Capillary Tube Scanning Applied to Cell Growth Kinetics

Metabolism and drug sensitivity can be monitored by optical scanning of cells growing in capillary tubes.

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Numerous assay techniques are employed in cell biology and oncology for following the growth, mitosis, and transformation of various cells in a diversity of chemical and physical environments. A major commitment of laboratory resources must be made to use current plating and counting methods because they require much time and technical expertise. Many of these methods are too slow to be practical for certain clinical applications, such as tailoring chemotherapeutic treatment to a given patient. We report here an instrumental approach to monitoring the growth of cells in tissue culture media. Our technique requires only minimal technical skill and affords rapid results.

Cells placed in a capillary tube filled with tissue culture medium scatter pulses of light as a sharply focused beam of light scans the tube along its length. Pulses of light scattered in a forward direction at about 30° from the incident beam can be measured and classified electronically for comparison with a later scan of the same tube after incubation. Pulses which increase in magnitude, and new pulses, indicate changes in optical properties that are related to cell growth and multiplication. Pulses caused by nonviable cells, other particulate matter, and optical imperfections remain constant and can be subtracted. Repetition of the scan gives information about the time course of growth and multiplication of the cells. Using several cell systems of current interest we will demonstrate that cell growth and mutiplication can be monitored with this instrument and, by varying the chemical environment in which the cells are tested, we will indicate how questions of research and clinical interest may be quickly and conveniently answered.

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Instruments, Methods, and Materials

We modified a prototype of a commercial instrument (*1*) based on the capillary tube scanner described by Bowman *et al.* for counting bacteria and testing antibiotic susceptibility (*2*). The linear filament light source is focused on the inner surface of the capillary tube from below; light scattered 30° from the illuminating beam is collected by a photomultiplier above the tube. Twelve tubes with their axes horizontal are held in a tray which is carried by the scanning mechanism of the instrument. A tube is scanned as the tray carries it through the light beam parallel to the axis of the tube at the rate of 5 millimeters per second. After one tube is scanned, the mechanism moves the tray to bring the next tube on the tray into the light beam. Two dozen trays of 12 tubes each occupy only 30 by 15 by 15 centimeters in an incubator or on the laboratory bench.

We used one or more of four separate ways to record the signal of scattered light from the photomultiplier. (i) We recorded the signal directly as a function of time; this permitted us to make visual correlation of the growth patterns within the tubes. (ii) We integrated the signal electronically and recorded the integral for each tube at the end of each scan. (iii) We counted all pulses larger than some selected value. (iv) We counted all pulses exceeding one or more of five different amplitudes. The last method provided a coarse measure of the pulse-height (amplitude) distribution.

To prepare a sample for scanning, an aliquot of cells was dispersed in fresh medium containing antimetabolites or additional nutrients, or both, to give a final cell concentration of approximately 10^4 cells per milliliter. A sterile glass capillary tube of 1.8 mm external diameter, 1.5 mm internal diameter, and 102 mm length was filled with the cell suspension from a 1-ml pipet. Approximately 150 microliters of suspension was required to fill each capillary tube. The tube was sealed at both ends by means of a small oxyhydrogen torch or with commercially available hematocrit tube caps, and then cleaned with lens cleaning tissue to remove fingerprints and dust, and placed in a carrier tray. We made the initial scan of the tubes about 1 hour after they had been filled; the cells had settled and adhered to the lower surface of the tubes within this time. The data from the initial scan provided the baseline for each tube and the net changes from the baseline during the experiment indicated cellular response to the experimental conditions.

Preliminary investigations had been conducted to evaluate the feasibility of growing cells from tissue cultures in agar. Rat hepatoma cells which had been established in suspension culture were suspended in Spinner's 77 medium supplemented with glutamine and containing 0.4 percent bacterial agar (Difco) for sample preparation. These cells maintained their position within the agar, and scattered pulses of light from the cells could be detected. Approximately 40 percent of the observed cells appeared to have undergone mitosis within the agar after 55 hours. The generation time for these cells in suspension culture is about 24 hours. It was observed that cell mitosis could be suppressed by cycloheximide ($10^{-4}M$) added to the media, and that the effects of media composition, such

as the content of fetal calf serum, on cell growth could be studied with this system.

In the experiments described here we used liquid media. This appeared preferable to the use of an agar matrix because the rate of mitosis is not apparently depressed in liquid media as it is in the agar, and the background light scattering from the liquid is less than that from the agar. Furthermore, the preparation of media and samples is simpler with liquid media.

We used lymphoma cells (E.L.4) from mice of strain C57BL/6N for the studies described here. The cells were from a 9,10-dimethyl-1,2-benzanthrene–induced tumor and had been established in suspension culture (*3*). These studies were intended to demonstrate the utility of the scanning system and the consistency of the information provided by the scanner with the known characteristics of these cells. The cells were 30 to 40 μm in diameter and were mitotically active, with a generation time of approximately 20 hours in culture.

Two sublines of the lymphoma cells were used: wild-type lymphoma cells, and lymphoma cells which had been selected for resistance to 5-bromodeoxyuridine (BrdU) at concentrations of up to 100 micrograms per milliliter by the method of Littlefield (*4*). The cells were grown in Eagle's minimal essential medium as modified by Mishell and Dutton (*5*) and supplemented with fetal calf serum. Two groups of the BrdU-resistant cells were used: one had been maintained for more than 6 weeks in media containing 100 μg per milliliter of BrdU and others, having been selected for BrdU resistance, had been maintained subsequently in media without BrdU.

Conditions for growth preparation procedures, settings for the electronic amplification and counter thresholds, and typical growth behavior with the capillaries were determined by growing the cells in the modified Eagle's medium already described. Phenol red was added to indicate when cellular metabolism produced a large change in the *p*H of the medium. Methotrexate, BrdU, and a mixture (HAT) of hypoxanthine ($10^{-4}M$), methotrexate (amethopterin) ($5 \times 10^{-7}M$), and thymidine ($2 \times 10^{-5}M$) (final concentrations) were added to the medium to demonstrate of the effects of metabolic modifiers.

Experiments and Results

Preliminary experiments were performed to assess the distribution of cells and the pattern of their growth in the capillary tubes, the response of the scanning system to increases in the cell concentration, and the factors limiting the continued growth of cells in the capillary tubes.

Microscopic examination of the capillary tubes revealed that the cells settle from the medium within 1 hour of sample preparation and grow along the bottom wall of the capillary tube. Initially, the cells were virtually monodisperse, but within 72 hours the cell density increased and cell aggregates were evident. The cells and cell aggregates appeared to adhere to the glass surface once they had settled and their position within the capillary remained constant despite repeated movement of the carrier. This observation was consistent with serial chart recordings of the scattered light signal made every 3 hours for 72 hours of the same tubes. These recordings indicated that the location of the pulses did not change while they grew in amplitude, and new pulses were observed to appear and grow. Cell growth continued in sealed capillary tubes for periods in excess of 72 hours, as shown by periodic microscopic examination and by the increased number of pulses and pulse amplitude detected by the scanner. The factors limiting continued cell growth were apparently the limited nutrient supply and the increasing acidity of the medium secondary to cellular metabolic activity. Phenol red in the medium indicated that tubes containing initial cell concentrations in excess of 5×10^4 cells per milliliter became acidic after 2 to 3 days, and cell growth in these tubes ceased at this point. In tubes containing 10^4 cells per milliliter or fewer the *p*H was maintained at a level compatible with cell growth for 4 or 5 days. Careful sealing of the capillary tubes prevented gas exchange with the atmosphere.

A monotonic relationship was found to exist between the initial cell concentration and the number of pulses detected by the scanner 1 hour after sample preparation. Between a concentration of 10^4 cells per milliliter and 2.5×10^5 cells per milliliter the simple linear correlation coefficient of this relationship was found to be greater than 0.95. For the first 12 hours after sample preparation, the integrated out-

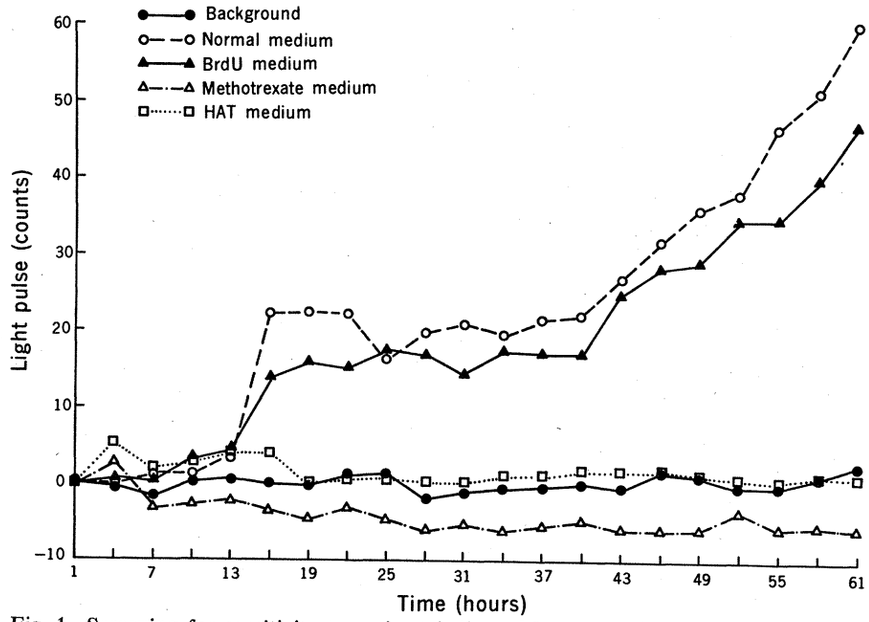

Fig. 1. Screening for sensitivity to antimetabolites. Mouse lymphoma cells selected for resistance to 5-bromodeoxyuridine (BrdU) were tested with $5 \times 10^{-7}M$ sodium methotrexate; BrdU, 100 μg/ml; and $10^{-4}M$ hypoxanthine, $5 \times 10^{-7}M$ methotrexate, and $2 \times 10^{-5}M$ thymidine (HAT medium). Scanner readings of cell growth in media free of antimetabolite (normal medium) and from medium without cells (background) illustrate normal growth and background noise, respectively. The ordinate shows the increase in number of pulses (or counts) detected by the scanner over the initial reading taken 1 hour after sample preparation. Abscissa units are hours from sample preparation time.

put of the scattered light signal correlated very closely (0.98) with the number of pulses detected; however, the integrator readings increased thereafter more rapidly than did the number of pulses detected. In fact, the number of pulses detected tended to plateau and even began to decline while the integrator reading was still increasing.

Figure 1 shows the results of an experiment to determine the antimetabolite sensitivity of mouse lymphoma cells which had been selected for resistance to BrdU at concentrations up to 100 μg per milliliter. Tubes containing BrdU or normal medium showed increased counts at 16 hours while tubes with methotrexate or HAT medium showed no increase or slight loss. The tubes without cells showed no significant increase or loss of counts, indicating the absence of growing organisms or significant accumulation of other light-scattering material. The difference in the observed count from the initial count (net count) was obtained by counting pulses at a preselected threshold value. This counter recorded pulses which exceeded the background fluctuation by about threefold; it could register light pulses scattered by individual cells. Counting conducted at slightly higher, more optimum thresholds revealed that significant growth could be detected within 10 to 15 hours.

The critical dosage of an antimetabolite needed to suppress mitosis was also determined with this instrument. Rather than exposing the cells to a battery of antimetabolites, we exposed them to a series of concentrations of a single agent. Sodium methotrexate, which was seen on screening to suppress mitosis of BrdU-resistant cells at concentrations of $5 \times 10^{-7}M$, was tested at concentrations from $10^{-11}M$ to $10^{-4}M$ in decade steps to identify the critical dosage for both BrdU-resistant cells and nonresistant wild-type lymphoma cells. Both cell types grew at methotrexate concentrations of up to $1.1 \times 10^{-8}M$, but were completely suppressed at concentrations of $1.2 \times 10^{-7}M$ and higher. In Fig. 2, the intregrator measurements for the BrdU-resistant cells are plotted against time. Within 8 to 12 hours the effects of the methotrexate were evident from these readings, attesting to the sensitivity of the signal integrator as a discriminant of growth. As is evident in Fig. 2, integrator readings from cell growth in media containing methotrexate at $10^{-11}M$ to $10^{-8}M$ concentrations is apparently greater than in media free of the antimetabolite. This observation of the behavior of the BrdU-resistant lymphoma cells has been observed consistently, but its biological significance, if any, is not clear. Once the critical concentration had been identified as being between $1.1 \times 10^{-8}M$ and $1.2 \times 10^{-7}M$ for the resistant cells, we conducted subsequent experiments using concentrations within this range. The lowest concentration needed to suppress mitosis of these cells was found to be approximately $1.0 \times 10^{-7}M$.

We evaluated the effects of BrdU on the lymphoma cells which had been selected for their resistance to BrdU concentrations of 100 μg per milliliter. The cells were grown in culture in two

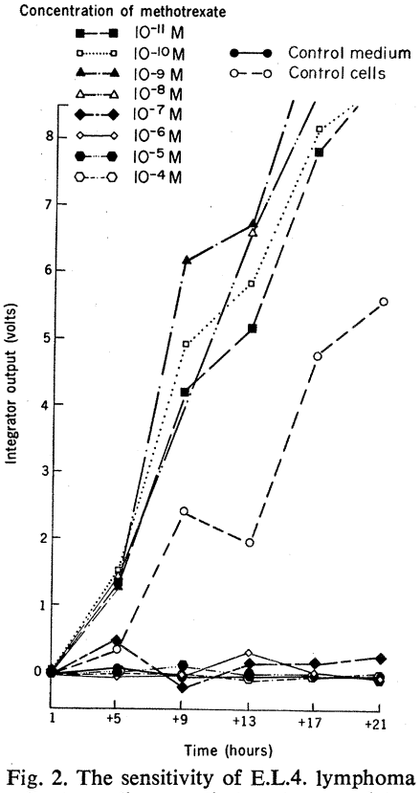

Fig. 2. The sensitivity of E.L.4. lymphoma cells to sodium methotrexate was determined by monitoring the growth of cells at antimetabolite concentrations ranging in decade steps from $10^{-11}M$ to $10^{-4}M$. Mitosis is suppressed at concentrations greater than or equal to $1.2 \times 10^{-7}M$, while growth continues at methotrexate concentrations less than or equal to $1.1 \times 10^{-8}M$. The ordinate represents the increase in the integral of the scattered light signal over the initial reading taken 1 hour after sample preparation. Each unit on the ordinate axis corresponds to 1 volt of output from the signal integrator. Abscissa units are hours from sample preparation time. Tubes filled with control medium contained no cells.

different environments prior to testing. One group of cells was grown for 6 weeks in media containing 100 μg of BrdU per milliliter, while a second group of the same cells was grown for 8 weeks in media free of BrdU. Both populations of cells were tested for their rates of growth distribution at concentrations of 0, 50, 100, 250, and 500 μg of BrdU per milliliter during a period of 3 days. The cells which had been grown in the presence of BrdU continued to grow in the capillary tubes, but they had consistently lower growth rates, as measured by signal integration, in all BrdU concentrations tested, than did those which had not been grown in the presence of the antimetabolite. Further, microscopic examination and analysis of pulse amplitudes demonstrated that the cells grown previously in BrdU did not grow in the tubes to cell densities where aggregates form, while the cells grown without BrdU did so up to concentrations of 250 μg per milliliter in the capillary tubes. The growth rates of both sets of cells were significantly reduced at a concentration of 500 μg per milliliter.

Wild-type lymphoma cells, lymphoma cells selected for their resistance to BrdU, and lymphoma cells once selected for BrdU resistance but grown for 10 weeks without exposure to BrdU were tested to determine their differential responses to methotrexate in medium supplemented with thymidine and hypoxanthine. Aliquots of cells from each of the three populations were dispersed in media containing $2 \times 10^{-5}M$ thymidine and $10^{-4}M$ hypoxanthine. Methotrexate was added to the suspensions to provide a final antimetabolite concentration of $5 \times 10^{-7}M$ without appreciably altering the cell concentration. The samples were monitored for growth over a period of 94 hours. Logarithmic plots of the integrator readings versus time for each of the three cell types are presented in Fig. 3. Nonresistant (wild-type) lymphoma cells continued to grow in the capillary tubes containing $5 \times 10^{-7}M$ methotrexate, albeit with integrator readings 30 to 40 percent lower than those recorded for the control tubes containing this cell population (Fig. 3A). The cells selected for their resistance to BrdU showed reduced growth for the first 24 hours in the presence of methotrexate and then died, while the cells in the control tubes continued to grow for over 52

hours (Fig. 3B). The cells which had been selected for BrdU resistance but grown without the compound for 10 weeks, demonstrated behavior intermediate between the wild and resistant cells. These cells continued to grow in the tubes containing methotrexate for 35 hours before dying, while the cells in the control tubes continued to grow for more than 80 hours. (Fig. 3C). Cell death was observed microscopically as cell lysis and electronically as a rapid decline in signal inetegrator reading.

Discussion

There are several discriminants of growth and mitosis which can be obtained by processing the photomultiplier signal. A computer interface for the photomultiplier would allow the optimum in interpretation; however, much information can be extracted from the signal with only simple electronic circuitry. One index of growth is the total number of pulses which exceeds a given threshold. The number of points of growth is reflected by counting, and if the cells are monodisperse this index should, and apparently does, correlate with the concentration of cells in the capillary tube. However, because several cells growing in a single aggregate may produce only one pulse, counting does not reflect the size of the aggregates or the total growth once the system is no longer monodisperse. This may explain the divergence of the integrator readings from the number of pulses counted once the cells have had sufficient time to undergo mitosis and form aggregates. The number of pulses may be further reduced if a number of growth points coalesce. By analyzing pulse amplitude, the sizes of the particles scattering the light can be evaluated qualitatively. In our system five channels of pulse amplitude were recorded, the least sensitive channel counted pulses 50 times larger than the most sensitive channel. This was adequate to distinguish the relative sizes of scattered pulses of light and to observe the growth of cells into multicellular aggregates. It appears that analysis of pulse ampltiude may be useful for evaluating biological processes where size changes are significant.

The discriminant most directly related to overall growth appears to be the integral of the photomultiplier signal. The integral reading reflects pulse frequency, amplitude, and width, which are themselves related to the number and size of the points of growth, respectively. All of these parameters have been used only as relative indications of growth. More quantitative and rigorous interpretation of the photomultiplier output is possible, and may increase the versatility of the scanner.

The studies presented here were conducted to demonstrate that this instrumental system can quickly and conveniently provide useful biological data which are consistent with the known properties of the cells under study. Screening of the antimetabolite sensitivity of the BrdU-resistant lymphoma cells revealed that these cells were unable to continue growing in the presence of $5 \times 10^{-7}M$ methotrexate plus thymidine ($2 \times 10^{-5}M$) and hypoxanthine ($10^{-4}M$). However, wild-type lymphoma cells which the scanner indicated could not tolerate more than $10^{-7}M$ methotrexate, could tolerate methotrexate concentrations of $5 \times 10^{-7}M$ in media supplemented with $2 \times 10^{-5}M$ thymidine and $10^{-4}M$ hypoxanthine. As an inhibitor of folic acid metabolism, methotrexate in sufficient concentrations blocks the direct pathways of purine and pyrimidine biosynthesis (*6*, *7*). Hypoxanthine allows cells containing pyrophosphorylase to bypass the block in purine biosnythesis

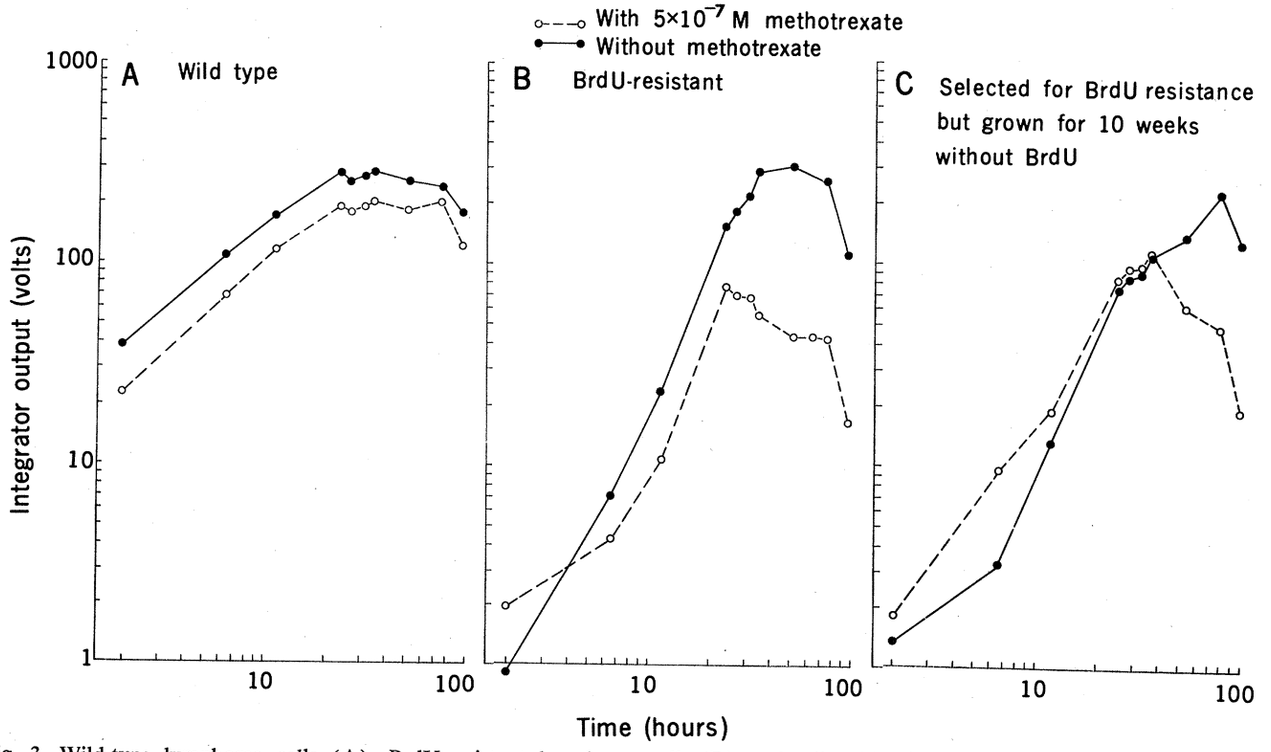

Fig. 3. Wild-type lymphoma cells (A), BrdU-resistant lymphoma cells (B), and cells which had been selected for BrdU resistance but had been grown without BrdU for 10 weeks (C) were tested to determine their differential responses to methotrexate ($5 \times 10^{-7}M$) in media supplemented with thymidine ($2 \times 10^{-5}M$) and hypoxanthine ($10^{-4}M$). The control tubes (no methotrexate) contained the thymidine and hypoxanthine but no methotrexate.

(*7*, *8*), while thymidine allows cells containing thymidine kinase to bypass the block in pyrimidine biosynthesis (*4*, *6*). Wild-type mammalian cells usually contain both enzymes necessary to bypass the metabolic blocks caused by methotrexate, provided they have exogenous sources of thymidine and hypoxanthine. Resistance or tolerance to BrdU, however, results from complete or almost complete absence of thymidine kinase activity. Thus, BrdU-resistant cells should not be expected to survive exposure to methotrexate despite the presence of exogenous thymidine and hypoxanthine. Hence, the observations made with the optical scanner are consistent with the proposed biochemical basis of BrdU resistance and the action of methotrexate. Further, the wild-type cells and the BrdU-resistant cells should be almost equally sensitive to methotrexate in media which do not contain thymidine, as there is no possible bypass of the methotrexate block for either type of cell. This expectation was also supported by data obtained with the scanner (Figs. 1 and 2).

Methotrexate sensitivity of the lymphoma cells employed in this investigation, determined by trypan blue exclusion tests, was found to be between $10^{-7}M$ and $5\times10^{-7}M$ (*9*). These values are within the limits of experimental error with the critical concentration of $1.0\times10^{-7}M$ determined with the optical scanner. Further, as would be anticipated, lymphoma cells which had been selected for their resistance to BrdU at concentrations up to 100 μg per milliliter were observed by the scanner to continue growing in media containing 100 μg of BrdU per milliliter at a rate comparable to that observed for the same cells scanned in media free of BrdU.

Reversion of BrdU-resistant cells to wild-type cells when the cells are grown in media free of BrdU has been reported by Littlefield (*4*) to occur in mouse fibroblasts. The examination of lymphoma cells which were once selected for BrdU resistance, but were grown subsequently without exposure to BrdU, offers the possibility of evaluating reversion rates for these cells. If the cells are grown in media containing methotrexate ($5\times10^{-7}M$), hypoxanthine ($10^{-4}M$), and thymidine ($2\times10^{-5}M$) those cells which have reverted to wild type should survive, while those which are still resistant should not. Lymphoma cells which were once selected for BrdU resistance and were then grown for 10 weeks without exposure to BrdU continued to show sensitivity to methotrexate in a medium supplemented with thymidine and hypoxanthine, thereby indicating that the population had not completely reverted to wild type. However, it appeared that these cells were less sensitive to methotrexate than were cells which had been grown continuously in a medium containing 100 μg of BrdU per milliliter. These results indicate that it may be possible to monitor the reversion of a BrdU-resistant population to a wild-type population with the optical scanner.

Summary

We have developed an optical scanning instrument which may become a valuable aid in a variety of cell studies. Cell multiplication can be readily monitored with the instrument. By varying the chemical environment in which the cells are tested, questions of research and clinical interest may be quickly and conveniently answered. It appears possible to investigate the chemotherapeutic value of experimental agents, and to scan the sensitivity of a given population of neoplastic cells to a variety of chemotherapeutic agents at several concentrations. The optical scanning system may also be used to investigate certain metabolic pathways of cells in tissue culture, to determine drug resistance, and to assess the effects of medium composition on cell growth.

Our studies of BrdU resistance in mouse lymphoma cells were done primarily to demonstrate the potential of this optical scanning method, and we do not intend to continue the cytokinetic studies at present. We trust that others will be stimulated to investigate further applications of this instrumental approach to problems in cell biology.

References and Notes

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