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An improved selection procedure for the rescue of hybridomas

A comparative study of methotrexate versus aminopterin

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Several of aminopterin's drawbacks such as photosensitivity and high toxicity prompted us to compare the ability of methotrexate to select for hybridomas. Assessing the effect of both drugs on X63/Ag 8.653 myeloma cells and hybrid cells secreting monoclonal antibodies by tritiated thymidine incorporation and percentage of viable cells, we have shown that methotrexate could be used in place of aminopterin for the rescue of hybrid cells in selective medium. In addition, methotrexate hybrids develop more rapidly and can therefore be processed earlier. The stability and low toxicity of methotrexate also favor its use in the selection of hybridomas.

Key words: Hybridoma; Selective medium; Methotrexate

Introduction

The basic technology for the production of hybridomas (Köhler and Milstein, 1975) consists of the following steps: (a) fusion of myeloma with spleen cells of immunized mice; (b) 'rescue' of hybrids in selective media; (c) clonal isolation of identified antibody-secreting hybrids and (d) their further expansion for antibody production.

Virtually all studies reported to date have based the selection of the hybrids on the inability of several myeloma cell lines to survive in hypoxanthine/aminopterin/thymidine (HAT) selective medium (Littlefield, 1964). Although the selection of the hybrid cells using aminopterin (AMT) has been well documented (Melchers et al., 1979; Goding, 1980), the drug suffers from the drawbacks that it is photosensitive, highly toxic and a potent carcinogen (Schnitzer and Hawking, 1966).

Methotrexate (MTX) belongs to the aminopterin family and like AMT is a powerful inhibitor of dihydrofolate reductase, an enzyme involved in cellular DNA synthesis (Bleyer, 1978). It has replaced AMT in cancer chemotherapy due to its low toxicity and chemical stability (Matherley et al., 1985; NCI Investigational Drugs, 1985).

This has prompted us to examine and compare the ability of MTX to select for antibody-forming cells following the basic approach described elsewhere (Galfrè and Milstein, 1981).

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Materials and methods

Experimental cell cultures

X63/Ag 8.653 mouse myeloma (Kearney et al., 1979) or a hybridoma clone secreting antibodies specific for a viral antigen (Horenstein et al., 1986c) were cultured in HT medium composed of RPMI 1640 (Gibco), 20 mM Hepes (pH 7.2), 15% fetal calf serum (Gibco), 2 mM glutamine, 2.2 mM sodium bicarbonate, 2 mM sodium pyruvate, antibiotics, 10^{-4} M hypoxanthine and 1.6×10^{-5} M thymidine supplemented when indicated with increasing concentrations of AMT (Sigma Chemical Co., U.S.A.) or MTX (Lederle, Arg.) from 4×10^{-8} M to 4×10^{-6} M.

[^{3}H]thymidine uptake and cell viability assays

The effects of AMT and MTX on the growth of cells were assessed by measuring the degree of DNA activity. Cells from log-phase growth were seeded at a concentration of 1×10^4 cells/well in 96-well microtiter plates (Nunc, Denmark) and kept in a 5% CO_2 humidified incubator at 37°C. For the first 24 h cells were grown in HT medium in order to eliminate any differential effects that experimental parameters might have on seeding efficiency. After 24 h the HT medium was replaced with one containing adequate concentrations of AMT (HAT medium) or MTX (HMT medium). Cells were pulse labelled every 24 h up to a maximum of 4 days with 0.5 μCi/well of [^{3}H]thymidine (TdR, NEN, 20 Ci/mmol) for 3 h at 37°C in triplicate cultures. The cultures were processed using an automatic cell harvester (MASH II) and the [^{3}H]TdR content in 10% trichloroacetic acid-insoluble cell material determined by liquid scintillation (400CL/D, Packard). At the end of the labelling period the density and percentage of viable cells were determined in parallel cultures by phase microscopy and the trypan blue exclusion assay according to Hudson and Hay (1980). The data were analyzed by the Student's *t*-test for statistical significance.

Production of new hybridomas

The details of the methods used in our laboratory to produce hybridomas have been published elsewhere (Horenstein et al., 1986b). In brief, cell fusion was carried out using mouse spleen cells (10^8) and myeloma cells X63/Ag 8.653 (10^7) by means of polyethylene glycol (PEG 1500, 30% w/v). After fusion the cells, resuspended in HT medium, were distributed in 1 ml aliquots into 8×24-well Falcon trays containing feeder layers of 5×10^4 BALB/c peritoneal macrophages established one day before the addition of the cells. After 24 h incubation one-half of the medium was replaced by HT containing 4×10^{-7} M AMT or MTX on days 2, 3 and 4. When clones had grown to about 30% confluence, aliquots of the supernatants were removed and tested for activity using a solid-phase RIA (Horenstein and Feinstein, 1985). Positive cultures were cloned at limiting dilutions on 96-well microtiter plates (Nunc) in the presence of macrophage feeder layers. After repetitive minicloning and expansion the hybridomas were frozen in liquid nitrogen.

Results

The effect of AMT and MTX on myeloma cells is shown in Fig. 1*a*, *b* and *c*. It can be observed that AMT and MTX inhibited [^{3}H]TdR incorporation into myeloma cells and this inhibition was dose and time dependent. Although the incorporation was totally supressed after 72 h of incubation, even at the lower concentration used, it falls more drastically at 4×10^{-6} M (Fig. 1*c*). As with AMT, MTX decreased myeloma cell survival 'in vitro' in parallel with DNA synthesis activity. When 96 h-treated cultures were shifted to HT medium, no viable cells were found in a follow-up for 20 days after DNA blockade release.

The effect of AMT and MTX on a hybridoma cell line is shown in Fig. 2*a*, *b* and *c*. AMT and MTX did not suppress [^{3}H]TdR incorporation into hybrid cells. However, DNA synthesis was lower in the presence of AMT, an effect which could be due to cell death. This would be expected to occur earlier and more rapidly with hybrids exposed to the higher dose of the anti-metabolite. To assess whether the AMT or MTX were toxic to hybrid cells, viability counts were performed on the wells. At the lower doses used (Fig. 2*a* and *b*), cell viability was 80% or more in both cultures throughout the time period, as ascertained by trypan blue exclusion. However, hybrids culti-

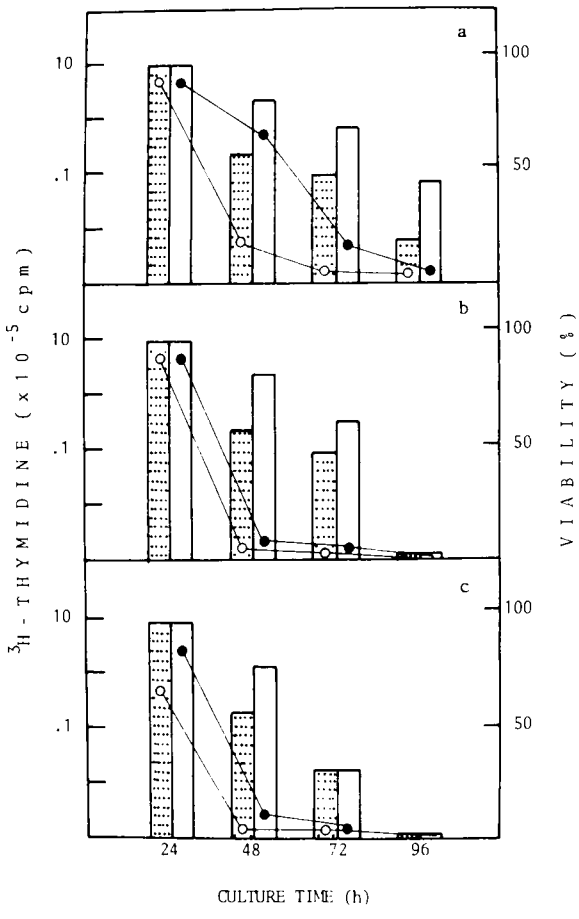

Fig. 1. Effect of AMT and MTX concentrations on DNA synthesis and viability of the myeloma cell line as a function of time. Cells were exposed to 4×10^{-8} M (*a*), 4×10^{-7} M (*b*) and 4×10^{-6} M (*c*) AMT or MTX and harvested at times indicated on the abscissa to measure: cell viability in AMT (dotted bars) or MTX (open bars) and [^{3}H]TdR uptake in AMT (open circles) or MTX (closed circles), as described in the text. Each value represents the mean of three experiments. The standard error of the means were generally less than 10%.

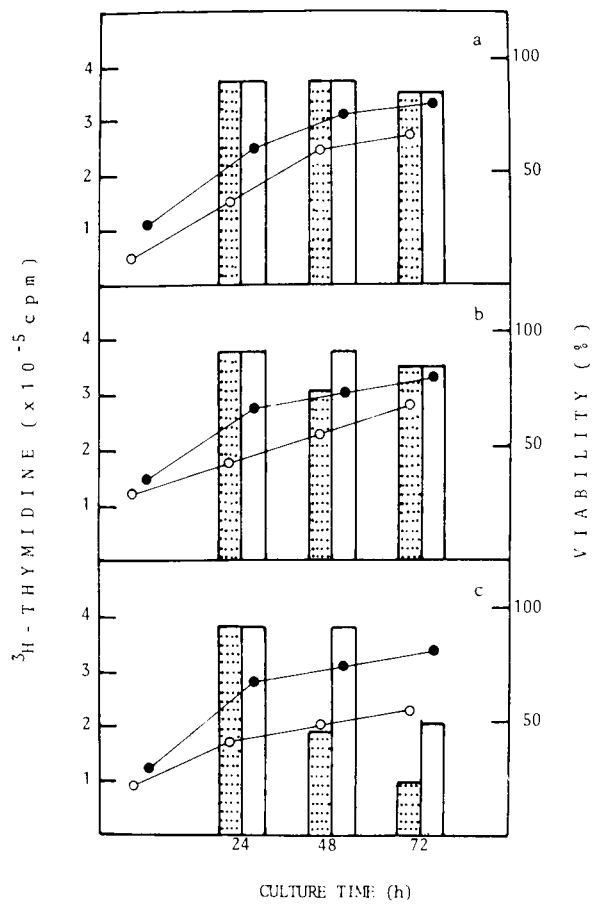

Fig. 2. Effect of AMT and MTX concentrations on DNA synthesis and viability of a hybridoma clone as a function of time. Hybrids were exposed to 4×10^{-8} M (*a*), 4×10^{-7} M (*b*) and 4×10^{-6} M (*c*) AMT or MTX and harvested at times indicated on the abscissa to measure: cell viability in AMT (dotted bars) or MTX (open bars) and [^{3}H]TdR uptake in AMT (open circles) or MTX (closed circles), as described in the text. Each value represents the mean of three different experiments. The standard error of the means were generally less than 10%.

vated with MTX at 4×10^{-6} M (Fig. 2*c*) demonstrated a mean viability percentage significantly higher after 48 h (93% ± 5 vs. 49% ± 4) and 72 h (50% ± 5 vs. 25% ± 3) of culture ($P < 0.01$).

In view of these results it was of interest to determine whether the effect exhibited by MTX could be used to select for hybridomas. This experiment was carried out as outlined in the materials and methods section using cells from two independent fusions. The recovery of hybrids and antibody production after selection in HAT or HMT medium are shown in Table I. As can be seen, both analyzed parameters were not statistically different at 4×10^{-7} M AMT or MTX, the concentration overwhelmingly used for the selection of hybridomas. However, the growth of hybrid cells in HMT was observed microscopically between 4–7 days after fusion and the development of the HAT-resistant cell population was not detected before 12–14 days post fusion.

TABLE I

EFFECT OF HAT- AND HMT-SELECTIVE MEDIA ON RECOVERY AND YIELD OF ANTIBODY PRODUCTION BY HYBRIDOMAS AFTER FUSION [a]

Selective media	Number of wells containing growing hybrid clones [b]	Number of specific Ig producing wells [b,c]
HAT	80 (70–82)	23 (18–28)
HMT	85 (80–91)	27 (24–30)

[a] Results were expressed as the number of wells containing hybridoma clones growing over a total of 8×24-well Falcon trays.

[b] Mean of two fusions. The number of growing wells is given as a range in parentheses.

[c] Hybridomas producing anti-CEA (Horenstein et al., 1986b) antibodies 30 days after fusion in the two different fusions. The yield of antibody-producing hybrids was measured by a solid-phase RIA.

Discussion

Numerous protocols to optimize the environment for hybrid cell growth and survival have been published (Westerwoudt et al., 1983). Nearly all these studies have been devoted to the analysis of factors that interact with cells during hybridization and cloning. The well known drawbacks of AMT, commonly used for hybrid cell selection, prompted us to evaluate the ability of MTX in the rescue of hybridomas.

MTX is distinguished from AMT only by the presence of a methyl group at position N-10, a change which profoundly reduces the anti-folate potency and toxicity (Rosenblatt et al., 1982). Nevertheless, the ability of MTX to block myeloma X63/Ag 8.653 DNA synthesis parallels that of AMT (Fig. 1*a–c*). Accordingly, no viable cells were found in a follow-up for 20 days after DNA blockade release, demonstrating the absence of a MTX-resistant myeloma cell population.

In common with AMT, MTX did not supress [^{3}H]TdR incorporation into the hybrid clone (Fig. 2), showing that the hybridomas were adapted to the DNA synthesis 'rescue' pathway used to overcome the metabolic blockade. However, a different effect of both drugs was observed at the highest concentration tested (Fig. 2*c*). The degree of viability and the reduced cytotoxic damage observed in HMT medium strongly argue in favor of the MTX dose effect being less toxic than AMT. Moreover, an LD_{50} for MTX mice 30 times higher than for AMT mice has been reported (Schnitzer and Hawking, 1966).

It is important to achieve the selection and growth of hybrid cells in HMT medium immediately after fusion. An accelerated proliferating rate in the presence of MTX was suggested by the observed time of appearence of hybrid cells, but we saw no evidence of increased specific efficiency above the levels observed with HAT (Table I). Nevertheless, it is noteworthy that MTX proved to be superior to AMT with regard to toxicity only when compared at the higher doses tested (Fig. 2*c*).

Finally, monoclonal antibody production remained constant when MTX was removed during expansion and hybridomas recovered from frozen storage were found to be stable (Glait et al., 1986; Horenstein et al., 1986b).

In summary, MTX may be used in place of AMT in the selective medium used in hybridoma production. In addition, assuming that a precise screening for specific antibody is dependent on the number of cells secreting antibody into the culture medium, the faster growth of MTX hybrids allows earlier detection and cloning, which is advantageous for preserving stable clones (Melcher et al., 1979). This property, combined with low toxicity, high stability and the lack of any carcinogenic activity suggest that MTX should prove useful in the selection of hybridomas. A preliminary account of a part of the data here reported has been presented (Horenstein et al., 1985a).

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