ABSTRACT

A rapid method has been developed which combines immunofluorescence and autoradiography and permits the double labeling of DNA. P388 murine leukemic cells were incubated with bromodeoxyuridine and tritiated thymidine simultaneously. After fixation, the sample was first processed with a monoclonal antibody to bromodeoxyuridine (RPMB I) so that any cell in S-phase was brightly fluorescent (RPMB technique). Next, tritiated thymidine grains were developed by autoradiography, and the result demonstrated fluorescence as well as black grains in each S-phase cell. P388 cells sensitive (P388S) and resistant (P388R) to 1-β-D-arabinofuranosylcytosine (ara-C) were incubated with bromodeoxyuridine and [3H]ara-C simultaneously. Processing by autoradiography and RPMB techniques revealed that all S-phase cells in the P388S sample demonstrated vivid "double labeling," whereas P388R cells only revealed bright green fluorescence in S-phase cells, but no grains, confirming a lack of ara-C incorporation into the DNA by this line. Finally, a computerized digital analysis system attached to a microphotometer was used to quantitate fluorescence and grains per cell, and the data demonstrated that the number of [3H]ara-C grains in each P388S cell was inversely proportional to the degree of fluorescence in that cell, indicating that DNA synthesis was inhibited by ara-C. In conclusion, a simple, easy-to-use double-labeling method has been introduced which will be useful to a wide variety of researchers, because this technique together with the digital analysis system offers the possibility of measuring drug sensitivities in individual cells.

INTRODUCTION

[3H]dThd has been used extensively to define cell cycle kinetics, and efforts have been made to detect 2 isotopes in the same cell simultaneously such as tritium and 14C to further refine cell cycle measurements (1). Since the β-energies of the 2 differ by a factor of 10, tritium β-particles traverse 1 μm in the emulsion, whereas 14C electrons penetrate beyond 10 μm so that double layers of emulsion separated by celloidin could help distinguish the 2 types of grains (2). Since both isotopes form black grains, whereas 14C electrons penetrate beyond 10 μm so that double layers of emulsion separated by celloidin could help distinguish the 2 types of grains, the only way to differentiate one type from the other is the depth of the image formed. Although extremely important in concept, the technique is so laborious in addition to being fraught with unreliable results that it has remained a rarely used, highly sophisticated tool in the domain of a few researchers.

We have reported a method using BrdUrd and a monoclonal antibody (RPMB I) against BrdUrd which can provide an accurate estimate of S-phase (fluorescent) cells within 4 h of acquiring the sample (3) without the tedious darkroom procedures involved in ARG. We have now combined this method with autoradiographic procedures so that radiolabeled compounds incorporated into DNA can be detected simultaneously with BrdUrd incorporated into DNA. The development of this dual-label methodology should permit reinstitution of the double-label cell cycle methodology described above but without the problems attendant in the past with this approach to cell cycle measurement. Additionally, we have extended this approach to the study of ara-C and have demonstrated the utility of this method in recognizing S-phase cells which are resistant to ara-C by virtue of their failure to incorporate ara-C into DNA and have also demonstrated the feasibility of assessing the relationship between ara-C incorporation into DNA and the effects of this agent on DNA synthesis.

MATERIALS AND METHODS

P388 murine leukemic cells were maintained in RPMI 1640 culture medium and passed every seventh day. A cell line resistant to ara-C was established by the addition of increasing doses of this drug to the liquid culture medium every week. After 20 passages, it was repeatedly confirmed that cells were able to grow in spite of the continuous presence of ara-C (5 μg/ml), and this line was subsequently designated as the P388AR line. P388S and P388R cells at a concentration of 1 x 10⁶ cells/ml were incubated for 2 h at 37°C in 5% CO₂ in the following groups: Group 1, [3H]dThd alone using 10 μCi/ml (specific activity, 77 Ci/mmol) along with 10⁻⁹ M FdUrd. Cells were washed once in cold dThd and 3 times in PBS and were processed for ARG as described below; Group 2, BrdUrd alone using 10⁻⁴ M BrdUrd and 10⁻⁵ M FdUrd and processed by RPMB technique described below; Group 3, [3H]ara-C alone using 10⁻⁶ M FdUrd and processed for ARG as described below; and Group 4, [3H]dThd (10 μCi/ml), 10⁻⁴ M BrdUrd, and 10⁻⁵ M FdUrd and processed for double labeling described below; and Group 5, [3H]ara-C (10 μl), 10⁻⁴ M BrdUrd, and 10⁻⁵ M FdUrd and processed for double labeling.

RPMB I Antibody. This is a monoclonal antibody prepared by standard cell fusion procedures for producing hybridomas using spleen cells from mice immunized with bromouridine and X-63/AG-8.653 HAP-sensitive myeloma cells. The monoclonal antibody has been shown to be highly specific for BrdUrd. Concentrations of dThd as high as 10⁻⁹ M did not inhibit binding in a radioimmunoassay, while BrdUrd at 10⁻⁴ M effectively competed with the antigen in a radioimmunoassay.

RPMB Technique. Groups 2, 4, and 5 were processed as follows. After completing incubations, cells were resuspended in PBS. Three drops of this suspension were pipetted onto Alcian blue-coated coverslips and fixed in 70% ethanol for 10 min. After a washing, 4 N HCl were poured on coverslips for 20 min at room temperature. Cells were next exposed to Tris buffer containing 0.1% bovine serum albumin for 1 h at 37°C in 5% CO₂. RPMB I diluted 1:100 with PBS:0.05% NP40 was
RENCE microscope.

For 30 min was carried out in PBS/Triton X-100 followed by a rinse in PBS:1% goat serum for 30 min at room temperature. A final rinse in PBS:Triton X-100 0.1% for 30 min and exposed to fluorescein isothiocyanate-conjugated goat anti-mouse antibody (Tago) diluted 1:20

DMA synthesis in that cell).

The 2 values are then displayed on the computer screen as a point in relation to their intensity of fluorescence and grain count. Each point, therefore, represents a single cell on the display screen.

RESULTS AND DISCUSSION

Table 1 summarizes the results of 3 separate experiments. The [3H]Thd labeling index of both P388S and P388R cells are almost the same (30% versus 35%, 29% versus 35%, and 19% versus 19%), and these values correlate with results obtained by the RPMB technique (Table 1). The correlation coefficient between the labeling index and RPMB processed BrdUrd-positive cells for individually labeled cells is $r = 0.99$. Fig. 1 demonstrates the vividly fluorescent P388S cells which incorporated BrdUrd into the DNA as detected by the monoclonal antibody (RMPI 1).

In the double label group, when [3H]dThd and BrdUrd were used simultaneously, every cell in all 3 experiments demonstrated the presence of bright fluorescence and black grains in both P388S and P388R lines (Table 1). The double label is vivid and unmistakable as seen in Fig. 2, where P388S cells incubated with [3H]dThd and BrdUrd demonstrate the presence of markedly double-labeled cells, and their distinction from nonlabeled cells is clear. When the automated DADS system was used to quantify grains and fluorescence over individual cells from these slides, a direct relationship between the 2 was noted both in the P388S and P388R groups. This was not unexpected, since both [3H]dThd as well as BrdUrd measure the synthesis of DNA.

In the [3H]ara-C experiment (Table 1, Group 3) developed by ARG, almost all S-phase cells from the P388S group incorporated [3H]ara-C into their DNA (e.g., labeling index of 30% with a [3H]ara-C index of 25% in Experiment 1, and so on), whereas none of the 35% S-phase P388R cells incorporated [3H]ara-C. This observation has already been reported by our group (4) and appears to be due to very low levels of deoxycytidine kinase in the P388R cells. When [3H]ara-C and BrdUrd were used simultaneously, [3H]ara-C grains were similarly detected in almost all fluorescent cells (Table 1; Fig. 3) of P388S group, but in none of

<table>
<thead>
<tr>
<th>Experiment</th>
<th>% of [3H]dThd-positive cells</th>
<th>% of BrdUrd-positive cells</th>
<th>% of [3H]ara-C-positive cells</th>
<th>% of BrdUrd-positive cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td>P388S 30 29 25 26/26 29/24</td>
<td>P388R 35 33 0 31/31 0/33</td>
<td></td>
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<tr>
<td>Experiment 2</td>
<td>P388S 29 33 23 26/26 27/25</td>
<td>P388R 35 32 0 31/31 0/31</td>
<td></td>
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<tr>
<td>Experiment 3</td>
<td>P388S 19 20 16 19/18 20/24</td>
<td>P388R 19 16 0 17/17 0/18</td>
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</table>
Chart 2. Automated quantitation of grains and fluorescence from a slide containing P388R cells incubated with BrdUrd and [3H]ara-C. Note a complete lack of [3H]ara-C incorporation and the resultant, relatively brighter fluorescent pattern of the cells, indicating the unhampered continuation of DNA synthesis. The lack of ara-C incorporation in this line is due to almost complete absence of the enzyme deoxycytidine kinase (see text for details).

In other words, the more [3H]ara-C a cell incorporated, the greater was the suppression in its DNA-synthetic ability. On the other hand, when P388R slides with BrdUrd and [3H]ara-C were examined in the DADS system, the positive cells revealed only bright fluorescence and no grains (Chart 2).

Gratzner and Leif (5, 6) reported the use of monoclonal antibodies raised against iododeoxyuridine, but their work was primarily in flow systems. In this study, we used a monoclonal antibody against BrdUrd, attached the cells firmly to coverslips by cationizing glass (7), and used bovine serum albumin to decrease nonspecific binding of the antibody. These latter 2 steps were important factors in allowing us to distinguish between fluorescent and nonfluorescent cells. The entire RPMB technique can be completed within 4 h to provide a rapid labeling index, and with the use of high-speed ARG (8), double labeling can be achieved within 24 h. The ability to place 2 easily distinguishable probes into cells which are actively synthesizing DNA will greatly facilitate cell cycle time estimates and will permit direct studies of the S-phase dependent incorporation of chemotherapeutic agents into DNA and other cellular macromolecules. Finally, with the digital analysis system, objective measurements of the amount of drug incorporated and the effects of the drug on the DNA synthesis of the cell under study were made possible. The ability to analyze the drug sensitivities of individual cells rather than the population as a whole opens up a new area of research, since we have the potential of recognizing even rare drug-resistant cells with this method.

REFERENCES

RAPID DOUBLE LABELING TECHNIQUE

Fig. 1. P388S cells incubated for 2 h with BrdUrd and processed by the RPMB technique demonstrate clearly fluorescent (S-phase) and nonfluorescent (non-S-phase) cells.
Fig. 2. P388R cells incubated for 2 h with BrdUrd and $[^3H]dThd$ and developed by ARG and RPMB technique reveal distinct double labeling in the S-phase cell. The other cells appear hazy because, as we focus on the grains, the rest of the field goes out of focus.

Fig. 3. P388S cells incubated with $[^3H]ara-C$ and BrdUrd demonstrate double labeling in the S-phase cells. Note the degree of fluorescence is less than for $[^3H]dThd$ and BrdUrd (Fig. 2).
Double Labeling of S-Phase Murine Cells with Bromodeoxyuridine and a Second DNA-specific Probe

Azra Raza, Charalampos Spiridonidis, Kalust Ucar, et al.

*Cancer Res* 1985;45:2283-2287.

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