Short Communication

Rapid fluorometric detection of drug resistant tumour cells

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A rapid estimation of mutation frequency in tumours would be invaluable in cancer management. Considerable evidence suggests that drug resistance in tumours frequently arises as a consequence of spontaneous somatic mutation (Goldie & Coldman, 1979) and therefore the mutation rate of a tumour will be an index of the potential to develop drug resistance. Previous studies have compared the mutation rates of cell lines using cloning assays. Cifone & Fidler (1981) reported a higher mutation rate in metastatic variant of UV-2237 fibrosarcoma cells than in a clone of the same cell line with lower metastatic potential. Warren et al. (1981) reported that fibroblasts from patients with Bloom syndrome, which predisposes individuals to various cancers, had a 10–15 fold higher mutation rate than did fibroblasts from normal individuals. These findings have been important in correlating malignant capacity with genetic instability, but unfortunately, the techniques used have limited general application since few human tumour cells will form colonies on plastic. An alternative assay, soft agar cloning, has also been used for the determination of mutation rates in a variety of mammalian cell lines, including Chinese hamster ovary cells (Li & Shimizu, 1983) and the L5178Y mouse lymphoma cell line (Irr & Snee, 1982). Again, the low cloning efficiency of human tumour cells in soft agar (Hamburger et al., 1978) and the fact that this method selects only mutant cells which clone in agar limits the use of this assay to determine mutation frequency in human tumours. This latter source of error might bias the estimation of the mutation rate in favour of more malignant cells, which generally have higher cloning efficiencies (Elmore et al., 1983).

Morley et al. (1983) and Albertini et al. (1982) have reported a limiting dilution technique for the measurement of mutation frequency in human lymphocytes. Culture conditions have been optimised for peripheral blood lymphocytes and a cloning efficiency of 20–60% has been obtained. Problems have been encountered, however, in studies of cultured lymphoblast lines with variable cloning efficiencies and different growth factor requirements (Seshadri et al., 1984). We have prepared a monoclonal antibody to bromodeoxyuridine (BrUdR) which recognises BrUdR-substituted DNA. BrUdR is a thymidine analogue readily incorporated into DNA by proliferating cells. Cells which incorporate BrUdR after exposure to a lethal concentration of a cytotoxic drug must be drug resistant and may be identified as such by the monoclonal antibody using immunofluorescence techniques. If the drug used is a selective agent for cells with a specific somatic mutation e.g. the purine analogue 6-thioguanine (6-TG) which selects for cells with a mutation at the hypoxanthine-guanine phosphoribosyltransferase (HGPRT) locus, then a measure of mutant frequency is obtained.

The monoclonal antibody to BrUdR was derived from the fusion of SP2/0-Ag14 cells (Shulman et al., 1978) to spleen cells from BALB/c mice immunized with bromouridine conjugated to bovine serum albumin (BSA) (Köhler & Milstein, 1975; Erlanger & Beiser, 1964). The antibody with highest affinity and specificity for BrUdR in the DNA strand was chosen using a competitive enzyme linked immuno-sorbent assay (ELISA) (Voller et al., 1978). Although this antibody identifies cells incorporating BrUdR after only minutes of exposure, an incubation period equivalent to one cell cycle time ensures that all cycling cells will have traversed S-phase and thus incorporated BrUdR into DNA. A fluorescence histogram of cells exposed to BrUdR for one cell cycle time is shown in Figure 1. Superimposed on this histogram of a highly fluorescent cell population is the background fluorescence peak obtained from stained cells which had not been exposed to BrUdR. This fluorescence is identical to the autofluorescence produced by unstained cells (not shown), indicating negligible non-specific binding of the anti-BrUdR antibody.

BrUdR incorporation and subsequent fluorescent staining, was used to investigate the 6-TG dose response relationships flow cytometrically. The dose response curves illustrated in Figure 2 show that for CCRF-CEM cells a concentration of over 30 μM 6-TG for 72h is necessary to stop DNA synthesis completely in 'wild type' cells. The dose-response
relationship detected by this assay is in close agreement with the results for peripheral blood lymphocytes and human skin fibroblast lines reported by others using limiting dilution and cloning assays, respectively (Albertini et al., 1982; Elmore et al., 1983). Using this information, conditions chosen for the detection of resistant cells were 72 h exposure to 100 μM 6-TG. This treatment is toxic to wild-type CCRF-CEM cells, but the growth rate of a mutant CCRF-CEM sub-line, HGPRT-1 (Waddell & Ullman, 1983) which is deficient in the enzyme HGPRT, is identical to that of untreated control cells under these conditions (data not shown).

Figure 3 illustrates fluorescence histograms of mutant, wild type and mixed cell populations exposed to 6TG. Histogram (A) shows that the HGPRT-1 mutants continue synthesizing DNA normally in the presence of a high concentration of 6-TG which stops DNA synthesis in the wild-type CCRF-CEM cells (B). In the latter case there is no fluorescence peak indicating that the cells are not incorporating BrUdR. Mixtures of mutants and wild type CCRF-CEM cells in varying proportions were exposed to 6-TG in order to investigate the sensitivity of the BrUdR assay in the rapid detection of low numbers of drug-resistant cells. The histogram (C) is obtained from a mixture of one HGPRT-1 mutant in 10⁴ wild-type CCRF-CEM cells. A small but easily distinguishable peak of fluorescent cells was obtained, representing
cycling mutant cells. The possibility of metabolic co-operation between the resistant and sensitive cells (Ochi et al., 1982) in mix experiments was examined but no evidence of this phenomenon was found. Accurate quantitation of rare cell sub-populations such as the HGPRT-cells in a mix experiment is being attempted using fluorescent beads to determine sample volume and thus cell number (Stewart & Steinkamp, 1982).

The BrUdR antibody method of identifying drug-resistant cells has many potential advantages. The method does not require high cloning efficiency conditions and provided adequate concentrations of 6-TG are used for a sufficient exposure period to kill sensitive cells, cells continuing to cycle can be assumed to be HGPRT-. As in the autoradiographic method for detection of 6-TG resistant lymphocytes described by Strauss and Albertini (1979), cells identified as 6-TG resistant by BrUdR fluorescence cannot be proved to be mutants since they cannot be clonally expanded and the HGPRT activity measured. However Dempsey and Morley (1983) have shown, in parallel cloning and autoradiographic mutation assays in peripheral blood lymphocytes, that provided a 6-TG concentration well along the plateau of the dose-response curve is used, resistant cells measured autoradiographically usually completely lack HGPRT.

This rapid and sensitive assay can be applied in a wide range of cells. It will find an important place not only as a drug resistance assay, measuring the proportion of cells that are resistant to a chemotherapeutic agent, but also as a mutation assay measuring the rates with which tumour cells acquire drug resistance. Clearly the precise conditions for studying drug resistance in human tumours remain to be defined and currently we are using this reagent to study biopsies of xenografted tumours. The duration of drug exposure in vitro and the timing of BrUdR addition may need to be varied depending upon the cytotoxic drug being tested and the growth fraction of the tumour. A similar antibody (Gratzner, 1982) has recently been used to measure the proliferating cell fraction in vivo (Morstyn et al., 1983) and to study cell cycle perturbations (Dolbeare et al., 1983).

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Figure 3 Fluorescence histograms obtained from a) HGPRT-1, a mutant subline of CCRF-CEM, b) the 'wild type' CCRF-CEM cells and c) a mixture of 1 mutant in 10⁵ non-mutant CCRF-CEM cells after exposure to 10⁻⁴ M 6-TG for 72 h with 10⁻⁵ M BrUdR added for the final 24 h. The total number of exponentially growing cells in each flask at the time of 6-TG addition was 1.7 × 10⁷. The HGPRT-1 cells (a) continued to cycle normally and at 72 h, 2 × 10⁶ cells were washed, fixed and stained for BrUdR incorporation, as described in the legend to Figure 1. The entire contents of flasks depicted in b and c, were harvested and stained, as substantial cell death had decreased the total number of whole cells (as determined by haemocytometer count) to <2 × 10⁶. The mutant cell population in c, has clearly expanded during this period and although accurate quantitation is not yet possible at least 800 fluorescent cells are detected in the histogram shown.
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