. 2). In addition, among the cells with high F>600 there appears a subpopulation of cells having increased F530. In 3-day-old phytohemagglutinin cultures (Fig. 1b), there is a further increase in the number of cells with high F530 values. Inhibition of DNA synthesis by hydroxyurea prevents this increase (Fig. 1c). Actinomycin D, on the other hand, precludes the appearance of cells with increased F>600 (Fig. 1d). In vinblastine-treated cultures, there is an accumulation of cells with F530 values twice as high as in the nonstimulated cultures (Fig. 1e).

The above results confirm the data of DNase and RNase treatments (Table 1), indicating that stainability of individual cells with acridine orange is closely related to the content of DNA and RNA per cell. Thus, the method makes it possible to
The lymphocytes from 2-day-old phytohemagglutinin-treated cultures were treated with a solution containing 0.1% (vol/vol) Triton X-100, 0.2 M sucrose, and 20 mM citrate-phosphate buffer, at pH 3.0, for 1 min. The cells were then centrifuged, suspended in 0.25 M sucrose–5 mM MgCl₂–20 mM Tris-HCl (pH 6.5), and incubated for 20 min at 37°C in the absence of nucleases (control), with 10³ units/ml of RNase A (Worthington Biochemical Corp., Freehold, N.J.), or with 4 × 10³ units/ml of DNase I (Worthington). After centrifugation the cells were resuspended in acidine orange solution; the fluorescence of cells (mean values of G₀ + G₁ population for F₅₀₀; of G₁ for F₆₀₀ ± SD) are given in arbitrary units. Percent decrease of F₅₀₀ or F₆₀₀ as a result of nuclease treatment is given in parentheses. Pretreatment of cells with Triton X-100 was necessary to see the effect of nucleases.

analyze the progression of the cells through the cell cycle and to express the degree of stimulation in terms of the percentage of cells in a particular phase of cell cycle (Fig. 2).

In addition to information regarding the quantity of DNA per cell, simultaneous staining of RNA (F₆₀₀) allows one to distinguish stimulated (G₁) from nonstimulated (G₀) cells (Fig. 1f). Assuming that the intensity of F₆₀₀ remains in proportion to the quantity of RNA per cell, this parameter provides a measure of the progressive accumulation of RNA in the cell during stimulation prior to the onset of DNA synthesis. As can be seen from Figs. 1 and 2, the content of stainable RNA of the stimulated G₁ cells increases during 24–72 hr of stimulation; addition of 0.05 μg/ml of actinomycin D at 24 hr prevents this increase (Fig. 1d). Since actinomycin D at this low concentration selectively inhibits synthesis of large-molecular-weight rRNA, rather than mRNA or tRNA (14), it appears that it is mostly rRNA that contributes to the total cellular F₆₀₀ after acridine orange staining. The cells treated with actinomycin D do not enter S phase. This confirms the findings of Kay et al. (15) that inhibition of RNA synthesis by low doses of actinomycin D precludes DNA replication in stimulated lymphocytes.

The content of stainable RNA nearly doubles in G₂ + M cells in comparison with the G₁ lymphocytes (Fig. 2, the mean values of F₆₀₀). On the other hand, the S phase lymphocytes have more stainable RNA than the G₁ cells, but less than cells in the G₂ + M phase. During the fourth day of stimulation, the content of stainable RNA decreases in S and G₂ + M cells.

It is interesting to note that the lymphocytes entering S phase belong mostly to the middle and the right ("tail") region of the G₁ locus (Fig. 1 b and f), i.e., they have accumulated more RNA than the G₁ cells that are immediately adjacent to the G₀ locus. However, after a long-lasting block with vinblastine (Fig. 1e), while all the cells with higher RNA content appear already in the G₂ + M locus, the cells that enter S phase at that point have less RNA than the S cells in control cultures (left region of G₁ locus). The experiments on cultures blocked in G₁ by hydroxyurea and then released from the block reveal that the entrance of G₁ cells into S phase after the release is asynchronous; the first cells appearing in the S locus are the lymphocytes with the highest RNA content (far right "tail" of the G₁ locus) and are followed 2–3 hr later by cells from the middle region of the G₁ locus (not shown). All these experiments indicate that under normal conditions most cells entering S phase have already accumulated a relatively high content of RNA. The large RNA content per cell, however, does not seem to be an absolute prerequisite for cells to enter S phase. In vinblastine-treated cultures, the low RNA stainable cells may finally enter S phase, after the high RNA stainable cells have already passed the S phase and are prevented from entering the cycle by a block in mitosis.

By choosing appropriate thresholds for F₅₀₀ and F₆₀₀ (Fig. 1f) it is possible to obtain in a single measurement the following parameters for analyzing the progress of lymphocyte stimulation: (i) total number of measured cells; (ii) total number of stimulated cells, i.e., cells that initiated RNA synthesis as de-
terminated by $F>600$ values that exceed those in control cultures; 

(\textit{it}) number of nonstimulated cells (G0); (\textit{is}) number of G1, 
stimulated cells; (\textit{it}) number of cells in S phase; (\textit{ot}) number of 
cells in G2 + M phase; (\textit{ui}) the extent of RNA accumulation per 
cell in particular classes of stimulated cells (mean or histogram 
distributions of $F>600$ values for G1, S and G2 + M); and (\textit{ott}) number of dead cells. 

The method thus provides simultaneous analysis of the pa-
parameters of lymphocyte stimulation related both to DNA 
transcription and replication. Since it is quite possible that in 
certain immunodeficiencies or in immune reactions a lack of 
correlation between the proliferative and transcriptional re-
response of lymphocytes exists (16, 17), the present method might 
be of unique value in such situations. Likewise, the method may 
be helpful in recognizing lymphocyte differentiation into 
blastoid compared to plasmacytoid cells (18, 19). The quanti-
tation of the number of G0 compared to G1 cells at the stage of 
stimulation prior to cell division may also be of special value in 
evaluating the absolute number of cells that are triggered to 
stimulation in cultures. To this end, considering that cellular 
commitment to stimulation (as evaluated by DNA synthesis) 
is asynchronous (20) by applying DNA synthesis blocks (i.e., 
hydroxyurea, see Fig. 1c), it is possible to quantitate the maxi-
mal number of cells in culture that undergo the G0 to G1 tran-
sition. 

Other advantages of this method, when compared to those 
using radioisotopes, include its safety and economy as well as 
the absence of any ecological impact. Moreover, scintillation 
counts do not provide information regarding individual cells, 
while autoradiography is tedious and time consuming. 

The present method does not require cell rinsing, fixation, 
or centrifugation. Consequently, there is no cell loss during the 
steps preceding the measurements, and since virtually all cells 
from a culture may be analyzed, the method is applicable to 
microcultures. Thus, $10^5$ cells per culture may be stained and measured in a commercially available cytofluorometer within 
8 min. The method can easily be made fully automatic, short-
ening the time to 1–2 min per sample of $10^4$–$10^5$ cells, and is 
presently being adapted to analyze lymphocyte stimulation in 
allogenic mixed lymphocyte cultures. 

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